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• BASIC RESEARCH •

Effects of platelet-derived growth factor and interleukin-10 on Fas/Fas-ligand and Bcl-2/Bax mRNA expression in rat hepatic stellate cells *in vitro*

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Abstract

AIM: To investigate the effects of platelet-derived growth factor(PDGF) and interleukin-10 (IL-10) on Fas/Fas-ligand and Bcl-2/Bax mRNA expressions in rat hepatic stellate cells.

METHODS: Rat hepatic stellate cells (HSCs) were isolated and purified from rat liver by *in situ* digestion of collagenase and pronase and single-step density Nycodenz gradient. After activated by culture *in vitro*, HSCs were divided into 4 groups and treated with nothing (group N), PDGF (group P), IL-10 (group I) and PDGF in combination with IL-10 (group C), respectively. Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was employed to compare the mRNA expression levels of Fas/FasL and Bcl-2/Bax in HSCs of each group.

RESULTS: The expression levels of Fas between the 4 groups had no significant differences ($P>0.05$). FasL mRNA level in normal culture-activated HSCs (group N) was very low. It increased obviously after HSCs were treated with IL-10 (group I) (0.091±0.007 *vs* 0.385±0.051, $P<0.01$), but remained the low level after treated with PDGF alone (group P) or PDGF in combination with IL-10 (group C). Contrast to the control group, after treated with PDGF and IL-10, either alone or in combination, Bcl-2 mRNA expression was down-regulated and Bax mRNA expression was up-regulated, both following the turn from group P, group I to group C. Expression of Bcl-2 mRNA in group C was significantly lower than that in group P (0.126±0.008 *vs* 0.210±0.024, $P<0.01$). But no significant difference was found between group C and group I, as well as between group I and group P ($P>0.05$). Similarly, the expression of Bax in group C was higher than that in group P (0.513±0.016 *vs* 0.400±0.022, $P<0.01$). No significant difference was found between group I and group P ($P>0.05$). But compared with group C, Bax expressions in group I tended to decrease (0.449±0.028 *vs* 0.513±0.016, $P<0.05$).

CONCLUSION: PDGF may promote proliferation of HSCs but is neutral with respect to HSC apoptosis. IL-10 may promote the apoptosis of HSCs by up-regulating the expressions of FasL and Bax and down-regulating the expression of Bcl-2, which may be involved in its antifibrosis mechanism.

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INTRODUCTION

Liver fibrosis is a progressive pathological process involving multi-cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular space. It is generally accepted that hepatic stellate cells (HSCs) are central to the process of fibrosis as the major source of extracellular matrix (ECM) components[1-10]. Following acute or chronic liver tissue injury, HSCs undergo a process of activation towards a phenotype characterized by increasing proliferation, motility, contractility and synthesis of ECM components. Cytokines play an important role in the formation, development and reversibility of fibrosis[9-14]. Activated HSCs secrete many important cytokines through autocrine and paracrine, of which platelet-derived growth factor (PDGF) can activate secretory cells and those quiescent HSCs around[15,16] and promote the proliferation of HSCs[17]. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines by T helper type 1 T cells and mono/macrophages. Previous studies have shown that endogenous IL-10 has the ability to inhibit the inflammation in injured liver and block the advance of fibrosis[18-21]. Previous works by our group have demonstrated that exogenous IL-10 has an anti-fibrogenic function[22]. But the underlying mechanism remains obscure. In this study, in order to investigate the effects of IL-10 and PDGF on the proliferation and apoptosis of rat HSCs, culture-activated HSCs were treated with IL-10 and PDGF. Fas/FasL and Bcl-2/Bax mRNA expressions in each group were assayed by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Materials

Male Wistar rats, weighing 450-500 g, were provided by Shanghai Center for Laboratory Animals. Total RNA isolation kit was obtained from Jingmei Biotechnology Company of Shenzhen. Moloney murine leukemia virus (M-MLV) reverse transcriptase was purchased from Promega. PCR reagent and Dulbecco's modified Eagle's medium (DMEM) were respectively provided by Shanghai Biotechnology Company and GibcoB. PCR primers were synthesized by Shanghai Biotechnology Company.

