—Regular Article—

Protective Effect of Sinomenine on Cartilage Degradation and Chondrocytes Apoptosis

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Sinomenine (SIN), an alkaloid extracted from the stem of the Chinese medicinal plant sinomenium acutum, has been used for treating rheumatoid arthritis. But little is known whether SIN has a protective effect on osteoarthritis (OA). In this study, we investigated the protective effect of SIN on IL-1β-induced proteoglycan degradation and apoptosis in rabbit articular cartilage and chondrocytes. Treatment with 10 ng/ml IL-1β increased the level of glycosaminoglycan (GAG) released into the culture media, and up-regulated the activity and mRNA expression of matrix metalloproteinase 13 (MMP-13) and down-regulated the activity and mRNA expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) in cartilage explants, as confirmed by the methods of GAG quantitation, MMP-13/TIMP-1 enzyme-linked immunosorbent assay (ELISA) and real-time quantitative RT-PCR. Treatment with 10 ng/ml IL-1β resulted in marked apoptosis in chondrocytes, as demonstrated by decreased cell viability, occurrence of DNA laddering and increased caspase-3 activity and annexin V binding of phosphatidylserine. However, simultaneous treatment with SIN (10, 50 or 250 μM) inhibited the GAG release and the activity and mRNA expression of MMP-13, and enhanced the activity and mRNA expression of TIMP-1 in a dose-dependent manner in cartilage explants. Furthermore, DNA fragment, caspase-3 activity and apoptosis rate were down-regulated, and cell viability was up-regulated dose-dependently in chondrocytes. Thus, SIN has the protective capacity to antagonize cartilage degradation and chondrocyte apoptosis, which suggest that SIN may act as an agent for pharmacological intervention in the progress of OA.

Key words——sinomenine; cartilage; chondrocyte; apoptosis; matrix metalloproteinase-13; tissue inhibitor of metalloproteinase-1

INTRODUCTION

Osteoarthritis (OA) is one of the most common chronic diseases and a major source of functional disability in elderly persons. With worldwide population ageing, the incidence of OA is rapidly increasing, and it is anticipated that OA will become the fourth leading cause of disability in the coming decades.[1] OA is characterized by progressive degradation of articular cartilage and chondrocytes apoptosis and death.[2] Chondrocytes apoptosis has been identified as a vital reason for cell loss and is now considered as an important factor contributing to degradation of extracellular matrix (ECM) in OA cartilage.[3] Many researches have also demonstrated that enzymatic cleavage by matrix metalloproteinases (MMPs) together with cytokines plays critical roles in the initiation and progression of cartilage destruction.[4,5] An imbalance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) is an determinant of the extent of ECM turnover.[6] Hence, a therapeutic approach regulating the MMPs/TIMPs balance and apoptosis may be effective in the treatment of OA.

Currently, most treatments for OA are directly to targeted symptoms of disease rather than the underlying causes. As therapeutic agents, analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) represent the mainstay.[7] Limitations of these drugs due to great side effects, and lack of specificity and benefit to the underlying cartilage changes suggest a need to develop therapeutic strategies focusing on natural agents or supplements that could modify or reverse the progression of cartilage degradation in the affected joints. Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) (SIN) is an alkaloid extracted from the stem of the Chinese medicinal plant sinomenium acutum.[8] Previous studies have demonstrated that sinomenine has significant anti-inflammatory, analgesic and immunosuppressive properties.[9] However, all these researches mainly focused on the effect of SIN on rheumatic or rheumatoid arthritis, little is known about its effect

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on cartilage and chondrocytes in OA.

The proinflammatory cytokine IL-1β, which is one of the most potent catabolic factors and plays an vital role in the pathogenesis of OA,[10] can induce the enhanced production of MMPs and inhibit the level of TIMPs[11,12] and also suppress synthesis of ECM and increase the apoptosis of chondrocytes thus inhibiting the repair process in cartilage.[5] It is always used on cartilage and chondrocyte to establish an OA model.[13,14] Thus, in the present study, we investigated the potential of SIN to protect rabbit cartilage and chondrocytes against imbalance of MMP-13/TIMP-1 and apoptosis in an IL-1β-mediated OA model.

