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Interleukin-10 gene therapy reverses thioacetamide-induced liver fibrosis in mice

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Abstract

Hepatic fibrosis represents a process of healing and scarring in response to chronic liver injury. Interleukin-10 (IL-10) is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis. The aim of this study was to investigate whether IL-10 gene therapy possesses anti-hepatic fibrogenesis in mice. Liver fibrosis was induced by long-term thioacetamide administration in mice. Human IL-10 expression plasmid was delivered via electroporation after liver fibrosis established. IL-10 gene therapy reversed hepatic fibrosis and prevented cell apoptosis in a thioacetamide-treated liver. RT-PCR revealed IL-10 gene therapy to reduce liver transforming growth factor-β1, tumor necrosis factor-α, collagen α1, cell adhesion molecule, and tissue inhibitors of metalloproteinase mRNA upregulation. Following gene transfer, the activation of α-smooth muscle actin and cyclooxygenase-2 was significantly attenuated. In brief, IL-10 gene therapy might be an effective therapeutic reagent for liver fibrosis with potential future clinical applications.

Keywords: IL-10; Gene therapy; Liver fibrosis; Thioacetamide; COX-2

Progressive accumulation of fibrillar extracellular matrix (ECM) in the liver is the consequence of reiterated liver tissue damage due to infective (mostly hepatitis B virus and hepatitis C virus), toxin/drug-induced, metabolic and autoimmune causes, and the relative chronic activation of the wound healing reaction [1–3]. The process may result in a clinically evident liver cirrhosis. Cirrhosis is defined as an advanced stage of fibrosis, characterized by the formation of regenerative nodules of liver parenchyma which are separated by and encapsulated in fibrotic septa, and is associated with major architectural changes. In cirrhotic liver, an imbalance takes place in the excess synthesis of ECM (fibrogenesis) and/or the reduction in the removal (fibrolysis), with consequent fibrotic scarring [4,5]. The pathophysiology of ECM formation during hepatic fibrosis is multifaceted and complex [6,7]. Fibrogenesis involves a change in the expression of ECM proteases (matrix metalloproteinases; MMPs) or their inhibitors (tissue inhibitors of metalloproteinases; TIMPs) and an increase in the synthesis of interstitial and basement membrane collagens, fibronectin driven by signaling pathways mediated by proinflammatory cytokines such as transforming growth factor (TGF-β1), tumor necrosis factor-α (TNF-α) [8–11].

Interleukin (IL)-10 is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis. Recombinant human IL-10 has been produced and tested in clinical trials, suggesting that IL-10 may become a new therapeutic target of chronic

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hepatitis C and other liver diseases [12]. Besides, IL-10 gene therapy had been studied in the animal models of pancreatitis, colitis, and liver transplantation [13–15]. Therefore, it may be possible to utilize its therapeutic properties to create a gene-based drug treatment. In this study, we had investigated IL-10 gene therapy against TAA-induced liver fibrosis in mice and explored its underlying mechanism.

Materials and methods

Animals. Male 6- to 8-week-old ICR mice were purchased from National Science Council, Taiwan, and allowed to acclimate for 5 days before use. Mice were housed in the Kaohsiung Chang-Gung Memorial Hospital Animal Facility under standard temperature, and light and dark cycles. All procedures were performed under the approval of Kaohsiung Chang-Gung Memorial Hospital Animal Care and Use Committee.

IL-10 expression plasmid preparation. The human IL-10 expression plasmid (pCYIL-10 vector) was a kind gift from Dr. Xianmin Meng (Thomas Jefferson University, Philadelphia, PA) [16]. In brief, the full length of human IL-10 cDNA was subcloned into a pCY4B expression vector driven by chicken β-actin promoter with a cytomegalovirus immediate early enhancer. pCMV-Lacz was used as a vehicle control. These plasmids were purified using the EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA).

