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Effects of salvianolic acid-A on NIH/3T3 fibroblast proliferation, collagen synthesis and gene expression

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Abstract

AIM To investigate the mechanisms of salvianolic acid A (SA-A) against liver fibrosis *in vitro*.

METHODS NIH/3T3 fibroblasts were cultured routinely, and incu bated with 10^{-4}mol/L-10^{-7}mol/L SA-A for 22 h. The cell viability was assayed by[^{3}H]proline incorporation, cell proliferation by [^{3}H]TdR incorporation, cell collagen synthetic rate was measured with[^{3}H] proline impulse and collagenase digestion method. The total RNA was prepared from the control cells and the drug treated cells respectively, and α (1) I pro-collagen mRNA expression was semi-quantitatively analyzed with RT-PCR.

RESULTS 10^{-4} mol/L SA-A decreased cell viability and exerted some cytotoxiciy, while 10^{-5} mol/L-10^{-7} mol/L SA-A did not affect cell viability, but inhibited cell proliferation signi ficantly, and 10^{-6} mol/L SA-A had the best effect on cell viability among these concentrations of drugs. 10^{-5} mol/L-10^{-6} mol/L SA-A inhibited intracellular collagen synthetic rate, but no significant influence on extracell ular collagen secretion. Both 10^{-5} mol/L and 10^{-6} mol/L SA-A could decrease α (1) I pro-collagen mRNA expression remarkably.

CONCLUSION SA-A had potent action against liver fibrosis. It inhibited NIH/3T3 fibroblast proliferation, intracellular collagen synthetic rate and type I pro-collagen gene expression, which may be one of the main mechanisms of the drug.

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INTRODUCTION

Radix salviae miltiorrhizae, one of the most frequently used Chinese herbs, is regarded to have effects on both blood production and circulation by traditio nal Chinese medicine, and is widely applied in clinical therapy for liver diseas es, such as chronic hepatitis, hepatic cirrhosis, etc. Salvianolic Acid-A is one of the water soluble components from Radix salviae miltiorrhizae. It was reported to have good actions on peroxidation [1]. Lipid peroxidation could stimulate hepatic stellate cell (HSC) transformed into myofibro blast like cell (MFBC) and collagen gene expression *in vivo* and *in vitro*, and played an important role in liver fibrogenesis[2]. In our previous work[3], it was found that SA-A could protect hepatic lipid peroxidation, and had marked effects against liver injur y and fibrosis in carbon tetrachloride induced fibrotic rats. In order to inves tigate the mechanism by which SA-A protects against liver fibrosis, we observed the effects of SA-A on NIH/3T3 fibroblast proliferation, collagen protei n production and procollagen gene expression.

MATERIALS AND METHODS

Drug

SA-A, molecular formular as $C_{26}H_{22}O_{10}$, molecular structure as shown in Figure 1, molecular weight 494, was extracted and identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

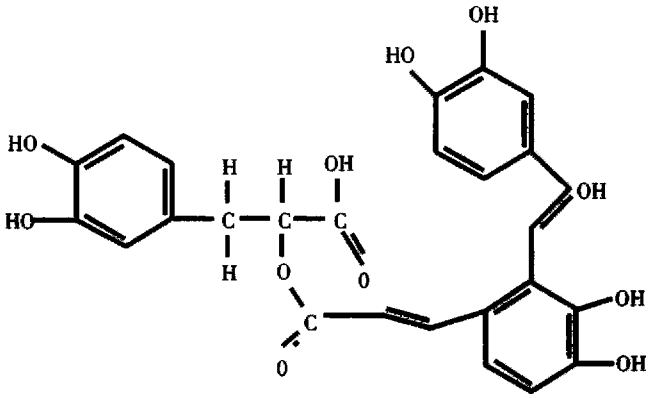

Figure 1 SA-A molecular structure.

Main reagents and solutions

PRMI-1640 Medium and Dubocal modified Eagle Medium (DMEM) were purchased from Gibco BRL

Co., new brown serum (NBS) from Shanghai Sino-American Co., purified type III collagenase (specific activity, 960U/mg), N-ethylmaleimide (NEM) and β-aminopropionitrile from Sigma Co. [5^{3}H]proline ([^{3}H] Pro) from Amersham Co. methyl-[^{3}H] thymidine (TdR) from Shanghai Inst itute of Atomic Energy, guanidium thiocynate from Serva Co. Access RT-PCR System Kit, PCR marker from Promega Co., Diethypyrocarbonate, saturated phenol/chloroform mix and agarose from Shanghai Sangon Biotech Co. Other reagents all were of analytical grade.

