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Phenotypic Changes in Hepatic Stellate Cells in Response to Toxic Liver Injury

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Abstract Hepatic stellate cells (HSCs) are the main extracellular matrix-producing cell type in the liver. These cells exist in a quiescent state in normal liver and an activated state in damaged liver. During quiescence, HSCs store and release various retinoids. After injury to the liver, however, various protein and non-protein mediators are released from cells that stimulate HSCs to differentiate into myofibroblasts, a process termed activation. Activated HSCs begin to express α-smooth muscle actin and migrate to sites of injury through chemotaxis. Within the damaged region of liver, these cells become contractile and produce growth factors that promote hepatocyte replication and angiogenesis, produce extracellular matrix that forms the scaffold for liver repair, and produce proteins that modulate immune cell function. Once liver repair is complete and liver function is restored, HSCs revert back to a quiescent phenotype or die by apoptosis. If liver injury becomes chronic, however, these processes persist resulting in the formation of an extensive scar (i.e., fibrosis) that ultimately leads to liver failure. Because of the importance of these cells to the development of liver fibrosis, there has been extensive research into understanding the mechanisms that drive HSC activation after injury and the mechanisms that regulate reversion of these cells back to quiescence after resolution of injury. In addition, there has been great interest in identifying the various functions of HSCs after acute and chronic injury. The aim of this review is to briefly discuss the phenotypic changes that occur in HSCs after acute and chronic liver injury and to highlight some of the important functions of these cells during repair of the liver.

Keywords Hepatic stellate cells · Liver · Fibrosis · Regeneration · Inflammation

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Introduction

After many years of research, Friedman and colleagues identified hepatic stellate cells as the principle extracellular matrix-producing cell in the liver, and subsequent studies have confirmed their importance in the development of liver fibrosis [1]. Although other cells have been proposed to contribute to collagen deposition during chronic injury, such as hepatocytes and bile duct epithelial cells through epithelial to mesenchymal transition, bone marrow-derived fibrocytes, and portal fibroblasts, recent studies have solidified HSCs as the predominant collagen-producing cell in the liver during injury [2•, 3••].

HSCs are located between the sinusoidal endothelium and hepatocytes in the Space of Disse. These cells exist in two main phenotypes termed quiescent and activated. During quiescence, HSCs store vitamin A and play an important role in vitamin A homeostasis. During liver injury, however, a number of protein and non-protein mediators are produced that stimulate HSCs to differentiate into myofibroblasts, a process termed activation [4]. Once activated, these cells migrate to sites of injury, proliferate, produce collagen, and regulate immune cell function by producing various immunomodulatory proteins. After acute liver injury, secretion of extracellular matrix by HSCs forms the initial scar, which may protect parenchymal cells from further damage and provide the initial scaffold for repair. Once repair is completed, HSCs revert

back to a quiescent state or die by apoptosis. During chronic injury, HSC activation and collagen deposition persists resulting in the formation of an extensive scar (i.e., fibrosis) that ultimately impairs liver function. Because these cells are crucial to the development of liver fibrosis, a disease for which there remains no effective therapy, there has been extensive research into understanding the mechanisms that drive HSC activation and the mechanisms that regulate reversion of these cells back to quiescence. In addition, there has been great interest in identifying HSC functions after injury. Surprisingly, although these cells were initially described over a century ago, the list of HSC functions continues to grow today with the advent of new methods to study these cells in vivo. The aim of this review is to discuss the phenotype and function of HSCs after acute and chronic liver injury. It will begin with a discussion of HSC function and phenotype during chronic injury, and end with a discussion of HSC phenotype and function during acute injury, where exciting, new functions of HSCs have recently been uncovered.

Activation and Function of HSCs During Chronic Liver Injury

During chronic liver injury, cells in the liver, such as hepatocytes, macrophages, and others, begin to secrete a variety of proteins that stimulate HSCs to differentiate into myofibroblasts, a process termed "activation." These mediators also render HSCs more responsive to paracrine and autocrine growth factors that stimulate HSCs to proliferate and secrete collagen, and stimulate HSC contraction. Furthermore, cytokines are produced that stimulate activated myofibroblasts to migrate to injured regions of liver where they deposit collagen that ultimately forms the scar. The mediators that govern these processes during chronic injury are briefly described in the following sections.