Liver fibrosis induction and gene therapy. Hepatic fibrosis was induced by giving 300 mg/L thioacetamide (TAA, Sigma–Aldrich, St Louis, MO) in the drinking water of the mice for an 18-week period modified from a previous study [17,18]. Sixteen mice were sacrificed at the end of 14 weeks for confirmation of liver fibrosis (group I). For evaluating the anti-fibrotic effect of IL-10, gene therapy was started at the end of 14 weeks. Briefly, 30 μl of bovine hyaluronidase (0.4 IU/μl) (Sigma–Aldrich) was injected into anterior tibialis (AT) muscle of mice 2 h before electroporation. Plasmid (pCYIL-10) was injected into the bilateral AT muscle by a 27 G needle (30 μl of each leg, 4 μg/μl) (group II, $n = 16$). Electroporation was delivered by electrical pulses (8 pulses of 20 ms, 175 V/cm, and 1-s interval) with Tweezertrode electrode disks and an electrical pulse generator (T830, BTX, San Diego, CA) [19]. Another 32 mice were gene electrotransferred in the same manner as above by pCYIL-10 (group III, $n = 16$) or pCMV-LacZ (group IV, $n = 16$) at the end of 14 and 16 weeks. All these mice (group II–IV) were sacrificed at the end of an 18-week treatment. Another five mice were sacrificed before TAA intoxication as normal control (group N).

Histopathology and immunohistochemistry. For histopathology, mice were sacrificed 0, 14, and 18 weeks after TAA. Liver was removed and fixed in 10% formalin solution. Five-micrometer sections were stained with hematoxylin–eosin and 0.1% sirius red in picric acid (Sigma–Aldrich), with matrix density quantified by a computerized image analysis system as previously described [20]. For immunohistochemistry, sections were washed in 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) or COX-2 (cyclooxygenase-2) (Abcam, Cambridge, MA) antibody. Negative control of immunohistochemical studies was incubated as above without primary antibodies. Immunoreactivity was visualized using the Vectastain Elite ABC Peroxidase method (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) as the chromagen. Furthermore, apoptosis in the liver was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) using an apoptosis detection kit (Oncogene Research Products, Cambridge, MA). TUNEL staining was performed according to the manufacturer's instructions.

Soluble collagen measurement. For soluble collagen analysis, the Sircol collagen assay (Biocolor, Belfast, UK) was performed following the manufacturer's instructions as given in a previous study [21]. Briefly, 50 mg of liver was homogenized, and total acid pepsin-soluble collagens were extracted overnight using 5 mg/ml pepsin in 500 μl of 0.5 M acetic acid. One milliliter of Sircol dye reagent was added to 100 μl of each sample, in duplicate, and incubated at 25 °C for 30 min. After centrifugation, the pellet was suspended in 1 ml of alkali reagent, and the absorbance was read at 540 nm.

Immunoblotting. Liver specimens were homogenized in lysis buffer with complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany). For analysis of α-SMA (α-smooth muscle actin) expression after thioacetamide intoxication, 20 μg of protein extracts was electrophoresed on a 10% acrylamide SDS–PAGE gel and immunoblotted onto PVDF membranes. 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 a horseradish peroxidase (HRP)-linked IgG. Bands were visualized using an ECL detection system (Amersham-Pharmacia Biotech, Little Chalfont, England). Band intensities were quantified by using an image analyzer (Densitograph AE-6900M, Atto, Tokyo, Japan).

Reverse transcription-polymerase chain reaction. Liver specimens were harvested 0, 14, and 18 weeks after TAA. The expressions of TGF-β1, collagen α1, fibronectin, tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), TIMP-1, and TIMP-2 mRNA were analyzed by the reverse transcription-polymerase chain reaction (RT-PCR) technique. Total RNA was extracted from harvested tissues with chloroform and the TRIzol reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed into cDNA. PCR was performed at a final concentration of 1× PCR buffer, 1.0 μM each of the 3′ and 5′ primers, and 10 U of Advan-*Taq* Plus DNA polymerase (Clontech, Palo Alto, CA) in a total volume of 50 μl. The mixture was amplified for 32 cycles in a thermal cycler (Stratagene, La Jolla, CA). And β-actin was amplified to verify equal loading. The primer sequence and expected product size are described in Table 1. Amplification products were separated by agarose-gel electrophoresis and visualized by ethidium bromide staining. The gel was scanned at a NucleoVision imaging workstation (NucleoTech, San Mateo, CA) and quantified using GelExpert release 3.5.

