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Electroporative interleukin-10 gene transfer ameliorates carbon tetrachloride-induced murine liver fibrosis by MMP and TIMP modulation[1]

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Key words

interleukin-10; gene therapy; liver cirrhosis; gelatinase A; tissue inhibitor of metalloproteinases; cyclooxygenase 2

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Abstract

Aim: Liver fibrosis represents a process of healing and scarring in response to chronic liver injury. Effective therapies for liver fibrosis are lacking. Interleukin-10 (IL-10) is a cytokine that downregulates pro-inflammatory responses and has a modulatory effect on hepatic fibrogenesis. The aim of this study was to investigate whether electroporative IL-10 gene therapy has an hepatic fibrolytic effect on mice. **Methods:** Hepatic fibrosis was induced by administering carbon tetrachloride (CCl_4) for 10 weeks in mice. The human IL-10 expression plasmid was delivered via electroporation after hepatic fibrosis was established. Histopathology, reverse transcription polymerase chain reaction (RT-PCR), immunoblotting, and gelatin zymography were used to investigate the possible mechanisms of action of IL-10. **Results:** Human IL-10 gene therapy reversed CCl_4-induced liver fibrosis in mice. RT-PCR revealed that IL-10 gene therapy attenuated liver TGF-β1, collagen α1, fibronectin, and cell adhesion molecule mRNA upregulation. Following gene transfer, both the activation of α-smooth muscle actin and cyclooxygenase-2 were significantly attenuated. Furthermore, IL-10 significantly inhibited matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of matrix metalloproteinase (TIMP) activation after CCl_4 intoxication. **Conclusions:** We demonstrated that IL-10 gene therapy attenuated CCl_4-induced liver fibrosis in mice. IL-10 prevented upregulated fibrogenic and pro-inflammatory gene responses. Its collagenolytic effect may be attributed to MMP and TIMP modulation. IL-10 gene therapy may be an effective therapeutic modality against liver fibrosis with potential clinical use.

Introduction

Oxidative stress, an important factor that induces liver fibrosis, represents a key feature of hepatitis induced by various conditions, including anoxic/reoxygenation injury, autoimmune hepatitis, viral hepatitis and alcoholic hepatitis[1]. Less severe oxidative stress may sustain fibrosis progression by causing activation and morphological changes in hepatic stellate cells (HSC), including promoting proliferative activity, synthesis and degradation/remodeling of the extracellular matrix (ECM), chemotaxis, contractility, proinflammatory activity and retinoid loss[1,2].

Carbon tetrachloride (CCl_4) is a xenobiotic used extensively to induce oxidative stress. It is assumed to initiate free radical-mediated lipid peroxidation, leading to the accumulation of lipid-derived oxidation products that cause liver injury and excess collagen deposition in the liver, resulting in liver fibrosis[3,4]. During hepatic fibrogenesis, there is an imbalance between excess synthesis of ECM and/or its removal, with consequent fibrosis and scarring[5,6]. The pathophysiology of ECM formation during liver fibrosis is multifaceted and complex[7,8]. It involves a change in the expression of ECM proteases (matrix metalloproteinases; MMP) or their inhibitors (tissue inhibitors of metalloproteinases;

TIMP) and an increase in the synthesis of collagen and fibronectin driven by signaling pathways mediated by pro-inflammatory cytokines such as transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α)[9–12].

Interleukin (IL)-10 is a cytokine that downregulates pro-inflammatory responses[13]. Human IL-10 is a 160 amino acid protein (molecular weight=18.5 kDa), and murine IL-10 is a 157 amino acid protein with 80% homology to the human form[14]. Recombinant human IL-10 has been produced and tested in clinical trials. Studies suggest that IL-10 may be effective against chronic hepatitis C and other liver diseases[15]. Further, IL-10 gene therapy has been studied extensively in animal models for autoimmune diabetes, thyroiditis, and colitis[16–18]. Because the elimination half-life of recombinant IL-10 is relatively short (=2 h)[19], it may be possible to utilize its therapeutic properties to develop a gene-based treatment regimen. In the present study, we investigate whether IL-10 gene therapy is effective against CCl_4-induced liver fibrosis in mice.

