

POINT OF VIEW

Molecular targets in the design of antifibrotic therapy in chronic liver disease

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INTRODUCTION

Hepatic fibrosis, the most characteristic histological feature of chronic liver diseases, is the result of a marked increase in the so-called extracellular matrix (ECM). This is composed of three types of proteins: a) *collagens*; b) *noncollagen glycoproteins* (fibronectin, laminin); and c) *proteoglycans* (1,2). Although all these components are increased in liver fibrosis, type I and III are particularly abundant in septa of fibrotic liver. In the normal liver and when fibrosis is not severe, the quantity of both types of collagen is small and the proportion between them is similar. However, when fibrosis is severe, not only the quantity of both types of collagens is increased, but also collagen type I is more abundant than type III. These collagens are complex proteins formed by three polypeptide chains that are wrapped around each other along their longitudinal axis. Only at their ends, these three chains remain free, separated from each other, not intertwined with the neighbor chains. These free ends are called the *globular segment* of the collagen molecule. Amino acids of these chains are distributed so that the electric charge of collagen molecules changes from positive to negative every 234 amino acids. This alternating electric charge is responsible for the tendency of collagen

molecules to group in parallel forming collagen bundles when they deposit in tissues. Another ECM protein whose production is greatly increased during liver fibrogenesis is *fibronectin*. Its deposition in the liver tissue precedes that of other ECM components. It is made up of two polypeptide chains tied together by a disulfide bond. It is believed that fibronectin plays an important role as a cohesion factor among all components of the ECM, since along its molecule there are contact points for cells, cytokines (TNF α), and other components of the ECM. *Proteoglycans* consist of a proteic axis to which one or more side chains of glycosaminoglycans (GAG) are bound. These are polymers of aminosulphated disaccharides or uronates with a potent negative charge. For this reason, proteoglycans bind osmophilic cations, are intensely hydrophilic, retain water, and are able to finally form a gel. This environment favors intercellular communication, the relationship between cells and ECM, diffusion of hydrophilic metabolites, and allows cell migration. In addition, proteoglycans represent a reservoir for cytokines and growth factors that are binded to the central proteic axis or to the GAG branches. In this reservoir, cytokines remain inactive but protected from degradation. When ECM is degraded, growth factors and cytokines may be released and regain their biological activity.

Although many types of liver cells are involved in the process of liver fibrogenesis, including portal fibroblasts and cells originated in the bone marrow, without a doubt, the most important contributors to this process are the so called *hepatic stellate cells* (HSC) (3,4). These cells are located between hepatocytes and endothelial cells of sinusoids and, in the normal, resting state, they have low metabolic activity and contain a large number of retinoid esters stored in their cytoplasm. Their morphology and biological activity change dramatically when the liver suffers an injury, as their size increases, retinoid droplets disappear, express smooth muscle actin in their cytoskeleton and receptors for growth factors and hormones in their plasma membrane. They also produce growth factors, proinflammatory and profibrogenic cytokines,

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and chemokines, proliferate, contract, and synthesize and secrete virtually all components of the ECM (3-5).

LIVER FIBROGENESIS

Activation of hepatic stellate cells

HSC play an essential role in the pathogenesis of liver fibrosis. In the absence of these cells or when they remain inactive, liver fibrosis is not possible. Knowledge of mechanisms leading to the activation, proliferation, inactivation and death of these cells may be essential to design new drugs to prevent and treat liver fibrosis.

During the process of HSC activation, the expression of the genes required by these cells to be able to perform their function and to respond to the factors involved in regulating their biological activity is increased. Although our understanding on the HSC activation is not complete, we know that, if liver injury occurs, hepatocytes, biliary cells, monocytes, including Kupffer cells, and other inflammatory cells, endothelial cells, and platelets release multiple factors that contribute to HSC activation. Thus, for example, injured hepatocytes often suffer oxidative stress and release factors that activate Kupffer cells or attract inflammatory cells to the injured area (6). Oxidative stress contributes to activate HSC through NF κ B. When, as a result of oxidative stress, this factor is liberated from its cytoplasmic inhibitor (I κ B, *Inhibitor of nuclear factor κ B*), it migrates into the nucleus where it induces the expression of multiple genes, some of them involved in the activation and survival of HSC (7,8). Platelets, inflammatory cells (mainly macrophages and Kupffer cells) and endothelial cells release a large number of cytokines, chemokines and growth factors that are essential for the activation and proliferation of HSC. Among these factors stand out PDGF (*Platelet Derived Growth Factor*), TGF β (*Transforming Growth Factor beta*), TNF α (*Tumor Necrosis Factor alpha*), IGF-1 (*Insulin-Like Growth Factor-1*), EGF (*Epidermic Growth Factor*), IFN γ (*Interferon gamma*), bFGF (*Basic Fibroblast Growth Factor*), HGF (*Hepatocyte Growth Factor*), IL-1, IL-5 (9,10), IL-6, IL-8, MIPs, RANTES, MCP-1, ICAM-1 (11). In addition, macrophages express NADPH oxidase (NOX) that in combination with the oxidative stress caused by the formation of *reactive oxygen species* (ROS) may contribute to activate HSC (12). Moreover, fibronectin secreted by endothelial cells interacts with integrin molecules situated in the membrane of HSC and triggers a cascade of intracellular signals that promote proliferation, migration, survival, and activation of these cells (13). Finally, degradation of the ECM as a result of tissue injury allows the release of numerous cytokines and growth factors retained in it, regaining their functional capacity.

Considering the factors that may facilitate the activation of CEH, it has been suggested that the use of anti-in-

flammatory or anti-oxidant agents might be useful in preventing the activation of these cells and, consequently, in the development of liver fibrosis.

Anti-inflammatory drugs

Because of their anti-inflammatory nature, it has been considered that corticosteroids, IL-10, curcumin, silymarin, colchicin, malotilate, antagonists of IL-1 or TNF α receptors, and anti-TNF α , might also have antifibrogenic effects. Here, we will comment only the effects of corticosteroids and IL-10, since the other listed agents will be discussed later.