The non-homogeneous scintillation liquid was dimethylbenzene solution containing 5 g/L 2,5-diphenyloxazol (PPO) and 0.5 g/L 1,4-bis[5-phen yloxazol-2]benzene (POPOP), the homogeneous scintillation liquid was dimethy lbenzene solution containing 7 g/L PPO, 0.5 g/L POPOP, 100 g/L naphthalene and 400 mL/L$_2$-ethoxy-ethanol.

Cell line

Mouse NIH/3T3 fibroblasts were purchased from Shanghai Institute of Cell Biolo gy, Chinese Academy of Sciences, and cultured with PRMI-1640 medium containing 100 g/L NBS, 100KU/L penicillin and 100 mg/L streptomycin. After the cell growth became confluent, they were digested with trypsin-EDTA and subcultured.

PCR Primers

The PCR primers for pro-collagen α 2(I) and β-actin were designed according to the published sequences and references in Table 1[4], and were synthesized by Gibco BRL Co.

Table 1 PCR primer sequences and expected size of amplified products

Primers	Sequence	Size
α 2(I) collagen upstream	5'TGT TCG TGG TTC TCA GGG TAG3'	
α 2(I) collagen downstream	5'TTG TCG TAG CAG GGT TCT TTC3'	254 bp
β-actin upstream	5'ACA TCT GCT GGA AGG TGG AC3'	
β-actin downstream	5'GGT ACC ACC ATG TAC CCA GG3'	163 bp

Cell proliferation assay

Confluent NIH/3T3 fibroblasts in 24 well plates were incubated with 10^{-4}mol/L-10^{-7}mol/L SA-A diluted in PRMI-1640 medium containi ng 100 mL/L NBS for 22 h, and[^{3}H]TdR (55.5KBq/well) was impu lsed in the last 16 h. Then cells were harvested with trypsin digestion and collected on the filtration membrane, then sample radioactivity (cpm) in the non-homogeneous scintillation liquid was measured by Backman Wallac 1410 Scintillator. All tests were repeated 3 times.

Cell viability assay

According to *Mallat's* method[5], confluent NIH/3T3 fibroblast s in 24-well plates were incubated with 10^{-4}mol/L-10^{-7}mol/L SA-A re solved in PRMI-1640 medium without NBS for 22 h, and[^{3}H]Pro (55.5KB q/well) was impulsed in the last 16 h. Then cells were collected and the cpm was measured as above.

Assay of cell collagen synthetic rate

According to Greets' method[6], confluent NIH/3T3 fibroblasts in 6 well plates were incubated with 10^{-5}mol/L-10^{-6}mol/L SA-A diluted in PRMI-1640 without NBS for 22 h, during the later 16h the culture media were changed to DMEM containing 185KBq/mL [^{3}H]Pro, 100 mg/L-β-aminopropionitrile, 50 mg/L asc orbic acid as well as the same drugs. Then the culture media and cell layer extract were collected respec tively, dialyzed thoroughly and reacted with collagenase, etc. The total radioa ctivity in the samples (cpm_t), radioactivity in the samples treated with colla genase (cpm_c) and not treated with collagenase (cpm_b) were counted in the ho mogeneous scintillation liquid by Backman Wallac 1410 Scintillator. The new col lagen that cell produced, i.e. the fraction of collagenous protein expressed as percentage of total radiolabeled protein, was calculated using the formula:

$$\% \text{ of collagen} = 100 \div \left(5.4 \times \frac{cpm_t - cpm_c}{cpm_c - cpm_b} + 1\right)$$

RNA extraction and RT-PCR (reverse transcription and polymerase chain reaction)

The total RNA was extracted from the control cells and SA-A incubated cells by the acid guanidium thiocynate-phenol-chloroform method[7]. The RNA quantity was determined by absorption at 260 nm, its purity was confirmed with A_{260}/A_{280} specrophoto meter readings that ranged from 1.6 to 1.9, and its integrity was checked by 9 g/L agarose gel ele ctrophoresis with ethidium bromide (EB) staining of 18S and 28S ribosomal RNA (Figure 2). With Access RT-PCR system kit, the cDNA synthesis and amplification was done in one tube following the manufacturer's instructions. In brief, 1 μg RNA, 50 pmol/L primers for α (1) I pro-collagen or β-actin were added to each reaction mixture respectively, which included 10 mmol/L dNTPs 1 μL, 25 mmol/L $MgSO_4$ 2 μL, AMV reverse transcriptase 5U, Tfl DNA polymerase 5U, AMV/Tfl-5 × buffer 10 μL. The reaction final volume was 50 μL and was covered with 20 μL mineraloil. Then with PCR Touchdown thermal cycler (Hybaid, England), RT-PCR reaction was run in the following procedures: ① 48℃ for 45 min, 1 circle. ② 94℃ for 2 min, 1 circle. ③ 94℃ for 30s, 60℃ for 1 min, 38℃ for 2 min, 30 circles. ④ 68℃ for 7 min, 1 circle. Five μL PCR product was run on 15 g/L agarose gel and observed by EB

