

Chronic liver injury, TGF- β , and cancer

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Abbreviations: ECM, extracellular matrix; HSC, hepatic stellate cell; HCC, hepatocellular carcinoma

Abstract

Cells termed *myofibroblasts* are prominent in the injury response of all epithelial tissues. They exhibit proliferation, migration, production of collagen and other extracellular matrix (ECM) molecules, and contraction, all for containing the injury and closing the wound. When the injury is limited in time, the final stage of the repair involves a dismantling of the cellular apparatus and restoration of normal tissue structure. With multiple cycles of repair, however, there is net accumulation of ECM, to the detriment of tissue structure and function. Repair-related ECM coalesces into fibrous bundles and, over time, undergoes changes that render it resistant to degradation. The result is a scar. In the skin, a scar may have cosmetic importance only. In the liver, however, extensive scarring is the setting for unregulated growth and neoplasia; also, fibrous bands disrupt normal blood flow, leading to portal hypertension and its complications. With regard to therapy for fibrosis, the first consideration is elimination of the injury factor. However, given that many liver diseases do not have effective therapies at present, strategies targeting fibrogenesis *per se* are under development. The main source of myofibroblast-like cells and ECM production in the liver is the perisinusoidal stellate cell, which responds to injury with a pleiotypic change termed *activation*. Activation is orchestrated by cytokines and the ECM itself. Among the cytokines involved in this process, transforming growth factor-beta (TGF- β) is particularly prominent. The early changes in ECM include *de novo* production of a specific "fetal" isoform of fibronectin, which arises from sinusoidal endothelial cells. It is stimulated by TGF- β and acts directly on stellate cells to promote their activation. Based on these and other advances in understanding the fundamentals of the injury response, several strategies now exist for

altering fibrogenesis, ranging from agents that block TGF- β to traditional Chinese herbal extracts. Arrest of fibrogenesis, even with underlying cirrhosis, is likely to extend life or prolong the time to transplant. Whether it reduces the risk of hepatocellular carcinoma remains to be proven. Although TGF- β antagonists are effective anti-fibrogenic agents, they will require detailed safety testing because of the finding that several forms of epithelial neoplasia are associated with altered regulation of TGF- β .

Keywords: liver cirrhosis, TGF- β , hepatocellular carcinoma, anti-fibrotic

Hepatic stellate cell (HSC) activation

At one time the origin of the *myofibroblasts* in liver injury was obscure, because "fibroblasts" are rare in normal liver, consisting only of a few that reside in the periportal stroma (Knittel *et al.*, 1999). Initial clues derived from histological studies that found "fat-storing cells" (now termed *hepatic stellate cells*, HSC) in the vicinity of injury and fibrosis (Kent *et al.*, 1976). Definitive proof came with the isolation of this cell type. When placed in conventional culture, on plastic with a serum-containing medium, HSC from normal rats underwent a rapid and striking phenotypic change, exhibiting features of myofibroblasts (Friedman *et al.*, 1985). The findings led to the concept of stellate cell activation and the postulate that myofibroblasts in the injured liver *in vivo* represent activated stellate cells (Bissell, 1990). Independently, studies of the kidney found that mesangial cells similarly give rise to myofibroblasts in injury, and subsequent work has demonstrated very similar regulation of mesangial cells and HSC (Abboud, 1995). The working thesis is that individual epithelia, while differing in function, use similar strategies for injury repair (Friedman, 1993).

Although HSC may be the most important source of myofibroblasts in injury, they are not the sole source. The portal area of the normal liver contains fibroblast-like cells, and the proximal bile ductules, up to the canals of Hering, are encircled by smooth muscle cells, which may proliferate, migrate and contribute to ECM production in response to biliary injury. The reaction to centrilobular injury may involve initially perivenular mesenchymal cells that differ morphologically from typical HSC. Consistent with this is evidence that the myofibroblast population is heterogeneous, at least with regard to the expression of desmin and smooth-muscle α -actin (Ballardini *et al.*, 1994). Further work is needed to evaluate the role

and regulation of the non-HSC-derived myofibroblasts in injury.

The possibility that a variety of cells may contribute to ECM production in the injury response does not alter the fact that HSC, by virtue of their perisinusoidal location in the liver lobule, play a key role. Injury that is confined to the periportal area is often silent until it extends into the lobule and involves stellate cells. The ensuing perisinusoidal fibrosis constitutes the lesion known as "capillarization of the sinusoids". It is associated with loss of the open fenestrae of sinusoidal endothelial cells (McGuire *et al.*, 1992) and effacement of the microvilli on the sinusoidal surface of hepatocytes (Orrego *et al.*, 1979), and it correlates clinically with significantly deranged liver function (Schaffnet and Popper, 1963).