Corticosteroids

There are numerous experimental evidence showing that corticosteroids can reduce liver fibrosis by acting at multiple levels of the process of liver fibrogenesis, as they, not only reduce the inflammatory response, but also influence HSC activation, transcription of ECM genes, and activity of prolyl- and lysyl-hydroxylases, among others. Clinically, most experience with corticosteroids has been acquired in patients with autoimmune hepatitis. In these patients, it has been found that this treatment is usually followed by a clinical, analytical and histological improvement, and a longer survival (14). Moreover, progression of fibrosis is slower, although this not always prevents the evolution to cirrhosis (15). Despite this possible beneficial effect on the development of fibrosis, this improvement is more likely due to their effect on the pathogenesis of the disease than to their effects on liver fibrogenesis. In any case, prolonged administration of these drugs is accompanied by side effects, sometimes serious. Therefore, corticosteroids should not be used as antifibrosing agent in the treatment of chronic liver diseases.

Interleukin 10 (IL-10)

Its antifibrosing effects are not unique, because, although it is a potent anti-inflammatory cytokine, it has also been suggested to influence directly on liver fibrogenesis. Its anti-inflammatory effect is exerted by shifting the pattern of cytokines from proinflammatory (Th1) to anti-inflammatory (Th2) and, therefore, IL-10 decreases the production of TNF α , IL-1, IFN γ , and IL-2. This cytokine has been used with antifibrosing purposes in the treatment of patients with chronic hepatitis C and advanced stages of fibrosis (16), and, although the stage of fibrosis decreased during treatment, the viral load increased markedly. The latter result seems to contraindicate the use of this cytokine in the treatment of liver fibrosis in patients with infectious liver dis-

eases, but not when fibrosis had other etiology, e.g., autoimmune or alcoholic.

Anti-TNF α

Although TNF α behaves as an antifibrogenic factor in cell cultures of fibroblasts and HSC, since it reduces collagen gene expression (17,18) and increases expression of MMPs (19), in experimental animals, TNF α acts in the opposite direction, as it is a powerful proinflammatory cytokine and therefore acts as a profibrogenic factor. This role is supported by the fact that TNF α inhibition results in a lower degree of inflammation and fibrosis after intoxication of rats with CCl₄ or in alcoholic and nonalcoholic experimental steatohepatitis (20,21). In humans, not many studies are available on the effects of suppressing TNF α synthesis (pentoxifyllin) (22) or inhibition of its activity by the use of monoclonal antibodies (infliximab, etanercept) (23). However, these studies show that inhibition of TNF α decreases the grade of inflammation and hepatocellular injury, but not the stage of liver fibrosis or HSC activation. The use of *etanercept*, antibody that binds soluble TNF α , in association with corticosteroids in a group of patients with alcoholic hepatitis was interrupted because mortality increased significantly in these patients mainly due to infectious complications (24). These results have forced to be cautious in the use of TNF α blockers in the treatment of (non-viral) liver diseases, mainly alcoholic and nonalcoholic steatohepatitis.

Antioxidants

The use of these agents is justified because of the role assigned to the ROS and lipid peroxidation by-products (malondialdehyde, 4-hydroxy-nonenal) in HSC activation (25,26), chemotaxis, cell toxicity (27), and in the stimulation of ECM gene expression (28). Oxidative stress has been found in a large number of liver diseases, including, chronic hepatitis C, hemochromatosis, alcohol related liver disease, porphyria cutanea tarda, Wilson's disease, and acetaminophen toxicity (29). These conditions are often associated with liver fibrosis. Oxidative stress is originated, in addition to injured hepatocytes, in neutrophils, macrophages and Kupffer cells, which, by inducing NOX, produce ROS (30) responsible for lipid peroxydation, formation of malonyl-dialdehyde and 4-hydroxynonenal, and increased TGF β . The significance of this stress in the liver fibrogenesis is supported by the beneficial effects observed with the use of several antioxidants, such as vitamin E (31), d-a-tocopherol (32), malotilate, trolox, propyl-tiouracile, silymarin, So-saiko-to, N-acetyl-cysteine, penicilamine, S-adenosyl-metionine, or curcumin. The antifibrosing effects of antioxidants have been proven "in vitro" and in experimental animals; however, there is limited evidence on these agents in humans.

N-acetyl-cysteine (NAC)

The antioxidant effects of NAC are exerted by reducing cystine to cysteine, by forming reduced glutathione (GSH), the main endogenous antioxidants, by increasing glutathione-S-transferase, and by capturing and neutralizing ROS (33). As in the case of other antioxidants, we have large quantities of evidence demonstrating the antifibrosing effects of NAC in cell cultures and in experimental animals (34,35), but clinical experience with this agent demonstrating its antifibrosing properties is limited. In humans, NAC has been used for decades as mucolytic and as antidote to acetaminophen poisoning and its use has been proposed in ischemic cardiopathy, colitis, pancreatitis, alcoholic liver disease, and NASH (36,37). In the latter condition, NAC administrated with metformin improved fibrosis stage (38). Among factors that may reduce the antioxidant capacity of NAC are its low diffusion capacity into the cells and its short half-life (39).

Cu-Zn superoxide dismutase (SOD Cu-Zn)

This enzyme is a potent, intracellular antioxidant that after reducing TGF β expression and activity, acts as antifibrosing agent, not only in experimental animals but also in patients (40). At present, there is a clinical trial intended to assess the usefulness of 8 mg SOD, i.m., three times a week for 7 weeks in patients with chronic hepatitis C not responding to antiviral treatment (41). This study has not yet been completed.

Melatonin (N-acetyl-5-metioxitriptamine)

It is a hormone secreted by the pineal gland that, besides being a regulator of circadian rhythm, has a potent antioxidant effect, since it is able to neutralize ROS (42), increases expression, synthesis and activity of glutathione peroxidase, superoxide dismutase and glutathione (43), three antioxidant agents. Although we have no experience on its antifibrotic potential in human, a number of studies have shown that after experimental liver injury, melatonin reduces fibroblast proliferation, collagen synthesis, and degree of liver fibrosis (44).

