

Effect of salvianolic acid B on Smad3 expression in hepatic stellate cells

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BACKGROUND: Salvianolic acid B (SA-B), one of water soluble compounds derived from *Radix salviae miltiorrhizae*, had good action against liver fibrosis of patients with chronic hepatitis. Hepatic stellate cells (HSCs) is the cellular resource for liver fibrogenesis, while transforming growth factor- β 1 (TGF- β 1) is most potent fibrogenic factor. In this study we investigated the mechanism of SA-B action against liver fibrosis relating to the interference with TGF- β 1 signaling at HSC.

METHODS: Hepatic stellate cells (HSCs) were isolated, cultured, and incubated with SA-B. The TGF- β 1 content in the supernatant of subcultured HSCs was assayed with ELISA. Type I collagen and Smad3 protein in TGF- β 1-stimulated primarily cultured HSCs for 4 days were detected by Western blot.

RESULTS: TGF- β 1 secreted in activated HSCs was more than in primary HSCs, and SA-B significantly decreased TGF- β 1 secretion in activated HSCs. TGF- β 1 increased the expression of type I collagen and Smad3 protein in d4 primary HSCs, while SA-B inhibited their expression.

CONCLUSIONS: SA-B inhibits TGF- β 1 secretion in activated HSCs and counteracts the expression of TGF- β 1 stimulated type I collagen and Smad3. These actions are associated with the effect of SA-B on liver fibrosis.

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KEY WORDS: salvianolic acid B; liver fibrosis; transforming growth factor- β ; Smads

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Introduction

Hepatic fibrosis is a reversible wounding-healing response that occurs in almost patients with chronic liver injury, which ultimately leads to cirrhosis.^[1,2] Although the causes of liver fibrosis are multiple and include congenital, metabolic, inflammatory and toxic factors, the pathological features and underlying mechanisms are similar. Liver fibrosis is characterized by the increased expression and over deposition of extracellular matrix (ECM) in the liver,^[3-5] hepatic stellate cells (HSCs) are crucial producer of ECM in the liver, and transforming growth factor- β 1 (TGF- β 1) is one of the most potent fibrogenic cytokines. TGF- β 1, oxidative stress and other factors activate HSCs.^[6-9] Once HSC is activated, it transdifferentiates into myofibroblast like cells, increases cell proliferation and ECM production, and down-regulates matrix metalloproteinases (MMP) activity through improving the expression of tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitor-1 (PAI-1),^[10-13] by which ECM production surpasses the degradation and results in fibrosis. TGF- β stimulates HSC activation by both autocrine and paracrine pathways,^[14] and it is known that this function is exerted through Smads molecules signal transductions.^[15] Inhibition of TGF- β function and HSC activation has been regarded as an important target for the development of antifibrotic strategies.^[16-19]

Salvianolic acid B (SA-B), one of water soluble ingredients from *Radix salviae miltiorrhizae*. In our previously clinical trial, SA-B was found to have a good action against liver fibrosis.^[20] Therefore, in this study, we investigated the mechanism of SA-B antifibrotic action relating to TGF- β 1 secretion in activated HSCs and TGF- β 1 signaling in primary HSCs.

Methods

Animals

Male Wistar rats, SPF grade, weighing 550 g, were purchased from the Animal Center of Shanghai University of Traditional Chinese Medicine, and used for isolation of HSCs.

Reagents

Pronase E, collagenase IV, DNase, Nycodenz and type I collagen were purchased from Sigma Co., USA, Medium 199 (M199) from Gibco Co., USA, TGF- β 1 kit with anti-mouse TGF- β 1 from R & D Co., USA, Tween-20 from Bio-Rad Co., USA, and Rabbit anti-mouse Smad3 from ZYMED Laboratoris Inc., USA. Rabbit antibody against mouse type I collagen was purchased from CalBiochem Co., USA.