Soluble regulators of fibrogenesis

These comprise a growing list of heterogeneous molecules dominated by biological response mediators, most of which are small proteins known generally as cytokines but include lipids (prostaglandins, retinoids) and oxyradicals. Other than an association with the injury response, what these molecules have in common is a short range of action. Unlike endocrine factors that act on distant targets *via* the vascular space, these are either paracrine (acting on cells in contact with, or directly adjacent to, the source of the factor) or autocrine (acting on the cells that produce the factor). Thus, their actions are strictly local, the role being to 'fine-tune' the cell's response rather than to switch it on or off. Cells are subject to combinations of these factors, which creates difficulties in analyzing the role *in vivo* of an individual factor.

To define individual cytokines that play a role in promoting or suppressing the repair response, investigators have used cell cultures in which the purified or recombinant factor is introduced. A particularly well-studied cytokine is TGF- β , which is widely regarded as pro-fibrogenic in liver injury (Bissell *et al.*, 2001). It exists in mammals as three isoforms (TGF- β 1, - β 2 and - β 3), which have very similar properties according to current evidence (Massague and Chen, 2000). All three are present in liver but, interestingly, are not expressed uniformly in individual cell types (Bissell *et al.*, 1995), posing the possibility of distinct individual roles; this remains to be explored. TGF- β is produced as a latent factor that requires proteolytic processing to become active. Candidate proteases that may accomplish this task include plasmin or cell-specific and membrane-associated proteases. In addition to proteolytic activation of latent TGF- β , a number of pericellular factors act to sequester or limit the activity of this cytokine. These consist of binding proteins, including the ECM, integrins that participate in TGF- β receptor activation and regulators of intracellular signaling from the receptor (Bissell *et al.*, 2001).

TGF- β stimulates ECM production not only by HSC but also by sinusoidal endothelial cells (SEC) (see below). However, its effects vary with the scenario. In the context of hepatic regeneration, TGF- β is anti-proliferative rather than pro-fibrogenic (Russell *et al.*, 1988). It is anti-proliferative also for lymphocytes. Thus, mice lacking TGF- β 1 exhibit histological inflammation, with infiltration of many tissues by mononuclear cells (Shull *et al.*, 1992). Viewed solely as a modulator of immune cells, TGF- β could be designated as anti-inflammatory and potentially anti-fibrogenic. Indeed, this characterizes its apparent role in inflammatory bowel disease (Monteleone *et al.*, 2001), in contrast to its pro-fibrogenic role in liver and other tissues.

This underscores the point that attempts to study a factor in isolation may be misleading with respect to its *in vivo* role. Work with cell culture is particularly prone to artifact, because culturing induces changes in *endogenous* cytokine expression. It is well-established, for example, that stellate cells in early primary culture exhibit strikingly increased expression of TGF- β (Bissell *et al.*, 1995). The background of endogenous expression complicates the interpretation of studies on the effect of added cytokines and may account in part for the rather modest changes in collagen expression that are seen with addition of TGF- β to HSC in culture (Gressner, 1996). *In vivo* transgenic models are important but only a partial answer to the problem. Results from transgenic animals may be misleading or irrelevant if the gene of interest is not expressed in the appropriate cell type within the liver. Also, if the alteration in cytokine expression is fixed and present throughout development, it may have unanticipated secondary effects on several aspects of homeostasis. Conditional mutants, in which the genetic alteration can be imposed under external control at the desired time may circumvent the problem. At present, however, this kind of model in the liver has been developed with hepatocyte-specific promoters only (Wang *et al.*, 2001). Promoters specific for HSC or other non-parenchymal cells are needed.

