Full Length Research Paper

Sinomenine suppresses expression of interleukin-1beta-induced matrix metalloproteinases in human osteoarthritic chondrocytes

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Sinomenine (SN) is an alkaloid extracted from the medicinal plant, *Sinomenium acutum* which has been utilized to treat rheumatoid arthritis (RA) in China for centuries. Previous studies showed that SN had anti-inflammatory and anti-rheumatic effects. The aim of the present study was to investigate the effect of SN on interleukin-1beta (IL-1beta)-induced expression of major cartilage damaging proteases in human osteoarthritic chondrocytes. Human osteoarthritic chondrocytes were obtained from the knee of patients undergoing total knee arthroplasty surgery due to OA. Human osteoarthritic chondrocytes and SW1353 cells were plated in monolayer culture. Cell viability was evaluated using MTT assay. SN was added before stimulation with 10 ng/mL human recombinant IL-1beta. mRNA expression of MMPs was measured by RT-PCR. Protein expression was detected by Western blotting. SN suppressed IL-1beta-induced MMP-1, MMP-3, MMP-9, and MMP-13 mRNA and protein expression in SW1353 cells and human osteoarthritic chondrocytes. SN inhibited the catabolic effect of IL-1beta by interception of proteolytic enzymes expression. The cartilage catabolic proteases inhibitory ability and the well-known anti-inflammatory activity make SN a potentially novel therapeutic agent for OA.

Key words: Sinomenine, interleukin-1, osteoarthritis, chondrocytes, matrix metalloproteinases.

INTRODUCTION

Osteoarthritis (OA) is one of the most common disabling diseases affecting millions of people worldwide. Patients with OA suffer from joint pain, limitation in joint function, destruction and extensive loss of articular cartilage. More and more evidence showed that OA was the result of joint inflammation at the molecular level (Attur MG et al., 2002). Interleukin-1(IL-1) was the main cytokine responsible for the signs and symptoms present in patients with OA, which upregulated the expression of metalloproteinases (Pelletier JP et al., 2006; Attur MG et al., 2000). Matrix metalloproteinases (MMPs) and aggrecanases (a disintegrin and metalloproteinase with thrombospondin motif, ADAMTS) that comprise two families of proteolytic enzymes play an important role in

the destruction of cartilage main structural components in OA (Clark and Parker, 2003).

Nowadays, most pharmacological interventions for OA are still directed to the treatment of signs and symptoms. The nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed drugs worldwide, which generally decrease pain, stiffness, and thereby improve joint function. However, NSAIDs do not reverse cartilage destruction or restore functional integrity to the OA joint (Steinmeyer and Konttinen, 2006; Andreas et al., 2009). The side effects of NSAIDs have limited their use in many patients. Recently, great effort was made to develop disease-modifying OA drugs (DMOADs) (Pelletier and Martel-Pelletier, 2007; Abramson and Yazici, 2006). DMOADs are directed to pathogenic events that drive progressive joint destruction, thereby provide long-term symptomatic relief and joint function improvement in patients with OA..

Sinomenine (SN) is an alkaloid extracted from the

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Table 1. Primers for RT-PCR.

Gene	Accession number	Forward primers (5'-3')	Reverse primers (5'-3')	Size (bp)	Cycles
MMP-1	NM_002421	AAAGGGAATAAGTACTGGG	GTTTTTCCAGTGTTTTCCTCAG	137	25
MMP-3	NM_002422	GTTCCGCCTGTCTCAAGATG	TTGGAGGGAAACCTAGGGTG	406	24
MMP-9	NM_004994	AGACCTGAGAACCAATCT	GGCACTGAGGAATGATCTAA	1146	26
MMP-13	NM_002427	GATAAAGACTATCCGAGAC	CGAACAATACGGTTACTC	146	25
GAPDH	NM_002046	ACAACAGCCTCAAGATCATCA	GTCCAGGGGTCTTACTCC	670	25