Statistical analysis. All data are presented as means ± SEM (at least three separate experiments). Statistical analyses were performed using one-way ANOVA, followed by *t* test. *p* values less than 0.05 were considered significant.

Results

Liver histopathology and collagen content

There were no significant differences in food and water intake throughout the entire study period between groups. After a 14-week TAA administration, liver fibrosis was already seen. H&E staining of liver sections revealed extensive fibrosis, portal-to-portal fibrous bridging, and nodular transformation in group I and IV. IL-10 gene therapy significantly abrogated hepatic fibrogenesis (Fig. 1). Sirius red staining also demonstrated that IL-10 gene therapy (group II, III) reduced matrix density (Fig. 2). This was further confirmed by the measurement of liver collagen content (Table 2).

IL-10 and COX-2 immunohistochemistry

There was scanty staining for IL-10 without gene therapy (group N, I, and IV). Strong positive staining for human IL-10 was detected in the liver following electroporative gene transfer (group II and III) (Fig. 3). COX-2 was not detected in the normal group. COX-2 expression was upregulated after TAA intoxication (group I, IV). IL-10 gene therapy significantly diminished this COX-2 expression (Fig. 4).

Table 1
The PCR primer sequence and expected product size

Gene	Primer sequence	Product size (bp)
TGF-β1	F 5′-TGAGTGGCTGTCTTTTGACG-3′ R 5′-ACTTCCAACCCAGGTCCTTC-3′	350
Collagen α1	F 5′-GAAACCCGAGGTATGCTTGA-3′ R 5′-GACCAGGAGGACCAGGAAGT-3′	274
Fibronectin	F 5′-TGTGACAACTGCCGTAGACC-3′ R 5′-GACCAACTGTCACCATTGAGG-3′	386
TNF-α	F 5′-GGCAGGTCTACTTTGGAGTCATTG-3′ R 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′	300
ICAM-1	F 5′-TGCGTTTTGGAGCTAGCGGACCA-3′ R 5′-CGAGGACCATACAGCACGTGCAG-3′	326
VCAM-1	F 5′-CCTCACTTGCAGCACTACGGGCT-3′ R 5′-TTTTCCAATATCCTCAATGACGGG-3′	442
TIMP-1	F 5′-TCTTGGTTCCCTGGCGTACT-3′ R 5′-GTGGCAGGCAAGCAAAGTG-3′	151
TIMP-2	F 5′-TGC CCT GGG ACA CGC TTA-3′ R 5′-GTACCACGCGCAAGAACCAT-3′	151
β-Actin	F 5′-TCAGCAAGCAGGAGTACGATGA-3′ R 5′-TGCGCAAGTTAGGTTTTGTCAA-3′	117

