Antifibrotic Effects of a Recombinant Adeno-Associated Virus Carrying Small Interfering RNA Targeting TIMP-1 in Rat Liver Fibrosis

Min Cong, Tianhui Liu, Ping Wang, Xu Fan, Aiting Yang, Yanfeng Bai, Zhen Peng, Peng Wu, Xiaofei Tong, Jing Chen, Hai Li, Rui Cong, Zhuzhen Tang, Baoren Wang, Jidong Jia, and Hong You

From the Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing, China

Elevated tissue inhibitor of metalloproteinase 1 (TIMP-1) expression contributes to excess production of extracellular matrix in liver fibrosis. Herein, we constructed a recombinant adeno-associated virus (rAAV) carrying siRNA of the TIMP-1 gene (rAAV/siRNAeTIMP-1) and investigated its effects on liver fibrosis in rats. Two models of rat liver fibrosis, the carbon tetrachloride and bile duct ligation models, were treated with rAAV/siRNAeTIMP-1. In the carbon tetrachloride model, rAAV/siRNAeTIMP-1 administration attenuated fibrosis severity, as determined by histologic analysis of hepatic collagen accumulation, hydroxyproline content, and concentrations of types I and III collagen in livers and sera. Levels of mRNA and active matrix metalloproteinase (MMP) 13 were elevated, whereas levels of mRNA and active MMP-2 were decreased. Moreover, a marked decrease was noted in the expression of α-smooth muscle actin, a biomarker of activated hepatic stellate cells (HSCs), and transforming growth factor-β1, critical for the development of liver fibrosis. Similarly, rAAV/siRNAeTIMP-1 treatment significantly alleviated bile duct ligation-induced liver fibrosis. Furthermore, this treatment dramatically suppressed TIMP-1 expression in HSCs from both model rats. These data indicate that the administration of rAAV/siRNAeTIMP-1 attenuated liver fibrosis by directly elevating the function of MMP-13 and diminishing activated HSCs. It also resulted in indirect decreased expression of type I collagen, MMP-2, and transforming growth factor-β1. In conclusion, rAAV/siRNAeTIMP-1 may be an effective antifibrotic gene therapy agent. (Am J Pathol 2013, 182: 1607–1616; http://dx.doi.org/10.1016/j.ajpath.2013.01.036)

Liver fibrosis is a common outcome of many chronic liver diseases, including viral and autoimmune hepatitis, iron deposition, alcohol consumption, and biliary obstruction. It results from dysregulation of the normal healing process and scar formation. Progression to liver fibrosis is characterized by increased synthesis and decreased degradation of extracellular matrix (ECM) proteins in the injured tissue. Liver injury leads to hepatic stellate cell (HSC) differentiation, which is associated with loss of their quiescent phenotype corresponding functionally to vitamin A storage. Activated HSCs acquire a myofibroblast-like phenotype and up-regulate the expression of cytoskeletal proteins, such as α-smooth muscle actin (α-SMA) and type I collagen.1–3 HSCs are the main fibrogenic cell population of the liver. Another population that has been shown to contribute to liver fibrosis is portal fibroblasts. They are located adjacent to bile duct epithelia and, thus, play a particularly significant role in biliary fibrosis.4,5

Along with the change of phenotype in activated HSCs, the most important roles of these cells in liver fibrosis are the increased synthesis of procollagen type I mRNA (mRNA) and protein,6 together with increased cellular proliferation, migration, and contractility.7,8 ECM degradation is mediated by matrix metalloproteinases (MMPs), a class of zinc- and calcium-dependent enzymes that are secreted as zymogens

Supported by the National Natural Science Foundation of China (grants 81000172, 81100287, and 30972602), the Basic-Clinic Cooperation project of Capital Medical University (grant 09JL-L02), and the Program for New Century Excellent Talents in University (grant NCET-09-0008), Municipal Key Laboratory of Beijing for Regulation of Liver Protection and Regeneration.
and activated by cleavage of their propeptide. Once secreted, MMP activity is regulated by the binding of specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). In addition to the increased production of type I collagen of activated HSCs, elevated production of TIMPs by HSCs shifts the balance between ECM synthesis and degradation toward ECM synthesis and fibrogenesis.

