Interaction between insulin-like growth factor binding protein-related protein 1 and transforming growth factor beta 1 in primary hepatic stellate cells

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BACKGROUND: **We previously showed that insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) is a novel mediator in liver fibrosis. Transforming growth factor beta 1 (TGFβ1) is known as the strongest effector of liver fibrosis. Therefore, we aimed to investigate the detailed interaction between IGFBPrP1 and TGFβ1 in primary hepatic stellate cells (HSCs).**

METHODS: **We overexpressed TGFβ1 or IGFBPrP1 and inhibited TGFβ1 expression in primary HSCs for 6, 12, 24, 48, 72, and 96 hours to investigate their interaction and observe the accompanying expressions of α-smooth muscle actin (α-SMA), collagen I, fibronectin, and phosphorylated-mothers against decapentaplegic homolog 2/3 (p-Smad2/3).**

RESULTS: **We found that the adenovirus vector encoding the TGFβ1 gene (AdTGFβ1) induced IGFBPrP1 expression while that of α-SMA, collagen I, fibronectin, and TGFβ1 increased gradually. Concomitantly, AdIGFBPrP1 upregulated TGFβ1, α-SMA, collagen I, fibronectin, and p-Smad2/3 in a time-dependent manner while IGFBPrP1 expression was decreased at 96 hours. Inhibition of TGFβ1 expression reduced the IGFBPrP1-stimulated expression of α-SMA, collagen I, fibronectin, and p-Smad2/3.**

CONCLUSIONS: **These findings for the first time suggest the existence of a possible mutually regulation between IGFBPrP1 and TGFβ1, which likely accelerates liver fibrosis progression. Furthermore, IGFBPrP1 likely participates in liver fibrosis in a TGFβ1-depedent manner, and may act as an upstream regulatory factor of TGFβ1 in the Smad pathway.**

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KEY WORDS: **insulin-like growth factor binding protein-related protein 1;
transforming growth factor β1;
primary hepatic stellate cells;
α-smooth muscle actin;
extracellular matrix;
Smad pathway**

Introduction

The insulin-like growth factor binding protein-related protein 1 (IGFBPrP1), also known as insulin-like growth factor binding protein 7 (IGFBP7),[1] is a secreted protein.[2] IGFBPrP1 has distinct characteristics[3] and shows tumor suppressor functions by participating in cell proliferation, senescence, and apoptosis in numerous cancers.[4-6] IGFBPrP1 increases the epithelial-to-mesenchymal transition (EMT) phenotype of malignant mesenchymal and epithelial cells.[7] There is evidence that IGFBPrP1 stimulates α-smooth muscle actin (α-SMA) expression in human brain endothelial cell (HBEC).[8] In addition, IGFBPrP1 plays a role

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in hepatic morphogenesis and shows the highest expression in the mid phase of the hepatic stellate cell (HSC) transdifferentiation process.[9, 10] We previously found that IGFBPrP1 activated and transdifferentiated HSCs *in vitro*.[11] Moreover, IGFBPrP1 protein levels markedly increased in the fibrotic and cirrhotic human liver specimens *in vivo*.[12]

Liver fibrosis is a progressive pathological process characterized by remodeling of the extracellular matrix (ECM) and excessive deposition of collagen.[13] Multiple cytokines participate in HSC activation and liver fibrosis, and the most established mediator is transforming growth factor beta 1 (TGFβ1).[14] TGFβ1 acts as a major profibrotic cytokine that potently promotes fibroblast recruitment, proliferation, differentiation into myofibroblasts and the production of ECM.[15] We previously also found that the expressions of both IGFBPrP1 and TGFβ1 were enhanced in mouse liver samples with thioacetamide (TAA)-induced fibrosis.[16] Moreover, IGFBPrP1 overexpression induced the excessive expressions of both ECM and TGFβ1 in the HSC-T6 cell line or mice.[12, 17] However, the interaction between IGFBPrP1 and TGFβ1 and the contribution of IGFBPrP1 to ECM expression in HSCs is unknown.

