The Effects of Interleukin-10 on the Expression of Fas and FasL in Rat Hepatic Stellate Cells

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Abstract: Objective: To study the effects of interleukin-10 on the expression of fas and fasL in hepatic stellate cells in experimental rat hepatic fibrosis.

Methods: Sixty clean SD rats were divided into control group (8 in group N), the model group (28 in group C) and the IL-10 treated group (24 in group I) randomly. The rats were administered CCl_4 with or without IL-10 treatment. Hepatic stellate cells (HSCs) were isolated from these rats at the beginning of the seventh and eleventh weeks during the course of liver fibrosis, respectively. Semi-quantitative RT-PCR and Western-blot were used to analyze mRNA and protein expressions of Fas and FasL from freshly isolated HSC. The liver tissues were harvested from three groups.

Results: The CCl_4- induced experimental rat hepatic fibrosis model was established successfully. The IL-10 could decrease the fibrotic degree of rat liver. The Fas and FasL mRNA can be measured in HSC of 3 groups. The mRNA of Fas and FasL in group C were significantly increased time-dependently compared to those of control group. In the 7th week, the expression level of Fas and FasL in group C was 0.66±0.02 and 0.45±0.33 respectively, and in the group I, the level was 0.74±0.02 and 0.52±0.05 respectively. In the 11th week, the level in group C was 0.72±0.02 and 0.62±0.04 respectively, and in the group I, the level was 0.73±0.04 and 0.83±0.04 respectively. The western-blot analysis showed that there was no FasL expression in group N, the expression of Fas and FasL in group C was significantly increased time-dependently compared to those of control group. After being treated with IL-10, the expression level of Fas and FasL was higher than those of group C. In group C, the expression level of Fas and in the 11th week was 0.92±0.02and 0.99±0.02 respectively, and in group I, the level was 0.96±0.16 and 1.22±0.03 respectively. In group C, the level of FasL in the 7th week and in the 11th week was 1.24±0.03 and 1.33±0.03 respectively, and in group I, the level was 1.36±0.16 and 1.39±0.19 respectively.

Conclusions: The expression of Fas and FasL increased in the course of the liver fibrosis, and would be furthered by IL-10. The IL-10 could cause the apoptosis of activated HSC, and making antifibrogenic come into effect in these ways.

Key Words: Liver fibrosis, rat, hepatic stellate cell, interleukin-10, Fas/FasL.

INTRODUTION

Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins including collagen that occurs in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation [1-3]. Activated hepatic stellate cells, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-producing cells in the injured liver. These cells are activated by fibrogenic cytokines such as TGF-β1. Cellular investigations of liver fibrosis have been suggested that the hepatic stellate cells (HSCs) are central to the fibrotic process, and HSCs are the main resource of ECM [4-6]. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, fibrogenic properties, secreting cytokines, and expressing α-smooth muscle actin (α-SMA)[7-8]. HSCs are the final target cells of liver injury factors. Many of the mechanisms of liver fibrosis showed that liver fibrosis is generally considered irreversible condition inhibit or block the process of liver fibrosis could prevent the liver cirrhosis effectively, which has stimulated researchers to develop antifibrotic drugs. The drug that inhibits the activation of HSCs or promotes the activated HSCs into apoptosis could inhibit the liver fibrosis, which maybe an ideal route to treat the liver fibrosis. During the fibrosis resolution, apoptosis was the main way to remove the activated HSCs. Some data demonstrate apoptosis of HSCs in the course of activation accompanied by an increased expression of FasL by the HSCs themselves [9]. Fas/FasL system is the key pathway for HSCs apoptosis. Cytokines are soluble autocrine and paracrine mediators [10]. Interleukin-10 could produce by several cells and have several functions, the main effects of IL-10 was shown to downregulate the inflammatory [11]. *In vitro* experiments have shown that HSCs express IL-10 receptor and produce IL-10 [12-13]. In the model of CCl_4 induced chronic liver injury, IL-10 deficient animals had a persistent increased inflammatory infiltrate, and developed a more extensive fibrosis than the ani-

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1573-4064/06 $50.00+.00

mals able to produce IL-10, indicating that IL-10 is involved in the control of fibrogenesis [14-16]. In the present study, the expressions of Fas and FasL in HSCs were examined and their changes were investigated in the presence of interleukin (IL-10), to study the effects of interleukin-10 on the liver tissues and hepatic stellate cells in experimental rat hepatic fibrosis, and to investigate the potential anti-fibrogenesis mechanism of IL-10.

