Fibrosis-Dependent Mechanisms of Hepatocarcinogenesis

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Hepatocellular carcinoma (HCC) is a rising worldwide cause of cancer mortality, making the elucidation of its underlying mechanisms an urgent priority. The liver is unique in its response to injury, simultaneously undergoing regeneration and fibrosis. HCC occurs in the context of these two divergent responses, leading to distinctive pathways of carcinogenesis. In this review we highlight pathways of liver tumorigenesis that depend on, or are enhanced by, fibrosis. Activated hepatic stellate cells drive fibrogenesis, changing the composition of the extracellular matrix. Matrix quantity and stiffness also increase, providing a reservoir for bound growth factors. In addition to promoting angiogenesis, these factors may enhance the survival of both preneoplastic hepatocytes and activated hepatic stellate cells. Fibrotic changes also modulate the activity of inflammatory cells in the liver, reducing the activity of natural killer and natural killer T cells that normally contribute to tumor surveillance. These pathways synergize with inflammatory signals, including telomerase reactivation and reactive oxygen species release, ultimately resulting in cancer. Clarifying fibrosis-dependent tumorigenic mechanisms will help rationalize antifibrotic therapies as a strategy to prevent and treat HCC. (HEPATOLOGY 2012;56:769-775)
led to effective antifibrotic therapies. The risk of HCC may be reduced by abrogating the initial, inflammatory insult, but increasing evidence suggests that persistent fibrosis confers its own carcinogenic risk. In other words, clearing hepatitis C in a cirrhotic patient might halt progression of the disease, but may not reduce the risk of HCC. Currently, there is a paucity of clinical data to address this possibility.

Potential mechanisms of fibrosis-dependent carcinogenesis include increased integrin signaling by the fibrotic matrix, paracrine signaling between hepatic stellate cells (HSCs) and hepatocytes, increased stromal stiffness, growth factor sequestration by extracellular matrix (ECM), and reduced tumor surveillance by natural killer (NK) and natural killer T (NKT) cells.

Enhanced Integrin Signaling. Fibrosis is characterized by changes in the amount and composition of ECM components, which contribute to tumorigenesis. Increased deposition of fibrillar collagens types I and III, as well as fibronectin, in hepatic fibrosis provokes cellular responses through the integrin family of transmembrane receptors. When organized into focal adhesions on the cell surface, integrins promote growth and survival by activating the phosphoinositol 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling cascades. Increased ECM may stimulate integrin signaling in hepatocytes, thereby enhancing the growth and survival of precancerous cells. This prospect is supported by studies that correlate collagen expression, integrin expression, and tumorigenicity in both human HCC samples and mouse HCC models.

Other proposed mechanisms for integrin-mediated tumorigenesis include increased migration and enhanced survival through antiapoptotic signaling. In tumor lines, overexpression of integrin β1 actually leads to growth arrest, attributed to up-regulation of the cyclin-dependent kinase inhibitors p21 and p27. In addition, human HCC samples have decreased expression of integrin β3, and its overexpression in a human HCC cell line leads to apoptosis. In future studies the specific ligands and downstream pathways of the integrin heterodimers need to be characterized in both premalignant and cancerous cells in order to clarify their combined impact on hepatocarcinogenesis.

In addition to the fibrillar collagens, other ECM molecules including laminin, fibronectin, and several nonfibrillar collagens may also amplify carcinogenic signaling. Although these proteins are in relatively low abundance compared to the fibrillar collagens, their potential function as growth factor receptor ligands could amplify their carcinogenic impact.
**Paracrine Crosstalk Between Tumorigenic and Stromal Cells.** Rich intercellular signaling networks exist between tumors and tumor-associated fibroblasts: tumor secretion of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) stimulates myofibroblast activation, leading to changes in ECM composition and organization. Reciprocally, activated fibroblasts promote tumor growth and invasion, not only in primary tumors but also in early stages of metastasis. This crosstalk has been emphasized in HCC, where stromal gene expression profiles have been correlated with patient survival. As the primary fibrogenic cells in the liver, activated hepatic stellate cells (HSCs) and myofibroblasts may directly support hepatic tumorigenesis. Stellate cells produce growth factors, including hepatocyte growth factor, interleukin 6, and Wnt ligands, fostering an environment conducive to hepatocyte proliferation. Similarly, hepatic myofibroblasts can enhance the growth and migration of malignant hepatocytes, at least partially through PDGF- and TGF-β-mediated mechanisms. In addition, hepatic stellate cells secrete more angiopoietin 1 when activated, facilitating an angiogenic milieu that is supportive of tumor growth. Reciprocally, tumors may signal to surrounding stroma. For example, elevated hedgehog signaling has been associated with liver injury in mice and humans, and promotes liver regeneration. Hedgehog activity has been implicated in the formation and maintenance of malignancies, yet hedgehog ligands fail to drive proliferation in several tumor cell lines. Instead, hedgehog signaling from tumors to the stromal microenvironment may be responsible for promoting tumor progression. Because hedgehog signaling may induce epithelial-to-mesenchymal transition, the tumorigenic effect of hedgehog could be mediated by increased myofibroblast activation and fibrosis. This prospect is supported by a hedgehog antagonist-mediated reduction of myofibroblasts in a mouse model of biliary injury and HCC. Several studies have identified cells resembling activated stellate cells associated with the liver progenitor cell niche, suggesting that these cells may provide paracrine signals that promote stem cell expansion. The nature of these paracrine signals, and the mechanisms underlying the supportive role of HSCs in stem cell expansion, are currently unknown and of intense interest.