staining under UV light, the electrophores is photo was transformed into computer, and α 1 (I) pro-collagen intensity was analyzed with MPIAS500 image system, while the β-actin band intensity was subtracted as an internal standard.

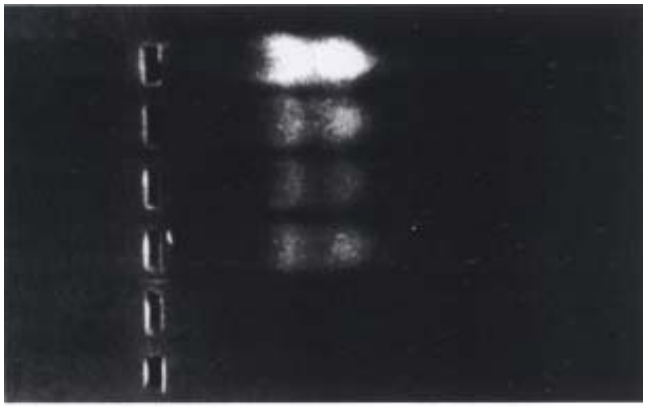

Figure 2 Total RNA gel electrophoresis photograph. 28S and 18S of total RNA run on 9 g/L agarose gel stained with EB.

Statistical analysis

Data were analyzed by *Student's t* test.

RESULTS

Effects on cell morphology and viability

10^{-5}mol/L-10^{-7}mol/L SA-A had no marked effects on cell morphology, but 10^{-4}mol/L SA-A led to shrinkage and detachment of some cells, showing cytotoxicity to some degree. 10^{-4}mol/L-10^{-7}mol/L SA-A did not decrease intercellular [^{3}H] Pro incorporation, wh ile 10^{-6}mol/L SA-A could increase[^{3}H]Pro impulse(P<0 .05) and enhance cell viability (Table 2).

Effects on cell proliferation

10^{-4}mol/L-10^{-6}mol/L SA-A remarkably decreased intercellular [^{3}H]TdR incorporation and inhibited cell proliferation(P<0.05), 10^{-4}mol/L SA-A showed more significant effect (P<0.01), but it induced some cell death, which may be associated with its cytotoxic action. 10^{-7}mol/L SA-A had no obvious effect on cell [$_3$H] TdR incorporation (Tabl e 2).

Table 2 Effects of SA-A on cell intracellular[^{3}H]TdR and[^{3}H] Pro incorporation (cpm/well, $\bar{x}$ ± s, n = 4)

Group	[^{3}H]TdR	[^{3}H]Pro
Control	1482 ± 486	21018 ± 5473
10^{-4}mol/L SA-A	675 ± 201[b]	18659 ± 2363
10^{-5}mol/L SA-A	969 ± 183[a]	23761 ± 5430
10^{-6}mol/L SA-A	868 ± 183[a]	31408 ± 4981[a]
10^{-7}mol/L SA-A	1056 ± 187	26080 ± 4504

[a]P<0.05, [b]P<0.01, *vs* control.

Effects on cell collagen synthetic rates

10^{-5}mol/L-10^{-6}mol/L SA-A could inhibit intracellular collagen synthetic rate significantly (P<0.01), but did not influence extracellular synthetic rate (Table 3).

Table 3 Effects of SA-A on NIH/3T3 fibroblast collagen synthetic rates (%, $\bar{x}$ ± s, n = 4)

Group	Intracellular	Extracellular
Control	0.78 ± 0.03	2.57 ± 0.37
10^{-5}mol/L	0.48 ± 0.24[b]	2.54 ± 0.91
10^{-6}mol/L	0.43 ± 0.26[b]	3.02 ± 0.69

[b]P<0.01, *vs* control.

Effects on procollagen α 2(I) mRNA expression

Both 10^{-5}mol/L and 10^{-6}mol/L SA-A decreased procollagen α 1(I) mRNA expression significantly (P<0.05), but there was no difference between the two different concentration groups (Table 4, Figure 3).