MATERIALS AND METHODS

Materials

Sixty clean male Sprague-Dawley rats (used for isolating HSCs), weighing 400-500g were divided into 3 groups, 8 in control group (group N2), 28 in fibrogenesis group (group C2) and 24 in fibrosisintervention group (group I2). All rats were bred under routine conditions (room temperature, 22 ±2℃; humidity, 55%±5%; light, 12 hrs per day; drinking tap water and eating in any time; animal feed was provided by BK Company in Shanghai, China). The control rats were injected intraperitoneally with saline at a dose of 2 ml·kg^{-1}, twice a week. The rats in the other groups received intraperitoneal injection of 50% CCl_4 (2 ml·kg^{-1}), twice a week, as described previously. From the third week, the rats in intervention group were given intraperitoneally IL-10 (4 mg·kg^{-1}, dissolved in saline) 20 minutes before CCl_4 administration, as proposed by Nelson *et al*. [17]. All injections were performed at Monday and Thursday, with their body weights being determined before each injection. To the end of the experiment, 12 rats in fibrogenesis group and 8 in fibrosisintervention group died. No animals died in control group. In the seventh and eleventh weeks, 3 rats in control group and 5 rats in other two groups were sacrificed to collect their hepatic stellate cells.

Methods

Hepatic Stellate Cells Isolation and Culture

Nonparenchymal cells were isolated from rats of the experimental groups by means of sequential perfusion with collagenase and pronase E as described previously [18-19]. Briefly, at the beginning of the 7th and 11th wk, five rats of each group were selected randomly to perfuse successively with 0.13% pronase E and 0.025% type-IV collagenase through a portal vein catheter. The liver tissue suspension was incubated with 0.02% pronase E and 0.025% type-IV collagenase with agitation. Then the suspension obtained from the digested liver was spun by centrifugation with 11% Nycodenz density gradient to purify HSCs. Thereafter, HSCs were seeded at approximately 1×10^6 cells/mL of Dulbecco's modified eagle medium (DMEM) with 20% fetal calf serum in 96-well plates. Then HSCs were kept in culture at 37 °C in a 50 mL/L CO_2 atmosphere for 24 h. They were identified by their typical phase-contrast microscopic appearance and by fluorescence microscope. Cell vitality was checked by trypan blue exclusion.

RNA Extraction and RT-PCR

Total RNA was extracted from freshly isolated HSCs, according to the RNA isolation kit instructions (Jingmei Biotechnology Company of Shenzhen). Measuring the optical density at 260 and 280 nm assessed its quantity and purity. After measurement of RNA amount, samples were either used immediately for reverse transcription (RT) or stored at -70 °C.

For RT, 1μg total RNA was reversely transcribed following the instructions of first strand cDNA synthesis kit (Jingmei Biotechnology Company of Shenzhen). Reaction mixtures of 20 μl were transcribed using the following program: at 42 °C for 60 min, 99 °C for 5 min, and stored at -20 °C

PCR system contained 2 μl cDNA, 5 μl 10×buffer, 5 μl 25 mmol/L $MgCl_2$, 1 μl 10 mmol/L dNTP, 1 μl 20 pmol/μL target gene sense and anti-sense primer, 1 μl 20 pmol/μL β-actin primer pair, 3 U Taq DNA polymerase. PCR was carried out as follows: initial denaturation at 95 °C for 5 min, 30 amplification cycles (denaturation at 94 °C for 45 s, annealing T see Table **1**, extension at 72 °C for 1 min) and final extension at 72 °C for 7 min.

Electrophoresis and Semi-Quantitative Analysis

The PCR products were run on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Bioimagine system was employed to detect the density of bands of PCR products. The values of detected products expression were semi-quantified by scanning densitometry using the ratios of detection/β- actin to assess the relative level. The detection was analyzed five times.