Isolation, culture and evaluation of HSCs

HSCs were isolated from normal male Wistar rats by *in situ* digestion of collagenase and pronase and single-step density Nycodenz gradient as Ramm GA[23] and Friedman SL[24] previously described, and cultured in DMEM supplemented with 100 mL/L FBS. Desmin immunocytochemistry was employed to determine the

isolated HSCs' purity. HSCs were subcultured 4 d after primary culture. Alpha smooth muscle actin (α-SMA) immunocytochemistry and electron microscope were employed to confirm that HSCs were activated by culture *in vitro* and transformed into myofibroblasts.

Intervention and division of HSCs

The subcultured HSCs were diluted to a concentration of 5×10^4/mL with DMEM containing 100 mL/L FBS and seeded onto the 24-well plastic tissue culture plates. When HSCs spread the plate fully, the culture medium was replaced with DMEM containing 10 mL/L FBS. After incubated for 24 h, HSCs were divided randomly into 4 groups: one as control group cultured in 1 mL DMEM containing 10 mL/L FBS, the other three were cultured in the same medium and treated with 20 ng PDGF or 20 ng IL-10, either alone or in combination, respectively. We named them group N, group P, group I and group C, respectively. Each group included 5 wells.

RNA extraction

Total RNA was extracted from the above treated HSCs after incubated for 24 h according to the RNA isolation kit instructions. The content and purity of total RNA were determined by spectrophotography. A_{260}/A_{280} of total RNA was between 1.8-2.0.

RT-PCR for Fas/FasL and Bcl-2/Bax

For RT-PCR, total RNA was reverse-transcripted using M-MLV reverse transcriptase and oligo (dT) at 37 °C for 60 min, followed by at 70 °C for 10 min. Approximately 2 μg total RNA was used in each reverse transcription reaction and the final volume was 25 μL. β-actin was used as internal control. The PCR reaction volume was 50 ul, including 5 μL 10×PCR buffer, 2 mmol/L $MgCl_2$, 1 μL 10 mmol/L dNTP, 1 μL 20 pmol/μL target gene sense and anti-sense primers, 1 μL 20 pmol/μL β-actin primer pair, 2 μL RT product, 1.5 U Tag DNA polymerase. The specific sets of primers and the target gene amplification conditions are shown in Table 1.

Result determination

PCR products were run on 20 g/L agarose gel eletrophoresis and visualized with ethidium bromide staining. Bio imagine system was used to detect the densities of bands of the PCR products. The ratio of target gene density to β-actin density was used to represent the relative levels of Fas/FasL and Bcl-2/Bax mRNA expressions. The semi-quantitative detection was analyzed 5 times repeatedly.

Statistical analysis

All data were expressed as mean±SE. The significance for the difference between the groups was assessed with SPSS 10.0 by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Evaluation of HSCs

Freshly isolated HSCs were round-shaped with many yellow droplets in cytoplasm. After cultured for 5-6 d, the spread cells showed a typical 'star'-like configuration. Desmin immunocytochemistry showed that the positive percentage was about 95% (Figure 1A), indicating that 95% of the isolated cells were HSCs. α-SMA immunocytochemistry showed that 98% of the cells were α-SMA positive (Figure 1B), indicating that most of the cells were activated. The myofilament could be seen in cytoplasm under the electron microscope, confirming that HSCs were activated and transformed into myofibroblasts after cultured *in vitro* (Figure 2).