Materials and methods

Subjects Male 6- to 8-week-old ICR mice were purchased from the National Science Council, Taiwan, China, and were allowed to acclimatize for 5 d before experimentation. The mice were housed in Kaohsiung Chang Gung Memorial Hospital Animal Facility under standard temperatures, and with a standard light and dark cycle. All procedures performed on the mice were approved by the Kaohsiung Chang Gung Memorial Hospital Animal Care and Use Committee.

IL-10 expression plasmid preparation A human IL-10 expression plasmid (pCYIL-10 vector) was used in the present study[20]. In brief, full-length human IL-10 cDNAs were subcloned into a pCY4B expression vector driven by a chicken β-actin promoter with a cytomegalovirus immediate early enhancer. pCMV-Lacz was used as the vehicle control. These plasmids were purified using the EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA, USA).

Liver fibrosis induction and gene therapy Based on the method used in a previous study, but with some modifications, the mice were administered CCl_4 (1 mL/kg body weight) dissolved in olive oil (1:1) twice a week for 10 weeks[21]. Sixteen mice were killed at the end of 6 weeks to confirm that liver fibrosis was established (group I). To evaluate the anti-fibrotic effect of IL-10, gene therapy administration was started at the end of 6 and 8 weeks of CCl_4 treatment. Briefly, 30 μL bovine hyaluronidase (0.4 IU/μL) (Sigma-Aldrich, St Louis, MO, USA) was injected into the anterior tibialis (AT) muscle of the mice 2 h before electroporation. pCYIL-10 was injected into the bilateral AT muscles using a 27G needle (30 μL into each leg; 4 μg/μL; group II, n=16). Electroporation was carried out using electrical pulses (8 pulses of 20 ms, 175 V/cm, and 1 s intervals) with Tweezertrode electrode disks and an electrical pulse generator (T830; BTX, San Diego, CA, USA)[22].

Sixteen mice received gene electro-transfer therapy using the same procedure as described above using pCMV-LacZ (group III) as a vehicle control at the end of 6 and 8 weeks. All surviving mice (group II, n=12; group III, n=7) were killed at the end of the 10-week CCl_4 treatment. Five mice were killed before CCl_4 intoxication as normal controls (group N).

Histopathology and immunohistochemistry For histopathology studies, mice were killed at 0, 6, and 10 weeks after CCl_4 administration. The liver was removed and fixed in 10% formalin solution. Five-micrometer sections were stained with 0.1% Sirius red in picric acid (Sigma-Aldrich). Matrix density was quantified using a computerized image analysis system as previously described[23]. For immunohistochemical studies, the sections were washed in phosphate-buffered saline (PBS), and incubated in 3% normal goat serum with 0.3% Triton X-100 in PBS for 1 h. The sections were incubated free-floating at 4 °C with IL-10 (specific for human origin; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclooxygenase-2 (COX-2), MMP-2, and TIMP-1 (Abcam, Cambridge, MA, USA) antibodies. Immunoreactivity was visualized using the Vectastain Elite ABC Peroxidase method (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as the chromagen.

Soluble collagen measurement For soluble collagen analysis, the Sircol collagen assay (Biocolor, Belfast, UK) was performed following the manufacturer's instructions as described in a previous study[24]. Briefly, 50 mg of liver was homogenized. Total acid pepsin-soluble collagens were extracted overnight using 5 mg/mL pepsin in 500 μL of 0.5 mol/L acetic acid. One milliliter of Sircol dye reagent was added to every 100 μL of each sample, in duplicate, and the mixture was incubated at 25 °C for 30 min. After centrifugation, the pellet was suspended in 1 mL of alkali reagent. The absorbance was read at 540 nm.

