Sphingosine-1-phosphate inhibits interleukin-1β-induced inflammation in human articular chondrocytes

MYUNG-HEE MOON, JAE-KYO JEONG, YOU-JIN LEE, JAE-WON SEOL and SANG-YOUEL PARK

Biosafety Research Institute, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea

Received June 24, 2012; Accepted July 26, 2012

DOI: 10.3892/ijmm.2012.1135

Abstract. Sphingosine-1-phosphate (S1P) is a pluripotent lipid mediator that transmits signals through a family of G-protein-coupled receptors (GPCRs) to control diverse biological processes including inflammation and wound-healing. In this study, a novel biological activity of S1P in articular chondrocytes was identified. Human primary chondrocytes were cultured in a monolayer. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting were performed to detect genes and proteins involved in inflammation and cartilage degradation when human primary chondrocytes were stimulated by interleukin (IL)-1β. Matrix metalloproteinase (MMP)-2 and MMP-9 activity was evaluated by gelatin zymography. Glycosaminoglycan (GAG) degradation was evaluated using the dimethylene blue method. Prostaglandin E_2 (PGE_2) was measured by enzyme-linked immunosorbent assay (ELISA). By using the $S1P_1$ receptor agonist and antagonist, we discovered the key role played by $S1P_1$ in the S1P-dependent inhibition of IL-1β-induced inflammation in human chondrocytes. S1P dose-dependently inhibited IL-1β-induced NF-κB p65, cyclooxygenase (COX)-2, MMP-1, MMP-3, MMP-13 and MMP-14 mRNA expression in human chondrocytes and IL-1β-induced PGE_2 synthesis and GAG degradation in human cartilage explants. W146, a known $S1P_1$ receptor antagonist, inhibited the active form of NF-κB p65 and COX-2 expression induced by IL-1β. The anti-inflammatory action of the $S1P_1$ receptor agonist SEW2871 was similar to that of S1P. This study demonstrates that S1P has anti-inflammatory effects on chondrocytes via the $S1P_1$ receptor. Our data suggest that targeting S1P and $S1P_1$ may be a potential therapy for arthritis.

Correspondence to: Professor Sang-Youel Park, Biosafety Research Institute, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea
E-mail: sypark@chonbuk.ac.kr

Key words: chondrocyte, cyclooxygenase-2, inflammation, prostaglandin E_2, sphingosine-1-phosphate

Introduction

Osteoarthritis (OA), the most common joint disorder, is characterized by progressive degenerative structural changes in articular cartilage and excessive production of several inflammatory mediators (1). Among these mediators, the pro-inflammatory cytokine interleukin (IL)-1β plays a central role in inducing cartilage damage in arthritis (2,3). IL-1β enhances the degradation of the extracellular matrix (ECM), including proteoglycan, through the activation of matrix metalloproteinases (MMPs). Degradation of the ECM in articular cartilage is a central event leading to joint destruction in several arthritic conditions, including OA (4). The pro-inflammatory effects may be mediated through cyclooxygenase (COX)-2 induction, which produces prostaglandin E_2 (PGE_2) that is responsible for the pain and swelling of inflamed joints by enhancing MMP expression and activity (5,6).

The expression and biological activity of the pro-inflammatory factors mentioned are regulated by the transcription factor nuclear factor κ-light chain-enhancer of activated B cells (NF-κB). NF-κB regulates the expression of a number of genes involved in immune responses and inflammation (7). In unstimulated cells, NF-κB is discovered in the cytoplasm (8). When these cells are stimulated by cytokines such as IL-1β, which play important roles in the initiation and development of OA, the NF-κB protein p65/p50 enters into the nucleus where it regulates the expression of a number of genes involved in inflammatory responses such as COX-2 (5,9).

At present, with the exception of anti-inflammatory corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX-2, a specific therapy based on fundamental intracellular pathways of chondrocytes does not exist for the medical management of OA (10); therefore, safe and efficacious drugs are needed to treat this debilitating disease.

Sphingosine-1-phosphate (S1P) is a member of an important group of signaling sphingolipids recognized to play a role in a diverse array of cellular processes, including apoptosis, survival, motility, calcium signaling and differentiation in a variety of cell types (11). S1P is generated by the phosphorylation of sphingosine kinases 1 (Sphk-1) and 2 (Sphk-2). S1P exerts most of its activity as a ligand of G-protein-coupled receptors (GPCRs) (12,13). At present, 5 members of the S1P receptor family have been identified in mammals, $S1P_{1-5}$, possessing distinct expression profiles and affinities toward

S1P (13). Stradner *et al* (14) demonstrated that the expression of $S1P_1$, $S1P_2$ and $S1P_3$ was observed in human articular chondrocytes.

Of the various cellular physiological actions of S1P, we focused on its ability to markedly influence inflammation. The S1P pathway has recently been associated with a variety of inflammatory-based diseases, and several studies have reported on the anti-inflammatory effects of S1P. Ogawa *et al* (15) discovered that the novel $S1P_1$ receptor agonist KRP-203 reduced experimental autoimmune myocarditis in rats. Hughes *et al* (16) demonstrated that S1P significantly reduced pro-inflammatory cytokine secretion such as ILs in macrophages. S1P is known to have potent anti-inflammatory actions.

In this study, we investigated the effects of S1P on human arthritis, as well as cellular responses using an *in vitro* model. Furthermore, the potential of $S1P_1$ to reduce the inflammation of human chondrocytes induced by IL-1β was evaluated using a specific agonist and antagonist.

Materials and methods

Reagents. S1P was purchased from Cayman Chemical (Ann Arbor, MI, USA) and prepared as a 2 mM solution in 0.3 M NaOH and was further diluted in cell culture medium. SEW2871, an S1P receptor 1 ($S1P_1$) agonist and W146, a $S1P_1$ antagonist, were purchased from Cayman Chemical. Recombinant human IL-1β was purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA).

