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Effect of sinomenine on the expression of rheumatoid arthritis fibroblast-like synoviocytes MyD88 and TRAF6

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ABSTRACT. The effect of sinomenine (SIN) on the toll-like receptor (TLR) signal transduction pathway as well as the expression of myeloid differentiation factor 88 (MyD88) and tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) was investigated. SIN inhibition of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) proliferation and RA cartilage and subchondral bone destruction was also investigated. RA-FLS were cultured *in vitro* and the intracellular alkaline phosphatase (ALP) activity was determined in order to obtain the optimal drug concentration. The rate of cell proliferation was determined. Fluorescence quantitative polymerase chain reaction (PCR) was applied to determine the MyD88 and TRAF-6 gene expression and western blot was used to detect the MyD88 and TRAF-6 protein expression. The ALP activity in the SIN groups was lower than that in the control group, among which the 0.5 mM SIN group had the lowest ALP activity ($P < 0.01$). The rate of RA-FLS proliferation detected by CCK-8 assay in the 0.5-mM SIN group was lower than that in

the control group (P < 0.01) and was the highest 4 days after SIN induction. Gene and protein expression of MyD88 and TRAF-6 were downregulated significantly in the 0.5-mM SIN group compared to that in the control group (P < 0.01). SIN effectively inhibited MyD88 and TRAF-6 expression in RA-FLS, which may be one of the important molecular mechanisms involved in RA treatment and prevention of cartilage and subchondral bone destruction.

Key words: Tumor necrosis factor receptor associated factor-6; Sinomenine; Rheumatoid arthritis; Fibroblast-like synoviocytes; Myeloid differentiation factor 88

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune and chronic disease of unknown etiology characterized by symmetric, multi-joint synovitis and joint structure destruction (Sandoo et al., 2011). With the development of immune genetics research, it has been found that innate immunity plays a significant role in the pathogenesis and progression of RA (Hoffmann et al., 1999). The toll-like receptor (TLR) signal transduction pathway is the classical pathway mediating innate immunity (Kaishoand Akira, 2006). For most TLR pathways, tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) can be activated via MyD88, the most important adaptor protein in the TLR signal pathway. Phosphorylated TRAF6 can induce transforming growth factor-activated kinase-1 (TAK1) to activate the downstream NF-/kB signal pathway so as to induce inflammatory cytokine expressions (Huang et al., 2012). For instance, deletions of TRAF6 result in defects in TLR signals, inhibition of nuclear factor (NF)-kB activation, and decreased production of inflammatory cytokines (Potter et al., 2007). The inflammatory cells release the induced degradation enzymes of pro-inflammatory cytokines and chemokines and induce the activation of osteoclasts, leading to destruction of the bone and cartilage (Shiozawa et al., 2011). Sinomenine (SIN) is an alkaloid monomer with multiple pharmacological effects such as immune inhibition, anti-inflammation, and analgesic action, which is extracted from the traditional Chinese medicine Caulis Sinomenii (Wangand Li, 2011). There is limited research on the inhibitory effect of SIN on RA-FLS MyD88 and TRAF6 expression. In this experiment, RA-FLS was processed using SIN to investigate its inhibitory effect on RA-FLS MyD88 and TRAF6 gene and protein expression, which may be one of the important molecular mechanisms of SIN inhibition of RA-FLS proliferation and RA-induced cartilage and subchondral bone destruction, resulting in joint deformity and stiffness.

MATERIAL AND METHODS

Cell strain, main reagent, and instrument

FLS were obtained from articular capsule disposals of RA patients (clinical diagnosis in line with 2009 American College of Rheumatology Standards) who underwent minimally invasive surgery on the limb joint in the past two years (conforming to the ethical requirements of the experimental specimens), which were provided by the Department of Orthopedics of the Affiliated Hospital of Jilin University. Sinomenine (purity >98%) was purchased from Chengdu Chroma-Biotechnology Co. Ltd.; fetal bovine serum (FBS) from Thermo company (USA); DMEM

(Dulbecco's Modified Eagle Medium) from Gibco (USA); the alkaline phosphatase (ALP), CCK-8 cell proliferation-toxicity assay kit, RNA-OUT total RNA extraction kit, total protein quantitative assay kit (BCA), and radio immunoprecipitation assay (RIPA) cell lysates were from Nanjing Jiancheng Biological Engineering Institute; rabbit anti-human MyD88, TRAF-6, and mouse anti-human beta-actin monoclonal antibody werefrom Santa Cruz (USA); goat anti-rabbit secondary antibody was from Bioworld Technology Corporation; CO_2 incubator was from ESCO (Singapore); Model 550 Elisa was from BLO-RAD (USA); the ABI7500PCR amplification instrument was from Biometra (Germany); the vertical slab electrophoresis unit Mini-Protein III was from BIO-RAD (USA); the Model DYY40B trans-Blotting electrophoresis groove was from Beijing Liuyi Instrument Factory; and the Chemi Imager 5500 gel electrophoresis imaging analysis system was from Alphainno-tech Chemi Imager (USA).

