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RAPID COMMUNICATION

Down-regulation of transforming growth factor β1/activin receptor-like kinase 1 pathway gene expression by herbal compound 861 is related to deactivation of LX-2 cells

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Abstract

AIM: To investigate the effect of herbal compound 861 (Cpd861) on the transforming growth factor-β1 (TGFβ1)/activin receptor-like kinase 1 (ALK1, type I receptor) signaling-pathway-related gene expression in the LX-2 cell line, and the inhibitory mechanism of Cpd861 on the activation of LX-2 cells.

METHODS: LX-2 cells were treated with TGFβ1 (5 ng/mL) Cpd861 (0.1 mg/mL), TGFβ1 (5 ng/mL) plus Cpd861 (5 ng/mL) for 24 h to investigate the effect of Cpd861 on the TGFβ1/ALK1 pathway. Real-time PCR was performed to examine the expression of α-SMA (α-smooth muscle actin), *ALK1*, *Id1* (inhibitor of differentiation 1). Western blotting was carried out to measure the levels of α-SMA and phosphorylated Smad1, and immunocytochemical analysis for the expression of α-SMA.

RESULTS: In LX-2 cells, TGFβ1/ALK1-pathway-related gene expression could be stimulated by TGFβ1, which led to excessive activation of the cells. Cpd861 decreased the activation of LX-2 cells by reducing the expression of α-SMA mRNA and protein expression. This effect was related to inhibition of the above TGFβ1/ALK1-pathway-related expression of genes such as *Id1* and *ALK1*, and phosphorylation of Smad1 in LX-2 cells, even with TGFβ1 co-treatment for 24 h.

CONCLUSION: Cpd861 can restrain the activation of LX-2 cells by inhibiting the TGFβ1/ALK1/Smad1 pathway.

Key words: Herbal compound 861; LX-2 cell; Activin receptor-like kinase 1; Inhibitor of differentiation 1; Smad1

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INTRODUCTION

Hepatic fibrosis is a reversible scarring process that is characterized by increased and altered deposition of extracellular matrix in the liver. During the past 20 years, much research has proved that the activation of hepatic stellate cells (HSCs) triggers fibrogenesis[1-3] and TGFβ1 has a pivotal regulating role in this process[1,4,5]. Currently, TGFβ1/ALK1/Smad1 signaling is found in HSCs and is involved in their activation[6-8].

Herbal compound 861 (Cpd861) is a traditional Chinese medicines that is used to treat liver diseases and has been demonstrated to have anti-fibrotic effects and to reverse cirrhosis, especially in the early stage[9-11]. Cpd861 is an extract of 10 herbs. A randomized, double-blinded, placebo-controlled clinical trial has demonstrated that Cpd861 can significantly delay and reverse the process of clinical hepatic fibrosis in patients with liver fibrosis and early cirrhosis due to HBV infection, as diagnosed with liver biopsy performed before and after treatment[11,12].

Our previous studies have shown that Cpd861 can inhibit the activation of HSCs to exert its anti-fibrotic effect[13]. In this study, we selected the TGFβ1/ALK1/Smad1 signaling pathway in an attempt to elucidate the molecular mechanism of Cpd861 in the deactivation of LX-2 cells. The effect of Cpd861 on the expression of

genes in this pathway, such as *ALK1*, *Id1* and the protein level of phosphorylated Smad1 was investigated.