Curcumin (diferuloilmetano)

It is a substance well known in the East, since, as a component of the curry, it has been used for many centuries both in the traditional diet of these countries as well as in the treatment of some illnesses. In the last years, it has been shown to have anti-inflammatory and antioxidant effects, and, therefore, it has been considered that it may also be an antifibrosant agent. As in the case

of other antioxidants, this effect is due not only because of its capacity to neutralize ROS, but because its power to induce the production of antioxidant factors (45). Evidence showing its antifibrotic effects are exclusively experimental, since its usefulness in humans is waiting to be proven. Curcumin decreases activation and proliferation of HSC and induces cell death by apoptosis.

Trolox

It is a vitamin E analog, with an antioxidant activity up to eight-times higher than α -tocopherol, which has been used in preventing oxidation of vegetable oils. In rats, it has been shown that trolox decreases the severity of liver lesions and, as in the case of other antioxidants, its antifibrogenic effects have been ascribed to its ability to reduce TGF β expression (46).

Trans-resveratrol

This is a natural antioxidant present in tea, grapes and other plants, which has shown to be capable of reducing activation and proliferation of HSC, and, therefore, is able to stop liver fibrogenesis.

Silymarin

It is another natural product, in this case derived from the milk thistle (*Silybum marianum*), with potent antioxidant and anti-inflammatory properties. Studies in several animal models of liver lesion have shown that silymarin behaves as an antifibrosing agent (47). These studies have demonstrated that silymarin reduces activity of HSC by decreasing their activation, proliferation, migration, and production of ECM and chemokines (IL-8, MCP-1). However, its antifibrosing effects in human remain to be demonstrated, since the results obtained in the different published studies have been conflicting. While some studies demonstrated that silymarin reduces mortality (48), other studies were unable to confirm these benefits (49). There are two multicenter, controlled studies trying to determine whether or not silymarin is beneficial in the treatment of patients with NASH or with chronic hepatitis C nonresponders to antiviral therapy (<http://clinicaltrials.gov/>; ClinicalTrials.gov NCT00680407 and NCT00680342); however, these studies have not yet been completed.

Polyunsaturated phosphatidylcholine

This is a phospholipid component of cell membranes, that has antioxidant effects and reduces the severity of liver lesions, including liver fibrosis, caused by alcohol

or CCl₄ in experimental animals. In spite of that, its administration to alcoholic patients, did not improve the severity of liver lesions (50). However, there is also a phase II study aiming to determine its usefulness in patients with liver diseases (<http://clinicaltrials.gov/>; ClinicalTrials.gov NCT00211848).

Proliferation of hepatic stellate cells

After liver injury, a marked increase in the number of HSC can be observed. This increase is due, in part, to the proliferation of existing activated HSC and partially to the arrival to the injured area of new HSC coming from the neighborhood areas. Among factors involved in the proliferation of HSC are: PDGF, leptin, EGF (*Epidermal Growth Factor*), CTGF (*Connective Tissue Growth Factor*), VEGF (*Vascular Endothelial Growth Factor*), IGF (*Insulin-like Growth Factor*), bFGF (*basic Fibroblast Growth Factor*), renin-angiotensin-aldosterone system (RAAS), endothelin (ET), thrombin, TGF α (*Transforming Growth Factor alpha*), IL-1, and some endocannabinoids.

Platelet derived growth factor (PDGF)

This factor is released mainly by platelets and Kupffer cells in injured areas. Its proliferative effects are mediated by specific receptors located on the surface of activated HSC and by tyrosine kinases and PI3K (*phosphatidylinositol-3-kinase*). The role of this factor in the liver fibrogenesis has been demonstrated by gene silencing of PDGF and PDGF-receptor, tyrosine kinase inhibition with *genisteine* or *imatinib* mesylate, or inhibition of PI3K. In all these cases, HSC proliferation declines and experimental fibrosis attenuates.

Renin-angiotensin-aldosterone system (RAAS)

HSC are sensitive to this system, since they possess angiotensin II receptors on their surface (51) and produce angiotensin II. The profibrogenic effects of this system are not simple, since, although they behave as inducers of HSC proliferation, they also increase cell survival by preventing apoptosis, and promote inflammation, and oxidative stress by inducing gene expression of NOX (52). This stress plays a key pathogenic role as mediator of all mentioned effects, since ROS, through PI3K and MAPK pathways, stimulate cell proliferation, activate NF κ B, force nuclear translocation of this factor and induce gene expression of proinflammatory cytokines. Moreover, in this process, RAAS increases gene expression of ECM and MMP inhibitors, also known as TIMPs. In addition, angiotensin II has been shown to increase TGF β (52), which may potentiate the profibrogenic effects of RAAS.

A large number of experimental studies have demonstrated the significance of this system in the pathogenesis of liver fibrosis, since its blockade consistently resulted in a reduction of the fibrous response (53). In spite of that, the therapeutic or preventive ability against liver fibrosis of the blockade of this system have not yet been sufficiently demonstrated in humans. In pilot studies, both inhibitors of angiotensin II or of its receptors (54,55), as well of the ACE (*Angiotensin-converting enzyme*) (56) administered to patients with a variety of liver diseases seem to reduce the stage of liver fibrosis. Thus, treatment of cirrhotic patients with candesartan, an AT1 receptor blocker, diminished serum levels of hyaluronic acid, a fibrosis marker (57), and losartan administered to patients with chronic hepatitis C for 18 months decreased gene expression of ECM, TIMPs, and utPA (*urokinase type plasminogen activator*), a TGF β activator, in addition to inflammation and fibrosis stage. The results of these pilot studies have justified initiating controlled trials seeking to assess the ability of these hypertensive agents, namely candesartan and irbesartan, to treat or prevent liver fibrosis in patients with chronic hepatitis C (<http://clinicaltrials.gov/>; ClinicalTrials.gov, NCT00930995 y NCT00265642).