Table 4 The relative expression amount of α 2(I) procollagen mRNA ($\bar{x}$ ± s, % of β-actin)

Group	n	Col α 1(I) mRNA
Control	3	98.71 ± 9.96
10^{-5}mol/L SA-A	3	76.23 ± 12.02[a]
10^{-6}mol/L SA-A	3	68.44 ± 8.06[a]

[a]P<0.05, *vs* control.

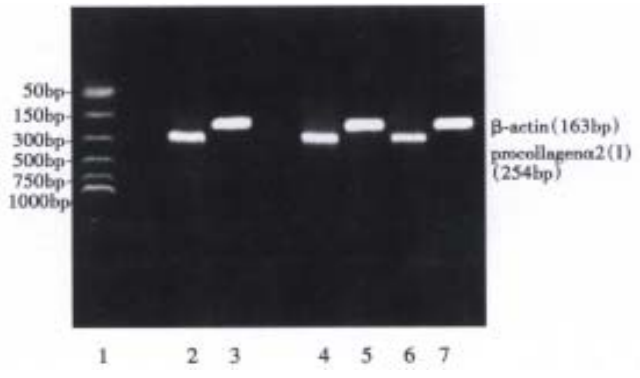

Figure 3 RT-PCR product gel electrophoresis photog raph. Five μL RT-PCR products of procollagen α 2(I) and β-actin run on 1.5% agarose gel stained with EB. Lane 1 as PCR marker, lane 2 and 3 as the control for procollagen α 2(I) and β-actin respectively, lane 4 and 5 SA-A 10^{-6}mol/L for procollagen α 2(I) and β-actin respectively, lane 6 and 7 as SA-A 10^{-5}mol/L for procollagen α 2(I) and β-actin respectively.

DISCUSSION

Hepatic fibrosis, a precursor of cirrhosis, is a common and important pathological feature of chronic liver diseases, which involves the abnormal accumulation of extracellular matrix (ECM)

proteins, particularly collagen[8]. In fi brotic liver, ECM components are mainly produced by HSC and fibroblasts. It is known that during fibrogenesis, HSC undergoes a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation and ECM production, especially type I collagen synthesis. The mouse NIH/3T3 fibroblast also shared the features that active HSC (MFBC) presented, such as remarkable prolife ration and substantial production of collagen, and stable cell line. In practice, NIH/3T3 fibroblast is often used as a desirable cell model for inves tigation of antifibrotic drugs.

In order to rule out the possibility of SA-A cytotoxic influence *in vitro*, the intracellular[^{3}H] Pro incorporation was measured, and inverted microscopic observation was done. It was found that only 10^{-4}mol/L SA-A caused some cell detachment, decreased [^{3}H] Pro incorporation, and showed cytotox icity to some extents. 10^{-5}mol/L-10^{-7}mol/L SA-A did no t influence cell morphology or inhibit cell viability. However, 10^{-6}m ol/L SA-A enhanced cell viability. Both 10^{-5}mol/L-10^{-6} mol/L SA-A could inhibit intracellular[^{3}H]TdR impulse that NBS stimul ated. It is suggested that SA-A had an effective action against NIH/3T3 fibr oblast proliferation.

Type I collagen is the predominant component of ECM during liver fibrosis. Its production involve two processes: the first is intracellular synthesis, includi ng gene transcription, translation and modification to form procollagen, then procollagen alpha chains are secreted to the outside of the cell to form helix collagen by sorting and alignment etc. In the study, it was found that SA-A downregulated procollagen α 2(I) steady-state mRNA expression, and intracellular collagen synthetic rate, but exerted no effect on extracellular synthetic rate. It is suggested that SA-A influence on collagen production through the intracellular synthetic process. The fibrogenic cells have two predo minant features: one is active in cell proliferation, which led to increase in cell number, another is strong fibrogenic ability percell, which led to accumula tion of ECM. In the study, SA-A not only inhibited NIH/3T3 fibroblast prolif eration, but also decreased collagen synthesis, showing a good action against liver fibrosis.

Salvianolic radix is widely used as an important component in Chinese herbal for mulas for the treatment of chronic liver diseases. Salvianolic Acid-A, one of water-soluble ingredients from Salvianolic radix, had effective actions on hepa tic peroxidation and fibrosis *in vivo*[3]. In the paper, it is for the first time found that SA-A has the potential action against hepatic fibrosi s *in vitro*, and its main mechanisms of antifibrotic action perhaps was assoc iated with the inhibition of fibrogenic cell proliferation, collagen gene expres sion and protein synthesis.

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