

ORIGINAL ARTICLE

siRNA-mediated knockdown of connective tissue growth factor prevents N-nitrosodimethylamine-induced hepatic fibrosis in rats

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Hepatic fibrosis is a dynamic process that involves the interplay of different cell types in the hepatic tissue. Connective tissue growth factor (CTGF) is a highly profibrogenic molecule and plays a crucial role in the pathogenesis of hepatic fibrosis. The aim of the present investigation was three-fold. First, we studied the expression of CTGF in the cultured hepatic stellate cells using immunohistochemical technique. Second, we induced hepatic fibrosis in rats through serial intraperitoneal injections of N-nitrosodimethylamine (NDMA; dimethylnitrosamine, DMN) and studied the upregulation of CTGF and TGF- β 1 during hepatic fibrogenesis. Third, we downregulated CTGF expression using CTGF siRNA and examined the role of CTGF siRNA to prevent the progression of NDMA-induced hepatic fibrosis. The results depicted strong staining of CTGF in the transformed hepatic stellate cells in

culture. Serial administrations of NDMA resulted in activation of hepatic stellate cells, upregulation of CTGF and TGF- β 1 both at mRNA and protein levels and well-developed fibrosis in the liver. Immunostaining, Western blot analysis, semiquantitative and real-time RT-PCR studies showed downregulation of CTGF and TGF- β 1 after treatment with CTGF siRNA. The results of the present study demonstrated that CTGF gene silencing through siRNA reduces activation of hepatic stellate cells, prevents the upregulation of CTGF and TGF- β 1 gene expression and inhibits accumulation of connective tissue proteins in the liver. The data further suggest that knockdown of CTGF upregulation using siRNA has potential therapeutic application to prevent hepatic fibrogenesis.

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Keywords: hepatic fibrosis; N-nitrosodimethylamine; dimethylnitrosamine; connective tissue growth factor; CTGF; siRNA

Introduction

Hepatic fibrosis refers to the accumulation of connective tissue proteins especially interstitial collagens in the extracellular space of liver parenchyma.^{1,2} Hepatic fibrosis is a dynamic process that involves the interplay of different cell types in the hepatic tissue. The transformation of quiescent hepatic stellate cells into myofibroblast-like cells with the expression of smooth muscle actin filaments initiates the chronic process of hepatic fibrosis that may end with the fatal stage of liver cirrhosis. Several factors such as toxins, viruses, oxidative stress, necrosis, apoptosis and growth factors are responsible for the activation of resting hepatic stellate cells.^{3,4} A cascade of signaling and transcriptional events in the activated stellate cells underlies the pathogenesis of hepatic fibrosis. Although the understanding of the pathophysiology of hepatic fibrosis development has increased significantly over the past few years, an effective treatment has not yet been developed. Limits to the efficacy of current treatment modalities call for the

development of novel therapeutic approaches targeting the specific molecular features of hepatic fibrosis. Regulation of the several steps involved in the activation and transformation of hepatic stellate cells offers a potential therapeutic target to prevent hepatic fibrosis and liver cirrhosis.

Connective tissue growth factor (CTGF) is a multifunctional protein involved in the regulation of cell growth and tissue remodeling. CTGF expression is generally very low in normal liver. However, CTGF expression gradually increases with the progression of hepatic fibrosis and other chronic liver injury, exhibiting a significant correlation with fibrogenesis. CTGF plays a key role in the pathogenesis of hepatic fibrosis and stimulates the transformation of resting hepatic stellate cells into myofibroblasts, which leads to the production of more CTGF.^{5,6} CTGF also stimulates the production of collagens, fibronectin and laminin, the predominant molecules of the extracellular matrix (ECM) of the liver.^{7,8} The inhibition of CTGF-mediated hepatic stellate cell activation and the related ECM production may be a promising strategy to prevent hepatic fibrosis and alcoholic cirrhosis.

Transforming growth factor-1 (TGF- β 1) regulates various biological activities including cell differentiation, proliferation, apoptosis, angiogenesis, ECM synthesis and fibrosis. TGF- β 1 stimulates the synthesis of

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connective tissue components and inhibits ECM degradation through autocrine and paracrine mechanisms and plays a key role in the pathogenesis of hepatic fibrosis.^{7,9} TGF- β also plays a crucial role in triggering the cascade of events that culminates in the production of CTGF, which causes the formation of nodular fibrosis in the liver. CTGF not only directly mediates hepatic stellate activation and ECM production but also specifically mediates the profibrogenic activity of TGF- β *in vitro* and *in vivo*.^{10,11} CTGF synergizes with TGF- β to promote a sustained fibrogenic response *in vivo*.^{12,13} Downregulation of CTGF expression can inhibit both CTGF- and TGF- β 1-mediated ECM production both *in vitro* and *in vivo*.^{14,15} So blocking the overexpression of CTGF could be a potential strategy to prevent the accumulation of ECM proteins and thus hepatic fibrosis.

The small interfering RNA (siRNA) is an incredibly powerful tool to specifically knockdown a gene's message, and subsequently the protein level of the targeted gene. The siRNA-mediated gene knockdown is a process of highly sequence-specific, post-transcriptional gene silencing initiated by a synthetic double-stranded RNA (dsRNA) molecule. Introduction of the CTGF siRNA into cells triggers degradation of CTGF endogenous mRNA. However, this is a challenging process and it is necessary to administer CTGF siRNA repeatedly for the continuous knockdown of CTGF mRNA *in vivo* in order to prevent the progression of hepatic fibrosis.

It has been demonstrated that *N*-nitrosodimethylamine (NDMA)-induced hepatic fibrosis in rats is a quick and reproducible animal model for studying the biochemical and pathophysiological alterations associated with the development of hepatic fibrosis.^{16–18} This model is highly appropriate for investigating the molecular mechanism of the development of hepatic fibrosis and also to screen antifibrotic agents.^{19,20} The aim of the present investigation was three-fold. First, we were interested to know whether activated hepatic stellate cells in culture could express CTGF. Second, we induced hepatic fibrosis in rats through serial administrations of NDMA and studied whether CTGF is upregulating during NDMA administration. Third, we downregulated CTGF expression at the translational level using CTGF siRNA and examined whether CTGF mRNA knockdown could prevent the progression of NDMA-induced hepatic fibrosis. As TGF- β 1 has synergetic action on the expression of CTGF, we monitored the expression of TGF- β 1 also both at the gene and protein levels. As systemic administration of siRNA is not effective to the target organs, we used intraperitoneal route to deliver siRNA to the liver. This is the first study to demonstrate that intraperitoneal administration of CTGF siRNA effectively prevents the progression of hepatic fibrosis in an animal model.

Materials and methods

Isolation, purification, characterization and culture of stellate cells from rat liver

Hepatic stellate cells (perisinusoidal cells) were isolated from about 12-month-old albino male rats of the Wistar strain. The animals were anaesthetized with 0.4 ml of sodium pentobarbital (Nembutal, 50 mg/ml) through

intraperitoneal injection. The liver was first perfused through the portal vein with Hanks balanced salt solution (HBSS) at 37°C for about 10 min at a flow rate of 10 ml/min followed by 0.2% pronase E (Calbiochem, EMD Biosciences, San Diego, CA, USA) in HBSS for 5 min. The perfusion was continued with HBSS containing 0.05% collagenase (Sigma, St Louis, MO, USA) and 0.05% pronase E at 37°C for 30 min at a flow rate of 10 ml/min. After perfusion, the liver was excised carefully and the Glisson's capsule was removed. The tissue was transferred to a sterile beaker, cut into small pieces and gently made into a paste. It was agitated gently on a magnetic stirrer with 50 ml of HBSS containing 0.02% pronase E and 0.05% collagenase for 30 min at 37°C. The suspension was filtered through double-layer cotton gauze and centrifuged at 1800 r.p.m. for 7 min. The cell pellet was resuspended in 10 ml of HBSS and dispersed through a syringe to further dissociate the cells.

The stellate cells were isolated from the cell suspension containing endothelial and Kupffer cells using a triple layer density (18, 13 and 8%) cushion of metrizamide (Sigma-Aldrich, St Louis, MO, USA) prepared in HBSS solution. A stock of 30% (w/v) metrizamide solution was diluted appropriately with HBSS to obtain the 18 and 13% metrizamide solutions. The gradient was prepared carefully with 7.5 ml each of 18 and 13% metrizamide on a 50-ml corning tube. The top layer of the gradient was prepared with 4.0 ml of 30% metrizamide and 11.0 ml of cell suspension in HBSS. It was centrifuged carefully at 3300 r.p.m. for 17 min. The stellate cells, which formed as a pale white ring at the top were carefully removed and washed three times in HBSS to remove the remnants of metrizamide. The purity of the isolated stellate cells was confirmed through immunohistochemical staining of desmin, a characteristic antigen of stellate cells. The desmin antibody was procured from Nichirei Corporation, Tokyo, Japan. More than 95% of the isolated cells were desmin positive. The purified stellate cells were suspended in Dulbecco's modified Eagles medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and antibiotics on 100-mm culture dishes at 37°C in a humidified atmosphere containing 5% CO₂ in air. About 50% confluent stellate cells were subcultured to collagen-fibronectin coated chamber slides until the cells were fully activated and transformed into myofibroblasts through autocrine mechanism.