Immunoblotting The liver specimens were homogenized in a lysis buffer with complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany). For analysis of α-smooth muscle actin (α-SMA) expression after CCl_4 administration, 20 μg of protein extracts were electrophoresed on a 10% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and immunoblotted onto PVDF membranes. The membranes were blocked for 1 h at

room temperature and incubated overnight with a 1:1000 dilution of α-SMA, and α-tubulin antibodies (Abcam). Antibody binding was detected using horseradish peroxidase (HRP)-linked immunoglobulin G (IgG). Bands were visualized using an ECL detection system (Amersham-Pharmacia Biotech, Little Chalfont, UK). Band intensities were quantified using an image analyzer (Densitograph AE-6900M; Atto, Tokyo, Japan).

Reverse transcription-polymerase chain reaction Livers were harvested at 0, 6, and 10 weeks after CCl_4 administration. The expression levels of TGF-β1, collagen α1, fibronectin, TNF-α, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), TIMP-1, and TIMP-2 mRNA were analyzed using reverse transcription-polymerase chain reaction (RT-PCR) techniques. The total RNA was extracted and then reverse-transcribed into cDNA. PCR was performed at a final concentration of 1× PCR buffer, 1.0 μmol/L of each of the 3' and 5' primers, and 10 U of Advan-*Taq* Plus DNA polymerase (Clontech, Palo Alto, CA, USA) in a total volume of 50 μL. The mixture was amplified for 32 cycles in a thermal cycler (Stratagene, La Jolla, CA, USA). The β-actin was amplified to verify equal loading. The primer sequence and expected product size were as previously described[25]. The amplification products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. The gel was scanned at a NucleoVision imaging workstation (NucleoTech, San Mateo, CA, USA), and quantified using GelExpert release 3.5.

Gelatin zymography Gelatin zymography was carried out to explore MMP activity. Briefly, liver tissues were homogenized in a protein extraction buffer. The supernatant of a centrifuged liver sample (20 μg of protein extract per line) was mixed 1:3 with a sample buffer and separated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis in 8% polyacrylamide gel copolymerized with 1 mg/mL gelatin (Sigma-Aldrich) as described elsewhere[26]. Gels were incubated at 37 °C overnight in an MMP activation buffer. After Coomassie blue staining, the extent of gel digestion localized to bands of active-MMP-2 (64-kDa) were quantified by densitometry.

Statistical analysis All data (from at least 3 separate experiments) are presented as mean±SEM. Statistical analysis was performed using one-way ANOVA followed by the *t*-test. $P<0.05$ was considered significant.

Results

Long-term IL-10 expression following electroporative gene transfer There was only scanty staining of cells for human IL-10 in the non-gene transfer groups (groups N, I, and III; Figure 1). Strong positive staining of cells for human IL-10 was seen in the livers of the gene transfer group II and 4 weeks after electroporation (group II; Figure 1C).

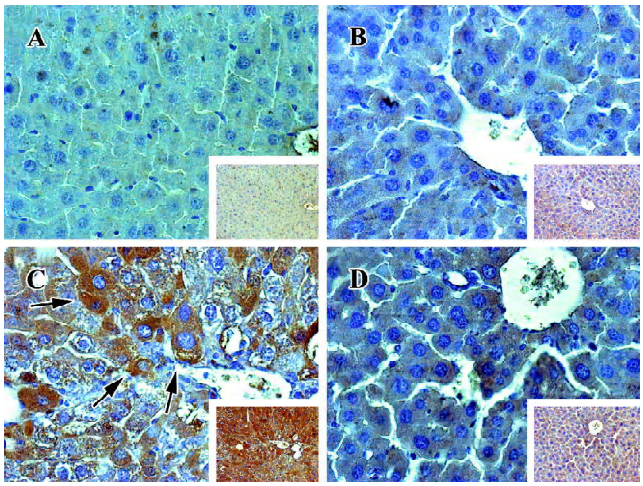

Figure 1. Representative photomicrographs of IL-10-stained mouse liver sections. There was scanty staining for human IL-10 in the non-gene therapy groups (groups N, I, and III). Strong positive staining for human IL-10 was detected in the livers of the gene transfer group (group II). (A) Normal control; (B) Group I; (C) Group II; (D) group III. Arrows indicate IL-10-positive cells. Magnification, ×400 (inset, ×200).