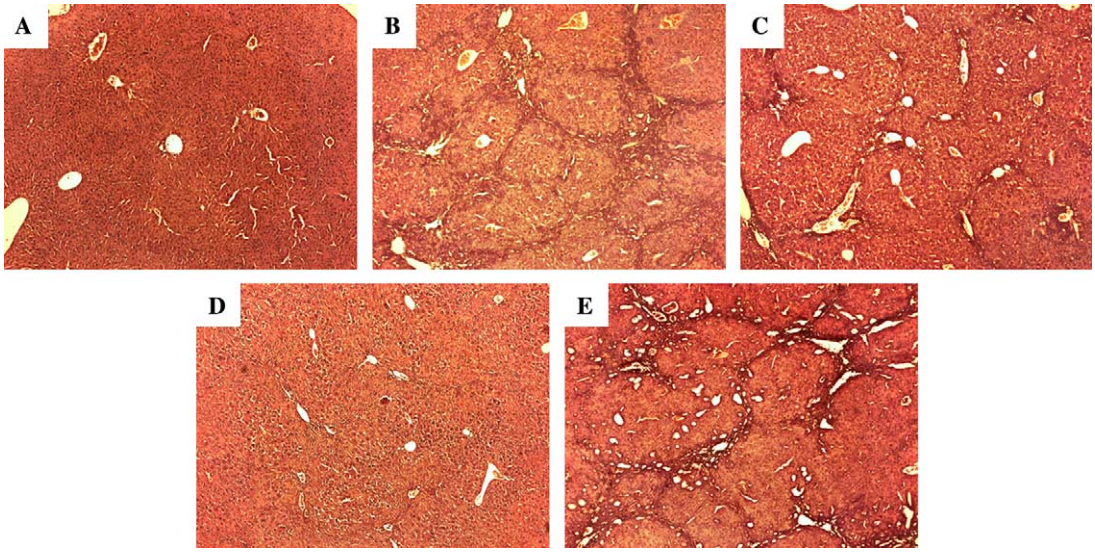

Fig. 1. Representative photographs of H&E-stained liver sections in mice. Extensive fibrosis, portal to portal fibrous bridging, and nodular transformation were demonstrated in the group I and IV after chronic TAA administration. IL-10 gene therapy (group II and III) significantly abrogated hepatic fibrogenesis. (A) Normal control, (B) group I, (C) group II, (D) group III, (E) group IV. Magnification 100×.

Tunel staining

Apoptosis in the liver was examined by TUNEL study. There was almost no TUNEL staining in the normal control mice. TUNEL-positive cells were observed in the fibrotic liver (group II, IV). There were remarkable fewer TUNEL-positive cells after gene therapy (Fig. 5). This result indicated IL-10 gene therapy inhibits TAA-induced cell apoptosis in the liver.

Immunoblotting of α-SMA

α-SMA (marker of activated hepatic stellate cell—HSC) was known to activate after acute TAA administration [22,23]. In this study, the expression of α-SMA increased after chronic TAA as measured by immunoblotting (Fig. 6). IL-10 gene therapy (group II, and III) significantly reduced this upregulation by implicating HSC inactivation ($p < 0.001$ vs group I, IV). α-Tubulin was used as internal control.

RT-PCR

The expressions of TGF-β1, collagen α1, fibronectin, TNF-α, ICAM-1, VCAM-1, TIMP-1, and TIMP-2 mRNA were upregulated in the fibrotic liver as semi-quantified by RT-PCR (Fig. 7). β-Actin was amplified as internal control. IL-10 gene therapy (group II, III) significantly attenu-

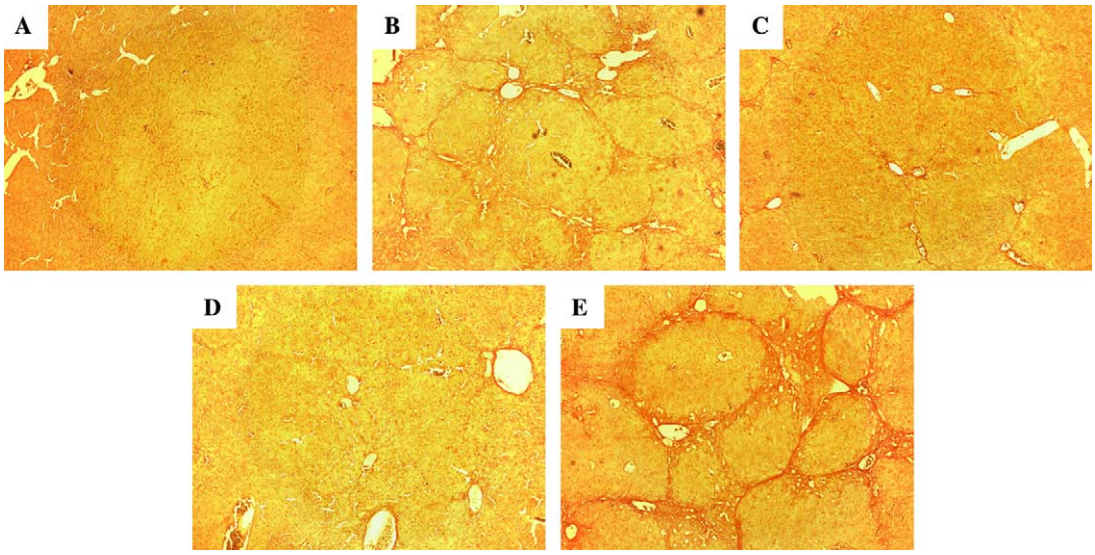

Fig. 2. Representative photographs of Sirius red stained liver sections in mice. Sirius red staining (specific for collagen) was significantly increased in the group I and IV after chronic TAA administration. IL-10 gene therapy (group II and III) significantly reduced Sirius red matrix density. (A) Normal control, (B) group I, (C) group II, (D) group III, (E) group IV. Magnification 50×.