Among the four identified TIMPs, TIMP-1 is significantly up-regulated by several inflammatory cytokines, including IL-1β, IL-6, tumor necrosis factor α, and transforming growth factor (TGF)-β1. It has been identified that TIMP-1 is the most relevant TIMP involved in toxic liver injury and cholestasis. The hepatic mRNA content of TIMP-1 and procollagen type I has been reported to decrease significantly in the first week of spontaneous recovery from liver fibrosis in carbon tetrachloride (CCl4)—treated rats, which coincided with the most rapid phase of collagen degradation. In support of the role of TIMP-1 in vivo, transgenic mice over-expressing human TIMP-1 showed increased fibrosis in response to long-term CCl4 administration. Conversely, the use of MMP-9 mutant proteins as TIMP-1 antagonists, which were designed to retain binding to TIMP-1 without enzymatic activity, reduced fibrosis accumulation by enhancing matrix resorption. Many studies have shown that TIMP-1 can act not only to prevent the degradation of the accumulated ECM by blocking MMP function but also to inhibit apoptosis of activated HSCs, the cellular source of type I collagen. Thus, declining TIMP-1 levels may lead to an increase in MMP activity and a decrease in the number of activated HSCs by inducing apoptosis, resulting in liver fibrolysis.

A previous study showed that delivering siRNA targeted to rat TIMP-1 using a recombinant adenovirus (rAAV) could suppress TIMP1 gene expression in the rat HSC-T6 cell line for approximately 3 months and result in elevated MMP-13 expression, the main substrate of TIMP-1. In the present study, we further examined the antifibrotic effects of rAAV/siRNA—TIMP-1 in rat liver fibrosis models.

Materials and Methods

Construction and Dilution of rAAV/siRNA—TIMP-1 and rAAV/EGFP

Five siRNA duplexes were designed using an Ambion online tool for siRNA design (http://rnaiexpress.extend.do?pid=4838267262238482408). After transfection with siRNAs into rat HSCs, we screened one pair of siRNAs that effectively inhibited expression of the TIMP-1 gene in HSC-T6 cells. RAASirNA—TIMP-1 and rAAV/EGFP were constructed and packaged by Benyuan Zhengyang Gene Technology (Beijing, China) at titers of $1 \times 10^{12}$ and $2 \times 10^{12}$ vector genomes per milliliter (v.g/mL), respectively, after being purified. Preparation of the final injectable dose of $4 \times 10^{10}$ v.g per rat was performed by diluting $80 \mu$L of rAAV/siRNA—TIMP-1 and $40 \mu$L of rAAV/EGFP in 2 mL of sterile saline.

Animal Use and Care

Male Wistar rats (Xiehe Institute of Animal Research, Beijing, China) weighing a mean ± SD of 200.0 ± 20 g were subjected to a 12:12-hour light/dark environment and were allowed unlimited access to food and water. Study protocols complied with the guidelines for the use of laboratory animals drawn up by the Beijing Friendship Hospital, Capital Medical University (Beijing, China).

For the administration of CCl4, 38 rats were randomly divided into four groups. The control group ($n = 8$) was injected (i.p.) with 0.2 mL/100 g olive oil twice a week for 4 weeks. The the model group ($n = 10$), the rAAV/siRNA—TIMP-1 group ($n = 10$), and the rAAV/EGFP group ($n = 10$) were injected (i.p.) with 40% CCl4 in olive oil (4:6, v/v) at a dose of 0.2 mL/100 g twice a week for 4 weeks. For bile duct ligation (BDL), 35 rats were randomly divided into sham ($n = 7$), BDL ($n = 8$), rAAV/siRNA—TIMP-1 ($n = 10$), and rAAV/EGFP ($n = 10$) groups. Rats were anesthetized, and the common bile duct was ligated twice using silk sutures. Sham operation was performed similarly except that the bile duct was not ligated. The day after the first injection of CCl4 or BDL operation, rats in the control/sham group and the model group were injected with 2 mL of normal saline via the tail vein, and rats in the rAAV/siRNA—TIMP-1 and rAAV/EGFP groups were injected with 2 mL of normal saline containing $4 \times 10^{10}$ v.g of the respective recombinant viruses. After 7 days, rats were administered (i.v.) a maintenance dose. At the end of the fourth week, animals were sacrificed to obtain liver and serum samples. Samples were kept frozen at −80°C until being assayed.