IL-10 gene therapy reversed CCl_4-induced liver fibrosis There was no significant difference in food and water intake throughout the study period between groups. After 6 weeks of CCl_4 administration, liver fibrosis was seen histopathologically. Sirius red staining of liver sections revealed extensive fibrosis, portal-to-portal fibrous bridging, and nodular transformation in groups I and III (Figure 2). Human IL-10 gene therapy (group II) significantly ameliorated hepatic fibrogenesis and reduced matrix density (Figure 2C). These findings were further confirmed by measurements of liver collagen content (Table 1).

IL-10 gene therapy attenuated COX-2 increment after CCl_4 COX-2 was not detected immunohistochemically in the normal group. COX-2 expression was upregulated after CCl_4 administration (groups I and III; Figure 3). IL-10 gene therapy significantly diminished this COX-2 expression (Figure 3C).

IL-10 gene therapy suppressed hepatic stellate cell activation after CCl_4 α-SMA [activated hepatic stellate cell (HSC) markers] are known to be activated after acute liver injury[27,28]. In the present study, the expression of α-SMA increased after chronic CCl_4 administration as measured using immunoblotting (Figure 4). IL-10 gene therapy (group II)

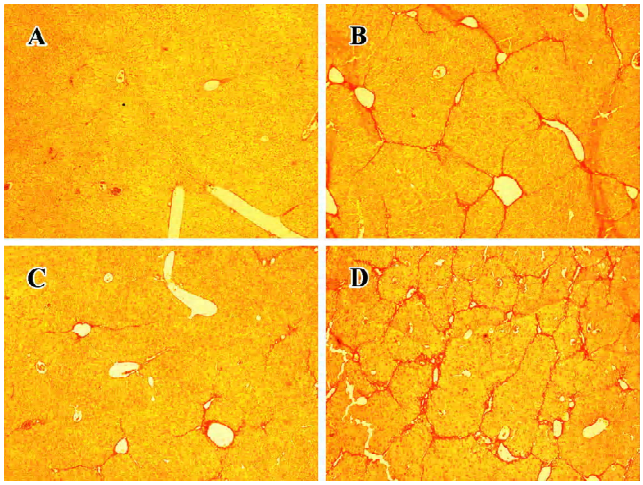

Figure 2. Representative photomicrographs of Sirius red-stained mouse liver sections. Extensive fibrosis, portal-to-portal fibrous bridging, and nodular transformation were seen in groups I and III after chronic CCl_4 administration. IL-10 gene therapy (group II) significantly reduced matrix density and abrogated hepatic fibrogenesis. (A) Normal control; (B) Group I; (C) Group II; (D) Group III. Magnification, ×50.

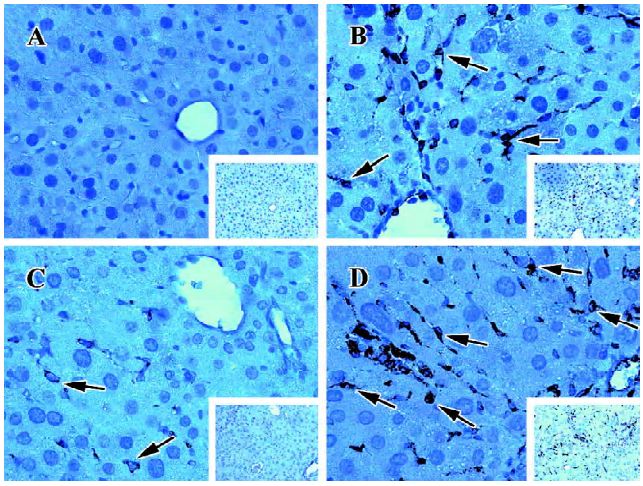

Figure 3. Representative photomicrographs of COX-2-stained mouse liver sections. COX-2 was not detected in the normal control group. COX-2 expression was increased in groups I and III after CCl_4 administration. IL-10 gene therapy (group II) significantly attenuated this increase. (A) Normal control; (B) Group I; (C) Group II; (D) Group III. Arrows indicate COX-2 positive cells. Magnification ×400 (inset ×200).

