Review

Hepatitis C and liver fibrosis

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Received 23.9.02; revised 14.10.02; accepted 17.10.02
Edited by M Piacentini

Abstract

Chronic hepatitis C progresses to cirrhosis within 20 years in an estimated 20–30% of patients, while running a relatively uneventful course in most others. Certain HCV proteins, such as core and NS5A, can induce derangement of lipid metabolism or alter signal transduction of infected hepatocytes which leads to the production of reactive oxygen radicals and profibrogenic mediators, in particular TGF- β 1. TGF- β 1 is the strongest known inducer of fibrogenesis in the effector cells of hepatic fibrosis, i.e. activated hepatic stellate cells and myofibroblasts. However, fibrogenesis proceeds only when additional profibrogenic stimuli are present, e.g. alcohol exposure, metabolic disorders such as non-alcoholic steatohepatitis, or coinfections with HIV or *Schistosoma mansoni* that skew the immune response towards a Th2 T cell reaction. Furthermore, profibrogenic polymorphisms in genes that are relevant during fibrogenesis have been disclosed. This knowledge will make it possible to identify those patients who are most likely to progress and who need antiviral or antifibrotic therapies most urgently. However, even the best available treatment, the combination of pegylated interferon and ribavirin, which is costly and fraught with side effects, eradicates HCV in only 50% of patients. While the suggestive antifibrotic effect of interferons (IF- $\gamma > \alpha, \beta$), irrespective of viral elimination, has to be proven in randomised prospective studies, additional, well tolerated and cost-effective antifibrotic therapies have to be developed. The combination of cytokine strategies, e.g. inhibition of the key profibrogenic mediator TGF- β , with other potential antifibrotic agents appears promising. Such adjunctive agents could be silymarin, sho-saiko-to, halofuginone, phosphodiesterase inhibitors, and endothelin-A-receptor or angiotensin antagonists. Furthermore, drug targeting to the fibrogenic effector cells appears feasible. Together with the evolving validation of serological markers of hepatic fibrogenesis and fibrolysis an effective and individualised treatment of liver fibrosis is anticipated.

Cell Death and Differentiation (2003) 10, S59–S67. doi:10.1038/sj.cdd.4401163

Keywords: antifibrotic; cirrhosis; collagen; CTGF; extracellular matrix; endothelin; fibrogenesis

Abbreviations: CTGF, connective tissue growth factor; ET, endothelin; ET_AR, endothelin A receptor; HGF, hepatocyte growth factor; HCC, hepatocellular carcinoma; MF, myofibroblastic cells; MMP, matrix metalloproteinase; MF, myofibroblast; PDGF, platelet derived growth factor; HSC, hepatic stellate cell; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases

Natural evolution of hepatitis C infection

Acute infection with the hepatitis C virus is uneventful in most of the cases, but runs a chronic course in an estimated 70% of patients. Because of the paucity of early symptoms, most infections are either diagnosed by chance due to transaminase elevations or in an advanced disease stage, when complications have occurred. Development of cirrhosis is the main determinant of morbidity and mortality, presenting either in a compensated state with only minor abnormalities of liver function or with fully decompensated liver disease which usually portends a dire prognosis. Furthermore, compensated as well as decompensated cirrhosis is a crucial precondition for the development of hepatocellular carcinoma (HCC), with an estimated yearly incidence of 2–5% in cirrhotics with hepatitis C. Consequently, only those patients whose HCV infection has led to cirrhosis or tends to progress to cirrhosis within a fairly short time period pose a major health problem, whereas patients who do not progress or who progress slowly usually do not require antiviral or antifibrotic treatment.

The natural evolution of chronic hepatitis C has been analyzed in a large retrospective study from France.¹ Here, roughly one third of patients progressed to cirrhosis in approximately 20 years, one third within approximately 50 years, and one third did not show any progression (Figure 1). Apart from duration of infection, factors associated with advanced fibrosis were male gender, age > 40 years, older age at infection, genotype 1 and alcohol abuse. However, the fraction of HCV patients without progression may be higher in nonselected patients, since the French study mainly considered cases that came to attention due to clinical or laboratory abnormalities. Thus the expected number of HCV infections without such abnormalities, e.g. including cases with persistently normal transaminases which predicts a less severe course of the disease, is higher.^{2,3}

Antiviral treatment

Antiviral treatment is given to patients who fulfil the criteria of chronic hepatitis (>6 months duration), with elevated transaminases and at least some degree of histological inflammation or fibrosis. In hepatitis C, transaminases and histology do predict insufficiently whose disease will progress

Figure 1 Natural course of hepatitis C and sustained response (SR) to antiviral therapy. Numbers as to the natural course of the disease are derived from retrospective studies; GT, genotype; NASH, non-alcoholic steatohepatitis

in a short time. But additional predictive parameters of disease progression are being validated the clinical application of which is pending.