Non-cytokine soluble factors undoubtedly contribute also to HSC activation, although they have been less well examined. Oxidant stress and oxyradicals figure prominently in several scenarios of fibrogenesis, notably injury from alcohol administration and iron overload (Casini *et al.*, 1997). The work to date has focused on regulation of collagen transcription by oxidant stress; the role of oxyradicals in initiating HSC activation is uncertain. The prostaglandins are another class of injury response modulators that may have direct effects on HSC (Kawada *et al.*, 1992). Finally, retinoids represent the most tantalizing and least well-understood soluble regulators of HSC activation. Retinoic acid has profound effects on cellular metabolism; the liver is unusually rich in retinoids due to the fact that HSC store roughly 90% of the body's total in the form of retinoid esters. With

injury, a remarkable shift in retinoids occurs with hydrolysis and release (as retinol) of a substantial fraction of the HSC stores (Friedman *et al.*, 1993). Nonetheless, it has been difficult to arrive at a clear picture of how, if at all, this process plays a role in fibrogenesis.

The ECM and activation of HSC

The ECM has undergone a conceptual transformation in the last 20 years. Once viewed as relatively simple proteinaceous material rich in collagen and termed 'ground substance', it is now viewed as a highly dynamic complex that varies in composition according to its tissue localization and physiological circumstances. Its essential nature is indicated by deletion experiments in mice. For example, the absence of fibronectin is incompatible with development beyond the early embryo stage (Georges-Labouesse *et al.*, 1996). Because the cells of an organism must adapt to changing circumstances, the ECM is highly dynamic. Changes in ECM during embryonic development have been related to cell migration and organogenesis. Similarly, alterations of the ECM during epithelial repair have important effects on cellular function as well as on blood flow and portal pressure.

The ECM interacts with cells *via* specific surface receptors, many of which are integrins, heterodimeric transmembrane proteins consisting of α and β subunits. Their long extracellular segment binds ECM (Ruoslahti and Engvall, 1997); the cytoplasmic side consists of a short segment that is physically connected to the cytoskeleton. Through this linkage, external ligands (*e.g.*, ECM components) may affect directly the internal structure of the cell. Also, when an integrin is engaged by the appropriate ligand, its cytoplasmic domain undergoes activation, initiating events that impact on the regulatory machinery of the cell. Thus, the ECM exerts its effects through both "mechanical" and "chemical" processes.

Alteration of ECM can occur either by addition of specific proteins or selective turnover of ECM constituents. Studies of cutaneous wound repair have demonstrated marked induction in the granulation base of a specific isoform of fibronectin that contains an "extra" type III domain known as EDA (in the rat, EIIIA); this arises by alternative splicing of the primary transcript (French-Constant *et al.*, 1989). A similar change occurs early in experimental liver injury (Ramadori *et al.*, 1992). Quantitatively the most important form of fibronectin is the circulating form known as plasma fibronectin, which is produced largely by hepatocytes and lacks the EDA domain. The EDA-containing form may be quantitatively minor as a proportion of the total fibronectin but nonetheless exerts local regulatory effects. The latter was demonstrated by studies of the cellular source and biological activity of EDA-containing fibronectin, which revealed that EDA-fibronectin arises at the onset of injury and is a product of sinusoidal endothelial cells. Production of this isoform by

endothelial cells is stimulated by TGF- β (George *et al.*, 2000). Given that endothelial cells are contiguous with stellate cells, the impact of this isoform on stellate cells was examined, and it was found to stimulate activation (Jarnagin *et al.*, 1994). Thus, an important early change in the local ECM in response to injury appears to be addition of EDA-fibronectin.

ECM may be altered by locally released proteinases. Matrix metalloproteinases (MMPs) comprise a large family of enzymes specialized in degradation of ECM. Stellate cells produce MMP2 and MMP9 (Arthur *et al.*, 1989), and Kupffer cells produce MMPs as well as other matrix-degrading enzymes (Arthur, 1995). Proteinases are prominent not only early in injury, in conjunction with stellate cell activation, but also in the late stages of wound repair, for ECM remodeling towards restoration of normal tissue structure (Arthur, 1995). Proteinases are subject to regulation by soluble inhibitors termed TIMPs (tissue inhibitors of metalloproteinases). TIMP expression appears to increase as acute fibrosis becomes chronic (Iredale, 1997).

Sustained stellate cell activation and scar formation

"Normal" and "pathological" repair are likely one and the same in their initial stages, based on the observation that experimental injury can progress to pathological cirrhosis and still resolve completely when the injury stimulus is withdrawn (Rojkind and Dunn, 1979). Stellate cell functions that may contribute to the formation of permanent scar include *proliferation, migration, contraction* and *angiogenesis*. Although overlapping *in vivo*, they can be examined in isolation and will be discussed individually.

