Original Article

Interleukin-26 promotes the proliferation and activation of hepatic stellate cells to exacerbate liver fibrosis by the TGF-β1/Smad2 signaling pathway

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Abstract: Liver fibrosis is a wound-healing process of liver featured by the activation of hepatic stellate cells (HSCs) and the deposition of extra cellular matrix (ECM). Accumulating facts have suggested that interleukin (IL) 26 is involved in the pathogenesis of liver fibrosis by the modulation of HSCs. However, the biological roles of IL-26 in liver fibrosis are still unclear. The present study aimed to determine the effect and mechanism of IL-26 on the proliferation and activation of HSCs in vitro. By cell counting kit (CCK)-8 assay, we observed that IL-26 significantly promoted the proliferation of HSCs by increasing S phase and decreasing G0/G1 phase. Annexin V-FITC/PI double staining showed that IL-26 could suppress the apoptosis of HSCs by inhibition of caspase 3 (CASP3) and Bcl-2 associated X protein (BAX). Furthermore, quantitative real-time PCR (qRT-PCR) assay and western blotting analysis revealed that IL-26 exacerbated the degree of hepatic fibrosis, which was associated with the upregulation of the mRNA levels and protein concentrations of IL-6, IL-10, tumor necrosis factor (TNF)-α, matrix metallopeptidase (MMP)-9, and α-smooth muscle act in (SMA). Mechanistically, western blotting analysis showed that IL-26 upregulated the protein expression levels of transforming growth factor (TGF)-β1 and SMAD family member 2 (Smad2) in HSCs. In summary, the data demonstrated a key role of IL-26 on the proliferation and activation of HSCs in liver fibrosis and the underlying mechanism might be related to the TGF-β1/Smad2 signaling pathway. The finding will provide a proof that targeting IL-26 may be developed as therapeutics for liver fibrosis.

Keywords: Liver fibrosis, IL-26, proliferation, activation, TGF-β1/Smad2

Introduction

Hepatitis B virus (HBV) infection is one of the serious problems that threaten public health worldwide [1, 2]. HBV infection leads to persistent inflammatory reaction in the liver, causing secretion and deposition of extracellular matrix (ECM) and eventually leading to liver fibrosis [3]. The current overall rate of reversal of liver fibrosis by anti-HBV drugs (nucleosides and interferons) is only 30-40% and fibrosis may still exist and continue to progress after virological response [4]. Because the pathogenesis of liver fibrosis is still unclear, this directly affects the development of prevention strategies and measures. Therefore, it is necessary and urgent to identify the potential mechanism of liver fibrosis.

Previous studies confirmed that hepatic stellate cells (HSCs) play a key role in the process of hepatic fibrosis [5, 6]. The activation and proliferation of HSCs lead to the secretion of a large amount of ECM, resulting in an imbalance between synthesis and degradation in liver [7]. The secretion of ECM by HSCs is regulated by various factors in the liver microenvironment, especially various cytokines [8]. The intrahepatic immune cells are the source of mostly regulated cytokines. Studies have shown that monocytes/macrophages accumulate in large areas of liver fibrotic lesions and have been identified as an important immune cell population that promote liver fibrosis [9]. Mononuclear/macrophage release profibrotic inflammatory cytokines such as transforming growth factor (TGF)-β1, interleukin (IL)-1β, and tumor necro-
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The IL-26 gene is located on chromosome 12q15 region, between interferon gamma (IFNG) and IL-22. IL-26 is highly conserved in mammalian species and more weakly similar to nonmammalian species. Emerging evidences have suggested that IL-26 contributes to host defense against intracellular and extracellular bacteria [11, 12]. At present, many studies have proved that multiple cells can produce IL-26, including Th17, NK subgroup, and monocytes [13-15]. In rheumatoid arthritis (RA), IL-26 is abundantly presented in the synovial fluid and plasma of patients and can promote the secretion of pro-inflammatory cytokines or aggravate local damage [16]. IL-26 is significantly elevated in multiple sclerosis (MS) and psoriasis lesions and is closely related to the degree of injury [17]. In inflammatory bowel disease (IBD), IL-26 synergizes with other pro-inflammatory factors to mediate tissue-damaging immune responses [18]. Thus, IL-26 plays an important role in the inflammation and injury process of various diseases. However, the relationship between IL-26 and liver fibrosis has not been illustrated. The purpose of this study was to observe the effect of IL-26 on the proliferation and activation of HSCs in vitro, and to elucidate the potential mechanism of IL-26 on liver fibrosis.

