Salvia miltiorrhiza injection restores apoptosis of fibroblast-like synoviocytes cultured with serum from patients with rheumatoid arthritis

QING-SONG LIU¹,², XIONG-YAN LUO³, HONG JIANG³, YAN XING², MING-HUI YANG³, GUO-HUA YUAN³, ZHONG TANG² and HE WANG¹

¹Laboratory of Genetics, West China Institute of Women and Children's Health, West China Second Hospital, Sichuan University, Chengdu, Sichuan 610041; ²Department of Clinical Laboratory; ³Institute of Rheumatology and Immunology, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan 637000, P.R. China

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Abstract. Salvia miltiorrhiza injection (SMI) is a water-soluble agent, derived from Salvia miltiorrhiza (SM), that is traditionally used to treat cardiovascular and cerebrovascular diseases. Furthermore, it has been demonstrated to possess the ability to induce apoptosis of tumor cells. However, it remains unclear whether SMI can induce apoptosis of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS), which are hyperplastic in RA due to defective apoptosis. There is also evidence that allogenic serum may be associated with the induction of apoptosis. The aim of the present study was to investigate the involvement of serum during SMI-induced apoptosis in RA FLS. The results demonstrated that SMI could induce apoptosis of RA FLS, cultured with fetal bovine serum (FBS), in a dose-dependent manner. In addition, SMI decreased the expression of nuclear factor-κB in RA FLS nuclear extracts and inhibited the secretion of tumor necrosis factor-α. Fas ligand expression was not detected in RA FLS, in either the presence or absence of SMI. The pro-apoptotic genes B-cell lymphoma 2 (Bcl-2) associated X protein (Bax) and Fas were shown to be upregulated after SMI stimulation, whereas the expression levels of the anti-apoptotic gene Bcl-2 were downregulated. Upon replacement of FBS with normal human serum, the apoptotic rate and Bax mRNA expression levels following SMI stimulation, were unchanged. However, culturing RA FLS with patient serum (RPS), restored the apoptotic rate and Bax mRNA expression levels following SMI stimulation. There may be numerous mechanisms by which SMI inhibits RA FLS proliferation. The present study demonstrated that SMI can restore apoptosis of RA FLS cultured with RPS. These results indicate that SMI may have a potential role in the treatment of synovial hyperplasia of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by synovial hyperplasia and local invasion of the bone and cartilage (1). Defective apoptosis of the RA fibroblast-like synoviocytes (FLS) is an important contributing factor of synovial hyperplasia (2). Previous research has indicated that hyperplastic RA FLS possess unique characteristics in RA pathogenesis, and have a key role in the development of sustained inflammation and angiogenesis in arthritic joints (3). Numerous studies have demonstrated that normal apoptosis is rare in RA FLS, due to the aberrant expression levels of apoptosis-associated genes, including B-cell lymphoma 2 (Bcl-2) (4-6), Fas (7,8) and various oncoproteins (9,10). Therefore, impaired apoptosis may have an important role in the pathogenesis of RA and restoration of FLS apoptosis may improve the RA joint destruction.

Salvia miltiorrhiza (SM) is a well-known herb used in traditional Chinese medicine. It has been successfully used in the treatment and prevention of aging diseases, such as cardiovascular and cerebrovascular diseases, and cancers, and has previously been considered as a ‘Super grade’ drug, according to Shen-Nung’s Pen-Ts’ao (11). Currently, SM is approved and used in Japan, the United States and some European countries (11-13). Products derived from SM include a dripping pill and an injection (SMI); these formulations have been developed and used clinically in China and other Asian countries. The pharmacologically active components of SM include the lipophilic diterpenoid tanshinones and water-soluble phenolic acids (11). The active ingredients of SM include tanshinol/danshensu, salvianolic acid B, tanshinone, dihydrotanshinone, ursolic acid and cryptotanshinone. Danshensu and salvianolic acid B have the highest concentrations within SM, accounting for >1 and 3-5% of the total dry weight, respectively (14,15). Previous reports have demonstrated that SMI-derived compounds can inhibit the growth of tumor cells,
by inducing apoptosis (16-18). RA FLS display certain unique features that are similar to transformed cells. Observations of a tumor-like phenotype were initially made by Fassbender and Simmling-Annefeld (19) in 1983, who noted the distinctive morphological features of RA FLS, including: Abundant cytoplasm, large pale nuclei with several prominent nucleoli and a dense rough endoplasmatic reticulum. Whether SMI can induce RA FLS apoptosis remains unknown. Furthermore, it has been reported that nuclear factor-κB (NF-κB), which regulates the expression of apoptotic genes, including the Bel family (20) and Fas (21), is activated in RA FLS, where it has an anti-apoptotic role (22,23). Therefore, the aim of the present study was to examine the effects of SMI on the proliferation and apoptosis of cultured human RA FLS, and to investigate the underlying mechanisms.

