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Effect of salvianolic acid B on collagen production and mitogen-activated protein kinase activity in rat hepatic stellate cells[1]

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KEY WORDS *Salviae miltiorrhizae*; salvianolic acid B; liver cirrhosis; cell division; collagen; transforming growth factor beta; mitogen-activated protein kinases; signal transduction

ABSTRACT

AIM: To investigate the mechanism of salvianolic acid -B (SA-B) action against liver fibrosis relating to mediating hepatic stellate cell (HSC) activation and transforming growth factor-β1 (TGF-β1) intracellular signal transduction. **METHODS:** HSC was isolated from normal rat through *in situ* perfusion of liver with pronase E and density-gradient centrifugation with 11 % nycondenz, then cells were subcultured. Cell proliferation was observed by [^{3}H]TdR uptake. Cellular collagen deposition was measured with Ponceau S stain and semi-quantified with image analytic system. Type I collagen secretion in the supernatant was detected with ELISA. The gene expression of type I pro-collagen was analyzed by RT-PCR. The supernatant was acidified and active TGF-β1 contents were assayed with ELISA. Mitogen-activated protein kinase (MAPK) activity was analyzed with immunoprecipitation and Western blot. **RESULTS:** SA-B 0.1, 1, 10, and 100 μmol/L suppressed HSC proliferation concentration-dependently as determined by [^{3}H]TdR uptake by 94.1 %, 82.4 %, 62.7 %, and 4 % of the control respectively (P<0.05 or P<0.01). SA-B 1, 10, and 100 μmol/L inhibited soluble type I collagen secretion by 75.3 %, 69.8 %, and 63.5 % of the control and decreased the matrix collagen deposition to 86.2 %, 75.4 %, and 73.4 % (P<0.05 or P< 0.01). SA-B 1 and 10 μmol/L decreased the cell active TGF-β1 secretion by 63.3 % and 15.6 % of the control, down-regulated pro-collgen α_1(I) mRNA expression to 77.0 % and 51.8 % respectively (P<0.05). SA-B 1 and 10 μmol/L also inhibited MAPK activity by 1 to 2 fold respectively. **CONCLUSION:** SA-B inhibited HSC proliferation and collagen production as well as decreased the cells' TGF-β1 autocrine and MAPK activity, which might contribute to the mechanism of SA-B action against hepatic fibrosis.

INTRODUCTION

Liver fibrosis, characterized by the overproduction and accumulation of hepatic extracellular matrix (ECM), was the common and important pathological changes in the chronic liver diseases, through which

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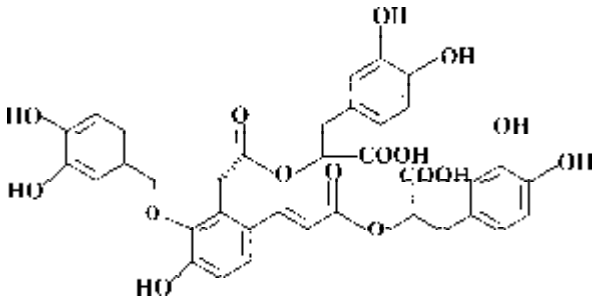
the chronic hepatitis developed into cirrhosis. Hepatic stellate cells (HSC), located at Disse's space in liver, was widely recognized as the main resource of hepatic ECM and the cellular basis for liver fibrosis. Transforming growth factor-β1 (TGF-β1) was one of the most potent pro-fibrogenetic cytokines and can stimulate HSC activation through paracrine or/and autocrine by mediating Ras/mitogen-activated protein kinase (MAPK) and other signal transduction pathways[1].

Radix Salviae Miltiorrhizae, one of frequently used Chinese herbs, had good action against liver fibrosis[2]. Salvianolic acid B (SA-B), one of water soluble component from Radix Salviae Miltiorrhizae, has molecular formula $C_{36}H_{30}O_{16}$ with M_r 718. In our previous study[3,4], it is found that SA-B can effectively reverse liver fibrosis induced by tetrachloride carbon (CCl_4) or di-methylnitrosamine (DMN). But the mechanism of SA-B action against liver fibrosis was still unclear. In the study, we investigated SA-B effects on HSC collagen production, TGF-β1 expression, and TGF-β1-mediated MAPK activity, to elucidate the mechanism of SA-B effect on liver fibrosis.



Chemical structure of SA-B. $C_{36}H_{30}O_{16}$, M_r 718.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing (360±20) g (SPF, Certificate № 980004), were obtained from the Animal Center of Shanghai University of Traditional Chinese Medicine, and were fed with food and water *ad libitum*.