Proliferation

Both parenchymal and non-parenchymal cells proliferate during wound repair. Proliferation of stellate cells is presumably a mechanism for amplifying the repair response and may contribute to its extent and duration. An important stellate cell mitogen is platelet-derived growth factor (PDGF). The stellate cell response depends on expression of the cognate receptor, the PDGF- β receptor, which occurs very early in the repair response (Friedman and Arthur, 1989; Failli *et al.*, 1995). Stellate cells in culture (*i.e.*, *activated* cells) respond also to epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) by increasing their DNA synthesis (Bachem *et al.*, 1992). Interesting questions revolve around the role of TGF- β . Hepatocyte proliferation is part of both acute and chronic injury. In culture, it is influenced markedly by the particular ECM substratum (Bissell and Choun, 1988), and emerging evidence from *in vivo* study of the partial hepatectomy model indicates that proteinase activity increases at the onset of regeneration (Kim *et al.*, 1997), suggesting that modification of the local ECM is a necessary prelude to hepatocyte

proliferation. The capacity for proliferation is determined in part by the length of chromosomal telomeres. Telomere length has been examined in human cirrhosis (Urabe *et al.*, 1996; Ogami *et al.*, 1999). Telomerase is an enzyme that maintains telomere length, and in mice lacking this function, the liver is unusually sensitive to chemical injury (Rudolph *et al.*, 2000).

Migration

Part of the response to injury is migration of activated stellate cells (myofibroblasts) to the wound area. Chemotactic signals may be involved (Marra *et al.*, 1999; Failli *et al.*, 2000; Kinnman *et al.*, 2000; Bonacchi *et al.*, 2001; Cassiman *et al.*, 2001; Tangkijvanich *et al.*, 2001) as well as specialized cell-ECM interactions. The specific composition of the ECM that supports migration has yet to be defined. The large polysaccharide, hyaluronic acid, has been implicated in migration of other cells, tumor cells in particular (Lara-Pezzi *et al.*, 2001).

Contraction

Stellate cells are closely associated with the capillary endothelium and exhibit markers of smooth muscle, specifically the intermediate filament, desmin. As such, they represent an extension of the arteriolar or venular smooth muscle layer. One report indicated that normal stellate cells are contractile (Zhang *et al.*, 1994). However, stellate cells in culture have no demonstrable contractility until they undergo activation, indicated by the expression of smooth muscle α -actin (Rockey *et al.*, 1993). A technical challenge with studies *in vivo* or in the isolated perfused liver is distinguishing intrahepatic (perisinusoidal) contraction from contraction of portal vessels (Kaneda *et al.*, 1998). In culture, the principal contractile agonists are the endothelins, a group of small vasoactive peptides. Their receptor has two forms termed A and B (ET_A and ET_B); ET_A is associated with contractile cells and within the liver is detectable only on stellate cells (Housset *et al.*, 1993). Stellate cells also express endothelin itself. Moreover, the mRNA for endothelin increases with injury, suggesting autocrine regulation of stellate cell contraction (Housset *et al.*, 1993). With activation, the relative expression of the two receptors changes in favor of ET_B (Pinzani *et al.*, 1996; Gabriel *et al.*, 1999). There is evidence that endothelins modulate stellate cell activation through the ET_B receptor (Rockey and Chung, 1996). NO and CO are believed to modulate the contractility of activated HSC (Failli *et al.*, 2000).

Contraction presumably serves to aid wound closure in the case of injury to the skin. Whether it is critical to repair of wounds to internal organs such as the liver is unclear. In some respects, contraction appears to be a facet of the general repair response that is unnecessary in liver and possibly detrimental. For this reason, its

regulation is an active area of investigation. Competitive antagonists of endothelins and endothelin receptors have been shown to block contraction of stellate cells in culture (Rockey and Chung, 1996; Cho *et al.*, 2000) but are as yet untested in humans. An open question concerns the effect of contraction on hepatic blood flow in the setting of acute and chronic injury, respectively. The autonomic nervous system also may influence on stellate cell contraction. It is known that neurons of the coeliac plexus terminate on stellate cells (Bioulac-Sage *et al.*, 1990). Also, in common with brain astrocytes, stellate cells display glial fibrillar acidic protein (Buniatian *et al.*, 1996; Neubauer *et al.*, 1996) and the neural crest marker, nestin (Niki *et al.*, 1999).