Using the best available therapy, i.e. pegylated interferon alfa and optimal doses of ribavirin, virus negativity 6 months after discontinuation of treatment (sustained response) can be achieved in 40–50% and 80% of patients with genotype 1 and 2/3, respectively.^{4,5} Retrospective analysis suggests that viral elimination stops disease progression and may even induce reversion of fibrosis.⁶ Despite these promising results, at least 50% of patients with genotype 1 which is the most frequent genotype in the Western world (60–90% of those infected), or with genotype 4 (which is prominent in Egypt) do not respond to this optimised antiviral therapy, let alone the patients who have contraindications to interferon or ribavirin. Moreover, the costs of interferon and ribavirin are out of reach for many health care systems and this combination has significant side effects. Therefore, reasonable pharmacological alternatives that retard fibrosis progression are needed.

Mechanisms of hepatic fibrogenesis

Chronic liver diseases frequently lead to scarring (cirrhosis) which is often accompanied by progressive loss of liver function despite the use of immunosuppressive, antiviral or antiinflammatory agents.

Fibrosis results from excessive accumulation of extracellular matrix (ECM). The collagens are the most important molecular targets, since (1) they represent the major matrix proteins, (2) they form important mechanical scaffolds and (3) their proteolysis by specific proteases appears to be rate-limiting for ECM removal. The fibril forming interstitial collagens type I and III, and the sheet-forming basement membrane collagen type IV are the most abundant ECM components in liver. In cirrhosis their content increases up to 10-fold.⁷ A variety of adverse stimuli such as toxins, viruses, bile stasis, hypoxia can trigger *fibrogenesis*, i.e.,

the excess synthesis and deposition of ECM, usually by activation of cytokine release, or simply by mechanical stress. In the acute phase of liver disease fibrogenesis is balanced by *fibrolysis*, i.e., the removal of excess ECM by proteolytic enzymes, the most important of which are the matrix metalloproteinases (MMPs). MMP-1, -2, -3, -8, -9, -12, -13 and -14 are expressed in human liver.⁸ With repeated injury of sufficient severity, fibrogenesis prevails over fibrolysis, resulting in excess ECM deposition, i.e. fibrosis. Fibrogenesis is characterised by an upregulation of ECM synthesis, a downregulation of MMP secretion and activity, and by an increase of the physiological inhibitors of the MMPs, the tissue inhibitors of MMPs (TIMPs). Among the four known TIMPs, the universal MMP-inhibitor TIMP-1 is most important.⁹ However, an increase of certain MMPs may also be detrimental. Thus activation of MMPs at the wrong place and time can lead to removal of the regular, differentiation-inducing ECM, such as basement membranes, with subsequent unfavourable tissue remodelling, architectural distortion and a fibrogenic response. An example is MMP-2 which mainly degrades basement membrane collagen and denatured collagens and which is upregulated during fibrogenesis. Collagens, MMPs and TIMPs are mainly produced by myofibroblastic cells (MF) which either derive from activated hepatic stellate cells (HSC) or from activated (portal and perivascular) fibroblasts^{10,11} (Figure 2). Activated liver macrophages, i.e. Kupffer cells, or proliferating bile ductular epithelia, but also endothelia, other mononuclear cells and myofibroblasts themselves are sources of fibrogenic cytokines and growth factors that can stimulate HSC and perivascular fibroblasts to become MF. The prominent profibrogenic cytokine is transforming growth factor β 1 (TGF- β 1) which is released from almost any cell during inflammation, tissue regeneration and fibrogenesis. Apart from immunosuppressive and, in most cell types, antiproliferative effects TGF- β 1 strongly upregulates production and deposition of the major ECM molecules.^{11–13} Therefore, TGF- β 1 as well as activated HSC and MF are the prime targets for antifibrotic therapies (Figure 2).