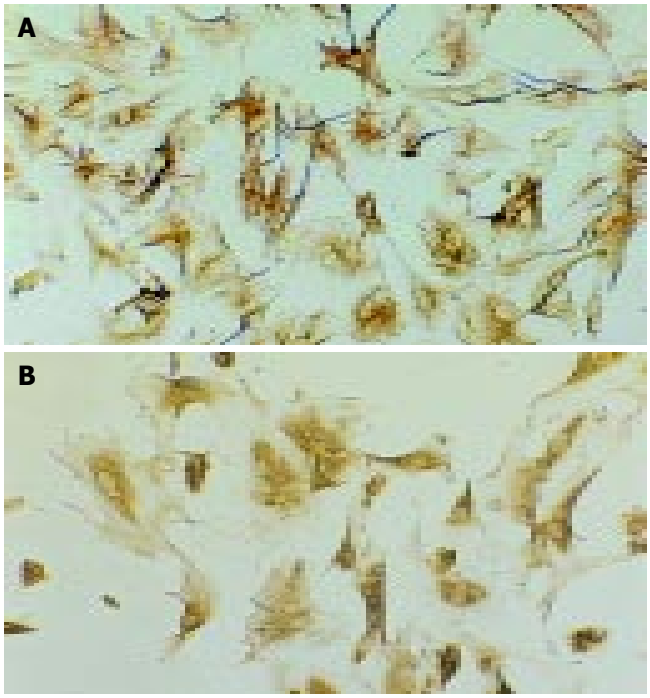

Figure 1 Desmin and α-SMA immunocytochemistry (SP, original magnification: ×100). A: Desmin immunocytochemistry of HSCs 7 d after isolation; B: α-SMA immunocytochemistry of HSCs 7 after isolation.

Table 1 Primer sequences for PCR and amplification conditions for each target gene

Primer (base)	Sequence	Amplification conditions
Fas 414	5'-GAATGCAAGGGACTGATAGC-3'	Denaturation at 94 °C for 45 s,
	5'-TGGTTCGTGTGCAAGGCTC-3'	Annealing at 55 °C for 30 s and synthesizing
		at 72 °C for 1 min for 25 cycles
FasL 239	5'-GGAATGGGAAGACACATATGGAACTGC -3'	Denaturation at 94 °C for 45 s,
	5'-CATATCTGGCCAGTAGTGCAGTAATTC-3'	Annealing at 55 °C for 30 s and synthesizing
		at 72 °C for 1 min for 33 cycles
Bcl-2 525	5'-TATGATAACCGGGAGATCGTGATC-3'	Denaturation at 94 °C for 45 s,
	5'-GTGCAGATGCCGGTTCAGGTACTC-3'	Annealing at 60 °C for 30 s and synthesizing
		at 72 °C for 1 min for 33 cycles
Bax 310	5'-GACACCTGAGCTGACCTTGG-3'	Denaturation at 94 °C for 45 s,
	5'-GAGGAAGTCCAGTGTCCAGC-3'	Annealing at 60 °C for 30 s and synthesizing
		at 72 °C for 1 min for 30 cycles
β-actin 660	5'-CCAACCGTGAAAAGATGACC-3'	Changed according to different target genes
	5'-CAGGAGGAGCAATGATCTTG-3'	

All initial denaturations were at 94 °C for 5 min. Finally an additional extension step at 72 °C for 7 min was done.

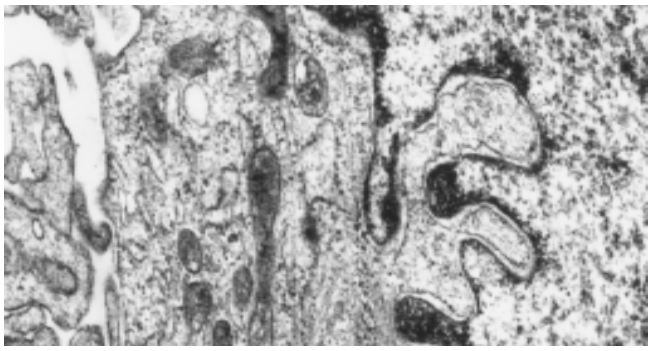

Figure 2 Activated HSCs under the electron microscope. The myofilament can be seen in the cytoplasm as the arrow point shows.