Mediators that Stimulate HSC Proliferation

Several mediators have been shown to stimulate HSC proliferation, including growth factors, such as PDGFs and FGFs, neurotransmitters, such as norepinephrine and serotonin, and the protease thrombin. Within this group of HSC mitogens, PDGF-B is the most potent.

Treatment of activated HSCs in vitro with PDGF-B stimulates proliferation [5]. This requires prior HSC activation, because this process upregulates PDGF receptor β on HSCs [6]. Similarly, activation of HSCs in vivo by chronic treatment with carbon tetrachloride increases expression of PDGF receptor β on these cells [6]. Activation of the PDGF receptor stimulates signaling through various pathways in HSCs that contribute to proliferation, including protein kinase C, Erk1/2, and phospatidylinositol-3 kinase [7]. In support of a role for PDGF-B in the development of liver fibrosis, transgenic overexpression of PDGF-B stimulates development of liver fibrosis [8]. Similarly, transgenic expression of PDGF-A in mice promotes liver fibrosis [9]. Compared to PDGF-B, however, PDGF-A is far less potent at simulating HSC proliferation in vitro. Accordingly, it is unclear whether PDGF-A achieves concentrations during chronic injury that affect HSC function. PDGF-C is the least potent stimulator of HSC proliferation, and studies have shown that liver fibrosis is not affected in PDGF-C knockout mice [10, 11]. However, similar to PDGF-A transgenic mice, transgenic expression of PDGF-C in mice produces liver fibrosis [12]. Whether the levels of PDGF-C achieved in this manner are pathologically relevant, however is not known. PDGF-D is also upregulated in animal models of fibrosis, and stimulates HSC proliferation in vitro with similar potency to that of PDGF-B. It still remains to be determined whether PDGF-D contributes to the development of liver fibrosis in animal models or humans.

Studies have shown that FGF-2 stimulates HSC proliferation in vitro, and that the mitogenic effects of TGF-β1 in vitro may be mediated by the release of FGF-2 from HSCs [13]. Liver fibrosis, induced by chronic carbon tetrachloride treatment, is reduced in FGF-1 and FGF-2 double-knockout mice [14]. Interestingly, similar numbers of α-smooth muscle actin-positive cells were observed in wild-type and FGF-1 and FGF-2 double-knockout mice, suggesting that FGF-1 and FGF-2 may stimulate collagen production by HSCs but not proliferation in vivo [14]. It is possible though that FGF-2 may be an important HSC mitogen in other models of liver fibrosis, although this remains to be determined.

Thrombin is most well-known for its ability to stimulate fibrin deposition after injury by cleaving fibrin and other factors in the coagulation cascade. Thrombin can also activate signal transduction pathways in cells by activating protease-activated receptor-1 (PAR-1) [15]. This receptor is expressed on HSCs, and studies have shown that HSCs proliferate in vitro when treated with thrombin, an effect that was prevented in HSCs from PAR-1 knockout mice [16]. In addition, liver fibrosis is reduced in PAR-1 knockout mice and in mice treated with a PAR-1 antagonist [17, 18]. Interestingly, recent studies showed that fibrin is deposited in the livers of patients with fibrosis indicating that even though coagulation factors may be depleted in patients with diminished liver function, sufficient liver function is present to activate the coagulation system locally in the liver, which may stimulate HSC proliferation in a PAR-1-dependent manner [19].