Genetic predisposition for hepatic fibrosis

Genetic polymorphisms may explain the widely differing individual rates of fibrosis progression in chronic hepatitis C. Several polymorphisms of genes that are involved in inflammation, immune regulation and regeneration have been implicated in autoimmune diseases, and some of these are candidates for the regulation of hepatic fibrogenesis (Table 1). While there is some minor contribution by IL-10 and TNF- α gene polymorphisms, a recent study from Australia showed that a promoter polymorphism at position -6 of the angiotensin gene and a mutation causing a proline to arginine substitution at amino acid position 25 of the TGF β 1 precursor confer susceptibility to accelerated fibrogenesis.¹⁴ Thus HCV patients with both polymorphisms exhibited fibrosis stage 2.5 (with four being cirrhosis) compared to 0.5 for patients without either of these polymorphisms. Furthermore, the HLA-DRB1*11 allele appears to protect HCV patients from progression to cirrhosis after liver transplantation.¹⁵

Figure 2 Initiation and maintenance of fibrogenesis. With continuous injury, primarily to hepatocytes or bile duct epithelia, and/or mechanical stress the normally quiescent hepatic stellate cells and portal fibroblasts undergo activation and transdifferentiation to myofibroblasts. These myofibroblasts produce excessive amounts of collagens, downregulate matrix metalloproteinases (MMPs) and show an enhanced expression of the physiological inhibitors of the MMPs (TIMP-1 and -2). TIMP-1 can also promote myofibroblast proliferation and inhibit their apoptosis

Table 1 Genetic predisposition for hepatic fibrosis. These can predispose for hepatic inflammation, viral persistence or fibrosis. Many mutations or polymorphisms remain to be identified

Gender (protection by high dose estrogens)
Enzyme polymorphisms (ADH2 ₁₋₁ , ALDH2 ₁₋₁ , CYP2E1) for alcoholic liver fibrosis
Metabolic syndrome – polygenetic (NASH)
Immune system (Th1 vs Th2 response)
Genes for regulation of regeneration and apoptosis (to be defined)
Gene polymorphisms (IL-10, TNF- α , IL-4R, TGF- β 1, AT-II, CTLA-4, MMP-3)

Contribution of a second hit

There is increasing evidence that severity and progression of fibrosis in chronic liver diseases is not only caused by repetitive exposure to a single detrimental external or internal factor, but by additional events that exacerbate the condition. This has been shown for the combination of hemochromatosis with alcohol consumption or with chronic viral hepatitis,^{16–18} of alcoholic liver disease with chronic viral hepatitis,¹⁹ and for the contribution by non-alcoholic steatohepatitis, a disorder related to the metabolic syndrome.^{19–21} Thus even the consumption of as little as 10 g of alcohol per day was associated with a significant effect on histological fibrosis progression in HCV patients.¹⁹ In contrast, the natural course of two large cohorts of young women from Ireland and Germany who were infected during Rhesus immunoprophylaxis more than 20 years ago, has been uneventful, with less than 2% developing cirrhosis and an estimated mean progression to cirrhosis in more than 60 years.^{22,23} Similarly, among asymptomatic HCV-infected blood donors, none developed cirrhosis 19–24 years after exposure.²⁴

The fibrogenic immune response

Immune-mediated damage is linked to fibrogenesis, but the character of the immune response determines fibrosis progression. Contrary to general thinking, fibrosis appears not to be the logical consequence of significant macroscopic inflammation as reflected by the mononuclear infiltrate, but rather by the associated intrinsic or extrinsic immunosuppression. Here cytokines (growth factors) play a prominent role. They can be subdivided into a fibrogenic, antifibrogenic and neutral group. The latter has by definition no effect on the fibrogenic effector cells, i.e. activated HSC and MF. As a rule of thumb certain Th1 cytokines, i.e. those that stimulate cellular immune responses, rather trigger matrix dissolution, i.e., fibrolysis, whereas Th2 cytokines, which stimulate the humoral immune response and can suppress Th1 T cells, promote fibrogenesis. Examples are the interferons (Th1) on one hand,^{6,25,26} and transforming growth factor β 1 (TGF- β 1),^{11–13} interleukin-4 (IL-4) and most likely IL-10 on the other hand.²⁷ Evidence comes from patients coinfecting with both HIV (Th2) and HCV (Th1 in acute, Th2 in chronic infection) who progress more quickly to cirrhosis than patients infected with HCV alone.²⁸ Similarly, accelerated fibrosis progression is found in HCV patients coinfecting with *Schistosoma mansoni* which triggers a Th2 cytokine profile.²⁹

Are there fibrogenic HCV proteins?