Table 1. Effects of IL-10 gene therapy on regression of hepatic fibrosis. IL-10 gene therapy significantly reduced the degree of liver fibrosis caused by chronic CCl_4 administration in mice as measured using Sirius red matrix density and collagen content. Mean±SEM. $^bP<0.05$, $^cP<0.01$ *vs* group I. $^fP<0.01$ *vs* group III.

	Sirius red matrix density (%)	Collagen content (μg/mg)
Group N	0^{cf}	10.6 ± 1.7^{cf}
Group I	6.1±0.5	48.3±2.0
Group II	4.1 ± 0.5^{bc}	35.7 ± 3.0^{bc}
Group III	8.2±0.6	59.7±5.0

significantly reduced this upregulation, indicating HSC inactivation ($P<0.01$ *vs* group I; $P<0.01$ *vs* III). α-Tubulin was used as an internal control.

IL-10 gene therapy attenuated fibrogenic, proinflammatory, and cell adhesion molecule gene responses after CCl_4 treatment Expression of TGF-β1, collagen α1, fibronectin, TNF-α, ICAM-1, and VCAM-1 mRNA were all upregulated in the fibrotic liver as semi-quantified using RT-PCR (Figure 5). β-actin was amplified as an internal control. IL-10 gene therapy (group II) significantly attenuated these increase. In brief, IL-10 gene transfer suppressed the fibrogenic, proinflammatory, and cell adhesion molecule gene responses after CCl_4 administration.

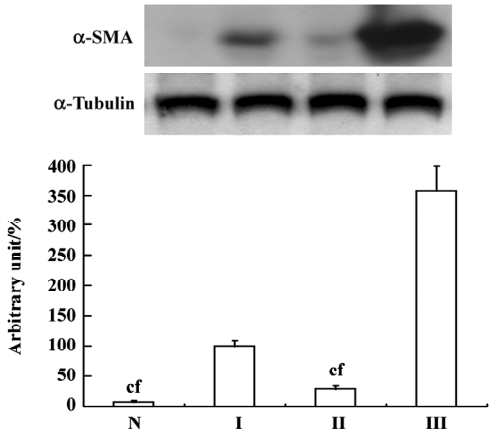

Figure 4. Representative immunoblots showing α-SMA expression in CCl_4-treated mouse liver. When compared with normal controls, α-SMA activation was seen in groups I and III after CCl_4 administration. IL-10 gene therapy (group II) significantly abrogated this activation. Mean±SEM. $^cP<0.01$ *vs* group I, $^fP<0.01$ *vs* group III. The arbitrary units are defined as α-SMA/α-tubulin band density.

IL-10 gene therapy attenuated MMP-2 activation in the fibrotic liver The expression of MMP after CCl_4 treatment was evaluated by using immunohistochemical and gelatin zymography methods. Immunohistochemical studies showed that when compared with normal livers, MMP-2 levels were significantly increased in the fibrotic livers (groups I and III; Figure 6). IL-10 gene therapy attenuated this upregulation