HSCs express many neural proteins, including neurotrophins and their receptors, glial fibrillary acidic protein,

and many others [20], and studies have shown that exposure of HSCs to various neurotransmitters stimulates HSC proliferation. Serotonin enhances PDGF-induced proliferation in vitro [21]. Norepinephrine is mitogenic for HSCs in vitro, an effect which is enhanced by co-stimulation with neuropeptide Y [22]. In support of a role for norepinephrine in the development of fibrosis, Dbh knockout mice, which are deficient in norepinephrine, showed reduced liver fibrosis [23]. Similar to norepinephrine, the neurotransmitter acetylcholine is an HSC mitogen in vitro [24]. Taken together, these studies suggest that neural input to the liver may regulate HSC proliferation and ultimately liver fibrosis. The importance of this process in patients with liver fibrosis, however, remains to be investigated.

Mediators that Stimulate Production of Extracellular Matrix by HSCs

The growth factor TGF-β has a number of functions, including regulation of the immune system and regulation of collagen deposition during tissue repair [25]. TGF-β stimulates collagen synthesis by activated HSCs, and increased levels of TGF-β are detected in humans and animals with fibrosis [26]. TGF-β is secreted in a latent form by Kupffer cells and HSCs during liver injury. Once secreted, TGF-β is converted to its active form by thrombospondin-1 or plasmin or by interaction with the integrin, αVβ6 [25, 27]. TGF-β activates the Smad transcription factor pathway and mitogen-activated protein kinases in HSCs which increases expression of collagen [28, 29]. Activation of these pathways in HSCs by TGF-β increases expression of procollagen. In mice, overexpression of TGF-β by an adenovirus increased collagen in the liver [30]. Furthermore, liver fibrosis was reduced in TGF-β knockout mice confirming that this growth factor is essential for collagen deposition in vivo during chronic injury [30].

Another mechanism by which TGF-β may stimulate collagen production in the liver during chronic injury is through the upregulation of connective tissue growth factor (CTGF). Studies have demonstrated increased levels of CTGF mRNA in patients with primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia, and in patients infected with hepatitis B [31–34]. Similarly, CTGF mRNA is upregulated in the livers of rodents treated chronically with carbon tetrachloride or subjected to bile duct ligation [35]. In fibrotic livers, CTGF is predominantly expressed in HSCs and bile duct epithelial cells. In cultured HSCs, CTGF expression is increased in activated HSCs and in HSCs treated with TGF-β [36]. PDGF also stimulates the production of CTGF by HSCs, an effect that is blocked by neutralization of TGF-β [36]. Treatment of HSCs with CTGF stimulates collagen production and HSC proliferation and migration [36]. In support of a role for CTGF in the development of liver fibrosis, inhibition of CTGF in vivo with an siRNA decreases collagen levels [37].

In addition to growth factors, recent studies have shown that the proinflammatory cytokine, interleukin-17A (IL-17A), stimulates HSCs to produce collagen. In these studies, treatment of HSCs in vitro with IL-17A increased expression of collagen-α1(I) in a Stat3-dependent manner [38]. In support of a role for this cytokine in the development of liver fibrosis, collagen deposition was reduced in IL-17RA knockout mice after bile duct ligation or chronic carbon tetrachloride treatment. Considering that IL-17A has also been linked to tissue damage from autoimmune diseases, targeting IL-17A in autoimmune liver diseases, such as primary biliary cirrhosis, may be an effective way to reduce not only liver injury, but also fibrosis.

In addition to the protein mediators described above, studies indicate that products of oxidative stress stimulate HSCs to produce collagen. Schneiderhan et al. showed that oxidized low-density lipoproteins (LDL), which are increased in baboons fed ethanol and in alcoholic patients, stimulated collagen production by HSCs in a CD36-dependent manner [39]. In addition, these studies showed that CD36 is upregulated on activated HSCs, and that CD36 is expressed on HSCs in the livers of alcoholic patients. Similar to oxidized LDL, products of lipid peroxidation, such as 4-hydroxy-2,3-nonenal, stimulate HSCs to produce collagen [40]. Collectively, these studies suggest that oxidative stress, potentially from immune sources or xenobiotics, may stimulate collagen production by activated HSCs.