MATERIALS AND METHODS

Materials

LX-2 cells were a gift from Dr. Friedman of Mount Sinai School of Medicine, New York, USA. They are activated human HSCs that are generated by spontaneous immortalization in low-serum conditions[14]. Cpd861 powder (*Radix Salviae Miltiorrhiae*, *Radix Astragali*, *Suberect Spatholobus*, *Flos Carthami*, *Rhizoma Chuanxiong*, *Radix Paeoniae Rubra*, *Rhizoma Cyperi*, *Pericarpium Citri Reticulatae*, *Radix Angelicae Sinensis*, *Radix bupleuri*, *patent No. 99103265.9)* was from Jiangyin Pharmaceutical Company, JiangSu Province, China. Powder (2500 mg) was dissolved in 100 mL PBS, and the final concentration was 0.1 mg/mL in LX-2 cell-culture medium. After centrifugation at 3000 r/min for 10 min, the solution was sterilized at 105℃ for 20 min. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, streptomycin and fetal bovine serum (FBS) were purchased from Gibco, NY, USA. Penicillin was from Sigma, St. Louis, USA. Oligo (dT) primers, M-MLV (Moloney murine leukemia virus) reverse transcriptase, recombinant RNasin ribonuclease inhibitor and dNTP were acquired from Promega (Madison, WI, USA). TRIzol reagent was from Invitrogen, Carlsbad, CA, USA. Power SYBR Green PCR Master Mix was purchased from Applied Biosystems, Warrington, UK. The CO_2 incubator was from SANYO, Sakata, Japan. Cell culture wells (6 cm^2) were from Corning Incorporation, NY, USA. Phospho-Smad1 antibody (60 kDa) was from Cell Signaling Company, Danvers, MA, USA. Mouse anti-human α-SMA (42 kDa) was from Zhongshan Goldenbridge Biotechnology, Beijing, China.

Cell culture and group design

LX-2 cells were cultivated in DMEM supplemented with 50 mL/L FBS, 200 mmol/L L-glutamine, penicillin G (100 U/mL) and streptomycin (100 U/mL) in a humidified incubator at (37℃, 5% CO_2). Cells were cultivated in 25 cm^2 culture flasks to 70% confluence or on glass coverslips in six-well culture dishes to 50% confluence. After serum starvation for 16 h, LX-2 cells were treated with Cpd861 0.1 mg/mL[13], TGFβ1 (5 ng/mL)[15] and TGFβ1 (5 ng/mL) plus Cpd861 0.1 mg/mL. Untreated cells served as a control group. All four groups were harvested after 24 h treatment to investigate the related gene expression and phosphorylation of Smad1 protein. Cells on glass coverslips in six-well culture dishes were fixed in 4% paraformaldehyde in PBS after 24 h for immunocytochemical analysis. Every group included six samples.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. They were incubated with blocking solution (5% Bovine Serum Albumin in PBS) for 30 min. Primary antibodies (mouse anti-human α-SMA, diluted 1:100 in blocking solution) were incubated with the cells overnight.

Table 1 Primers sequence for real time-PCR

Gene	Oligonucleotide primer	Fragment sequence size (bp)
α-SMA (target)		
Sense	CGCATCCTCATCCTCCCT	268
Anti-sense	GGCCGTGATCTCCTTCTG	
ALK1 (target)		
Sense	AGACCCCCACCATCCCTA	67
Anti-sense	CGCATCATCTGAGCTAGG C	
Id1 (target)		
Sense	CCAGAACCGCAAGGTGAG	62
Anti-sense	GGTCCCTGATGTAGTCGATGA	
Gapdh (household)		
Sense	GGCTCTCCAGAACATCATCC	187
Anti-sense	GCTTCACCACCTTCTTGATG	

After three washes with PBST (0.2% Tween 20 in PBS) for 15 min, cells were incubated with the secondary antibody (goat anti-mouse IgG labeled with biotin, with streptavidin labeled with horseradish peroxidase), and then with DAB (Diaminobenzidine), Beijing Zhongshan Goldenbridge Co.). PBS (0.01 mol/L) was substituted for primary antibody as a negative control. The α-SMA positive cells presented as brownish yellow. Cells were counterstained with hematoxylin to identify nuclei. Cells were viewed with an Olympus DP71 microscope (Tokyo, Japan) at 20 × magnification.

RNA isolation and reverse transcription

Total RNA was extracted from LX-2 cells using TRIzol reagent as the lysis buffer. cDNA was synthesized using the Reverse Transcription System (Promega). RNA (1 μg) in 7.7 μL nuclease-free water was added to 2.5 μL 10× transcriptase buffer, 2.5 μL 10 mmol/L dNTP, 0.5 μL RNase inhibitor, and 1 μL M-MLV reverse transcriptase. The reaction was performed for 60 min at 42℃ (cDNA synthesis), and 5 min at 95℃ (enzyme denaturation).