Immunohistochemical staining of α -smooth muscle actin and CTGF

The immunohistochemical staining for α -smooth muscle actin (α -SMA) and CTGF for cultured stellate cells was carried out using horseradish peroxidase-conjugated streptavidin system (Dako, Carpinteria, CA, USA). In brief, the transformed stellate cells on chamber slides were fixed in ice-cold methanol for 10 min, washed twice in PBS and treated with 3% hydrogen peroxide for 5 min to quench the endogenous peroxidase activity. The cells were then washed and treated with α -SMA antibody (Nichirei Corporation, Tokyo, Japan) at room temperature for 1 h. The CTGF antibody (Abcam, Cambridge, MA, USA) was diluted 1:200, added to the slides and incubated overnight at 4°C. The slides were washed in PBS and further treated with biotinylated second anti-

body and streptavidin peroxidase. The final color was developed using the substrates 3-amino-9-ethylcarbazole (AEC) for α -SMA and with 3,3'-diaminobenzidine (DAB) for CTGF which produce red and brown color respectively. The cells were counterstained with Mayer's hematoxylin and mounted using an aqueous-based mounting medium and photographed.

Construction of siRNA expression vector

The siRNAs for rat CTGF mRNA were procured from GenScript (Piscataway, NJ, USA). We prepared five siRNAs and the most effective one was selected for the present investigation on the basis of *in vitro* studies on hepatic stellate cells. The selected siRNA sequence targeting rat CTGF mRNA begins at nucleotide 890 (NM_022266), 5'-CAA UAC CUU CUG CAG GCU GGAtt-3' (sense) and 3'-ttGUU AUG GAA GAC GUC CGA CCU-5' (antisense). The scrambled siRNA sequence was, 5'-GGG ACG CAC UAC CUA GAC UUUtt-3' (sense) and 3'-ttCCC UGC GUG AUG GAU CUG AAA-5' (antisense). The loop selected was 5'-TTG ATA TCC G-3'. The linear siRNA construct – with the sense and antisense strand, termination signal, *Bam*HI and *Hind*III restriction sites – was annealed with the complimentary strand in 6 × SSC (NaCl–sodium citrate) by heating to 90°C and then slow cooling to 37°C. The double-stranded oligo was then ligated into a mammalian siRNA expression vector, pRNA-CMV3.1/Neo (GenScript, Piscataway, NJ, USA) between the *Bam*HI and *Hind*III sites. The powerful cytomegalovirus (CMV) promoter drives the expression of CTGF siRNA. The plasmid vector carrying the siRNA was transfected into JM109 competent cells (Promega Corporation, Madison WI, USA), and the positive colonies were screened using Qiagen miniprep (Qiagen, Valencia, CA, USA) plasmid DNA purification system. The highly expressing colony was propagated several times and the plasmid DNA expressing CTGF siRNA was purified using maxiprep (Qiagen, Valencia, CA, USA).

Delivery of CTGF siRNA into rat livers

CTGF siRNA was delivered into the animal liver through intraperitoneal injections. The siRNA-recombinant plasmid DNA vector was mixed with a suitable amount of lipophilic transfection reagent, *in vivo* jetPEI (Qbiogene, Carlsbad, CA, USA) in 5% glucose solution. The siRNA was absorbed quickly, entered the circulatory system and reached the liver through hepatic portal veins. The highly lipogenic CTGF siRNA entered both parenchymal and non-parenchymal cells of the liver within a short time. We have studied the absorption of a mammalian expression plasmid, the pHCMV-FSR vector contains a reporter gene luciferase (5.9 kb) into the liver. The animals were injected with luciferase plasmid vector, pHCMV-FSR (procured from Genlantis, San Diego, CA, USA and propagated in JM109 competent cells) intraperitoneally at a concentration of 1 mg/kg body weight and screened at 2, 6 and 24 h after the injection to study the absorption and distribution of the reporter gene. At 2, 6 and 24 h, the animals were injected with D-luciferin (Gold Biotechnology, St Louis, MO, USA) at a concentration of 25 mg/rat and scanned after 10 min on a Xenogen *in vivo* imaging system (IVIS) using Xenogen image analysis software (Xenogen IVIS-200, Alameda, CA, USA). In the present study, we used a dose of 1 mg

CTGF siRNA plasmid vector per kilogram body weight of the animal. The dosage was selected on the basis of prior dose–response study.

Induction of hepatic fibrosis on experimental animals

The animal experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals, Kanazawa Medical University, Japan. About 2-month-old albino male rats of the Wistar strain, weighing around 200 g, were used for the induction of hepatic fibrosis. The animals were procured from the local vendor and maintained with commercial rat feed (Nihon Nousan Industry, Yokohama, Japan) and water available *ad libitum*. They were housed in polypropylene cages with a wire mesh top and hygienic animal beddings with proper humane care. A total of 36 animals were used in the present investigation. The animals were divided into four groups: (A) control group of six animals, which received intraperitoneal injections 0.15 M saline without NDMA; (B) NDMA group comprises 12 animals, which received intraperitoneal injections of NDMA in doses of 10 mg/kg body weight daily for seven consecutive days;³ (C) CTGF siRNA control group of six animals, which received scrambled CTGF siRNA daily for up to 14 days; and (D) NDMA-CTGF siRNA group of 12 animals, which received intraperitoneal injections of CTGF siRNA plasmid vector in doses of 1 mg/kg body weight daily 2 h before the administration of NDMA (as in group B) and afterwards daily until the killing of the animals on day 14. All the injections were given without anesthesia.

The experimental animals (six each from groups B and D) were killed by decapitation on days 7 and 14 from the start of the administration of NDMA as well as CTGF siRNA. Owing to the unacceptable death rates based on our previous studies, we did not keep the experimental animals for more than 14 days. In the present investigation, none of the animals died. Three animals each from both control groups (groups A and C) were killed along with the experimental animals on days 7 and 14, and the pooled samples were used as controls. All the animals were anaesthetized with isoflurane before killing. The liver tissue was quickly removed and a portion was instantly frozen in liquid nitrogen for PCR analysis. Another portion was simultaneously fixed in 10% phosphate-buffered formalin for histopathological studies. The remaining liver tissue was stored at –80°C for Western blot analysis.

Evaluation of hepatic fibrosis

The pathogenesis of NDMA-induced hepatic fibrosis and the effects of CTGF siRNA were evaluated through hematoxylin and eosin (H&E) as well as Masson's trichrome staining. The paraffin-embedded tissues were cut into sections of 5 μ m thickness, heated in a vacuum oven at 60°C for 20 min, deparaffinized and hydrated. The serial sections were then stained for H&E and Masson's trichrome as per standard protocols. The stained sections were examined under an Olympus research microscope, BX61 (Olympus corporation, Tokyo, Japan) and photographed. The activation of hepatic stellate cells indicated by the staining of α -SMA filaments is also considered as a marker for the degree of hepatic fibrosis and also as the effects of CTGF siRNA for the inhibition of fibrogenesis.

Immunohistochemistry for α -SMA and CTGF on liver sections

The immunohistochemical staining for α -SMA and CTGF on paraffin sections was carried out as cultured stellate cells on chamber slides. The serial liver sections were deparaffinized using xylene and alcohol and hydrated to water. The sections were then treated with α -SMA and CTGF primary antibodies, followed by biotinylated second antibody and streptavidin peroxidase. The final stain was developed using AEC for both α -SMA and CTGF. The cells were counterstained with Mayer's hematoxylin, treated with ammonia water and mounted using aqueous-based mounting medium and photographed. The staining intensity of α -SMA and CTGF was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Analysis of hyaluronic acid and TGF- β 1 in serum

Hyaluronic acid (HA) concentrations in rat serum were determined using an ELISA-based sandwich HA-binding protein assay kit (Chugai Diagnostics Science, Tokyo, Japan) as per the manufacturer's instructions, which follows the method of Chichibu *et al.*²¹ The TGF- β 1 levels in rat serum were determined using a quantitative sandwich enzyme assay kit (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions. In brief, 50 μ l of activated and 60-fold diluted serum samples were added to a microplate pre-coated with TGF- β 1 monoclonal antibody followed by 50 μ l of assay diluent provided in the kit. It was mixed gently and incubated for 2 h at room temperature (RT). The wells were washed four times with wash buffer provided in the kit and 100 μ l of TGF- β 1 conjugate was added and incubated for 2 h at RT. Finally, 100 μ l of substrate solution was added and incubated for 30 min in the dark for color development. The reaction was terminated and the intensity of the color was measured on a microplate reader at 450 nm.

