BASIC RESEARCH

Effects of interleukin-10 on activation and apoptosis of hepatic stellate cells in fibrotic rat liver

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Abstract

AIM: To study the effects of interleukin-10 (IL-10) on the expression of α-smooth muscle actin (α-SMA), nuclear factor-κB(NF-κB) and Fas/Fas ligand (FasL) in hepatic stellate cells of experimental rats with hepatic fibrosis.

METHODS: Sixty clean SD rats were randomly divided into control group (group N), liver fibrotic group (group C) and IL-10 treatment group (group I). Control group received intraperitoneal injection of saline ($2ml \cdot kg^{-1}$), twice a week. Fibrotic group was injected intraperitoneally with 50% carbon tetrachloride (CCl_4) ($2 ml \cdot kg^{-1}$), twice a week. IL-10 treatment group was given IL-10 at a dose of $4 \mu g \cdot kg^{-1}$ 20 minutes before CCl_4 administration from the third week. Hepatic stellate cells (HSCs) were isolated from these rats at the seventh and eleventh weeks during the course of liver fibrosis, respectively. The expression of α-SMA and NF-κB in HSCs was measured by S-P immunohistochemistry. The expression of Fas and FasL mRNA was measured by RT-PCR. Furthermore, liver tissues were harvested from three groups at the same time.

RESULTS: The CCl_4- induced experimental rat hepatic fibrosis model was established successfully. The purity of extracted hepatic stellate cells was about 95% and the yield of hepatic stellate cells was $1.2-2.3 \times 10^6$/g liver tissue averagely. The positive expression of α-SMA and NF-κB was 36.5% and 28.5% respectively in group N. The positive levels of α-SMA and NF-κB were increased significantly in group C compared to group N ($P < 0.01$). The positive signals decreased significantly ($P < 0.05$) in group I. In the 11th week, the HSCs of group I became round with visible pyknotic nuclei. The expression of NF-κB in group C was significantly increased in a time-dependentmanner ($P < 0.01$), but there was no difference in the α-SMA expression ($P > 0.05$). The mRNA of Fas and FasL in group C was significantly increased in a time-dependent manner compared to that in control group. After treated with IL-10, the expression level of Fas and FasL was higher in group I than in group C.

CONCLUSION: The positive expression of α-SMA and NF-κB in hepatic stellate cells is decreased by ectogenic IL-10 in liver fibrosis induced by CCl_4. The expression of Fas and FasL is increased in the course of liver fibrosis, and is further increased by IL-10. IL-10 could inhibit the activation of HSCs and cause apoptosis of activated HSCs.

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INTRODUCTION

Liver fibrosis is a model of wound-healing responses to chronic liver injury and is the excessive accumulation of extracellular matrix proteins in most types of chronic liver diseases[1]. The cellular and molecular mechanisms of liver fibrosis have greatly advanced since hepatic stellate cells (HSCs) were identified as the main collagen-producing cells in the liver. HSCs are the central event in hepatic fibrosis[2-3]. In normal liver, HSCs reside in the Disse's space and are the major storage sites of vitamin A. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile and fibrogenic properties, secreting cytokines and expressing α-smooth muscle actin(α-SMA)[4-5]. The mechanisms involved in cytokine secretion by HSCs include activation of the transcription nuclear factor-κB (NF-κB). NF-κB, a key transcription factor induces genes involved in inflammation, responses to infection, and stress[6]. DNA binding activity of NF-κB is demonstrated in activated but not in quiescent HSCs, and activation of HSCs is associated with the nuclear translocation of