Real-time PCR of mRNA

The cDNA samples were analyzed with the Applied Biosystems 7300/7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA) for detecting the gene expression of α-*SMA*, *ALK1* and *Id1*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The primer sequences were obtained from SaiBaiSheng Biocompany, Beijing, China (Table 1). Each experiment was performed in 20 μL reaction volume. The PCR program consisted of an initial activation step at 50℃ for 2 min, followed by 95℃ for 10 min, then 40 cycling steps of denaturing for 15 s at 95℃, annealing and extension at 60℃ for 1 min (data collected at this stage). The PCR data were analyzed using SDS 2.1 software (Applied Biosystems). mRNA levels were normalized relative to GAPDH values. Fold expression changes and standard errors were calculated using the equation $2^{-\Delta\Delta Ct}$ (Ct, threshold cycle). Each group had six wells of cells for examining the relative fold change of gene expression. Three replicate reactions per sample and endogenous control were used to ensure statistical significance.

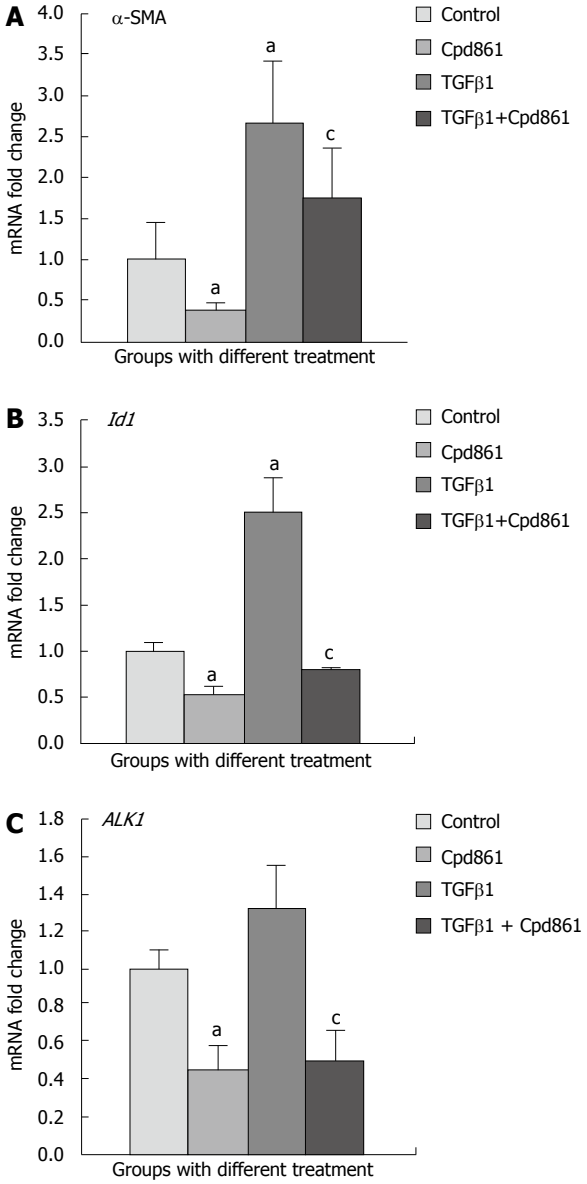

Figure 1 Effect of different treatment on α-*SMA*, *Id1*, *ALK1* expression after 24 h. Cpd861 could decrease the level of all these genes compared with untreated LX-2 cells ($^aP < 0.05$ *vs* control LX-2 cell group). Even with TGFβ1, Cpd861 could also exert its inhibitory roles in these genes expression ($^cP < 0.05$ *vs* TGFβ1 treated group). Samples were obtained from 6 wells of cells for examining the relative fold change of α-*SMA* (**A**), *Id1* (**B**), *ALK1* (**C**) expression .Three replicate reactions for per sample. Error bars, SD.