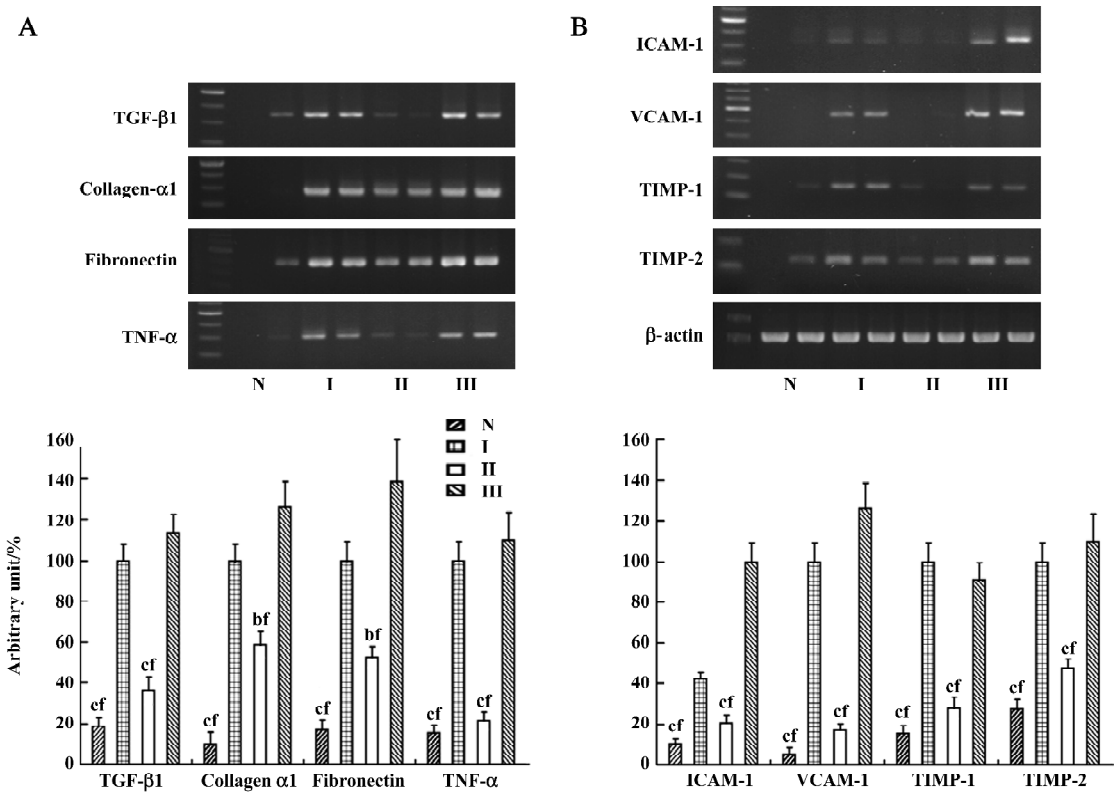

Figure 5. Representative photographs of semi-quantified RT-PCR in the CCl_4-treated mouse liver. IL-10 gene therapy (group II) significantly attenuated TGF-β1, collagen α1, fibronectin, TNF-α, ICAM-1, VCAM-1, TIMP-1, and TIMP-2 mRNA activation after CCl_4 administration when compared with groups I and III ($^bP<0.05$, $^cP<0.01$ *vs* group I. $^fP<0.01$ *vs* group III). β-Actin was amplified to verify equal loading. The arbitrary units are defined as target gene/β-actin. Mean±SEM.

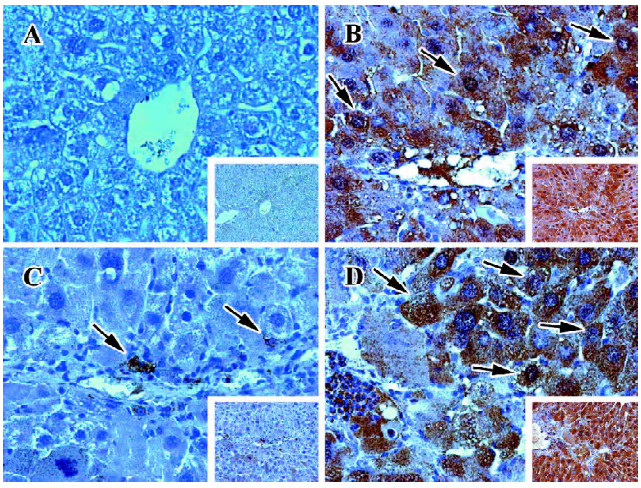

Figure 6. Representative photomicrographs of MMP-2-stained mouse liver sections. After CCl_4 administration, MMP-2 staining was significantly accentuated in groups I and III. IL-10 gene therapy (group II) decreased this upregulation. (A) Normal control; (B) Group I; (C) Group II; (D) Group III. Arrows indicate MMP-2-positive cells. Magnification, ×400 (inset, ×200).

