Reversibility of hepatic fibrosis: from the first report of collagenase in the liver to the possibility of gene therapy for recovery

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Abstract. Since the authors reported the presence of collagenase in the liver as well as its increased activity in the early stage of hepatic fibrosis and its reduced activity in advanced fibrosis in rats induced by chronic CCl_4 intoxication, in baboons fed alcohol chronically and in patients with alcoholic fibrosis, other investigators have demonstrated the same tendency of collagenase activity biologically and histochemically. Very recently, the authors demonstrated definite gene expression of collagenase during the recovery from experimental hepatic fibrosis using Northern blotting and *in situ* hybridization. The findings of *in situ* hybridization not only demonstrated the cells expressing collagenase, but also suggested much information on the mechanism of the recovery from fibrosis. Hepatic stellate cells play a key role not only in fibrogenesis but also in fibrolysis. The authors' recent observation revealed that collagenase (matrix metalloproteinase-13 (MMP-13)) gene expression appears very early in the process of recovery from liver fibrosis, and that both stellate cells and hepatocytes express MMP-13. Recovery from liver cirrhosis requires the gene expression of collagenase, increased production of the collagenase enzyme, and activation of the enzyme balanced with the specific inhibitors of collagenase. The understanding of molecular mechanisms of MMP-1 gene expression which is under investigation in our laboratory may provide us a new strategy for the treatment of liver fibrosis including the possibility of gene therapy. (Keio J Med 50 (2): 58–65, June 2001)

Key words: collagenase, matrix metalloproteinase (MMP), MMP-13, recovery from liver fibrosis, liver cirrhosis

Introduction

The reversibility of liver fibrosis has been observed experimentally[1–7] and clinically[8–12] since Cameron and Karunaratne[1] observed this phenomenon in the carbon tetrachloride-induced cirrhosis model after removal of the toxic agent. Hepatic fibrosis induced by thioacetamide,[2] α-naphthylisothiocyanate,[3] ethionine,[4] choline-deficient diet[5] as well as by the ligation of bile duct[6] were demonstrated to be reversible after removal of the causative agent. Recovery from hepatic fibrosis has been observed among patients with alcoholic liver disease, hemochromatosis and other liver diseases.[8–10] The authors demonstrated the disappearance of liver fibrosis during recovery from hepatitis B virus-positive subacute hepatitis with massive fibrosis.[11] More recently, treatment with interferon reversed hepatic fibrosis in patients with hepatitis C virus antibody-positive chronic hepatitis and cirrhosis.[12]

Recovery from liver fibrosis involves the destruction of newly formed fibrous bands in the liver. Since Gross and Lapiere[13] found tadpole collagenase in 1962, interstitial collagenase has been isolated from skin, uterus, granulocytes, macrophages, and other organs

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and cells,[14,15] and it has been noted that this enzyme is synthesized de novo and excreted extracellularly, can attack the collagen molecule at three-quarters from the amino terminal end under neutral pH,[14,15] and plays an important role in growth, inflammation, tumor development and other physiological and pathological conditions.[14,15] The authors previously postulated the presence of interstitial collagenase in the liver and demonstrated the first report of collagenase activity in experimental liver fibrosis.[16] This review discusses the roles of interstitial collagenase in liver fibrogenesis and fibrolysis; recent advances in the understanding of matrix metalloproteinases (MMPs) and their specific tissue inhibitors of metalloproteinases (TIMPs); and the possibility of gene therapy causing MMP-1 gene expression.

The First Report of Collagenase in Experimental Liver Fibrosis

In 1974 the authors demonstrated collagenolysis around the explant of a slice of rat fibrotic liver on a collagen gel film, and demonstrated the typical collagenase attack pattern against neutral salt-extracted collagen by disc electrophoresis of the sample collected from the reacted collagen gels.[16] This disc gel revealed β^A (3/4-length of β chain), α^A (3/4-length of α chain) and α^B (1/4-length of α chain) products, which are the typical products of limited collagen degradation by mammalian collagenase on polyacrylamide gels.