Human chondrocyte isolation and monolayer cultures. Cartilage tissue samples were obtained from the femoral condyle and tibial plateau of the knee from 6 OA patients at the time of joint replacement surgery. Cartilage samples were derived from human patients following full informed consent and local ethics committee approval. Full-thickness cartilage slices were obtained from above the subchondral bone from a relatively lesion-free area. Human OA chondrocytes were harvested from the discarded knee tissue as previously described (17). Briefly, the cartilage surfaces were first rinsed with sterile $NaCl/P_i$. The cartilage slices were chopped and incubated with 0.25% trypsin for 30 min, followed by 0.1% collagenase (Sigma-Aldrich, St. Louis, MO, USA) treatment for 6 h in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen-Gibco-BRL) and antibiotics. Cells were filtered through a 70-μm cell strainer (Falcon, Franklin Lakes, NJ, USA), washed twice with $NaCl/P_i$ and then seeded into tissue culture flasks in DMEM supplemented with 10% FBS and antibiotics. After ~7 days, confluent chondrocytes were split once and seeded at high density and these first passage chondrocytes were used within 2 days in subsequent experiments. Chondrocytes were incubated with DMEM containing 1% FBS prior to treatment with S1P, SEW2871, W146 and pro-inflammatory cytokines.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from human chondrocytes using the Easy-spin™ total RNA extraction kit (Intron Biotechnology, Seoul, Korea). cDNA synthesis was performed following the instructions of the Takara PrimeScript™ first-strand cDNA synthesis kit (Takara Bio, Inc., Tokyo, Japan). mRNA expression of COX-2 and MMPs was analyzed by RT-PCR using the specific primer sets. The primer sequences used for the RT-PCR were as follows:

COX-2: forward, 5'AGTCCCTGAGCATCTACGGTTTG3' and reverse, 5' CATCGCATACTCTGTTGTGTTCCC3'; MMP-1: forward, 5'TCCACTGCTGCTGCTGCTG3' and reverse, 5'TTTCAACTTGCCTCCCATCATTCTTC3'; MMP-3: forward, 5'TGAACAATGGACAAAGGATACAACAGG3' and reverse, 5'ATCATCTTGAGACAGGCGGAACC3'; MMP-14: forward, 5'GCTGGTTCTGGCGGGTGAG3' and reverse, 5'TCTCGTAGGCAGTGTTGATGGAC3'; β-actin (as an internal control): forward, 5'GCAAGCAGGAGTATGACGAG3' and reverse, 5'CAAATAAAGCCATGCCAATC3'. The PCR conditions were as follows: initial denaturation at 94˚C for 2 min; 35 cycles of denaturation at 94˚C for 30 sec; amplification at 60˚C for 30 sec; extension at 72˚C for 1 min. The size of the amplified products was examined by agarose gel electrophoresis. Images were captured using the Fusion FX7 acquisition system (Vilber Lourmat, Eberhardzell, Germany). For semi-quantitive analyses, relative band intensities against the internal control were calculated using Bio-1D (Vilber Lourmat, Marne La Vallee, France).

Quantitative (q)RT-PCR. Total RNA was extracted from human chondrocytes using the Easy-spin™ total RNA extraction kit. cDNA synthesis was performed following the instructions of the Takara PrimeScript™ first-strand cDNA synthesis kit (Takara Bio, Inc.). For qRT-PCR, 1 μl of gene primers with SYBR-Green in 20 μl of reaction volume was applied. The primer sequences used for the real-time PCR were as follows: COX-2: forward, 5'AGTCCCTGAGCATCTACGGTTTG3' and reverse, 5' CATCGCATACTCTGTTGTGTTCCC3'; MMP-1: forward, 5'TCCACTGCTGCTGCTGCTG3' and reverse, 5'TTTCAACTTGCCTCCCATCATTCTTC3'; β-actin (as an internal control): forward, 5'GCAAGCAGGAGTATGACGAG3' and reverse, 5'CAAATAAAGCCATGCCAATC3'. All reactions with iTaq SYBR-Green Supermix were performed on the CFX96 real-time PCR detection system (all were from Bio-Rad, Hercules, CA, USA).

Western blotting. Human chondrocytes were lysed in a lysis buffer. Equal amounts of lysate protein were electrophoretically resolved on a 10-15% SDS-PAGE, and the resolved proteins were transferred. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. Images were captured using the Fusion FX7 acquisition system. Densitometry of the signal bands was analyzed using Bio-1D. The antibodies used for immunoblotting were COX-2, p65 (Santa Cruz Biotechnology, Inc.) and β-actin (Sigma-Aldrich).

Immunofluorescence staining. Human chondrocytes, cultured on glass slides, were fixed with cold acetone and blocked by 5% FBS in TBST and incubated with mouse NF-κB (active p65 subunit) antibody (Millipore) and goat COX-2 antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C. After washing with TBST, the cells were incubated with anti-mouse