MATERIALS AND METHODS

Meterials Sinomenine was provided from Hunan Zhengqing Pharmaceutical Group. 1,9-dimethylmethylene blue (DMMB), collagenase were obtained from Sigma (St. Louis, USA). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from GIBCO (Carlsbad, USA). Z-Asp(OCH3)-Glu(OCH3)-Val-Asp(OCH3)-CH2F (Z-DEVD-FMK) was purchased from Calbiochem (La Jolla, USA). MMP-13 and TIMP-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (Minneapolis, USA). Reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from Invitrogen (Paisley, UK). The annexin V fluorescein isothcocyanate (FITC) apoptosis detection kit was purchased from Boehringer Mannheim (Mannheim, Germany). DNA extraction kit and caspase-3 assay kit was obtained from Promega (Madison, USA). All other reagents or drugs were of analytical grade.

Animals New Zealand rabbits (five-week-old, 1200-1400 g) were provided by Peking University Experimental Animal Center. All rabbit experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, 1996).

Cartilage Explants Culture Articular cartilages were obtained from the knee joints of rabbits. Briefly, after the articular surfaces were exposed surgically under sterile conditions, articular cartilage was removed and steeped in complete medium (DMEM, supplemented with heat-inactivated 10% FBS; penicillin 100 U/ml; streptomycin 100 μg/ml). The samples were then rinsed several times with complete medium and incubated for 2 days at 37℃ in a humidified 5% CO_2/95% air incubator for stabilization. The complete medium was replaced with a basal medium (DMEM, supplemented with heat-inactivated 1% FBS, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin). The cartilage explants were treated with 10 ng/ml IL-1β alone or with 10 ng/ml IL-1β+ various concentrations of SIN (10, 50 or 250 μM) or SIN for 72 h. Explants cultured in the absence of IL-1β and SIN were used as controls.

GAG Quantitation in Cartilage Explants Cultures Approximately 50 mg cartilage pieces were placed in 24-well plates and incubated in 10 ng/ml IL-1β with or without various concentrations of SIN (10, 50 or 250 μM). The culture media were harvested 72 h later and stored at −80℃ for the followed assays. The amount of GAG in the media at the end of reaction reflect the amount of proteoglycan degradation. GAG levels in the culture media were determined by the amount of polyanionic material reacting with DMMB, using shark chondroitin sulfate as the standard.[15] Samples were examined spectrophotometrically at 540 nm (Spectramax, Molecular Devices, Sunnyvale, CA, USA). Results were expressed as μg GAG released into the medium per mg wet weight of the cartilage

Activity of MMP-13 and TIMP-1 in Cartilage Explants Cultures The levels of MMP-13 and TIMP-1 activity in the harvested media were evaluated using ELISA kits according to the manufacturer's instructions. Plates were read at 450 nm with microplate reader. Results are expressed as -fold changes, considering the value of control as 1.

Expression of MMP-13 and TIMP-1 mRNA in Cartilage Explants To investigate the mRNA expression of MMP-13 and TIMP-1 in cartilage explants, real-time quantitative RT-PCR was performed. Cartilage explants were lysed with Trizol and total RNA was extracted in accordance with the manufacturer's instructions. First, 2 μg RNA was subjected to RT reaction for 60 min at 37℃ and was stopped by incubation at 95℃ for 5 min. Then 1 μl cDNA was subjected to real-time quantitative PCR to assess MMP-13 and TIMP-1 mRNA expression levels with an ABI 7700 Sequence Detection System, in accordance with the manufacturer's instructions (Applied Biosystems, Warrington, UK). Obtained threshold cycle (CT) values of each sample were normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and relative expression

level was expressed mean value of control group as 1.

Isolation and Culture of Chondrocytes Chondrocytes were obtained from articular of rabbit tibial plateaus and femoral condyle. Cartilage slices were digested in serum-free medium with sequential treatments of 0.2% pronase for 1 h, and then overnight with 0.025% collagenase type II in medium supplemented with 5% FBS in a humidified atmosphere of 5% CO_2 at 37°C with continuous agitation. After removing undigested cartilage using a 70 μm nylon sieve, the chondrocytes were collected by centrifugation, and then cultured at 10^5 cells/ml in DMEM with 10% Fetal Bovine Serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Chondrocytes were treated with 10 ng/ml IL-1β alone or with 10 ng/ml IL-1β+ various concentrations of SIN (10, 50 or 250 μM) or SIN for 24 h. Chondrocytes cultured in the absence of IL-1β and SIN were used as controls.