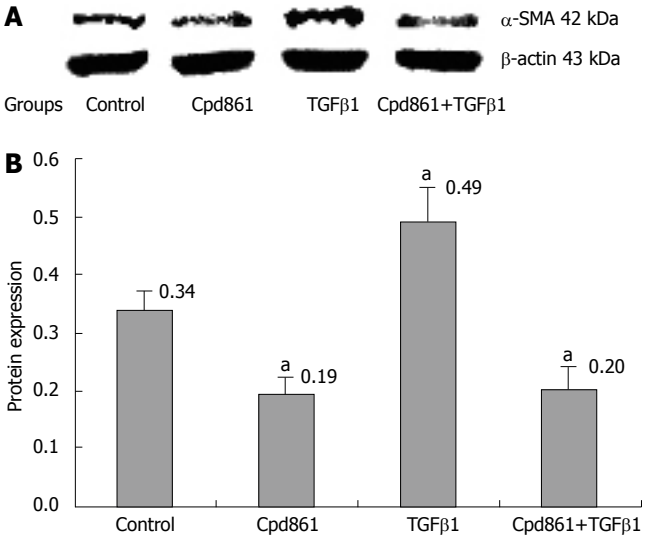

Figure 2 The level of α-SMA protein expression with different treatment after 24 h. Western blot was used as described in Materials and Methods. **A:** Representative Western blot results of α-SMA. The positions of protein size markers were given; **B:** Densitometry of Western blot analyzed by Gel-pro software. The levels of α-SMA were normalized to the level of β-actin protein. Six independent experiments were performed. $^aP < 0.05$ *vs* control LX-2 cell group. Error bars, SD.

Western blot analysis

Cells were lysed on ice by 80 μL lysis buffer for 30 min. The cell lysate was centrifuged at 10000 r/min for 10 min and the supernatant was collected for Western blot analysis. Protein concentration was measured using BCA Protein Assay kit (Pierce Company, Rockford, IL, USA) following the manufacturer's instructions. Protein samples (40 μg/lane) were subjected to 10% SDS-PAGE and then transferred onto a PVDF nitrocellulose membrane by electro-blotting. The membrane was incubated in 25 mL blocking buffer for 1 h at room temperature. After blocking, the membrane was incubated in 10 mL blocking buffer containing 1:1000 dilution of rabbit anti-phospho-Smad1 or anti-SMA at 4℃ overnight. After gentle washing with blocking buffer, the membrane was incubated in rabbit anti-β-actin polyclonal antibody (1:200 dilution). After vigorous washing, the membrane was incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody. The intensity of the bands was determined by scanning video densitometry. The levels of phospho-Smad1 and SMA were normalized to the level of β-actin protein. Six independent experiments were performed. The intensity of the bands was determined using Gel-Pro Analyzer Version 3.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data were expressed as mean ± SD and the statistical significance was assessed by one-way analysis of variance followed by Student-Newman-Keul tests. $P < 0.05$ was considered to be significant.

RESULTS

Effect of TGFβ1 and Cpd861 on α-SMA expression

Changes in gene or protein expression of α-SMA are often used to study the extent of HSC activation[16,17]. In this study, the effect of TGFβ1 and Cpd861 on the activation of the LX-2 cell line was investigated by examining changes in gene and protein expression of α-SMA.

Real-time PCR analysis showed that after exogenous TGFβ1 (5 ng/mL) stimulation for 24 h, α-SMA mRNA expression reached 2.65 fold ($P < 0.05$) compared with that in control LX-2 cells, whereas Cpd861 inhibited the expression to 0.38-fold (24 h, $P < 0.05$) (Figure 1A). α-SMA mRNA expression in Cpd861 and TGFβ1 co-treated cells was 1.73-fold compared to that in the controls.