Sinomenine solution formulation

Raw sinomenine powder (molecular weight 401.44 g/mol) was weighed on an electronic balance instrument and then dissolved in DMEM to formulate 10 mL at a concentration of 1 M. The solution was preserved under sterile conditions after filtration with a 0.22-μm millipore filter.

Primary culture of RA-FLS cells

Several small pieces were taken from the articular capsule tissues of RA patients who underwent minimally invasive surgery of the limb joint, placed in DMEM containing 20% FBS,and incubated with 5% CO_2 at 37°C. The culture liquid was renewed once every 2-3 days based on the cell growth. After reaching 90% confluence, cells were passaged using 0.25% trypsin. The culture medium was replaced with DMEM containing 10% FBS and changed once every 2 days. Cells at passage 3 were used for the experiment.

Cell grouping and drug delivery

The logarithmically growing RA-FLS were randomly divided into two groups: the control group, cultured in DMEM with 10% FBS and thedrug group, cultured in DMEM with 10% fetal bovine serum and SIN.

ALP activity assay

ALP, an indicator of the metabolism of living cells, has multiple enzyme-catalyzed reactions in organisms and maintains normal life activities. Through detection of the ATP content, the number, state, and proliferation of cells can be detected. Cells were seeded at a density of 1 x 10^4/mL on a 96-well plate. When cells reached confluence, the culture medium was replaced with the SIN medium. SIN medium with final concentrations of 0.125, 0.25, 0.5, and 1 mM was added to the drug group.The culture medium was added to the control group. The SIN culture medium was changed once every 2 days. After 4-day induction culture, the culture medium was discarded. Then, the ALP activity of the cells was tested using the ALP assay kit to identify the lowest ALP activity as the optimal SIN concentration, which was adopted in the subsequent experiments.

Detection of the cell proliferation rate using CCK-8 assay

The passage 3 RA-FLS were selected and seeded at a density of 1 x 10^4/mL on a 6-well plate with 100 µL medium. Using a total of 60 wells, the control group and 0.5-mM SIN groups were seeded in 5-well increments. The plate was placed in a culture incubator containing 5% CO_2 at 37°C, and 5 wells from each group were taken every 24 h for proliferation detection after cell attachment. During detection, the culture medium was removed from the well, and 100 µL of DMEM (excluding FBS) and 10 µL of CCK-8 were added to each well. After incubation for 2 h, light absorbance at a wavelength of 450 nm was detected using an enzyme-linked immunosorbent assay (ELISA) and was converted to the cell proliferation rate.

Fluorescence quantitative PCR

The RA-FLS and 0.5 mM SIN-processed RA-FLS were collected four days after culture induction at a density of 1 x 10^5/mL. The total RNA was extracted using RNA-OUT and reverse transcribed. Using GAPDH as the reference gene, MyD88 and TRAF-6 mRNA expression were detected using fluorescence quantitative PCR. The PCR reaction conditions included denaturation at 94°C (94°C, 30 s; 60°C, 30 s; 72°C, 30 s) for 5 min for 55 cycles and extension at 72°C for 5 min. Primers were obtained from Gene Bank and designed using Prime 5.0 software, which was synthesized by Takara Biotechnology (Dalian) Co., Ltd. The upstream MyD88 primer was 5'-ATA GGC ACC AGC ATG CAC-3', the downstream primer was 5'-TAG GGT CCT TAC CAG GTA-3', and the amplified product length was 181bp. The upstream TRAF-6 primer was 5'-AGC CAC AAT CCC ATG-3', the downstream primer was 5'-GTC ACG GAA AGG CGC-3', and the amplified product length was 224 bp. The upstream GAPDH primer was ATG 5'-CTC ACC ACA GTC CAT GC-3', the downstream primer was 5'-CAC ATT GGG GGT AGG AAC AC-3', and the amplified product length was 201bp. Using the threshold cycle (CT) value automatically calculated by the computer, the relative expression level of MyD88 and TRAF-6 mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