**Increased Matrix Stiffness.** Liver fibrosis increases ECM stiffness, which promotes cell proliferation and HSC activation. Increased stromal stiffness precedes and accompanies fibrosis in chronic liver disease, and elevated liver stiffness, as measured by transient elastography, is associated with enhanced risk of HCC. Similar paradigms exist in other systems: non-transformed 3T3 cells have increased proliferation on stiff polyacrylamide substrates, and enhanced stiffness has been correlated with malignancy in a mouse model of breast cancer. Experimentally, a stiff collagen gel inhibits primary hepatocyte differentiation and promotes proliferation. Hepatoma lines are affected similarly, with increased proliferation and chemotherapeutic resistance when grown on increasingly stiff polyacrylamide gels. This effect is mediated by the Fak, Erk, Pkb/Akt, and Stat3 pathways, primarily downstream of integrin β1 signaling. Stromal stiffness also increases activation of stellate cells and portal fibroblasts, creating a positive feedback loop that continues to promote fibrosis. Stromal stiffness is regulated in part by matrix metalloproteinases (MMPs) and their inhibitors, but MMPs can regulate cell proliferation independently of their effects on stromal stiffness. Although MMPs degrade the stroma, they paradoxically increase liver growth, HSC proliferation, and tumor progression. MMPs might also liberate sequestered growth factors (see next section). Alternatively, the production of reactive oxygen species in response to MMP activity may overcome the loss of stromal stiffness by promoting genomic instability. This type of reactive oxygen species induction is reportedly downstream of an alternatively spliced form of Rac1, which is induced after mammary epithelial exposure to MMP-3. A third possibility is that MMP induction of reactive oxygen species leads to enhanced stellate cell activation, also through a Rac1-mediated mechanism.

**Growth Factor Sequestration by the ECM.** Growth factors are sequestered by the ECM and signal in an autocrine or paracrine manner to nearby cells. Initial work focused on fibroblast growth factor (FGF) sequestration in the ECM, but many other cytokines are passively sequestered, including ligands from the FGF, TGF, BMP, Wnt, and interleukin families. MMPs can both activate and inhibit growth factor signaling; they liberate growth factors from the ECM, but can also remove the extracellular receptors by cleavage at the cell surface. Other signaling factors are actively recruited to the ECM by regulatory carrier proteins. For example, TGF-β signaling is highly dependent on ECM interactions. TGF-β is directly recruited to the ECM by latent TGF-β binding proteins (LTBPs), which have affinity for both TGF-β and ECM fibrils. When bound to LTBPs, TGF-β is unable to signal. This suggests that accumulation of ECM would lead to
increased proliferation and decreased apoptosis, because TGF-β signaling would be suppressed. However, LTBP3s contain multiple proteinase sensitive sites, and cleavage of those sites by MMPs leads to the release of TGF-β.55

In the setting of inflammation or increased migratory potential, elevated MMP activity can liberate sequestered TGF-β. Fibrotic ECM, containing more sequestered TGF-β, would release greater amounts of the cytokine. This could antagonize oncogenesis by inhibiting proliferation and promoting apoptosis. The nature of ECM-cytokine interactions may change depending on the particular cytokine, duration, and cellular context of each interaction. To clarify these relationships, there is a need for controlled, reproducible systems that model the interaction between cells and stroma.