NF-κB. Inhibition of NF-κB by proteosome inhibitors or by adenovirus expressing the IkB superrepressor can markedly blunt cytokine secretion by activated HSCs. Using differential display, investigators have shown that intercellular adhesion molecule 1 (ICAM-1) is expressed in HSCs activated in culture or *in vivo*, but not in quiescent HSCs[7]. The ICAM-1 gene contains a NF-κB binding site and its transcription is stimulated by NF-κB[8,9]. This observation provides functional support for a critical role of NF-κB in the activation of HSCs. As liver injury resolves, the number of activated stellate cells decreases through 1 of 2 potential pathways, namely spontaneous reversion or clearance by apoptosis[10,11]. To date, spontaneous reversion of myofibroblasts to quiescent cells has only been documented in culture, but not *in vivo*. In contrast, there is evidence that HSCs undergo apoptosis during resolution of liver injury *in vivo*. Some data demonstrate that apoptosis of HSCs in the course of activation is accompanied with increased expression of FasL by HSCs themselves[12]. Fas/FasL system is the key pathway for apoptosis of HSCs. Our work team found that ectogenic IL-10 could decrease liver fibrosis, but its mechanism still remains unclear. In the present study, α-SMA, NF-κB and Fas/FasL were selected as the targets to study the effects of interleukin-10 on activation and apoptosis of HSCs.

MATERIALS AND METHODS

Materials
Sixty clean male Sprague-Dawley rats, weighing 400-500g (provided by Shanghai Experimental Animal Center), were divided randomly into 3 groups. The control group (group N) included 8 rats, the fibrotic group (group C) included 28 rats and the IL-10 intervention group (group I) included 24 rats. All the rats were bred under clean conditions (room temperature: 22℃±2℃; humidity: 55%±5%) in a 12 h light/dark cycle with free access to drinking water and food. High voltage disinfectant animal food was provided by BK Company in Shanghai, China.

Reagent
Recombined rat interleukin-10 was provided by Jingmei Biotechnology Corporation. Collagenase type IV and Nycodenz were provided by Sigma Corporation. Pronase E was provided by Merk Corporation. DNase I was provided by Sino-American Biotechnology Corporation. DMEM was provided by Gibco Corporation. Mouse anti-rat α-SMA and NF-κB monoclonal antibodies were provided by Santa Cruz Biotechnology Inc. SP immunocytochemistry kit was provided by American Zymed Company. RNA isolation kit was provided by Gentra Company (USA) and PCR kit was provided by Promega Company. The primers were synthesized by Beijing Sanbo Company.

Animal models
The rats of group N were injected intraperitoneally with saline (2ml·kg⁻¹), twice a week. The rats of groups C and I were intraperitoneally injected with 50% CCl₄ (dissolved in castor oil) (2ml·kg⁻¹), twice a week. From the third week, the rats of group I were injected intraperitoneally with IL-10 (4μg·kg⁻¹) (dissolved in saline) 20 minutes before they were injected with CCl₄[13]. All injections were performed on Monday and Thursday with the body weights determined before each injection. By the end of the experiment, 12 rats in group C and 8 in group I died. No animals died in group N. In the seventh and eleventh weeks, 3 rats in group N and 5 rats in the other two groups were sacrificed to collect their hepatic stellate cells. The liver tissue was collected from 1 rat in each group at this time point and fixed in 10% formalin and embedded with paraffin.

Isolation and culture of hepatic stellate cells
Nonparenchymal cells were isolated from rats of the experimental groups by sequential perfusion with collagenase and pronase E as described previously[14,15]. Buoyant HSCs were separated from the resulting cell suspension by elutriation over a Nycodenz gradient. In brief, the liver of rats was routinely perfused through a portal vein catheter with Ca²⁺-free D-Hanks solution, then with pronase E and type IV collagenase dissolved in Ca²⁺ containing D-Hanks solution. The liver was homogenized and incubated with pronase E, type IV collagenase and DNase I dissolved in Ca²⁺ containing D-Hanks solution for 20 min at 37℃ with constant stiring. This suspension was centrifuged by 11% Nycodenz density gradient centrifugation for 15 minutes at 1500r/min after filtered. The cells were aspirated from the interface, washed twice in DMEM solution, and then resuspended in DMEM containing 20% calf serum. The HSCs were seeded onto plastic tissue culture bottle at $1×10^6$ cells/mL and incubated at 37℃ with 50mL/L CO₂ in air.