Western blot analysis confirmed the trend of α-SMA gene expression changes (Figure 2A and B). In the TGFβ1 treatment group, α-SMA protein expression

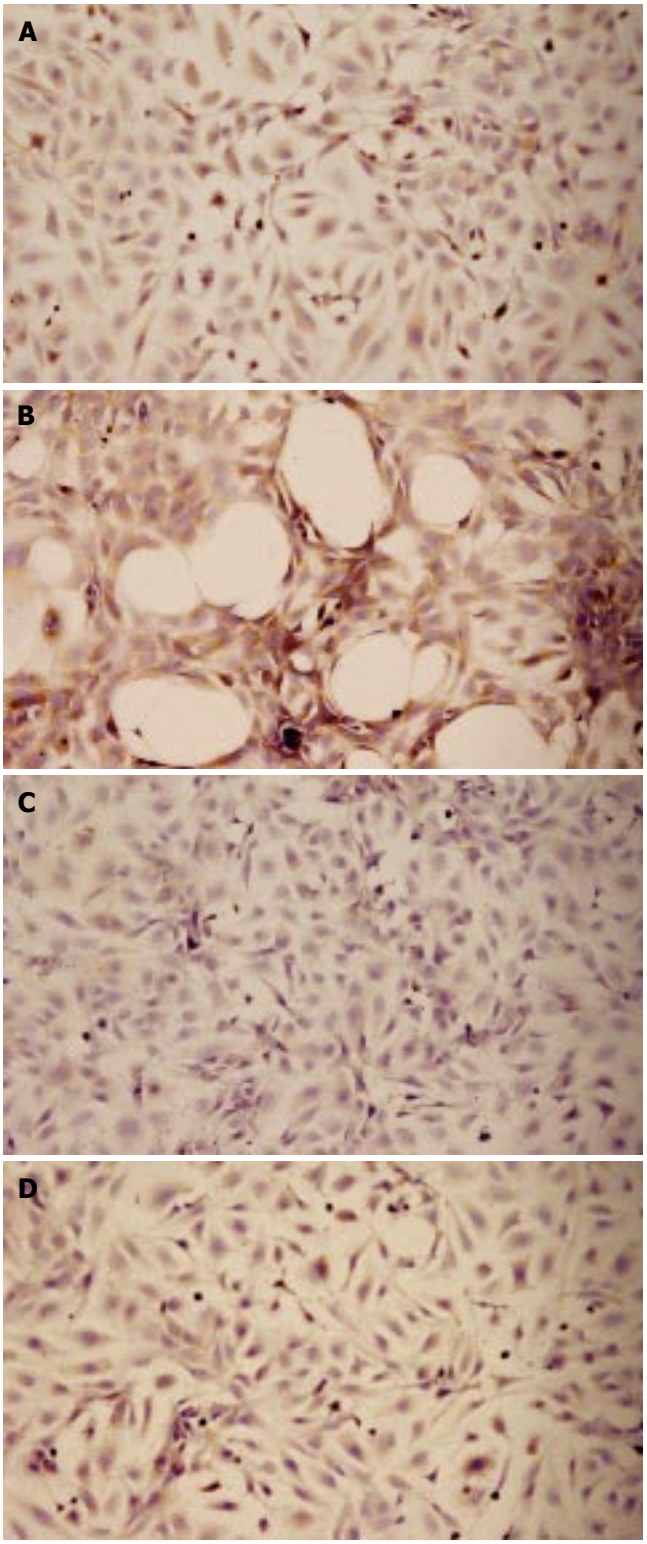

Figure 3 Expression of α-SMA in different treatment groups. Qualitative expression of α-SMA in control LX-2 cells (**A**), treated with TGFβ1 (**B**), Cpd861 (**C**) and Cpd861 together with TGFβ1 (**D**) for 24 h using immunohistochemical staining. α-SMA presented brown color in cytoplasm (× 200).

increased significantly compared with that in the controls (photodensity: 0.49 in TGFβ1 group and 0.34 in control respectively), whereas its expression was inhibited by Cpd861 (0.19 *vs* 0.49), even in the presence of TGFβ1 (0.20 *vs* 0.34) (Figure 2).