Drugs

SA-B was obtained from the Shanghai Medical Research Institute, Chinese Academy of Science, Shanghai, China. The purity of SA-B was 80%, molecular weight 718, and molecular formula $C_{36}H_{30}O_{16}$. SA-B was diluted to 10 μ mol/L with M199 containing 0.5% NBS. TGF- β 1 was purchased from R & D Co., USA, diluted to 100 pmol/L with M 199 containing 0.5% NBS.

HSCs isolation and culture

Primary rat HSCs were isolated through in situ perfusion of the liver with pronase E and collagenase, and gradient centrifugation with Nycodenz, as described previously.^[21]

ELISA assay for TGF- β 1 secretion in HSCs

After 4-day culture, primary HSCs were divided into 2 groups: SA-B and control. Primary HSCs were washed twice with serum free-M199, and then incubated in M199/0.5% NBS or 10 μ mol/L of SA-B for 24 hours. The supernatant was collected, and the TGF- β 1 amount was assayed according to the kit instruction.

Western blot

After culture for 4 days, primary HSCs were divided into 3 groups: normal, control and SA-B treated. They were incubated in 0.5% NBS/M199, 100 pmol TGF- β 1, or 100 pmol TGF- β 1/SA-B respectively for 24 hours. Fractions of cytoplasmic and nuclear proteins were collected by low-salt buffer or high-salt buffer respectively. Cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with antibody.

Results

Freshly isolated HSCs were round, and rich in retinol, with a viability of 95% and a purity of 90%. HSCs demonstrated an oval shape 24 hours later, and enlarged as the time prolonged. On day 7 the cells had few retinol and turned to be of myofibroblast-like phenotype. On day 4 they were of intermediate phenotype.

Effect of SA-B on TGF- β 1 secretion of HSCs

In primary HSCs, the content of TGF- β 1 in the supernatant was 44.7 ± 16.7 pg/ml, and after SA-B treatment it was 36.2 ± 10.1 pg/ml ($t=1.23$, $P > 0.05$). In the activated HSCs subcultured, the secretion of TGF- β 1 was increased more significantly in the supernatant than in primary HSCs, but decreased significantly after incubation with SA-B (Table 1).

Effect of TGF- β 1 on type I collagen expression and SA-B intervention

Type I collagen expression level was increased markedly after TGF- β 1 stimulation, but decreased significantly after SA-B treatment. The results indicated that TGF- β 1 stimulated the expression of type I colla-

Table 1. Effect of SA-B on TGF- β 1 secretion in activated HSCs (mean \pm SD)

Group	n	TGF- β 1 (pg/ml)
Primary HSC	6	39.5 \pm 16.0
Subcultured HSC	6	84.5 \pm 30.9 ^a
SA-B treated	6	47.9 \pm 24.1 ^b

a: Vs primary HSCs, $P < 0.05$; b: vs subcultured HSCs, $P < 0.05$.

Table 2. Effect of SA-B on expression of type I collagen and Smad3 in HSCs (OD, $n=3$, mean \pm SD)

Group	Type I collagen	Smad3	
		Cytoplasm	Nucleus
Normal	39.1 \pm 4.4	17.9 \pm 1.8	8.3 \pm 0.8
Control	52.6 \pm 4.1 ^a	24.3 \pm 1.9	20.2 \pm 4.2 ^a
SA-B treated	38.3 \pm 3.5 ^b	19.3 \pm 2.8	12.9 \pm 1.1 ^b

a: vs normal, $P < 0.05$; b: vs control, $P < 0.05$.

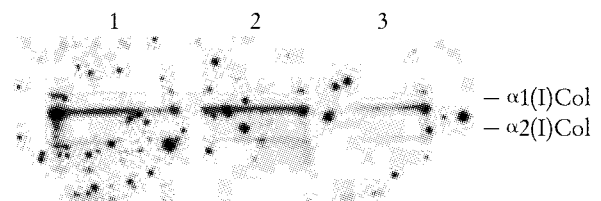


Fig. 1. Effect of TGF- β 1 on type I collagen expression and SA-B intervention. Lane 1: normal; Lane 2: control (100 pmol/L TGF- β 1 stimulated); Lane 3: SA-B treated.