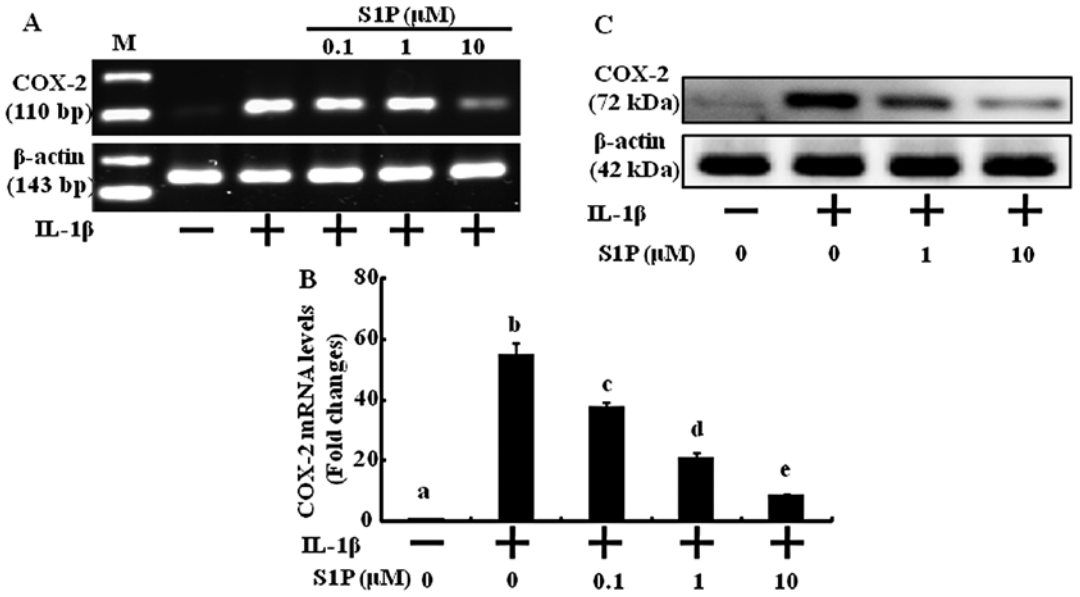

Figure 1. S1P prevents IL-1β-induced COX-2 gene and protein expression. (A and B) Chondrocytes were stimulated with IL-1β (10 ng/ml) and co-treated with increasing concentrations of S1P for 12 h and then stimulated with IL-1β (10 ng/ml) for 12 h. Total RNA was isolated, cDNA was synthesized and COX-2 mRNA (A and B) was quantified using real-time reverse RT-PCR. Base pair markers (M) denoting DNA sizes are displayed in the far left lane. mRNA fold changes were calculated relative to the control. The data represent samples from 3 different experiments expressed as the means ± SEM (n=3). The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P< 0.01$). Bars displaying different letters indicate significant differences between each group of bars according to Duncan's test at $P<0.01$. (C) Representative western blotting from total protein isolated after 24 h of treatment with IL-1β (10 ng/ml) alone or co-treated with indicated concentrations of S1P.

IgG conjugated with Alexa Fluor® 488 (green) and anti-goat IgG conjugated with Alexa Fluor® 350 (blue). The cells were washed with TBST, mounted with fluorescence mounting medium (Dako) and observed under a fluorescence microscope (Nikon ECLIPSE 80i; Nikon Corporation).

Gelatin zymography. Gelatin zymography was performed for the detection of MMP protein secretion and activation in conditioned medium. Conditioned media were collected and centrifuged to remove cellular debris, and the supernatant was collected and stored at -20˚C. Each sample suspension was mixed with SDS sample buffer without reducing agent, followed by gelatin zymography. The sample was resolved by SDS-PAGE gels containing 10% acrylamide and 1 mg/ml gelatin. The gels were then washed twice in 2.5% (w/v) Triton X-100 in distilled water for 30 min at room temperature and were incubated overnight at 37˚C in developing buffer containing 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl and 5 mM $CaCl_2$. The following morning, gels were stained with 0.2% Coomassie Blue R-250 in 50% ethanol and 10% acetic acid for 1 h at room temperature and destained with a buffer consisting of 20% methanol, 10% acetic acid and 70% distilled water for 30 min at room temperature to visualize these zones of digestion as light areas against the darkly stained protein background. The zymography gels were analyzed using the Fusion FX7 acquisition system.

Quantification of glycosaminoglycan (GAG) release. Cartilage explants were treated with IL-1β with or without S1P, SEW2871 and W146 for 72 h. GAG levels in the culture medium were determined by reaction with 1,9-dimethylmethylene blue. Twenty microliters of samples or chondroitin sulfate were mixed with 180 *μl* DMB reagents (48 mg/ml DMB, 40 mM glycine, 40 mM NaCl, 10 mM HCl and pH 3.0) for 10 min at room temperature. Absorbance at 525 nm was measured with a spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). All measurements were performed in quadruplicate. Quantification was performed using a standard curve of chondroitin sulfate in the range of 0-5 *μ*g/ml. Results were normalized to the protein concentration for GAG release. Culture supernatant was also measured for protein concentration by BCA reagent (Pierce Biotechnology, Inc., Rockford, IL, USA).

Analysis of PGE_2 levels: enzyme-linked immunosorbent assay (ELISA). Human chondrocytes were incubated with DMEM containing 1% FBS prior to treatment with or without IL-1β (10 ng/ml), S1P, SEW2871 and W146 for 24 h. Culture supernatants were collected and stored at -20˚C. PGE_2 levels in the culture medium were quantified using an immunoenzymatic method (PGE_2 EIA kits; Cayman Chemical) according to the manufacturer's instructions.

Statistical evaluation. All data are expressed as the means ± SEM, and the data were compared using the ANOVA and Duncan's test using the SAS statistical package.

Results

S1P inhibits IL-1β-induced COX-2 expression in human chondrocytes. The pro-inflammatory cytokine IL-1β contributes to the pathogenesis of OA and is a potent inducer of COX-2 expression which plays a pivotal role in the pathogenesis of cartilage inflammation. We treated human chondrocytes with various concentrations of S1P to define its anti-inflammatory effects via COX-2 expression