A major impediment to development of specific antiviral drugs has been the lack of a highly replicative and low cost *in vitro* or *in vivo* model of HCV infection. This may change after the recent generation of human hepatoma cell lines that persistently replicate HCV³⁰ and of a mouse model that harbours HCV-replicating human hepatocytes.³¹ Several studies with HCV-transfected, preferably hepatocytic cell lines and HCV-transgenic mouse models have emerged in recent years³² that enable some conclusions to be drawn as to possible direct profibrogenic and procarcinogenic mechanisms of certain HCV-proteins, irrespective of the host's immune response. However, only some transgenic mouse strains develop liver damage, pointing to additional, genetic predispositions. Furthermore, numerous studies used a highly expressed single gene or an incomplete set of HCV-genes, a setting that does not mimic human infection which is usually characterised by moderate viral replication in liver. Finally, expression of a limited set of genes ignores the interactive potential of HCV proteins with each other.³³

Overexpression of the cytoplasmatically located HCV core has been analysed in detail. Sensitive C57BL/6 mice with the core transgene driven by the albumin promoter develop hepatic lipid accumulation (steatosis) after 6 months and hepatocellular carcinoma (HCC) after 16 months.^{34,35} In cell culture the core protein has transforming potential.³⁶ It can interact with the intracytoplasmic TNF- α type 1 and the lymphotoxin β receptors, enhancing their pro-apoptotic signal transduction,^{37,38} and activate the tumour suppressor p53.³⁹ In addition, HCV core can repress the cell cycle regulator p21WAF⁴⁰ and via inhibition of the p38 mitogen activated kinase pathway promote Fas-induced apoptosis.⁴¹ Taken together, these data rather

suggest a pro-apoptotic effect of core protein in hepatocytes, favouring the elimination of infected and potentially transformed cells. On the other hand, anti-apoptotic Bcl-xl can be upregulated.⁴² While the jury is open as to a predominantly anti- or proapoptotic effect of core protein, the data regarding lipid accumulation in core-transfected hepatocytes,²⁰ are more homogeneous. Thus it interacts with apolipoprotein II and reduces microsomal triglyceride transfer protein *in vivo*, inhibiting assembly and secretion of regular VLDL particles.⁴³ In transfected hepatoma cells core activates the retinoid X receptor alpha which dimerises with the peroxisome proliferator activated receptor alpha to upregulate genes of lipid metabolism like cellular retinol binding protein II and acetyl CoA-reductase.⁴⁴ Notably, by interaction with mitochondria core protein induces reactive oxygen intermediates and thus oxidative stress which can induce steatohepatitis.^{45,46}

The non-structural protein NS5A has also been implicated in HCV pathogenicity. It can compromise the antiviral and hypothetically antifibrotic effect of interferon, obviously either by repression of protein kinase R or alternative pathways^{47,48}. NS5A enhances the acute phase response via activation of NF-kappa B and STAT-3 and, as core, causes oxidative stress.⁴⁹ In addition, NS5A has been implicated in favouring cell cycle progression to the G2/M by binding to the cyclin dependent kinase 1 (cdk1)/cdk2-complex.⁵⁰

Transgenic mice that express very low levels of the complete reading frame of the HCV polyprotein developed significantly more spontaneous HCCs after 13 months compared to their nontransgenic controls (5/38 vs 0/16), while transgenic mice expressing significant levels of the HCV structural proteins (Core, E1 and E2) only developed HCC in a single case (1/43 vs 0/35).⁵¹ While steatosis developed in all animals, inflammation, apoptosis and histochemical fibrosis were not enhanced in both models, and increased hepatocellular proliferation was only found in the transgenic mice harbouring the complete HCV genome. Matrix gene expression remains to be investigated in these models which should also be challenged, to test how far a second hit can promote both fibrosis and hepatocarcinogenesis. Potential profibrogenic actions of HCV-proteins are illustrated in Figure 3.

