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Effects of salvianolic acid-A on rat hepatic stellate cell proliferation and collagen production in culture[1]

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KEY WORDS *Salviae miltiorrhizae*; salvianolic acid-A; liver cirrhosis; cell division; collagen; cultured cells; gene expression; rats; hepatic stellate cells

ABSTRACT

AIM: To investigate the effects of salvianolic acid-A (SA-A), one of main effective components of Salvia miltiorrhiza for its antifibrotic action, on the cell proliferation and collagen production in cultured hepatic stellate cells (HSC). **METHODS**: HSC were isolated through *in situ* perfusion of liver with pronase E and collagenase, and gradient centrifugation with Nycodenz. The cultured HSC were incubated with SA-A 0.1 - 100 μmol/L for 24 h. MTT spectrometric assay and intercellular incorporation of methyl-[^{3}H]thymidine ([^{3}H]TdR) was used to assess the cell proliferation. The amount of collagen was semi-quantified by ponceau staining and image analysis, the amount of type I collagen secretion was measured with ELISA and normalized by the total protein of cell layer. The total RNA was prepared from the control cells and the drug treated cells respectively, and the expression of pro-collagen α_2(I) mRNA was semi-quantitatively analyzed with RT-PCR. **RESULTS**: SA-A 100 μmol/L showed a little cytotoxity, SA-A 0.1 - 10 μmol/L did not influence cell morphology, and SA-A 1 - 100 μmol/L decreased the cell proliferation significantly in a concentration-dependent manner ($P<0.05$). SA-A 1, 10, 100 μmol/L decreased the cell collagen deposition by 78.6 %, 71.8 %, and 61.3 % of the control respectively ($P<0.05$), and decreased type I collagen secretion to 53.1 %, 52.6 %, and 49.5 % ($P<0.01$ or $P<0.05$). Both SA-A 1 and 10 μmol/L downregulated procollagen α_2(I) mRNA expression remarkably ($P<0.05$). **CONCLUSION**: SA-A inhibited HSC proliferation and collagen expression. The inhibitory effect on HSC activation is the main mechanism of SA-A action against liver fibrosis.

INTRODUCTION

Salviae miltiorrhizae, one of the commonly used Chinese herbs, promotes both blood production and circulation according to the theory of traditional Chinese medicine, and is widely applied in clinical therapy for the liver diseases, such as chronic hepatitis and hepatic cirrhosis. Salvianolic acid-A (SA-A) one of water-soluble components from Salvia miltiorrhizae, has good antioxidant actions[1]. It is widely recognized that hepatic stellate cell (HSC) plays a central role in the pathogenesis of hepatic fibrosis[2]. Following liver injury of any etiology, such as cytokines, free radical species, lipid peroxidative production etc, HSC undergoes a process of activation, transmitting into a myofibroblast-like phenotype associated with increased proliferation and extracellular matrix (ECM) production, especially type I collagen synthesis. The overproduced ECM is deposited in liver, and leads to fibrosis. In our previous work[3], it was found that SA-A could protect hepatic lipid peroxidation, and had marked effects against liver injury and fibrosis in carbon tetrachloride induced fibrosis in rats. In order to investigate the mechanism of SA-A actions against liver fibrosis, we observed effects of SA-A on HSC proliferation, collagen production, and procollagen gene expression.

MATERIALS AND METHODS

Drug SA-A, $C_{26}H_{22}O_{10}$ and M_r 494, was extracted and identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. SA-A was freshly diluted with Medium 199 (M199) containing 10 % newborn bovine serum (NBS) before use.

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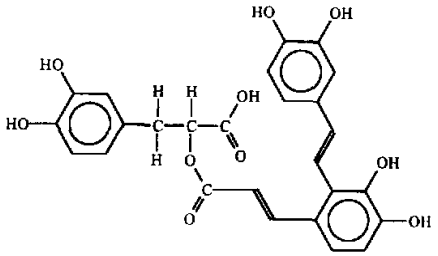

Salvianolic acid A
$C_{26}H_{22}O_{10}$
M_r 494

Animals Wistar rats, grade Ⅱ, male, 400 – 500 g, were purchased from the Shanghai Experimental Animal Center, and maintained with food and water *ad lib*.