Materials and methods

Ethics statement

The study was approved by the ethics committee of Navy Military Medical University (Shanghai, China). All experimental procedures on rats were approved by the ethics committee of Animal Experiments of Navy Military Medical University. The experiment was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Cell culture and IL-26 stimulation

Normal male Sprague-Dawley rats weighing at 100-130 g (5-6 weeks) were obtained from Laboratory Animal Center of Navy Military Medical University (Shanghai, China). All rats were fed with available diet and water in an air-conditioned environment with temperature at 23-25°C, humidity at 50 ± 2%, and 12 h light/dark cycle. Primary rat HSCs were isolated from rat livers by pronase/collagenase digestion followed by subsequent density gradient centrifugation as previously reported [19]. The HSCs (passage at 3-5) were cultured in a humidified incubator at 37°C with 5% CO₂ atmosphere containing DMEM/Nutrient Mixture F-12 Ham (Sigma, St Louis, MO, USA), 10% FBS (Gibco, CA, USA), 1% of 100 U/ml penicillin + 100 mg/ml streptomycin (Sigma, St Louis, MO, USA), and 1% fungizone (Gibco, CA, USA). Recombinant human IL-26 protein was obtained from Abcam (#ab163232; Cambridge, MA, USA). HSCs were serum starved for 24 hand then stimulated with human IL-26 protein at 0, 50, 100, and 200 pg/ml for 12 h. After that, HSCs were collected for further experiments.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with the TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers’ protocols. First strand cDNA was synthesized from 1 µg total RNA in a 20 µl reaction, using a Reverse Aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocols. The qPCR reaction was performed using a Go Taq® qPCR Master Mix (Promega, Madison, WI, USA) on Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturers’ protocols. The thermocycling conditions were as follows: 95°C for 10 sec; followed by 40 cycles at 95°C for 5 sec, 60°C for 30 sec, and 72°C for 30 sec. Specific primers for IL-6, IL-10, TNF-α, matrix metalloproteinase (MMP)-9, α-smooth muscle act in (SMA), caspase 3 (CASP3), and Bcl-2 associated X protein (BAX) were designed from Ribo Bio Co., Ltd (Guangzhou, China). The sequences of each primer were shown in Table 1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The relative expression level of each target gene was quantified by the $2^{ΔΔCT}$ method [20].
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Western blotting analysis

Total protein was extracted with RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers’ protocols. The concentration of protein was determined by DC protein assay (Bio-Rad, CA, USA). Total protein (30 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk at 37°C for 2 h and primary mouse monoclonal anti-TGF-β1 (#ab27969, 1:1000; Abcam, Cambridge, MA, USA) and anti-Bcl-2 associated X protein (BAX) (#ab77566, 1:500; Abcam) antibodies, and primary rabbit monoclonal anti-SMAD family member 2 (Smad2) (#ab40855, 1:500; Abcam), anti-caspase 3 (CASP3) (#9662, 1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-cleaved CASP3 (#9661, 1:1500; Cell Signaling Technology) antibodies were prepared in blocking solution and incubated overnight at 4°C. After washing three times in 0.1% Tween-20/PBS buffer, membranes were incubated with anti-mouse and rabbit horseradish peroxidase (HRP) conjugated secondary antibodies at 37°C for 1 h. Protein of GAPDH (#ab181602, 1:2000; Abcam, Cambridge, MA, USA) was used as loading control. Protein signals were detected using a Chemiluminescence plus western blot analysis kit (GE Healthcare, Waukesha, USA).

Enzyme-linked immunosorbent assay (ELISA)

Total protein was extracted from HSCs using an ELISA kit (Sen BeiJia Biotechnology Co., LTD, Nanjing, China) in accordance with the manufacturer’s protocols. A Synergy™ HT Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA) was applied to analyze optical density values at a wavelength of 450 nm, with a standard curve drawn. After that, the protein concentrations of human TNF-α (#SBJ-H0038), IL-6 (#SBJ-H0465), IL-10 (#SBJ-H0481), MMP-9 (#SBJ-H0154), and α-SMA (#SBJ-H1419) were calculated.

Cell proliferation assay

The proliferation of HSCs was detected by Cell counting kit (CCK)-8 assay. The HSCs at 6 × 10⁴/well were used to plate onto 96-well culture plates. After grown overnight, the cells were stimulated with IL-22 at 0, 50, 100, and 200 pg/ml for 12 h, respectively. After incubation for 0, 24, 48, and 72 h, each well was added with 10 µl CCK-8 solution (Dojindo, Kumamoto, Japan), and HSCs were incubated for 4 h in a humidified incubator at 37°C with 5% CO₂ atmosphere. Optical density values were measured at 450 nm by a Synergy™ HT Multi-Mode Microplate Reader (Biotek) as reading reference wavelength at 690 nm.

