Effect of IL-10 on the expression of HSC growth factors in hepatic fibrosis rat

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INTRODUCTION

Hepatic fibrosis, which represents the wound healing response of the liver, is a common sequel to diverse liver injuries, characterized by increased deposition and altered composition of extracellular matrix (ECM). Hepatic stellate cells (HSCs) are the major source of ECM, and believed to be the crucial cell type in the development of hepatic fibrosis[1,2] and growth factors were considered to exert their effects through autocrine or paracrine on HSCs during the process[3]. It has been reported that interleukin-10 (IL-10) could relieve the degree of rat hepatic fibrosis induced by carbon tetrachloride (CCL4). In the present study, based on the established hepatic fibrosis rat model and IL-10 treated model, we isolated HSCs, detected the expression of TGF-β1, EGF, HGF and PDGF of HSC, and tried to explore the relationship between growth factors and hepatic fibrosis and the possible mechanisms of the anti-fibrogenic activities of exogenous IL-10 in vivo.

MATERIALS AND METHODS

Establishment of animal models

Sixty clean male SD rats, weighing 200-300 g, were divided randomly into three groups: normal control group (GN, 8 rats), hepatic fibrosis model group (GC, 28 rats) and IL-10 treated group (GI, 24 rats). All rats were bred under routine conditions (room temperature, 22±2 ℃; humidity, 55±5%; light, 12 h per day; drinking tap water and food...
ad libitum. Animal food was provided by BK Company in Shanghai, China. Rats in Gn were injected intra-peritoneally with saline at a dose of 2 mL/kg twice a wk; rats in the other two groups received intra-peritoneal injection of 500 mL/L CCl₄ dissolved in castor oil 2 mL/kg twice a week as described previously[40]. From the 3rd wk, rats in the treated group were given intra-peritoneally IL-10 dissolved in saline 4 µg/kg, 20 min before CCl₄ administration, as proposed by Nelson et al.[41]. The intervention stated above lasted to the end of the experiment.

**Histological examination**

At the beginning of the 7th and 11th wk, two rats of each group were selected randomly for histological examination. The liver tissues were fixed in 40 g/L formaldehyde and embedded with paraffin. Sections were stained with hematoxylin and cosin (HE) and examined under a light microscope.

**Isolation, culture and identification of HSCs**

Isolation, culture and identification of HSCs have been described thoroughly in our previous experiments[6]. 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 1×10⁶ 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 CO₂ atmosphere for 72 h. They were identified by their typical phase-contrast microscopic appearance and by immunocytochemistry using antibody directed against desmin. 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). Its quantity and purity were assessed by measuring the optical density at 260 and 280 nm. After measurement of RNA amount, samples were either used immediately for reverse transcription (RT) or stored at -70°C.

For RT, 1 µg 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.

For PCR, primers coding for the house-keeping gene β-actin was added to the reaction mixture spontaneously to standardize the results. Reactive systems contained cDNA 2 µL, 10×buffer 5 µL, 25 mmol/L MgCl₂ 5 µL, 10 mmol/L d NTP 1 µL, 20 mmol/L target gene sense and anti-sense primers 1 µL, 20 mmol/L β-actin primer pairs 1 µL, Taq DNA polymerase 3 U, with a total volume of 50 µL by adding aqua. Then PCR was performed with cycle parameters of 45 s at 94°C, 30 s at 55°C (TGF-β1) or 58°C (EGF) or 66°C (HGF) or 62°C (PDGF) and 60 s at 72°C after pre-denaturation for 5 min at 94°C; final elongation time was 7 min at 72°C. The number of corresponding cycles for TGF-β1, EGF, HGF and PDGF were 30, 32, 34 and 34, respectively. Primers were designed with reference to GenBank (Table 1). PCR products were immediately analyzed by 2% agarose gel electrophoresis and the density of resultant bands was semi-quantified by scanning densitometry with the ratio of TGF-β1/β-actin1, EGF/β-actin1, HGF/β-actin1 and PDGF/β-actin2.