medicinal plant, *Sinomenium acutum*. SN exhibited a wide range of pharmacological actions, including anti-inflammation (Liu et al., 1994), arthritis amelioration (Liu et al., 1996a), immunosuppression (Vieregge B et al., 1999; Feng et al., 2007), anti-angiogenesis (Kok et al., 2005), and protection against hepatitis induced by lipopolysaccharide (Kondo et al., 1994). The *in vitro* studies showed that SN potently suppressed the proliferation of rat synovial fibroblasts, murine, and human lymphocytes (Liu et al., 1994; Liu et al., 1996b). Moreover, SN inhibited mRNA expression of tumor necrosis factor-α (TNF-α) and Interleukin-1β(IL-1β)in peritoneal macrophages and synoviocytes in animal model for rheumatoid arthritis (RA) (Liu et al., 1994; Wang et al., 2005), and showed therapeutic efficacy in patients with RA (Yamasaki, 1976). It is known that at the molecular level, joint tissues in OA are the site of active production of cytokines and inflammation mediators. Human osteoarthritic chondrocytes overproduced metalloproteinases which were one of the key protagonists in the destruction of particular cartilage in OA (Kobayashi et al., 2005). Therefore, the present study investigated whether SN inhibited IL-1β-induced cartilage damaging proteases expression in human osteoarthritic chondrocytes.

MATERIALS AND METHODS

Chemicals

Sinomenine (purity > 99% by HPLC) was obtained from the State Institute for the Control of Pharmaceutical and Biological Products.

Cell culture and treatment

Human osteoarthritic cartilage was obtained with informed consent from patients with OA at the time of knee arthroplasty. All patients with OA met criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (Altman et al., 1986). The study protocol was approved by Medical Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University. Cartilage slices were rinsed 5 times with saline, and minced. Then, human osteoarthritic chondrocytes were released by trypsin, followed by 2 mg/mL type II collagenase (Worthington, Lakewood, NJ, USA) overnight in DMEM/Ham's F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). Human osteoarthritic chondrocytes were plated in high density monolayer culture in DMEM/Ham's F-12 containing 10% FBS. SN was added at the final concentration of 1 mmol/L,

3 mmol/L, or 5 mmol/L 30 min before stimulation with 10 ng/mL human recombinant IL-1β (PeproTech, Rocky Hill, NJ, USA) for 24 h. The human chondrosarcoma cell line SW1353 (ATCC; Manassas, VA, USA) was cultured in DMEM/Ham's F-12 containing 10% FBS, when treated with IL-1β, is an appropriate model for human osteoarthritic chondrocytes (Vincenti MP and Brinckerhoff CE, 2001). SW1353 cells were treated in the same manner as human osteoarthritic chondrocytes.

Cell viability

Cell viability was evaluated in a 96-well culture plate using the 3-[4,5-dimethylthiazol-2yl] 2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, USA). Briefly, after starvation in serum-free DMEM/Ham's F-12 for 24 h, cells were treated with SN. MTT was added into each well 20 h later followed by incubation for an additional 4 h. Supernatants were removed and 200 μL of DMSO was added. After insoluble crystals were completely dissolved, the optical density was read at a wavelength of 450 nm.

RNA extraction and RT-PCR

Total RNA was extracted using the TRIzol Reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. For the RT reaction, 2 μg RNA was combined with 0.5 μg oligo (dT) 15 (15 μL total volume). The mixture was incubated at 70°C for 5 min and placed on ice. Then 5 μL 5× M-MLV (moloney murine leukemia virus) reaction buffer, 1.25 μL 4× dNTP (10 mmol/L), 1 μL M-MLV Reverse Transcriptase (Promega, Madison, WI, USA; 200 U/μL), 0.625 μL RNaseOUTTM (40 U/μL) was added (25 μL total volume). The tube was incubated at 42°C for 90 min and then at 75°C for 10 min for termination. The PCR reaction was performed in the presence of Taq DNA polymerase, dNTP mix, PCR buffer and primers (all from Invitrogen, USA). After denaturation at 94°C for 3 min, the samples underwent the amplification (30 s at 94°C, 30 s at 50°C for MMP-1, -13 and GAPDH, or 55°C for MMP-3, or 52°C for MMP-9, and at 72°C 30 s for MMP-1, -3, -13, or 90 s for MMP-9, or 60 s for GAPDH) with a 15 min extension at 72°C following the last cycle. PCR primers were listed in Table 1 (Kobayashi et al., 2005; Pérez et al., 2005). PCR products were electrophoresed on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference to demonstrate equal gel loading.