Main reagents M199 and minimum essential medium Eagle (MEM) were purchased from Gibco BRL Co. Nycodenz, collagenase (type Ⅳ), DNase (type Ⅰ), type Ⅰ collagen standard from mouse tail and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from Sigma Co. Pronase E was from Merck Co, Germany. DC protein kit was from Bio-Rad Co. Rabbit antibody to mouse type Ⅰ collagen was from Calbiochem Co. Access RT-PCR kit and PCR marker was from Promega Co. Diethylpyrocarbonate, guanidium thiocyanate, saturated phenol/chloroform mix, and agarose were from Shanghai Sangon Biotech Co. NBS was from Shanghai Sino-American Co. Methyl-[^{3}H] thymidine (TdR) was from Shanghai Institute of Atomic Energy. All other reagents were of analytical grade.

PCR primers The PCR primers for procollagen α_2(Ⅰ) and β-actin were adopted or designed according to the published sequences and references as in Tab 1[4], and were synthesized by Gibco BRL Co.

Tab 1. PCR primer sequences and expected size of amplified products.

Primers	Sequence	Size
α_2(Ⅰ) Procollagen upstream	5′-*GTT CGT GGT TCT CAG GGT* AG-3′	
α_2(Ⅰ) Procollagen downstream	5′-TTG TCG TAG CAG GGT TCT TT-3′	254 bp
β-Actin upstream	5′-ACA TCT GCT GGA AGG TGG AC-3′	
β-Actin downstream	5′-GGT ACC ACC ATG TAC CCA GG-3′	163 bp

HSC isolation and cultivation HSC were isolated from the rat liver as described previously[5]. Briefly, the liver was perfused *in situ* through the portal vein with Ca^{2+} free D-Hanks' solution at 37 ℃ at a flow rate of 9 mL/min for 10 min, then with 100 mL of 0.1 % pronase E, finally with 225 mL of 0.015 % collagenase. The digested liver was excised, minced, and agitated in 100 mL solution of 0.015 % pronase E, 0.015 % collagenase, and 0.002 % DNase in 37 ℃ water bath at 40 × *g* for 30 min. The resulting suspension was filtered through double layers of gauze, resuspended with 12 % Nycodenz and centrifuged at 1450 × *g* at 4 ℃ for 20 min. The cells in the interface were collected and suspended in M199 supplemented with 10 % NBS, penicillin 10^5 U/L, streptomycin 0.1 g/L, and 1 % glutamine.

The cell purity was more than 90 %, which was identified by positive immunocytochemical staining for desmin, typical appearance with the cytoplasm full of lipid drop via phase-contrast microscope and intrinsic vitamin A autofluorescence. The cell recovery was $2 \times 10^7 - 5 \times 10^7$ cells per liver, and the viability was more than 98 % as examined by trypan blue exclusion. The cells were cultured in humidified atmosphere containing 5 % CO_2, and passaged with 0.25 % trypsin-0.02 % edetic acid after growing confluent, at this time the cell purity increased to 98 %. The subcultured HSC were used in all the tests of the study and observed for the morphology with phase-contrast microscope.

Cell proliferation assay 1) MTT spectrometric assay[6]: HSC were planted on 96-well plate at a density of 1×10^5 cells/L. When the cells were confluent, the culture medium was replaced with the drug for a 24-h incubation (the following drug incubation as the same). Then 10 μL of MTT (5 g/L in PBS) was added per 100 μL of medium, and the plates were incubated at 37 ℃ for 4 h. After the supernatant was aspirated, 100 μL of HCl 0.01 mol/L -isopropanol was added to dissolve the blue crystals, and the values of absorbence A_{570} were read on the microplate reader (Labsystem, Finland) while A_{690} was kept as a background. Each sample had triplicates or four replicates, all tests were repeated 2 – 3 times. 2) [^{3}H] TdR incorporation: confluent HSC in 24 well plates were incubated with SA-A 0.1 – 100 μmol/L diluted in M199 containing 10 % NBS for 24 h, and [^{3}H] TdR (55.5 kBq/well) was added in the last 16 h. Then cells were harvested with trypsin digestion and collected on the filter membrane, and sample radioactivity was measured by Backman Wallac 1410 Scintillator.

Semi-quantification of cell collagen deposition The media of cells in 24 well plates were aspirated and cell layers were rinsed twice with PBS. After fixing with 10 % formaldehyde (containing NaH_2PO_4 0.033 mol/L and Na_2HPO_4 0.092 mol/L, pH 7.0) for 10 min, the cell layer was stained with 0.5 % Victorial blue for 15 min and 0.05 % ponceau for 2 min, and washed with alcohol. The collagens was stained with ponceau and appeared to be red in color, and the images of ponceau-stained cells under microscope were transformed to the computer and semi-quantitatively measured with MPIAS 500 image system for the collagen deposition by the optical density values.