The RA-FLS and 0.5 mM SIN-processed RA-FLS were washed twice with PBS 4 days after culture induction at a density of 1 x 10^5/mL. After the addition of RIPA lysis buffer, the cells were placed on ice for 15 min. Then, the lysis buffer was collected and stored at -20°C. After three repeated freeze-thaw cycles of the pyrolysis products, centrifugation was performed at 12,000 rpm for 30 min. The protein was quantified according to the BCA-200 Protein Quantitative Kit manual. Sixty micrograms of protein was taken and separatedusing SDS-PAGE gel electrophoresis and then transferred to a PVDF membrane to be sealed with blocking buffer. Rabbit anti-human MyD88 and TRAF-6 antibodies were added at a concentration of 1:200. After being stored overnight at 4°C, the membrane was washed three times with TBSTfor 15 min per wash. After addition of peroxidase-labeled goat anti-rabbit secondary antibody (1:1000), the membrane was stored at 25°C in the dark for 2 h for color rendering. β-actin protein was also detected. The protein expression level = specific protein gray value/β-actin protein gray value.

Statistical analysis

SPSS 13.0 statistical software was employed for data analysis. The ALP activity, cell

proliferation, and MyD88 and TRAF-6 protein expression levels are presented as means ± SD. One-way analysis of variance was utilized to compare the mean among multiple groups.

RESULTS

ALP activity of SIN-processed RA-FLS

The ALP activity detected 4 days after culture induction of RA-FLS in each SIN group (drug concentration: 0.125, 0.25, 0.5, and 1 mM) was 3.286 ± 0.180, 3.102 ± 0.127, 2.716 ± 0.097 and 2.94 ± 0.126, respectively, which were lower than that in the control group ($P < 0.05$). Among all the groups, the 0.5-mM SIN group had the lowest ALP activity compared with the control group (3.875 ± 0.106) ($P < 0.01$). Therefore, a SIN concentration of 0.5 mM was utilized in the subsequent experiments.

Efficacy of SIN on the RA-FLS proliferation rate at different time points

The cell proliferation rates of RA-FLS and 0.5 mM SIN-processed RA-FLS were detected using the CCK-8 assay. Substantial inhibitory effects on cell growth were observed in the 0.5-mM SIN group compared with that in the control group. The differences in growth speed appeared 24 h after cell attachment and became apparent afterwards. On day 4, the RA-FLS cell proliferation in the control group and SIN group reached the highest level and then entered a plateau phase (Table 1).

Table 1. Proliferation rates of fibroblast-like synoviocytes in control group and SIN group (N = 6, mean ± SD, η/%).

Group	Proliferation rate				
	1 day	2 days	3 days	4 days	5 days
Control	41.8 ± 0.3	96.8 ± 0.1	131.9 ± 0.3	172.8 ± 0.0	168.0 ± 0.1
SIN (0.5 mM)	30.4 ± 0.1*	42.7 ± 0.0*	61.7 ± 0.2*	73.2 ± 0.2*	72.8 ± 0.1*

*P < 0.01 versus control group.

Detection of MyD88 and TRAF-6 mRNA expression using fluorescent quantitative PCR

The effect of SIN on MyD88 and TRAF-6 mRNA expression was detected 4 days after induction. A considerable reduction in the expression of MyD88 and TRAF-6 mRNA was observed in the SIN Group ($P < 0.01$, Table 2).

Table 2. Expression of MyD88 and TRAF-6 mRNA in fibroblast-like synoviocytes in SIN group and control group (N = 6, mean ± SD).

Group	MyD88	TRAF-6
Control	3.82 ± 0.36	3.74 ± 0.68
SIN (0.5 mM)	2.24 ± 0.28*	2.11 ± 0.43*

*P < 0.01 versus control group.

Detection of MyD88 and TRAF-6 protein expression

Abundant MyD88 and TRAF-6 protein expression was detected in the control group

using western blotting at four days after culture induction. However, MyD88 and TRAF-6 protein expression in the 0.5 mM SIN drug group decreased. According to the gray analysis, the MyD88 and TRAF-6 protein expression levels in the control group were notably higher than that in the 0.5-mM SIN group ($P < 0.01$, Table 3 and Figure 1).

Table 3. Expression levels of MyD88 and TRAF-6 protein in fibroblast-like synoviocytes in SIN group and control group (N = 6, mean ± SD).