Immunocytochemical analysis showed that α-SMA protein was expressed in the cytoplasm, which was stained brown in low-power fields (Figure 3A). An interesting phenomenon was that after TGFβ1 treatment for 24 h, many various-sized, round spaces were present. Cells around the spaces were fusiform in shape, with dark brown cytoplasm. We presumed that it was a result of contraction of adjacent cells, which acquired the contractive property from excessive activation (Figure 3B). Cells treated with Cpd861, however, had a lighter brown cytoplasm (Figure 3C). In the Cpd861 and TGFβ1 co-treatment group, the cytoplasm presented lighter brown than in the TGFβ1-treated group and no spaces were seen (Figure 3D).

These results confirmed that gene and protein expression and function of α-SMA in LX-2 cells, markers of activation of LX-2 cells, were enhanced by TGFβ1 and inhibited by Cpd861. This is consisted with a previous study, which showed that Cpd861 can inhibit activation of LX-2 cells[13].

Effect of TGFβ1 and Cpd861 on TGFβ1/ALK1 signaling pathway in LX-2 cell line

TGFβ1/ALK1/Smad1 signaling has been found in HSCs[18,19]. In this pathway, ALK1 phosphorylated Smad1 leads to gene expression of *Id1*[20]. Idl proteins have a helix-loop-helix (HLH) domain but lack ability to bind DNA that acts as an important dominant negative antagonist of the bHLH family of transcription factors[21,22]. Recently, the *Id1* gene was identified as a novel target gene that promotes the expression and polymerization of α-SMA, therefore involving the transdifferentiation of HSCs.

Here, we examined the level of phosphorylated Smad1 (the marker of activation of the pathway) using Western blot analysis. The level of phosphorylated Smad1 increased after TGFβ1 treatment and was reduced by Cpd861 or TGFβ1 and Cpd861 co-treatment (photodensity was 0.66 in control, 0.45 in Cpd861-treated cells, 0.84 in TGFβ1-treated cells, and 0.55 in TGFβ1 and Cpd861 co-treated cells, Figure 4).

Stimulation of LX-2 cells with exogenous TGFβ1 strongly induced *Id1* gene expression by 2.5-fold, whereas Cpd861 reduced *Id1* expression by 0.53-fold ($P < 0.05$). The fold change of *Id1* expression in Cpd861 and TGFβ1 co-treatment was 0.79 compared with that in the controls, but it was significantly lower than that with TGFβ1 treatment alone (Figure 1B).

The *ALK1* gene was constitutively expressed in LX-2 cells and TGFβ1 did not further increase its expression, but Cpd861 reduced it significantly (Figure 2C). The change in gene expression of the receptor might be an important inhibitory mechanism of Cpd861 in the pathway.

DISCUSSION

Our data indicated that the TGFβ1/ALK1/Smad1 pathway could be activated by TGFβ1 and abrogated by Cpd861 in LX-2 cells. The TGFβ1/ALK1/Smad1 pathway is one of the TGFβ superfamily signaling branches[23]. Several studies have indicated that activation of the