Immunocytochemistry measurement
The HSCs cultured for 24 hours were fixed by 4% paraformaldehyde at 4℃ for 24 hours, then incubated in PBS containing 3%H₂O₂ to remove endogenous perxoidase activity and then in PBS containing 0.1mol/L citrate to saturate nonspecific binding sites. After incubation with 1:100 mouse anti-rat α-SMA and NF-κB monoclonal antibody, the reaction was carried out with the instant S-P immunocytochemistry reagents. The primary antibody was replaced with PBS as blank contrast. The plates were incubated in a buffer containing 3,3-diaminobenzidine tetrahydrochloride (DAB) and H₂O₂ to produce a brown reaction product, then imaged under microscope.

Result assessment
Two hundred cells were calculated under the microscope. The reactions were graded and scored according to their intensities and percentage of the positive cells as follows: zero score for negative reaction, 1 score for pale yellowish staining, 2 scores for dense yellow staining and 3 scores for brown staining. The eventual result was produced by the two scores for staining intensity and positive cell percentage.

Reverse transcription- polymerase chain reaction (RT-PCR) for Fas and FasL
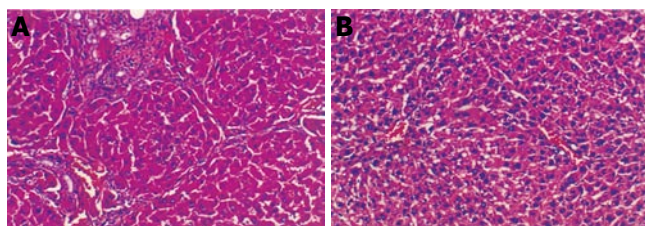Total RNA was isolated from HSCs using Gentra

Figure 1 Liver of rats in groups C (**A**) and I (**B**) (week 11, H-E staining, ×100)

Figure 2 Autofluorescence (**A**) and desmin staining (**B**) of HSCs.

reagent (USA) according to the protocol provided by the manufacturer. The A260/A280 of total RNA was between 1.8 and 2.0. After treated with DNase-I, 1-2μg of total RNA was reversely transcribed into complementary DNA (cDNA) with oligo (dT) using cDNA synthesis kit. Then 2 μL of cDNA product was used as the template to amplify specific fragments in a 25μL reaction system. PCR was performed with an initial denaturation at 94℃ for 5 min, followed by 25 cycles at 94℃ for 45 s, annealing at 55℃ for 30 s (FasL at 58℃), at 72℃ for 60 s, with a final extension at 72℃ for 7 min. The primer sequences used are sense: 5' -GAATGCAAGGGACTGATAGC-3' and antisense: 5' -TGGTTCGTGTGCAAGGC TC-3' for Fas; sense: 5' -GGAATGGGAAGACACATATGGAACTGC-3' and antisense: 5'-CATATCTGGCCAGTAGTGCAGTAAT TC-3' for FasL; sense: 5'-GAGCTATGAGCTGCCTGA CG-3' and antisense: 5'-AGCACTTGCGGTCCAC GATG-3' for β-actin (660bp) ; sense: 5' -GAGCTATGAGCTGC CTGACG-3' and antisense: 5' -AGCACTTGCGGTCCACGATG-3' for β-actin (410bp).

Electrophoresis and semi-quantitative analysis

The PCR products were run on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. The expected product sizes were Fas (414bp), FasL (239bp), β-actin (660bp and 410bp). Bioimagine system was used to detect the density of bands of PCR products. The values of Fas and FasL expression were calculated from percentage of the ratio of band density of PCR products and the band density of β-actin.

Statistical analysis

All data were expressed as mean±SD. The significance for the difference between the groups was analyzed with SPSS10.0 by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Animal model