Protein Extraction and Western Blot Analysis

Total protein was extracted from freshly isolated HSCs according to the protein isolation kit instructions (Promega Co. USA). HSCs specimens were homogenized, sonicated

Table 1. Primer Sequences Used in this Study

mRNA	Sense primers	Antisense primers	product length (bp)	annealing T(℃)
Fas	GAATGCAAGGGACTGATAGC	TGGTTCGTGTGCAAGGCTC	414	55
FasL	GGAATGGGAAGACACATATGGAACTGC	CATATCTGGCCAGTA GTGCAGTAATTC	239	58
β-actin1	AGC ACT TGC GGT CCA CGA	GAG CTA TGA GCT GCC TGA	410	55
β-actin2	CCA ACC GTG AAA AGA TGA CC	CAG GAG GAG CAA TGA TCT TG	660	55

for 15s twice in 300μl of lysis buffer, and placed on ice for 30 min. The lysate was centrifuged at 25 000 g for 15 min at 4 °C, the supernatant was collected. Measuring the optical density at 260 and 280 nm assessed their quantity and purity. Each 20 μg protein sample was treated with SDS sample buffer, denatured in boiling water for 5 minutes, separated by SDS-PAGE on a 10% acrylamide gel, and transferred onto a nitrocellulose (NC) membrane. After blocking for 1 hour at room temperature with 3% skim milk, the NC membrane was incubated for 1 hour at room temperature with Fas and FasL primary antibody (1:300) respectively diluted in antibody buffer (10 mmol/L Tris-HCl pH 7.4, 0.8% NaCl, 0.02% KCl, 0.1% Tween 20, and 3% skim milk). The NC membrane was washed three times with washing buffer and incubated for 30 minutes at room temperature with horseradish peroxidase-conjugated secondary antibody diluted (1: 1000) in antibody buffer. After 4 washes in washing buffer, the NC membrane was processed for autoradiography using chemilumi nescence techniques (ECL kits), according to the manufacturer's instructions. The molecular mass of the protein was estimated to be relative to pre-stained size marker (Bio-Rad Laboratories, USA).

Statistical Analysis

All data were expressed as mean±SD; t test was used for comparison between groups. SPSS10.0 was used to describe the difference between groups. P values less than 0.05 were regarded as statistically significance.

RESULTS

Animal Model

Liver fibrosis, as shown histologically, became remarkable during the treatment with CCl_4. In the fifth week, steatosis and ballooning degeneration were obvious. In the seventh week, the collagen fibers increased and began to extend to the parenchyma. In the ninth week, complete fibrous septa were seen and pseudobulbar structures were also present occasionally. In the IL-10-intervention group, the CCl_4-caused alterations as described above seemed to be markedly alleviated, with no evident changes observed in the fifth week, less profound steatosis and necrosis noted in the seventh week, and only early-stage fibrosis found in the ninth week (Fig **1A-C**).

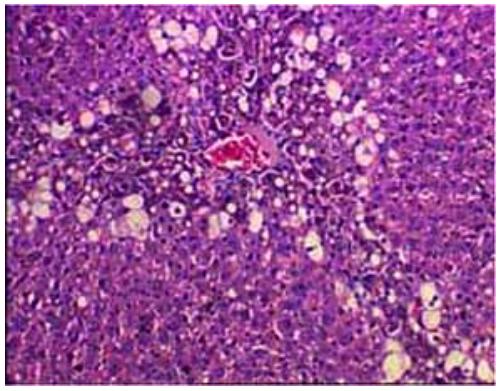

Fig (1A). Liver of rats in group C (7th week, H-E staining, ×100).

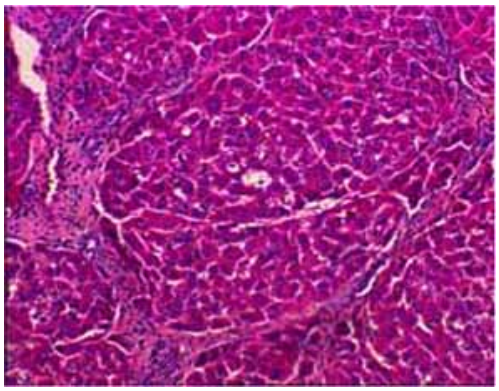

Fig (1B). Liver of rats in group C (11th week, H-E staining, ×100).

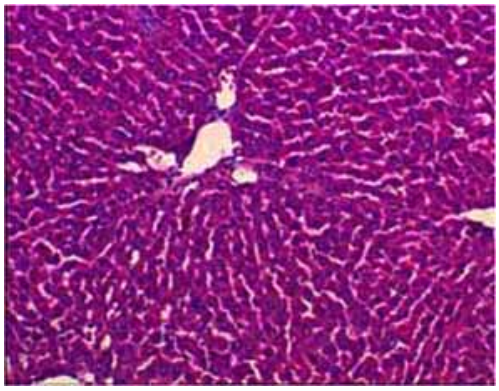

Fig (1C). Liver of rats in group I (11th week, H-E staining, ×100).