Assay of type I collagen secretion The media and cell layers were collected. The media were assayed for the amounts of type I collagen according to modified *Rennard SI method*[7]. *Briefly*, 100 μL *of medium* sample was coated in 96-well plates in carbonate-bicarbonate buffer at 4 ℃ overnight. After aspirating, each well was incubated with 100 μL of 1∶500 dilution of rabbit polyantibody to mouse type I collagen in PBS at 37 ℃ for 2 h. After washing 3 times with PBS containing 0.05 % Tween 20, each well was incubated with 100 μL of 1∶1000 dilution of goat anti-rabbit IgG-HRP at 37 ℃ for 2 h. Then 100 μL of 0.02 % *o*-phenylene-diamine and 0.01 % H_2O_2 in citrate-phosphate 0.05 mol/L (pH 5.0) was added for 30 min at room temperature. The reaction was quenched by adding sulfuric acid 1 mol/L, and the plate was read in a microplate reader at 492 nm, the sample concentration was determined by a plot of optical density versus concentration (0 - 1000 mg/L) of type I collagen standards. While the cell layer was measured for the total protein with Bio-Rad DC protein kit, the medium amounts of type I collagen was normalized with the cell layer protein and expressed as "mg/g protein".

RNA extraction and reverse transcription and polymerase chain reaction (RT-PCR) The total RNA was extracted from the control cells and SA-A incubated cells by the acid guanidium thiocyanate-phenol-chloroform method[8]. The RNA amount was determined by A_{260}, its purity was confirmed with A_{260}/A_{280} read by spectrophotometer that ranged from 1.6 to 1.9. Its integrity was checked by 1 % agarose gel electrophoresis with ethidium bromide (EB) staining of 18 S and 28 S ribosomal RNA. With RT-PCR kit, the cDNA synthesis and amplification were made in one step following the manufacturer's instructions. Briefly, 1 μg RNA, primers 50 pmol/L for procollagen α_2(I) or β-actin were added to each reaction mix respectively, which included dNTPs 10 mmol/L 1 μL, $MgSO_4$ 25 mmol/L 2 μL, AMV reverse transcriptase 5U, Tfl DNA polymerase 5U, AMV/Tfl 5 × buffer 10 μL. The final volume was 50 μL and was covered with 20 μL mineral oil. Then with PCR Touchdown thermal cycler (Hybaid, England), RT-PCR reaction was run as follows: (1) 48 ℃ for 45 min, 1 cycle. (2) 94 ℃ for 2 min, 1 cycle. (3) 94 ℃ for 30 s, 60 ℃ for 1 min, 38 ℃ for 2 min, 30 cycles. (4) 68 ℃ for 7 min, 1 cycle. Five μL PCR product was run on 1.5 % agarose gel and visualized by EB staining under UV light, the electrophoresis photo was transmitted to computer, and pro-collagen α_2(I) intensity was analyzed with image system (MPIAS-500), and normalized by β-actin band intensity taken as an internal standard.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Effects on cell morphology and proliferation In the group incubated with SA-A 100 μmol/L, parts of cells shrunk and died, and the amount of intercellular [^{3}H]TdR *incorporation was very low, just* 2.58 % *of the* control. The cell morphology in other groups had no marked changes compared with the control. SA-A 1 - 100 μmol/L remarkably decreased both the cellular MTT transformation and intercellular [^{3}H]TdR incorporation in a concentration dependent manner, and SA-A 0.1 μmol/L had no obvious influences. (Tab 2).

Tab 2. Effects of SA-A on MTT transformation and [^{3}H] TdR incorporation in HSC. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Groups	MTT (A_{570}-A_{690})	[^{3}H]TdR incorporation (Bq/well)
Control	0.48 ± 0.04	663 ± 48
SA-A 0.1 μmol/L	0.48 ± 0.06	665 ± 97
SA-A 1 μmol/L	0.43 ± 0.03^b	562 ± 11^b
SA-A 10 μmol/L	0.42 ± 0.02^b	357 ± 20^c
SA-A 100 μmol/L	0.32 ± 0.07^c	17 ± 9^c

Effects on cellular collagen deposition and type I collagen secretion SA-A decreased both cellular collagen deposition in culture dishes and type I collagen secretion in a concentration-dependent manner. SA-A 1- 100 μmol/L decreased cellular collagen deposition

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by 78.6 %, 71.8 %, and 61.3 % of the control ($P < 0.05$), and type I collagen secretion by 53.1 %, 52.6 %, and 49.5 % of the control ($P < 0.01$ or $P < 0.05$). (Tab 3, Fig 1).