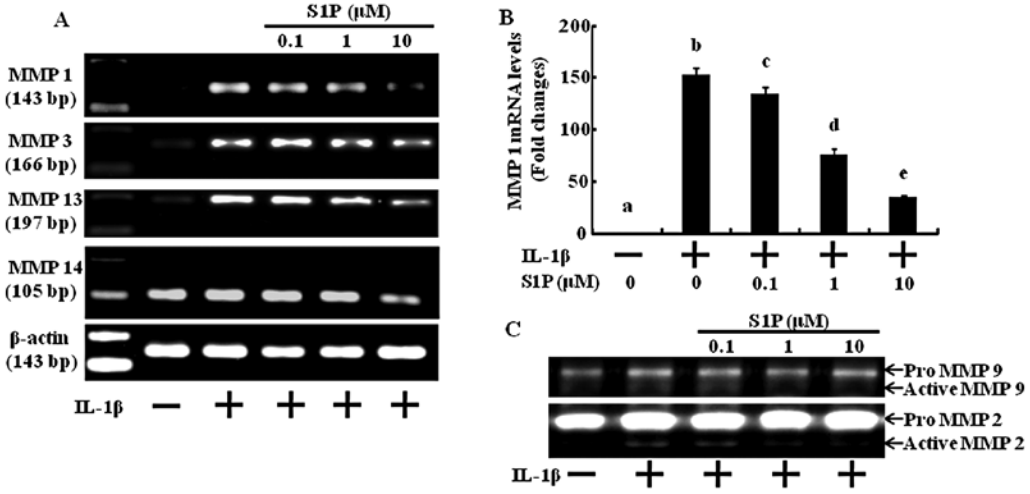

Figure 2. Inhibitory effect of S1P on the MMP expression and activation induced by IL-1β. (A) Chondrocytes were treated with various concentrations of S1P with IL-1β (10 ng/ml) for 12 h. Total RNA was isolated, cDNA was synthesized and MMP-1, -3, -13 and -14 mRNA was quantified using RT-PCR. β-actin gene expression was used for normalization. Base pair markers (M) denoting DNA sizes are displayed in the far left lane. (B) Chondrocytes were treated as described in (A). MMP-1 gene expression was analyzed by real-time RT-PCR. β-actin gene expression was used for normalization. mRNA fold changes were calculated relative to the control. Bar graph indicates the means ± SEM (n=3). The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P<0.01$). Bars displaying different letters indicate significant differences between each group of bars according to Duncan's test at $P<0.01$. (C) Chondrocytes were treated as described in (A) and MMP activation was analyzed 24 h after IL-1β treatment from the concentrated conditioned media using gelatin gel zymography. The data represent samples from 3 different experiments.

inhibition. Under basal conditions, expression of COX-2 mRNA and protein was undetectable. Treatment with IL-1β resulted in significant increases in mRNA (Fig. 1A and B) and protein expression (Fig. 1C). S1P prevented the induction of COX-2 mRNA as well as protein expression by IL-1β in a concentration-dependent manner (Fig. 1). The effect of S1P was significantly detected at 0.1 μM and was maximal at 10 μM. These results indicate that S1P has anti-inflammatory effects by decreasing the expression of COX-2 at the protein level as well as at the transcriptional level.

S1P decreases matrix degradation gene products as well as MMP activity induced by IL-1β in human chondrocytes. Since the loss of cartilage matrix is due to the upregulation of MMP expression and activation (18), we investigated whether S1P inhibits MMP gene expression and activation induced by pro-inflammatory cytokines in human articular chondrocytes. In the control situation, MMP mRNA was undetectable, but treatment with IL-1β resulted in a substantial increase in mRNA expression (Fig. 2A and B). Dose-dependent treatment with S1P led to significant inhibition of all 4 MMP gene products measured (MMP-1, -3, -13 and -14) (Fig. 2A and B). Next, we examined the inhibitory effect of S1P on the MMP activation induced by IL-1β using gelatin zymography (Fig. 2C). Under basal conditions, MMP-2 and -9 were not activated, but the addition of IL-1β activated these MMPs (Fig. 2C). Increasing concentrations of S1P significantly decreased the pro-inflammatory cytokine-induced activities of MMP-2 and -9 (Fig. 2C). These results indicate that S1P inhibits MMP expression and activation and may potentially block cartilage degradation.

$S1P_1$ receptor is involved in the chondrocyte anti-inflammatory effect of S1P. The biological action of S1P is largely ascribed to ligation to specific S1PRs that may evoke distinct biological responses. According to Ogawa *et al*, the novel $S1P_1$ receptor agonist, KRP-203, reduces experimental autoimmune myocarditis in rats (15). Therefore, among S1P-specific receptors, we first investigated the involvement of $S1P_1$ in anti-inflammation. Human chondrocytes, stimulated by IL-1β, were treated with SEW2871, a selective $S1P_1$ agonist, to activate $S1P_1$. With increasing concentrations of SEW2871, COX-2 mRNA expression was significantly diminished (Fig. 3C). In addition, the anti-inflammatory action of S1P was prevented by blocking the $S1P_1$ receptor using W146, a selective $S1P_1$ antagonist (Fig. 3A, B and F). The inhibition of COX-2 mRNA and protein upregulation by IL-1β was blocked by the addition of 10 μM of W146. Also, the anti-inflammatory action of $S1P_1$ receptor-specific activation using SEW2871 was disturbed by blocking the $S1P_1$ receptor using W146 (Fig. 3D, E and G).

NF-κB is a transcription factor that regulates the expression of several genes involved in immune responses and inflammation including COX-2 and MMPs (7). S1P and SEW2871 both decreased the IL-1-induced active form of NF-κB p65, but W146 reversed the anti-inflammatory actions of S1P and SEW2871 (Fig. 3B and E-G).

In order to determine whether only $S1P_1$ selective activation affects MMP expression and activation and if blocking the $S1P_1$ receptor abolishes the effect of S1P and SEW2871 on MMP expression and activation, RT-PCR and gelatin zymography were performed. In human chondrocytes exposed to 10 ng/ml IL-1β and treated with SEW2871, the levels of MMP-1 and -13 were significantly decreased back to the levels of the control (Fig. 4). W146 abolished the action of S1P and SEW2871 that had decreased MMP-1 and -13 mRNA levels (Fig. 4). These results suggest that the regulation of NF-κB and MMP activation induced by S1P is mediated through the $S1P_1$ receptor.