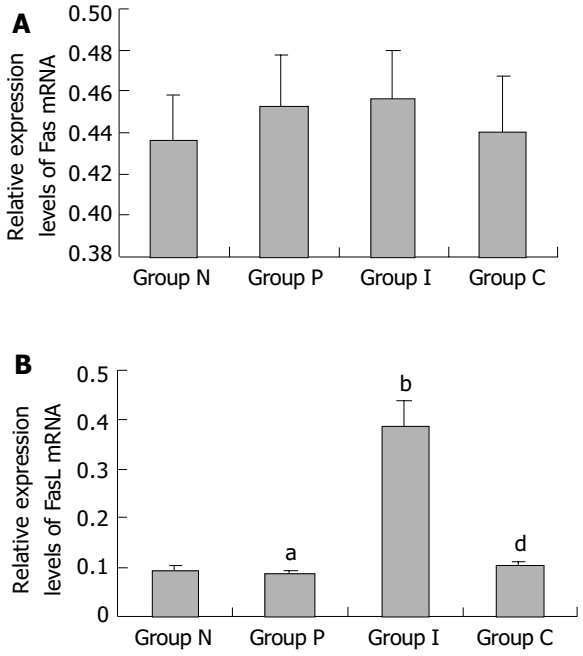

Figure 3 Relative Fas/ FasL mRNA expression levels in HSCs of different groups assessed by RT-PCR. A: Relative Fas mRNA expression levels (P>0.05 between random two groups.); B: Relative FasL mRNA expression levels (^aP>0.05 *vs* group N, ^bP<0.01 *vs* group N, ^dP<0.01 *vs* group I.); group N: Normal group as control; group P: PDGF treated group; group I: IL-10 treated group; group C: Combined PDGF and IL-10 treatment group.

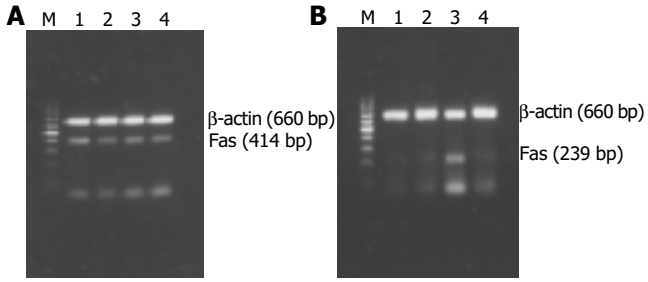

Figure 4 RT-PCR results of Fas/FasL mRNA expression in HSCs of different groups. A: RT-PCR results of Fas mRNA expression; B: RT-PCR results of FasL mRNA expression; M: 100 bp DNA ladder (upper to lower: 1 000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp); Lane 1: Normal group as control; Lane 2: PDGF treatment group; Lane 3: IL-10 treatment group; Lane 4: Combined PDGF and IL-10 treatment group.

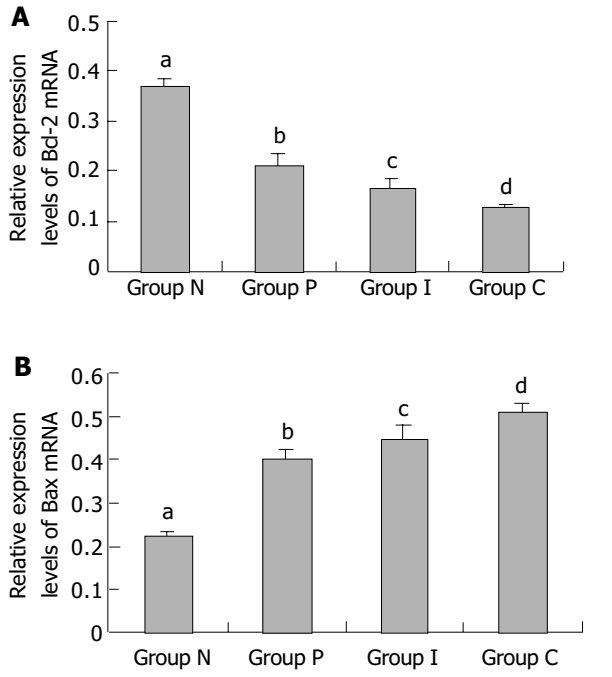

Figure 5 Relative Bcl-2/Bax mRNA expression levels in HSCs of different groups assessed by RT-PCR. A: Relative Bcl-2 mRNA expression levels (^bP<0.01 *vs* group P, group I and group C, respectively; ^aP>0.05 *vs* group I, ^cP>0.05 *vs* group C, ^dP<0.01 *vs* group P.). B: Relative Bax mRNA expression levels (^bP<0.01 *vs* group P, group I and group C, respectively; ^aP>0.05 *vs* group I, ^cP = 0.045<0.05 *vs* group C, ^dP<0.01 *vs* group P.). Group N: Normal group as control; group P: PDGF treated group; group I: IL-10 treated group; group C: Combined PDGF and IL-10 treatment group.