**Immunocytochemistry**

Most of HSCs were attached to the dishes after 72 h of primary culture. Then the 96-well plates were washed twice with 0.1 mol/L PBS and fixed with poly-formaldehyde at 4°C overnight. The following procedures were performed according to the instructions of streptavidin-peroxidase (S-P) kit (Beijing Zhongshan Company). Briefly, cells were washed with PBS, incubated with bovine serum albumin in PBS, reacted with primary antibody dissolved in PBS, washed, incubated with peroxidase-conjugated second antibody, washed, and reacted for 20 min with S-P. Color reaction was developed by incubation with DAB. For negative controls, the primary antibody was replaced by PBS. Table 2 summarizes the characteristics of the antibodies used in this study.

**Statistical analysis**

All data were expressed as mean±SE. The significance for the difference between the groups was analyzed with SPSS10. 0 by one-way ANOVA.

**RESULTS**

**Histological examination**

Hepatic fibrosis, as shown histologically, became remarkable with the treatment of CCl₄. During the 7th wk, specimens from GC showed steatosis and ballooning degeneration, the collagen fibers increased and began to extend to the parenchyma, a great number of mononuclear cells and unusual neutrophils surrounded the centrilobular veins and fibrotic septa (Figure 1A), while only a few inflammatory

| mRNA      | Sense primers | Anti-sense primers | Product length (bp) |
|-----------|---------------|--------------------|---------------------|
| TGF-β1    | CTC TGC ACG GCC AGC TCT G | GGA CTC TCC ACC TGC AAG AC | 392                |
| EGF       | GAC AAC CTC CTT AAG GCT TA | CATGCA CAC GCC ACC ATT | 567                |
| GAG       | TCT TGA CCC TGA CACC CCC | GCAA GTC ACC CCC CAT CCG G | 269                |
| HGF       | TTC TGA CCC TGA CACC CCC | GCT CTC AACT TGG CAT CCC G | 435                |
| PDGF      | CAT CCG CTC TTT TGA TGA TC | GCTCCA CAC TGG CAT CCC AG | 410                |
| β-actin1  | AGC ACT TCC CTT CCA CGA | GAG CTA TGA GCT GCC TGA | 660                |
| β-actin2  | CCA ACC GTG AAA AGA TGA CC | CAG GAG GAG CAA TGA TCT TG |
cells infiltrated around centrilobular veins without evident changes of lobular structure in GI (Figure 1B). At the 11th wk, the reticular fibers extended into hepatic plate and full delimitation was developed in GC (Figure 1C), while less fibrous and inflammatory infiltrates were seen in GI (Figure 1D). Specimens from GN showed normal lobular structure (Figure 1E). Due to the limit of samples, no statistical data could be obtained to present disparity between the two groups, but it still illustrated the trend that fibrogenesis of GI was much less severe than that of GC.

**Isolation and identification of HSCs**

Totally 2-4.5×10^7 cells per rat were harvested by the present method. HSCs were identified by immunoreaction for desmin (Figure 2), the mean purity of freshly isolated HSCs was (95±5)%. Cell vitality checked by trypan blue exclusion was higher than 95%.

**Table 2 Antibodies used for immunocytochemistry**

| Antibody                  | Type           | Source                          | Working solution |
|---------------------------|----------------|---------------------------------|------------------|
| Desmin                    | Polyclonal, mouse | Beijing Zhongshan Co.           | 1:100            |
| TGF-β1                    | Polyclonal, rabbit | BOSTER Co.                     | 1:20             |
| EGF                       | Polyclonal, rabbit | BOSTER Co.                     | 1:50             |
| PDGF                      | Polyclonal, rabbit | Beijing Zhongshan Co.         | 1:20             |
| Antibody to rabbit IgG    | Goat            | Beijing Zhongshan Co.          | Ready to use     |
| Antibody to mouse IgG     | Goat            | Beijing Zhongshan Co.          | Ready to use     |

**Expression of mRNA**

RNA purity was determined by the ratio of A_{260nm}/A_{280nm}, which ranged from 1.8 to 2.0. At the 7th and 11th wk, TGF-β1, EGF, and HGF mRNA in GC increased obviously compared with GN and GI (P<0.01). For TGF-β1, no difference was seen between GI and GN. For EGF, mRNA level in GI increased compared with GN at the 7th wk (P<0.01) and 11th wk (P<0.05). For HGF, mRNA level in GI
Protein expression and immunophenotyping of cells

TGF-β1, EGF and PDGF positive expressions were localized in cytoplasm and nuclei of HSCs in all groups by immunocytochemistry after primary culture for 72 h. By then, most of the cells were attached and spread over the plastic sub-stratum. At the 7th wk, the size of HSCs in GC was slightly larger than GN, as well as the number and length of pseudopodium. At the 11th wk, cell phenotype in GI was in the form of a circle or an ellipse and slightly smaller than the 7th wk, no obvious change of phenotype was seen in GC compared to the 7th wk. Despite the small number of samples and lack of statistical data, we could still find the tendency that the expression of TGF-β1, EGF and PDGF in GC was higher than that of GN and decreased after treatment with IL-10 (Figure 4A-F).