In the following study, the authors used type I collagen that had been purified from acid-soluble collagens extracted from rabbit skin.[17,18] The enzyme source was the liver of baboons fed an alcohol-containing diet. The reaction mixture containing type I collagen as a substrate, the homogenate of baboon liver as an enzyme, and 3mM p-chloromercuribenzoate to inhibit thiol proteinase activity and to convert procollagenase into the active form, was incubated under neutral $pH^{7,6}$ at 27°C, assayed by viscometer, and monitored by disc electrophoresis.[17] The levels of the reaction products of β^A and α^A and their ratio showed the predicted reaction pattern by interstitial collagenase. Using this assay method and the same substrate, the authors demonstrated increased activity of interstitial collagenase in the early stage of hepatic fibrosis in baboons that had been fed alcohol chronically[17,18] and in patients with alcoholic hepatic fibrosis.[18] This research was performed 20 years ago, but the results are still relevant today. The authors also measured the level of collagenase activity in the liver of rats fed alcohol chronically[19,20] and in rats that had been chronically treated with carbon tetrachloride.[21] However, the level of collagenase activity in these rats was quite low and there was a slight, but not significant, increase in collagenase activity in the early stage of fibrosis. Therefore, the increased collagenase activity during early fibrosis in these rats had been observed only by a semi-quantitative, unspecific tissue culture method.[21,22] Other researchers subsequently demonstrated the presence of increased collagenase activity in the early stage of liver fibrosis and reduced collagenase activity in advanced fibrosis.[23–25]

Possible Participation of MMPs and TIMPs in Extracellular Matrix Metabolism in the Liver

Advances in genetic research have changed the method of identification of enzymes. For example, interstitial collagenase was originally defined as the enzyme that can degrade interstitial collagen at a specific site under physiological conditions as mentioned above.[14,15] However, recent advances have revealed that MMP-1, MMP-8 and MMP-13 can also degrade interstitial collagenase. These MMPs belong to the family of MMPs. They can degrade type I, type III and type X collagens, but cannot degrade other types of collagen such as type IV, type V and type VI, nor other components of extracellular matrix such as proteoglycans and glycoproteins. Presently, eighteen MMPs have been identified and are grouped into four subclasses according to their structure and function.[26,27] The activity of MMPs is regulated by several mechanisms including regulation of gene expression by cytokines or hormones; extracellular cleavage of the proenzyme to make the active form of the enzyme; and specific inhibition of the active enzyme by endogenous proteins known as TIMPs. The metabolism of extracellular matrix is regulated by MMPs in close association with TIMP-1, 2, 3, and 4.[28–31] In the case of rats, sequence homology analysis revealed that except for human MMP-13, there is no sequence in rats that shows more than 90% similarity with the sequence of MMP-1 in humans. The cDNA of rat MMP-1 has not yet been cloned, and rat interstitial collagenase should be considered to be the rat homologue of human MMP-13.[32,33]

MMPs other than MMP-1, MMP-8 and MMP-13 cannot degrade type I collagen which is very stable, and a net deposition of type I collagen has been observed in progressive hepatic fibrosis.[34–41] Arthur, *et al.*[42,43] reported that hepatic stellate cells secrete a neutral metalloproteinase that can degrade type IV collagen (a component of the basement membrane), and the metalloproteinase that they isolated seems to be MMP-2. MMP-2, a potent gelatinase, and membrane type 1 (MT1)-MMP, an activator of MMP-2, can cleave native type I collagen,[44,45] but with less efficiency than MMP-1.[44] Interstitial collagenase is a key enzyme involved in the degradation of fibrosis, and the expres-

sion of interstitial collagenase in the fibrous liver may be important from the standpoint of ameliorating liver fibrosis.

Advanced liver fibrosis is associated with the appearance of perihepatocellular fibrosis, which contributes to the formation of sinusoidal capillarization. Intralobular shunt vessels between portal vein tributaries and hepatic vein tributaries are formed and there is no metabolic exchange of the blood with hepatocytes, leading to the irreversibility of liver fibrosis. MMP-2, MMP-3 and MMP-9 can cleave type IV collagen. The authors previously developed an assay method for type IV collagenase in the liver and demonstrated reduced activity of type IV collagenase in human liver cirrhosis.[46–48] Generally, however, in the process of wound healing, type V- and type IV-collagens appear first, followed by the appearance of large amounts of type III- and type I-collagens. The authors observed reduced activity of type IV collagenase in liver cirrhosis as mentioned above;[46–48] however, the most stable and most abundant collagen is type I collagen. Therefore, the authors has investigated the MMP-1/MMP-13 gene expression in the process of liver cirrhosis.