Hepatocyte, HSC, and Kupffer Cell Isolation and Culture

To verify the infection efficiency in different cells, hepatocytes, HSCs, and Kupffer cells (KCs) were isolated from healthy rats 2 weeks after infection with rAAV/EGFP, as previously described with some modification. Briefly, hepatocytes were isolated by one-step collagenase perfusion of the rat liver, and HSCs were isolated by two-step collagenase-pronase perfusion of the rat liver followed by 8.2% Nycodenz medium (Accurate Chemical and Scientific Corp., Westbury, NY) two-layer discontinuous density gradient centrifugation. To get a higher yield of and more pure KCs, collagenase perfusion was performed, followed by 17.6% iodixanol gradient (OptiPrep; Nycomed Pharma, Oslo, Norway) layered onto 11.5% iodixanol gradient two-layer discontinuous density gradient centrifugation. To demonstrate the specificity of gene knockdown in different cells, hepatocytes, HSCs, and KCs were isolated from rats infected by rAAV/siRNA—TIMP-1 and rAAV/EGFP that were administered CCl4 or underwent BDL for 4 weeks by the same procedure. All the cells were cultured in suitable medium containing 10% fetal bovine serum. Twelve hours later, all the cells were collected for RNA and protein assay.
Histologic Examination

Liver samples were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. H&E and Masson staining were performed. Each sample was independently assessed and scored by two pathologists (Xiaoyan Shi and Guangyong Chen, Beijing Friendship Hospital) blinded to the study protocol according to a fibrosis scoring system recently published by Zhao et al. The severity of fibrosis was categorized into seven stages (0 to 6), where 0 indicates no fibrosis and 6 indicates cirrhosis. Additional sections were stained for α-SMA (dilution 1:500; Sigma-Aldrich, St. Louis, MO) and TGF-β1 (dilution 1:500; Abcam, Cambridge, UK). Immunostaining was quantified using Image-Pro Plus software version 7.0 (Media Cybernetics, Rockville, MD). All the staining was averaged from 10 randomly selected fields per slide.

ELISA for Types I and III Collagen, TIMP-1, and TGF-β1

Serum types I and III collagen, TIMP-1, and TGF-β1 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (types I and III collagen: Bluegene, Shanghai, China; TIMP-1 and TGF-β1: R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Hydroxyproline, Alanine Aminotransferase, and Aspartate Aminotransferase Measurement

Commercially available hydroxyproline detection kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and were used according to the manufacturer’s instructions. Serum alanine aminotransferase and aspartate aminotransferase activities were measured by kits from Sigma-Aldrich.

RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

Total cellular RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) extraction according to the manufacturer’s protocol. The reverse transcription reaction was performed using a reverse transcription kit (Promega, Madison, WI) and was treated with DNase I (Promega). Real-time quantitative PCR was performed using a 7500 real-time PCR instrument (Applied Biosystems, Carlsbad, CA). RNA normalization was performed against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative RT-PCR was performed at least three times, and primer sequences are summarized in Table 1.

Western Blot Analysis

The preparation of protein extracts from frozen livers or isolated cells, electrophoresis, and subsequent blot analysis were performed as previously described. We incubated blots with a rat antibody to GFP (dilution 1:200; Abcam), TGF-β1 (dilution 1:1000; Abcam), SMAD3 (dilution 1:1000; Cell Signaling Technology, Irvine, CA), and β-actin (dilution 1:2000; Sigma-Aldrich) and then visualized them using the enhanced chemiluminescence light method (Thermo Scientific, Logan, UT).