Culture medium has an important role in the growth of RA FLS. Serum is added to provide mitogenic peptide growth factors, attachment factors and undefined factors for cell growth, division and differentiation maintenance. Numerous types of basal culture media supplemented with animal serum have been widely used to provide basic nutrients for human RA FLS. However, the use of animal serum does not reflect the internal environment of the human body, because the cells and the serum are isolated from different species. The culturing of human cells with human serum avoids the interference of allogeneic serum, and ensures that the experimental conditions are similar to the natural environment. Therefore, in the present study, foetal bovine serum (FBS) was replaced with serum from patients with RA (RPS) and normal human serum (NHS), in the culturing of RA FLS. Furthermore, a concentration of SMI was added, which had previously been proved to induce apoptosis of RA FLS cultured with FBS, to investigate the mechanism of inhibition of RA FLS proliferation by SMI.

Materials and methods

Cell culture and treatment. Synoviocytes were isolated from four patients (three female, one male) with long-standing advanced RA, as confirmed by the 1987 American College of Rheumatology diagnostic criteria (24). The patients were sourced from the Affiliated Hospital of North Sichuan Medical College (Sichuan, China) and their ages ranged between 41 and 72 years old, with an average age of 60 years.

Human serum was obtained from the Department of Clinical Laboratory, Affiliated Hospital of North Sichuan Medical College. The NHS was isolated from a patient undergoing a health examination, whose laboratory results were normal and excluded diabetes, cardiovascular disease and autoimmune disease. Approval for the present study was obtained from the Local Ethics Committee of the Affiliated Hospital of North Sichuan Medical College and written informed consent was obtained from the patients.

The synoviocytes were isolated from the synovial fluid obtained from the patients with RA, by knee joint aspiration. The synovial fluid was centrifuged at 840 x g, 4°C and then washed with cold phosphate-buffered saline (PBS). Cell aggregates were removed using a 100 µm mesh nylon mesh filter. The isolated cells were then resuspended in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (HyClone Laboratories, Inc., Logan, UT, USA), supplemented with 10% FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China), 100 U/ml penicillin and 100 µg/ml streptomycin. Following an overnight culture, the non-adherent cells were removed and synovial fibroblasts were obtained from the adherent cells. The medium was refreshed every two days, until the cells approached confluence by seven days. The synovial cells used for the following experiments were obtained from the third to sixth passages.

Cells cultured with human serum. The stable grown cells were washed twice with cold PBS and trypsinized. The cells (5x10^6/l, 5x10^7/l or 5x10^7/l) were resuspended in DMEM supplemented with 10% NHS, RPS or FBS, as a control. The cells were then cultured in six- or 96-well plastic culture plates, or a 25 cm² plastic culture flask, in an automatically controlled incubator with a humidified atmosphere of 5% CO₂, at 37°C for 24 h. According to previous results, 0.39 mg/ml of freeze-dried SMI (Batch no: 090313; Harbin Pharmaceutical Group Co., Ltd., Harbin, China), was used to stimulate the cells for a further 24 h. The morphology of the RA FLS was confirmed using an Olympus BX51 optical microscope (Olympus Corporation, Tokyo, Japan).

Cell density. MTT reagent (Amresco LLC Solon, OH, USA) was used to measure the density of the cells. The cells were grown in 96-well plates overnight and treated with different concentrations of SMI (0, 0.195, 0.39, 0.78, 1.56 and 3.12 mg/ml). Following the 24 h incubation the cells were centrifuged at 1,260 x g for 5 minutes, the supernatant was removed and the cells were washed twice with PBS. The labeling solution (20 µl) was then added to each well. The solubilization solution (200 µl) was added to dissolve the purple crystals produced by the MTT substrate, following a 4 h incubation in a CO₂ incubator. The absorbance was measured at 450 nm, using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Apoptosis analysis. The synoviocytes were cultured in 25 mm plastomer culture flasks. Following a 24 h incubation, different concentrations of SMI were added (0, 0.195 and 0.39 mg/ml) and the cells were cultured for a further 24 h, at 37°C. The cells were trypsinized and collected for apoptosis detection, using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Keygen Biotech, Nanjing, China). Briefly, the cells were washed twice with cold PBS and resuspended in 500 ml of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂), at a concentration of 1x10⁶ cells/ml. Following the addition of 5 µl Annexin V-FITC solution and propidium iodide (PI) (1 mg/ml), the cells were incubated for 15 min at room temperature and analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA) (25).

