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Octreotide attenuates hepatic fibrosis and hepatic stellate cells proliferation and activation by inhibiting Wnt/β-catenin signaling pathway, c-Myc and cyclin D1

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ABSTRACT

Fibrosis is the common results from an excessive wound-healing response to chronic liver injury. Otreotide (OCT), an analogue of somatostatin, was reported to have an anti-hepatic fibrosis effect. However, its anti-fibrosis mechanisms have not been well characterized to date. The present study aimed to investigate the protective effects of OCT on carbon tetrachloride (CCl_4)-induced rat liver fibrosis and activation and proliferation of transforming growth factor-β1 (TGF-β1)-treated hepatic stellate cells (HSCs) and explore its anti-hepatofibrotic mechanisms. Our results indicated that treatment with OCT markedly down-regulated the protein and mRNA expression of liver fibrosis markers including α-smooth muscle actin (α-SMA) and collagen I in CCl_4-induced rat model of liver fibrosis, accompanied by decreasing aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL) activities and increasing the serum level of albumin (ALB). In addition, in vitro results revealed that OCT inhibited the activation and proliferation of TGF-β1-treated LX-2 cells in a concentration-dependent manner and decreased in parallel the expression of Wnt1, β-catenin, c-Myc and cyclin D1, indicating that OCT might attenuate liver fibrosis, at least in part, by inhibiting Wnt/β-catenin signaling pathway. Overall, these results provide a novel anti-fibrotic mechanism of OCT, which might be associated with its ability to repress Wnt/β-catenin signaling pathway.

1. Introduction

Hepatic fibrosis (HF) is the result of chronic liver injury from multiple causes, including viral infection, continuous alcoholic toxicity and non-alcoholic steatohepatitis [1]. HF is characterized by the excessive accumulation of extracellular matrix (ECM), leading to a wound healing response to the inflammation injury and eventually develop into cirrhosis [2,3]. Quiescent HSCs in the healthy liver reside in the space of Disse between hepatocytes and sinusoidal endothelial cells [4,5]. Activated HSCs are the primary source of ECM, which lead to the occurrence of liver fibrosis [6–8]. Therefore, the activation and proliferation of HSC is an important step in the development of liver fibrosis [9]. Inhibiting activation and proliferation of HSCs has become the most crucial treatment strategy for hepatic fibrosis.

Accumulating evidence demonstrate that increased activation of Wnt/β-catenin signaling might have a pivotal role in fibrogenesis. So far, Wnt/β-catenin signaling has been reported to be involved in multiple fibrosis disease, including heart [10], lung [11], kidney [12] and bone [13]. Furthermore, a number of studies have confirmed that Wnt/β-catenin is a key signal pathway to regulate HSCs proliferation in liver fibrosis, and the expressions of some molecules in Wnt/β-catenin signaling pathway are increased and implicated in the process of this disease [14–16]. Therefore, inhibiting Wnt/β-catenin signaling could down-regulate the activation and proliferation of HSCs [17] and attenuate CCl_4-induced liver fibrosis [18].

Somatostatin is a natural peptide that negatively regulates cell proliferation and differentiation. Octreotide has been widely used in clinics to control variceal hemorrhage effectively [19]. Previous studies have shown its antifibrotic effects via reducing the degree of liver fibrosis and inhibiting the activation of HSCs. However, the mechanism

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Table 1
Primer sequence used for RT-PCR.

Target gene	Race	Forward sequence	Reverse sequence
α-SMA	Human	5′-TCATGGTCGGTATGGGTCAG-3′	5′-CCGTGCTCGATAGGGTACTT-3′
	Rat	5′-CAATGGCTCCGGGCTCTGTA-3′	5′-CTCTTGCTCTGCGCTTCGTC-3′
Collagen I	Human	5′-CACCAATCACCTGCGTACAG-3′	5′-GCAGTTCTTGGTCTCGTCAC-3′
	Rat	5′-ACCTCAGGGTATTGCTGGAC-3′	5′-GACCAGGGAAGCCTCTTTCT-3′
Wnt1	Human	5′-CAGAGCCACGAGTTTGGATG-3′	5′-AGTGGAGAGGGATTGGGTTG-3′
	Rat	5′-CCTACCTCCCTCCCTCTTCT-3′	5′-ACAATACCACAGGGACAGCA-3′
β-catenin	Human	5′-TGTTCAGCTTCTGGGTTCAG-3′	5′-TATACCACCCACTTGGCAGA-3′
	Rat	5′-TTCCTGAGCTGACCAAACTG-3′	5′-GCACTATGGCAGACACCATC-3′
GAPDH	Human	5′-CTGCCTCGATGGGTGGAGTC-3′	5′-AGGCGCCCAATACGACCAAA-3′
	Rat	5′-AAGGCTGTGGGCAAGGTCAT-3′	5′-TTTCTCCAGGCGGCATGTCA-3′

by which OCT exerts these antifibrosis effects has not been clearly elucidated. Whether OCT could attenuate liver fibrosis and HSCs activation by inhibiting Wnt/β-catenin signaling pathway remains unclear. Therefore, the present study was designed to investigate the effects of OCT on liver fibrosis in rats in vivo and in immortalized HSC cell line LX-2 in vitro.