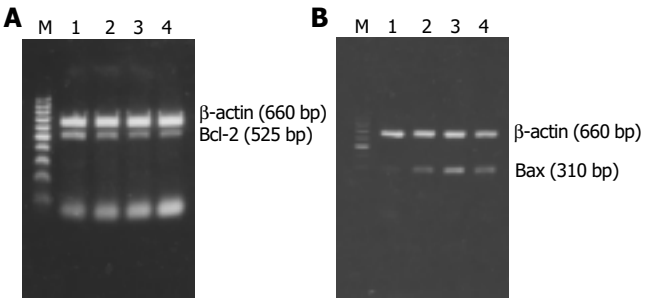

Figure 6 RT-PCR results of Bcl-2/Bax mRNA expression in HSCs of different groups. A: Bcl-2 mRNA expression. B: Bax mRNA expression. M: 100 bp DNA ladder (upper to lower: 1 000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp); Lane 1: Normal group as control; Lane 2: PDGF treatment group; Lane 3: IL-10 treatment group; Lane 4: Combined PDGF and IL-10 treatment group.

Effects of PDGF and IL-10 on Fas and FasL expressions in HSCs

Fas mRNA was expressed in HSCs of each group and the expression levels had no significant difference among the 4 groups, as shown in Figures 3A, 4A, indicating that neither PDGF nor IL-10 had effect on Fas mRNA expression in HSCs. As it could be informed from Figures 3B, 4B, FasL mRNA level in normal culture-activated HSCs (group N) was very low. It increased obviously after HSCs were treated with IL-10 (group I) (0.091±0.007 *vs* 0.385±0.051, P<0.01), but remained the low level after treated with PDGF alone (group P) or PDGF in combination with IL-10 (group C) (0.085±0.006, 0.101±0.008, respectively). The data suggested that IL-10 could improve FasL mRNA expression in culture-activated HSCs and PDGF could not. Furthermore, PDGF tended to abolish this effect of IL-10.

Effects of PDGF and IL-10 on Bcl-2 and Bax expressions in HSCs

Bcl-2 and Bax mRNA were expressed in normal culture-activated HSCs. Both of their expression levels were significantly changed after treated with PDGF and IL-10, either alone or in combination. Bcl-2 mRNA expression was down-regulated and Bax mRNA expression was up-regulated, following the turn from group P, group I to group C. The expression of Bcl-2 in group C was significantly lower than that in group P (0.126±0.008 *vs* 0.210±0.024, $P<0.01$). But no significant difference was found between group C and group I, as well as between group I and group P (0.210±0.024 *vs* 0.166±0.017, 0.166±0.017 *vs* 0.126±0.008, $P>0.05$) (Figures 5A, 6A). Similarly, the expression of Bax in group C was higher than that in group P (0.513±0.016 *vs* 0.400±0.022, $P<0.01$). No significant difference was found between group I and group P (0.400±0.022 *vs* 0.449±0.028, $P>0.05$). But compared with combined treatment group, Bax expressions in group I tended to decrease (0.449±0.028 *vs* 0.513±0.016, $P = 0.045<0.05$) (Figures 5B, 6B). These results showed that both PDGF and IL-10 promoted the Bax mRNA expression in HSCs and inhibited the Bcl-2 expression, but the differences of their effects were not significant. Intervention with PDGF and IL-10 seemed to be able to manifest effects on Bax expression than intervention alone. IL-10 showed similar influences on culture-activated HSCs and reactivated HSCs by PDGF.

DISCUSSION

It is generally accepted that hepatic stellate cells (HSCs) are central to the process of hepatic fibrosis. They are the major source of extracellular matrix and during fibrogenesis undergo an activation process characterized by increased proliferation and collagen synthesis[1-10,24]. So the activation, proliferation and apoptosis of HSCs have close relationship with the formation and development of liver fibrosis. To inhibit the activation and proliferation of the HSCs and promote their apoptosis has become the most important therapeutic approach for liver fibrosis[7-10,14,25-28].