Table 2
Effect of IL-10 gene therapy on the regression of hepatic fibrosis

	Sirius red matrix density (%)	Collagen content (μg/mg)
Group N	$0^{**,\#\#}$	$7.4 \pm 1.0^{**,\#\#}$
Group I	8.3 ± 1.1	56.2 ± 7.3
Group II	$5.1 \pm 1.0^{*,\#\#}$	$34.6 \pm 6.1^{*,\#\#}$
Group III	$4.4 \pm 1.1^{*,\#\#}$	$29.8 \pm 6.6^{*,\#\#}$
Group IV	11.1 ± 1.0	65.2 ± 7.3

IL-10 gene therapy significantly reduced the degree of liver fibrosis induced by chronic TAA administration in mice as measured by Sirius red matrix density, and collagen content.
* $p < 0.05$ vs group I.
** $p < 0.01$ vs group I.
$^{\#\#}$ $p < 0.01$ vs group IV.

ated this increment [TGF-β1: $p < 0.05$ (II vs I, II vs IV), $p < 0.01$ (III vs I, III vs IV); collagen α1: $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV); TNF-α : $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV); ICAM-1: $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV); VCAM-1: $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV); TIMP-1: $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV); and TIMP-2: $p < 0.01$ (II vs I, II vs IV, III vs I, and III vs IV)]. Interestingly, IL-10 gene therapy did not suppress fibronectin activation ($p > 0.05$). In brief, IL-10 gene transfer suppressed fibrogenic, proinflammatory, and cell adhesion molecule gene response after TAA.

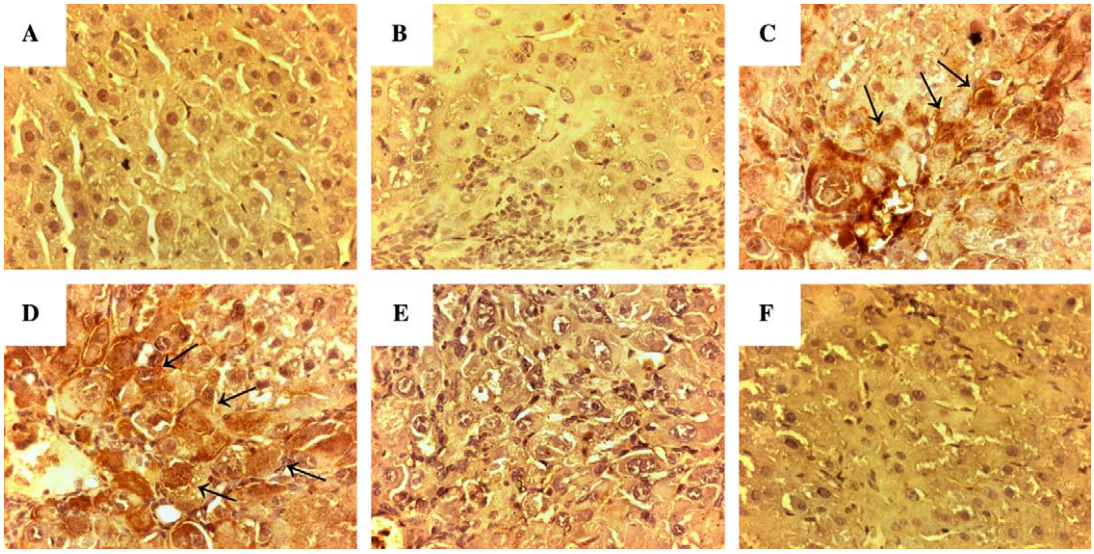

Fig. 3. Representative photographs of IL-10-stained liver sections in mice. There was no staining for human IL-10 without gene therapy (group N, I, and IV). Positive staining for human IL-10 was detected in the liver following gene transfer (group II and III). (A) Normal control, (B) group I, (C) group II, (D) group III, (E) Negative control (omit the first antibody) of group III. (F) Group IV. Arrows indicate human IL-10-positive cells. Magnification 400×.