Statistical Analysis

Data are expressed as means ± SD. The t-test was used to determine statistical significance between groups. A P < 0.05 was considered statistically significant.

Table 1 Primers Used for Real-Time Quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers Forward</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timp1</td>
<td>5′-TCCTCCTGGCTGCTATGATAAGCTT-3′</td>
<td>NM_053819</td>
</tr>
<tr>
<td></td>
<td>5′-CGCTGTTATAAGGGTGCTG-3′</td>
<td>NM_133530.1</td>
</tr>
<tr>
<td>Mmp13</td>
<td>5′-GGAAGACCTCCTCTTTCTCA-3′</td>
<td>NM_031054.2</td>
</tr>
<tr>
<td></td>
<td>5′-TCATAGACAGCACTCTTTTG-3′</td>
<td>NM_03304.1</td>
</tr>
<tr>
<td>Mmp2</td>
<td>5′-CACCAACGGAGCTATGACC-3′</td>
<td>NM_032085.1</td>
</tr>
<tr>
<td></td>
<td>5′-TGTTGCCCAAGGAAGTGAAG-3′</td>
<td>NM_032085.1</td>
</tr>
<tr>
<td>Col1a1</td>
<td>5′-CATGTTCAAGCTTTGAGG-3′</td>
<td>NM_03034.1</td>
</tr>
<tr>
<td></td>
<td>5′-GCCAGCTGACTCATGAGATGC-3′</td>
<td>NM_03034.1</td>
</tr>
<tr>
<td>Col3a1</td>
<td>5′-GGATATGACGATGGAGAATG-3′</td>
<td>NM_021578</td>
</tr>
<tr>
<td></td>
<td>5′-GCCCTATTGAGGATGTTAC-3′</td>
<td>NM_021578</td>
</tr>
<tr>
<td>Acta2</td>
<td>5′-GAGCATCCGGACGTGGTTAC-3′</td>
<td>BC158550</td>
</tr>
<tr>
<td></td>
<td>5′-ACATGGCAAGGACATGAGA-3′</td>
<td>BC059110</td>
</tr>
<tr>
<td>Tgfβ1</td>
<td>5′-ATAACCGCTGATGGCTGCTT-3′</td>
<td>NM_021578</td>
</tr>
<tr>
<td></td>
<td>5′-TGAGACTGATCCATGATT-3′</td>
<td>NM_021578</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5′-CTATCCATGGATAAAAG-3′</td>
<td>NM_053819</td>
</tr>
<tr>
<td></td>
<td>5′-GTAGGCCAGGATGCCCTTT-3′</td>
<td>NM_053819</td>
</tr>
</tbody>
</table>

Results

rAAV/siRNA–TIMP-1 Decreased Histologic Fibrosis and Collagen Deposition in the CCl₄-Induced Fibrosis Model

Livers from control rats showed normal H&E and Masson staining around vessels. In contrast, livers from the model and rAAV/EGFP groups showed severe fibrosis, including septal fibrosis, extensive bridging, and fatty degeneration. Compared with the fibrosis model group, the rAAV/siRNA–TIMP-1 group showed good preservation of liver acini architecture and only mild bridging fibrosis (Figure 1A). Semiquantitative analysis of the fibrosis stage indicated that most rats in the model and rAAV/EGFP groups were at stages 3 and 4 (80%), whereas 20% of rats were at stage 5. In contrast, most rats (90%) in the rAAV/siRNA–TIMP-1 group were at stages 2 and 3, with only one at stage 4. The average stage of rats in the rAAV/siRNA–TIMP-1 group was 1.3 stages less than the model and rAAV/EGFP groups (Table 2).