Effects on procollagen α_2(I) mRNA expression SA-A 1 and 10 μmol/L decreased procollagen α_2 (I) mRNA expression greatly ($P < 0.05$), but there was no difference between the two groups. (Tab 4, Fig 2).

DISCUSSION

Hepatic fibrosis, a precursor of cirrhosis, is a common and important pathological feature of the chronic liver diseases, and consists of the abnormal accumulation and deposition of extracellular matrix (ECM) proteins in liver. The collagens are the major components of the normal and fibrotic liver, and type I collagen is particularly produced predominantly during fibrogenesis[8]. It is widely documented that HSC is the major cellular resource of ECM in liver injury, and HSC activation plays an important role in liver fibrosis. The HSC activation has two predominant features[9]: proliferation and fibrogenesis, the former led to increase of the cell number, the later increases the fibrogenic ability per cell, both contribute to the accumulation of ECM. HSC cultured in uncoated plastic well *in vitro* can spontaneously undergo the activation similar to the *in vivo* process, and is a reliable cell model for the investigation of antifibrotic drugs[9].

In order to rule out the possibility of SA-A's cytotoxic influence *in vitro*, the cell morphology was observed by microscopy as well as the intracellular [^{3}H]TdR incorporation. In SA-A 100 μmol/L group, parts of cells died and [^{3}H]TdR incorporation was very low, it indicated SA-A at the concentration of 100 μmol/L was cytotoxic on HSC to some extent. SA-A 0.1 – 100 μmol/L had no obvious influence on the cell morphology, but SA-A 1 – 100 μmol/L obviously

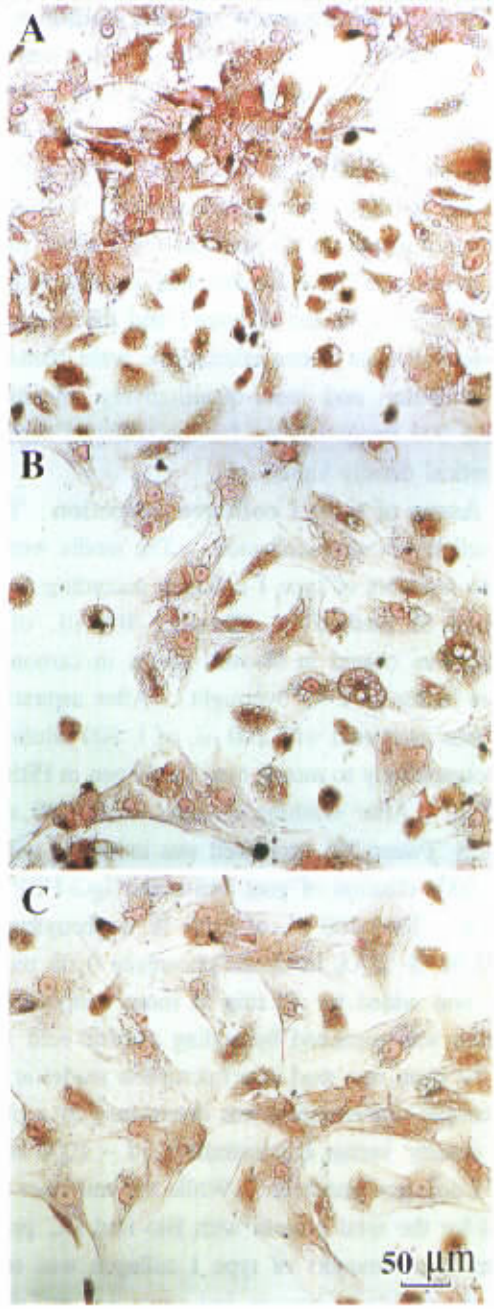

Fig 1. Hepatic stellate cells stained with ponceau. (× 200). (A) Control. (B) SA-A 1 μmol/L treated for 24 h. (C) SA-A 10 μmol/L treated for 24 h.

Tab 3. Effect of SA-A on collagen deposition and type I collagen secretion in HSC. $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Group	Collagen deposition (Optical density, $n = 9$)	% of Control	Type I collagen secretion (mg/g protein, $n = 4$)	% of Control
Control	3328 ± 697	100	288 ± 37	100
SA-A 0.1 μmol/L	2983 ± 365	89.6	207 ± 64	71.8
SA-A 1 μmol/L	2618 ± 316^c	78.6	152 ± 44^b	53.1
SA-A 10 μmol/L	2391 ± 275^c	71.8	151 ± 36^b	52.6
SA-A 100 μmol/L	2040 ± 233^c	61.3	143 ± 40^c	49.5

Tab 4. The relative amount of procollagen α_2(I) mRNA expression (% of β-actin). $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control.