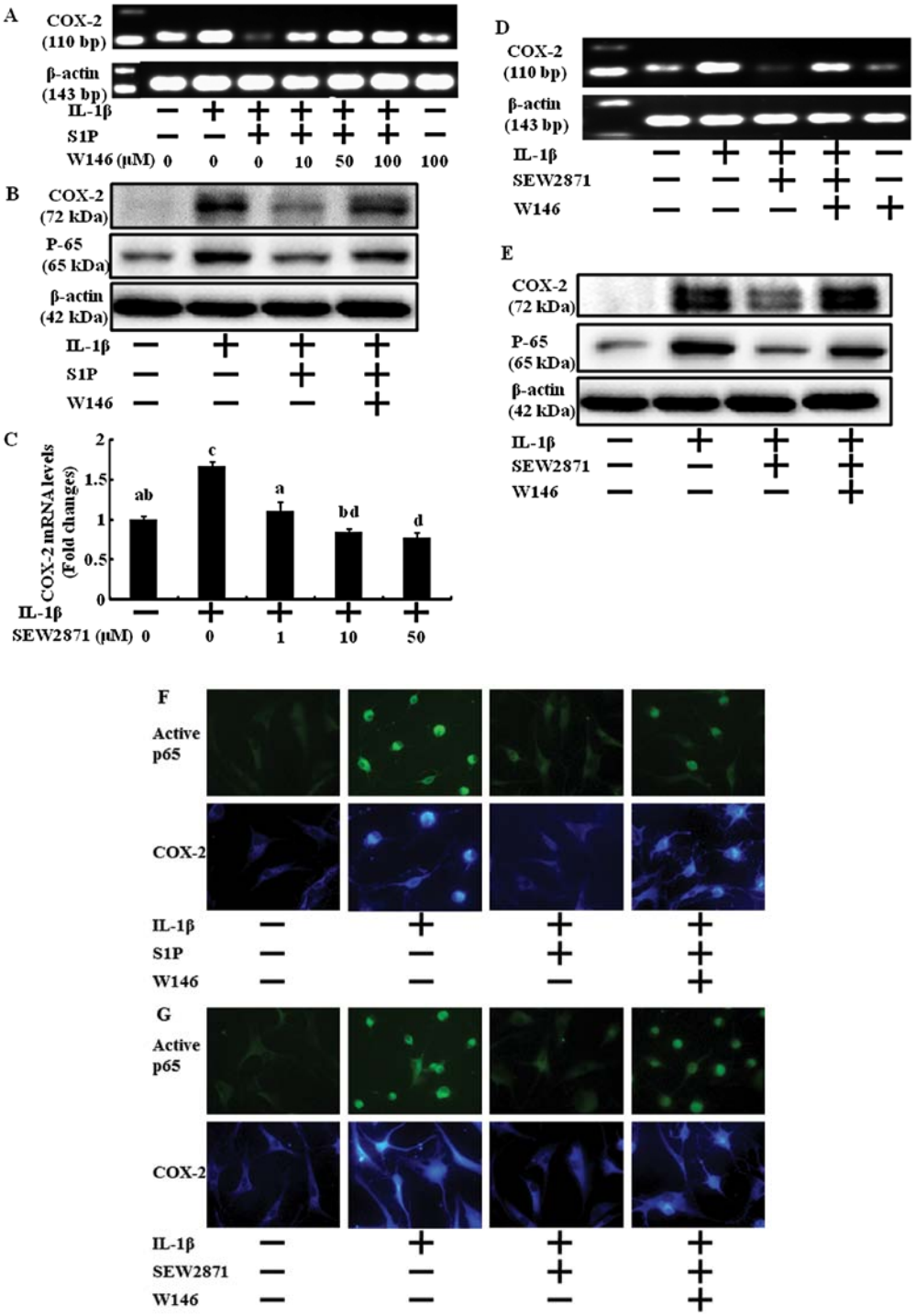

Figure 3. Selective activation of the $S1P_1$ receptor is involved in the action of S1P on decreasing COX-2 and active p65 expression in human chondrocytes. (A and B) Human chondrocytes were pretreated with increasing concentrations (10-100 μM) of W146 for 1 h and then stimulated with IL-1β (10 ng/ml) and/or S1P (10 μM) for 12 h. COX-2 mRNA expression in cells was assessed by (A) RT-PCR. COX-2 and active p65 protein expression was determined by immunoblotting using (B) human anti-COX-2, anti-active p65 and β-actin antibodies. (C) Cells were stimulated with SEW2871 (0-50 μM) and IL-1β (10 ng/ml) for 12 h. COX-2 gene expression was assessed by real-time quantitative PCR. β-actin gene expression was used for normalization. mRNA fold changes were calculated relative to the control. Bar graph indicates the mean ± SEM (n=3). The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P<0.01$). Bars displaying same letters indicate significant differences between each group of bars according to Duncan's test at $P<0.01$. (D and E) Cells were treated with or without W146 (10 μM) 1 h prior to stimulation with SEW2871 (50 μM) and IL-1β (10 ng/ml). After 12 h, (D) COX-2 mRNA expression in cells was assessed by real-time quantitative PCR and (E) COX-2 and active p65 protein expression by immunoblotting and (F and G) immunocytochemistry. The data represent samples from 3 different experiments.

S1P protects cartilage explants from IL-1-induced GAG loss and PGE_2 production. Cartilage explants were treated with S1P in combination with IL-1β. GAG release to the media was significantly and dose-dependently inhibited by S1P (Fig. 5A). At 10 μM of S1P, GAG release was reduced compared to the control. GAG release induced by IL-1β was reduced by adding 50 μM of SEW2871 (Fig. 5B). However, W146 abolished the inhibitory effect of the GAG degradation action of S1P and