Cell Viability Assay Cell viability was determined by use of an MTT assay.[16] Chondrocytes were seeded in 96-well plates at 1×10^4 cells per well and grown to 70% confluence in culture medium. The medium was replaced by medium containing various concentrations of SIN and/or 10 ng/ml IL-1β. A total of 5 mg/ml MTT was added to each well after 24 h, and the culture continued to incubate for another 4 h at 37°C. After the medium had been removed, cells and dye crystals were solubilized with 200 μl dimethylsulfoxide (DMSO), and optical density was measured at 570 nm by use of a model ELX-800 microplate assay reader (One Lambda Inc.).

DNA Fragmentation in Chondrocytes DNA was extracted by use of a DNA extraction kit according to manufacturer's instructions after treated with various concentrations of SIN and/or 10 ng/ml IL-1β for 24 h. Briefly, a 10 μl DNA sample was loaded onto 1.5% horizontal agarose gels containing ethidium bromide. Gels were run at 60 V for 1 h submerged in Tris acetic acid-EDTA buffer and DNA fragments were visualized using ultraviolet (UV) illumination.[17]

Chondrocytes Apoptosis After being exposed to various concentrations of SIN and/or 10 ng/ml IL-1β for 24 h, We used annexinV-FITC apoptosis kit to detect chondrocytes apoptosis.[18] In addition, to examine the apoptotic pathways involved in IL-1β-induced apoptosis and the anti-apoptosis mechanism of SIN, chondrocytes also were treated with 100 μM Z-DEVD-FMK (caspase-3-specific inhibitor) alone or Z-DEVD-FMK+IL-1β for 24 h. Chondrocytes were harvested and suspended in binding buffer at a final cell concentration of 10^6 cells/ml. Approximately 10^5 cells were incubated in the dark with annexin V and propidium iodide for 15 min. Then the suspension was analyzed with FACS scan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin V FITC and propidum iodide-related fluorescence was recorded on FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

Caspase-3 Activity in Chondrocytes Caspase-3 activity was detected with Apo-ONETM Homogeneous Caspase-3 Assay kit, according to the manufacturer's instructions. Briefly, chondrocytes were seeded into 96-well plates at 10^4 cells/well. After being exposed to 10 ng/ml IL-1β and/or various concentrations of SIN, 100 μM Z-DEVD-FMK for 24 h, cells were washed with ice-cold PBS. Then, 1 μl Z-DEVD-R110 and 99 μl caspase buffer were mixed to make the homogeneous caspase-3 reagent. A total of 100 μl homogeneous caspase-3 reagent was added to each well. The contents were gently mixed at 300–500 rpm for at least 30 s and incubated for 4 h at room temperature in the dark. The intensity of fluorescence was measured at an excitation wavelength of 498 nm and an emission wavelength of 521 nm with microplate spectrofluorometer (Wallac Victor2TM 1420 Multilabel Counter, USA).

Statistical Analysis Results are expressed as the mean±S.E.M of triplicate values for each experiment. The significance of statistical differences among groups was determined using ANOVA and post hoc test. Values at $p<0.05$ were considered to be statistically significant.

RESULTS

Effect of SIN on Proteoglycan Degradation in Cartilage Explants Cultures To study whether SIN affects proteoglycan degradation, cartilage explants were cultured with 10 ng/ml IL-1β and/or various concentrations of SIN for 72 h. The results showed that untreated cartilage explants also had a basal level of GAG release, however it was also partly blocked by 50 or 250 μM SIN. This indicated that SIN on its own could not induce the enhanced GAG release and has no toxic effect on cartilage in the scope of 0–250 μM (Fig. 1). After incubation with IL-1β, the amount of GAG released into the culture media increased significantly compare to the control group (6.79 ± 0.88 μg/mg *vs.* 1.26 ± 0.21 μg/mg). How-