Western blot analysis

The secreted proteins in conditioned medium of cultured cells were concentrated by Centricon YM 10 filter devices (Millipore, Bedford, MA). Cells were collected and lysed with radio immuno precipitation assay (RIPA) buffer. The protein concentration was determined by Bradford assay. The samples containing equal amounts of total
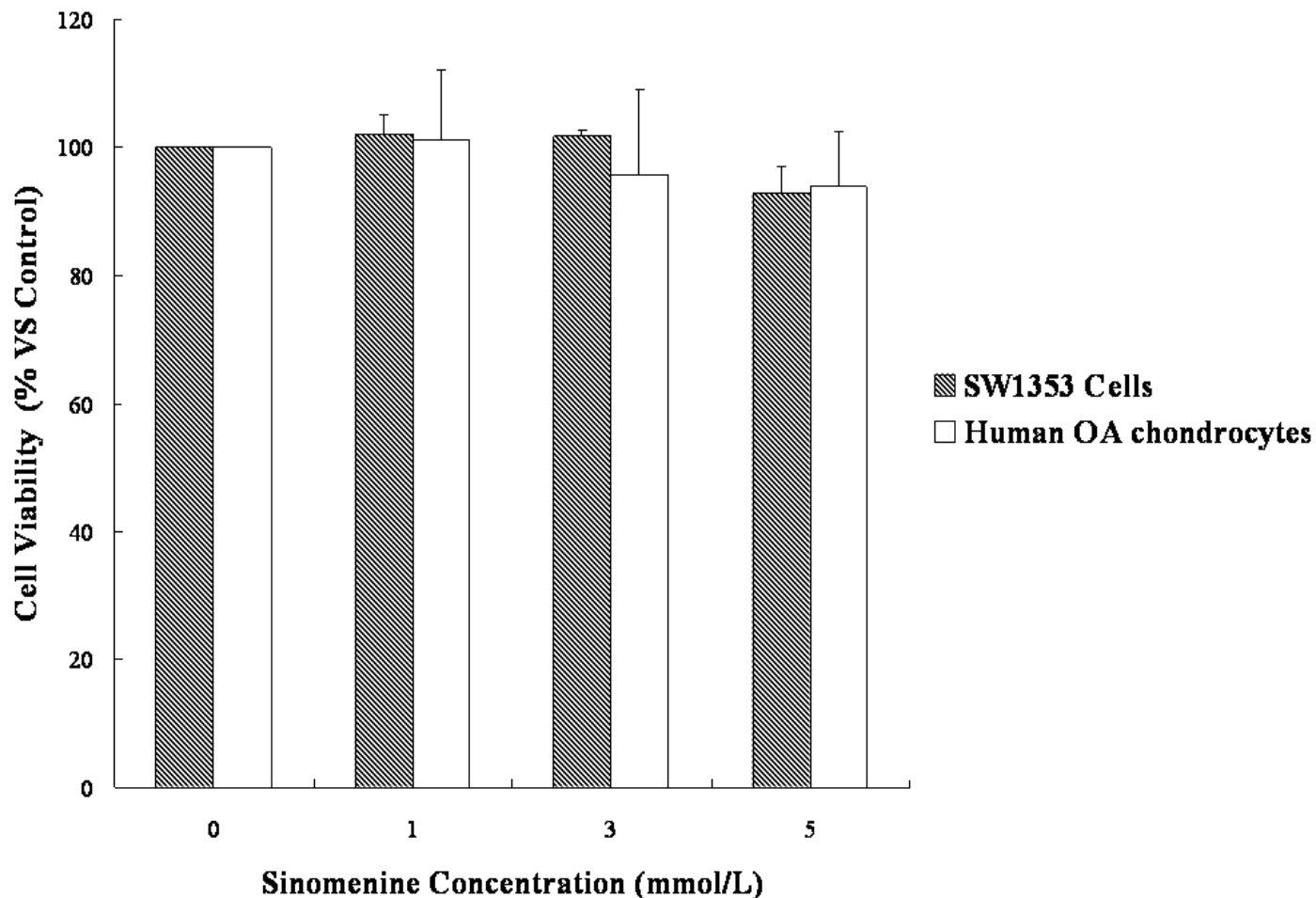
Figure 1. Effect of SN on SW1353 cells and human osteoarthritic chondrocytes viability. Quiescent SW1353 cells and human osteoarthritic chondrocytes were either treated with saline (0 mmol/L) or exposed to the indicated doses of SN for 24 h, incubated with the MTT reagent. The precipitate was solubilized, and subjected to spectrophotometry. Data are expressed as percentages with respect to control conditions, and represent the mean ± SD of at least three experiments. The viability of SW1353 and human osteoarthritic chondrocytes was not affected by 1 - 5 mmol/L SN. P > 0.05.