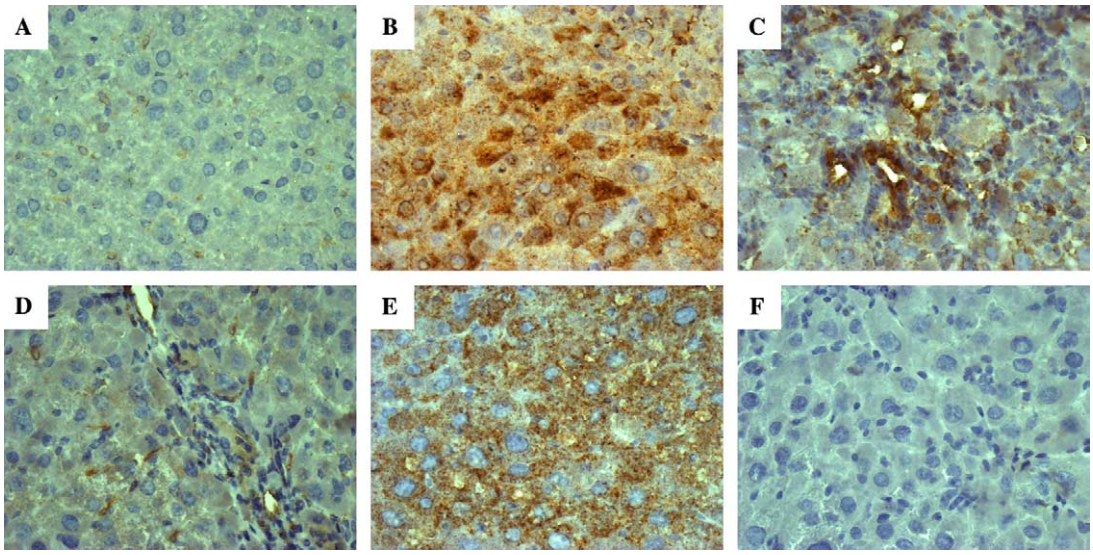

Fig. 4. Representative photographs of COX-2-stained liver sections in mice. COX-2 is not detected in the normal control group. COX-2 expression was increased in the group I and IV after TAA administration. IL-10 gene therapy (group II and III) significantly attenuated this expression. (A) Normal control, (B) group I, (C) group II, (D) group III, (E) group IV. (F) Negative control (omit the first antibody) of group IV. Magnification 400×.