Endothelin

Effects of this factor on HSC vary depending on the activation stage in which these cells are. In the initial stages, these cells express ET_A receptors on the cell membrane, which are mediators of the fibrogenic response. Therefore, in these cells, endothelin increases proliferation and ECM production. By contrast, fully activated HSC, as they are in advanced stages of fibrosis, express on their surface type ET_B receptors, which cause the opposite effects, inhibition of cell proliferation and prevention of fibrogenesis. The use of endothelin blockers in a variety of experimental models of liver injury have produced conflicting results, likely due to the divergent role of the two types of endothelin receptors (58).

Endocannabinoids

Like ET, the effects of endocannabinoids depend on the type of receptors on which they act. CB2 receptor agonists behave as antifibrogenic, as they diminish HSC proliferation and survival by promoting cell apoptosis or necrosis. By contrast, stimulation of CB1 receptors, for example, by marijuana, resulted in an enhanced liver fibrosis. This is what has been observed in patients with chronic hepatitis C who consume marijuana (59). Therefore, it would be possible to prevent or treat liver fibrosis either with the use of CB2 receptor agonists or by inhibiting CB1 receptors (60). The latter approach seems not to be recommended, as CB1 receptors are abundant in the central nervous system, neurological adverse effects are

frequent and these receptors are uncommon in the cirrhotic liver (61). On the contrary, stimulation of CB2 receptors seems more reasonable, as these receptors are abundant in myofibroblasts of the injured liver and uncommon in central nervous system. The experience with CB2 agonists is limited to their use in cirrhotic rats. These experiments have shown that, after liver injury, the grade of fibrosis and inflammation, as well as the number of active HSC diminishes, with an increase in cell apoptosis and MMP-2 expression (61). As far as we know, this type of agonists has not been used in humans.

Gene transcription

Among the functions acquired by HSC during activation, the increased gene expression and synthesis of ECM components stands out. At present, the internal organization of all the genes involved in fibrogenesis is quite well known, particularly the structure of collagen I gene (62). In the collagen α 1(I) promoter, a region of 220 bp that seems to be essential for the regulation of its transcription has been identified. A similar region has been found in the promoter of collagen α 2(I). In both promoters, it has been found consensus elements for transcription factors implicated in the regulation of gene expression? Activity of these factors is regulated by a number of cytokines, growth factors, and other agents, some of which are activators, while others are inhibitors of gene expression. The first group includes TGF β , acetaldehyde, iron, and lipid peroxidation by-products. The second group includes TNF α , IL-1 and 10, vitamin E, glucocorticoids, retinoids, IFN γ , prostaglandins, adrenergic agonists, calcium, and terminal peptides of collagen I and III.

Transforming growth factor (TGF β)

This is the most powerful fibrogenic factor known. Added to cell cultures, TGF β increases ECM synthesis and administered to mice causes an increase in the degree of fibrosis after experimental liver injury. Likewise, transgenic mice expressing large amounts of TGF β develop fibrosis in many organs. The opposite occurs in TGF β -deficient mice by gene silencing, as they develop little fibrosis after experimental liver injury. TGF β acts not only on gene transcription, but also favoring HSC activation and reducing ECM degradation. High serum levels of this factor have been found in patients with chronic hepatitis C, alcoholic liver disease, and autoimmune hepatitis. Moreover, after experimental liver injury, serum levels of TGF β increase. Sources of this factor are inflammatory cells, platelets, macrophages, and activated HSC of the injured tissue. In addition, it has been shown that some viral proteins, particularly the "core" protein of the HCV, are able to increase the synthesis of this factor. TGF β is secreted by cells as an inactive precursor, as it is

bound to other proteins [LTBP (*Latent TGF β 1 Binding Protein*)] or peptides [LAP (*Latency-Associated Peptide*)] that inhibit its function. It recovers its activity when plasmin, generated from plasminogen by *urokinase type plasminogen activator*; and metalloproteinases (MMPs) release TGF from its inhibitors. Once freed, TGF β starts an intracellular signal that is initiated by its union to the T β RII a cell surface receptor and ends by the formation of a transcriptional complex (Sp1-C300-CBP) that binds the promoter of a large number of profibrogenic genes. These include ECM genes, CTGF (*Connective Tissue Growth Factor*), PAI-1, PDGF, TIMP-1, MMP-2 and TGF β . In this signaling chain, participates T β 2RI that binds serine/threonine phosphorylated Smad2, Smad3 messengers and Smad4 to activated T β RII. This complex (Smad2/Smad3/Smad4), together with the previously mentioned complex (Sp1/C300/CBP), binds to specific consensus elements of the target genes. Formation of this transcriptional complex may be interfered by PPAR γ (*Peroxisome Proliferator Activated Receptor- γ*), its gene inducers, caffeine and other xanthines, and its agonists, e.g., natural prostaglandins like 15dPGJ2 (*15-Deoxy-D^{12,14}-Prostaglandin J2*). Although most of these genes are profibrogenic, Smad7 gene is also target of TGF β . This factor binds to T β RI and blocks TGF β signal transmission and favors degradation of TGF β receptors.

Blocking TGF β or disrupting its signaling may be an effective way to prevent liver fibrosis and to promote ECM degradation. Indeed, profibrogenic effects of TGF β can be blocked by the addition of molecules that bind to extracellular TGF β and prevent effects of this growth factor on HSC. This group of blocking molecules includes LAP, decorin, anti-TGF β 1 blocking antibody, TGF β soluble receptors, or manose-6-phosphate receptor. Although the efficacy of these extracellular TGF β inhibitors has been demonstrated in "in vitro" studies, the experience with them in experimental animals is limited (63) and have never been used in humans. Also TNF α , IFN γ , and HGF oppose TGF β effects, since, for instance, TNF α increases expression of the inhibitor of signal transduction, Smad7. More experience is available with the use of inhibitors of its intracellular signal transduction. Both cell cultures and experimental animals have been demonstrated that these inhibitors decrease the fibrogenic response. Thus, in rats, development of fibrosis has been successfully prevented by silencing T β RII (64) or by overexpression of Smad7 (65).