Serum markers of liver fibrosis

An index that uses α 2-macroglobulin, γ -globulin, haptoglobin, γ -GT and bilirubin could predict the absence or the presence of significant fibrosis/cirrhosis (metavir stage 2–4) in 46% of patients with chronic hepatitis C.⁵² While 54% of the patients could not be allocated, this index will not be suited to detect day-to-day changes in the active processes of fibrogenesis or fibrolysis. Classical serum fibrosis markers are matrix constituents that are released into the circulation during matrix remodelling. The majority of these markers appear to reflect fibrogenesis rather than fibrolysis⁵³ (Figure 4). These surrogate markers may open the possibility to assess the future evolution of fibrosis and the effect of potential antifibrotic treatment in an individual patient and on a frequent

Figure 3 HCV proteins as triggers of inflammation, apoptosis and fibrogenesis. Core, NS3 and NS5A have been studied most intensively. All three can interact with mitochondria and increase the formation of reactive oxygen species (ROS). Core and NS5A associate with the membranes of the endoplasmic reticulum and golgi apparatus. In particular, they can enhance intracellular lipid accumulation via interactions with apolipoprotein A1 (apoA1) or A2 (apoA2) which cause inhibition of lipid transfer protein or defective synthesis and transport of very low density lipoprotein (VLDL). Core has also been shown to activate the retinoid X receptor (RXR α) which by dimerisation with the peroxisome proliferator activated receptor alpha (PPAR- α) induces proinflammatory NF κ B, cytochrome P450A4 (CYP4A) (which combined with steatosis favours lipid peroxidation and thus ROS formation) and lipid degradation via enhanced β -oxidation (leading to reduction of VLDL production). Taken together, the HCV proteins appear to induce both lipid accumulation and degradation, with consequent derangement of lipid compartmentalisation and metabolism, favouring ROS production. ROS lead to induction of TGF- β , the strongest known promoter of fibrogenesis. Core has also been shown to trigger apoptosis by interaction with TNF receptors and via downregulation of p21 and p38, and upregulation of p53, while NS5A can induce inflammation via NF κ B and stat-3, and cell cycle progression via activation of the cyclin dependent kinase cdk1/cdk2 complex

Figure 4 Serum markers of fibrogenesis and fibrolysis. Procollagen precursors released by fibrogenic cells are processed by procollagen peptidases. Removal of the bulky propeptides allows the formation of collagen fibrils in the extracellular space. Thus circulating propeptide levels should reflect *de novo* synthesis and deposition of collagen, i.e. fibrogenesis. On the other hand, action of matrix metalloproteinases (MMPs) is expected to generate fragments of already deposited matrix proteins the levels of which should reflect matrix dissolution, i.e. fibrolysis. The diagnostic and predictive value of these markers is currently evaluated

basis. Several studies to validate these markers in large prospective follow-up studies of patients with liver diseases are currently under way. The availability of serological fibrosis markers is central to the rapid clinical development and validation of antifibrotic therapies.

Antifibrotic drug development

A major obstacle to antifibrotic drug development is the slow evolution of significant fibrosis which takes years or even decades in man. Sequential biopsy with semiquantitative and preferably quantitative assessment of fibrous tissue remains the gold standard to monitor fibrosis progression, but sampling error remains a problem, since due to large regenerative nodules that consist mainly of hepatocytes up to 25% of patients with advanced fibrosis or cirrhosis may be wrongly categorised as only slightly fibrotic.⁵⁴ Consequently, prospective studies in patients have to be large and testing of the large spectrum of potential antifibrotic agents is impossible. First proof of efficacy has to come from cell culture data that show inhibition of proliferation, induction of apoptosis and/or downregulation of collagen production in the key fibrogenic liver cells, i.e. activated HSC and MF. This has to be followed by suitable animal models of hepatic fibrosis to show the antifibrotic effect *in vivo* in the absence of general toxicity. Rat models are preferable, since significant fibrosis can be produced within 3–10 weeks and, most importantly, total liver collagen, the gold standard for fibrosis, can be determined easily in a representative tissue sample using biochemical methods. However, the *in vivo* models must include a sizeable number of animals per treatment group ($n=10-20$) and should be devoid of major hepatocyte necrosis. This is important, since drugs with anti-inflammatory, anti-necrotic or radical scavenging properties can prevent necrosis and collapse, as is characteristic of the models induced by carbon tetrachloride, dimethylnitrosamine or galactosamine, but are not truly antifibrotic. Thus fibrosis should evolve chronically and reproducibly, with no or little inflammation and necrosis, as in biliary cirrhosis due to bile duct occlusion. In addition, models of fibrosis reversion, e.g. after induction by carbon tetrachloride or thioacetamide, are alternatives to predict a true antifibrotic drug effect. Many reports on so-called antifibrotic agents do not fulfil the above-mentioned criteria, and the following examples will refer mainly to those studies that provide sufficient *in vivo* evidence for an antifibrotic effect. Importantly, on the long term a decrease of fibrosis should be followed by an improvement of portal hypertension and liver function.