There were no significant differences in serum levels of aspartate aminotransferase and alanine aminotransferase in the CCl₄, rAAV/siRNA–TIMP-1, and rAAV/EGFP groups (Figure 1B). Fibrotic rats showed a twofold increase in hepatic hydroxyproline levels compared with controls, whereas rats in the rAAV/siRNA–TIMP-1 group showed a 38% decrease (Figure 1C). Compared with the model group, types I and III collagen mRNA expression in the liver showed a 50% and 42% reduction, respectively, in the rAAV/siRNA–TIMP-1 group (Figure 1, D and E). A similar decrease in the serum concentrations of types I and III collagen was noted in the rAAV/siRNA–TIMP-1 group (Supplemental Figure S1, A and B).

Table 2 Stage of Liver Fibrosis by Treatment Group

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Liver fibrosis stage (No.)</th>
<th>Average stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>8 0 0 0 0 0 0 0</td>
<td>2.5</td>
</tr>
<tr>
<td>Model</td>
<td>10</td>
<td>9 0 0 0 4 4 2 0</td>
<td>3.8</td>
</tr>
<tr>
<td>rAAV/EGFP</td>
<td>10</td>
<td>0 0 0 4 4 2 0</td>
<td>3.8</td>
</tr>
<tr>
<td>rAAV/siRNA–TIMP-1</td>
<td>10</td>
<td>0 0 6 3 1 0 0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Figure 1 rAAV/siRNA–TIMP-1 treatment decreased histologic fibrosis and collagen deposition in CCl₄-induced fibrotic livers. Liver sections from control and CCl₄-treated rats administered the indicated therapeutic agent were stained for collagen deposition by H&E and Masson staining. A: Representative microscopic views of livers of control and CCl₄-treated rats treated with rAAV/EGFP or rAAV/siRNA–TIMP-1. Original magnification, ×100. Scale bars: 200 μm. B: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum were measured. C: Liver segments from control or CCl₄-treated rats administered the indicated treatments were analyzed for hydroxyproline levels. D and E: mRNA expression of types I and III collagen (COL) in liver tissues was measured by real-time PCR. *P < 0.05 versus the control group; †P < 0.05 versus the CCl₄ and rAAV/EGFP groups. Each sample was analyzed in duplicate (n ≥ 8 per group). Data are given as means ± SD. Dark brown shows the α-SMA positive cells, which was stained by DAB.
protein expressions of TIMP-1 in the rAAV/siRNA—TIMP-1 group were decreased by nearly 60% compared with the model and rAAV/EGFP groups (Figure 2, B and C).

Increased Activated MMP-13 Expression and Decreased Activated MMP-2 Expression by rAAV/siRNA—TIMP-1 Therapy in CCl₄-Induced Fibrotic Livers

We next assessed the effect of rAAV/siRNA—TIMP-1 treatment on the expression of MMP-13, the main substrate of TIMP-1, using real-time PCR for mRNA and Western blot analysis for pro- and active—MMP-13 expression. Accompanied by the increase in mRNA, expression of pro- and active—MMP-13 in the rAAV/siRNA—TIMP-1 group increased by 27% and 60%, respectively, compared with the CCl₄ and rAAV/EGFP groups (Figure 3, A and C). Although the expression of MMP-2 is not directly regulated by TIMP-1, we used the same methods to detect the expression of MMP-2 owing to its major role in fibrogenesis. Compared with the CCl₄ and rAAV/EGFP groups, mRNA and protein levels of MMP-2 in the rAAV/siRNA—TIMP-1 group were relatively decreased (Figure 3, B and D).

Decreased α-SMA Expression by rAAV/siRNA—TIMP-1 Therapy in CCl₄-Induced Fibrotic Livers

Although mRNA expression of α-SMA, which is a biomarker of activated HSCs in the liver, could be detected in all four groups, Western blot analysis for α-SMA revealed expression in only CCl₄-treated rats. The expression level of α-SMA was significantly decreased (70%) by treatment with rAAV/siRNA—TIMP-1 (Figure 4, A and B). To further address this finding, immunostaining of tissue sections for α-SMA expression revealed intense staining patterns in rats from the model and rAAV/EGFP groups. Administration of rAAV/siRNA—TIMP-1 resulted in 50% decreased positive staining in the sinusoids, demonstrating less activated HSCs after rAAV/siRNA—TIMP-1 treatment (Figure 4, C and D).