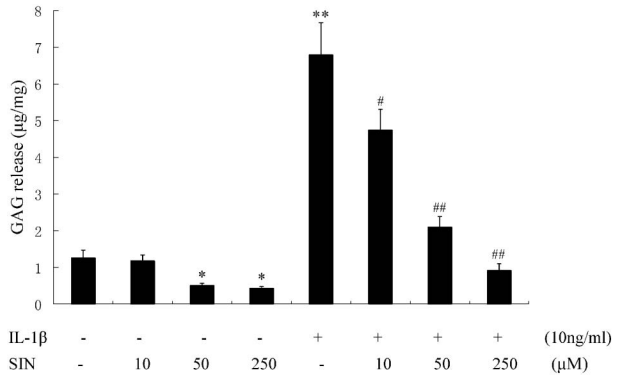

Fig. 1. Effect of SIN on Proteoglycan Degradation in Cartilage Explants Cultures

Cartilage explants were treated with 10 ng/ml IL-1β or/and various concentrations of SIN (10–250 μM) for 72 h. Then GAG concentrations in culture media were determined by the DMMB assay. Results were expressed as μg GAG released into the medium per mg wet weight of the cartilage. $n=8$. Data are mean±S.E.M. $^{*}p<0.05$, $^{**}p<0.01$ as compared to control; $^{\sharp}p<0.05$, $^{\sharp\sharp}p<0.01$ as compared to IL-1β.

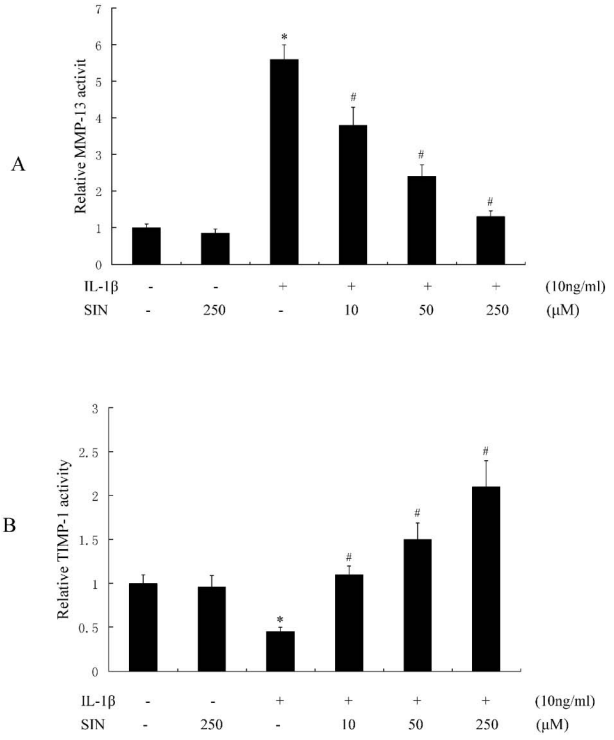

Fig. 2. Effect of SIN on the Activity of MMP-13 (A) and TIMP-1 (B) in IL-1β-induced Cartilage Explants Cultures

Cartilage explants were treated with 10 ng/ml IL-1β or/and various concentrations of SIN (10–250 μM) for 72 h, followed by ELISA. Relative levels of activities were expressed mean value of control group as 1. $n=8$. Data are mean±S.E.M. $^{*}p<0.01$ as compared to control; $^{\sharp}p<0.01$ as compared to IL-1β.

ever, SIN treatment caused a significant and dose-dependent reduction in IL-1β-induced release of GAG (Fig. 1). Thus, the critical observation of the present studies is that SIN appeared to be an effective agent for blocking the IL-1β-induced release of GAG from cartilage explants.

Effect of SIN on the Activity of MMP-13 and TIMP-1 in Cartilage Explants Cultures ELISA clearly showed an increase of MMP-13 activity and a decline of TIMP-1 activity in IL-1β-induced cartilage culture media compared to control. SIN at three concentrations markedly suppressed MMP-13 activity in a dose-dependent manner. Furthermore, TIMP-1 activities were significantly increased dose-dependently in three SIN groups. 250 μM SIN alone had no obvious effect on the MMP-13 and TIMP-1 activity (Fig. 2).

Effect of SIN on the mRNA Expression of MMP-13 and TIMP-1 in Cartilage Explants To evaluate the effect of SIN on IL-1β-induced mRNA expression of MMP-13 and TIMP-1, we treated cartilage explants with IL-1β alone or with IL-1β plus various concentrations of SIN for 24 h. As shown in Fig. 3, analysis of real-time quantitative RT-PCR revealed that the levels of mRNA in cartilages treated with IL-1β alone were about 3.9-fold for MMP-13 and 0.6-fold for TIMP-1 when compared with the level of control. Importantly, IL-1β-mediated increase of MMP-13 mRNA was suppressed by SIN in a dose-dependent manner, and SIN enhanced the production of TIMP-1 mRNA in IL-1β-treated cartilages in a dose-dependent manner. In addition, 250 μM SIN alone had no obvious effect on the mRNA expression of MMP-13 and TIMP-1 (Fig. 3).

