Tanshinone IIA Induces Apoptosis in Fibroblast-Like Synoviocytes in Rheumatoid Arthritis via Blockade of the Cell Cycle in the G2/M Phase and a Mitochondrial Pathway

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and progressive joint destruction.1) Although various cell populations may participate in the pathogenesis of RA, fibroblast-like synoviocytes (FLS) are considered crucial in both the initiation and progression of arthritis. The mechanisms of synovial hyperplasia are not fully understood, although the paucity of the apoptosis may contribute to the pathogenesis of RA. Recent evidence suggests that the activation of RA-FLS is associated with a reduced level of apoptosis in vivo, especially at sites of invasion into cartilage and bone.9–11) Apoptosis induction of RA-FLS is therefore suggested as a potential therapeutic approach for RA.7)

Salvia miltiorrhiza (Labiatae) is used primarily for the treatment of coronary heart diseases in China, and has long been used to treat arthritis.1,3) Tanshinone IIA (Tan IIA; 14,16-epoxy-20-nor-5(10),6,8,13,15-abietapentaene-11,12-dione) is a major component of Salvia miltiorrhiza and Chemical structure of Tan IIA is shown in Fig. 1. Moreover, recent studies have documented anti-tumor, pro-apoptotic, and anti-inflammatory activities of Tan IIA.5–8) Tan IIA induces apoptosis in human cancer cell lines via the mitochondrial-dependent pathway involving Bcl-2, p53, and Bax. Activation of p53 leads to the release of many mitochondrial proteins, such as cytochrome c (Cyt-c) via translocation of Bax from the cytosol to mitochondria, overcoming the regulation by Bcl-2 of mitochondrial membrane protein permeability. An apoptosome complex then forms through the binding of Cyt-c, apoptotic protease activating factor 1 (Apaf-1), procaspase-9, procaspase-3, caspase-9, and caspase-3. The results support the conclusion Tan IIA treatment likely induces apoptosis of RA-FLS through blockade of the cell cycle in the G2/M phase and a mitochondrial pathway. These data suggest that Tan IIA may have therapeutic potential for RA.

Key words  rheumatoid arthritis; fibroblast-like synoviocyte; Tanshinone IIA; apoptosis; cell cycle

The authors declare no conflict of interest.

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Fig. 1. Chemical Structure of Tanshinone IIA

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cellular signaling in RA have not yet been examined. We performed this study to investigate whether Tan II A treatment of RA-FLS promotes apoptosis through blockade cell cycle and a mitochondrial pathway.

MATERIALS AND METHODS

Culture of RA-FLS   RA-FLS were ordered from Cell Applications Inc. (San Diego, CA, U.S.A.). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, U.S.A.) supplemented with fetal 10% bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, and 100 mg/L streptomycin. Cells were cultured at 37°C in a humidified incubator containing 5% CO₂. RA-FLS obtained from passages three to six were used for experiments.

Assessment of Cytotoxicity by 3-(4,5-Dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl-2,4-sulfophenyl-2H-tetrazolium (MTS) Assay   Tan II A (purity≥98%; HPLC) was purchased from Shanghai Yuanye Biotech Co. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO; Sigma). RA-FLS were seeded in 24-well plates at a density of 2×10⁵ cells/well, and were treated with 2.5, 5, 10, 20, 40, and 80 μg Tan II A or DMEM (control). After 24-, 48-, or 72-h incubation, 2.5 mg/mL MTS solution (Promega) was added to the wells, cells were incubated for 2 h, and absorbance at 490 nm was measured using a microplate reader. MTS assay results informed the selection of Tan II A concentrations that did not adversely affect cell viability for use in subsequent experiments.

Induction of RA-FLS Apoptosis by Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay   After 48 h incubation in various Tan II A concentrations, RA-FLS were detached and placed in serum-free medium for 24 h. Apoptotic events were measured by an DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer’s protocol. Briefly, cells were seeded on glass cover slides previously coated with gelatin 1% in 24 wells plates and cultivated to reach 80% confluency. After proper treatment, culture medium was removed and fixation was performed with 4% neutral formalin in phosphate buffered saline (PBS) for 25 min at 4°C. After washing two times with PBS, the cells were kept in 70% ethanol at −20°C overnight. Cells were subsequently saturated and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Next, 100 μL buffer including 45 μL equilibrium buffer, 5 μL nucleotide Mix, and 1 μL rdT Enzyme was added for 10 min. After washing 15 min with saline sodium citrate (SSC) and two times with PBS, cells were incubated for 4,6-diamidino-2-phenylindole (DAPI) staining at room temperature for 15 min at dark. Acquisition of the images was performed with a fluorescence microscope. Cells in each slide were counted in no less than five fields, and the apoptosis ratio was calculated as the number of positive cells divided by the number of DAPI-staining cells.