Angiogenesis

This is an important but insufficiently studied aspect of chronic liver injury. With progression of the scarring process, the native endothelial lining of the sinusoids undergoes conversion to a non-fenestrated cell, leading to an appearance that has been termed "capillarization of the sinusoids" (Schaffner and Popper, 1963). "True" angiogenesis also may occur with budding of large-vessel endothelium in areas of injury. The expression of pro-angiogenic factors (*e.g.*, VEGF) (Ankoma-Sey *et al.*, 1998) and angiogenesis inhibitors (Musso *et al.*, 2001) has been documented in liver, although their involvement in injury repair is unclear.

Time-course and reversibility of fibrosis

The rate of fibrosis progression varies widely, depending in part on the nature of the injury agent and probably also on genetic differences that determine the vigor of the injury response (Powell *et al.*, 2000). Malignant biliary obstruction in adults or biliary atresia in infants can lead to cirrhosis within one year (Scobie and Summerskill, 1965). Progression can be rapid also in protoporphyria (Bloemer, 1988). In contrast, hepatitis C typically requires more than 25 years to reach cirrhosis, in the absence of cofactors such as alcohol use (Poynard *et al.*, 1997). Progression in non-alcoholic steatohepatitis also appears to be characteristically slow.

With elimination of the injury factor, the healing process concludes with dismantling the repair apparatus including activated stellate cells and excess ECM. Activated stellate cells disappear largely *via* apoptosis (Iredale *et al.*, 1998); the ECM presumably is degraded by specific proteinases, which increase in parallel with the resolution of injury.

The fact that fibrosis resolves completely after a time-limited injury, with essentially perfect restoration of tissue structure, indicates its reversibility. This applies also to acute fibrosis occurring in a liver with significant chronic fibrosis or cirrhosis. Indeed, the dramatic improvement in clinical status that follows successful treatment of hepatitis B in the setting of cirrhosis (Villeneuve *et al.*,

2000; Yao *et al.*, 2001) suggests that the acute component of acute-on-chronic fibrosis is disproportionately responsible for hepatocellular dysfunction. Over time, changes occur that render fibrosis irreversible. The biochemical basis for this event remains unclear; cross-linking of collagen fibrils appears to be involved (Vater *et al.*, 1979). Also, chronic disease is associated with increased expression of TIMPs (Iredale *et al.*, 1996), which may reduce the activity of endogenous matrix proteinases.

Fibrosis and cancer

Hepatocellular carcinoma (HCC) and cholangiocarcinoma comprise almost all primary liver cancer. HCC arises from transformed hepatocytes and cholangiocarcinoma from the biliary epithelium. Mesenchyme-derived neoplasms, such as angiosarcoma, are rare. Hepatoblastoma occurs in infants and children. HCC is clinically the most important cancer of the liver because of its association with chronic hepatitis B or C, which has a high prevalence in countries of the Middle East, Asia and Africa. It is associated also with genetic diseases (*e.g.*, hemochromatosis, α 1-antitrypsin deficiency, acute intermittent porphyria) and with chronic exposure to hepatotoxins (*e.g.*, aflatoxin, ethanol). Common to all forms of HCC is advanced fibrosis or cirrhosis, although the strength of the association varies by etiology. In chronic hepatitis C, as much as 95% of HCC is in a cirrhotic liver, whereas in chronic hepatitis B, the figure is ~60%; in a series of patients with acute intermittent porphyria, less than half of HCC were associated with cirrhosis (Andersson *et al.*, 1996).

TGF- β represents a potentially important link between fibrosis and neoplasia in the liver. As already noted, it is well-established that this cytokine drives fibrosis. However, its impact on the initiation or progression of neoplasia is controversial. This is due in large part to the multiple actions of TGF- β (Table 1). In HCC, expression of TGF- β appears to be increased (Abou-Shady *et al.*, 1999; Matsuzaki *et al.*, 2000), suggesting a tumor-promoting effect. Experimental models suggest that this could be direct: transgenic mice overproducing TGF- β have an increased susceptibility to chemical carcinogens (Factor *et al.*, 1997; Schnur *et al.*, 1999). Alternatively, the effect could be pericellular and indirect, involving (for example) suppression of local immune cells (Letterio

and Roberts, 1998). Mutations of the TGF- β receptor or signaling intermediates (members of the Smad family) have been found in various epithelial cancers (Zhu *et al.*, 1998; de Caestecker *et al.*, 2000). Reports differ, however, on the importance of receptor mutations (Vincent *et al.*, 1996; Furuta *et al.*, 1999; Kawate *et al.*, 1999). Smad mutations appear to be rare (Kawate *et al.*, 1999). Overall, various factors point to a role for TGF- β in tumorigenesis. At the same time, the effects of the cytokine appear to be context-dependent as in injury repair. The role of TGF- β during neoplastic initiation may well differ from its role in progression (Rossmann and Schulte-Hermann, 2001).