Western blot analysis for CTGF and TGF- β

The frozen liver tissue was cut into very small pieces and transferred to 3 ml of freshly prepared ice-cold RIPA buffer (Tris-HCl (50 mM, pH 7.4) containing 1% Nonidet P-40, 150 mM NaCl, 1 mM activated sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 1 mM EDTA, 5 μ g/ml aprotinin and 5 μ g/ml pepstatin) per gram of tissue and homogenized on an ice bath with a Polytron homogenizer (Brinkmann Instruments, Burlingame, CA, USA). It was centrifuged at 14 000 r.p.m. for 10 min at 4°C and the supernatant was collected. The protein concentration in the supernatant was determined using bicinchoninic acid protein assay by Smith *et al.*²²

About 100 μ g protein was used for resolving CTGF and 50 μ g for TGF- β . All the samples were mixed with 6 $\times$ loading buffer, which contained 600 mM dithiothreitol. The samples were boiled for 3 min and separated on a 10% SDS-PAGE. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The non-specific sites on the membrane were blocked with 4% BSA in Tris-buffered saline containing 0.05% Tween-20 at RT for 30 min. The membranes were then incubated at 4°C overnight with CTGF antibody (Abcam, Cambridge, MA, USA) and TGF- β antibody (Cell Signaling Technology, Danvers, MA, USA) that had been diluted 1:5000

and 1:1000, respectively, in PBS containing 0.1% Tween-20 and 1% bovine serum albumin. The membranes were washed three times in 0.05% Tween-20 and treated with 1:2000 diluted anti-rabbit HRP conjugated second antibody (Biomedex, Foster City, CA, USA) at RT for 1 h. The membranes were washed thrice in 0.05% Tween-20 and visualized using an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

The membranes were reprobed using Western reprobe buffer (Gbiosciences, St Louis, MO, USA) and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content to demonstrate that similar amounts of protein had been loaded in each lane. Mouse monoclonal GAPDH antibody procured from Novus Biologicals, Littleton, CO, USA was used.

Real-time and semiquantitative RT-PCR for CTGF and TGF- β mRNA

The total cellular RNA was isolated from the frozen liver tissue using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The gene-specific primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA). The following are the primer sequences used: CTGF (No. NM_022266, forward 5'-AAA TGC TGT GAG GAG TGG-3', reverse 5'-GCA GAA GGT ATT GTC ATT GG-3'), TGF- β 1 (No. NM_021578; forward 5'-ATT CCT GGC GTT ACC TTG G-3', reverse 5'-CCT GTA TTC CGT CTC CTT GG-3'); GAPDH (No. NM_017008; forward 5'-TCA ACG GCA CAG TCA AGG-3', reverse 5'-TTC TGA GTG GCA GTG ATG G-3'). Real-time RT-PCR was carried out using a one-step RT-PCR kit with SYBR green (Bio-Rad Laboratories, Hercules, CA, USA) on a real-time PCR machine (iCycler, Bio-Rad Laboratories, Hercules, CA, USA) with the following reaction conditions: cDNA synthesis, 10 min at 50°C; reverse transcriptase inactivation, 5 min at 95°C; thermal cycling and detection (up to 35 cycles), 10 s at 95°C; 30 s at 56°C (data collection). About 100 ng of total isolated RNA was transcribed. GAPDH was used as a housekeeping gene.

Semiquantitative RT-PCR was also carried out for CTGF and TGF- β 1 mRNA expression for the visualization of PCR products on ethidium bromide gels. The above primers were transcribed with 100 ng of isolated total RNA using a one-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) on a thermocycler (GeneAMP PCR Systems 9700, Applied Biosystems, Foster City, CA, USA) with the following reaction conditions: cDNA synthesis, 50°C for 30 min; inactivation, 94°C for 2 min; PCR amplification of 35 cycles, denature at 94°C for 20 s, annealing at 56°C for 30 s, chain extension, 72°C for 45 s and a final chain extension at 72°C for 10 min. The amplified products were separated on 1.5% agarose gels with ethidium bromide and visualized using a transilluminating UV (Alpha Innotech Corporation, San Leandro, CA, USA) integrated with a computer.

Statistical analysis

Arithmetic mean and standard deviation (s.d.) were calculated for the data. The results were statistically evaluated using Student's paired *t*-test. The NDMA values on days 7 and 14 were compared with the respective CTGF siRNA values. The value of $P < 0.05$ was considered as statistically significant.

Results

Immunohistochemical staining of α -SMA and CTGF

Our previous studies demonstrated that NDMA-induced model of hepatic fibrosis in rats is an excellent and potential model for studying the pathobiochemical and molecular biological events associated with the development of early human cirrhosis. The expression of α -SMA is a characteristic feature of activated liver stellate cells and considered as a marker for the triggering of hepatic fibrosis and related cellular events. The resting stellate cells isolated and cultured from healthy adult rat livers were transformed into myofibroblasts through autocrine mechanism. Immunohistochemical staining for α -SMA demonstrated strong positive staining of actin filaments in the transformed stellate cells (Figure 1a). The activation of hepatic stellate cells is associated with the upregulation of numerous genes associated with the transcription of several cytokines and growth factors involved in the triggering of hepatic fibrosis. The activated stellate cells dramatically upregulate the expression of CTGF and TGF- β 1. Immunohistochemical staining of cultured activated hepatic stellate cells depicted strong positive staining for CTGF (Figure 1b). Therefore, our study proved that hepatic stellate cells express CTGF upon activation.

Absorption of CTGF siRNA into rat livers

siRNA can be delivered *in vivo* through different methods. We adopted the intraperitoneal method because it is the best method for the maximum absorption to the target organ, the liver. The plasmid vector carrying the reporter gene, luciferase, demonstrated that plasmid vectors ligated with a gene of interest or siRNAs can be absorbed quickly, enter the circulatory system and reach the liver as well as the other vital organs of the body within a short time after administration. The pRNA-CMV3.1/Neo vector carrying the CTGF siRNA was only 5.2 kb, compared to the pHCMV-FSR vector carrying the luciferase gene, which was 5.9 kb. This indicates a better absorption of the CTGF siRNA vector compared to the higher kb plasmid vector carrying the luciferase reporter gene. The live animal imaging using Xenogen IVIS-200, after administration of D-luciferin, demonstrated a strong bioluminescence signal in the liver tissue in all the three scanning at 2, 6 and 24 h with a maximum luminescence signal at 6 h. At 2 h, strong signal was observed in the liver and lungs with an increase at 6 h in both tissues. At 24 h, moderate signal was present in the eyes, liver, lungs, kidney, intestine and urinary bladder. This indicated that the luciferase gene has been expressed inside the cells and translated into the active luciferase enzyme, which oxidizes the substrate luciferin and produces the bioluminescence. This experiment clearly demonstrated that the intraperitoneal administration is an appropriate route to deliver siRNA or other gene of interest through plasmid vectors into experimental animals.

Histopathological evaluation of hepatic fibrosis and effect of CTGF siRNA

The histopathological evaluation of the progression of NDMA-induced hepatic fibrosis and the role of

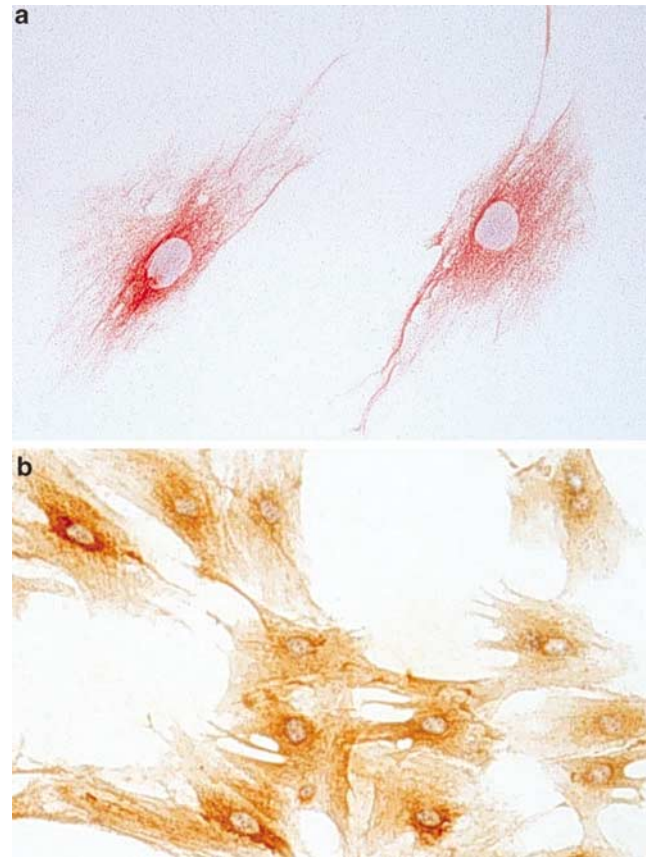


Figure 1 (a) Immunohistochemical staining of transformed hepatic stellate cells for α -SMA. The hepatic stellate cells were isolated from rat liver after pronase and collagenase digestion and metrizamide gradient centrifugation. The isolated cells were stained immunohistochemically for the presence of desmin antigen, which is characteristic for liver stellate cells. The desmin positive stellate cells were cultured on chamber slides for about 2 weeks and stained for α -SMA. (b) Immunohistochemical staining of activated hepatic stellate cells for CTGF. The hepatic stellate cells were isolated from rat liver and the desmin positive cells were cultured on chamber slides until it is transformed into myofibroblasts by autocrine mechanism.