Drugs and reagents SA-B was extracted and identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and diluted into the concentrations of 0.1–100 μmol/L with Medium 199 (M199). Pronase E, type IV collagenase, DNase I, Modified Eagle's Medium (MEM), and nycodenz were purchased from Sigma Co. M199 and Dulbecco's Modified Eagle's Medium (DMEM) were from Gibco/BRL. New bovine serum (NBS) was from Hyclone Co. [^{3}H]TdR was from Shanghai Atom Co. TGF-β1 immunoassay system kit, PCR marker, and Access RT-PCR Kit were from Promega Co. Rabbit-anti mouse type I collagen serum (1:500) and human recombinant TGF-β1 were from Calbiochem Co. Trizol reagent was from Shanghai Sangon Co.

HSC isolation and culture HSC was isolated as described previously[5]. Briefly, the liver was perfused *in situ* with a Ca^{2+}/Mg^{2+}-free MEM followed with pronase E and collagenase. The digested liver suspension was filtered through double layer gauze and was gradiently centrifuged (1450×*g*, 4 ℃, 20 min) with 11 % (w/v) nycodenz. HSC was obtained from the interface of gradient solution and seeded onto 100-mm uncoated plastic culture plates at a density of 1×10^9/L and incubated at 37 ℃ in a 5 % CO_2-95 % air humidified atmosphere. The medium was changed with M199 containing 10 % NBS after 48 h and every other day thereafter. The cells were trypsinized and passaged after they grew confluent and applied for the following experiments as the subcultured HSC grew confluent.

Cell proliferation assay HSC proliferation was measured by [^{3}H]TdR uptake[6]. Confluent HSC in 24-well plates was incubated with SA-B for 48 h (the following as the same), and impulsed with 74 kBq/well [^{3}H]TdR among the last 24 h. Then the cells were harvested by trypsinization and collected onto 0.45 mm membrane. The radioactivity of intracellular [^{3}H]TdR incorporation was counted by Beckman Wallac 1410 liquid-scintillator.

Collagen deposition measurement After treated with SA-B, cells on the 24 well-plate were washed with phosphate buffer saline (PBS), fixed with 10 % formalin, dehydrated with 70 % ethanol, and stained with Victorial blue for 15 min. After washing with distilled water twice, cells were stained with Ponceau S for 2 min and dehydrated with ethanol twice. The collagen deposition was analyzed according to its optical density with

computer MPIAS-500 image analytic system.

Type I collagen and active TGF-b1 content assay After drug treatment, cells were incubated with serum free M199 for 24 h and the supernatants were collected. Type I collagen contents were assayed with ELISA according to Rennard's method[7]. For active TGF-β1 content assay, the supernatants were acidified with HCl 1 mol/L and then measured by ELISA with the TGF-β1 immunoassay system kit according to the manufacturer's instructions.

Pro-collagen type I mRNA level analysis Total RNA was extracted from the cells by the acid-guandium -phenol-chloroform method as described[8]. mRNA levels of TGF-β1 and pro-collagen type I were analyzed by reverse-transcription polymerase chain reaction (RT-PCR), while house keeping gene-"β-actin" was used as the internal standard. The left and right primers for β-actin were respectively as follows: 5' -ACA TCT GCT GGA AGG TGG AC-3' and 5' -GGT ACC ACC ATG TAC CCA GG-3' (the expected product size 163 bp)[9]. The left and right primers for type I pro-collagen α_1(I): 5' -CAC CCT CAA GAG CCT GAG TC-3' and 5' -GTT CGG GCT GAT GTA CCA GT-3' (the expected product size 253 bp)[10]. The RT-PCR reaction was carried on with Access RT-PCR kit. The products were fractionated in 2 % agarose electrophoresis containing ethidium bromide (EB) 0.5 mg/L and photographed under UV transilluminator. Their density was semi-quantitatively analyzed with MPIAS-500 image system.

MAPK activity assay HSC was treated with TGF-β1 5 μg/L for 1 h and then incubated with SA-B. The cytoplasmic proteins were lysed and immunoprecipitated with p44/p42 MAPK antibody. After reacting with EIK-1 and ATP substrate, the immunoprecipitated antigen was fractionated by SDS/polyacrylamide gel electrophoresis, and were transferred onto PVDF membranes. After blocking with 5 % skim milk, the blot was incubated with p44/p42 MAPK primary antibody (1:1000) overnight at 4 ℃. Then the blot was washed and incubated with HRP-coupled second antibody and signals were detected by ECL film.

Statistical analysis All data are expressed as mean±SD and were analyzed with *t* test.

RESULTS

Effect of SA-B on HSC proliferation SA-B 0.1–100 μmol/L inhibited the HSC intracellular uptake of [^{3}H]TdR in a concentration-dependent manner and the inhibitory rates were 94.1 %, 82.4 %, 62.7 %, and 4 % of the control, respectively (Tab 1).