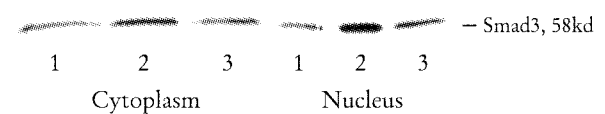


Fig. 2. Effect of SA-B on expression of TGF- β 1 stimulated cytoplasm and nucleus Smad3 in HSCs. Lane 1: normal; Lane 2: control (100 pmol/L TGF- β 1 stimulated); Lane 3: SA-B treated.

gen, while SA-B antagonized this effect (Fig. 1 and Table 2).

Effect of TGF- β 1 on Smad3 protein expression and SA-B intervention

The expression level of Smad3 in cytoplasm and nucleus increased evidently after TGF- β 1 stimulation, but reduced obviously after SA-B addition. This indicated that SA-B inhibited TGF- β 1 stimulated Smad3 protein expression and its nuclear translocation, and intervened with TGF- β 1 signaling in HSCs (Fig. 2 and Table 2.)

Discussion

After liver injuries, HSCs activate and transform into myofibroblasts phenotypically, functionally with extracellular matrix overproduction and deposition in the liver, resulting in liver fibrosis. TGF- β 1 is one of the most important cytokines that activate HSCs through paracrine and autocrine pathways,^[14] and the inhibition of TGF- β 1 function in HSCs has been regarded as a primary target for the development of antifibrotic strategies.^[19,22]

It has been proved that HSCs could activate spontaneously in vitro and at different stages respond to TGF- β 1 differently, whereas primary HSCs of intermediate phenotype are most sensitive to TGF- β 1.^[15,23] Therefore, HSCs cultured for 4 days or at their intermediate activation stage are used as a cell model.^[15] In ECM components in which activation is induced by HSCs, type I collagen is predominant. In this study, the content of TGF- β 1 in the activated HSCs supernatant was increased significantly than in primary HSCs, indicating that activated HSCs could autocrine TGF- β 1. Moreover, SA-B suppressed TGF- β 1 secretion in activated HSCs, and also inhibited the expression of TGF- β 1 stimulated type I collagen. These results suggest that SA-B inhibits HSC-activated functions.

It was well understood that TGF- β signaling exists outside and inside cell,^[24,25] and its signaling features in HSCs have also been elucidated.^[15,23,26] Smads as the special molecules for TGF- β intracellular signaling could move into the nucleus and regulate responsive gene expression when TGF- β receptor binds with ligands and phosphorylation. The family of Smads includes 3 distinct types: receptor-activated Smads (Smad2, 3), common-partner Smads (Smad4), and inhibitory Smads (Smad7). Smad3 is considered important for mediating TGF- β 1 signaling effect of collagen production in HSCs.^[15,27-29] We have found that Smad3 phosphorylation levels are parallel to the TGF- β 1 stimulated HSCs activation and α 1(I) collagen mRNA expression.^[15,30] Others proved that the maximal expression of collagen type I in activated HSCs requires Smad3 in vivo and in culture with Smad3 knockout mice.^[27]

We found that TGF- β 1 slightly increased the expression of cytoplasmic Smad3, but markedly increased the nuclear Smad3 levels in HSCs. This indicates that TGF- β 1 stimulates Smad3 phosphorylation and nuclear translocation, causing HSCs responses like increase of collagen production. SA-B suppresses Smad3 expression, while lowering its nuclear levels in particular. SA-B could inhibit TGF- β 1 intracellular signaling in HSCs. The inhibition of TGF- β 1 secretion and its intercellular signaling through down-regulation of Smad3 nuclear translocation at HSCs are the main mechanisms of SA-B actions against liver fibrosis.

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Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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