CTGF siRNA in the prevention of the pathogenesis of hepatic fibrosis are demonstrated through Figure 2a–f. The control livers depicted normal lobular architecture with central vein and radiating hepatic cords (Figure 2a). There was no significant structural alteration in the livers of animals treated with scrambled vector for 14 days (Figure 2b). Massive hepatic necrosis and collapse of the liver parenchyma were observed in animals administered with NDMA in doses of 10 mg/kg body weight for 7 consecutive days (Figure 2c). Only focal necrosis was present in animals administered with CTGF siRNA before the injection of NDMA (Figure 2d). Marked fibrosis and intense neutrophilic infiltration were present on day 14 after the start of daily administration of NDMA for 7 consecutive days. Focal bridging necrosis and hepatocyte regeneration were also present (Figure 2e). However, only moderate necrosis was present in animals administered with CTGF siRNA daily for 14 days after the start of the injection of NDMA for 7 consecutive days (Figure 2f).

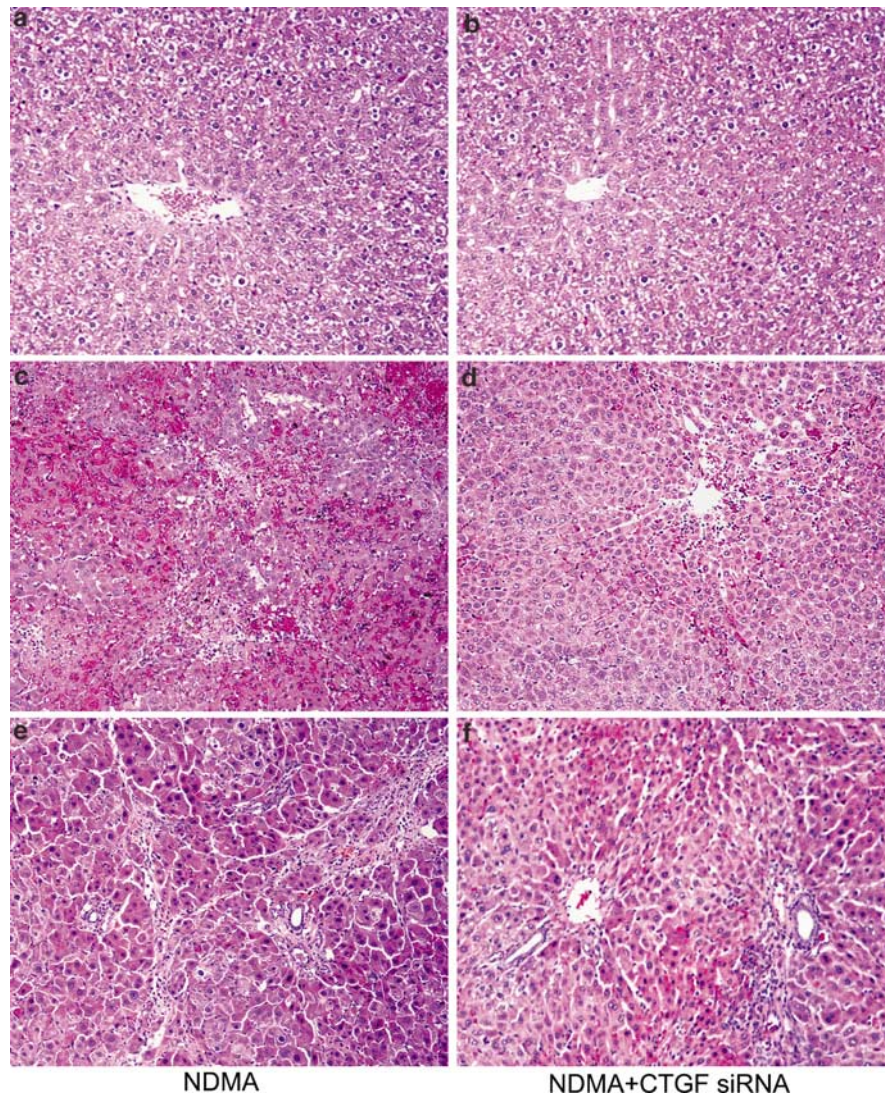


Figure 2 H&E staining of rat liver during NDMA-induced hepatic fibrosis and after concurrent administration of CTGF siRNA. (a) Normal liver ($\times 100$). (b) CTGF siRNA control liver ($\times 100$). Scrambled vector was administered for 14 days. (c) NDMA was administered daily in doses of 10 mg/kg body weight for 7 consecutive days ($\times 100$). Massive hepatic necrosis. (d) CTGF siRNA was administered daily 2 h before the administration of NDMA ($\times 100$). Focal necrosis. (e) Day 14 after the start of daily administration of NDMA for 7 consecutive days ($\times 100$). Marked hepatic fibrosis. (f) Day 14 ($\times 100$). CTGF siRNA was administered daily until day 14 after the start of the administration of NDMA for 7 consecutive days. Moderate necrosis.

CTGF siRNA prevents collagen accumulation in Masson's trichrome staining

Masson's trichrome staining is an excellent technique to demonstrate the accumulation of collagen fibers in the liver tissue during hepatic fibrosis and cirrhosis. The results of the Masson's trichrome staining demonstrating accumulation of matured collagen fibers during NDMA-induced hepatic fibrosis and also the role of CTGF siRNA to prevent collagen synthesis and deposition in the liver during NDMA administration is depicted through Figure 3a–f. Figure 3a demonstrates trichrome staining of normal liver. Masson's trichrome staining did not show any alteration or collagen deposition in the rat liver after the administration of scrambled vector for 14 consecutive days (Figure 3b). Massive hepatic necrosis and early fibrosis were present after the administration of NDMA for 7 consecutive days (Figure 3c). There was extensive bridging necrosis and initiation of the deposi-

tion of matured collagen fibers (Figure 3c). Only moderate necrosis was present in the rat livers treated with CTGF siRNA before the administration of NDMA (Figure 3d). Marked hepatic fibrosis with abundant matured collagen fibers in blue colour was present on day 14 after the start of the administration of NDMA for 7 consecutive days (Figure 3e). The normal architecture was totally distorted. However, the CTGF siRNA-administered animals exhibited only marked focal necrosis (Figure 3f). There was no fibrosis and deposition of blue collagen fibers (Figure 3f).

CTGF siRNA decreases hepatic stellate cell activation

The expression of α -SMA filaments by the transformed hepatic stellate cells is considered as a marker for the activation of stellate cells and initiation of fibrotic process. This process triggers the expression of numerous genes and also the upregulation of several con-