Effect of SIN on Cell Viability in Chondrocytes After incubation with IL-1β, approximately 49.3% of chondrocytes underwent death. Treatment with SIN (10, 50 or 250 μM) decreased the cell death rate in a dose-dependant manner; but three concentration of SIN alone did not cause any apparent cytotoxicity (Fig. 4). Thus, SIN showed a good chondroprotective effect on IL-1β-induced cell death.

Effect of SIN on DNA Fragmentation in Chondrocytes Exposed to IL-1β for 24 h, typically pronounced DNA laddering was observed. However, treatment with SIN inhibited IL-1β-mediated DNA laddering, especially at the concentration of 250 μM, which almost completely inhibit DNA fragmentation. In addition, 250 μM SIN alone had no obvious effect on the DNA fragmentation (Fig. 5).

Effect of SIN on Apoptosis in Chondrocytes The anti-apoptotic effect of SIN was also confirmed using annexin V staining to detect the presence of phos-

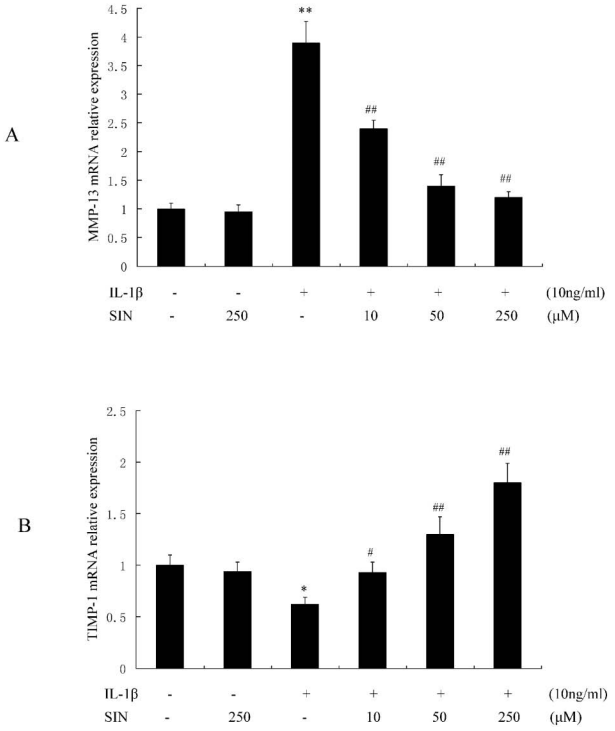

Fig. 3. Effect of SIN on the Gene Expressions of MMP-13 (A) and TIMP-1 (B) in IL-1β-induced Cartilage Explants

Cartilage explants were treated with 10 ng/ml IL-1β or/and various concentrations of SIN (10–250 μM) for 72 h. Then mRNA expression of MMP-13 and TIMP-1 were detected with real-time quantitative RT-PCR. Relative expression levels of mRNA were expressed mean value of control group as 1. $n=8$. Data are mean±S.E.M. $^{*}p<0.05$, $^{**}p<0.01$ as compared to control; $^{\sharp}p<0.05$, $^{\sharp\sharp}p<0.01$ as compared to IL-1β.

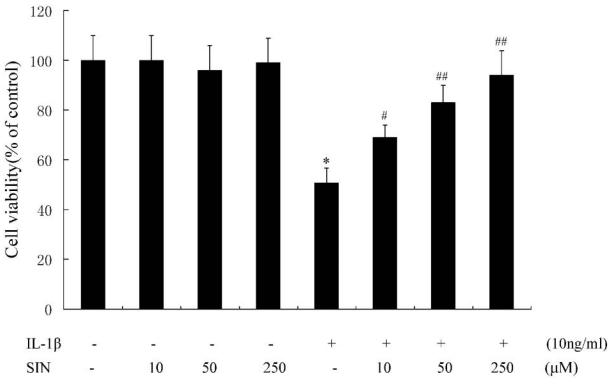

Fig. 4. Effect of SIN on IL-1β-induced Decrease in Chondrocytes Viability

Chondrocytes were treated with 10 ng/ml IL-1β or/and various concentrations of SIN (10–250 μM) for 24 h, followed by MTT method. $n=8$ Data are mean±S.E.M. $^{*}p<0.01$ as compared to control; $^{\sharp}p<0.05$, $^{\sharp\sharp}p<0.01$ as compared to IL-1β.