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR). RT and qPCR were conducted as described by previous methods (26,27). Briefly, the RA FLS were cultured in six-well plates with FBS (5x10⁵ cells/well). Following a 24 h incubation, SMI was added at different concentrations (0, 0.195, 0.39 mg/ml) and the cells were cultured at 37°C, for an additional 24 h. Total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).
and was reverse transcribed into cDNA using the Improm-II Reverse Transcription system (Promega Corporation, Madison, WI, USA). The RT and qPCR analyses were performed using the 2.5X Taq PCR MasterMix and 2X Taq real-PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China), respectively. The following primer pairs were used: Human Bcl-2 forward, 5'-TCC CAT CAA TCT TCA GCA CTC T-3' reverse, 5'-TGG ATC TGG AAA TCC TCC TAA T-3'; human Bcl-2 associated X protein (Bax) forward, 5'-TGT CGC CCT TTT CTA CTT TGC-3' reverse, 5'-GCT CCC GGA GGA AGT CCA AT-3'; human Fas forward, 5'-CCT CCC ATC CTC CTT ACC ACC G-3' reverse, 5'-CTG GTT GCC TGT GGA GGG TTG-3'; human Fas ligand (FasL) forward, 5'-CTG GTT GCC TTG GTA GGA TTG-3' reverse, 5'-TCA CTC GTA AAC CGC TTC CCT C-3'; and human β-actin forward, 5'-CCT CCC ATC CTC CTG ACC ACC TGC-3' reverse, 5'-GCT CCC GGA GGA AGT CCA AT-3'; mouse-anti-p65 or -p50 (1:200 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) (Tiangen Biotech Co., Ltd., Beijing, China), respectively. The primers' sequences were as described previously. The levels of Bcl-2 mRNA changed significantly after SMI stimulation, but those of Bax and Fas mRNA did not change significantly (data were not shown). Hence, qPCR was used to detect the expression levels of Bax and Fas mRNA. qPCR was performed on 2 µl cDNA, 0.25 µl of each primer (10 pmol/µl) and 10 µl 2X SYBR Green Master mix (Tiangen Biotech Co., Ltd., Beijing, China) to obtain a final reaction volume of 20 µl. Triplicate amplification reactions were performed with an ABI 7900TH Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primers' sequences were as described previously. The data was analyzed using the cycle threshold (Ct) 2-ΔΔCt method (28). Furthermore, based on the results of Bax mRNA expression in the cells cultured with FBS, the level of Bax mRNA in the cells cultured with RPS and NHS was detected by qPCR as described above.

Western blot analysis. A total of 50 µg of protein was separated using a 12% SDS containing PAGE and transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). The membranes were blocked with 5% non-fat milk and then incubated with the following primary antibodies: Rabbit-anti-p65 or -p50 (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C, or mouse-anti-β-actin (1:1,000 dilution; Santa Cruz biotechnology Inc.) for 1 h at room temperature. The membranes were then incubated with a horseradish peroxidase-conjugated rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at a 1:2,000 dilution for 1 h. The blots were developed using electrochemical luminescence (Engreen Biosystem Co. Ltd., Beijing, China) and an X-ray technique. The densitometry of the bands of western blot were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Determination of TNF-α. The secretion levels of TNF-α in the cell supernatants were determined using an ELISA (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions.

Statistical analyses. Statistical significance was assessed by one-way analysis of variance, or a two-sided Student's t-test using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

SMI promotes apoptosis of RA FLS cultured in vitro. To determine whether SMI altered the proliferation of RA FLS, an MTT assay was performed to assess the viability of the cells. SMI significantly decreased the viability of the RA FLS cultured with FBS, in a dose-dependent manner (Fig. 1A). To investigate whether the reduction in viability was due to the induction of apoptosis, Annexin V-FITC/PI double staining was used to assess the apoptotic rate. Treatment with 0.39 mg/ml SMI resulted in a significant increase in the rate of apoptosis of the RA FLS cultured with FBS, as compared with the cells cultured in the absence of SMI (Fig. 1B). These results indicate that SMI induced apoptosis of the RA FLS.