Group	Procollagen α_2(I) mRNA
Control	98 ± 8
SA-A 1 μmol/L	78 ± 13[b]
SA-A 10 μmol/L	72 ± 15[b]

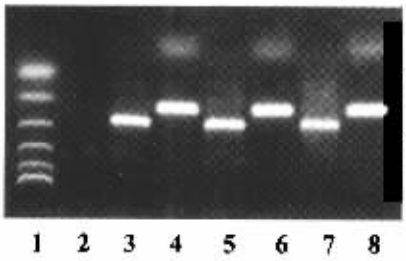

Fig 2. RT-PCR products of procollagen α_2(I) and β-actin 5 μL run on 1.5 % agarose gel stained with EB. Lane 1 as PCR marker, lane 2 as control, lane 3 and 4 as the control for procollagen α_2(I) and β-actin, respectively; lane 5 and 6 as SA-A 1 μmol/L for procollagen α_2(I) and β-actin, respectively; lane 7 and 8 as SA-A 10 μmol/L for procollagen α_2(I) and β-actin, respectively.

decreased both the cellular MTT transformation and [^{3}H]TdR incorporation. It suggests that SA-A has a potential action against NBS stimulated HSC proliferation, but this action is the pharmacological effect, not associated with the drug cytotoxity.

Type I collagen is composed of two α_1(I) chains and one α_2(I) chain. In the experiment, SA-A not only down-regulated procollagen α_2(I) steady-state mRNA expression, but also decreased the cell secretion of type I collagen and collagen deposition, suggesting that SA-A had inhibitory effect on HSC collagen synthetic process. In the study, SA-A not only inhibited HSC proliferation, but also decreased the cell collagen synthesis, indicating that SA-A was effective we against HSC activation and liver fibrosis.

In Asia especially in China, herbal medicines have been used for centuries to treat liver diseases, among which *Salvia miltiorrhiza* is a typical one. Recent studies have found that the herb inhibits fibrosis in animal models and down-regulates mRNA expression in pro-collagens, indicating an action on liver fibrosis[10,11]. Salvianolic acid-A, one of water-soluble ingredients from *Salvia miltiorrhiza*, has effective actions on hepatic peroxidation and fibrosis *in vivo* as found in our previous study[3]. Usually herbal medicines are prepared in boiling water and taken orally, thus the aqueous soluble components may play an important role[12]. In our study, SA-A has been found to have a potential action against hepatic fibrosis *in vitro*. It is deduced that SA-A is one of the major effective components of *Salvia miltiorrhiza*, and the main mechanisms of its antifibrotic action is associated with the inhibition of HSC proliferation and down-regulation of procollagen gene expression.

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丹酚酸 A 对培养的大鼠肝星状细胞增殖与胶原生成的作用[1]

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关键词 丹参；丹酚酸 A；肝硬化；细胞分裂；胶原；培养的细胞；基因表达；大鼠；肝星状细胞

目的：研究丹酚酸 A 对培养的大鼠肝星状细胞增殖与胶原生成的影响．**方法**：用链酶蛋白酶与胶原酶对肝脏进行原位灌流消化，Nycodenz 密度梯度离心分离大鼠肝星状细胞，传一代培养．MTT 法与 [^{3}H]TdR掺入法测定细胞增殖．丽春红染色、图象分析法半定量细胞胶原沉积量，ELISA 法测定细胞培养上清中 I 型胶原分泌量，检测细胞层总蛋白量校正细胞数．RT-PCR 法分析前胶原 α_2(I)基因的表达．**结果**：丹酚酸 A 100 μmol/L 引起部分细胞脱壁与死亡，有一定毒性反应．丹酚酸 A 0.1－10 μmol/L 对细胞形态无明显影响．丹酚酸 A 1－100 μmol/L 浓度依赖性抑制细胞增殖，降低胶原沉积量与 I 型胶原分泌量．丹酚酸 A 1－10 μmol/L 对前胶原 α_2(I) mRNA 表达均有明显抑制作用．**结论**：丹酚酸 A 抑制肝星状细胞增殖与胶原表达，是丹参抗肝纤维化的主要有效成分之一，抑制肝星状细胞活化是其抗肝纤维化的主要作用机制．

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