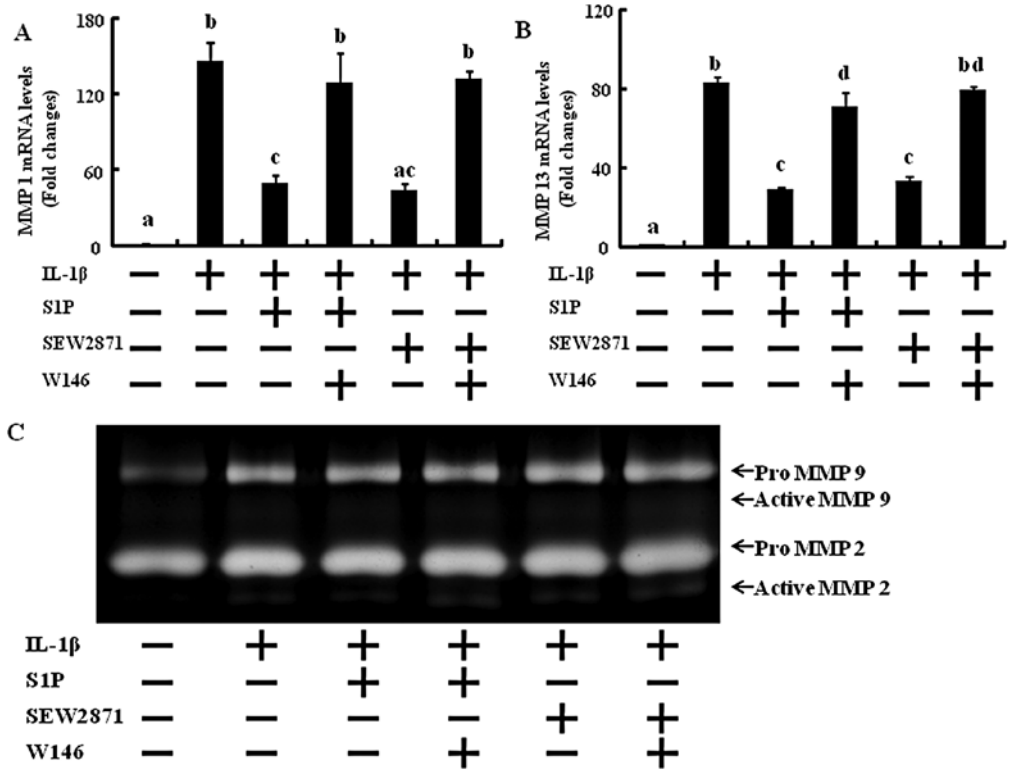

Figure 4. Inhibitory effect of S1P on MMP expression and activation is related to $S1P_1$ receptor-selective activation. (A and B) W146 (10 μM) was added to chondrocytes 1 h before stimulation with S1P (10 μM) or SEW2871 (50 μM) and IL-1β (10 ng/ml) treatment for 12 h. (A) MMP-1 and (B) MMP-13 mRNA was quantified by real-time RT-PCR. β-actin gene expression was used for normalization. mRNA fold changes were calculated relative to the control. Bar graph indicates the means ± SEM (n=3). The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P<0.01$). Bars showing same letters indicate significant differences between each group of bars according to Duncan's test at $P<0.01$. (C) MMP activation was analyzed 24 h after treatment as described in (A) from the concentrated conditioned media using gelatin zymography. The data represent samples from 3 different experiments.

SEW2871 and the contents of GAG release were similar to that with the IL-1β treatment. These results revealed that S1P protects cartilage degradation from inflammatory cytokine-induced GAG degradation via $S1P_1$ receptor activation.

PGE_2, which originates from the activation of the COX pathways, is produced by OA chondrocytes and may promote matrix degeneration (19,20). In *ex vivo* cultures of cartilage explants, the production of PGE_2 was increased by the stimulation of IL-1β in the supernatants of the conditioned media (Fig. 5C). After treatment with S1P, PGE_2 synthesis was significantly reduced to the level of the control (Fig. 5C). Next, we investigated the action of S1P through the $S1P_1$ receptor. Only $S1P_1$ selective activation decreased PGE_2 production, and blocking the $S1P_1$ receptor abolished the effect of S1P and SEW2871 on PGE_2 synthesis (Fig. 5C). These results suggest that S1P prevents inflammatory cytokine-induced PGE_2 synthesis through $S1P_1$ receptor activation.

Discussion

The main goal of this study was to determine the anti-inflammatory effects of S1P in human chondrocyte-induced inflammation. The data presented demonstrated that S1P acts as an anti-inflammatory regulator of chondrocytes and that the action of S1P is mediated by the $S1P_1$ receptor. Treatment with exogenous S1P effectively inhibited COX-2 expression by reducing the activation of NF-κB p65, which is considered one of the major regulators of OA pathogenesis. Also, S1P significantly decreased MMP-1, -3, -13 and -14 expression. Moreover, S1P protects cartilage explants from IL-1β-induced PGE_2 synthesis and GAG degradation. S1P effectively prevented cartilage explants from PGE_2 synthesis and GAG release caused by IL-1β. These S1P effects were abolished by the inhibition of the $S1P_1$ receptor using W146. The $S1P_1$ selective agonist, SEW2871, had anti-inflammatory effects similar to S1P. These anti-inflammatory effects of S1P via the $S1P_1$ receptor occurred through the regulation of PGE_2 production mediated by COX-2 expression and NF-κB and MMP activation.

OA is a painful and disabling disease that affects millions of people. Arthritic joints display an altered metabolism and an imbalance between anabolic growth factors and pro-inflammatory cytokines, TNF-α and IL-1β produced by inflammatory cells, synovial fibroblasts and chondrocytes in affected joints (21). In fact, IL-1β, a well-recognized pro-inflammatory cytokine, is increased locally during the OA process (22). IL-1β induces a large cascade of events that leads to cartilage damage, such as the synthesis of MMPs and ECM proteins that are absent in normal cartilage and the release of other inflammatory mediators including COX-2 (23). Several of these effects are mediated by eicosanoids, which are products of arachidonic acid metabolism. PGE_2 is the predominant eicosanoid synthesized by OA cartilage and mediates several IL-1β-induced effects (24). The goal of pharmacological treatment is usually to control symptoms of the disease, pain and limitation of function, which is traditionally accomplished by the use of analgesic agents and NSAIDs (25). However, while