Although inhibition of TGF β effects is possible and the use of this strategy reduces the degree of liver fibrosis in experimental animals, its use in human should be done very cautiously, as this inhibition may be associated with an elevated risk of malignancies, particularly in patients with liver cirrhosis (63) in whom the risk of hepatocellular carcinoma is already high. This elevated risk of tumors is due to the antiproliferative activity of TGF β . In addition, one of its intracellular mediators, Smad4, is considered to be a product of a tumor suppressor gene.

On the other hand, it should be considered that TGF β is active, not only on the liver, producing liver fibrosis, but also on many other organs. Therefore, the absence of TGF β effects may affect the tissue and organ architecture, including bone integrity. Finally, because TGF β has immunosuppressive and anti-inflammatory properties, blockade of this factor may cause an excessive inflammatory or immunological response.

Interferon gamma (IFN γ)

IFN γ is also a proinflammatory cytokine with antifibrosing potential, as it blocks TGF β signal transduction. Indeed, activation of Stat1 by the binding of IFN γ to its receptor on one hand induces Smad7 synthesis, which, as mentioned above, binds T β RI and blocks TGF β signal. On the other hand, binds and neutralizes the p300/CBP genetic coactivator. It has been proved that blockade of TGF β signaling results in a lesser activation and proliferation of HSC, a reduced collagen synthesis and a lower degree of experimental liver fibrosis. Despite this antifibrosing potential, this cytokine has rarely been used in humans. In some studies, it appears that IFN γ administration to patients with chronic viral hepatitis decreased fibrosis stage (66). However, a recent study including 502 patients with advanced stages of liver fibrosis could not demonstrate that IFN γ decreases fibrosis stage in patients with cirrhosis of the liver (67). However, that fact that most patients included in this study were cirrhotics does not exclude that patients with earlier stages of fibrosis may benefit from IFN γ therapy by reducing liver fibrosis.

Prostaglandins (PGs)

These molecules, which are synthesized in cell membranes from arachidonic acid by cyclooxygenase-2 (COX-2), have cytoprotective effects on hepatocytes, decrease HSC proliferation and collagen gene transcription, and increase ECM degradation. PGs increase intracellular levels of cAMP (*cyclic AMP*), which through PKA (*protein kinase A*) decreases ECM gene expression. TGF β 1 increases COX-2 levels and, as a result, this growth factor increases PGE₂ formation, which in turn inhibits TGF β effects. PGs, like Smad7, act as negative regulators of TGF β 1 effects. Therefore, PGs behave as antifibrosing agents, and consequently inhibition of COX-2 may potentiate TGF β 1 effects (68). Moreover, some PGs, such as the *15-deoxy-D12,14-prostaglandin J2*, (*15d-PGJ2*) formed from the PGD₂, are PPAR γ agonists and decrease TGF β effects on CTGF (*Connective Tissue Growth Factor*) as they are able to dissociate the transcriptional complex Smad2/3-CBP-p300 and to reduce collagen and fibronectin synthesis. The antiproliferative effect of PGs is likely due to their blocking effect on PDGF. This is suggested by the fact that ibuprofen, an in-

hibitor of COX and PGs synthesis, increases the proliferative response to PDGF. Despite these antifibrosing effects of PGs, there is no clinical experience with PGs as antifibrosing agents.

Although ibuprofen may contribute to increase fibrosis, the results from the use of COX-2 inhibitors are conflicting. While some authors state that blocking COX-2/prostanoid pathway results in an increase in ECM production (68), other authors find that it reduces experimental fibrosis secondary to liver lesion (69). Latter is the experience obtained from the use of celecoxib, a specific inhibitor of COX-2, since it has been observed that it reduces proliferation and activation of HSC, induces apoptosis, decreases production and secretion of collagen by HSC, and diminishes fibrosis stage after biliary duct ligation (70).

Collagen synthesis

The mRNA originated in the nucleus translates on ribosomes the corresponding polypeptide chains, which, in the endoplasmic reticulum, undergo some posttranslational changes before being secreted out of the cells. These changes include proline and lysine hydroxylation, as these hydroxylations are necessary for the three-polypeptide chains forming collagen I and III to combine longitudinally in order to form a triple-stranded helical molecule. If these hydroxylations do not occur, polypeptide chains remain individualized, they do not form the helical structure, collagen molecules do not leave the cells, they are retained in the endoplasmic reticulum, and are degraded within the cell. Polypeptide fragments resulting from collagen degradation act as suppressors of ribosomal transduction. One essential enzyme involved in these posttranslational modifications of collagen is the 4-prolylhydroxylase, which requires ascorbic acid to exert its optimal activity. Its inhibition should reduce formation and secretion of collagen and promote its intracellular degradation. Numerous experimental studies have shown that inhibition of 4-prolylhydroxylase reduces collagen production by HSC and decreases the degree of liver fibrosis. Therefore, this enzyme has been a target on which attempts to stop the development of liver fibrosis have been focused. However, considering that ECM is present in almost all organs and that architecture of these organs depends on this matrix, the use of effective antifibrosing agents with no specificity for the liver may result in serious side effects such as disorders of bone (osteoporosis), vessels (fragility, aneurysms, hemorrhages), eyes (lens subluxation), etc. Development of these adverse side effects has been observed in experimental animals. Hence, blockade of fibrogenesis must be focused exclusively or predominantly on the liver. More than ten years ago, studies were conducted looking for drugs with inhibitory properties on the 4-prolylhydroxylase. Thus, it was developed the *HOE-077* y *S0885*

HOE-277 (*Safironil*). They were inactive prodrugs that in the liver were activated and exerted their effects locally. Although the inhibitory effects of these drugs on prolylhydroxylase are undoubted, lately it was found that their preference for the liver was due to their capability to return HSC to their resting state (71). A number of studies confirmed that these drugs reduced experimentally induced liver fibrosis. Based on these experimental studies, approximately ten years ago, a multicenter, controlled, randomized trial in patients with chronic liver diseases was started and whose results have never been published. That lets one assume that the results of this study were not favorable.