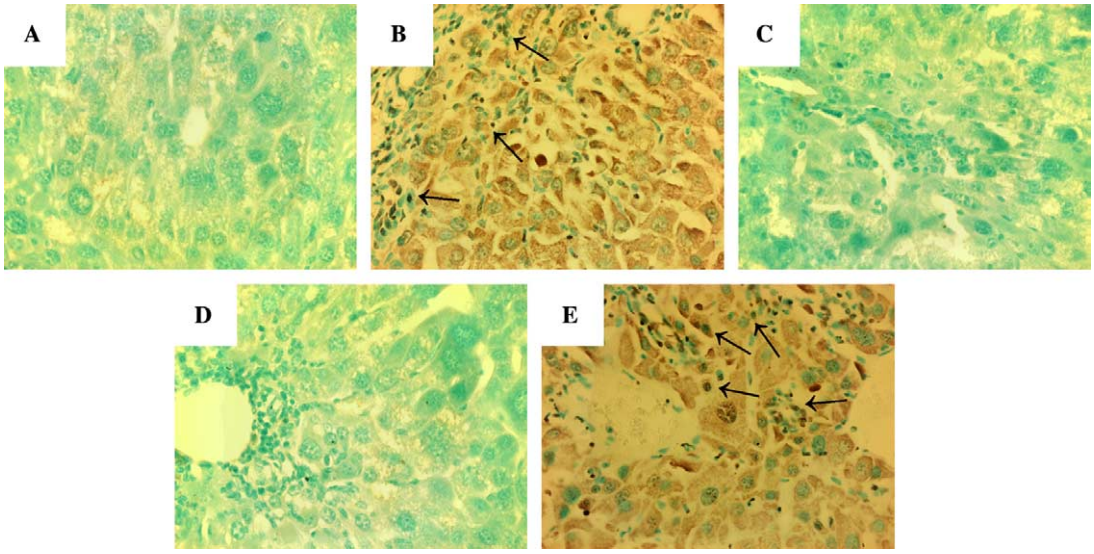

Fig. 5. Representative photographs of TUNEL-stained liver sections in mice. There was almost no TUNEL staining in the normal control group. TUNEL-positive cells were observed in the group II and IV. There were remarkably fewer TUNEL-positive cells after α-MSH gene therapy (group II and III). (A) Normal control, (B) group I, (C) group II, (D) group III, (E) group IV. Arrows indicate TUNEL-positive cells. Magnification 400×.

Discussion

Liver cirrhosis was considered to be an irreversible process characterized by excess ECM deposition in the liver with scar formation and destruction of the normal liver architecture [2,3,10]. The most effective mode to treat liver fibrosis at early stages is to remove the causative agent. Treatments that are currently under evaluation mainly inhibit HSC activation and proliferation or the release of cytokines and only few drugs have a direct effect on collagen synthesis or degradation [2,3,24].

Imbalance between MMPs and TIMPs in the ECM contributes to the pathogenesis of liver fibrosis. MMP activity is regulated by the TIMPs, which bind in substrate- and tissue-specific manners to MMPs, blocking their proteolytic activity [25]. During fibrosis, TIMP mRNA and protein levels dramatically increase while MMP levels increase modestly or remain relatively static [26,27]. Antibody and antisense oligonucleotide directed to TIMP-1 attenuated rat liver fibrosis [25,28]. Therefore, the interplay between MMPs and TIMPs provides a key point of regulation to target therapies for treating patients with ongoing fibrosis.

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of proinflammatory cytokines [29]. IL-10 was shown to downregulate the synthesis of collagen type

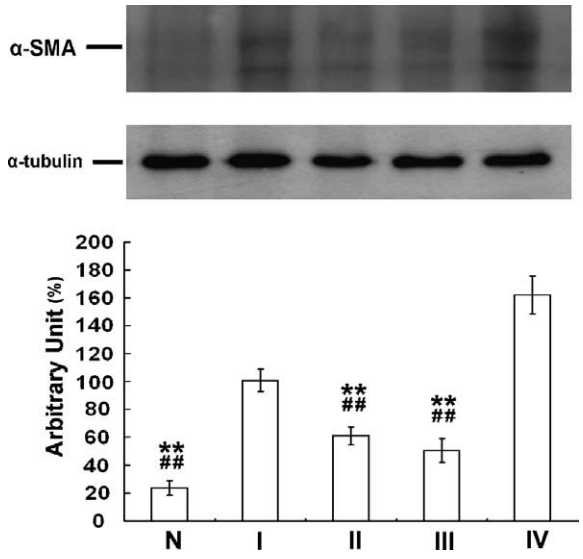

Fig. 6. Representative photographs of α-SMA expression in TAA-treated mice liver TAA by immunoblotting. Compared to normal control, α-SMA activation was demonstrated in the group I and IV after TAA. IL-10 gene therapy (group II and III) significantly abrogated this increment (**$p < 0.01$ vs group I, $^{\#\#}p < 0.01$ vs group IV). Arbitrary unit was defined as α-SMA/α-tubulin band density.

I and TIMP [30,31]. It could also play an antifibrogenic role by reducing the profibrogenic cytokines including TGF-β1 and TNF-α [30]. In this study, we had demonstrated that electroporative IL-10 gene therapy provides an effective expression for a long-term use. It could reverse establish liver fibrosis and reduce collagen synthesis in mice. IL-10 gene therapy could also inhibit HSC activation and prevent cell apoptosis after TAA. Fibrogenic gene (TGF-β1, TNF-α, and TIMP) response attenuation might be responsible for the IL-10 hepatoprotective effect.