Cell cycle analysis

The cell cycle of HSCs was determined by flow cytometry. The HSCs at 5 × 10⁵/well were used to plate onto 6-well culture plates. After grown overnight, the cells were stimulated with IL-26 at 0, 50, 100, and 200 pg/ml for 12 h, respectively. After incubation for 48 h, HSCs were collected and resuspended in 75% ethanol at -4°C for 60 min. After that, cells were stained with 5 µl of Annexin V-FITC and 10 µl of propidium iodide (PI). After incubated for 15 min at 37°C in the dark, FITC or PI fluorescent intensities were detected and analyzed by a BD FACS Calibur™ Flow Cytometer (Franklin Lakes, NJ, USA). The percentages of the cells in G0/G1, S, and G2/M phases were calculated.

Cell apoptosis assay

The apoptosis of HSCs was analyzed by annexin V-FITC/PI double staining. Cells were seeded in 6-well plates overnight and then treated with different concentrations of IL-26 for 48 h. A annexin V-FITC/PI apoptosis detection kit (BD Biosciences, CA, USA) were used for detection of cell apoptosis. Annexin V-FITC positive cells were regarded as apoptotic cells using a BD FACS Calibur™ Flow Cytometer (Franklin Lakes, NJ, USA).
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**Statistical analysis**

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). Each experiment was replicated three times, and data were expressed as means ± standard deviation (SD). Multiple-comparison tests were applied only when a significant difference was determined by ANOVA followed by an LSD post-hoc test. P<0.05 was considered significant.

**Results**

**IL-26 promoted the proliferation of HSCs by modulation of cell cycle**

The effect of IL-26 on the growth of HSCs was evaluated using multiple doses of human IL-22 protein at 0, 50, 100, and 200 pg/ml in vitro. We observed that treatment with IL-26 significantly increased the proliferation of HSCs in a dose-dependent manner (Figure 1A, P<0.05). To assess mechanism of IL-26 promoting HSCs proliferation, cell cycle analysis was performed in HSCs. The percentages of S and G0/G1 phases in each group are shown in Figure 1B and 1C. The results showed that IL-26 stimulation resulted in an increase of HSCs in S phase and a decrease of HSCs in G0/G1 phase in a dose-dependent manner (P<0.05). In contrast, there was no significant difference in percentages of G2/M phase in the different concentrations of IL-26 stimulation groups (Figure 1D). These data indicated that IL-26 promoted the proliferation of HSCs in a dose-dependent manner by increasing S phase and decreasing G0/G1 phase.

**Figure 1.** Interleukin (IL)-26 promoted the proliferation of hepaticstellate cells (HSCs) by modulation of cell cycle. A. Cell counting kit (CCK)-8 assay was applied to evaluated cell proliferation, and IL-26 treatment significantly increased the proliferation of HSCs in a dose-dependent manner. B. Flow cytometry was used to measure the proportion of HSCs in S phase. C. IL-26 stimulation markedly decreased G0/G1 phase of HSCs in a dose-dependent manner. D. There was no significant difference in percentages of G2/M phase in the 0, 50, 100, and 200 pg/ml groups. *P<0.05.
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IL-26 suppressed the apoptosis of HSCs by inhibition of CASP3 and BAX

In order to observe the effect of IL-26 on apoptosis of HSCs, we exposed HSCs to multiple doses of IL-26 for 48 h. Annexin V-FITC/PI double staining was used to evaluate apoptotic cells. The percentages of cell apoptosis in each group were shown in Figure 2A. The results showed that IL-26 treatment significantly decreased the apoptosis of HSCs in a dose-dependent manner \((P<0.05)\). To further inquire into the mechanism of IL-26-related apoptosis in HSCs, qRT-PCR was applied to detect the mRNA expression levels of CASP3 and BAX. We found that IL-26 stimulation markedly downregulated the mRNA expression levels of CASP3 and BAX in a dose-dependent manner \((P<0.05)\). In addition, the protein levels of CASP3, cleaved CASP3, and BAX in HSCs were decreased in a dose-dependent manner after IL-26 treatment \((P<0.05)\). The data suggested that IL-26 could decrease the apoptosis of HSCs by inhibition of CASP3 and BAX.

IL-26 induced the activation of HSCs to promote liver fibrosis

The activation of HSCs is regulated by various cytokines, including IL-6, IL-10, and TNF-α \([8]\). To explore the possible role underlying the IL-26-related activation of HSCs, we investigated IL-6, IL-10, and TNF-α expression. The qRT-PCR assay and western blotting analysis revealed IL-26 stimulation significantly upregulated the mRNA levels and protein concentrations of IL-6, IL-10, and TNF-α in HSCs in a dose-dependent manner \((P<0.05)\). Furthermore, we also investigate the effect of IL-26 on the expression of MMP-9 and α-SMA (a marker protein of HSCs activation).
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The results showed that treatment with IL-26 significantly increased the mRNA levels and protein concentrations of MMP-9 and α-SMA in a dose-dependent manner (Figure 3C and 3D, P<0.05). These data indicated that IL-26 induced the activation of HSCs to promote liver fibrosis.