Tab 1. Effect of SA-B on HSC intracellular [^{3}H]TdR uptake. *n*=4 samples. Mean±SD. ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* Control. The data is a representative of 2–3 experiments.

Group	[^{3}H]TdR (Bq/well)
Control	408±52
SA-B 0.1 μmol/L	384±48^b
1 μmol/L	336±24^b
10 μmol/L	256±28^c
100 μmol/L	16±4^c

Effect of SA-B on HSC collagen production Overproduction of extracellular matrix, particularly collagen, is an important feature of liver fibrosis. SA-B 1, 10, and 100 μmol/L suppressed soluble type I collagen to 75.3 %, 69.8 %, and 63.5 % of the control, and inhibited HSC total collagen deposition to 86.2 %, 75.4 %, and 73.4 % of the control, respectively (Tab 2). Also RT-PCR analysis showed that SA-B 1 and 10 μmol/L down-regulated the expression of type I pro-collagen mRNA to 77.0 % and 51.8 % of the control (Tab 3, Fig 1).

Tab 2. SA-B effect on HSC collagen secretion and deposition. *n*=4 samples. Mean±SD. ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* Control.

Group	Soluble type I collagen/mg·g^{-1} protein	Matrix collagen (*A*)
Control	475±74	812±196
SA-B 0.1 μmol/L	424±22	766±48
1 μmol/L	358±57^b	699±117^b
10 μmol/L	332±69^b	613±194^c
100 μmol/L	302±56^c	596±134^c

Tab 3. Effect of SA-B on type I procollagen mRNA and active TGF-b 1 level. *n*=4 samples. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* Control.

Group	Pro-collagen α_1(I)mRNA /% of β-actin	Active TGF-β1 /μg·L^{-1}
Control	54±8	3451±661
SA-B 1 μmol/L	42±6[b]	2185±734[b]
10 μmol/L	28.0±1.5[c]	539±282[c]

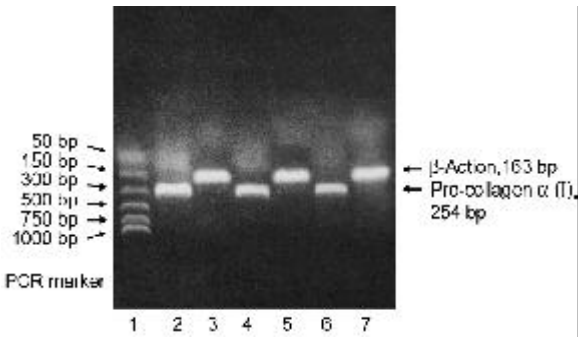


Fig 1. Effect of SA-B on the gene expression of type I pro-collagen in HSC. RT-PCR product 5 μL was run on 2 % agarose gel stained with EB. Lane 1: PCR markers; Lane 2 and 3: a $_1$(I) pro-collagen and b -actin in SA-B 1 μmol/L treated HSC; Lane 4 and 5: a $_1$ (I) pro-collagen and b -actin in SA-B 10 μmol/L treated HSC; Lane 6 and 7: a $_1$(I) pro-collagen and b -actin in the control respectively.

Effect of SA-B on HSC TGF-b 1 secretion TGF-β1 is one of the most potent cytokines for extracellular matrix synthesis and HSC is main effector and producer of TGF-β1 in the liver. On acidification, the active TGF-β1 was released from the complex of TGF-β1 and latency-associated peptide (LAP), while SA-B 1 and 10 μmol/L significantly decreased the active TGF-β1 content in the culture medium (Tab 3).

Effect of SA-B on HSC MAPK activity Ras/MAPK is an important pathway of TGF-β signaling in HSC. To elucidate the possible effects of SA-B on TGF-β1 signaling in HSC, we examined MAPK activity. As shown in Fig 2, the TGF-β1 5 μg/L increased MAPK phosphorylation level remarkably, while SA-B 1 μmol/L and 10 μmol/L reduced TGF-β1 stimulated-MAPK

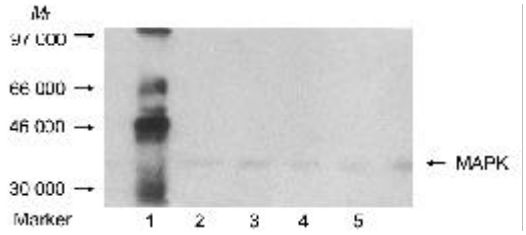


Fig 2. SA-B effect on phosphorylated MAPK level in HSC. Lane 1: protein markers; Lane 2: normal HSC; Lane 3: control HSC stimulated with TGF-b 1 5 μg/L; Lane 4: the HSC treated SA-B 1 μmol/L and TGF-b 1 5 μg/L; Lane 5: the HSC stimulated with SA-B 10 μmol/L and TGF-b 1 5 μg/L.

phosphorylation level by 1- and 2-fold respectively.