Induction of RA-FLS Apoptosis by Flow Cytometry   After 48 h incubation in various Tan II A concentrations, RA-FLS were detached and placed in serum-free medium for 24 h. Apoptosis was analyzed by two-dimensional flow cytometry using annexin V and propidium iodide (PI), according to the manufacturer’s instructions (Keygen), in a Becton Dickinson FACSort flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Dead cells were not gated out; rather, after gating out debris, RA-FLS were gated for an annexin V-positive versus PI-positive contour plot. Numbers in the dot plots represent the percentages of annexin V-positive, PI-positive cells and annexin V-positive, PI-negative cells. Data are expressed as mean±S.D. of three independent experiments.

Analysis of Cell Cycle by Flow Cytometry   After 48 h incubation in various Tan II A concentrations, RA-FLS of all groups were digested and collected using 0.25% trypsin, and then washed with PBS solution. Cells were fixed at 4°C with 75% cold ethanol overnight and washed with PBS solution. The cell density was adjusted to 1×10⁶ cells/mL and the final volume was 100 μL. DNAStain comprehensive dye liquor (500 mL; Sigma, St. Louis, MO, U.S.A.) was added for storage at room temperature in a dark place for 30 min prior to testing with flow cytometry. The DNAStain contained RNase, PI and Triton X-100 at end concentrations of 50 mg/L, 100 mg/L, and 1 mL/L, respectively.

Western Blot Analysis   After 48 h incubation in various Tan II A concentrations, RA-FLS were washed with ice-cold PBS, collected and homogenized with radio immunoprecipitation assay (RIPA) lysis buffer containing 1×PBS, 1% Nonidet P-40, 0.5% sodium deoxocholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors. Total protein was extracted and measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Equal amounts of proteins (50 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.). The following antibodies were used for the Western blot analysis: a mouse monoclonal antibody (1:600) against Bel-2, Bax, Apaf-1, caspase-3, caspase-9, procaspase-3, and procaspase-9 obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and a rabbit polyclonal antibody (1:1000) against β-actin was bought from Cell Signaling Technology (Beverly, MA, U.S.A.). Immunoreactive proteins were detected using the ELC Western blot detection system kit (Amer sham, Braunschweig, Germany). Band intensity was analyzed by densitometry.

**Fig. 2. Cell Viability Rate of Tan II A on RA-FLS Detected by a MTS Assay**   RA-FLS were treated with different concentrations of Tan II A (2.5, 5, 10, 20, 40, and 80 μg), and Con (control group cells were treated with DMEM using the same volume as Tan II A treatment). The cell viability rate were analyzed at three time points (24, 48, and 72 h) using a MTS assay. No changes of RA-FLS viability at concentrations from 2.5 to 20 μg of Tan II A were observed, compared to vehicle-treated control cells (p>0.05). Significant decreased cell viability were noted at both 40 and 80 μg of Tan II A for 24, 48, and 72 h, *p<0.05 versus control.
Cyt-c Release Measurements by Western Blot Analysis

RA-FLS treatment with various concentrations of Tan II A (0, 2.5, 5, 10, or 20 μM) for 48h. Release of Cyt-c from mitochondria to cytosol was measured by Western blot analysis. Cells were harvested, washed once with ice-cold phosphate-buffered saline and gently lysed for 30s in 80mL ice-cold lysis buffer (250 mM sucrose, 1mM ethylenediaminetetraacetic acid (EDTA), 0.05% digitonin, 25mM Tris, pH 6.8, 1mM dithio-
threonine, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 12000 × g for 3 min to obtain the supernatant (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). Supernatants (40 mg) and pellets (40 mg) were electrophoresed on 15% SDS-PAGE and then analyzed by Western blot using anti-cytochrome c antibody (Santa Cruz, CA, U.S.A.).