Therapy of fibrosis

The first consideration is eliminating the etiologic agent. Successful treatment of hepatitis C leads to histological improvement with reduced fibrosis (Reichard *et al.*, 1999; Poynard *et al.*, 2000; Shiratori *et al.*, 2000). As the pathogenesis of fibrosis has come to light, so have new possibilities for preventing progression of fibrosis, notably for diseases in which the etiology is unknown or for which specific therapy does not exist. Antifibrotic strategies can be grouped as: (a) Anti-inflammatory agents and antioxidants; (b) Antagonists of cytokines or cytokine receptors; (c) Inhibitors of stellate cell activation; and (d) Anti-collagen agents (Table 2).

Anti-inflammatory and anti-oxidant agents

Inflammation is a nearly constant accompaniment of fibrosis. Colchicine is an anti-inflammatory agent that under some conditions suppresses collagen formation and/or stimulates collagenase. An early report on its use in patients with advanced fibrosis or cirrhosis was encouraging (Kershenovich *et al.*, 1988) but has not been replicated, and randomized trials of patients with primary biliary cirrhosis (Kaplan *et al.*, 1986; Kaplan *et al.*, 1999) or chronic viral hepatitis (Wang *et al.*, 1994) have been negative. Corticosteroids are useful for diseases that benefit from broad immunosuppression, such as autoimmune hepatitis. Immunomodulatory cytokines also are being explored as antifibrotic agents: IL-10 (Thompson *et al.*, 1998; Louis *et al.*, 1998; Nelson *et al.*, 2000), IL-12 (Wynn *et al.*, 1995) and antagonists of IL-13 (Chiaramonte *et al.*, 1999; Chiaramonte *et al.*, 2001).

Given the experimental evidence that oxyradicals

Table 1. Actions of TGF- β in Liver

Action	References
Fibrogenesis	Friedman and Dooley, 2000
Growth inhibition (of normal hepatocytes and stellate cells)	Russell <i>et al.</i> , 1988; Dooley <i>et al.</i> , 2000
Mitogenesis (?? of HCC)	Schnur <i>et al.</i> , 1999; Factor <i>et al.</i> , 1997
Pro-apoptosis	Grasl-Kraupp <i>et al.</i> , 1998
Chemoattraction	Nilsson <i>et al.</i> , 1995; Stolz <i>et al.</i> , 1998

Table 2. Proposed anti-fibrotic agents

Compound	Comment	Reference
Anti-inflammatory or anti-oxidant agents		
Corticosteroids	Used as an immunosuppressive drug	Davis <i>et al.</i> , 1984
Colchicine	Well-established safety profile but no established efficacy in controlled trials	Kaplan <i>et al.</i> , 1986; Bodenheimer <i>et al.</i> , 1988; Kaplan <i>et al.</i> , 1999; Kershenovich <i>et al.</i> , 1988
IL-10	Encouraging preliminary data in patients with hepatitis C	Louis <i>et al.</i> , 1998; Thompson <i>et al.</i> , 1998; Nelson <i>et al.</i> , 2000;
IL-12	Data from experimental models only	Wynn <i>et al.</i> , 1995
Malotilate	Anti-oxidant effects; mixed results in human trials	Ala-Kokko <i>et al.</i> , 1989; Group TEMS, 1993; Keiding <i>et al.</i> , 1994
Silymarin	Anti-oxidant; mixed data in human trials	Ferenci <i>et al.</i> , 1989; Boigk <i>et al.</i> , 1997; Pares <i>et al.</i> , 1998; Angulo <i>et al.</i> , 2000
Vitamin E	No controlled trials in humans	Parola <i>et al.</i> , 1992
Anti-cytokine agents		
Anti-IL-13	Not yet studied in humans	Chiaromonte <i>et al.</i> , 1999, 2001
Anti-TGF- β	Anti-fibrogenic in animal models; no human trials	George <i>et al.</i> , 1999; Qi <i>et al.</i> , 1999; Ueno <i>et al.</i> , 2000; Nakamura <i>et al.</i> , 2000
Endothelin antagonists	Some human safety data; no trials in fibrosis	Rockey and Chung, 1996; Cho <i>et al.</i> , 2000
Hepatocyte growth factor	Blocks expression of TGF- β in animal models	Matsuda <i>et al.</i> , 1995, 1997, Yasuda <i>et al.</i> , 1996; Ueki <i>et al.</i> , 1999
Inhibitors of stellate cell activation		
Pentoxifylline	Approved for human use but not yet tested as an anti-fibrogenic agent	Peterson, 1993; Peterson and Neumeister, 1996; Pinzani <i>et al.</i> , 1996; Preaux <i>et al.</i> , 1997; Desmouliere <i>et al.</i> , 1999
Sho-saiko-to	No controlled trials in humans, as yet	Sakaida <i>et al.</i> , 1998; Shimizu <i>et al.</i> , 1999
Halofuginone	Not yet tested as an anti-fibrogenic agent in humans	Pines <i>et al.</i> , 1997; Bruck <i>et al.</i> , 2001
Fumagillin	Not yet tested as an anti-fibrogenic agent in humans	Wang <i>et al.</i> , 2000
Angiotensin II blockers	Well-established safety profile in humans	Jonsson <i>et al.</i> , 2001; Yoshiji <i>et al.</i> , 2001
Trichostatin A	Animal tests only	Niki <i>et al.</i> , 1999
Safironil	Animals tests only	Sakaida <i>et al.</i> , 1996; Matsumura <i>et al.</i> , 1997; Wang <i>et al.</i> , 1998