Decreased TGF-β1 Expression by Administration of rAAV/siRNA—TIMP-1 in the CCl₄-Induced Fibrosis Model

Among many inflammatory cytokines involved in liver fibrosis, TGF-β1 is the most potent profibrogenic factor involved in the initiation and maintenance of hepatic fibrogenesis. Real-time PCR, Western blot analysis, and serum
ELISA for TGF-β1 were performed to determine the effect of TGF-β1 on the development of liver fibrosis. Compared with the model and rAAV/EGFP groups, fibrotic rats administered rAAV/siRNA—TIMP-1 showed a significant decrease in TGF-β1 expression. The decrease in serum (20%) was less than that in liver tissue (70%) (Figure 5, A and B, and Supplemental Figure S1C). Similarly, immunostaining of tissue sections for TGF-β1 expression demonstrated intense staining patterns in rats in the model and rAAV/EGFP groups, whereas administration of rAAV/siRNA—TIMP-1 decreased TGF-β1 staining in liver sections compared with the CCl4 and rAAV/EGFP groups. Quantiﬁcation of TGF-β1—positive cells. D: A representative image from each group is shown. Original magniﬁcation, ×200. Scale bar = 100 μm. *P < 0.05 versus the control group; †P < 0.05 versus the CCl4 and rAAV/EGFP groups. Each sample was analyzed in duplicate (n ≥ 8 per group). Data are given as means ± SD.
resulted in approximately 44% reduced positive staining (Figure 5, C and D). There were no significant differences in the expression of SMAD3 in the CCl4, AAV/EGFP, and rAAV/siRNA–TIMP-1 groups (Supplemental Figure S2).

Administration of rAAV/siRNA–TIMP-1 Dramatically Suppressed TIMP-1 Expression and Up-Regulated MMP-13 Expression in HSCs from CCl4 Rats

After 4 weeks of CCl4 administration, TIMP-1, MMP-13, and MMP-2 expression in hepatocytes, HSCs, and KCs isolated from rats exposed to rAAV/EGFP or rAAV/siRNA–TIMP-1 treatment was detected by real-time PCR and Western blot analysis (Figure 6A). mRNA expression of TIMP-1 in HSCs isolated from rAAV/siRNA–TIMP-1 rats showed a 37% reduction compared with the levels in HSCs from rAAV/EGFP rats, and the protein level of TIMP-1 in HSCs isolated from rAAV/siRNA–TIMP-1 rats showed a >90% decrease (Figure 6, B and C). TIMP-1 expression in KCs was less reduced in rAAV/siRNA–TIMP-1 rats compared with rAAV/EGFP rats. MMP-13 expression was increased in HSCs from rAAV/siRNA–TIMP-1 rats compared with those from rAAV/EGFP rats (a >90% increase in mRNA and an approximately 20% increase in protein). Although MMP-13 could also be produced by KCs, the increased expression was not so significant from rAAV/EGFP rats compared with rAAV/siRNA–TIMP-1 rats (a 47% increase in mRNA and an approximately 10% increase in protein). MMP-2 expression showed no significant changes in HSCs and KCs from these two groups of rats (Figure 6, B and C).

rAAV/EGFP Efficiently Infected HSCs in Vivo and Administration of rAAV/siRNA–TIMP-1 Attenuated BDL-Induced Liver Fibrosis

Two weeks after rAAV/EGFP infection in healthy rats, hepatocytes, HSCs, and KCs were isolated (Figure 7A), and GFP expression was detected. Although rAAV/EGFP could infect hepatocytes and KCs, the efficiency in HSCs was higher, as detected by Western blot analysis (Figure 7B), demonstrating that rAAV could efficiently infect HSCs in vivo.