Gene Expression of MMPs and TIMPs in the Progression of Liver Fibrosis

The increased collagenase activity in the early stage of liver fibrosis, which had been observed by the authors[16–22] and others,[23–25] initially could not be correlated with the results of gene research. For example, Iredale, *et al.*[28,49,50] did not observe an increase in MMP-13 mRNA transcription in the liver. Instead, they demonstrated an increase in TIMPs mRNA transcripts and postulated that the balance between the downregulation of MMP-13 expression and the upregulation of the expression of TIMPs may result in the deposition of type I collagen in experimental hepatic fibrosis. The question is which MMPs are responsible for the increased collagenase activity in the early stage of liver fibrosis.

Takahara, *et al.*[51] showed that the level of MMP-2 expression increased during the process of experimental hepatic fibrosis as well as during the process of hepatic fibrosis in chronic hepatitis, and that it decreased during the process of cirrhosis. Takahara, *et al.*[52] also demonstrated the dual expression of MMP-2 and MT1-MMP in chronic hepatitis and cirrhosis, and further demonstrated cytoplasmic and membranous immunodeposits of both MMPs in endothelial cells, Kupffer cells, capillary endothelial cells and lymphocytes. In particular, they observed the over-expression of MMPs in stellate cells and fibroblasts, and suggested that MT1-MMP activates Pro-MMP-2 and the activated MMP-2 may remodel the liver parenchyma during the process of liver fibrosis. Thus, the gene expression of MMP-2 increased during the process of liver fibrosis and decreased with cirrhosis. MMP-2 expression is stimulated by transforming growth factor (TGF)-β, and MMP-2 expression is regulated in a different manner from the expression of interstitial collagenase. TGF-β upregulates MMP-2 expression while it downregulates MMP-1 expression. Therefore, the increased activity of collagenase in the early stage of liver fibrosis has been considered to be mediated by MMP-2 and MT1-MMP.

Recently, the authors demonstrated the gene expression of MMP-13 in the CCl_4-treated rat liver by reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization.[53] In the normal rat liver, no signal for MMP-13 mRNA was observed by *in situ* hybridization. In the liver of rats with fatty change induced by 4-week CCl_4 treatment, positive signals for MMP-13 mRNA were observed in scattered cells, and these cells were negative for α-smooth muscle actin but seemed to be hepatic stellate cells because they were positively stained with desmin. In the rats treated with CCl_4 for 8 weeks, signals for MMP-13 mRNA were observed in a few cells lining the fibrous septa. Some of these cells were stained with α-smooth muscle actin antibody. On the other hand, the cirrhotic liver of rats treated with CCl_4 for 12 weeks revealed very weak expression of MMP-13 mRNA in stellate cells. No hepatocyte in the liver revealed MMP-13 mRNA transcripts regardless of the length of CCl_4 treatment.

As mentioned above, generally there was very weak MMP-13 gene expression during the process of rat hepatic fibrosis induced by chronic CCl_4 intoxication. The authors, however, postulated that an increase in MMP-13 gene expression, even if the increase is very slight, may be necessary for the destruction of the tissue in order to deposit newly formed matrix. Liver fibrosis in baboons and humans shows a prominent increase in MMP-1 biological activity at the early stage. In fact, Iredale, *et al.* reported that the activated stellate cells in autoimmune chronic active hepatitis produced matrix components as well as MMP-1.[49] The gene expression of MMP-1/MMP-13 appears in the early stage of liver fibrosis before the deposition of fibrosis in the liver.[53] Therefore, the increased collagenase activity that the authors reported in 1974[16] seems to be mediated by MMP-13 as well as MMP-2 and MT1-MMP.

Gene Expression of MMPs and TIMPs in Recovery from Liver Fibrosis

The authors demonstrated that MMP-13 expression was markedly enhanced during the recovery phase of liver fibrosis, and cells strongly positive for MMP-13 were observed mainly at the interface between the resolving fibrous septa and the parenchyma by *in situ*