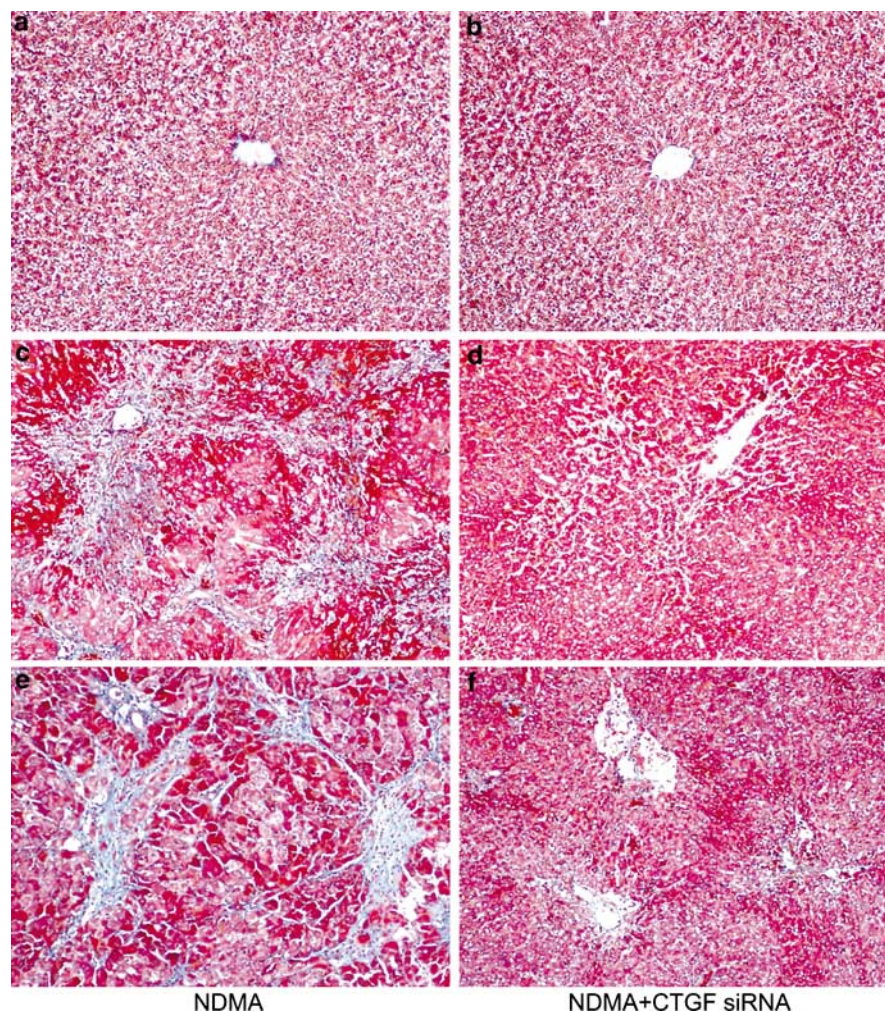


Figure 3 Masson's trichrome staining of rat liver during NDMA-induced hepatic fibrosis and after concurrent administration of CTGF siRNA. (a) Normal liver ($\times 100$). (b) CTGF siRNA control liver ($\times 100$). Scrambled vector was administered for 14 days. (c) NDMA was administered daily in doses of 10 mg/kg body weight for 7 consecutive days ($\times 100$). Massive hepatic necrosis with early fibrosis. (d) CTGF siRNA was administered daily 2 h prior to the administration of NDMA ($\times 100$). Moderate necrosis. (e) Day 14 after the start of daily administration of NDMA for 7 consecutive days ($\times 100$). Marked hepatic fibrosis. (f) Day 14 ($\times 100$). CTGF siRNA was administered daily until day 14 after the start of daily administration of NDMA for 7 consecutive days. Moderate marked necrosis.

nective tissue proteins including collagens.²³ We have studied the rate of activation of hepatic stellate cells during NDMA-induced hepatic fibrosis and also during the concurrent administration of CTGF siRNA. Figure 4a–f demonstrates the immunohistochemical staining α -SMA and the related activation of hepatic stellate cells as well as the effect of CTGF siRNA to decrease the rate of activation of stellate cells. Activated α -SMA-positive stellate cells were absent in control livers (Figure 4a) as well as in livers treated with scrambled vector for 14 days (Figure 4b). Enormous numbers of α -SMA-positive activated stellate cells were present in the liver after administration of NDMA for 7 consecutive days (Figure 4c). The number of α -SMA-positive stellate cells in the liver reduced dramatically after concurrent administration of CTGF siRNA (Figure 4d). On day 14, there was remarkable staining of α -SMA-positive stellate cells in the fibrotic zone (Figure 4e). This implies the prominent role of activated stellate cells in the pathogenesis of hepatic fibrosis. The daily administration of CTGF siRNA before the administration of NDMA – and also

afterwards throughout the study – prevented the development of hepatic fibrosis and also significantly reduced the number of activated stellate cells (Figure 4f). This indicates that CTGF plays a significant role in the activation of hepatic stellate cells and pathogenesis of liver fibrosis. Quantitative evaluation of the staining intensity of activated stellate cells using computer-assisted Image-Pro Plus software depicted significant decrease ($P < 0.001$) of the staining on days 7 and 14 after treatment with CTGF siRNA (Figure 4g).

CTGF siRNA prevents expression of CTGF as indicated by immunohistochemistry

CTGF plays a significant role in the upregulation of several connective tissue proteins that play a prominent role in the pathogenesis of hepatic fibrosis. The results of the immunohistochemical staining of CTGF during NDMA-induced hepatic fibrosis and the effects of CTGF siRNA are demonstrated through Figure 5a–g. CTGF staining was totally absent in the normal liver (Figure 5a)

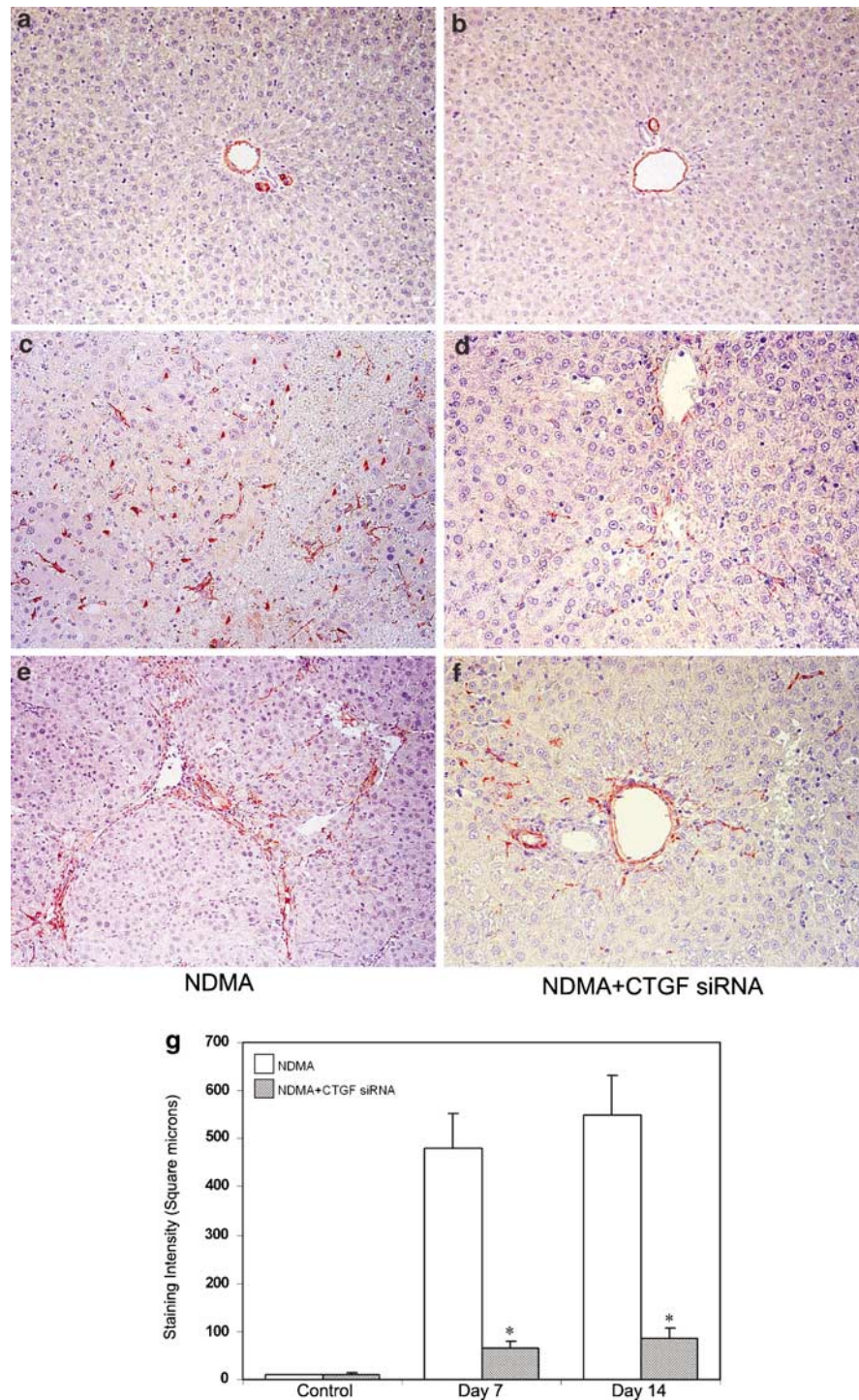


Figure 4 Immunohistochemical staining of α -SMA demonstrating activated hepatic stellate cells in the rat liver during NDMA-induced hepatic fibrosis and after concurrent administration of CTGF siRNA. (a) Normal liver ($\times 200$). The absence of α -SMA staining. (b) CTGF siRNA control liver ($\times 200$). Scrambled vector was administered for 14 days. The absence of α -SMA staining. (c) NDMA was administered daily in doses of 10 mg/kg body weight for 7 consecutive days ($\times 200$). Abundant staining of α -SMA demonstrating large number of activated stellate cells especially in the necrotic zone. (d) CTGF siRNA was administered daily 2 h before the administration of NDMA ($\times 200$). A few activated stellate cells in the necrotic areas. (e) Day 14 after the start of daily administration of NDMA for 7 consecutive days ($\times 100$). Remarkable staining of α -SMA demonstrating enormous number of activated stellate cells especially in the fibrotic areas. (f) Day 14 ($\times 200$). CTGF siRNA was administered daily until day 14 after the start of daily administration of NDMA for 7 consecutive days. Staining of few activated stellate cells in the necrotic areas. (g) Quantitative evaluation of the activated stellate cells using computer-assisted Image-Pro Plus software. Data are representative of 10 randomly selected microscopic fields from six samples ($*P < 0.001$ when compared with NDMA samples without CTGF siRNA on respective days).