DISCUSSION

It is widely known that HSC is the key cellular basis for liver fibrosis. On liver injury, free radicals, cytokines, *etc*, would stimulate HSC activation. HSC cultured in uncoated plastic plate can spontaneously activate *in vitro*, which mimics the activation processes *in vivo*. As HSC activated, they transformed from the quiescent state full of cytoplasmic lipid droplets to the myofibroblast like cell phenotypically, produced a lot extracellular matrix in liver and led to liver fibrosis[11]. Activation of stellate cells were mediated by cytokines, which include platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), hepatic growth factor (HGF), and transforming growth factor-β1 (TGF-β1), *etc*. Theses cytokines promoted HSC proliferation and matrix production. Moreover, activated HSC could secrete TGF-β1 itself, which help to perpetuate HSC activation. Therefore, cultured HSC is a good and reliable cell model for hepatic fibrosis research and interference with HSC activation and cytokines effect on HSC was usually regarded as the main strategy for curing liver fibrosis.

SA-B is one of main phenol acid compounds from Radix Salviae Miltiorrhizae[12]. It had good effect against liver injury and fibrosis[2,3]. HSC activation had two obvious features, proliferation and ECM production. The deposition and accumulation of ECM, particularly

type I collagen, is the main feature of liver fibrosis. In this study, we found that SA-B inhibited the proliferation of HSC in a concentration-dependent manner, decreased excessive type I collagen secretion and ECM deposition, and down-regulated pro-collagen gene expression. It suggests that SA-B can inhibit or reverse the HSC activation and this effect is one of the important mechanisms of SA-B action against hepatic fibrosis.

TGF-β1 is known to be the most potent fibrogenic cytokine[13]. HSC was the main producer and effector of TGF-β1 during liver fibrogensis[14]. TGF-β1 was synthesized in HSC as precursor that includes LAP, proteolytic cleavage of the precursor occurs intracellularly, but active peptide (M_r 25 000) and noncovalently associated LAP are secreted as a complex that lacks biological activity. With plasmin or acid condition, *etc*, the active TGF-β1 is secreted[15]. The active TGF-β1 could bind its cell membrane receptors, then downstream the signal through the signal molecules such as MAPK from plasma to the nucleus, and regulate the target gene transcription, through combinatoril binding of transcription factors (such as AP1 and SP1) to the gene regulatory elements[16]. Pro-collagen gene is one of main target genes of TGF-β1. In this study, SA-B 1 and 10 μmol/L suppressed active TGF-β1 secretion and MAPK phosphorylation in HSC. It indicated that SA-B can inhibit TGF-β1/MAPK signal transduction, and that is important reason for SA-B down-regulation of collagen gene and protein expression.

In summary, SA-B could effectively inhibit HSC proliferation and collagen production and interfere with TGF-β1/MAPK signal transduction. These actions underlie the mechanism of SA-B effect against hepatic fibrosis.

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丹酚酸 B 对大鼠肝星状细胞胶原生成与丝裂原激活蛋白激酶活性的影响[1]

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关键词　丹参；丹酚酸 B；肝硬化；细胞分裂；胶原；转化生长因子 β；促细胞分裂剂活化的蛋白激酶；信号转导

目的：研究丹酚酸 B 影响肝星状细胞活化与转化生长因子β1胞内信号转导的抗肝纤维化作用机制．方法：正常大鼠肝脏链酶蛋白酶原位灌流消化与 11 % nycondenz 密度梯度离心分离肝星状细胞，传一代培养．[^{3}H]TdR 掺入法测定细胞增殖，丽春红染色、图像分析半定量细胞胶原沉积量，ELISA 法测定细胞培养上清 I 型胶原分泌量，培养上清酸化处理后，ELISA 法测定活性 TGF-β1 含量．RT-PCR 法分析细胞前胶原 α_1(I)基因的表达，免疫沉淀与蛋白印迹法分析丝裂原激活蛋白激酶(MAPK)活性．结果：丹酚酸 B 0.1-100 μmol/L 浓度依赖性抑制星状细胞增殖，丹酚酸 B 1-100 μmol/L 浓度依赖性抑制细胞的 I 型胶原分泌量与总胶原的沉积．丹酚酸 B 1-10 μmol/L 抑制 TGF-β1 自分泌量，下调 α_1(I)前胶原的基因表达．而且丹酚酸 B 1-10 μmol/L 明显抑制 TGF-β1 刺激的 MAPK 活性．结论：丹酚酸 B 可抑制肝星状细胞的增殖与胶原生成，抑制 TGF-β1 的自分泌与 MAKP 活性，这些作用是丹酚酸 B 抗肝纤维化的主要作用机制．

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