phatidylserine on the cell membrane. Treatment with IL-1β significantly increased the percentage of apoptotic chondrocytes to 46.7%, but SIN markedly decreased the percentage of cell apoptosis to 25.1%,

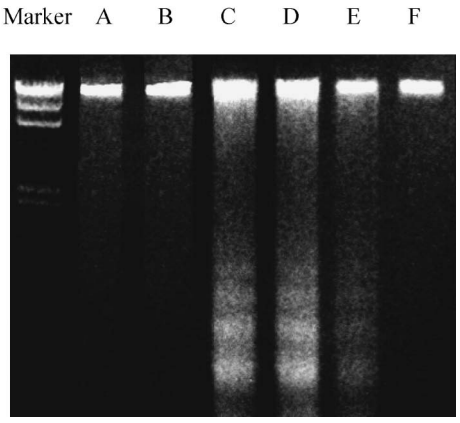

Fig. 5. Effect of SIN on IL-1β-induced DNA Fragmentation in Chondrocytes

Chondrocytes were treated with 10 ng/ml IL-1β or/and various concentrations of SIN for 24 h. Then 10 μl DNA sample of each group was loaded onto agarose gels and run at 60 V for 1 h. DNA fragments were visualized using ultraviolet (UV) illumination. (A) control; (B) treatment with 250 μM SIN; (C) treatment with 10 ng/ml IL-1β; (D) treatment with 10 ng/ml IL-1β and 10 μM SIN; (E) treatment with 10 ng/ml IL-1β and 50 μM SIN; (F) treatment with 10 ng/ml IL-1β and 250 μM SIN.

14.8% and 5.2% at 10, 50 or 250 μM respectively (Fig. 6). To further support these results and also to understand the apoptotic pathways, caspase-3 inhibition experiments was performed. Z-DEVD-FMK alone had no toxic effect on chondrocytes apoptosis (data not shown), however this caspase-3-special inhibitor greatly blocked IL-1β-induced apoptosis, showing that it was a caspase-3 dependent apoptosis.

Effect of SIN on Caspase-3 Activity in Chondrocytes Figure 7 showed that caspase-3 activity was increased by 7.4-fold compared with control after IL-1β incubation. In contrast, chondrocytes treated with 10, 50 or 250 μM SIN showed a significant decrease in caspase-3 activity compared with IL-1β-treated cells at the same time point. And Z-DEVD-FMK also completely inhibited the caspase-3 activity induced by IL-1β. Combined with the results of chondrocytes apoptosis, these data exhibited that SIN did block chondrocytes apoptosis by inhibiting IL-1β-induced activation of caspase-3.

DISCUSSION

Sinomenium acutum has been used for the treatment of joint pain and arthritis by Chinese medical doctors for over 2000 years.[19] SIN is the most pharmacologically active compound in sinomenium acutum in terms of anti-inflammatory, analgesic, anti-arthritic, and immunosuppressive properties, and is a promising drug that has been used for treating rheu-