After 4 weeks of BDL, TIMP-1 expression in hepatocytes, HSCs, and KCs isolated from rats exposed to rAAV/EGFP or rAAV/siRNA–TIMP-1 treatment was detected by Western blot analysis. TIMP-1 expression in HSCs isolated from rAAV/siRNA–TIMP-1 rats showed a 68% reduction compared with the levels in HSCs from rAAV/EGFP rats (Figure 7, C and D). H&E and Masson staining of liver sections demonstrated that BDL resulted in a disruption of
Histologically, less fibrosis was observed after rAAV/siRNA–TIMP-1 treatment compared with the BDL group (Figure 7E).

**Discussion**

Hepatic fibrosis, when moderate, is usually reversible when an etiologic treatment exists. However, reversibility of cirrhosis is much more problematic and depends on the etiology and stage of the liver disease. The development of fibrosis, especially cirrhosis, is associated with significant morbidity and mortality. Thus, developing antifibrotic strategies that are applicable to liver fibrosis is of considerable importance.

Based on the mechanisms of liver fibrosis, two strategies are possible: increase the levels of ECM-degrading MMPs and decrease the levels of their natural inhibitors, TIMPs.

Recent studies using adenoviral introduction of human MMP-1 or administration of an antibody against TIMP-1 in a rat model of liver fibrosis have shown attenuation in fibrosis levels. A previous study compared the suppressive effects of antisense RNA and siRNA targeting the TIMP-1 gene in HSCs. These results confirmed that RNA interference exerted suppressive effects on the TIMP-1 gene, thus increasing MMP-13 expression in cultured HSCs for a longer time when an rAAV was used as the gene delivery vector. Administration of rAAV/siRNA–TIMP-1 may, therefore, be an effective strategy to treat liver fibrosis.

To evaluate the effects of rAAV/siRNA–TIMP-1 administration on CCL4-induced hepatic fibrosis, three main methods were performed: histologic analysis of collagen accumulation, evaluation of hydroxyproline content in the liver, and determination of types I and III collagen concentrations in the liver and serum. As demonstrated by H&E and
Masson staining, extensive fibrosis, including bridging or even pseudolobular fibrosis (stages 4 and 5), was observed in rats from the model and rAAV/EGFP groups. In contrast, the level of collagen fibers was considerably less in the therapy group than in the model group.

Moreover, hepatic hydroxyproline content, which is the gold standard for determining collagen accumulation, was dramatically reduced by rAAV/siRNA—TIMP-1 treatment and paralleled the levels of types I and III collagen mRNA expression in the liver and collagen protein in serum. These data indicate that rAAV/siRNA—TIMP-1 therapy alleviates the degree of liver fibrosis induced by CCl₄.

Using another liver fibrosis model based on BDL, the antifibrotic effect of rAAV/siRNA—TIMP-1 was histologically confirmed.

One of the most important roles of TIMP-1 is to inhibit activated MMPs by binding to the active site of the MMPs after they activated from the prometalloproteinases form, subsequently protecting newly synthesized collagen from degradation by MMPs. Because of the predominance of types I and III collagen in fibrotic liver, expression of the interstitial collagenase activity would be necessary to initiate collagen degradation.⁹,²⁷ After having demonstrated TIMP-1 suppression by rAAV/siRNA—TIMP-1, we focused on the main substrate of TIMP-1 in liver tissue, MMP-13, which is the major interstitial collagenase in rats.²⁷ Associated with the inhibition of TIMP-1 in liver tissue, mRNA and active protein levels of MMP-13 were elevated in the rAAV/siRNA—TIMP-1 group. This elevation could be partially responsible for the attenuation of liver fibrosis. The increased expression of MMP-13 was also detected in isolated HSCs from rats treated with rAAV/siRNA—TIMP-1, which was induced by positive feedback from the decreased expression of TIMP-1. Although KCs also contributed to MMP-13 synthesis, the expression of MMP-13 in these cells was not significantly increased owing to less infection efficiency by rAAV/siRNA—TIMP-1. MMP-2, unlike the expression of other MMPs that increase immediately after insult and diminish over time, is secreted mainly by activated HSCs. In addition, MMP-2 expression and activity are greatly increased during fibrogenesis and remain elevated during fibrotic progression.²⁷–³⁰ Increased MMP-2 activity is believed to be associated with increased degradation of normal liver matrix, leading to increased HSC activation and synthesis of type I collagen.³¹ TIMP-1 is not important for the direct regulation of MMP-2 but may indirectly regulate MMP-2 expression through apoptosis stimulation of activated HSCs. The present data showed decreased mRNA and active protein levels of MMP-2 in liver tissues, likely related to a reduced number of activated HSCs. As an indirect regulator of MMP-2, down-regulation of TIMP-1 could not reduce its expression in the isolated HSCs from rats treated with rAAV/siRNA—TIMP-1.