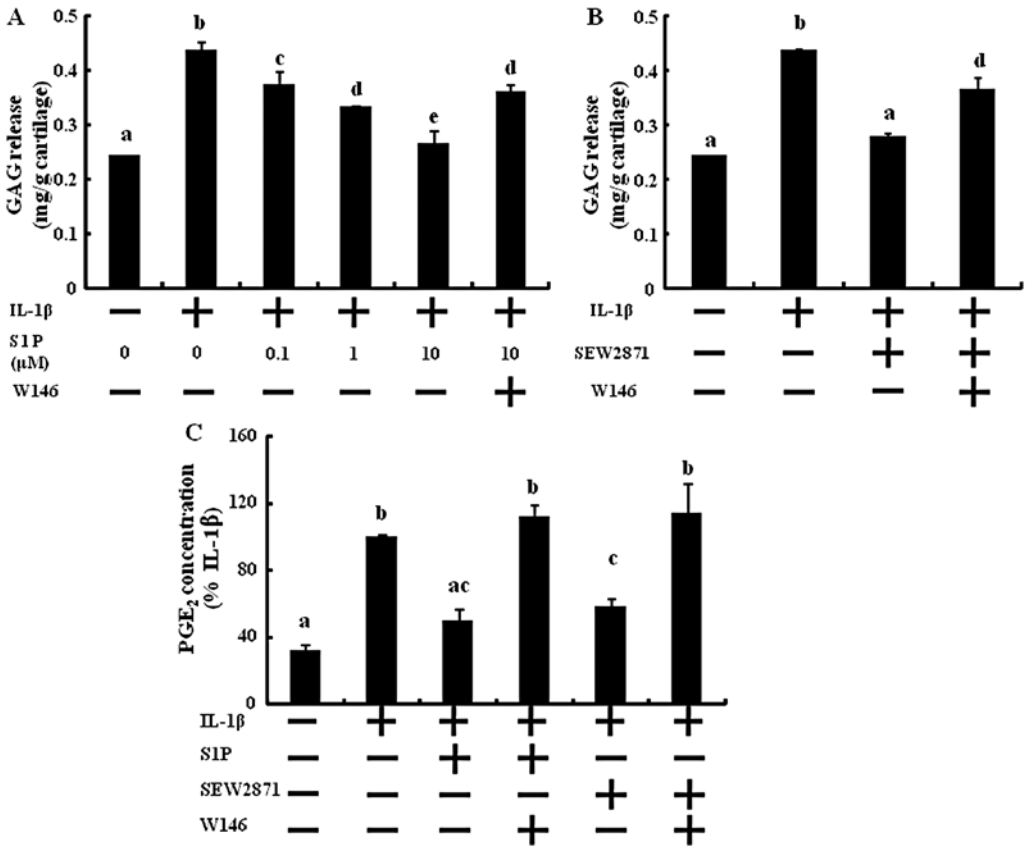

Figure 5. Inhibitory effect of S1P on GAG release and PGE_2 synthesis via $S1P_1$ receptor-selective activation. (A) Human cartilage explants were treated for 72 h with the indicated concentrations of S1P and IL-1β (10 ng/ml) with or without W146 (10 μM). GAG released to the culture media was assessed using dimethylmethylene blue. (B) Human cartilage explants were treated for 72 h with SEW2871 (50 μM) and IL-1β (10 ng/ml) with or without W146 (10 μM). (C) Human cartilage explants were treated as described in A and B. The quantification of PGE_2 production was determined by ELISA. Bar graph indicates the means ± SEM (n=3). The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P<0.01$). Bars displaying same letters indicate significant differences between each group of bars according to Duncan's test at $P<0.01$.

providing relief from pain, none of these agents inhibit cartilage breakdown or disease development; and they also have varying degrees of GI toxicity (26). Therefore, new and safe therapeutics which inhibit disease progression are required.

The beneficial effects of S1P on inflammation in different tissues and cells have been demonstrated (15,16). Therefore, S1P was used as an anti-inflammatory agent for IL-1β-induced OA in a human chondrocyte *in vitro* model. The results of this study demonstrated that inflammation of human chondrocytes by IL-1β-induced expression of COX-2 was effectively inhibited by S1P (Fig. 1). In contrast to our results, Masuko *et al* demonstrated that S1P increased COX-2 expression and PGE_2 production in chondrocytes. They treated chondrocytes with S1P and the increase in COX-2 was 40 pg/ml (32). Similarly, we observed that only S1P treatment increased the COX-2 expression and PGE_2 production slightly (not significant). However, when the chondrocytes were pretreated with S1P prior to IL-1β treatment, S1P significantly decreased IL-1β-induced COX-2 expression and PGE_2 production (Figs. 1 and 5). Although S1P only slightly increased COX-2 expression and PGE_2 production under a normal condition, S1P effectively functions as a COX-2 inhibitor in arthritic joint tissue induced by IL-1β.

An immediate cause of the destruction of joint tissue in OA is the augmentation of MMP family enzymes (27). In normal tissue, these enzymes are expressed at low levels to maintain cartilage homeostasis, but in pathological states such as OA, they are expressed at abnormally high levels (28). S1P significantly reduced MMP expression and activation (Fig. 2). MMPs are subdivided into several subtypes. Among them, MMP-1 and -13 preferentially degrade native type II collagen and are synthesized in increased amounts by OA chondrocytes; thus, they are postulated to have an important role in the destruction of cartilage (4). S1P was found to dose-dependently decrease MMP-1 and -13 gene products, whereas proteoglycan loss involves MMP-3 and -14 expression (4). Treatment with exogenous S1P reduced MMP-3 and -14 gene products (Fig. 2). These results are consistent with the findings that high levels of GAG released from cartilage explants caused by IL-1β were diminished by S1P treatment (Fig. 5A), suggesting that S1P downregulates MMP-3 and -14 expression at the transcriptional level, leading to suppression of GAG degradation by pro-inflammatory cytokines. Together these data suggest that S1P may prevent cartilage destruction in arthritis by suppressing COX-2 and MMPs.

Several of the biological effects of IL-1β on chondrocytes (i.e. upregulation of MMPs and COX-2) are also mediated by NF-κB (29,30). In the present study, we demonstrated that IL-1β enhanced the activation of NF-κB p65. Interestingly, treatment with S1P downregulated the expression of the active form of NF-κB p65 (Fig. 3B) suggesting that the anti-inflammatory action of S1P (i.e. downregulation of MMPs

and COX-2) is due to the inhibition of NF-κB activation. These results are in accordance with reports revealing that S1P suppresses NF-κB in germ cells (31).

Several signaling pathways that are activated in response to the stimulation of cells by S1P are initiated by activation of S1P-specific receptors. The results of this study demonstrated that the anti-inflammatory action of S1P was associated with the $S1P_1$ receptor (Figs. 3-5). Treatment with $S1P_1$ selective agonist SEW2871 inhibited the active form of NF-κB p65 (Fig. 3E), PGE_2 production (Fig. 5C), COX-2 expression (Fig. 3A-C), expression and activation of MMPs (Fig. 4) and GAG degradation (Fig. 5B) similar to S1P treatment (Fig. 3C-E). In addition, the S1P1 antagonist W146 significantly reversed the actions of S1P and SEW2871 (Figs. 3-5). Collectively the evidence in the current study indicates that the selective activation of S1P and $S1P_1$ may potentially improve the therapeutic effect in arthritis.

In conclusion, we identified the anti-inflammatory action of S1P via the $S1P_1$ receptor by inhibiting NF-κB p65 activation, COX-2 expression, MMP activation, PGE_2 production and GAG degradation in articular chondrocytes. These results suggest that $S1P_1$ activation may be a therapeutic target for OA. Therefore, S1P and the development of $S1P_1$ receptor subtype-specific ligands may result in a promising new class of drugs for the treatment of OA.