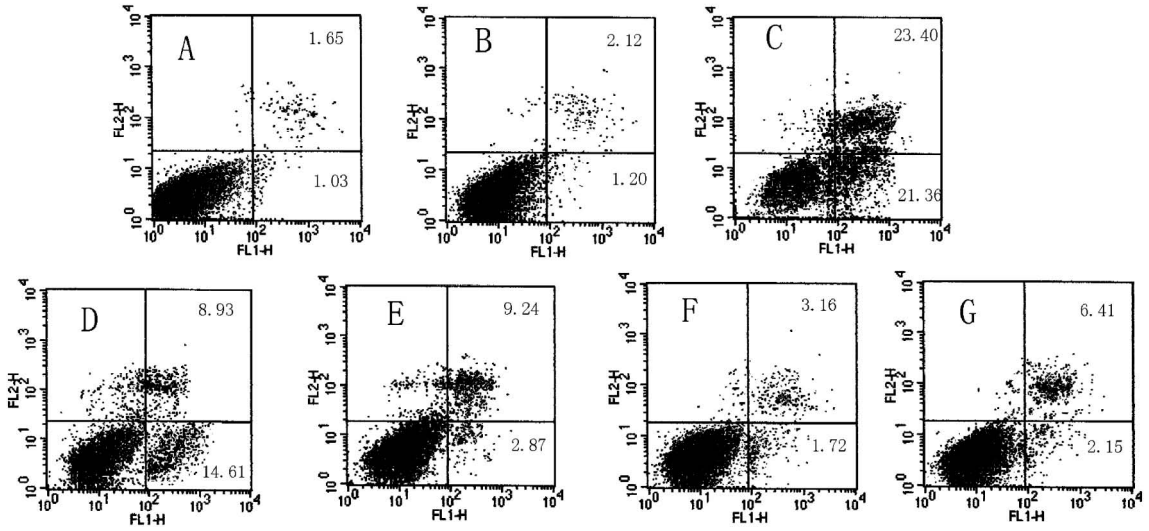

Fig. 6. Effect of SIN on IL-1β-induced Apoptosis in Chondrocytes

Chondrocytes were treated with various concentrations of SIN and 100 μM Z-VAD-FMK for 24 h in the presence of 10 ng/ml IL-1β, followed by flow cytometric analysis. Z-DEVD-FMK is a kind of caspase-3-specific inhibitor, which is used to examine the apoptotic pathways involved in IL-1β-induced apoptosis and the anti-apoptosis mechanism of SIN. (A) control; (B) treatment with 250 μM SIN; (C) treatment with 10 ng/ml IL-1β; (D) treatment with 10 ng/ml IL-1β and 10 μM SIN; (E) treatment with 10 ng/ml IL-1β and 50 μM SIN; (F) treatment with 10 ng/ml IL-1β and 250 μM SIN; (G) treatment with 10 ng/ml IL-1β and 100 μM Z-DEVD-FMK. Numbers in each quadrant indicate the percentages of chondrocytes in that particular quadrant with respect to total. The percentage data were obtained from eight independent experiments.

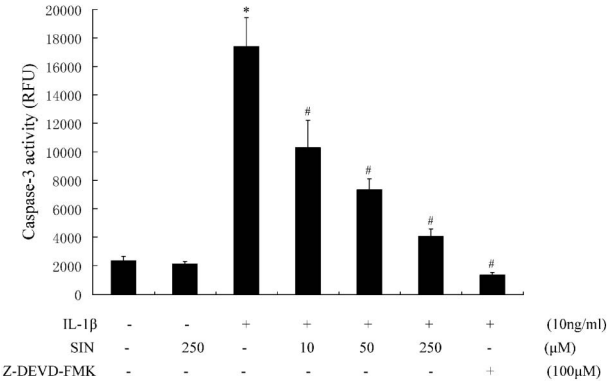

Fig. 7. Effect of SIN on the Caspase-3 Activity in IL-1β-induced Chondrocytes

Chondrocytes were treated with various concentrations of SIN and 100 μM Z-VAD-FMK for 24 h in the presence of 10 ng/ml IL-1β. Then caspase-3 activity in chondrocytes were measured with caspase-3 assay kit. $n=8$. Data are mean$\pm$S.E.M. $^{*}p<0.01$ as compared to control; $^{\sharp}p<0.01$ as compared to IL-1β.

matoid arthritis for its excellent therapeutic effects and litttle side effects.[8,20] Previous mechanistic studies revealed that SIN could protect the arthritic joints by decreasing mRNA expression of TNF-α and IL-1β, inhibiting production of prostaglandin E2 (PGE2) and nitric oxide (NO), and so on.[8,21] Much of the effects of SIN are related to the etiological factors of OA, but up to now, little is known whether SIN has a protective effect on OA. In the present study, we demonstrated the obvious effectiveness of SIN in protecting rabbit cartilage and chondrocyte against cartilage degradation and chondrocyte apoptosis in an IL-1β-mediated OA model. These cartilage-protective and anti-apoptotic effects of SIN appeared to result from its ability to down-regulate expression of MMP-13 and up-regulate expression of TIMP-1, and inhibit the activity of caspase-3.

The pathogenesis of OA involves multiple etiologies, including aging, obesity, mechanical, genetic and biochemical factors.[22] Although much progress has been made on identifying the molecular events responsible for OA, it is still not fully understood,[23] and there are also no effective drugs that can modify or reverse cartilage destruction and restore the functional integrity of a joint. It is widely accepted that OA is characterized by the destruction of articular cartilage due to an imbalance between biosynthesis and degradation of ECM. Inhibition of cartilage degeneration is a priority in the effective treatment of OA. In this study, we confirmed that proteoglycan was markedly degraded by IL-1β and SIN dose-dependently inhabited IL-1β-induced GAG release in cartilage explants cultures. These results suggest that SIN can be effective for reduction of cartilage degradation.