Mediators that Stimulate HSC Chemotaxis

After injury, HSCs migrate to damaged regions of liver. Chemokines, released at the site of injury, stimulate HSC migration by forming a chemotactic gradient. This process is similar to immune cell chemotaxis, and many of the chemokines that stimulate migration of immune cells also stimulate HSC chemotaxis, such as MIP-1α, MIP-1β, RANTES, and MCP-1 [41–43]. In addition to these traditional chemokines, various growth factors, such as VEGF and PDGF, and the protein osteopontin stimulate HSC chemotaxis [44–46].

HSCs express the traditional chemokine receptors, Ccr2 and Ccr5, which are activated by MIP-1α, MIP-1β, RANTES, and MCP-1 [42, 43]. In vitro, HSCs migrate toward these chemokines in a Ccr2- or Ccr5-dependent mechanism, and in vivo, liver fibrosis is reduced in Ccr2 and Ccr5 knockout mice [42, 43]. To distinguish between the role of these receptors on immune cells and non-immune cells, bone marrow from wild-type mice was transplanted into

Ccr2 and Ccr5 knockout mice in these studies. Although the chemokine receptors remained functional on immune cells in these mice, liver fibrosis was decreased, suggesting that activation of these receptors on a non-immune cell type was profibrotic. Since these receptors are expressed by HSCs, it was proposed by the authors that activation of these receptors on HSCs promoted fibrosis.

Osteopontin is another chemokine that is chemotactic for HSCs [46]. The receptor for this protein is $\alpha_v\beta_3$ integrin, which is expressed by HSCs [47]. Conflicting results exist, however, with regard to the role of this chemokine in the development of liver fibrosis. Whereas liver fibrosis was reduced in osteopontin knockout mice fed a methionine/choline-deficient diet to model nonalcoholic steatohepatitis, liver fibrosis was not reduced in mice subjected to bile duct ligation [48, 49]. In addition, treatment of these mice chronically with carbon tetrachloride resulted in enhanced liver fibrosis, which may have resulted from increased liver injury in these mice [50]. In a separate study, inhibition of osteopontin in mice with a neutralizing antibody decreased fibrosis in mice fed a methionine/choline-deficient diet and in mice treated chronically with carbon tetrachloride [51]. It is not completely clear why these studies are conflicting, although the difference may have resulted from the method of osteopontin inhibition (i.e., knockout mouse vs. neutralization). Lastly, the growth factors, VEGF and PDGF, have been shown to be chemotactic for HSCs in vitro, although their importance in vivo remains to be investigated [44, 45].

Mediators that Stimulate HSC Contraction

A key step in the wound-healing process is a contraction of the wound to facilitate closer. This task is performed by myofibroblasts in the skin and in other tissues. In the liver, activated HSCs contract when exposed to various vasoactive mediators. Contraction of HSCs has been shown to modulate sinusoidal blood flow, and it has been proposed that this process in fibrotic livers may lead to increased portal resistance and ultimately portal hypertension [52–54]. Endothelin-1 is the most well-characterized mediator of HSC contraction. HSCs express endothelin-1 receptors, and these cells contract when exposed to endothelin-1 [55, 56]. In addition, endothelin-1 levels are increased in cirrhotic human livers and studies suggest that endothelin-1 antagonists, such as bosentan, are useful for the treatment of portopulmonary hypertension in patients with cirrhosis [57–59].

Activation and Function of HSCs During Acute Liver Injury

Similar to chronic liver injury, HSCs are activated in the liver during acute liver injury. In rodents, levels of α-SMA and type I collagen, hallmarks of HSC activation, are increased in centrilobular lesions after a single dose of carbon tetrachloride [60]. Similarly in humans suffering from acute liver failure from viral hepatitis, drug toxicity, or autoimmune hepatitis, there is an increase in α-SMA-expressing cells within necrotic lesions indicating activation of HSCs [61]. While persistent HSC activation is detrimental to liver function during chronic liver injury, activation of HSCs during acute liver injury may benefit liver function by producing pro-reparative growth factors, by depositing extracellular matrix that provides the scaffold for repair, and by modulating immune cell function. The role of HSCs in these processes during acute liver injury is described in brief below.