**Reduced Tumor Surveillance by NK and NKT Cells.** Reduced NK cell function may also contribute to the emergence of HCC in chronic liver disease. NK cells induce apoptosis in cells that have either down-regulated class I major histocompatibility complex expression or up-regulated stress-induced ligands. These expression changes are usually present in tumor cells, allowing NK cells to function in tumor surveillance and control.56 In addition to killing tumor cells, NK cells down-regulate fibrosis by inducing apoptosis of activated stellate cells,57,58 without affecting quiescent stellate cells.59

NK cells are enriched in the liver,60 but have reduced activity in chronic liver disease.61-63 Fibrosis may inhibit NK cell function by separating them from their tumor and stellate cell targets; NK cells in the tumor microenvironment remain in the stroma, unable to function, instead of making cell-cell contact.64 NK cells express MMPs, and migrate more slowly in the presence of MMP inhibitors,65 further suggesting that NK function, and subsequently tumor surveillance, is inhibited by the ECM accumulation in fibrosis.

NKT cells are a distinct population of cells that can both direct class switching and induce Fas/perforin-mediated apoptosis.66 Like NK cells, NKT cells home to the liver. CD1d-tetramer+CD4+ populations can promote stellate cell activation,67 but CD45RB+B220+TCRβ+CD1d-tetramer-reactive iNKT cells are antifibrotic.68 The endogenous activity of NKT cells most likely reflects their level of activation.69 CD1d+ and CD3+DX5+NKT cell surveillance of HCC has been established using mouse hepatoma implantation models,70-72 but the effect of fibrosis on NKT tumor surveillance is less clear—although CD1d-tetramer+CD4+NKT cells are increased in the setting of cirrhosis67 and CD3+Vα24+Vβ11+iNKT cells are increased in hepatic malignancy,73 little is known about their interactions with the ECM.

**Conclusions**

Several pathways link chronic liver disease, fibrosis, and carcinogenesis (Fig. 2), yet a coherent model linking fibrosis to HCC remains elusive. Importantly, key experimental challenges continue to stall therapeutic progress.

Each tumorigenic mechanism may operate across a limited range of the natural history of HCC, a concept that can greatly inform the most appropriate models and patients to study. For example, whereas stromal stiffness promotes cell growth, it only contributes to oncogenesis when cells are unable to proliferate without a stiff stroma. This might be true for premalignant hepatocytes, but not tumor cells—carcinoma cell populations have limitless replicative potential and relative independence from extracellular growth signals, allowing them to proliferate independently of stromal stiffness. Although stromal stiffness is most likely influential early in the development of HCC, angiogenic factors become increasingly important as solid tumor size increases. It follows that different models or timepoints should be used for the study of stromal stiffness and angiogenic potential. Many studies focus on cirrhosis patients, presumably because they are easier to define and have more obvious disease phenotypes. This restricts the scope of the study to more advanced pathogenic events. Similarly, molecular studies often use immortalized lines from advanced tumors. Due to the diverse natural history of chronic liver disease, an ongoing challenge is to identify when particular oncogenic mechanisms are contributing to HCC, and to use experimental models that accurately reflect liver pathology at that point.

To fully clarify the role of fibrosis in HCC development, there is a pressing need for the experimental separation of fibrosis and inflammation, which will facilitate the ability to determine how fibrosis per se contributes to hepatocarcinogenesis. A few existing models may prove useful. For example, a transgenic mouse with hepatic overexpression of PDGF-B74 leads to activation of hepatic stellate cells, without additional inflammatory stimuli. Alternatively, mice expressing collagenase-resistant collagen have delayed fibrosis regression after sustained injury,75 offering the potential to study fibrotic influences after the majority of inflammatory sequelae have resolved. A reciprocal approach would be to induce fibrosis in an immunocompromised animal, although the feasibility of this approach is not established—strong inflammatory
stimuli normally accompany myofibroblast activation.  

Models of liver disease are especially lacking in several areas. First, whereas hedgehog-mediated crosstalk with stroma may facilitate progression in mouse xenograft models, the contribution of stromal-tumor hedgehog signaling in the liver is not clear. In addition, no models specifically interrogate the immune defects resulting from fibrosis, which purportedly contribute to HCC—whereas NK cells contribute to tumor surveillance and their activity is reduced with progressive fibrosis, the actual effect of fibrosis-related NK dysfunction has not been clarified. Lastly, mechanisms linking fibrosis to cancer development in other tissues have been described in breast and several other tumors. These may apply to hepatocarcinogenesis, but must be tested directly in liver models.

In hepatocarcinogenesis, the convergence of chronic liver disease, inflammation, and fibrosis is likely complex, nuanced, and varied. A recent study reports that liver fibrosis may be protective in the context of acute liver injury, suggesting that complete suppression of fibrosis might not be an optimal therapeutic approach. Instead, targeted manipulation of hepatocarcinogenic pathways should be more fruitful. This targeted approach will only be possible with an enhanced understanding of the genetic and epigenetic mechanisms in HCC. Ultimately, a deeper understanding of fibrotic influences will yield valuable insights for the prevention and treatment of hepatic neoplasia.

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