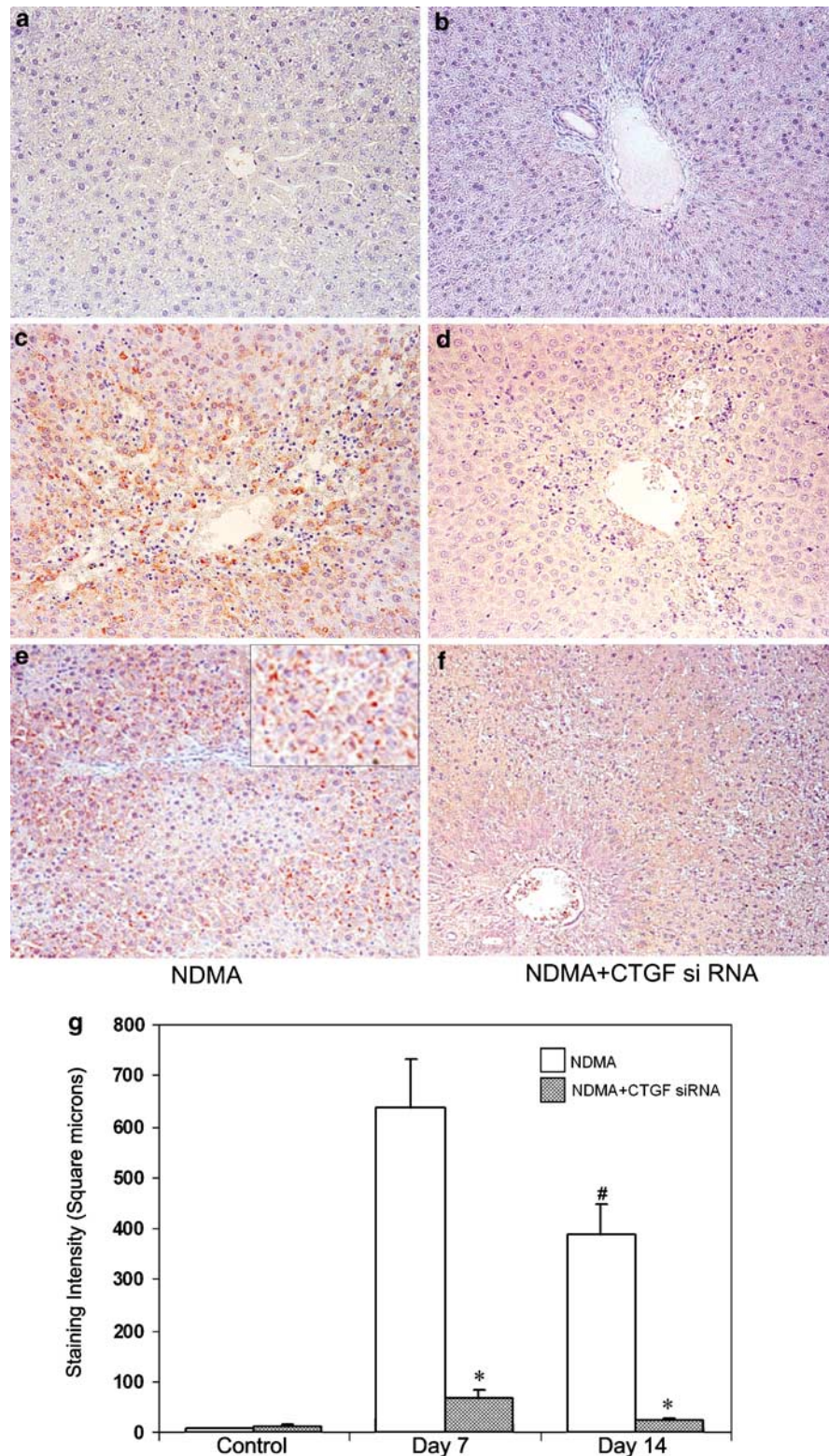


Figure 5 Immunohistochemical staining for CTGF in the rat liver during NDMA-induced hepatic fibrosis and after concurrent administration of CTGF siRNA. (a) Normal liver (×200). CTGF staining was absent. (b) CTGF siRNA control liver (×200). Scrambled CTGF siRNA was administered for 14 days. CTGF staining was absent. (c) NDMA was administered daily in doses of 10 mg/kg body weight for 7 consecutive days (×200). Enormous staining of CTGF especially in the necrotic zone. (d) CTGF siRNA was administered daily 2 h before the administration of NDMA (×200). Only marginal staining of CTGF. (e) Day 14 after the start of daily administration of NDMA for 7 consecutive days (×100). Marked staining of CTGF especially in the necrotic and fibrotic areas. inset: higher magnification of CTGF staining demonstrating expression of CTGF in stellate cells. (f) Day 14 (×100). CTGF siRNA was administered daily until day 14 after the start of daily administration of NDMA for 7 consecutive days. CTGF staining was absent or insignificant. (g) Quantitative evaluation of CTGF staining using Image-Pro Plus software. Data are representative of 10 randomly selected microscopic fields from six samples (* $P < 0.001$ when compared with NDMA samples without CTGF siRNA on respective days; [#] $P < 0.01$ when compared with respective NDMA values on day 7).

as well as in the livers treated with scrambled vector for 14 days (Figure 5b). Remarkable staining of CTGF was present in the livers treated with NDMA for 7 consecutive days (Figure 5c). The staining was more prominent in the necrotic zone. However, only focal marginal staining was present in the livers treated with CTGF siRNA (Figure 5d). Marked staining of CTGF was present on day 14 in the rat livers treated with NDMA for 7 consecutive days (Figure 5e). The inset depicts CTGF staining under higher magnification demonstrating the expression of CTGF in stellate cells. CTGF staining was absent or insignificant in livers treated with CTGF siRNA for 14 consecutive days (Figure 5f). These results indicate that CTGF expression is absent in normal livers and upregulated during hepatic fibrosis. Furthermore, it proved that treatment with CTGF siRNA could knockdown the upregulation of CTGF during NDMA-induced hepatic fibrosis. Quantitative evaluation of the CTGF staining intensity using computer-assisted Image-Pro Plus software showed significant decrease ($P < 0.001$) of the staining on days 7 and 14 after treatment with CTGF siRNA (Figure 5g).

CTGF siRNA decreases serum HA and TGF- β 1

Serum HA is a very early indicator of liver damage and appears much sooner than aspartate transaminase and alanine transaminase in the serum.^{1,3} TGF- β 1 is a strong profibrogenic molecule and highly upregulated during hepatic fibrogenesis. Serum HA and TGF- β 1 levels during NDMA-induced hepatic fibrosis and concurrent administration of CTGF siRNA are demonstrated in Figure 6a and b, respectively. As evident from Figure 6a, more than a 10-fold increase was observed in serum HA levels after the administration of NDMA for 7 consecutive days. Serum HA was dramatically decreased on day 14 after discontinuation of NDMA administration on day 7. However, serum HA levels were still significantly ($P < 0.001$) higher than the normal values indicating enhanced synthesis of HA in the liver and simultaneous release into the blood stream owing to the persistent necrosis in the liver. Serum HA levels were significantly reduced in CTGF siRNA-treated animals on day 7 when compared with the nontreated NDMA group but did not attain normal values. On day 14, serum HA levels returned to the normal values in CTGF siRNA-treated animals suggesting that CTGF siRNA could regulate the expression of liver HA through prevention of hepatic fibrogenesis. Serum TGF- β 1 levels determined by sandwich enzyme immunoassay elevated significantly on day 7, after the administration of NDMA for 7 consecutive days and the values were still significantly higher on day 14 when compared with normal values (Figure 6b). The concurrent administration of CTGF siRNA resulted in a significant ($P < 0.001$) decrease of serum TGF- β 1 levels on days 7 and 14 when compared with the NDMA group on respective days. However, the values did not return to normal levels on either day.