Liver fibrosis was remarkable during the treatment with CCl4. In the seventh week, steatosis and ballooning degeneration of hepatocytes were obvious, plentiful inflammatory cells infiltrated into the Disse's space, collagen fibers increased and extended to the parenchyma. In the eleventh week, collagen fibers formed widely, complete fibrous septa were seen and pseudolobular structures were also present occasionally. In the IL-10 treatment grou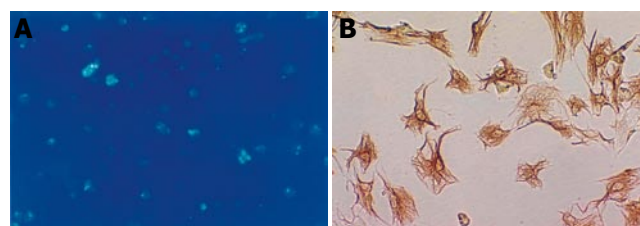p, the CCl4-caused alterations as described above seemed to be markedly alleviated, less profound steatosis and necrosis were noted in the seventh week, and no distinct change was found in the eleventh week compared to the normal group (Figures 1A, 1B).

Hepatic stellate cell identification

HSCs were isolated successfully. The yield of HSCs was 1.2-2.3×10^6/g liver tissue. HSCs were identified according to their typical autofluorescence at 328-nm excitation wavelength (Figure 2A) and by immunohistochemistry with monoclonal antibody against desmin. Greater than 95% of the isolated HSCs were stellate cells (Figure 2B). The cells' viability was determined by trypan blue exclusion staining with viability over 90%.

Expression of α-SMA and NF-κB in HSCs

The granular positive products of α-SMA were localized in cytoplasm of HSCs, and the NF-κB was in cytoplasm and nuclei. In control group, the expression was weak and pale yellowish, the positive rate of α-SMA and NF-κB was 36.5% and 28.5%, respectively. In group C, the expression increased obviously with the development of liver fibrosis (positive rate was 100% and the granular positive products were dense yellow or brown). The distribution of NF-κB was found mainly in nuclei. In group I, the changes were less pronounced than in group C. Especially in the eleventh week, the HSCs became round with visible pyknotic nuclei, the vigour of cells was weak (Figures 3A-3E). Comparison of α-SMA and NF-κB positive expression levels among 3 groups is shown in Table 1 and in Figures 4A, 4B. In group I, IL-10 treatment resulted in decreased immunoreactivties of α-SMA and NF-κB ($P < 0.05$). With the development of liver fibrosis, the intensities of NF-κB increased gradually, but that of α-SMA did not change significantly.

Expression of Fas and FasL in HSCs

The Fas and FasL mRNA could be measured in HSCs of control group. The mRNA of Fas and FasL in fibrotic group was increased in a time-dependent manner compared to that in control group. After treated with IL-10, the expression level of Fas and FasL mRNA was higher in fibrotic group than in control group. The expression of Fas and FasL mRNA was increased in the course of liver fibrosis and was further increased by IL-10 (Figures 4C, 4D). Comparison of Fas and FasL mRNA expression levels among 3 groups is shown in Table 2 and in Figures 4E, 4F.
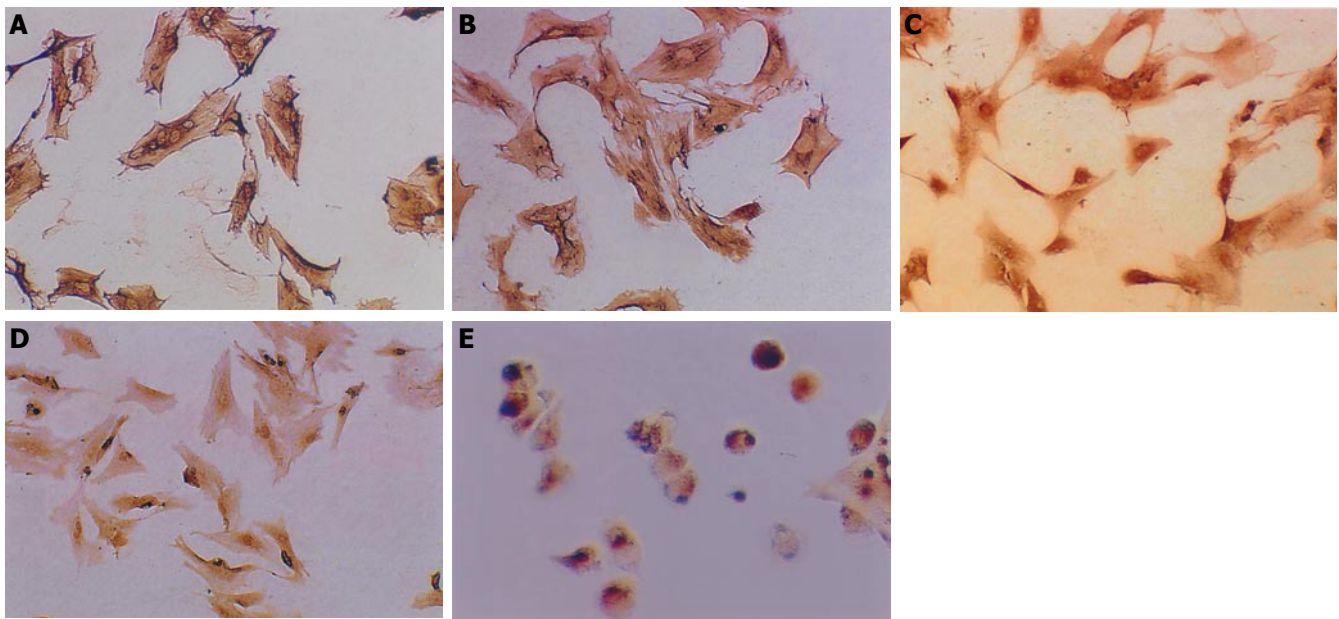
Figure 3 α-SMA in HSCs of group C (**A**) in week 11 and group I (**B**) in week 7; NF-κB in HSCs of group C (**C**) in week 11 and group I (**D**) in week 7; and changes of HSCs in group I (**E**) in week 11.