Role of HSCs in Liver Regeneration

Much of our knowledge regarding HSC function during liver regeneration has been discovered in rodents subjected to partial hepatectomy. Although this model does not typically involve substantial acute liver injury, information gained from partial hepatectomy studies may be applicable to liver repair after acute liver injury; however, this remains to be confirmed. A recent study utilizing gliotoxin demonstrated a potential role for HSCs in early cell proliferation after partial hepatectomy and a role in termination of liver regeneration [62•]. Gliotoxin is a fungal metabolite that reduces fibrosis in animal models by stimulating HSC apoptosis [63]. This chemical has been used to investigate HSC function in the liver, although gliotoxin can affect other liver cell types, including Kupffer cells [64]. Interestingly, though, pretreatment of rats with gliotoxin substantially reduced early cell proliferation after partial hepatectomy indicating a potential role for HSCs in liver regeneration [62•]. This was also associated with a decrease in hepatocyte growth factor (HGF) levels. Previous studies have shown that HSCs synthesize HGF, although interestingly, after partial hepatectomy, it was only unactivated HSCs that produced HGF [65]. Another mechanism by which HSCs may contribute to cell proliferation is through expression of TGF-α. TGF-α stimulates hepatocyte proliferation through an autocrine mechanism, and studies have shown that TGF-α levels are increased after partial hepatectomy [66]. In addition to hepatocytes, another cell type that expresses TGF-α is HSCs, which may facilitate regeneration through the release of this factor [67]. It was also recently shown that HSCs may promote liver regeneration through delta-like 1 homolog (DLK-1) [68]. DLK-1 is a transmembrane protein that is cleaved by ADAM-17 which releases the extracellular domain of DLK-1 [69]. This domain activates signal transduction pathways in cells by mechanisms that are not fully understood. A recent study showed that after partial hepatectomy, DLK-1 levels are increased in HSCs [68].

Furthermore, in this study, co-culture of HSCs isolated from mice subjected to partial hepatectomy with hepatocytes stimulated hepatocyte proliferation, an effect that was inhibited by a DLK-1-neutralizing antibody. Similarly, inhibition of DLK-1 in partial hepatectomized mice reduced cell proliferation indicating a key role for this protein in regeneration of the liver.

Another mechanism by which HSCs may facilitate liver regeneration after injury is through the regulation of angiogenesis, the process of new blood vessel formation. Key factors in this process include VEGF, angiopoietin-1 (Ang-1), and Ang-2. Angiogenesis is important for regeneration of the liver after partial hepatectomy, as studies have shown that neutralization of VEGF reduces sinusoidal endothelial cell proliferation and impairs liver repair [70]. During angiogenesis, hepatocyte nodules are invaded by sinusoidal endothelial cells to reform the sinusoidal vasculature during regeneration [71]. The importance of HSCs to this process is not fully understood, however, studies have shown HSCs express Ang-1 and Ang-2 after partial hepatectomy [72]. These two factors are important for remodeling and maturation of vessels, and it is possible that the release of these proteins from HSCs contributes to this process. In addition, deposition of extracellular matrix by HSCs may facilitate angiogenesis. Studies have shown that after partial hepatectomy, clusters of hepatocytes are first invaded by activated HSCs which deposit matrix [71]. This process is soon followed by the invasion of sinusoidal endothelial cells into the hepatocytes clusters

In addition to stimulating cell proliferation during repair, HSCs may be important for termination of cell proliferation once liver repair has completed. In support of this, treatment of rats with gliotoxin at 5 days after partial hepatectomy resulted in increased cell proliferation at 7 days, suggesting that termination of liver regeneration was affected [62•]. Associated with this was a decrease in type I collagen protein in the liver and a decrease in TGF-β. The authors proposed that the increase in hepatocyte proliferation may have resulted from a decrease in hepatocyte–extracellular matrix interactions which can increase hepatocyte proliferation, or a decrease in TGF-β which may be important for termination of hepatocyte proliferation [73, 74]. Once liver repair is completed, HSCs may also contribute to the maturation of newly formed hepatocytes through the release of epimorphin. Levels of epimorphin are increased in HSCs after partial hepatectomy, and studies have shown that epimorphin may increase differentiated features of hepatocytes in culture [75]. Whether this process is important for liver regeneration, however, remains to be determined.