(group II; Figure 6C). The collagenolytic activity of MMP protein in liver homogenates was examined by zymography (Figure 7). Gelatin zymography showed that the concentration of the 64 kDa active MMP-2 molecule increased in groups I and III after CCl_4 administration, and IL-10 gene therapy (group II) abrogated this increase ($P<0.01$).

IL-10 gene therapy attenuated TIMP activation after CCl_4 treatment Expression of TIMP in the fibrotic livers was also evaluated by RT-PCR and immunohistochemical methods. RT-PCR showed that TIMP-1 and TIMP-2 mRNA were significantly upregulated in the fibrotic liver. IL-10 gene therapy (group II) significantly attenuated these increase ($P<0.01$, Figure 5). Immunohistochemical studies revealed that the level of TIMP-1 was increased after chronic CCl_4 administration (Figure 8). IL-10 gene therapy (group II) significantly attenuated this activation (Figure 8C).

Discussion

Animal models of hepatic fibrosis provide a means to study the cellular and molecular mediators of fibrosis in a

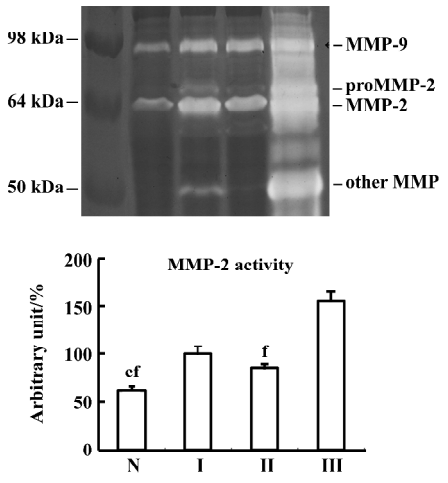

Figure 7. Representative photograph of gelatin zymography in CCl_4-treated mouse liver. When compared with the normal control group, levels of the active 64 kDa MMP-2 molecule were increased in groups I and IV after CCl_4 treatment. IL-10 gene therapy attenuated MMP-2 activation significantly ($^cP<0.01$ *vs* group I. $^fP<0.01$ *vs* group III).

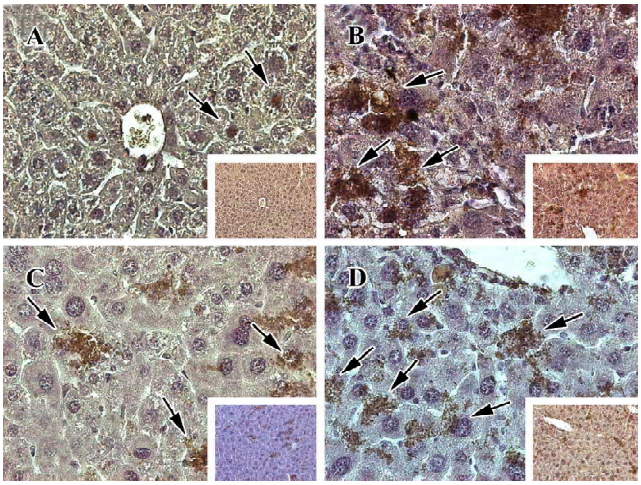

Figure 8. Representative photomicrographs of TIMP-1-stained mouse liver sections. After CCl_4 administration, TIMP-1 staining was significantly accentuated in groups I and III. IL-10 gene therapy (group II) attenuated this upregulation. (A) Normal control; (B) Group I; (C) Group II; (D) Group III. Arrows indicate TIMP-1-positive cells. Magnification, ×400 (inset, ×200).

serial manner during both progression and recovery. Several approaches to the induction of fibrosis have been described. Of these, CCl_4 intoxication in rats and mice is probably the most widely studied[29]. In addition, the CCl_4 model is the best characterized with respect to histological, biochemical, cellular, and molecular changes associated with the development of fibrosis[30,31]. CCl_4 can be given intraperitoneally or by oral gavage; it induces hepatocyte necrosis and apoptosis with associated HSC activation and tissue fibrosis. With ongoing treatment CCl_4 can be used to induce bridging hepatic fibrosis (4 weeks of twice-weekly treatment), cirrhosis (8 weeks of twice-weekly treatment) and advanced micronodular cirrhosis (12 weeks of twice-weekly treatment)[31].