Table 1 Positive expression levels of α-SMA and NF-kB in HSCs of 3 groups (mean ± SD)

	Group N		Group C		Group I	
	wk 7	wk 11	wk 7	wk 11	wk 7	wk 11
α-SMA	0.64±0.05	0.68±0.07[d]	2.43±0.03	2.47±0.14[b]	2.14±0.11	2.09±0.06[c]
NF-κB	0.43±0.08	0.42±0.05[d]	2.13±0.09	2.48±0.70[a]	1.92±0.42	1.62±0.10[a]

[a]*P*<0.05 *vs* week 7 of group N; [b]*P*<0.05 *vs* week 7 of group C; [c]*P*<0.05 *vs* group C; [d]*P*<0.05 *vs* week 7 of group N.

Table 2 Expression levels of Fas and FasL in HSCs of 3 groups (mean ± SD)

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]*P*<0.05 *vs* groups C and N; [b]*P*<0.05 *vs* week 11 of group C.

DISCUSSION

Liver fibrosis results from the excessive secretion of matrix proteins by HSCs. In normal liver, HSCs are nonparenchymal, quiescent cells whose main function is to store vitamin A[16]. In response to liver injury, HSCs undergo an "activation" process in which they produce cytokines and chemokines, express receptors of cytokines and chemokines, and synthesize ECM[17]. Activation of HSCs is the central event of liver fibrosis, which consists of 2 major phases: initiation and perpetuation[18]. The earliest changes in stellate cells are likely to result from paracrine stimulation by all neighboring cell types, including Kupffer cells, sinusiodal endothelium, etc. Perpetuation of stellate cell activation involves several discrete changes in cell behavior, such as proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, of which contractility of HSCs may be a major determinant during liver fibrosis. The activated HSCs show common phenotypic features of smooth muscle cells and myofibroblasts, shape of well-developed stress fibers of actin cytoskeleton. The microfilament protein α-SMA has been explored as a marker for activated HSCs. Quiescent cells are negative *in vitro* or *in vivo* and activated HSCs are clearly positive[19]. This suggests a close relationship between α-SMA induction and liver fibrosis. Our data show that α-SMA is expressed in activated hepatic stellate cells in the course of liver fibrosis. After the treatment with IL-10, the expression of α-SMA decreased, indicating that ectogenic IL-10 may release activated HSCs.