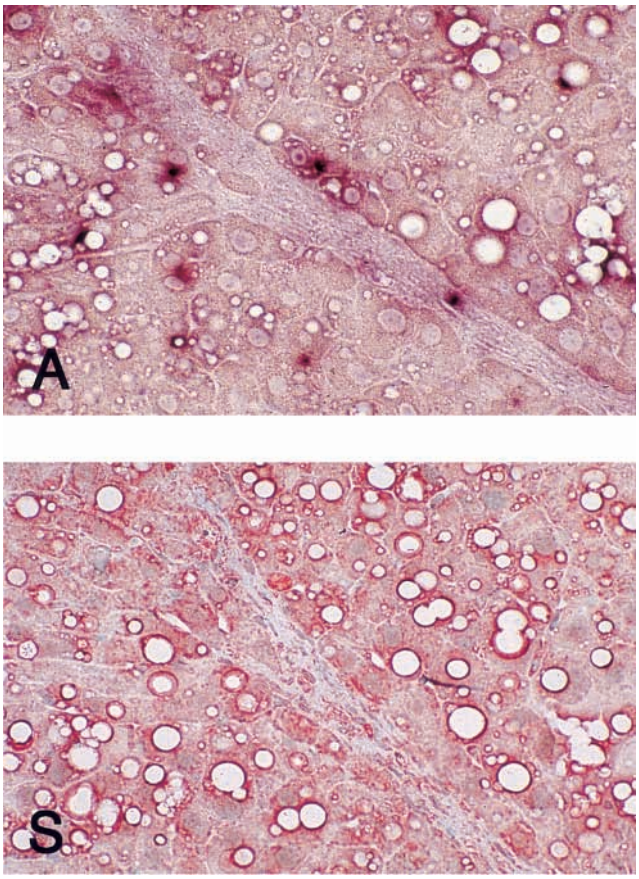

Fig. 1 *In situ* hybridization of MMP-13 mRNA in rat liver sections obtained 5 days after 8-week CCl4 treatment. Increased MMP-13 gene expression was observed with the antisense (AS) probe but not with the sense (S) probe in a serial section. MMP-13-positive cells were located along the border between the resolving fibrous band and the parenchyma. Original magnification: AS, S 50×.

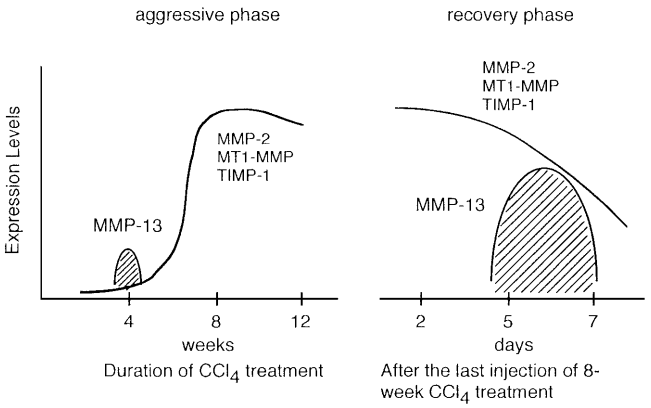

Fig. 2 The gene expression levels of MMPs and TIMPs in the aggressive phase of liver fibrosis (left) and its recovery phase (right). This figure is presented by the permission of the Japanese Society of Hepatology (originally appeared in Acta Hepatol Jap 2000; 41: 741–753).

hybridization (Fig. 1).[53] Overlapping the images of *in situ* hybridization and immunohistochemical staining with the help of a computer revealed that some, but not all, of the MMP-13-positive cells were stellate cells that were stained with α-smooth muscle actin antibody. This was the first report that provided direct evidence of definite MMP-13 gene expression during the recovery phase, which is in contrast with the downregulation of MMP-13 expression during the progression of fibrosis (Fig. 2).[53]

Takahara, *et al.*[51] showed that following the discontinuation of chronic CCl_4 treatment, there was increased MMP-2 expression on day 3 and day 7, and reduced expression on day 14, and suggested that the destruction of pericellular fibrosis may occur during the very early stage of recovery. *In situ* hybridization for MMP-2[54] revealed that vimentin-positive, CD-68-negative mesenchymal cells, which are assumed to be stellate cells, showed high transcript levels of TGF-β as well as MMP-2. Iredale, *et al.*[28] and Roeb, *et al.*[30] demonstrated that the TIMP-1 mRNA level increased during the early phase of CCl_4 treatment, and then decreased and remained at a low level during the recovery from liver fibrosis. The net activity of MMPs is determined by the balance between the activities of the MMPs and their inhibitors. Herbst, *et al.*[31] revealed that high levels of TIMP-1 and TIMP-2 transcripts were present in all fibrotic rat and human livers, predominantly in stellate cells. Iredale, *et al.*[50] reported that the levels of TIMPs decreased during the recovery phase of experimental hepatic fibrosis.