**TGF-β1/Smad2 signaling pathway was associated with IL-26 induced liver fibrosis**

To further determine the potential mechanism of IL-26 in liver fibrosis, the TGF-β1/Smad2 signaling pathway aroused our attention. TGF-β1 and Smad2 are two important molecules in the TGF-β1/Smad2 signaling. TGF-β1 is one of the strongest profibrotic factors expressed by activated HSCs. Western blotting analysis revealed that IL-26 upregulated the protein expression levels of TGF-β1 in HSCs in a dose-dependent manner (Figure 4, P<0.05). In addition, similar results of Smad2 protein expression levels were also found in HSCs (Figure 4, P<0.05). Taken together, our data demonstrated that IL-26 exacerbated liver fibrosis by the TGF-β1/Smad2 signaling pathway.

**Discussion**

Liver fibrosis is a complex fibrogenic and inflammatory process that results from chronic liver injury and represents an early step in the progression of liver cirrhosis [21]. HSCs mainly localize to the perisinusoidal space between sinusoidal endothelial cells and hepatocytes, and are the primary source of activated myofibroblasts and portal fibroblasts that start the fibrogenic process. Activated HSCs can secrete ECM, tissue inhibitors of metalloproteinases, and MMPs that cause liver structural remodel-
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Liver transplantation is currently regarded as the ideal treatment method for liver cirrhosis and is generally insufficient. Therefore, it is necessary to develop effective anti-fibrotic therapy for the treatment of fibrosis. In this study, we observed that IL-26 promoted the proliferation and activation of HSCs to exacerbate liver fibrosis in vitro. Further investigation demonstrated that IL-26 facilitated liver fibrosis via the TGF-β1/Smad2 signaling pathway. To date, several literatures showed potential effects of interleukin on liver fibrosis, including IL-11, IL-17, and IL-22 [5]. For instance, block IL-11 signaling decreases fibrosis, steatosis, hepatocyte death, inflammation and hyperglycemia in mice with diet-induced steatohepatitis [23]. IL-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice [24]. IL-22 suppressed HSCs activation and reduces liver fibrosis through downregulating expression of microRNA-200a and upregulating expression of β-catenin [25]. However, the role and mechanism of IL-26 in liver fibrosis remain unknown. In agreement with previous reports, our data indicated that treatment with IL-26 significantly promoted the development of liver fibrosis. Furthermore, similar to the Meng et al [24] research, we found that promotive effect of IL-26 on HSCs was majorly reflected by induction of cell proliferation and reduction of cell apoptosis in a dose-dependent manner. The activation of HSCs is regulated by various cytokines, including IL-6, IL-10, and TNF-α [8]. α-SMA is a marker for the detection of activated HSCs during liver fibrosis. We further observed that the mRNA levels and protein concentrations of IL-6, IL-10, TNF-α, and α-SMA were upregulated in a dose-dependent manner along with the IL-26 treatment, indicating that IL-26 induced the activation of HSCs to promote liver fibrosis.

Accumulating studies have indicated that TGF-β regulates ECM metabolism and tissue fibrosis through the mixed actions of MMPs [26]. In vitro, we found that IL-26 significantly increased them RNA and protein expression of MMP-9 in HSCs, and thus inhibited the degradation of ECM. Previous data have proved that the Smad2/3/4 complex can translocate to the nucleus and increase the expression of profibrotic genes in human [27]. A recent study has demonstrated that TGF-β/Smad signaling pathway plays a key role in the development of hepatic fibrosis [28]. TGF-β1 is transduced through the sequential activation of its two serine/threonine kinase receptors, which in turn Smad-2 to induce nuclear translocation [29]. The transformation of HSCs into myofibroblasts can be promoted by TGF-β1 [30]. The results of the present study revealed that treatment with IL-26 upregulated the protein expression levels of TGF-β1 and Smad2 in HSCs in a dose-dependent manner. These data indicated that IL-26 exacerbated liver fibrosis by the TGF-β1/Smad2 signaling pathway.

In conclusion, we first showed that IL-26 promoted the proliferation and activation of HSCs to exacerbate liver fibrosis in a dose-dependent manner.

Figure 4. IL-26 regulated the expression of TGF-β1/Smad2 signaling pathway in HSCs. The expression of transforming growth factor (TGF)-β1 and SMAD family member 2 (Smad2) was detected by western blotting analysis in the different concentrations of IL-26 groups. The results showed that IL-26 upregulated the protein expression levels of TGF-β1 and Smad2 in HSCs in a dose-dependent manner. *P<0.05.
manner, and the underlying mechanism might be related to the TGF-β1/Smad2 signaling pathway. These results might help in developing more effective therapeutics for liver fibrosis.

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**Disclosure of conflict of interest**

None.

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