NF-κB exists in cytoplasm as an inactive form associated with regulatory proteins called inhibitors of kB (IkB)[20]. Phosphorylation of IkB, an important step in NF-κB activation, is mediated by IkB kinase (IKK). Appropriate stimuli induce selective IkB phosphorylation, which is then degraded by the proteasome pathway. Free NF-κB migrates to nuclei by virtue of its nuclear localization signal and induces transcription of multiple kB-dependent genes. Newly synthesized IkB both in cytoplasm and in nuclei inactivates NF-κB. NF-κB regulates the transcription of a number of proinflammatory molecules involved in acute responses to injury and chronic liver inflammation, including TNF-a, IL-6, ICAM-1 etc[21]. The induction of NF-κB during liver regeneration after partial hepatectomy appears to be a required event to prevent apoptosis and allow for normal cell cycle progression. NF-κB is a key transcription factor involved in activation of HSCs[22,23]. Inhibition of the IKK/NF-κB pathway is sufficient to increase the rate at which activated hepatic stellate cells undergo
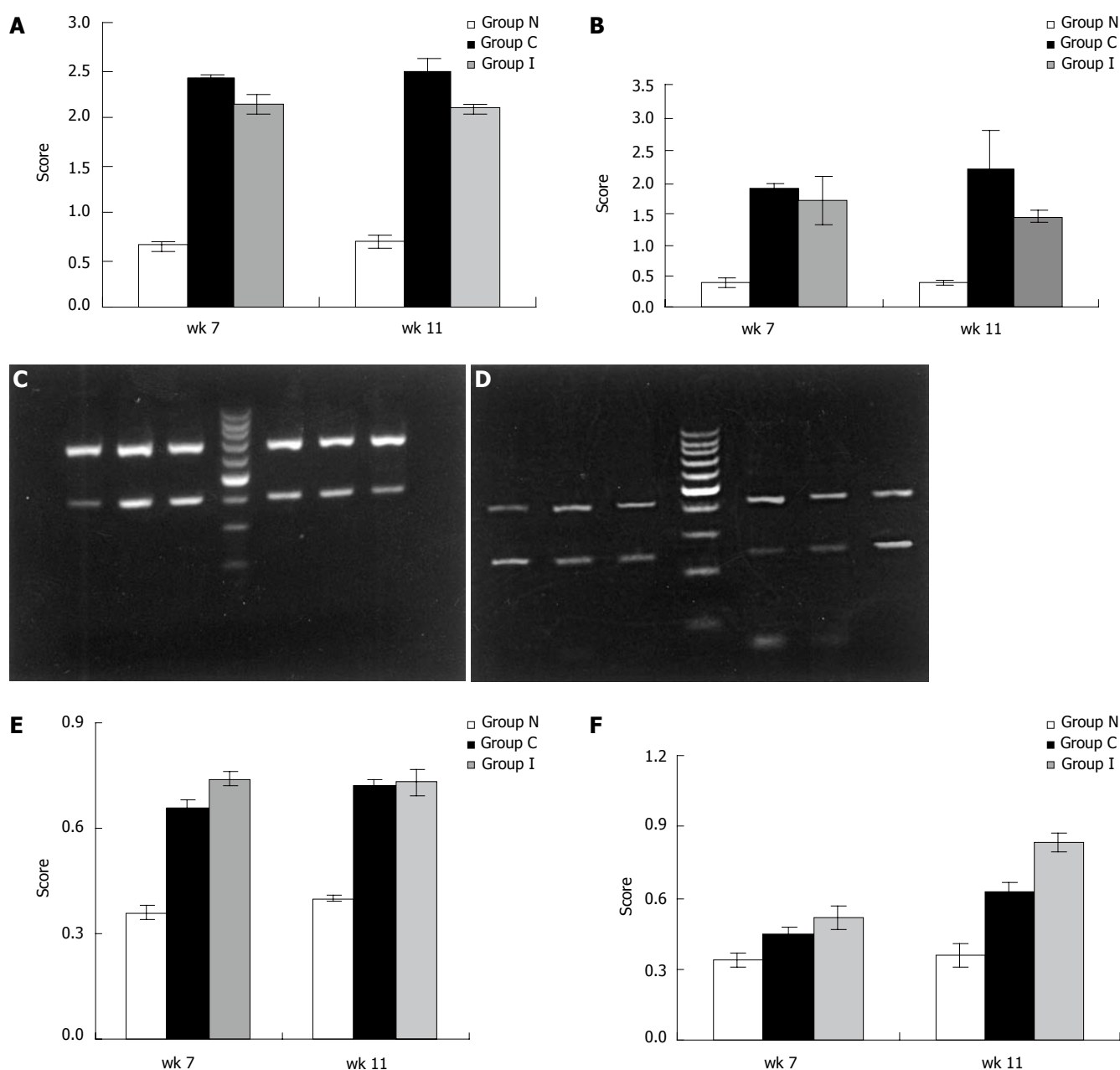
Figure 4 Expressions of α-SMA (**A**), NF-κB (**B**), Fas mRNA (**C**), FasL mRNA (**D**), Fas (**E**), and FasL (**F**) in HSCs.