Group	MyD88/β-actin	TRAF-6/β-actin
Control	0.785 ± 0. 012	0.708 ± 0.016
SIN (0.5 mM)	0.228 ± 0. 010*	0.274 ± 0.021*

*P < 0.01 versus control group.

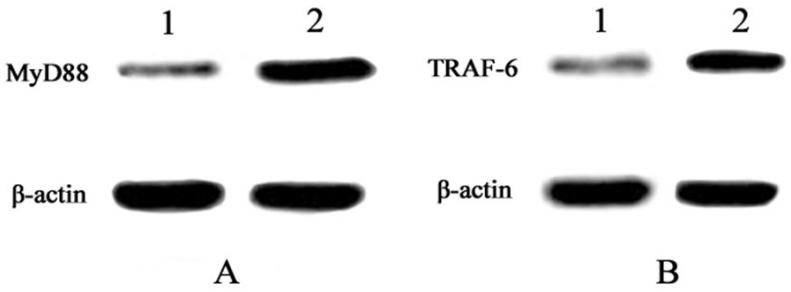

Figure 1. Expression of MyD88 and TRAF-6 proteins of fibroblast-like synoviocytes in SIN and control groups. **A.** MyD88 protein. **B.** TRAF-6 protein. *Lane 1* = SIN group; *lane 2* = control group.

DISCUSSION

RA is a systemic autoimmune disease characterized by symmetric arthritis, and it is the most prevalent of all autoimmune connective tissue diseases. In the course of its pathology, synovial cell proliferation, inflammatory reaction, and subsequent invasion of cartilage and bone tissue result in the destruction of the joints, which eventually leads to joint deformity, rigidity, and functional incapacitation (Feldmann et al., 1996; Szekanecz et al., 2009). Previous studies have revealed the involvement of a variety of inflammatory cytokines and inflammatory reactions. Recently, research has focused on the function of inflammatory factors and signal transduction pathways (Matsuno et al., 2002; Walker et al., 2006). In recent years, the TLR signal transduction pathway and autoimmune diseases have become popular research topics. MyD88 and TRAF6 are important adaptor proteins in the pathway. MyD88 functions mutually with IL-1R-related kinase (IRAK) and is involved in catalyzing the phosphorylation of IRAK. IRAK detached from MyD88 binds to TRAF6. Activated TRAF6 can stimulate NF-kB activation of the mitogen-activated protein kinase (MAPK) signal pathway, resulting in a variety of inflammatory factors transcription and secretion, thereby causing synovial inflammation as well as cartilage and bone destruction (Wesche et al., 1997; Loiarro et al., 2009). Studies have revealed the involvement of the TLR pathway in MyD88 gene defects, animal joint synovitis, and bone damage (Choe et al., 2003). Based on the close relationship between MyD88 and pro-inflammatory factor transcription in the TLR signal pathway,

MyD88 plays an important role in RA progression. In this signal transduction pathway, TRAF6 activation promotes the expression of inflammatory factors, resulting in the constant worsening of RA. For instance, TRAF6 deletions may result in TLR signal defects, inhibition of NF-kB activation, and a reduction in the production of inflammatory cytokines. SIN has many pharmacological effects including immune suppression, anti-inflammatory, and analgesic effects. On the cytokine level, SIN can inhibit the TNF-α synthesis of synovial lining macrophage-like cells, blocking the development of RA synovitis (Li et al., 2004). Sin also effectively inhibits the hyperplasia of synovial macrophage Type A cells and fibrous tissues in Type II collagen-induced arthritis rats and reduces the IL-6 mRNA expression of synovial cells (Wong et al., 2003). It down-regulates IL-1β and IL-8 mRNA expression in human peripheral blood mononuclear cells (Liu et al., 2002) and reduces IL-4 and IL-10 levels in serum and joint immersion liquid of rats with adjuvant-induced arthritis, thereby enhancing the anti-inflammatory effects of cytokines (Yang et al., 2006). In this study, RA-FLS cells were cultured with SIN to detect the gene and protein expression of intracellular MyD88 and TRAF6. The results showed that compared with the control group, MyD88 and TRAF6 gene and protein expression was significantly inhibited in RA-FLS cultured with 0.5 mm SIN. Hence, SIN could potentially delay articular cartilage and subchondral bone destruction and the subsequent joint deformity and stiffness caused by RA-FLS proliferation.

Conflicts of interest

The authors declare no conflict of interest.

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