protein in conditioned medium and cell lysate were detected by western blot. Protein was resolved by 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Pall, NY, USA). The proteins were detected with specific antibodies against MMP-1 (AB19140, Chemicon, USA), MMP-3 (AB19150, Chemicon, USA), MMP-9 (#2270, Cell Signaling Technology, USA), MMP-13 (AB19056, Chemicon, USA), GAPDH (sc-32233, Santa Cruz, USA) overnight at 4°C, followed with 3 times of washes in TBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were detected using enhanced chemiluminescence detection and exposed to X-ray film (Fujifilm, JP).

Statistical analysis

All experiments were performed at least three times. Data were expressed as the mean ± SD. The Student's t-test was used to make a statistical comparison between groups. The level of significance was set at $P < 0.05$. Statistical analysis was performed by using SPSS. version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Sinomenine has no adverse affect on the viability of SW1353 cells and human osteoarthritic chondrocytes

We first analyzed the effects of SN on cell viability by MTT assay. The results showed that cell viability of SW1353 cells and human osteoarthritic chondrocytes was not affected after treatment with various concentrations of SN (0, 1, 3, and 5 mmol/L) for 24 h (Figure 1).

Sinomenine inhibits IL-1β-induced matrix metalloproteinases mRNA and protein expression in SW1353 cells

SW1353 cells were pretreated with SN for 30 min alone or further treated with IL-1β-. Conditioned medium and cells cultured in monolayer were harvested after 24 h.
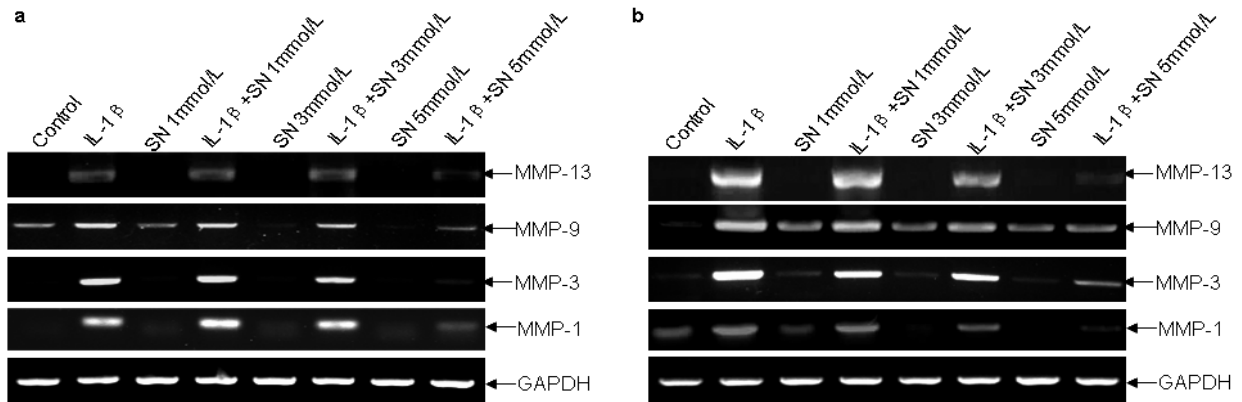
Figure 2. Expression of MMP-1, -3, -9, and -13 mRNA. (a) Quiescent SW1353 cells were pretreated with the indicated doses of SN for 30 min alone or followed by the treatment with IL-1β for 24 h. (b) Confluent human osteoarthritic chondrocytes were treated in the manner as SW1353 cells.
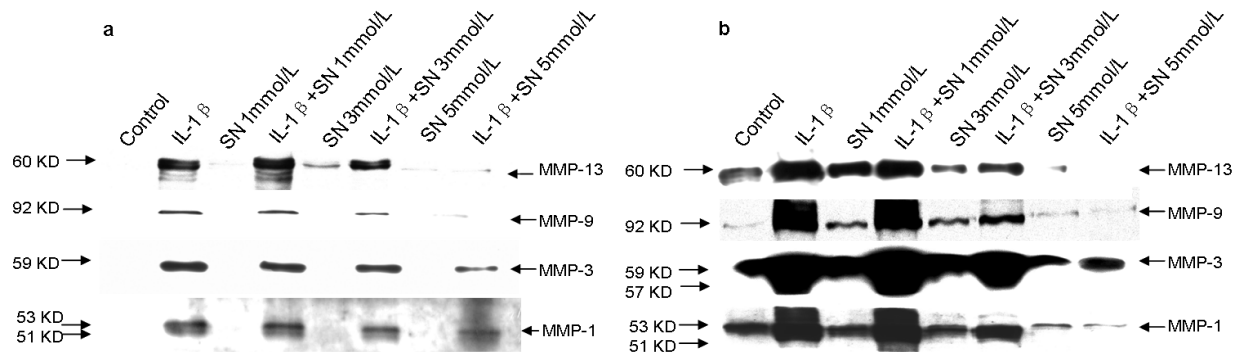