Collagen secretion

Another step in liver fibrogenesis that has been used to stop this process is the secretion of collagen by HSC. Collagen molecules synthesized by HSC use the microtubular system to leave these cells. This system is formed by the polymerization of tubulin, thus, it was supposed that arresting this polymerization by the use of *colchicin* might inhibit liver fibrogenesis. The antifibrosing effects of this drug were confirmed by a number of animal models of liver fibrosis (72). It is likely that these effects of colchicine are due not only to its ability to reduce collagen secretion, but also to its anti-inflammatory activity, and also because it increases metalloproteinase secretion and has cytoprotective activity. This is a drug that has been used for decades in the treatment of crisis of uric gout and, therefore, tolerance and side effects are well known. In men, it has been used in the treatment of some liver diseases, particularly in the treatment of primary biliary cirrhosis (PBC) and alcoholic cirrhosis. Although several studies have shown that colchicine (0.6 mg/bd) improves symptomatology, liver tests, and even severity of liver lesions and progression of disease (73,74), other authors failed to confirm such benefits. Moreover, a meta-analysis performed by the "Cochrane Central Register of Controlled Trials" concluded that colchicine should not be used for alcoholic, viral, or cryptogenic liver fibrosis outside randomized clinical trials (75).

The use of colchicine in patients with cirrhosis of the liver has also produced conflicting results. The classical, controlled, double-blind, randomized study of Kershenobich et al. showed that colchicine administered to patients with liver cirrhosis for ten years increased survival significantly and decreased fibrosis stage in some patients (76). However, these results were viewed with much skepticism, hence many other groups attempted to reproduce these results. In a study similar to that published by Kershenobich et al., which also included 100 cirrhotic patients treated with colchicine for 10 years, was found that survival of treated patients increased from 20 to 56% (77) and in another study performed in patients with chronic hepatitis B, progression to cirrhosis in a period of four

years decreased from 73.2%, in controls, to 32% in those receiving colchicine (78). Similarly, some other authors have reported that colchicine improved survival of patients with cirrhosis of the liver. Despite these favorable results, many other studies, including one meta-analysis conducted by the Cochrane Central Register of Controlled Trials that recruited 1138 patients were not able to confirm these beneficial effects of colchicines in the evolution of chronic liver diseases (79). Furthermore, colchicine was not able to reduce the degree of fibrosis in idiopathic pulmonary fibrosis. Thus, we have no clinical basis for the use this drug in the prevention and treatment of liver fibrosis.

Formation of collagen bundles and degradation of the ECM

The collagen secreted and deposited in the extracellular space undergoes a first digestion by tissue proteases that cut and released both ends where the three polypeptide chains of collagen are not intertwined. The middle portion of the collagen molecule is resistant to the effects of these proteases, is not soluble in water, and tends to precipitate in tissues. Because the electrical charge is alternating along the entire collagen molecule, as this charge changes from positive to negative every 234 aminoacids, these middle portions of collagen tend to group in parallel forming bundles. This newly formed fibrosis is very unstable; therefore, it can easily disappear. Animal experimentation has taught us that liver fibrosis induced by bile duct ligation or by CCl₄ intoxication can disappear spontaneously within one to three months if toxic is removed or bile duct ligation is released. This regression of fibrosis is not exclusive of experimental animal, as we have a large number of well documented examples of spontaneous regression of liver fibrosis in human when causative agent of disease is eliminated (80).

Collagen molecules are resistant to be digested by tissue proteases but are sensitive to some metalloproteinases (MMP). The latter are the enzymes that initiate ECM degradation. They are produced by neutrophils, Kupffer cells, macrophages, and by HSC themselves. All these cells secrete MMP as inactive proenzymes. In order to be activated, it is necessary to separate one of its ends. Several types of MMP have been differentiated. MMP-1, -8, -9, and -10 initiate degradation of fibrillar collagen I, III and V. These MMP bind to fibrillar collagen molecules and cut them in a very specific point, between a glycine and a lysine close to the carboxylic end. The two fragments resulting from this first digestion are sensitive to the effects of other tissue proteases and peptidases. These enzymes are actually causing the final degradation of collagen molecules. There are other MMPs, for example, MMP-2 that digests membrane-related collagens (collagen IV) and denatured proteins. MMP-3 and MMP-10