Although the reports of Vyas, *et al.*,[55] Herbst, *et al.*[56] and Winwood, *et al.*[57] showed that MMP-3 and MMP-9 are involved in perihepatocellular fibrosis, there has been no study on the gene expression of MT1-MMP and other MMPs in the context of fibrolysis in the liver.

The authors initially had considered that MMP-2 and MT1-MMP are not involved in the recovery from liver fibrosis because both MMP-2 and MT1-MMP gene expression decreased during the process of fibrolysis in the liver. Against our expectation, however, recent our observation suggests that both MMP-2 and MT1-MMP have a some role in fibrolysis although their gene expression decrease in the recovery phase (submitted). The hepatic stellate cell is a pivotal key player that produces extracellular matrix, secretes and deposits matrix with MMP-2 and MT1-MMP extracellularly, and sometimes secretes MMP-1/MMP-13 for fibrolysis after receiving the signal transduction following removal of the toxic reagent. The authors' observations led to the next hypotheses: 1) MMP-13 gene expression appears very early in the process of recovery, and both stellate cells and hepatocytes probably express MMP-13 at this stage. 2) The expression of MMP-2, MT1-MMP and

TIMPs are down-regulated during the recovery from liver fibrosis, but the cells that are expressing MMP-2, MT1-MMP or TIMPs change very quickly. Although the precise mechanism is not known, the co-ordination among MMP-13, MMP-2, MT1-MMP and TIMPs may participate in the destruction of newly formed fibrous tissue in the recovery phase of liver fibrosis.

Induction of MMP-1/MMP-13 Gene Expression in the Liver

The authors previously investigated the mechanism of collagenase production in liver cells using a monolayer culture of fibroblasts derived from the rabbit liver.[58–60] Different mechanisms of collagenase production were observed among fibroblasts derived from the synovium, gastric mucosa and liver of the same rabbit. All fibroblasts used in this experiment were the fourth passaged cells in order to exclude macrophages and to obtain uniform cell lines. Synovial fibroblasts secreted a low level of collagenase without any treatment, and those treated with phorbol myristate acetate (PMA) produced a high level of collagenase. Gastric mucosal fibroblasts produced a high level of collagenase without any treatment. Upon treatment with PMA, MMP-1 production dramatically increased. Liver fibroblasts did not produce collagenase, even with PMA treatment.[60]

The authors succeeded in inducing MMP-1 expression by co-culture of fibroblasts and hepatocytes at the cell number ratio of 3 : 1. After a long latent period, a remarkably high level of collagenase synthesis was observed.[59,60] The large quantity of collagenase produced by fibroblasts contributes to massive necrosis or tissue breakdown *in vivo*. As hepatic stellate cells can express high levels of MMP-1/MMP-13, MMP-1/MMP-13 production should be considered to be related to the activation of stellate cells.

Electron microscopic studies and culture studies of stellate cells revealed that myofibroblasts are cells that had been transformed from stellate cells, and may produce extracellular matrix.[34–41] This transformation is referred to as the activation of stellate cells. Stellate cells are activated by expressing c-myb and NFκB, which is induced by oxidative stress. This is evidenced by the following observations. Lee, *et al.*[61] demonstrated that stellate cells were activated by the generation of free radicals with ascorbate/FeSO4 and by malondialdehyde, a product of lipid peroxidation. Stellate cells were also activated by the addition of type I collagen or TGF-α. This activation was inhibited by an antioxidant (1-α-tocopherol) or butyrate hydroxytoluene. Their valuable findings were that oxidative stress, TGF-α and collagen type I each cause the proliferation of activated stellate cells by markedly stimulating NFκB activity and c-myb expression. Antioxidants and c-myb antisense oligonucleotide inhibited the activation of stellate cells by type I collagen and TGF-α; the expression of NFκB and c-myb; and the proliferation of activated stellate cells (myofibroblasts).[61] Moreover, they demonstrated that nuclear extracts from activated stellate cells formed a complex with the promoter E box of the α-smooth muscle actin gene, and this process was disrupted by antibodies against NFκB65 and c-myb. C-myb was also expressed in activated stellate cells in the fibrotic liver of rats that had been treated with chronic administration of carbon tetrachloride intoxication.[61]

Endothelin induces the activation of stellate cells, and an antagonist of endothelin (bosentan) inhibited the activation of stellate cells.[62] Platelet-derived growth factor (PDGF), a cytokine, stimulates collagen production, and pentoxifylline is now known to inhibit PDGF as well as the activation of stellate cells.[63] A recent study by Marra, *et al.*[64] clarified that phosphatidylinositol 3-kinase is involved in the PDGF-induced activation of stellate cells, and that this phosphorylation is necessary for the motility, proliferation and transformation of stellate cells to myofibroblasts.