Figure 3. Expression of MMP-1, -3, -9, and -13 in conditioned medium. (a) Human chondrosarcoma cells (SW1353) were maintained for 24 h in serum-free medium, pretreated with the indicated doses of SN for 30 min alone or stimulated further with IL-1β 24 h later, Conditioned medium was collected, concentrated and measured by western blot analysis. (b) Human osteoarthritic chondrocytes were treated in the same manner as SW1353 cells.

SN suppressed mRNA expression of MMP-1 and MMP-13 at 5 mmol/L, MMP-3 at 3 and 5 mmol/L, MMP-9 in a dose-dependent manner by RT-PCR (Figure 2a). Furthermore, MMP-1, -3, -9, and -13 proteins in conditioned medium were gradually down regulated with the increase of SN by western blot (Figure 3a). Although MMP-3 protein in cells was abundant, MMP-3 expression was upregulated after treatment with IL-1β-, and its production was inhibited by SN at 3 and 5 mmol/L (Figure 4a).

Sinomenine diminishes IL-1β-induced matrix metalloproteinases mRNA and protein expression in human osteoarthritic chondrocytes

Besides SW1353 cells, we investigated whether SN had inhibitory effect on the expression of MMPs in human osteoarthritic chondrocytes. The result showed that SN reduced mRNA expression of MMP-1, -3, -9, and -13. Inhibition of IL-1β--induced MMPs mRNA expression was observed when treated with 3 mmol/L SN. SN at 5 mmol/L strongly suppressed the expression of MMPs mRNA induced by the cytokine (Figure 2b). SN down regulated IL-1β-induced protein expression of MMP-3 at 3 mmol/L and 5 mmol/L in cells (Figure 3b), MMP-1, -3 and -9 at 3 and 5 mmol/L, MMP-13 in a dose-dependent manner in conditioned medium (Figure 4b).