Cytokine strategies to inhibit hepatic fibrosis

Antifibrotic cytokines

While immunosuppressive or Th2 cytokines are usually profibrogenic,²⁷⁻²⁹ most Th1 cytokines, especially the antiviral interferons are antifibrogenic. This effect is linked, at least in part, to the activation of stat-1 signalling pathways (Figure 5). In cell culture interferon- α (IF- α) blocks activation, proliferation and collagen synthesis of HSC and MF.²⁵

Figure 5 Effects of interferon- α and - β on fibroblast signaling and gene transcription. Interferon- α and - β activate the same dimeric receptor which leads to recruitment of the intracellular tyrosine kinases jak-1 and tyk-2. This receptor complex catalyses the assembly of stat-1, stat-2 and p48 to a trimeric complex that directs transcription of genes involved in the known interferon-induced effects including inhibition of proliferation and collagen production

Retrospective analyses and one small prospective study in patients with chronic hepatitis C suggest that IF- α therapy can prevent fibrosis progression, even in nonresponders to antiviral therapy.^{1,2,6,26} The effect was dependent on IF- α dose duration, and most pronounced in sustained responders. A recent analysis of four large international studies even found reversion of cirrhosis in 75 out of 153 patients.⁶ However, there might be a problem of sampling error and the number of initially noncirrhotic patients who had cirrhosis on the second biopsy is not stated. Several large prospective studies have been initiated (HALT-C, EPIC-3 and COPILOT, PROFI-C) (Table 2) that try to answer the question of an antifibrotic effect of longterm IF- α (up to 4 years) in nonresponders to IF- α /ribavirin combination therapy by using conventional follow-up histology. In PROFI-C nonresponders to combination therapy receive retarded (pegylated) IF- α at 100 or 25 g per week combined with high-dose (840 mg/day) silymarin or placebo for 2 years. Fibrosis progression is assessed by collagen morphometry of pre- and post-treatment biopsies and by sequential measurement of a spectrum of serum fibrosis markers.

Antagonising profibrogenic cytokines

TGF- β 1 is considered the most potent fibrogenic cytokine and its inhibition therefore appears attractive.¹¹⁻¹³ Soluble TGF- β 1 decoy receptors or adenoviral constructs that block TGF- β 1 signalling have been developed that show antifibrotic efficacy *in vitro* and *in vivo*^{13,55-57} (Figure 6). It appears that an approach targeting activated HSC and MF is necessary, since TGF- β receptors are expressed on most cell types and systemic inhibition that reaches sufficient levels to block hepatic fibrogenesis may trigger autoimmune diseases and cellular de-differentiation (Figure 7). Connective tissue growth

Table 2 Prospective studies to prevent disease progression in hepatitis C. Patients with no response or relapse after IF- α /ribavirin combination therapy are included. Primary end-points are fibrosis progression, survival, development of hepatocellular carcinoma or need for liver transplantation

HALT-C (USA)	Peginterferon α -2a 40 kD vs no treatment for 3.5 years, stage 3–6, 900 pts. 10 US-centers, 2000–2006
Copilot	Peginterferon α -2b 12 kD 50 μ g/w vs colchicine, stage 3–6, 800 pts. 2000–2007
EPIC-3 (worldwide)	Peginterferon α -2b 12 kD 50 μ g/w vs no treatment for 4 years, to be launched: 1000 pts. without, 800 pts. with cirrhosis
PROFI-C	Four arms: peginterferon α -2b 12 kD 25 μ g/w or 100 μ g/w plus silymarin 840 mg/d or placebo for 2 years, 320 pts. 2000–2005

Figure 6 Anti-TGF- β 1 strategies to inhibit fibrogenesis. Strategies have been developed that neutralize TGF- β 1, e.g. by blockage or inactivation of its signaling receptors (T- β RI or T- β RII), the major signal transducers of the TGF- β pathway, such as smad2 and smad3, or SARA (smad anchor for receptor activation), a protein that allows docking of smad2 to the activated T- β RII. Alternatively, the inhibitory smad7 which is induced as an endogenous feedback inhibitor late after activation of profibrogenic genes can be delivered by gene transfer