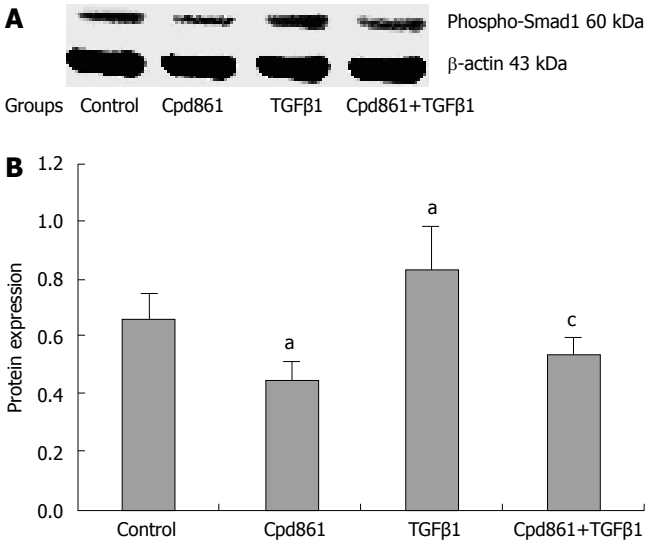

Figure 4 The level of Phosphorylated Smad1 with different treatment after 24 h. Western blot was used as described. **A:** Representative Western blot results of Phosphorylated Smad1. The positions of protein size markers were given; **B:** Densitometry of Western-blot analyzed by Gel-pro software. The levels of Phospho-Smad1 were normalized to the level of β-actin protein. Six independent experiments were performed. $^{a}P < 0.05$ *vs* untreated LX-2 cell group, $^{c}P < 0.05$ *vs* TGFβ1 treated group. Error bars, SD.

pathway might play an important role in development of organ fibrosis[24-27]. Recent research has demonstrated that the existence of ALK1 and the TGFβ1/ALK1/Smad1 pathway is critical during the transdifferentiation process of primary rat HSCs to myofibroblast (MFB)[20]. The present study demonstrated that activation of the TGFβ1/ALK1/Smad1 pathway may be enhanced by TGFβ1 in LX-2 cells. It was shown that Cpd861 inhibited activation of the TGFβ1/ALK1/Smad1 pathway, and this might have been mediated by reducing *ALK1* expression. However, effects of Cpd861 on ALK1 protein expression and on the affinity of TGFβ1 for ALK1 need further investigation.

Cpd861 has been demonstrated to be effective for treatment of patients with hepatic fibrosis[28]. Previous studies have shown that Cpd861 has multiple anti-fibrotic mechanisms, such as stimulating apoptosis of activated HSCs[29], down-regulating tissue inhibitor of metalloproteinase 1 gene expression[30], reducing expression of collagen and fibrosis-related cytokines. This study presented another possible anti-fibrotic mechanism of Cpd861, and also showed that the TGFβ1/ALK1/Smad1 pathway may represent a potential target for antifibrotic therapy. However, which components in the compound play the inhibitory roles needs to be further investigated.

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COMMENTS

Background

Activation of hepatic stellate cells (HSCs) triggers fibrogenesis and TGFβ1/ALK1/Smad1 signaling has been shown to be involved in the transdifferentiation of HSCs. Herbal compound 861 (Cpd861) can inhibit activation of HSCs to exert its anti-fibrotic effect, but the mechanism is not very clear.

Research frontiers

The effect of TGFβ1/ALK1/Smad1 signaling on the activation of HSCs has recently been reported. This study chose the pathway as a means to establishing the molecular mechanism of Cpd861 in the deactivation of LX-2 cells.

Innovations and breakthroughs

This is believed to be the first attempt to describe the molecular mechanism of the traditional herbal medicine Cpd861, which has been demonstrated to have anti-fibrotic effects and to reverse liver disease caused by HBV infection. This is believed to be the first time that TGFβ1/ALK1/Smad1 has been selected as a pathway to study the effects of deactivation of LX-2 HSCs.

Applications

This study presents another possible anti-fibrotic mechanism of Cpd861 and shows that the TGFβ1/ALK1/Smad1 pathway may represent a potential target for the anti-fibrotic therapy.

Terminology

ALK1 is activin receptor-like kinase 1, a type I receptor, which is typically activated by the bone morphogenetic proteins, and then phosphorylates Smad1, Smad5 and Smad8 for signal transduction. Id1 is inhibitor of differentiation 1, which has an helix-loop-helix (HLH) domain but lacks a DNA-binding domain. It was first shown to act as a dominant negative antagonist of the basic HLH family of transcription factors, which positively regulate differentiation in many cell lineages. It is an important part of signaling pathways involved in development, cell cycle and tumorigenesis.

Peer review

This study demonstrates that Cpd861 can down-regulate the TGFβ1/ALK1 pathway, which results in the deactivation of HSCs. The study presents novel findings on the anti-fibrotic mechanism of Cpd861 and its possible application in anti-fibrotic therapy.

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