Hepatic Stellate Cells Identification

The HSCs were isolated successfully. Stellate cells were identified by immunohistochemistry with a monoclonal antibody against desmin. Greater than 95% of the isolated cells were stellate cells (Fig. **2A**). The cells' viability was determined by trypan blue exclusion staining with viability over 90%. In group C, the cells contained pseudopodium and refracted particles. In the eleventh week, the HSCs of group I become surrounded with pyknotic nucleus, as the vigor of cells was weak (Fig. **2B**).

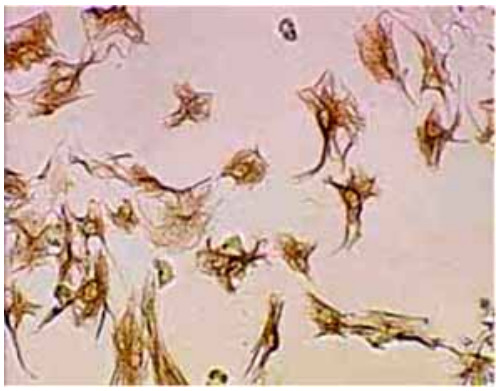

Fig (2A). Desmin staining of HSC (×100).

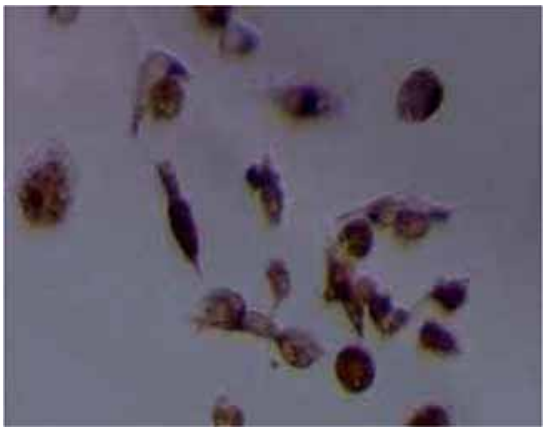

Fig (2B). HSC of rats in group I (11th week, ×200).

Relative Quantities of Fas and FasL mRNA in HSCs

The Fas and FasL mRNA can be measured in HSCs of control group. The mRNA of Fas and FasL in fibrotic group were significantly increased time-dependently compared to those of control group. After being treated with IL-10, the expression level of Fas and FasL mRNA was higher than those of fibrotic group. The expression of Fas and FasL mRNA increased in the course of the liver fibrosis, and would be furthered by IL-10 (Fig **3 A-B**). Comparison of Fas and FasL mRNA expression levels between 3 groups shown in Table **2**.

The Expressions of Fas and FasL Protein in HSCs

The western-blot analysis showed that no FasL protein was found in control group and that the expression of Fas and FasL protein (48KD and 40KD, respectively) in fibrotic group was significantly increased time-dependently compared to those of control group. After being treated with IL-10, the expression level of Fas and FasL protein was higher than those of fibrotic group. (Table **3** and Fig. **4 A-B**).

DISCUSSION

Hepatic fibrosis is a wound healing response comprising reversible scarring that occurs in almost all patients with chronic, but not self-limited liver injury. Ultimately, hepatic fibrosis leads to cirrhosis, characterized by nodule formation and organ contraction. The causes of cirrhosis are multiple and include congenital, metabolic, inflammatory, and toxic liver diseases. In all circumstances, the composition of the hepatic scar is similar. In normal liver, HSCs are nonparenchymal, quiescent cells whose main functions are to store vitamin A and probably to maintain the normal basement membrane-type matrix. However, numerous *in vivo* and *in vitro* studies indicate that in response to liver injury, HSCs undergo an activation process in which they lose vitamin A,

