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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.
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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).

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