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

RESULTS 10^{-4} \text{mol/L} SA-A decreased cell viability and exerted some cytotoxicity, while 10^{-5} \text{mol/L} - 10^{-9} \text{mol/L} SA-A did not affect cell viability, but inhibited cell proliferation significantly, and 10^{-6} \text{mol/L} SA-A had the best effect on cell viability among these concentrations of drugs. 10^{-5} \text{mol/L} - 10^{-9} \text{mol/L} SA-A inhibited intracellular collagen synthetic rate, but no significant influence on extracellular collagen secretion. Both 10^{-5} \text{mol/L} L and 10^{-6} \text{mol/L} SA-A could decrease \alpha (1) \text{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.

INTRODUCTION

*Radix salviae miltiorrhizae*, one of the most frequently used Chinese herbs, is regarded to have effects on both blood production and circulation by traditional Chinese medicine, and is widely applied in clinical therapy for liver diseases, 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 myofibroblast-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 injury and fibrosis in carbon tetrachloride induced fibrotic rats. In order to investigate the mechanism by which SA-A protects against liver fibrosis, we observed the effects of SA-A on NIH/3T3 fibroblast proliferation, collagen protein production and procollagen gene expression.

MATERIALS AND METHODS

**Drug**

SA-A, molecular formula 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., [3H]proline ([1H]Pro) from Amersham Co. methyl-[3H] thymidine (TdR) from Shanghai Institute of Atomic Energy, guanidium thiocynate from Serva Co. Access RT-PCR System Kit, PCR marker from Promega Co., Diethylypyrcarbonate, 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 (POPO) and 0.5 g/L 1,4-bis[5-phenyloxazol-2]benzene (POPOP), the homogeneous scintillation liquid was naphthalene and 400 mL/L 2-ethoxy-ethanol.

**Cell line**

Mouse NIH/3T3 fibroblasts were purchased from Shanghai Institute of Cell Biology, 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.

| Primers          | Sequence                        | Size  |
|------------------|---------------------------------|-------|
| α 2(I) collagen upstream | 5'TGT TCG TCT TTC TCA GGG TAG3' | 254 bp|
| α 2(I) collagen downstream | 5'TTG TCG TAG CAG GGT TCT TCT3' |       |
| β-actin upstream  | 5'ACA TCT GCT GGA AGG TGC AC3'  | 163 bp|
| β-actin downstream | 5'GCT ACC ACC ATG TAC CCA GG3'  |       |

**Cell proliferation assay**

Confluent NIH/3T3 fibroblasts in 24 well plates were incubated with 10⁻⁴mol/L-10⁻⁷mol/L SA-A diluted in 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.

**Cell viability assay**

According to Mallat’s method[5], confluent NIH/3T3 fibroblasts in 24-well plates were incubated with 10⁻⁴mol/L-10⁻⁷mol/L SA-A were solved in PRMI-1640 medium without NBS for 22 h, and [3H]Pro (55.5KBq/well) was impulused 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 10⁻⁴mol/L-10⁻⁶mol/L SA-A diluted in PRMI-1640 medium without NBS for 22 h, during the later 16h the culture media were changed to DMEM containing 18.5KBq/mL [3H]Pro, 100 mg/L -β-aminopropionitrile, 50 mg/L ascorbic acid as well as the same drugs. Then the culture media and cell layer extract were collected respecively, dialyzed thoroughly and reacted with collagenase, etc. The total radioactive activity in the samples (cpm), radioactivity in the samples treated with collagenase (cpmα), and not treated with collagenase (cpmβ) were counted in the homogeneous scintillation liquid by Backman Wallac 1410 Scintillator. The new collagen that cell produced, i.e. the fraction of collagenous protein expressed as percentage of total radiolabeled protein, was calculated using the formula:

\[
\text{% of collagen = 100 \div (5.4 \times \frac{\text{cpm}_\alpha - \text{cpm}_\beta}{\text{cpm}_\beta}) + 1)}
\]

**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} spectrophotometer readings that ranged from 1.6 to 1.9, and its integrity was checked by 9 g/L agarose gel electrophoresis with ethidium bromide (EB) staining of 18S and 28S ribosomal RNA (Figure 2). With Access RT-PCR system kit, the cDNA synthesis and amplification were done in one tube following the manufacturer’s instructions. In brief, 1 µg RNA, 50 pmol/L primers for α (1) pro-collagen or β-actin were added to each reaction mixture respectively, which included 10 mmol/L dNTPs 1 µL, 25 mmol/L MgSO₄ 2 µL, AMV reverse transcription 5U, Tfi 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°C for 45 min, 1 circle. 2. 94°C for 2 min, 1 circle. 3. 94°C for 30s, 60°C for 1 min, 38°C for 2 min, 30 circles. 4. 68°C 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 electrophoresis photos were transformed into computer, and α(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**

10−5mol/L–10−7mol/L SA-A had no marked effects on cell morphology, but 10−4mol/L SA-A led to shrinkage and detachment of some cells, showing cytotoxicity to some degree. 10−4mol/L–10−7mol/L SA-A did not decrease intercellular [3H] Pro incorporation, while 10−6mol/L SA-A could increase [3H]Pro impulse (P<0.05) and enhance cell viability (Table 2).

**Effects on cell proliferation**

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

**Effects on cell collagen synthetic rates**

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

**Effects on procollagen α2(I) mRNA expression**

Both 10−5mol/L and 10−6mol/L SA-A decreased procollagen α1(I) mRNA expression significantly (P<0.05), but there was no difference between the two different concentration groups (Table 4, Figure 3).

**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. In fibrotic 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 proliferation and substantial production of collagen, and stable cell line. In practice, NIH/3T3 fibroblast is often used as a desirable cell model for investigation of antifibrotic drugs.

In order to rule out the possibility of SA-A cytotoxic influence in vitro, the intracellular [3H] 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 [3H] Pro incorporation, and showed cytotoxicity to some extents. 10^{-5}mol/L-10^{-7}mol/L SA-A did not influence cell morphology or inhibit cell viability. However, 10^{-6}mol/L SA-A enhanced cell viability. Both 10^{-5}mol/L-10^{-6}mol/L SA-A could inhibit intracellular [3H]TdR impulse that NBS stimulated. It is suggested that SA-A had an effective action against NIH/3T3 fibroblast proliferation.

Type I collagen is the predominant component of ECM during liver fibrosis. Its production involves two processes: the first is intracellular synthesis, including 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 \( \alpha_2(I) \) 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 predominant features: one is active in cell proliferation, which led to increase in cell number, another is strong fibrogenic ability percell, which led to accumulation of ECM. In the study, SA-A not only inhibited NIH/3T3 fibroblast proliferation, 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 hepatic peroxidation and fibrosis in vivo. In the paper, it is for the first time found that SA-A has the potential action against hepatic fibrosis in vitro, and its main mechanisms of antifibrotic action perhaps was associated with the inhibition of fibrogenic cell proliferation, collagen gene expression and protein synthesis.

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