COX-2 is a key executor of uncontrolled inflammation [32]. Over-expression of COX-2 has been demonstrated in TAA-induced liver fibrosis and postviral human cirrhosis [33,34]. In addition, COX-2 could contribute to hepatic carcinogenesis by increasing necroinflammatory activity, promoting proliferation, and enhancing angiogenesis [35,36]. Selective COX-2 blocker is known to reduce TAA-induced liver fibrosis [33]. Hence, COX-2 might be a novel therapeutic target for liver cirrhosis. IL-10 is known as the central regulator of cyclooxygenase-2 [37]. Therefore, IL-10 gene therapy might exert anti-hepatic fibrogenesis through COX-2 inactivation.

Cell adhesion molecules are known as prognostic marker of liver fibrosis [38]. The expressions of ICAM-1 and VCAM-1 modulated by TNF-α are upregulated in alcoholic hepatitis, CCl_4-induced liver injury, and nutritional fibrosis [39–41]. A previous study has shown that ICAM-1 and VCAM are upregulated in the IL-10 knockout colitis mice [42]. Besides, IL-10 could attenuate ICAM-1 activation in cisplatin nephrotoxicity [43]. Therefore, cell adhesion molecule regulation might be involved in IL-10 anti-fibrotic effect.

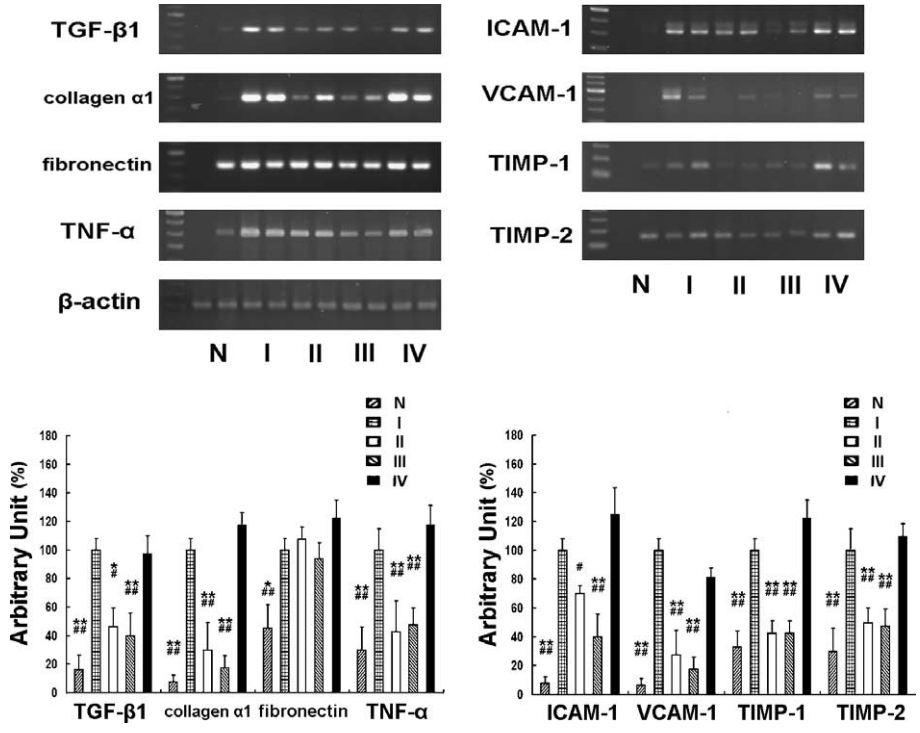

Fig. 7. Representative photographs of semi-quantified RT-PCR in the TAA-treated mice liver. IL-10 gene therapy (group II and III) significantly attenuated the TGF-β1, collagen α1, TNF-α, ICAM-1, VCAM-1, TIMP-1, and TIMP-2 mRNA activation after TAA as compared to group I and IV (*$p < 0.05$ vs group I, **$p < 0.01$ vs group I, $^{\#}p < 0.05$ vs group IV, and $^{\#\#}p < 0.01$ vs group IV). Interestingly, the fibronectin mRNA activation after TAA was not affected by IL-10 ($p > 0.05$). β-Actin was amplified to verify equal loading. Arbitrary unit was defined as target gene/β-actin band density.

In this study, we had delineated IL-10 effect on liver fibrosis. IL-10 gene therapy reversed established liver fibrosis in mice with fibrogenic gene response attenuation. In conclusion, IL-10 gene therapy might provide a novel therapeutic route for liver cirrhosis with clinical applications.

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