Statistical Analysis All statistical tests were performed using the SPSS statistical software version 13.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). The level of statistical significance was determined by one-way ANOVA, followed by Bonferroni correction for multiple comparisons. The results are presented as means ± standard error of the mean (S.E.M.) and a p value of less than 0.05 with a 95% CI was considered to indicate significance.

RESULTS

Effects of Tan IIA on Cytotoxicity of RA-FLS To investigate the effect of Tan IIA treatment on cytotoxicity, RA-FLS were incubated with various concentrations of Tan IIA (0, 2.5, 5, 10, 20, 40, or 80 µM) for 24, 48, or 72 h (Fig. 2). No significant suppression of cell viability was observed at up to 20 µM Tan IIA, compared with vehicle-treated control cells (p > 0.05). However, Tan IIA treatment at 40 and 80 µM significantly suppressed RA-FLS viability in a dose-dependent manner (p < 0.05); treatment with 40 and 80 µM Tan IIA decreased cell survival by 88 and 75% after 24 h, 86 and 72% of after 48 h, and 76 and 58% of after 48 h, respectively. As they did not adversely affect cell viability, we employed Tan IIA concentrations of 2.5, 5, 10, and 20 µM for subsequent experiments.

Induction of RA-FLS Apoptosis by Tan IIA We evaluated whether Tan IIA treatment influences the rate of apoptotic death of RA-FLS. Tan IIA treatment at 5, 10 and 20 µM for 48 h increased the number of TUNEL-positive cells, compared to vehicle-treated control cells (p < 0.05) (Fig. 3A). The apoptosis rates of cells treated with 5, 10, and 20 µM Tan IIA were 4.67 ± 1.02%, 7.46 ± 1.55%, and 13.38 ± 2.21%, respectively. We also determined the pro-apoptotic effects of Tan IIA following treatment of RA-FLS using a flow cytometry assay (Fig. 3B). Tan IIA (5, 10 and 20 µM) treatment enhanced the rate of apoptotic cells, compared to vehicle-treated control cells (p < 0.05) (Fig. 3C). These data suggest a pro-apoptotic effect of Tan IIA on RA-FLS.

Induction of RA-FLS Cell Cycle Blockade in G2/M Phase by Tan IIA Flow cytometry was conducted to investigate whether Tan IIA affected the cell cycle of RA-FLS. The results revealed that 48 h following the addition of different concentrations (5, 10, 20 µM) of Tan IIA to RA-FLS, a cell cycle blockade was observed in the G2/M phase compared with the control group (Fig. 4). This suggests that Tan IIA is able to increase the percentage of RA-FLS in the G2/M phase to induce apoptosis of RA-FLS.

Regulation of Mitochondrial Apoptosis Pathway-Related Proteins by Tan IIA We next investigated the effect of Tan IIA treatment on the levels of several mitochondrial apoptosis pathway-related proteins, which are important factors for RA-FLS resistance to apoptotic cell death. RA-FLS were treated with 0 to 20 µM Tan IIA for 48 h and the levels of Bel-2, Bax,
Apaf-1, cytosolic Cyt-c, mitochondrial Cyt-c, procaspase-9, procaspase-3, caspase-9, and caspase-3 were evaluated by Western blotting. As shown in Fig. 5A, the level of antiapoptotic Bcl-2 were reduced by Tan II A (5, 10, and 20 µM), compared with vehicle-treated control cells (p<0.05). The level of the proapoptotic Bax, Apaf-1 proteins were increased by Tan II A (10 and 20 µM), compared with vehicle-treated control cells (p<0.05); both responses were dose-dependent. As shown in Fig. 5B, Tan II A induced Cyt-c release in a dose-dependent manner. Both a significant increase in the amount of Cyt-c in the cytosol and a significant decrease in the mitochondria were detected. As shown in Fig. 5C, expression level of caspase-9 proteins was significantly increased by Tan II A at concentrations of 5, 10, and 20 µM (p<0.05), and those of caspase-3 was increased by Tan II A at concentrations of 10 and 20 µM (p<0.05), compared with vehicle-treated control cells (p<0.05). Expression level of procaspase-9, -3 proteins was significantly decreased by Tan II A at concentrations of 5, 10, and 20 µM (p<0.05). These results indicate that Tan II A treatment induces apoptosis of RA-FLS by regulating the production of pro/anti-apoptotic mitochondrial pathway-related proteins; these effects were also dose-dependent.