mediate fibrogenesis, there is new interest in administration of anti-oxidants for oxidant-stress injury. Iron depletion in hemochromatosis appears to prevent progression of fibrosis, although in patients with cirrhosis, treatment may not prevent hepatocellular carcinoma (Niederer *et al.*, 1996). Preliminary studies of experimental injury suggest a protective effect of vitamin E against oxidant stress injury (Parola *et al.*, 1992). Also, an herbal remedy, silymarin, which is the active principal in milk thistle extract, and malotilate have anti-oxidant activities; experimental studies have been encouraging (Boigk *et al.*, 1997), but the clinical data are as yet incomplete (Ferenci *et al.*, 1989; Pares *et al.*, 1998; Angulo *et al.*, 2000).

Cytokine antagonists

Cytokines can be blocked, in principle, by agents such as antibodies or peptide mimetics. Antibody to TGF- β prevented experimental kidney fibrosis (Border *et al.*, 1990), encouraging studies of fibrosis elsewhere. However, the kidney may have a particular susceptibility to injury from circulating TGF- β because of its filtration function. To

date, these results have not been replicated with hepatic fibrosis. For the liver, two other strategies to block TGF- β have proven effective in experimental models. One uses a "dominant-negative" receptor strategy, in which a type II TGF- β receptor with a truncated cytoplasmic domain is delivered *via* an adenovirus. It reduces fibrosis induced by dimethylnitrosamine in rats (Qi *et al.*, 1999; Ueno *et al.*, 2000). A second approach is administration of a "soluble TGF- β receptor", which consists of the extracellular portion of the TGF- β type II receptor spliced into IgG. The soluble receptor presumably functions as a competitive binder of TGF- β , preventing interaction of the cytokine with its receptor. As an IgG, it circulates for several days, a distinct advantage over a small peptide competitor, which would be expected to disappear within a few minutes of injection. The soluble receptor inhibited HSC production of collagen I in animals subjected to bile-duct ligation (George *et al.*, 1999).

Inhibition of stellate cell activation

This is the mode of action of several candidate antifibrotic compounds. Although their specificity is

unclear, they have no measurable effects on resting stellate cells. Thus, if the injury is limited to the liver, they may affect stellate cells selectively. Examples are interferon- γ (George *et al.*, 2000) and Safironil[®] (Wang *et al.*, 1998). Interferon- γ blocks stellate cell activation in culture but has not as yet proven useful *in vivo*. This may reflect the other immunological functions of this cytokine: animals that overexpress interferon- γ in the liver exhibit a diffuse inflammatory infiltrate (Toyonaga *et al.*, 1994).