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines[32]. IL-10 has been shown to downregulate the synthesis of collagen type I and TIMP in previous investigations[33,34]. It also plays an anti-fibrogenic role by decreasing the levels of pro-fibrogenic cytokines, including TGF-β1 and TNF-α[33]. In the present study, we demonstrated that electroporative IL-10 gene therapy provided an effective expression method for long-term use. This treatment reversed established liver fibrosis and reduced collagen synthesis in mice. IL-10 gene therapy also inhibited HSC activation after CCl_4 administration. The fibrogenic gene (TGF-β1 and TNF-α) response attenuation may be responsible for the hepatoprotective effect of IL-10.

COX-2 is a key executor of uncontrolled inflammation[35]. Overexpression of COX-2 has been demonstrated in CCl_4-induced liver fibrosis and post-viral human cirrhosis[36,37]. Further, COX-2 can contribute to hepatic carcinogenesis by increasing necroinflammatory activity, promoting proliferation, and enhancing angiogenesis[38,39]. Selective COX-2 blockers are known to reduce CCl_4-induced liver fibrosis[36]. Hence, COX-2 may be a new therapeutic target for treatments for liver cirrhosis. IL-10 is known as the central regulator of COX-2[40]. Therefore, IL-10 gene therapy might have exerted its anti-hepatic fibrogenesis effect through COX-2 inactivation.

Cell adhesion molecules are known as prognostic markers of liver fibrosis[41]. Expression of ICAM-1 and VCAM-1 modulated by TNF-α are upregulated in alcoholic hepatitis, CCl_4-induced liver injury, and nutritional fibrosis[42–44]. A previous study showed that ICAM-1 and VCAM were upregulated in IL-10 knockout-colitis in mice[45]. In addition, IL-10 can attenuate ICAM-1 activation in cisplatin nephrotoxicity[46]. Therefore, cell adhesion molecule regulation may be involved in the anti-fibrotic effect of IL-10.

The imbalance between MMP and TIMP in the ECM contributes to the pathogenesis of liver fibrosis. Matrix metalloproteinases are a family of zinc-dependent proteases capable of degrading hepatic ECM, thereby playing a central role in tissue remodeling and repair after injury[47]; however, persistent overexpression of MMP may contribute to the pathogenesis of liver diseases. Inhibition of MMP-2 produced by activated stellate cells blocks lethal hepatitis

and apoptosis induced by TNF-α[48]. Furthermore, MMP-2-deficient mice have decreased hepatocyte apoptosis and necrosis, and enhancsed survival in this model. Recent studies have also revealed a strong correlation between MMP-2 activity and severity of human liver disease[49]. MMP activity is regulated by the TIMP, which binds in a substrate- and tissue-specific manner to MMP, blocking their proteolytic activity[50]. Antibodies and antisense oligonucleotides directed at TIMP-1 attenuate rat liver fibrosis[50,51]. IL-10 is known to suppress MMP-2 and TIMP-1 expression in HSC during liver fibrosis[52]. In the present study, we demonstrated that IL-10 gene therapy attenuated MMP-2 and TIMP activation in the fibrotic liver. Therefore, its collagenolytic effect might be attributed to MMP and TIMP modulation.

In the present study, we demonstrated the anti-hepatic fibrogenic effect of IL-10 in mice. IL-10 gene therapy reversed established CCl_4-induced liver fibrosis in mice through fibrogenic gene response attenuation. In conclusion, IL-10 gene therapy may be a new therapeutic modality for liver cirrhosis with potential clinical use.

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