TIMP-1 is important for inhibiting the apoptosis of activated HSCs. It has been shown that TIMP-1 has a direct and significant antiapoptotic effect on human and rat HSCs.⁸ Although in this study we did not directly explore HSC apoptosis, we demonstrated, in the CCl₄ model, that rAAV/siRNA—TIMP-1 administration significantly decreased levels of α-SMA protein, which is a biomarker for activated HSCs. Moreover, by immunostaining, there was a marked reduction in α-SMA—positive cells in the rAAV/siRNA—TIMP-1 group accompanied by decreased collagen fibers. Collectively, these results support the view that rAAV/siRNA—TIMP-1 therapy could diminish the number of activated HSCs.

A further interesting finding in the present study was that rAAV/siRNA—TIMP-1 therapy decreased the expression of TGF-β1 in liver tissue and serum. TGF-β1 promotes wound healing and repair. Under pathologic conditions, TGF-β1 orchestrates cross talk among parenchymal, inflammatory, and collagen-expressing cells and plays a key role in stimulating fibrosis.³²–³⁵ Several studies have focused on the suppression of TGF-β1 activation and intervention of the TGF-β1/SMAD signaling pathway to treat liver fibrosis.³⁶,³⁷ However, because a generalized blockage of TGF-β1 activity may result in the promotion of carcinogenesis and excessive immune reactions, much attention has to be given to the selective intervention of the TGF-β1/SMAD signal specifically in collagen-producing cells. Herein, we confirmed that TGF-β1 mRNA and protein levels in the liver tissue decreased compared with the CCl₄ model and rAAV/EGFP groups. Similarly, immunostaining demonstrated that rAAV/siRNA—TIMP-1 administration could cause a decline in the number of TGF-β1—positive interstitial cells. Because TGF-β1 expression in activated HSCs was increased, the finding that TGF-β1 expression was decreased might be explained by the decreased number of activated HSCs, a major source of TGF-β1 in liver fibrosis.

Conclusions

Suppression of TIMP-1 is mechanistically important in promoting fibrolysis by directly elevating the function of MMP-13, which is a main metalloproteinase that degrades types I and III collagen, and by diminishing the number of activated HSCs, thus indirectly decreasing the expression of type I collagen, MMP-2, and TGF-β1, which are potent factors in liver fibrogenesis. The first and still most extensive clinical experience concerning the administration of AAV vectors comes from the treatment of cystic fibrosis.³⁸,³⁹ Although the transduction of rAAV may activate a host immune response, many ongoing genetic improvements should provide, in the near future, new important inputs for therapeutic approaches and pave the way for AAV-based drug commercialization.⁴⁰ The present results suggest that AAV vectors also have the potential for the therapeutic delivery of genes targeting HSCs, which is consistent with a previous study by Tsui et al.⁴¹ Using rAAV, the present approach to antifibrosis might avoid the possible destruction of plasmid or multiple administrations of antibodies. Thus, rAAV/siRNA—TIMP-1 treatment successfully attenuated...
fibrosis in rats, demonstrating its potential as an effective antifibrotic gene therapy.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.01.036.

References

34. Shiek FW, Benyon RC: How can transforming growth factor beta be targeted usefully to combat liver fibrosis? Eur J Gastroenterol Hepatol 2004, 16:123–126
35. Hayashi H, Sakai T: Biological significance of local TGF-beta activation in liver diseases. Front Physiol 2012, 3:12–21