DISCUSSION

OA is a painful and debilitating disease. Up to now, no medicine could stop cartilage destruction in OA. NSAIDs exert limited efficacy. Arthroplasty becomes the only acceptable option for late-stage OA patients to relieve disabling symptoms. More and more patients have to face revision surgery once or even twice in their lives. It is
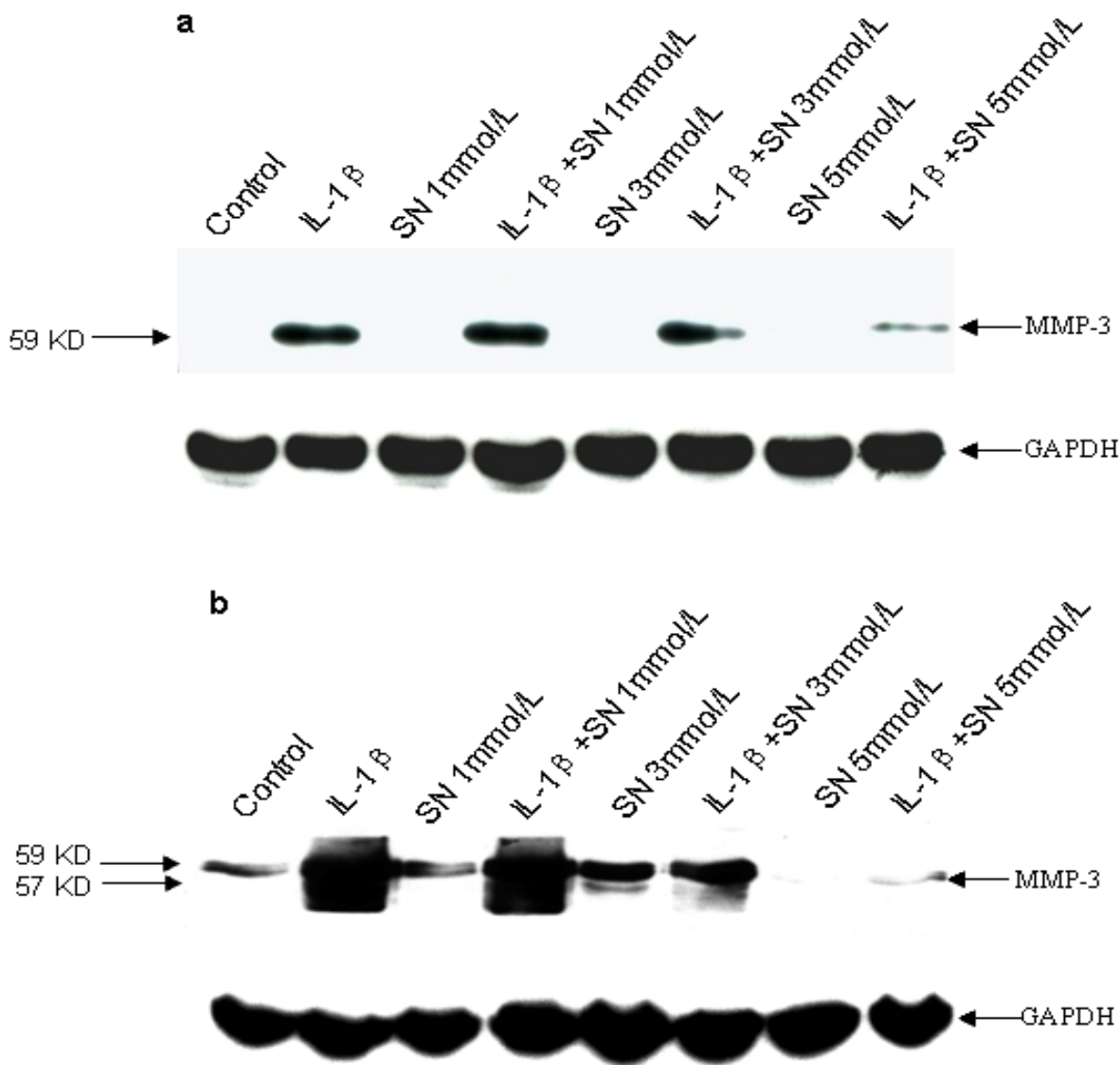
Figure 4. Expression of MMP-3 protein in cells. The inhibition of IL-1β-induced MMP-3 protein expression by SN in (a) SW1353 cells and (b) human osteoarthritic chondrocytes by western blot. The cells were grown to confluence, made quiescent for 24 h, and pretreated with the indicated doses of SN for 30 min alone or stimulated with the cytokine for 24 h.

well known that proinflammatory cytokines including IL-1β and TNF-α promote cartilage degradation and are responsible for clinical manifestations of OA. MMPs induced by proinflammatory cytokines are direct proteolytic enzymes damaging cartilage (Clark and Parker, 2003; Dickinson et al., 2003). Our study showed that SN effectively suppressed IL-1β-induced MMPs expression in SW1353 cells and human osteoarthritic chondrocytes.