It has been reported that imbalance between MMPs and TIMPs is determinant in the degradation of ECM. MMPs are a large group of enzymes in charge

of tissue remodeling as well as the destruction of cartilage in arthritic joints due to their ability to degrade a wide variety of ECM components.[24] Excessive MMPs production is a key mechanism by which cartilage matrix destruction occurs during the development of OA.[25] Among various MMPs, MMP-13 is one main member of the MMPs superfamily and is of particular importance because it is obviously elevated in osteoarthritic synovial fluid and can cleave proteoglycan and type II collagen more efficiently.[26,27] Endogenous TIMPs which bind tightly and irreversibly to active MMPs play an important role in controlling tissue breakdown by blocking MMPs activity.[28] TIMP-1, a glycoprotein from chondrocytes and synovial cells, inhibits MMP activation by forming high-affinity complexes with them at a stoichiometry of 1:1. In this study, we found an imbalance between MMPs and TIMPs characterized by the increase of activity and mRNA expression of MMP-13 and the decrease of activity and mRNA expression of TIMP-1 in IL-1β-induced cartilage explants, and determined that SIN has the protective effect to prevent cartilage degradation by reversing such an imbalance through MMP-13 suppression and TIMP-1 induction.

Besides its direct beneficial effect on cartilage tissue, we also investigated the inhibitory effect of SIN on chondrocyte apoptosis. Chondrocytes play a crucial role in maintaining cartilage homeostasis as the sole cell type in cartilage. In the previous researches, apoptosis has been identified as a critical reason for cell loss in aging OA cartilage. Outer stimuli such as IL-1β can initiate apoptosis and may converge on the caspase pathway to execute the final phase of the apoptotic process.[29] Caspase-3 is the vital downstream member of the caspase cascade and acts as a central effector to execute the final phase of the apoptotic process.[30] Several studies showed expression of caspase-3 is of high level in OA cartilage.[31] Nuttall *et al*. demonstrated that caspase inhibitors rescue chondrocytes from apoptotic death after exposure to the damaging agent.[32] Thus, substances that can inhibit the activity of caspase-3 might protect chondrocytes from apoptosis. In our study, we found the specific caspase-3 inhibitory peptide, Z-DEVD-FMK, can greatly block chondrocytes apoptosis, which indicate that caspase-3 take part in IL-1β-induced apoptosis. And SIN markedly decreased IL-1β-induced apoptotic chondrocytes and DNA fragment in a dose-dependent manner, accompanied with obviously inhibiting the enhanced activity of caspase-3 in IL-1β-treated chondrocytes in a dose-dependent manner. So, SIN might protect chondrocytes against apoptosis by directly inhibiting the activity of caspase-3.

Proinflammatory cytokine IL-1β can increase the synthesis of PGE2 and produce a variety of reactive oxygen species (ROS) such as NO, superoxide and peroxynitrite.[33,34] These mediators stimulate the chondrocyte to produce proteolytic enzymes, including aggrecanases and matrix metalloproteinases (MMPs) that contribute to destruction of the cartilage matrix. Moreover much evidence suggests that these mediators including hyperproduction of ROS have been proposed to be necessary for apoptosis of chondrocytes.[35] Much of the effects of SIN, such as anti-inflammation, inhibition of PGE2 and NO and removal of free radicals, are related to the pathogenetic factors of OA. Our results indicated that SIN showed the cartilage and chondrocytes protective effect through prevention of GAG degradation by increase of TIMP-1 activity and decrease of MMP-13 activity, and inhibition of apoptosis by down-regulation of caspase-3 activity in an IL-1β induced OA model. In view of the critical roles of PGE2, NO, and ROS in the pathogenesis of OA, it is strongly assumed that SIN may decrease the MMP-13 and caspase-3 activity by blocking the production of these important mediators. Besides, some researches proved that MAPK pathways (p38 and JNK) as well as the transcription factor NF-κB pathway are involved in IL-1-induced expression of MMP-13 in chondrocytes.[36] So, in the future, we will investigate the molecular mechanism of SIN to inhabit MMP-13 expression and chondrocytes apoptosis, and confirm the effect of PGE2, NO, ROS as well as MAPK and NF-κB pathways in the process. We suggest that SIN could act as an agent for pharmacological intervention in the progress of OA.

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