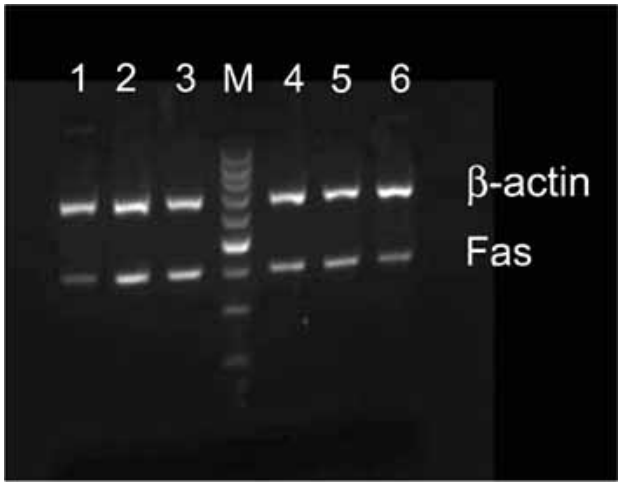

Fig (3A). Fas mRNA expression on HSCs (414bp).
Lane 1- group N, Lane 2- group I (11th week), Lane 3- group I (7th week), M- maker, Lane 4 - group C (7th week), Lane 5- group C (11th week), Lane 6- group N.

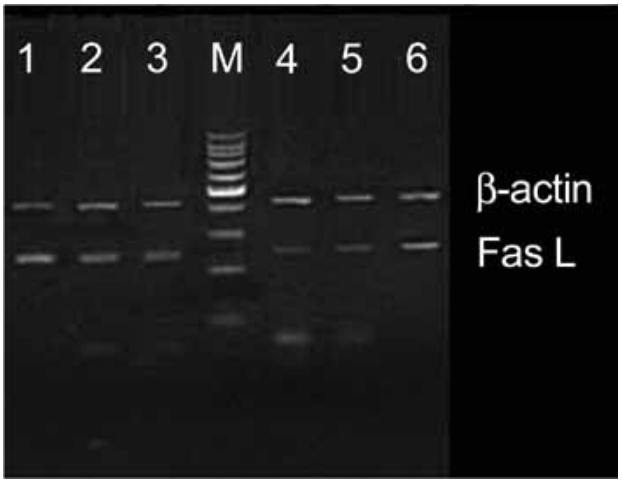

Fig (3B). FasL mRNA expression on HSCs (239bp).
Lane 1- group I (11th week), Lane 2- group I (7th week), Lane 3- group N, M- maker, Lane 4 –group N, Lane 5- group C (7th week), Lane 6- group C (11th week).

Table 2. Expression Levels of Fas and FasL mRNA in HSCs Between 3 Groups

Group	Fas			FasL		
	Group N	group C	group I	group N	group C	group I
7th week	0.36±0.02	0.66±0.02	0.74±0.02[a]	0.34±0.03	0.45±0.03	0.52±0.05[a]
11th week	0.40±0.01	0.72±0.02[a]	0.73±0.04[b]	0.36±0.05	0.62±0.04	0.83±0.04[a]

a: *vs.* group C and group N $P<0.05$, b: *vs.* 11th week of group C $P>0.05$.

Table 3. Expression Levels of Fas and FasL Protein in HSCs Between 3 Groups

Group	Fas			FasL		
	Group N	Group C	Group I	Group N	Group C	Group I
7th week	0.56±0.02	0.92±0.02	0.99±0.02[a]	…	1.24±0.03	1.36±0.16[a]
11th week	0.58±0.02	0.96±0.16	1.22±0.03[a]	…	1.33±0.03	1.39±0.19[a]

a P<0.05.

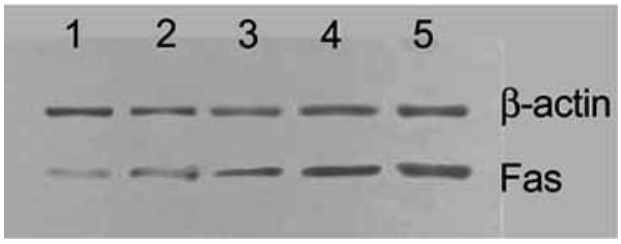

Fig (4A). Fas protein expression on HSCs (48kD).
Lane 1- group N, Lane 2- group C (7th week), Lane 3- group C (11th week), Lane 4 - group I (7th week), Lane 5- group I (11th week).