SMI induces RA FLS apoptosis through NF-κB activation. NF-κB activation has previously been reported to initiate both cell survival and death pathways (28). Therefore the expression levels of the NF-κB subunits p65 and p50, were assessed in the nucleus of the RA FLS by western blot analysis. SMI decreased the p65 and p50 protein expression levels in the RA FLS nucleus extracts (Fig. 2A and B). As NF-κB activation has been reported to stimulate TNF-α production (29-31), the levels of TNF-α in the cell
supernatants were also analyzed, by ELISA. The incubation of the RA FLS with 0.39 mg/ml SMI for 24 h reduced TNF-α production (Fig. 2C). To further explore whether the inhibition of NF-κB activation resulted in altered expression levels of apoptosis-related genes, RT and qPCR analyses were used to determine the mRNA expression levels of Bcl-2, Bax, Fas and Fas L. Treatment with SMI triggered a significant downregulation of Bcl-2, Bax, Fas and Fas L. These results demonstrate that the inhibition of NF-κB activation by SMI, is crucial for SMI-induced RA FLS apoptosis.

Serum source is associated with SMI-restored apoptosis of RA FLS. To further investigate whether SMI altered the serum-sensitivity of RA FLS, the effects of several sera were assessed on cell viability, in the presence and absence of SMI. A concentration of SMI (0.39 mg/ml) was selected that had previously resulted in ~50% inhibition of cell viability, as determined by MTT assay (Fig. 1A). The addition of SMI significantly decreased the viability of the RA FLS cultured with FBS, as compared with the cells cultured in the absence of SMI. These results demonstrate that the inhibition of NF-κB activation by SMI, is crucial for SMI-induced RA FLS apoptosis.

Discussion

RA FLS have been identified as being responsible for the invasion and destruction of cartilage and bone (32,33). Furthermore, RA FLS have shown evidence of transformation, indicated by excessive proliferation, loss of contact inhibition and increased migration (34). Therefore, the inhibition of FLS proliferation may provide a potential treatment strategy for RA. The pharmacologically active compounds of SMI have been shown to significantly inhibit the proliferation of cancer cells, including HepG2 cells (16), and head and neck squamous cell carcinomas (17,18). The present study was the first, to the best of our knowledge, to demonstrate that SMI significantly
Figure 3. *Salvia miltiorrhiza* injection (SMI) regulates expression levels of apoptosis-related genes. (A) The mRNA expression levels of apoptosis-related genes in the cells cultured with FBS, including Fas, Fas ligand (FasL), B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax), were determined by reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR). No expression of FasL mRNA was detected in the RA FLS. (B) SMI stimulation (0.39 mg/ml) resulted in a significant downregulation of Bcl-2 mRNA expression levels in the cells cultured with FBS, as detected using RT-PCR. (C and D) SMI stimulation (0.39 mg/ml) resulted in a significant upregulation of Fas and Bax mRNA expression levels in the cells cultured with FBS as detected using qPCR. The data are expressed as the means ± standard deviation of three independent experiments, each performed in triplicate are shown. *P<0.01 as compared with the samples in the absence of SMI.

Figure 4. Serum type is associated with *Salvia miltiorrhiza* injection (SMI)-induced apoptosis of rheumatoid arthritis fibroblast-like synoviocytes (RA FLS). (A) SMI stimulation reduced the viability of RA FLS, regardless of the serum source, as determined using an MTT assay. (B) SMI stimulation induced the apoptosis of RA FLS cultured with serum from patients with RA (RPS) and fetal bovine serum (FBS), but not with normal human serum (NHS), as determined using flow cytometry. (C) The mRNA expression levels of the pro-apoptotic gene B-cell lymphoma 2 associated X protein (Bax) were determined using quantitative polymerase chain reaction. SMI stimulation resulted in an upregulation of Bax mRNA expression levels in RA FLS cultured with RPS and FBS, but not with NHS. The data are expressed as the means ± standard deviation of three independent experiments, each performed ≥4 times. *P<0.01, as compared with the samples in the absence of SMI, or the samples treated with FBS. OD, optical density.
inhibited the proliferation of human RA FLS, cultured with FBS, by promoting apoptosis in a NF-κB-dependent manner. SMI stimulation was shown to significantly inhibit the proliferation of RA FLS and promote apoptosis. Furthermore, the addition of SMI inhibited the activation of NF-κB and the secretion of TNF-α, resulting in a significant downregulation of Bcl-2 and upregulation of Bax and Fas mRNA expression levels. These findings are consistent with the hypothesis that SMI triggers NF-κB-dependent apoptosis signaling pathways in RA FLS.