Studies demonstrated that pain management with NSAIDs was superior to that with simple analgesics (Pincus et al., 2004). Although the gastrointestinal safety was improved with the use of selective cyclooxygenase-2 inhibitors, serious cardiovascular risks with all NSAIDs

have made any incident adverse event being considered a significant problem (Jenkins and Seligman, 2005). Therefore, a growing number of researchers are searching for alternative agents that modify underlying pathological condition of OA with fewer side effects.

Medicinal plants are one of the important sources to develop new anti-OA drug. SKI306X was found to protect articular cartilage from degradation in the experimental OA model (Choi et al., 2002), and clinical trial suggested it had good pain relief effect in patients with RA (Song et al., 2007). Triptolide, a purified derivative of *Tripterygium wilfordii* Hook F, inhibited the proinflammatory cytokine-stimulated expression of major proteases responsible for cartilage degradation (Liacini et al., 2003). SN is the

purified extract of Chinese herb *S. acutum* which has been used to treat rheumatic diseases in China for over two thousand years (Yamasaki, 1976). Based on its molecular structure, SN belongs to the family of morphinans (Qian et al., 2007). Clinical trials in China demonstrated that SN had good pain relief efficacy, and improved joint function in patients with RA (Ke and Xiu, 1986). SN decreased mRNA expression of TNF-α and IL-1β by inhibiting the NF-κB binding activity in animal model for RA (Wang Y et al., 2005). SN significantly inhibited proliferation of IL-1β-activated synovial sarcoma cells and suppressed gene expressions which mediate inflammation, cell adhesion, proliferation, apoptosis and angiogenesis (Li et al., 2006).

Evidence indicated that increased cytokine production in OA was observed in synovial membrane, cartilage and subchondral bone. In this study, proinflammatory cytokine IL-1β was used to mimic catabolic action on human osteoarthritic chondrocytes. In SW1353 and human osteoarthritic chondrocytes, four MMPs mRNA and protein were expressed after the exposure to 10 ng/mL IL-1β. The metalloproteinase family includes over 20 members. MMP-1 and MMP-13 were two collagenases which primarily involved in type II collagen degradation in OA (Wu et al., 2002). MMP-3 played a primary role in the degradation of proteoglycan (Little et al., 1999). MMP-9 was widely expressed and broke down denatured interstitial collagen, collagens IV and V (Rannou et al., 2006). We found that SN down regulated IL-1β-induced MMP-1, -3, -9, and -13 mRNA and protein expression in SW1353. Moreover, the expression of MMPs in human osteoarthritic chondrocytes was inhibited in the same manner as in SW1353. This indicated that the repression of mRNA transcription caused the down regulation of MMPs. SN with 1 mmol/L or higher concentration could only be achieved by intra-articular injection in vivo. Higher administration skill was required, and this also may raise concern about possible complications. Although no adverse effects on the *in vitro* osteoarthritic chondrocyte viability were observed, such viability and cytotoxicity need to be validated in the *in vivo* models. In this study, the effect of SN on the inhibition of MMPs indicated that this alkaloid may intercept cartilage degradation process in the pathogenesis of OA. Further studies are needed to understand how SN inhibits IL-1β-induced MMPs expression in human osteoarthritic chondrocytes.

In summary, we demonstrated that SN inhibited IL-1β-induced expression of major proteases of MMP-1, -3, -9, and -13 in SW1353 cells and human osteoarthritic chondrocytes. Such catabolic proteases inhibitory ability, as well as its well-known anti-inflammatory activity makes SN an attractive and new therapeutic agent for OA.

ACKNOWLEDGEMENTS

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