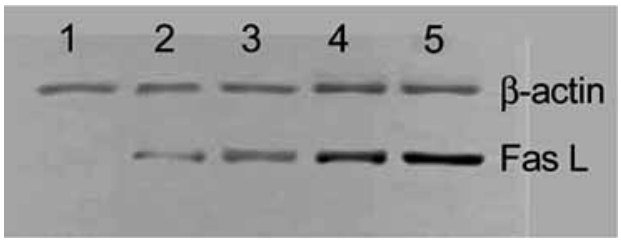

Fig (4B). FasL protein expression on HSCs (40kD).
Lane 1- group N, Lane 2- group C (7th week), Lane 3- group C (11th week), Lane 4 - group I (7th week), Lane 5- group I (11th week).

become highly proliferative, and synthesize fibrotic matrix rich in type I collagen. HSCs are central to the process of hepatic fibrosis; the activation, proliferation and apoptosis of HSCs have close relationship with the formation and development of liver fibrosis. This understanding has helped to identify underlying mechanisms, and will likely lead to new therapies for fibrotic diseases of many organs, including liver. To inhibit the activation and proliferation of the HSCs and promote their apoptosis has become the most important therapeutic approach for liver fibrosis. Attention is now increasingly focused on how liver fibrosis regresses, and in particular, on the fate of activated stellate cells as fibrosis recedes. The fate of activated stellate cells during resolution of liver injury is uncertain, but may include reversion to a quiescent phenotype and/or selective clearance by apoptosis [20]. Saile B [21] reported that resting HSCs displayed no sign of apoptosis and spontaneous apoptosis became detectable in parallel with HSCs activation, suggesting that apoptosis might represent an important mechanism terminating proliferation of activated HSCs. Recent animal studies have used gliotoxin, which provokes selective apoptosis of stellate cells in culture and *in vivo*, leading to reduced fibrosis [22]. So, as the liver injury resolves, apoptosis of activated HSCs may involve in the reversion of liver fibrosis [23]. Stellate cells contain several families of apoptotic mediators, including Fas/FasL, TNF receptors (including those for nerve growth factor), and Bcl/Bax, so that additional targets to promote apoptosis will likely be exploited in the future [24]. Fas is known as an important mediator of apoptosis, and act as an inducer of apoptosis in Fas-expressing cells in response to ligand binding (FasL) [25]. Apoptosis of HSCs in course of activation is accompanied by an increased expression of FasL by the HSCs themselves. The activated HSCs possess more Fas and FasL, compared with HSCs in the resting and transitional phase, the apoptosis of HSCs could largely be inhibited by blocking Fas, indicated that Fas/FasL system is the major tool for initiation of apoptosis [26]. Our data shows that with the development of liver fibrosis, the Fas/FasL system mRNA and protein express increased, this result is coincident with the literature, in addition, IL-10 could promote the expression of Fas and FasL in activated HSCs, implying that IL-10 may promote activated HSCs into apoptosis through binding FasL to Fas on the cell membranes of HSCs.

IL-10 is an important immunoregulatory cytokine produced by many cell populations, such as lymphocytes and macrophages as well as cells within liver such as Kufffer cells, hepatocytes and HSCs. It has profound inhibitory actions on macrophages and inflammation. The main biological function of IL-10 is to limit and terminate inflammatory responses and to regulate differentiation and proliferation of immune cells such as T cells, B cells, natural killer cells, and granulocytes [27-29]. Previous studies showed that IL-10 had additional effects on liver fibrogenesis, such as on HSCs [30]. In highly purified preparations of rat HSCs, messenger RNA (mRNA) for IL-10 was detected by reverse-transcription polymerase chain reaction (RT-PCR), from the time of isolation to 120 days of culture on plastic. Long-term cultures of unstimulated mouse HSCs secreted IL-10 protein as detected by immunoblotting and specific enzyme-linked immunosorbent assay (ELISA). IL-10 protein was undetectable by immunohistochemistry in mouse HSCs for the first 3 days in culture. After this, the percentage of IL-10-positive cells increased to 45% at day 7 and 100% by day 14, and expression of IL-10 continued in long-term cultures of up to 120 days. These indicated the important role of IL-10 in liver

fibrosis [31]. IL-10 could inhibit the activation of HSCs by inflammatory cells [32], relieve the inflammation of liver [14-15,33], suppress the function of NF-κB [34], affect the expression of collagen I and collagenase [35], and thus exerting an antifibrogenesis effect [36]. Failure for HSCs to sustain IL-10 expression might underlie pathologic progression to liver cirrhosis [15, 35]. IL-10 plays a role in inflammatory, malignant and autoimmune diseases and recombinant human IL-10 has been produced and tested in clinical trials, suggesting that IL-10 may become a new therapeutic target [37]. IL-10 may be useful in the treatment of chronic liver diseases regarding the prevention of advanced fibrosis and cirrhosis [38].

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Received: 12 December, 2005 **Revised: 30 April, 2006** **Accepted: 15 May, 2006**