apoptosis *in vitro* and *in vivo*. Drugs selectively targeting IKK have potential as antifibrotics[24]. Although in some studies IL-10 has been demonstrated to block NF-κB activation, our data suggest that IL-10 could decrease the NF-κB expression. The molecular target for IL-10-induced inhibition of NF-κB has not been established. There is evidence that IL-10 regulates NF-κB by dual mechanisms[25]. Firstly, IL-10 blocks IKK activity, thus inhibiting phosphorylation and degradation of IkBa. The preserved IkBa continues to bind to NF-κB in cytoplasm, prohibiting NF-κB nuclear translocation and NF-κB-dependent transcription. Secondly, IL-10 can directly block NF-κB DNA binding by a mechanism that is independent of NF-κB nuclear translocation.

Apoptosis associated with liver disease is increasingly viewed as a nexus through which many key pathways converge[26]. Pathologic apoptosis in liver may not only

result from inflammation and fibrosis, but also in turn amplify these responses. In particular, HSCs contribute to apoptosis and inflammation. As the liver injury resolves, apoptosis of activated HSCs may be involved in the reversion of liver fibrosis[27]. Fas is known as an important mediator of apoptosis and acts as an inducer of apoptosis in Fas-expressing cells in response to ligand binding (FasL)[28]. Saile *et al*[29] reported that resting HSCs display no sign of apoptosis and spontaneous apoptosis becomes detectable in parallel with HSC activation, suggesting that apoptosis might represent an important mechanism terminating proliferation of activated HSCs. They also demonstrated that apoptosis of HSCs in the course of activation is accompanied with 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, indicating that Fas/FasL system plays a major role in initiation of apoptosis. Thus, driving activated HSCs into apoptosis may be a way to resolve fibrosis. Our data show that with the development of liver fibrosis, the Fas/FasL system mRNA expression increases. In addition, IL-10 could promote the expression of Fas and FasL mRNA-activated HSCs, implying that IL-10 may promote activated HSCs into apoptosis through binding of FasL to Fas on the cell membranes of HSCs.

IL-10 is pleiotropic[30-31] and has multiple effects on diverse cell types. One of the most important properties of IL-10 is its anti-inflammatory action, which restrains the immune response under various stimuli, otherwise the individuals would have deleterious consequences. Evidence of *in vivo* function of IL-10 indicates that in the absence of IL-10 (in genetically IL-10 deficient animals), an exaggerated inflammatory response can lead to inflammatory states[32]. In our study, the ectogenic IL-10 could alleviate liver fibrosis induced by CCl4 in rats. During the course of liver fibrosis, the positive expression of α-SMA in HSCs was decreased by IL-10, suggesting that activation of HSCs can be inhibited by IL-10. The mechanisms may involve blocking NF-κB activation. At the same time, the expression of Fas and FasL mRNA was further increased by IL-10, suggesting that IL-10 may induce apoptosis of HSCs. In brief, IL-10 could inhibit activation and promote apoptosis of HSCs, which may be related with its mechanism against fibrosis.

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