Figure 7 Biological actions of TGF- β 1. Apart from initiating a profibrogenic response in mesenchymal cells, TGF- β 1 is an essential mediator controlling Th1 immune activation or cellular dedifferentiation. Its long-term untargeted inhibition may trigger autoimmunity and neoplasia

factor (CTGF),⁵⁸ a fibrogenic cytokine that is triggered by TGF- β , is expressed mainly in hepatic mesenchymal cells, but also in proliferating bile duct epithelia. Its inhibition may render a more specific antifibrotic strategy. Reports on the antifibrotic

activity of hepatocyte growth factor (HGF)⁵⁹ have to be interpreted with care, since HGF rather causes hypertrophy and hyperplasia of (liver) epithelia, thus reducing the relative and not the absolute collagen content in the organ, with an inherent risk of promoting epithelial malignancy. IL-10, an immunosuppressive cytokine, was reported to reduce liver fibrosis in a study of 22 patients with chronic hepatitis C who were treated for only 12 weeks. However, apart from IL-10 being a Th2-like cytokine for which a profibrogenic effect may be anticipated, there is an inherent problem of sampling error in a small study like this.²⁷

Other antifibrotic agents and combination therapies

Plant-derived drugs

Several promising drugs derive from plants. *Silymarin* from the milk thistle contains three prominent flavonoids, with silibinin representing up to 60% of the dried extract. Silibinin was shown to stimulate hepatocyte RNA synthesis, to act as a radical-scavenger and hepatoprotectant, and to suppress HSC proliferation and collagen synthesis *in vitro*. Importantly, it reduced hepatic collagen accumulation in rat biliary fibrosis secondary to bile duct occlusion, a model which leads to a 10–12-fold hepatic collagen accumulation after 6 weeks, by 30–40%, even when treatment was started in an advanced stage of fibrosis.⁶⁰ The major alkaloid baicalein from the traditional Chinese/Japanese plant extract *Sho-saiko-to* that displays a structure similar to silibinin has radical-scavenging but also antifibrotic properties in activated HSC *in vitro* and in rat porcine serum-induced fibrosis *in vivo*.⁶¹ *Halofuginone*, a semisynthetic alkaloid-derivative from the antimalarial plant *Dichroa febrifuga*, was shown to normalise hepatic collagen content in a fibrosis reversion model *after* induction of hepatic fibrosis by thioacetamide.⁶² These drugs appear to mainly act as anti-oxidants, though probably with different pharmacokinetics, pharmacodynamics and cellular specificities. Intracellular oxidative stress is a relevant contributor to fibrogenesis, and recent studies have shown the induction of profibrogenic TGF- β 1 by peroxide radicals,^{63,64} providing a rationale for the use of intracellular anti-oxidants as adjunctive antifibrotic agents (Figure 8).

Modulators of fibrogenic signal transduction

In vitro the phosphodiesterase inhibitor and cytokine antagonist pentoxifylline suppresses proliferation and collagen production by skin fibroblasts and HSC,⁶⁵ while in rat biliary fibrosis, oral pentoxifylline reduced hepatic collagen accumulation by only 20%. The drug induced a hitherto unmatched eightfold downregulation of hepatic procollagen I mRNA, the product of activated HSC and MF, but this was counterbalanced by a twofold increase of hepatic TIMP-1 mRNA expression, with pentoxifylline apparently stimulating bile duct epithelia and hepatocytes to express TIMP-1.⁶⁶ Better targeting of pentoxifylline to HSC and MF may prevent upregulation of the profibrogenic TIMP-1 without compromising the downregulatory effect on procollagen I expression.

Figure 8 TGF- β 1, oxidative stress and antioxidants. Oxidative stress and the generation of hydrogen peroxide activate the TGF- β 1 pathway and vice versa. Certain antioxidants may therefore exert antifibrotic effects. Drugs that act mainly extracellularly, like vitamin E, are expected to be less effective. NAc, N-acetyl cysteine; SAMe, S-adenosyl-methionine

Figure 9 Targeting of activation-dependent receptors. Receptors for collagen VI (CVI) or PDGF⁷⁵⁻⁷⁷ and other profibrogenic mediators are predominantly upregulated on activated hepatic stellate cells/myofibroblasts. Their respective ligands are in part produced in an autocrine and paracrine manner by the activated cells themselves. These receptors can either be blocked directly by use of antagonistic small peptides or peptide analogues, or serve as ideal targets to deliver potential antifibrotic agents exclusively to the fibrogenic focus. Antifibrotic agents can be coupled to these receptor-recognizing molecules, leading to highly efficient delivery to the fibrogenic cells, followed by internalisation with release of the antifibrotic principle. CTGF, connective tissue growth factor; antisense, antisense constructs directed to profibrogenic mRNAs