CTGF siRNA prevents upregulation of CTGF and reduces the expression of TGF- β 1 as indicated by Western blot, semiquantitative and real-time PCR

We carried out Western blot, semiquantitative and real-time PCR analyses to study the expression of CTGF and TGF- β 1 both at the protein and mRNA levels during

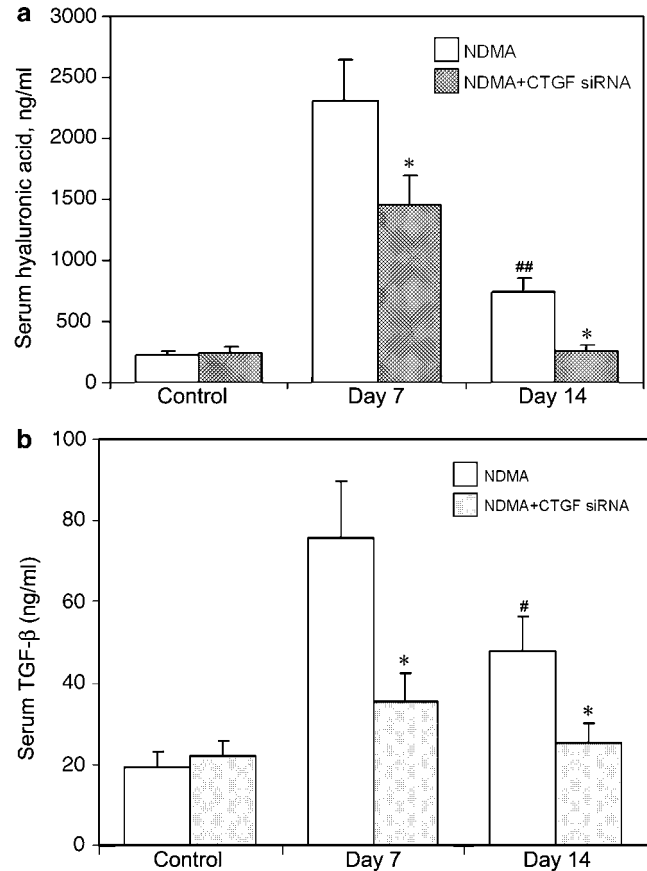


Figure 6 (a) Serum HA during NDMA-induced hepatic fibrosis and also after concurrent administration of CTGF siRNA. Serum HA was determined using an ELISA-based sandwich HA-binding protein assay kit (* $P < 0.001$ when compared with NDMA samples without CTGF siRNA on respective days; ** $P < 0.001$ when compared with respective NDMA values on day 7). (b) TGF- β in the rat serum during NDMA-induced hepatic fibrosis and also after concurrent administration of CTGF siRNA. The TGF- β in rat serum was determined using an enzyme immunoassay kit (* $P < 0.001$ when compared with NDMA samples without CTGF siRNA on respective days; * $P < 0.01$ when compared with respective NDMA values on day 7).

NDMA-induced hepatic fibrosis and concurrent administration of CTGF siRNA. Figure 7a demonstrates the Western blot analysis of CTGF and TGF- β 1 protein levels before and after the administrations of NDMA and also after knockdown of CTGF mRNA using cognate siRNA. Both CTGF and TGF- β 1 protein levels were significantly increased on days 7 and 14 after the administration of NDMA for 7 consecutive days. However, neither CTGF nor TGF- β 1 protein levels were altered either on days 7 or 14 in the animals treated with CTGF siRNA. Reprobing and analysis of the membrane for GAPDH demonstrated equal loading of the protein samples analyzed. Figure 7b depicts semiquantitative RT-PCR analysis for CTGF and TGF- β 1 mRNA using gene-specific primers during NDMA-induced liver injury and also after concurrent administration of CTGF siRNA. As evident from the figure, there was a dramatic increase in the levels of both CTGF and TGF- β 1 mRNA expression on days 7 and 14 after the start of the NDMA administration. However, CTGF siRNA treatment completely prevented the upregulation of the cognate mRNA

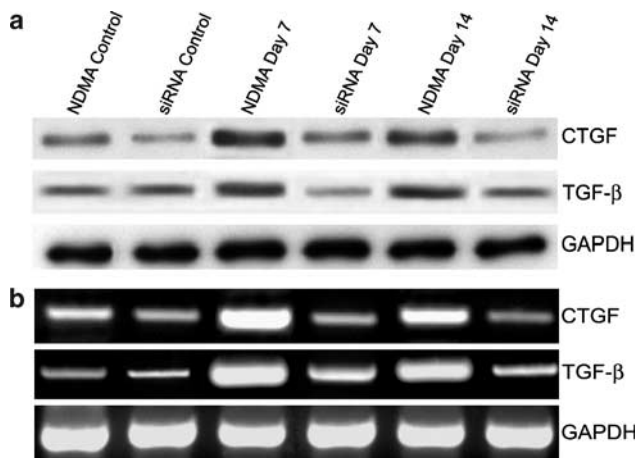


Figure 7 (a) Western blot analysis for CTGF and TGF- β in the rat liver during NDMA-induced hepatic fibrosis and also after simultaneous treatment with CTGF siRNA. The nitrocellulose membranes were reprobed and analyzed for GAPDH content to demonstrate that similar amounts of protein had been loaded in each lane. (b) Semiquantitative RT-PCR for the expression of CTGF and TGF- β mRNA. The total cellular RNA was isolated using Qiagen RNeasy kit. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used as a housekeeping gene.

during NDMA-induced hepatic fibrosis (Figure 7b). Even though CTGF siRNA treatment significantly reduced the elevated levels of TGF- β 1 mRNA, it did not return to the normal levels.

Real-time RT-PCR analysis for CTGF mRNA using SYBR green demonstrated similar data as semiquantitative RT-PCR (Figure 8a). These indicate the complete knockdown of the upregulated CTGF mRNA by the cognate siRNA expressed through the mammalian vector carrying the template DNA for the CTGF siRNA. The real-time RT-PCR analysis for TGF- β 1 mRNA also depicted similar results as of semiquantitative RT-PCR (Figure 8b). These data suggest that CTGF siRNA also regulates the expression of TGF- β 1 during NDMA-induced hepatic fibrosis to some extent. Analysis for GAPDH mRNA prominently revealed the presence of this important housekeeping gene in all the samples analyzed. Taken together, these data suggest that serial administrations of CTGF siRNA effectively prevented the upregulation of CTGF and TGF- β 1 both at the mRNA and protein levels, and thus prevented hepatic fibrogenesis.

Discussion

Gene silencing using siRNAs is a novel and promising technology for therapeutic applications of various diseases such as hepatic fibrosis or cancer that involves upregulation of several genes.^{24–26} Using this technology, the expression of a particular gene can be reduced often to 80–90% and thus its cognate protein. Introduction of a suitable mammalian expression vector carrying a template DNA strand into the cells and the subsequent expression of the corresponding siRNA molecules forms the basis of the mammalian RNA interference. The single-stranded siRNA molecules form a 20–25-nucleotide dsRNA on its loop, which is further processed by dicer enzyme. The siRNAs assemble into endonuclease

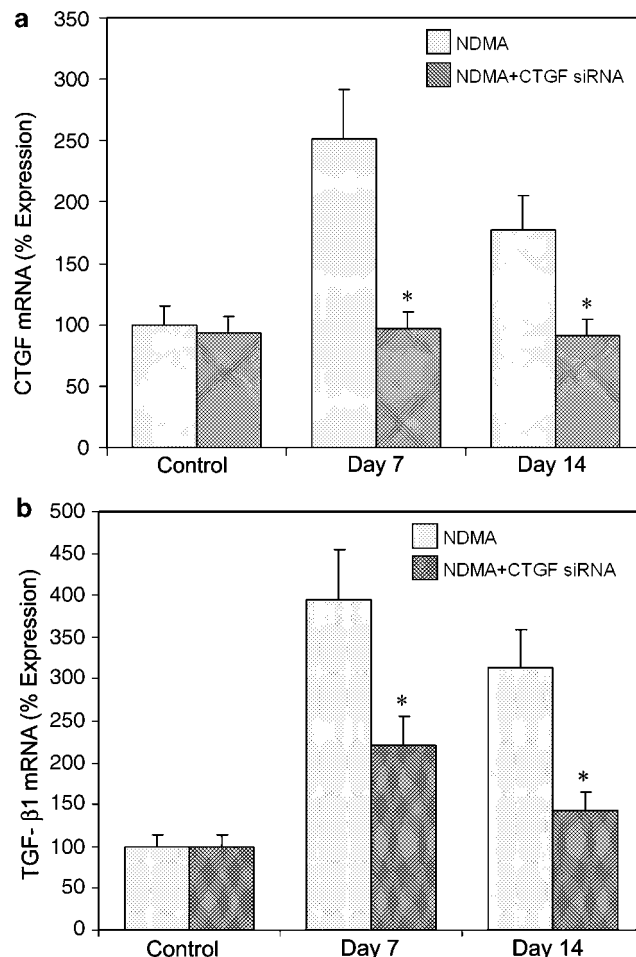


Figure 8 Real-time RT-PCR analysis using SYBR green for the quantitative evaluation of the expression of CTGF (a) and TGF- β (b) mRNA in rat liver. The animals were administered with NDMA at a dose of 10 mg/kg body weight for 7 consecutive days and simultaneously treated with CTGF siRNA. The data are mean $\pm$ s.d. of six samples (* P < 0.001 when compared with NDMA samples without CTGF siRNA on respective days).

containing complexes known as RNA-induced silencing complexes (RISCs) that unwind the duplex siRNA. The antisense strand subsequently guides the RISCs to complementary RNA molecules that initiate ATP-independent endonuclease activity resulting in cleavage and destruction of the cognate mRNA.