Other compounds that block stellate cell activation include endothelin antagonists (Rockey and Chung, 1996; Cho *et al.*, 2000), pentoxifylline (Pinzani *et al.*, 1996; Preaux *et al.*, 1997), halofuginone (Pines *et al.*, 1997; Bruck *et al.*, 2001), fumagillin (Wang *et al.*, 2000) and inhibitors of angiotensin II (Yoshiji *et al.*, 2001). Natural products also have yielded candidate compounds, based on studies in culture and in experimental models of fibrosis. In addition to silymarin extract from milk thistle (noted above), sho-saiko-to is an herbal mixture including glycyrrhizin, glycyrrhetic acid, baicalin and baicalein as active ingredients. It has anti-fibrogenic effects in experimental models (Sakaida *et al.*, 1998; Shimizu *et al.*, 1999) and directly blocks stellate cell activation in culture (Kayano *et al.*, 1998). Trichostatin A is a product of the *Streptomyces* fungus that blocks histone deacetylase and has differentiating effects on cells in culture. In culture, it inhibits the spontaneous activation of HSC without detectable toxicity (Niki *et al.*, 1999). Studies *in vivo* are awaited.

Anti-collagen agents

These have been examined longer than any other as therapy for fibrosis, because collagens – notably types I, III and IV – are the predominant constituents of the fibrotic scar. Modifiers of collagen cross-linking (lathyrogens) as well as inhibitors of collagen synthesis have been examined. None has achieved clinical use because of possible adverse systemic effects on collagen-rich tissues such as skin and the vasculature.

Cell targeting of antifibrotic therapy

The safety and efficacy of many proposed antifibrotic agents are likely to improve with effective targeting to the liver. A strategy that has been explored in some detail is synthesis of pro-inhibitors that require hepatic metabolism for their conversion to the active compound. This strategy takes advantage of the high concentration of cytochrome P-450 in the liver. However, the active agent will be generated predominantly, if not entirely, within hepatocytes, where it could undergo further metabolism or biliary excretion. For it to exert antifibrotic effects on HSC, it would need to cross the basolateral plasma membrane of the hepatocyte. To circumvent this problem, specific targeting of HSC has been explored,

with evidence that protein or DNA can be delivered with fairly high selectivity *via* the mannose-6-phosphate receptor (Beljaars *et al.*, 1999) or the collagen VI receptor (Beljaars *et al.*, 2000).

Delivery of therapeutic agents by way of recombinant viruses also has received extensive study. The adenovirus holds particular interest for liver because of the fact that this vector homes predominantly to the liver after its intravenous administration (Davern, 2001). Although uptake within the liver was thought to involve hepatocytes only, it is now clear that stellate cells are nearly as active as hepatocytes in this regard (Davern *et al.*, 1999). However, there are also important limitations to the use of adenoviral vectors for *in vivo* gene delivery. The first relates to the fact that expression of the gene of interest is transient only, because adenoviral DNA does not integrate into the host cell chromatin; thus, expression peaks within a few days and becomes undetectable after 1-3 weeks. Secondly, the virus is highly immunogenic, which may account for the brief duration of gene expression and precludes repetitive administration of virus. Finally, and most importantly, administration of high particle loads is associated with direct toxicity to the liver; one well-publicized fatal reaction has occurred in the United States (Stephenson, 2001). Its disadvantages notwithstanding, adenovirus has been used successfully in proof-of-principle studies of anti-fibrotic therapy in experimental liver injury. The virus was used to deliver a mutated TGF- β type II receptor, designed to act in a dominant-negative fashion to block signaling from the endogenous receptor. The strategy was very effective in preventing the onset or progression of liver fibrosis induced by administration of dimethylnitrosamine (Qi *et al.*, 1999; Nakamura *et al.*, 2000).

Gene transfer has been used also to deliver hepatocyte growth factor (HGF). Although characterized initially as a mitogen, it has been shown to have antifibrotic effects as well (Matsuda *et al.*, 1995; Yasuda *et al.*, 1996; Matsuda *et al.*, 1997). A cDNA encoding a soluble form of HGF was injected into muscle using a liposomal vector containing inactivated hemagglutinating virus of Japan (HVJ) (Ueki *et al.*, 1999). The fibrosis model was similar to that just described. Compared with control animal, rats receiving the HGF cDNA by gluteal injection showed reduced fibrosis. This was associated with decreased liver expression of TGF- β , decreased apoptosis of hepatocytes, increased hepatocyte proliferation and increased survival of the animal. Neoplasm is a potential concern with this form of therapy: mice that are transgenic for HGF frequently develop tumors (Takayama *et al.*, 1997).

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