IGFBPrP1 induces liver fibrosis through Smad-dependent and independent pathways.[17] We observed that phosphorylated-mothers against decapentaplegic homolog 2/3 (p-Smad2/3) expression was upregulated by an adenoviral vector encoding the IGFBPrP1 gene (AdIGFBPrP1) in cultured HSC-T6 cells.[18] Currently, it is not clear whether IGFBPrP1 directly induces p-Smad2/3 expression, thereby inhibiting TGFβ1 expression in primary HSCs. The present study was to investigate the relationship between IGFBPrP1 and TGFβ1, and the effect of their interaction on liver fibrogenesis.

Methods

Primary cell isolation, culture, and identification

The animals were obtained from Shanxi Medical University Laboratory Animal Center (Taiyuan, China). All the animal protocol procedures were approved by the Shanxi Medical University Animal Care and Use Committee (SCXK2009-0001). The healthy male Sprague-Dawley rats were anesthetized with chloral hydrate, their livers were sequentially perfused *in situ* using a pronase E and type IV collagenase solution via the portal vein, and then the primary HSCs were isolated and purified by Nycodenze density gradient centrifugation. The cell viability was determined by Trypan blue staining. The HSCs were seeded on plastic dishes in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1000 U/mL penicillin/streptomycin and incubated in a humidified atmosphere containing 5% CO_2 at 37 ℃ for 48 hours. Then, the medium was replaced with 10% FBS-DMEM, which was changed every 48-72 hours. The antibodies to both desmin (TransGen Biotech, Beijing, China) and α-SMA (Abcam, Cambridge, UK) were used to identify HSCs.

Cell transfection

The HSCs were transfected with AdIGFBPrP1 or AdTGFβ1 (GenePharma Company, Shanghai, China), with enhanced green fluorescent protein (EGFP) at a multiplicity of infection (MOI) of 10, 20, or 40 (number of virus/number of cells). Four short-hairpin RNAs (shRNAs) targeting the rat TGFβ1 mRNA (NM 021578) were designed and synthesized by Sangon Biotech Company (Shanghai, China). The most effective shTGFβ1 was used to construct the LvshTGFβ1 with red fluorescent protein (RFP). The HSCs were transfected with the LvshTGFβ1 at different MOI (1, 10, or 100). The transfection efficacy was assessed by detecting the number of EGFP- or RFP-positive cells. The optimized MOI was used in subsequent experiments.

Quantitative real-time polymerase chain reaction (qPCR) assays

Total RNA was extracted from the HSCs using a Qiagen Rneasy Mini kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions. Then, 1 μg of total RNA was reverse transcribed by using the PrimeScript RT Master Mix (TransGen Biotech, Beijing, China). The quantitative real-time polymerase chain reaction (qPCR) was performed with a Step One PCR System using SYBR Green PCR kit (Invitrogen, Carlsbad, CA, USA). The primer sequences were as follows; TGFβ1: 5'-ATT CCT GGC GTT ACC TTG G-3' (forward) and 5'-AGC CCT GTA TTC CGT CTC CT-3' (reverse); IGFBPrP1: 5'-TCA CCC AGG TCA GCA AAG-3' (forward) and 5'-TCA CCA GGC AAG AGT TCT GT-3' (reverse); and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-ACA GCA ACA GGG TGG TGG AC-3' (forward) and 5'-TTT GAG GGT GCA GCG AAC TT-3' (reverse). The PCR cycle used the following program: 95 ℃ for 10 minutes, followed by 40 cycles at 95 ℃ for 10 seconds, and 60 ℃ for 30 seconds.

Immunocytochemical staining

The HSCs were cultured on glass coverslips in a 6-well plate and incubated with the various treatments. The cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 30 minutes at 4 ℃,