Hepatic fibrosis is characterized by increased expression, biosynthesis and deposition of connective tissue components, especially collagens in the liver. NDMA-induced model of liver injury in rats is a well-established and valuable animal model for studying the biochemical, pathophysiological and molecular alterations associated with the development of human hepatic fibrosis.^{19,27,28} It is a quick, reproducible and highly reliable animal model to investigate the molecular events associated with the activation of hepatic stellate cells and also to screen antifibrotic agents. Furthermore, this model is suitable to study the upregulation of several genes involved in hepatic fibrogenesis and also to knockdown the upregulated mRNAs using cognate siRNAs. The 7-day course of serial administrations of NDMA produced centrilobular

necrosis and well-developed fibrosis, with a lot of collagen deposition in the liver within 14 days.

CTGF is a novel cysteine-rich, secreted peptide, which is implicated in several disorders such as atherosclerosis, fibrosis and cancer.^{29,30} During hepatic fibrogenesis, CTGF is expressed mainly in the activated hepatic stellate cells. In the present investigation, we have observed marked staining of CTGF in the cultured activated stellate cells. Strong staining of CTGF in the cytoplasm of cultured stellate cells was reported by Paradis *et al.*⁵ Furthermore, *in situ* hybridization demonstrated CTGF mRNA in the cytoplasm of cultured hepatic stellate cells.⁵ In cultured hepatic stellate cells, CTGF is localized exclusively to the Golgi apparatus and quantitatively secreted into the medium.³¹ In addition, CTGF protein has been demonstrated in cultured primary hepatocytes by Western blotting.⁵ Taken together, all these data along with the results of the present study proved that activated hepatic stellate cells express CTGF.

The activation of hepatic stellate cells is considered as a marker for the initiation of fibrogenesis. This process is accompanied with the expression and upregulation of several genes involved in the synthesis of connective tissue proteins especially collagens, fibronectin and laminin in addition to many cytokines and growth factors including CTGF. The observation of a large number of activated hepatic stellate cells in the present investigation corroborates with our previous study on NDMA-induced hepatic fibrosis.³ Our present study demonstrated that serial administrations of CTGF siRNA significantly arrest the activation hepatic stellate cells and thus hepatic fibrogenesis. The significant decrease in the number of activated stellate cells in the present study coincides with a recently reported study on CTGF siRNA during CCl₄-induced hepatic fibrosis in rats.³² During hepatic fibrogenesis, CTGF is highly upregulated from various cell types, which in turn stimulates the activation of hepatic stellate cells and production of more CTGF.²⁹ CTGF siRNA effectively knockdown the upregulation of CTGF expression during fibrogenesis and prevents the activation of hepatic stellate cells and further production of CTGF. Our study along with a recent report confirmed that CTGF siRNA could significantly decrease the activation of hepatic stellate cells and attenuates the pathogenesis of hepatic fibrosis.

It was reported that CTGF is a downstream mediator of TGF- β and thus a potential target for antifibrotic treatment strategies.³³ In the present study, we have noticed that CTGF expression was dramatically upregulated in rat livers treated with NDMA for 7 consecutive days. Immunohistochemical staining, Western blot analysis, semiquantitative and real-time RT-PCR studies demonstrated the same pattern of upregulation both at protein and gene levels. The CTGF upregulation was positively correlated with the enhanced expression of TGF- β in fibrotic livers. Marked upregulation of CTGF and TGF- β was reported in CCl₄-induced experimental liver fibrosis^{33,34} and human liver cirrhosis.^{8,10} It was also reported that HCV core protein promotes liver fibrogenesis via upregulation of CTGF with TGF- β .³⁵ In the present study, serial administrations of CTGF siRNA downregulated the increased expression of CTGF both at mRNA and protein levels. Furthermore, CTGF siRNA treatment also reduced the enhanced expression of TGF-

β 1 but not as that of CTGF. In the case of TGF- β 1, the downregulation was only about 50% in contrast to the almost 100% knockdown of CTGF mRNA and the cognate protein. Taken together, these data suggest that CTGF plays a significant role in the pathogenesis and progression of hepatic fibrosis and holds a key position in the network of events involved in the enhanced synthesis of ECM components. In addition, CTGF plays as a downstream effector for the best-known fibrogenic cytokines, the TGF- β .

Increased levels of serum HA and TGF- β 1 have been well documented in NDMA-induced hepatic fibrosis^{1,3,36} and alcoholic liver cirrhosis.³⁷ A five-fold increase of serum CTGF has been reported in patients with hepatic fibrosis.³⁸ It was also reported that CTGF increases in circulation of patients with active, fibrogenic liver diseases and suggested that measurement of serum CTGF as a potential new noninvasive marker of ongoing fibrogenesis in chronic liver diseases.³⁸ In the present investigation, we have observed significantly elevated levels of serum HA and TGF- β 1 in NDMA-induced hepatic fibrosis. Serial administrations of CTGF siRNA restored normal levels of serum HA in fibrotic animals. This indicates that elevation of serum HA is secondary to fibrotic process and the arrest of hepatic fibrosis through CTGF siRNA effectively prevents hepatic necrosis and simultaneous leakage of HA into circulation. In the present study, serum TGF- β 1 levels did not return to normal values after treatment with CTGF siRNA. TGF- β 1 production is ahead to or concurrent with CTGF during hepatic fibrogenesis and their action is always synergetic. Furthermore, serum levels of cytokines and other conventional markers of hepatic fibrosis depend mainly on the extent of liver necrosis and do not really reflect the network of events happening in the liver during fibrogenesis or CTGF siRNA treatment.

It is a controversy whether CTGF regulates TGF- β 1 expression or TGF- β 1 regulates CTGF expression. Previous studies using fibroblasts demonstrated that CTGF is a TGF- β 1-induced immediate early gene.³⁹ Expression of CTGF following TGF- β stimulation occurs within minutes in the absence of *de novo* protein synthesis, an effect that was initially attributed to a TGF- β response element in the CTGF promoter.⁴⁰ More recent studies have demonstrated that although this element may be important for basal CTGF expression, TGF- β -induced CTGF expression requires smad-binding sequences and a novel promoter sequence that is preferentially activated in fibroblasts.⁴¹ It is now well established that CTGF and TGF- β share profibrogenic properties and are highly upregulated in many fibrotic diseases including hepatic fibrosis and are fibrogenic in both *in vitro* and *in vivo*.⁴² The close association between TGF- β action and CTGF expression shows the role played by CTGF in mediating the biological activities of TGF- β .⁴³ It was reported that TGF- β -induced collagen production is antagonized by CTGF antibodies or antisense oligonucleotides in normal rat kidney cells and human fibroblasts.⁴⁴ Subcutaneous injection of CTGF into neonatal mice causes a rapid increase in the amount of granulation tissue comprising connective tissue cells and abundant ECM, resulting in enhanced levels of TGF- β mRNA and is not mimicked by other growth factors.⁴⁵ Our study and a similar recent study³² demonstrated that CTGF regulates the expression of TGF- β 1 both

at gene and protein levels to an extent. Collectively, these data support a role of CTGF as a downstream mediator in some of the fibrogenic actions of TGF- β 1, particularly the activation of hepatic stellate cells and ECM production.

In conclusion, the results of the present study demonstrated that CTGF siRNA attenuates the activation of hepatic stellate cells, prevents the deposition of matured collagen fibers in the liver and thus inhibits hepatic fibrogenesis. Our results further suggest that RNA interference using siRNAs has potential therapeutic application to prevent hepatic fibrogenesis.

Acknowledgements

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