While the above studies suggest that HSCs could play a key role in coordinating liver repair after injury by producing growth factors and extracellular matrix that regulate cell proliferation and migration, these findings require confirmation using more selective methods to inhibit HSC function during liver regeneration.

Potential Role of HSC–Macrophage Interactions in Regulation of Acute Liver Injury

With the recent development of methods to selectively deplete HSCs in the liver, previously unrecognized functions of HSCs have been identified. One finding that was quite surprising was the ability of HSCs to modulate liver injury. The first study to demonstrate this used transgenic mice in which the herpes simplex thymidine kinase gene was driven by the GFAP promoter [3]. Expression of this gene in HSCs makes them susceptible to killing by ganciclovir allowing for depletion of HSCs in mice. While these studies were conducted to confirm the importance of HSCs in collagen deposition during fibrosis, an unexpected finding was that the depletion of HSCs decreased liver injury after treatment with carbon tetrachloride and allyl alcohol [3]. This was also associated with an increase in the levels of the anti-inflammatory cytokine, interleukin-10, suggesting that the depletion of HSCs renders the liver less inflammatory during acute liver injury. Using the same system to deplete HSCs, another group showed recently that HSCs are important for liver injury after bacterial lipopolysaccharide (LPS) treatment and after ischemia/reperfusion [76•]. In these studies, depletion of HSCs decreased mRNA levels of tumor necrosis factor-α and CXCL1, and decreased hepatic neutrophil accumulation and liver injury in these two models of inflammatory liver injury. The authors proposed that HSCs are an important source of TNF-α and CXCL1, and that the depletion of HSCs reduced the pool of these cytokines which are important for neutrophil-dependent liver injury in these models. While this explanation is quite plausible, an alternative explanation may be that HSCs modulate macrophage function during acute liver injury resulting in an altered production of cytokines by these cells. Two recent studies lend support for this possibility. Chang and colleagues showed that exposure of macrophages to conditioned medium from activated HSCs increased IL-6 and IL-8 and decreased IL-10, indicating that activated HSCs polarize macrophages toward a proinflammatory phenotype [77••]. In a second study from our laboratory, selective deletion of the transcription factor, hypoxia-inducible factor-1α, in hepatic stellate cells in the liver prevented upregulation of the inflammatory cytokines TNF-α, Cxcl1, Cxcl2, Ccl3, and Ccl4 and decreased the percentage of $Gr1^{hi}$ $CD11b^{hi}$ $F4/80^{+}$ proinflammatory macrophages in the liver after treatment with a single dose of carbon tetrachloride [78]. Considering that these end-points are markers of proinflammatory macrophages, this study

indicated that HSCs polarize macrophages toward a pro-inflammatory phenotype during acute liver injury. Taken together, these four studies suggest that HSCs influence macrophage phenotype during acute injury, and that in the absence of HSCs, macrophages may skew toward an anti-inflammatory state, supported by the finding that IL-10 levels are higher in HSC-depleted mice treated with carbon tetrachloride and allyl alcohol [3]. While this remains to be fully investigated, additional research in this area could potentially uncover novel therapeutic targets that limit inflammatory liver injury in patients suffering from liver disease.

Conclusion

Although HSCs were initially described over a century ago, research continues today to understand the mechanisms that regulate HSC activation and to identify the critical functions of these cells after injury. Although great strides have been made in these areas, development of new methods to selectively deplete HSCs or to selectively knockout genes in these cells could lead to important new findings that have the potential to revolutionize treatment of liver disease.

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Compliance with Ethics Guidelines

Conflict of Interest Bryan L. Copple declares no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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