The Bcl-2 family is essential for the maintenance of mitochondrial homeostasis and cell density in FLS (5). SMI stimulation increased the mRNA expression levels of the pro-apoptotic protein Bax, and decreased the mRNA expression levels of the anti-apoptotic protein Bcl-2, in RA FLS. These results indicate that SMI likely induced apoptosis through modulating the balance of pro- and anti-apoptotic factors. Rheumatoid synoviocytes also undergo Fas-mediated apoptosis (23,35). The results of the present study revealed that FasL expression was not detected under any of the experimental conditions, which is consistent with the observations of other studies (36), however Fas expression levels were increased in response to SMI stimulation. These results indicate that SMI may also have the ability to interact with Fas, in order to promote RA FLS apoptosis.

The present study is the first, to the best of our knowledge, to demonstrate that the source of serum is important in SMI-induced RA FLS apoptosis in vitro. The apoptotic rate of the RA FLS was increased in the RPS-cultured cells in response to SMI stimulation, however this SMI-induced increase was not observed in the NHS-cultured cells. The Bax mRNA expression levels were significantly upregulated by SMI stimulation (0.39 mg/ml) in the RA FLS cultured with RPS, however there was no SMI-stimulated difference in the RA FLS cultured with NHS. These results suggest that: RA FLS can adequately grow and proliferate when cultured in human serum. The proliferation of RA FLS cultured with NHS was inhibited by 0.39 mg/ml SMI, however the apoptotic rate and Bax mRNA expression levels were not significantly altered, indicating that the inhibition of RA FLS proliferation, by SMI, may be due to changes in ionic strength and other factors. Although the apoptotic rate was not altered in the NHS-cultured RA FLS following SMI stimulation, it was significantly increased in the RA FLS cultured in RPS, indicating that SMI does have the ability to restore RA FLS apoptosis. In addition, in the RPS group, the baseline apoptotic rate of the RA FLS was lower than that of the cells cultured in NHS and FBS in the absence of SMI. This is consistent with the phenomenon that RA FLS apoptosis is rare in vivo. The apoptotic rate increased following SMI stimulation, indicating that SMI has the ability to promote apoptosis of RA FLS in vitro. Therefore, it is feasible to culture primary cells with FBS in vitro if patient serum is not available, whereas the use of NHS in the culture medium would produce an opposite result since NHS can promote apoptosis and inhibit drug activity. The finding that SMI was capable of increasing apoptotic rate in the RA FLS cultured with RPS, but not the RA FLS cultured with NHS, indicates that the ability of SMI to restore RA FLS apoptosis may be associated with the experimental environment. This function of SMI may due to improved blood environment (7,8). Finally, SMI restored the Bax mRNA expression levels in the RA FLS cultured with RPS. These findings, together with the previous results that SMI inhibited NF-κB activation, show that SMI has the ability to restore the apoptosis of RA FLS through a NF-κB-dependent pathway.

The findings of the present study demonstrate that SMI inhibited RA FLS proliferation and induced RA FLS apoptosis, providing a novel mechanism for the inhibitory effects of SMI on RA. However, it remains unknown which active component of SMI is responsible for inducing apoptosis of RA FLS. SMI consists of tanshinol/danshensu, salvianolic acid, tanshinone, dihydrotanshinone, ursolic acid, and cryptotanshinone. Numerous studies have demonstrated that salvianolic acid (18, 37), and tanshinone (38,39) have the ability to induce tumor cell apoptosis. Although there has been no previous evidence suggesting that salvianolic acid and tanshinone are capable of facilitating RA FLS apoptosis, it has been noted that RA FLS have a tumor-like phenotype (19). Therefore, salvianolic acid and tanshinone may be the possible substances that induce apoptosis of RA FLS. Furthermore, the target in RA FLS which may interact with the active components of SMI, remains to be elucidated. Previous studies have determined that epidermal growth factor receptor (EGFR) (40) and matrix metalloproteinase-9 (MMP-9) (41) are capable of interacting with salvianolic acid B. Yamane et al (42) found that there was no significant difference in the expression levels of EGFR in RA FLS, as compared with normal and osteoarthritis (OA) FLS; however amphiregulin, a member of the EGF family that can connect with EGFR, was significantly increased in RA FLS. MMPs are a family of zinc neutral endopeptidases, which can hydrolyze FasL into soluble FasL (43). Previous research has shown that MMP-9 is significantly increased in the joint effusion from patients with RA, as compared with those from patients with OA (44). In addition, in the present study, SMI could not induce apoptosis in the RA FLS cultured with NHS. It remains unknown whether there are cytokines that may inhibit SMI-induced apoptosis of RA FLS, which are present in NHS.

In conclusion, the findings of the present study demonstrate that SMI exerts anti-proliferative effects against RA FLS by promoting apoptosis through the induction of apoptotic signaling pathways and restoring normal apoptotic function. These results indicate that SMI may have potential therapeutic implications in the treatment of RA.

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References