2. Materials and methods

2.1. Animal models of liver fibrosis

Male Sprague Dawley rats weighing 160–200 g were all provided by Experimental Animal Center of Anhui Medical University (Hefei, Anhui, China). The animal experimental procedures were reviewed and approved by the University Animal Care and Use committee. All rats were randomly divided into three groups: control group, model group and octreotide group. Rats in model group and octreotide group were hypodermically injected with 40% CCl_4 diluted with olive oil at a dose of 3 mL/kg twice a week for 8 weeks. Additionally, rats in octreotide group were hypodermically injected with OCT (Novartis Pharma AG, Basel, Switzerland) diluted with saline (10 μg/kg) twice a day for 8 weeks. Meanwhile, animals in control group were treated with the same volume of saline at the same time intervals. 48 h after the last CCl_4 injection rats were anesthetized with 10% chloral hydrate and sacrificed. Serum samples were collected from each rat and stored at −80 °C to determine the serum biochemical parameters. Liver tissues were harvested for two uses: 1) fix with 10% formalin for H&E staining, Masson staining and immunohistochemistry staining; and 2) preserved at −80 °C for protein and RNA preparation.

2.2. Cell culture

Immortalized human HSC line, LX-2 cell line, was donated by Dr. Scott Friedman (Mount Sinai School of Medicine, USA). The LX-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 100 U/mL streptomycin. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium change was performed every 1–2 days. When cells grown to 80%–90% confluence, we plated the cells in 6-well plates and 96-well plates and incubated for quantitative real-time PCR analysis, western blot analysis and cell proliferation assays, respectively. Cells were divided into five group: 1) normal group: cells treated with DMEM; 2) control group: cells treated with DMEM containing TGF-β1 (10 ng/mL); 3) octreotide group: cells treated with DMEM containing TGF-β1 (10 ng/mL) and OCT (1×10^{-8} mol/L, 1×10^{-7} mol/L and 1×10^{-6} mol/L).

2.3. Cell viability assays

The growth inhibitory effect of OCT on LX-2 cells was determined by MTT assay. Briefly, exponential growing LX-2 cells were plated into 96-well plates (5000 cells per well). When cells grow overnight to reach ~70% confluence, the culture medium was changed and 10 ng/mL TGF-β1 was added to control group and OCT group well. 24 h after TGF-β1 administration, increasing concentrations of OCT in 100 μL culture medium (1×10^{-8} mol/L, 1×10^{-7} mol/L and 1×10^{-6} mol/L) were added to the corresponding wells. After exposure to OCT for 48 h, 20 μL MTT solution (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. Following careful removal of the supernatant, 100 μL dimethylsulfoxide (DMSO) was added to each well and plates were vibrated for 10 min. The optical density (OD) value of each well was measured in enzyme-linked immunity implement (Elx800, Bio-TEK, VT, USA) at 570 nm. Quintuplicate wells were analyzed at different concentration and experiments were carried out in triplicate.

2.4. Total RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from rat liver tissues and LX-2 cells, using TRIzol reagents (Invitrogen, USA) according to the manufacture's protocol. The extracted total RNA was eluted in 20 μL nuclease-free water and the concentration of the RNA was measured by NanoDrop 2000 (Thermo Fisher Scientific). A transcript first-strand cDNA synthesis kit (TaKaRa, Shiga, Japan) was used to generate cDNA from total RNA from each sample. Real time PCR was performed in a detection system with SYBR-Green Master Mix (TaKaRa). The mRNA level of GAPDH was used as an internal control. Primer sequences were listed in Table 1. Each measurement was repeated at least in triplicate and the $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of mRNA in cells and tissue samples.

2.5. Protein isolation and western blot analysis

Protein from liver tissues and cells were extracted with RIPA lysis buffer (Beyotime, China). After centrifugation for 30 min at 4 °C at 12,000 ×*g*, the supernatant was collected and protein concentration was measured using a BCA protein assay kit (Boster, China). Total protein from samples were separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore Corp, Billerica, MA, USA). After incubated with 5% non-fat milk, membranes were washed in TBS/Tween20 buffer and incubated with specific primary antibodies overnight. Rabbit polyclonal anti-α-SMA, collagen I, WISP1 (Wnt1), β-catenin (Proteintech, USA), c-Myc and cyclin D1 (Abcam) were diluted 1:500, 1:1000, 1:500, 1:1000, 1:10,000 and 1:30,000, respectively. Mouse monoclonal anti-β-actin (Abcam) was diluted 1:5000. Therefore, membranes were incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies used as secondary antibodies for 1 h. After three washes in TBS/Tween20 buffer, the membranes developed onto chemiluminescence Western blotting detection system. Protein was visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Fisher Scientific). The intensity of bands was