DISCUSSION

RA is a chronic inflammatory joint disease characterized
by synovial proliferation and erosion of the affected joint. During the past few decades, drugs therapy for RA were mainly based on two principal approaches: symptomatic treatment with non-steroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs. However, most of the currently available drugs were primarily directed towards the control of pain and/or the inflammation associated with joint synovitis, while did little to interfere with the underlying immunoinflammatory events, and consequently also did little to block the disease progression and reduce cartilage and bone destruction of joint, meanwhile, long-term side-effects and toxicity, high cost and incur hypersensitivity to medications and infections were also existence. Therefore, there was a dramatically growing interest in Chinese traditional medicines in therapy for RA. In fact, Chinese traditional medicines were widely used virtually around the world for treatment inflammatory diseases and in recent few decades considerable advance have been made in both clinical and basic research on the treatment of autoimmune disease. Salvia miltiorrhiza has been used widely in China for the treatment of RA. Danshen (Salvia miltiorrhiza) injection inhibits proliferation of FLS in RA. Tan IIA is a major component of S. miltiorrhiza and exhibits antitumor activities in many human cancer cell lines. Numerous studies demonstrate that Tan IIA also has pro-apoptotic activity, and the present work was undertaken to elucidate the effects of Tan IIA on apoptotic death of FLS derived from RA-affected joints.

Synovial hyperplasia is one of the major pathological characteristics of RA, and leads to marginal bony erosions and resultant joint destruction. Several lines of evidence suggest that RA-FLS are resistant to apoptotic signaling, yielding expansion capabilities similar to tumors. Therefore, previous studies suggested that induction of apoptosis was a promising therapeutic strategy to eliminate RA synovial pannus. In the present study, the result indicated that Tan IIA treatment could effectively induce apoptosis in RA-FLS in a dose-dependent manner. Moreover, Tan IIA has the potent effect of restraining the cell cycle transition of RA-FLS to blockade the cell cycle in G2/M phase, therefore altering the progression of the cell cycle to induce apoptosis.

Apoptosis, or programmed cell death, results from various signaling pathways. The mitochondrial pathway is one of the most important signaling pathways. Bcl-2 family proteins are key regulatory factors of the mitochondrial pathway. We demonstrated that that when RA-FLS had been treated with different doses of Tan IIA for 48 h, Bax and Bcl-2 migrated, the mitochondrial membrane potential increased and Cytc was released. These results indicated that the induction of RA-FLS apoptosis by Tan IIA was closely associated with the mitochondrial pathway. As demonstrated in previous studies, with the stimulation of pro-apoptosis factors, the Bax protein migrated from the cytoplasm to the outer mitochondrial membrane, changing the permeability of the outer mitochondrial membrane to promote the mitochondrial release of Cytc. Release of Cytc leads to the formation of the apoptosis complex by the binding of Cytc to Apaf-1, procaspase-9 and dATP. The dimer complex activates first caspase-9 and then caspase-3, and these activities results in apoptosis. In the present study, We analyzed the changes in Bcl-2, Bax, Apaf-1, cytosolic Cyt-c, mitochondrial Cyt-c, procaspase-9, procaspase-3, caspase-9, and caspase-3 proteins following treatment of RA-FLS with Tan IIA. A significant decrease in Bcl-2, mitochondrial Cyt-c, procaspase-3, and -9 expression and a significant increase in Bax, Apaf-1, cytosolic Cyt-c, caspase-3, and -9 expression were observed when RA-FLS apoptosis occurred. These results suggest that the pro-apoptotic effect of Tan IIA on RA-FLS is realized via the endogenous mitochondrial pathway.

In conclusion, our results demonstrate that Tan IIA stimulates apoptosis of RA-FLS through a mitochondrial pathway in vitro. These findings may lead to the development of Tan IIA as a novel therapeutic agent for RA. We are aware of the limitations of this study, which assessed apoptosis without simulating a pathological condition. Further research to establish the in vivo relevance of these findings is warranted. Moreover, Tan IIA has a variety of pharmacological activities and biological targets; therefore, further study to identify the targets responsible for its pro-apoptotic effects is necessary.

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