Acknowledgements

This study was supported by the National Research Foundation of the Korea Grant funded by the Korean Government (2010-E00019).

References

1. Chabane N, Zayed N, Afif H, *et al*: Histone deacetylase inhibitors suppress interleukin-1beta-induced nitric oxide and prostaglandin E2 production in human chondrocytes. Osteoarthritis Cartilage 16: 1267-1274, 2008.
2. Goldring MB and Marcu KB: Cartilage homeostasis in health and rheumatic diseases. Arthritis Res Ther 11: 224, 2009.
3. Goldring MB and Berenbaum F: Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. Osteoarthritis Cartilage 7: 386-388, 1999.
4. Kim HA, Yeo Y, Kim WU and Kim S: Phase II enzyme inducer sulphoraphane blocks matrix metalloproteinase production in articular chondrocytes. Rheumatology (Oxford) 48: 932-938, 2009.
5. Lianxu C, Hongti J and Changlong Y: NF-kappaBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1beta-induced and TNF-alpha-induced chondrocytes. Osteoarthritis Cartilage 14: 367-376, 2006.
6. Pelletier JP, Martel-Pelletier J and Abramson SB: Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum 44: 1237-1247, 2001.
7. Kopp EB and Ghosh S: NF-kappa B and rel proteins in innate immunity. Adv Immunol 58: 1-27, 1995.
8. Baldwin AS Jr: The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 14: 649-683, 1996.
9. Newton R, Kuitert LM, Bergmann M, Adcock IM and Barnes PJ: Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. Biochem Biophys Res Commun 237: 28-32, 1997.
10. Shakibaei M, Csaki C, Nebrich S and Mobasheri A: Resveratrol suppresses interleukin-1beta-induced inflammatory signaling and apoptosis in human articular chondrocytes: potential for use as a novel nutraceutical for the treatment of osteoarthritis. Biochem Pharmacol 76: 1426-1439, 2008.
11. Johnstone ED, Chan G, Sibley CP, Davidge ST, Lowen B and Guilbert LJ: Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G(i)-coupled receptor response. J Lipid Res 46: 1833-1839, 2005.
12. Schuppel M, Kurschner U, Kleuser U, Schafer-Korting M and Kleuser B: Sphingosine 1-phosphate restrains insulin-mediated keratinocyte proliferation via inhibition of Akt through the $S1P_2$ receptor subtype. J Invest Dermatol 128: 1747-1756, 2008.
13. Pyne S and Pyne NJ: Sphingosine 1-phosphate signalling in mammalian cells. Biochem J 349: 385-402, 2000.
14. Stradner MH, Hermann J, Angerer H, *et al*: Sphingosine-1-phosphate stimulates proliferation and counteracts interleukin-1 induced nitric oxide formation in articular chondrocytes. Osteoarthritis Cartilage 16: 305-311, 2008.
15. Ogawa R, Takahashi M, Hirose S, *et al*: A novel sphingosine-1-phosphate receptor agonist KRP-203 attenuates rat autoimmune myocarditis. Biochem Biophys Res Commun 361: 621-628, 2007.
16. Hughes JE, Srinivasan S, Lynch KR, Proia RL, Ferdek P and Hedrick CC: Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. Circ Res 102: 950-958, 2008.
17. Seol JW, Lee HB, Kim NS and Park SY: Tartrate-resistant acid phosphatase as a diagnostic factor for arthritis. Int J Mol Med 24: 57-62, 2009.
18. Goldring MB: The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. Connect Tissue Res 40: 1-11, 1999.
19. Thomas CM, Fuller CJ, Whittles CE and Sharif M: Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis Cartilage 15: 27-34, 2007.
20. Dave M, Attur M, Palmer G, *et al*: The antioxidant resveratrol protects against chondrocyte apoptosis via effects on mitochondrial polarization and ATP production. Arthritis Rheum 58: 2786-2797, 2008.
21. Liacini A, Sylvester J, Li WQ, *et al*: Induction of matrix metalloproteinase-13 gene expression by TNF-alpha is mediated by MAP kinases, AP-1, and NF-kappaB transcription factors in articular chondrocytes. Exp Cell Res 288: 208-217, 2003.
22. Wood DD, Ihrie EJ, Dinarello CA and Cohen PL: Isolation of an interleukin-1-like factor from human joint effusions. Arthritis Rheum 26: 975-983, 1983.
23. Largo R, Alvarez-Soria MA, Diez-Ortego I, *et al*: Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 11: 290-298, 2003.
24. Amin AR, Dave M, Attur M and Abramson SB: COX-2, NO, and cartilage damage and repair. Curr Rheumatol Rep 2: 447-453, 2000.
25. Reginster JY, Deroisy R, Rovati LC, *et al*: Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 357: 251-256, 2001.
26. Singh R, Ahmed S, Islam N, Goldberg VM and Haqqi TM: Epigallocatechin-3-gallate inhibits interleukin-1beta–induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes: suppression of nuclear factor kappaB activation by degradation of the inhibitor of nuclear factor kappaB. Arthritis Rheum 46: 2079-2086, 2002.
27. Burrage PS, Mix KS and Brinckerhoff CE: Matrix metalloproteinases: role in arthritis. Front Biosci 11: 529-543, 2006.
28. Martel-Pelletier J, Welsch DJ and Pelletier JP: Metalloproteases and inhibitors in arthritic diseases. Best Pract Res Clin Rheumatol 15: 805-829, 2001.
29. Shakibaei M, John T, Schulze-Tanzil G, Lehmann I and Mobasheri A: Suppression of NF-kappaB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: implications for the treatment of osteoarthritis. Biochem Pharmacol 73: 1434-1445, 2007.
30. Barnes PJ and Karin M: Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336: 1066-1071, 1997.
31. Suomalainen L, Pentikäinen V and Dunkel L: Sphingosine-1-phosphate inhibits nuclear factor κB activation and germ cell apoptosis in the human testis independently of its receptors. Am J Pathol 166: 773-781, 2005.
32. Masuko K, Murata M, Nakamura H, Yudoh K, Nishioka K and Kato T: Sphingosine-1-phosphate attenuates proteoglycan aggrecan expression via production of prostaglandin E2 from human articular chondrocytes. BMC Musculoskelet Disord 8: 29, 2007.