There is evidence that HSCs can be successfully isolated by *in situ* digestion of collagenase and pronase and single-step density Nycodenz gradient[23,24,29]. Desmin is a marker for muscle cells and expressed by all muscle lineages including HSCs (either quiescent or activated) in the liver. Alpha smooth muscle actin (α-SMA) is an intermediate filament protein that is expressed by activated HSCs and is widely accepted to be a marker of activation. Both of them were used to identify and quantify HSCs and their activation. The desmin immunocytochemistry result showed that the purity of the isolated HSCs by this method was satisfying (Figure 1A). The results of α-SMA immunocytochemistry (Figure 1B) and electron microscope (Figure 2) confirmed that HSCs were activated and transformed into myofibroblasts after cultured *in vitro*.

PDGF, which is produced by HSCs, Kuffer cells and platelets, is a major mitogen for connective tissues and certain other cells. It was viewed as one of the most important growth factors serving as the matrix-bound cytokines[11] and plays an important role in the pathogenesis of liver fibrosis via promoting the activation and proliferation of HSCs[12,15,25,30-32]. The best characterized chemotactic factor for HSCs identified so far is the PDGF-BB[33-35] which is also known as the most potent mitogen for HSCs over-expressed during active hepatic fibrosis[36]. But there is also evidence that PDGF is proapoptotic for fibroblasts in conditions of low serum[37]. Saile B[38] 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. He also found that Fas and Fas-ligand in HSCs became increasingly expressed during the course of activation. But our data demonstrated that PDGF alone had no effect on the expression of Fas and FasL during further activating the culture-activated HSCs, which was supported by Issa R[39]. Bax and Bcl-2 are known as the representatives of proapoptotic factor and contra-apoptotic factor of Bcl-2 family, respectively[40,41]. In our study, evidences showed that PDGF could promote Bax mRNA expression in HSCs and inhibited Bcl-2 mRNA expression as well, resulting in the apoptosis of HSCs[41]. All the above data demonstrated that PDGF can accelerate the apoptosis of HSCs through Bcl-2/Bax pathway in parallel with their proliferation[42]. In other words, PDGF may promote proliferation but is neutral with respect to HSCs apoptosis. But the proportion of apoptosis-inducing forces and apoptosis-inhibiting forces would determine that PDGF-activated HSCs tend to proliferate and increase[22].

Cytokine interleukin-10 (IL-10), produced by lymphocytes and macrophages as well as cells within liver such as Kufffer cells, hepatocytes and HSCs, has profound inhibitory actions on macrophages and inflammation. The present studies showed that IL-10 had additional effects on connective tissue cells, such as HSCs and fibroblast. IL-10 could inhibit the activation of HSCs by inflammatory cells[43], relieve the inflammation of liver[18,19,44], suppress the function of NF-κB[45] and affect the expression of collagen I and collagenase[20], thus exerting an antifibrogenesis effect[46]. Failure for HSCs to sustain IL-10 expression might underlie pathologic progression to liver cirrhosis[18,20] .Our previous studies also implied that IL-10 had an antagonism on CCL_4-induced rat hepatic fibrosis[22]. But the underlying mechanism remains obscure. In this study, our results showed that IL-10 could promote the expression of FasL and Bax mRNA in culture-activated HSCs and meanwhile could inhibit Bcl-2 mRNA expression, implying that IL-10 may induce the apoptosis of HSCs through binding FasL to Fas on the cell membranes of HSCs and increasing the proportion of Bax and Bcl-2. Saile B[38] found that apoptosis could be fully blocked by Fas-blocking antibodies in normal cells and HSCs already entering the apoptotic cycle, implying that Fas/FasL system is the key pathway for the apoptosis of HSCs. Our data, however, showed that Bax/Bcl-2 system was another important pathway involving in HSCs' apoptosis[40,41]. In short, IL-10 could promote the apoptosis of HSCs, which may be related to its mechanism of antifibrosis.

There is evidence that activated-HSCs could express IL-10 as well as its receptor[20,47]. In this study, PDGF had a similar effect to IL-10 on Bax/Bcl-2 mRNA expression in HSCs. This promotes us to hypothesize that PDGF may regulate the expression of Bax and Bcl-2 mRNA by affecting the expression of IL-10 in HSCs. But PDGF in combination with IL-10 did not show a satisfying synergistic action, thus we can not exclude the possibility that PDGF and IL-10 affect in different ways, and further works are demanded.

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