degrade glycoproteins and proteoglycans of the ECM, while MMP-14 and -25 are involved in the activation of other MMPs. Although in this article we are interested in the MMPs for their role in degradation of the ECM, in fact these enzymes have many other properties and are involved in a large number of biological processes (apoptosis, cell migration, angiogenesis, carcinogenesis and proliferation). On inflammation, they may act both as anti-inflammatory and as proinflammatory. The latter effect occurs because a MMP, called TACE (*TNF α -converting enzyme*), activates TNF α and consequently promotes inflammation. Considering the role of MMPs in the ECM degradation and fibrosis regression, it can understand the interest in knowing the factors involved in the regulation of their gene expression and activity. Factors that increase MMP gene expression are potential antifibrotic agents. They include some familiar antifibrotic factors as they act in the same way on other phases of fibrogenesis. This is the case of TNF α (19), IL-1, IL10, and IFN γ . In the same direction act IL-6 (81), IFN γ (82), fibronectin, polyunsaturated lecithin, and phorbol esters, a tumor promoter. TGF β appears among the factors that inhibit MMP gene expression and activity, and therefore contributes to increase the amount of ECM deposited in the tissues. Molecular mechanisms involved in the suppression of MMP gene expression are not well known; however, it is known that p38 MAPK is implicated. Its inhibitory effect on MMPs is exerted by enhancing TIMPs (*Tissue Inhibitors of Metalloproteinases*) gene expression. These glycoproteins play a major role in the regulation of liver fibrosis, as shown by the fact that their exclusion results in a substantial decrease in the degree of experimental fibrosis and that their gene overexpression is accompanied by a greater fibrogenic response to the CCl₄-induced liver injury. The disappearance of fibrosis that occurs upon cessation of liver injury is due both to the increase in MMPs and to a reduction in TIMPs. This decrease is ascribed to the disappearance of proinflammatory cytokines that promote MMP gene expression. TIMPs inhibit MMPs either by binding the catalytic region of these enzymes or by binding MMP precursors avoiding their activation. Because of the role ascribed to MMPs in the ECM degradation, it has been considered the induction of MMP gene expression in order to fight development of liver fibrosis. Among factors able to stop MMP gene expression are polyunsaturated lecithin, pentoxifylline, and IFN α . The antifibrotic effects of *polyunsaturated lecithin* have been proved in rats and other animal, but until now these effects have not been demonstrated in human. A study designed to determine the usefulness of this agent (NCT00211848) has not been completed and results are not available. *Pentoxifylline* decreases experimental liver fibrosis (83) by a variety of mechanisms, including reduction of collagen and TNF α production, and increase in ECM degradation as result of a reduced TIMP formation. Several studies carried out in patients with chronic hepatitis C have suggested that

IFN α may have antifibrosing effects not only in responder patients, but also in non responders (84-86). More recently, several controlled studies have evaluated the clinical usefulness of IFN α administered for three to five years in the prevention or regression of liver fibrosis in patients with chronic hepatitis C in advanced stages of fibrosis, non-responders to its antiviral effects (*ClinicalTrials.gov* number; NCT00006164) (87,88). The reported results have been disappointing, as they could not demonstrate whether this cytokine is able or not to slow or stop the development of fibrosis. However, this study does not definitely exclude that IFN α is able to stop fibrosis progression when administered to patients in earlier stages of fibrosis. As we will see later, it is very difficult to achieve regression of fibrosis when it is in an advanced evolutive stage. Therefore, a similar study should be done including only patients with mild or moderate fibrosis.

Inactivation and apoptosis of HSC

When liver aggression declines, in addition to a decrease in the intensity of inflammation and hepatocellular necrosis, it is observed a reduction in the number of HSC. Although mechanisms by which HSC disappear are not well understood, it is assumed that it occurs either by deactivation of these cells or by an increased rate of apoptotic death. PPAR γ (*Peroxisome Proliferator-Activated Receptors*), retinoic acid, IL-10, and the trans-resveratrol, among others, are factors involved in the deactivation of HSC. PPAR γ are members of a nuclear hormonal receptor superfamily involved in lipid metabolism, in adipocyte differentiation and in maintaining the inactive phenotype of HSC. PPAR expression is very high when HSC are at rest, but decreases when these cells are activated. The role played by these receptors in the regulation of HSC activation is shown when by the administration of PPAR γ ligands (polyunsaturated fatty acids, prostaglandin J₂, 15sPGJ₂, rosiglitazone) the inactivation of HSC is achieved and the fibrogenic response to TGF β is reduced. In vitro and in vivo studies have shown that treatment with *rosiglitazone*, a PPAR γ ligand, reduces activation of HSC, intensity of fibrosis, expression of TGF β and proinflammatory cytokines TNF α and IL-6, and increases PPAR γ expression in HSC (89). With the clinical use of this PPAR ligand in the treatment of nonalcoholic steatohepatitis (NASH) there has been a significant improvement in the NASH lesions, including fibrosis (90,91). It is possible, however, that these beneficial effects in this disease are due more likely to improve insulin sensitivity than to a direct effect on HSC. Several controlled, multicenter trials have been carried out including an important number of patients with NASH (<http://clinicaltrials.gov/>; ClinicalTrials.gov, NCT00063622, NCT00227110, NCT00699036) that demonstrated a beneficial effect on NASH lesions but not on fibrosis (92,93). Another, more recent, controlled study conducted in 200 patients with

chronic hepatitis C and moderate fibrosis (<http://clinicaltrials.gov/>; ClinicalTrials.gov, NCT00244751) was not able to demonstrate that farglitazar may reduce HSC activation and even less the stage of liver fibrosis (94).

Among the changes that occur in HSC during the activation process the disappearance of retinol esters droplets from their cytoplasm is one of the most striking features. Therefore, it was believed that *retinol* could act as an inhibitor of HSC activation and that it could prevent fibrosis development. Although these effects have been confirmed in cell cultures, in experimental animals, the opposite results were obtained; that is, worsening of liver fibrosis following, for example, ethanol administration. In humans, it is not advisable to use retinoids as antifibrosing agents, since it is well known that among the consequence of prolonged intake of elevated doses of vitamin A is liver fibrosis. This effect is ascribed to a higher activation of latent TGF β by plasminogen.

It is thought that the main mechanism by which HSC disappear after cessation of liver aggression is by apoptosis of these cells. We know very little about the factors that determine the death of these cells, but there are grounds to assume that among the changes that occur during activation of these cells there are some that also are involved in their death. For instance, the appearance of Fas and TNF α receptors (TNFR-1) on the cell surface during activation determines that these cells may respond to Fas ligand and TNF α , which appear as a result of the liver lesion and inflammation, and consequently these cells are candidates to die by apoptosis. Other factors that may also contribute to the death of these cells are p53, Bax, Bcl-2 proteins, NK and NKT $\gamma\delta$ cells, MMPs, IFN γ , and other proinflammatory cytokines (95). Among factors that favor survival of HSC, and therefore also act as fibrogenic, stand out TGF β and fibronectin (13). It is thought that selective induction of HSC apoptosis would be a way to control liver fibrogenesis without causing the death of other cell types. That is, it would be required to detect inducers that would only cause apoptosis in HSC, but not in the remaining liver cells, and particularly in hepatocytes. In this regard, it should be noted that HSC, not hepatocytes, express large amounts of TRAIL receptor 2 (*TNF-related apoptosis-induced ligand*) (TRAIL-R2) making them susceptible to death by TNF α (96). That is, TNF α also acts as antifibrogenic by this route. In spite of that, as we have already said, the clinical use of this cytokine is not advisable, among other reason because of it proinflammatory properties.