DISCUSSION

Although significant progress has been made in understanding the pathogenesis of hepatic fibrosis, a rational therapy that prevents the progression or even reverses established fibrosis remains elusive[7-9]. IL-10 is produced mainly by TH2 cells and inhibits functions of TH1 cells. It downregulates pro-inflammatory cytokine synthesis and is associated with amelioration of the inflammatory response[10-12] and fibrosis. The present study also found the trend of IL-10 relieving the degree of inflammation. Then, except for anti-inflammation, is there any other mechanism to fulfill its anti-fibrotic role? In this experiment, we detected the mRNA level of TGF-β1, EGF, HGF and PDGF of HSCs of rat in hepatic fibrosis group and IL-10 treated group at the 7th and 11th wk.

TGF-β1 is the most important growth factor involved in the fibrotic and cirrhotic liver. It is known to promote the development of liver fibrosis by inducing synthesis of ECM proteins and downregulating the expression of matrix metalloproteinases[13]. The study also found the trend of IL-10 relieving the degree of inflammation. Then, except for anti-inflammation, is there any other mechanism to fulfill its anti-fibrotic role? In this experiment, we detected the mRNA level of TGF-β1, EGF, HGF and PDGF of HSCs of rat in hepatic fibrosis group and IL-10 treated group at the 7th and 11th wk.

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a certain extent and that IL-10 exerted its anti-fibrotic activities by attenuating the expression of TGF-β1 of HSCs.

EGF is a potent mitogen for many cells. EGF up-regulation is a characteristic feature of fibrotic liver disease[17]. Apart from stimulating hepatocyte and bile duct epithelial proliferation, EGF also has a chemotactic mitogen effect on HSCs[18]. A recent study demonstrated that direct stimulation with TGF-β1 and EGF results in an increased migratory response of activated HSCs[19]. In the present study, EGF level increased with the presence of hepatic fibrosis or cirrhosis and dropped after intervention with IL-10. It showed that IL-10 inhibited HSCs from proliferating and migrating by reducing autocrine secretion of EGF, thus it limited the spread of disease, and retarded the progression of hepatic fibrosis.

HGF is secreted as a precursor polypeptide that requires proteolytic cleavage to a disulfide-linked heterodimer and binds to a specific receptor known as c-Met[20] for biological activity. Physiologically, HGF has potent hepatotrophic and cytoprotective functions[21]. Reports have shown treatment with HGF suppressed the increase of TGF-beta1, reduced mRNA levels of procollagen, inhibited fibrogenesis and cell apoptosis, and produced complete resolution of fibrosis in the cirrhotic liver, thereby improving the survival rate of rats with this severe illness[22-26]. In the present experiment, HGF mRNA level of HSCs increased in hepatic fibrosis rats which might reflect intra-hepatic inflammation or the compensation of steatosis of hepatocytes. After intervention with IL-10, HGF mRNA level dropped. So we postulated that IL-10 probably suppressed some cytokines that positively regulated HGF expression by anti-inflammation or immunoreaction or that it indicated normal liver function, or a manifestation of decreased degree of steatosis and degeneration.

PDGF has been identified as the most potent-polypeptide growth factor able to stimulate the proliferation of HSCs[27-29], which play a critical role in the development of hepatic fibrosis. Although no statistical data were obtained to prove any difference of PDGF mRNA in these groups, which probably resulted from the difference in experimental animals, drugs, reagents and methods, PDGF protein level seemed to show the disparity that it increased in GC and
decreased in GI compared to GN.

Our experiment showed IL-10 exerted anti-fibrotic effect not only by anti-inflammatory reaction but by suppressing the expression of growth factors. Whether IL-10 plays an adverse role in liver regeneration by downregulating the expression of HGF mRNA awaits further studies. Based on the biological effect model of HGF, it is necessary to elucidate its activities at protein and receptor levels, which should provide a promising prospect for the therapy of hepatic fibrosis.

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