The relationship between these activation processes mentioned above and the mechanism of production of MMP-1/MMP-13 has not been found. Activated stellate cells producing extracellular matrix seem not to express MMP-1/MMP-13 from the authors' observation.[53] Therefore, the mechanism of MMP-1/MMP-13 gene expression in stellate cell should be investigated.

The molecular mechanism of the transcriptional regulation of the MMP-1/MMP-13 gene has not been studied in hepatic stellate cells. Inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are known to be involved in the activation of hepatic stellate cells. Most such cytokines activate the retrovirus-associated DNA-mitogen activated protein kinase (Ras-MAPK) signaling pathway including c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which in turn activate the transcription of early genes such as c-fos and c-jun. The Fos and Jun proteins contribute to the induction of MMP-1 gene transcription by their binding to proximal AP-1 sites of the promoter in fibroblasts and immortalized cells.[65–67] In addition to the role of AP-1, Vincenti, *et al.*[68] recently reported the role of NF-κB in the induction of rabbit MMP-1 expression in IL1-treated synovial fibroblasts. TNF-α and IL-1β increase MAPK activity including JNK and ERK in rat HSCs, thereby stimulating AP-1 activity in the hepatic stellate cells.[69] Activation of stellate cells is also closely related to NF-κB activity.[70] Activation of transcriptional factors such as AP-1 and NF-κB activity may also contribute to gene regulation of MMP-1 in HCCs. The molecular mecha-

nisms of the regulation of MMP-1 expression are still unknown. It should be determined how such transcriptional factors as NF-κB or AP-1 may be involved in MMP-1 gene expression in stellate cells.

Possibility of Gene Therapy to Induce MMP-1 Gene Expression in Cirrhotic Liver

The recent topic is hepatocyte growth factor (HGF), that is, the fibrolytic effect of HGF on experimental liver fibrosis.[71–72] Ueki, *et al.*[72] showed that in dimethylnitrosamine-induced liver fibrosis, HGF down-regulated TGFβ expression, which was followed by the disappearance of pre-deposited fibrous tissue. Ozaki, *et al.*[73] proposed that HGF induces MMP-1 expression in LI 90 (cell line of human stellate cells) via Ets-1 in the promoter region of MMP-1. The recovery from liver fibrosis by external HGF treatment, indicates that fibrolysis induced by increased collagenase activity could be related to regeneration of the liver.

The participation of hepatocytes in the destruction of the extracellular matrix should be considered. The authors previously reported that transient expression of interstitial collagenase was observed in differentiated, very early hepatocellular carcinomas (less than 2 cm), but not in moderately and undifferentiated hepatocellular carcinomas.[74] From the results of our experiments,[53,74] we hypothesize that in the recovery phase of liver fibrosis, liver regeneration occurs through the proliferation of both mature pre-existing hepatocytes and stem cells, which may express MMP-1/MMP-13 in response to the elevated HGF level to destroy the extracellular matrix to complete the tissue repair. MMP-1/MMP-13 transcription is transient, and stem cells may differentiate into hepatocytes, stellate cells and other cells. The authors are now investigating the possibility of gene therapy to proliferate stem cells in the liver and to express MMP-1/MMP-13 gene in stem cells with or without transfusion of stem cells derived from bone marrow in the patients with liver cirrhosis.

Conclusion

The authors believe that MMP-1/MMP-13 can degrade collagen fibers and contribute to the recovery of liver fibrosis. Therefore, the gene expression of MMP-1/MMP-13 should be a key step in this process. Finally, the finding of collagenase expression in the recovery phase of experimental liver fibrosis by *in situ* hybridization as well as demonstration of the enzymatic activity of collagenase indicates that there is promising potential for the development of a new therapy which should focus on the gene expression of MMP-1/MMP-13, for the treatment of liver fibrosis, although we must be careful about tumor development.

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