Antagonizing vasoactive mediators

Oral endothelin A receptor (ET_AR) antagonists are attractive, since the ET_AR mediates HSC/MF contraction, proliferation and possibly also collagen synthesis, whereas the ET_BR induces MF relaxation and inhibition of proliferation. In rat biliary fibrosis the oral ET_AR antagonist LU135252 reduced hepatic collagen accumulation by up to 60% when given over the full 6 weeks of the experiment, being still effective when treatment was begun after week 3, a time point with an already fourfold increased liver collagen.⁶⁷ Angiotensin 1 receptor antagonists or angiotensin converting enzyme

Table 3 Potential antifibrotic drugs that may be useful in antifibrotic combination therapy. Examples of drugs for which antifibrotic activity has been shown in suitable animal models of liver fibrosis or for which an antifibrotic effect can be anticipated

Certain antioxidants: Silymarin, TJ-9 (Sho-saiko-to), (Halofuginone) etc.
Pentoxifylline, PDE-3/4-antagonists (Rolipram)
Prostaglandin E2
Endothelin A receptor antagonists
Angiotensin-system inhibitors
NO-donors (Pyrro-NO)
Certain immunosuppressants: Mycophenolate, Sirolimus, Leflunomide
Na ⁺ /H ⁺ -antiporter antagonists (Cariporide)
Histone diacetylase inhibitors: Trichostatin A, etc.
Growth factor antagonists: pirfenidone

inhibitors can retard liver fibrosis in suitable rat models.^{68,69} However, since the doses applied are up to 100-fold above the doses given in human hypertension, their antifibrotic effect in man remains controversial.

Modulation of growth factor bioactivity by matrix-derived peptides

Certain growth factors and cytokines can bind to matrix molecules. Basic fibroblast growth factor, some hematopoietic growth factors, platelet-derived growth factor (PDGF), HGF, keratinocyte growth factor (KGF) and others are immobilised and protected from proteolytic degradation by heparan sulphates. More importantly, the epithelial mitogens HGF and KGF, the profibrogenic mitogens PDGF, oncostatin M, and the pro-inflammatory cytokine IL-2 bind to the abundant collagens.⁷⁰⁻⁷² This binding is reversible and retains the factors in a biologically active form in the fibrotic matrix. The collagenous consensus peptide (Gly-Pro-Hyp)_n can liberate the collagen-bound factors from the matrix.⁷² It remains to be shown how far application of this peptide can perturb matrix metabolism in fibrogenesis.

Reversion of the fibrogenic phenotype

A promising target is the induction of stress relaxation of fibrogenic cells, a matrix (integrin) receptor-mediated process that is associated with a decrease in collagen synthesis and an increase in collagenase activity.^{73,74} Stress relaxation occurs once mesenchymal cells are placed from a stressed, two-dimensional environment (mimicking a situation of wounding) into a relaxed, three-dimensional context. Thus, the receptors for platelet-derived growth factor,⁷⁵ endothelin-1 (via the ET_AR) or the pericellular collagen VI⁷⁶ transmit stress signals that trigger proliferation and ECM synthesis in activated HSC and MF. Apart from the possibility to directly block these receptors, coupling receptor-ligands to a drug carrier allows a highly specific targeting to the activated fibrogenic cells in the liver. This has been shown both *in vitro* and *in vivo* with cyclic peptides recognising the receptors for PDGF, mannose-6-phosphate and collagen VI. More than 40% of the i.v. injected collagen VI receptor recognising construct was found bound to activated HSC of fibrotic rat livers, and *in vitro* data demonstrated its efficient internalisa-

tion.⁷⁷ Therefore, such carriers to receptors that are highly upregulated on activated HSC and MF are promising vehicles for targeted delivery of various antifibrotic agents (Figure 9).

Combination therapy for hepatic fibrosis

As in cancer therapy combination of several drugs that show different actions by either blocking fibrogenesis, stimulating fibrolysis, by inducing myofibroblast apoptosis, or reversion to a fibrolytic phenotype is most promising. This will make it possible to use lower, non-toxic amounts of single agents for a treatment that will have to be given for years or even life-long. Table 3 lists additional drugs that may be useful in such combinations.

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