The use of blocking *TIMP-1 antibodies* has been able to stop the progression of experimental liver fibrogenesis, not only by promoting ECM degradation, but also by inducing HSC apoptosis.

Phagocytosis of apoptotic bodies is one of the mechanisms that are involved in the activation of HSC. Therefore, it is thought that inhibition of hepatocyte apoptosis may help to arrest fibrogenesis. This is the rationale of some clinical trials (IDN-6556; PF-03491390) aimed to

analyze the effects of some caspase inhibitors in patients with various liver diseases, mostly, chronic hepatitis C. Although it is certain that such treatments are able to reduce the signs of hepatocellular injury, it could not be proved that this effect was associated with an improvement in the stage of liver fibrosis (97). Other studies are being conducted to assess the antifibrogenic potential of other apoptosis inhibitors (<http://clinicaltrials.gov/>; ClinicalTrials.gov, NCT00874796), But the results of these studies are not yet available. In any case, it should be noted that the use of such apoptosis inhibitors enhances the risk of carcinogenesis, as apoptosis is a mechanism used by the organism to eliminate cells with damaged DNA. This risk is particularly high when these inhibitors are used in cirrhotic patients in whom this risk is already high.

As we have seen, at present, we have a wide therapeutic arsenal capable of stopping the formation of liver fibrosis or of accelerating its degradation. These are measures by which we can prevent activation and proliferation of HSC or cause their death by inducing apoptosis. We also have means to stop gene transcription of ECM components or to prevent their synthesis and secretion and even to cause degradation of the ECM that could have been formed during the time the liver suffered the aggression. These are means that have proven their antifibrotic capacity, not only *in vitro*, when used in culture cells, but also *in vivo*, in a large number of experimental models of liver injury and fibrosis. However, despite this efficacy, when these drugs have been tested in humans, results have been always frustrating. Therefore, despite the great advances we have made over the last 30 years in the understanding of the mechanisms that determine development of fibrosis, There is still no effective treatment available to control the development of this lesion and even less to achieve its regression. This discrepancy between clinical and experimental results has several explanations. Among them, it must be noted that liver fibrosis found in chronic liver diseases is not similar to that induced in experimental animals. The latter is, in general, a newly formed fibrosis that has developed in only days or weeks. Fibrosis associated with chronic inflammatory liver diseases has been formed slowly over years. During this time, some molecular changes have taken place in the tissue collagen that prevent ECM to be degraded by MMPs. Several decades ago, Perez-Tamayo observed that spontaneous regression of fibrosis after liver injury cease was more difficult when liver fibrosis was old and collagen bundles were thick and compact (98).

Formation of cross-linking among collagen molecules

Collagen molecules, once deposited in the extracellular space, as it has already mentioned above, group together in parallel forming bundles attracted by electrochemical forces. Thereafter, these bundles are strengthened by the formation of covalent unions between aldehyde

and carboxylic groups in some collagen molecules, and amino group of hydroxylysine in others. In the formation of these bridges between hydroxylysine molecules from parallel collagen fibers, takes part *lysyl oxidase*, which, in the presence of copper, causes a desaminative oxidation and replaces an amino group of the hydroxylysine by an aldehyde group. Subsequently, a cross-linking is established between this aldehyde group and a hydroxylysine amino group belonging to another parallel collagen fiber. In the formation of cross-links between carboxylic groups of glutamic acid of some collagen fibers with hydroxylysine amino group of other fibers participates the *tissue transglutaminase-2* (tTG-2). This is an enzyme that plays a major role in the pathogenesis of fibrosis (99), as it can irreversibly reinforce the unions among collagen bundles by forming intra- and intermolecular bound. Thus, tissue repair is reinforced. This enzyme is produced by both hepatocytes and nonparenchymal cells, including activated HSC. In this process it is also involved TGF β (100). Formation of these cross-links among collagen fibers is of great importance, since the resistance of longstanding fibrosis to degradation by MMPs is attributed to them. Their formation requires usually a time much longer than the time normally needed to induce experimental fibrosis. Given the role attributed to the lysyl oxidase, its inhibition has been considered for therapeutic purposes. The activity of this enzyme can be reduced by *D-Penicillamine*, a copper chelator, and lathyrogen agents (*β -aminopropionitrile*). *D-penicillamine*, widely used successfully in the treatment of Wilson's disease, has a wide variety of biological effects; one of them is the prevention of formation of intermolecular cross-links among collagen fibers. In spite of this, the use of this drug in the treatment of chronic liver diseases has also been disappointing. A number of controlled studies carried out in patients with primary biliary cirrhosis have been unable to demonstrate that this drug can stop the progression of fibrosis. It is likely that this failure is due to the predominant role of tTG-2 in the formation of the intermolecular cross-links. We have no means to stop tTG-2 activity. It has been reported that *cystamine*, a component of garlic, can inhibit this tissue enzyme. Its use in rats exposed to CCl₄ decreases tTG gene expression and the grade of HSC activation and fibrosis (101), but we do not know whether these effects also occur in humans.

In conclusion, although in the last 30 years we have made considerable progress in the understanding of mechanisms and factors involved in the formation and progression of liver fibrosis, until now very little of this has resulted in the development of effective measures to prevent the development of this lesion or to achieve its regression. However, at present, there are several controlled, multicentric, randomized trials attempting to determine the usefulness of a large number of drugs, whose efficacy has been proven in experimental animals. Accepting the difficulties presented by old fibrosis to disappear, these studies should include a subset of patients,

clearly differenced from the rest of patients, with not advanced stages of fibrosis. In these patients, the response to these treatments can be expected to be more effective.

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