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Disease-Modifying Effects of Glucosamine HCl Involving Regulation of Metalloproteinases and Chemokines Activated by Interleukin-1β in Human Primary Synovial Fibroblasts

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Abstract The purpose of this study was to investigate the possible involvement of synovium in cartilage destruction in osteoarthritis (OA) patients. Using human primary synovial fibroblasts (HPSFs), we examined the effects of glucosamine (GLN) on the regulation of the expression of matrix metalloproteinases (MMP-1, -2, and -13) and chemokines (IL-8, MCP-1, and RANTES) as well as the involvement of MAPK signal pathways (JNK, ERK, and p-38) and the transcription factor of NF-κB on the present or absence of interleukin (IL)-1β. Our experiments showed that protein production and mRNA expressions of MMP-1, MMP-3, MMP-13, IL-8, MCP-1, and RANTES were downregulated by treatment with glucosamine in HPSFs. The results further showed that GLN could inhibit IκBα phosphorylation and IκBα degradation leading to inhibition of the translocation of NF-κB to nuclei. However, GLN upregulated MAPKs pathways in HPSFs cells with or without IL-1β. The results suggest that the inhibition of MMP-1, -3, and -13 expressions as well as IL-8, MCP-1, and RANTES productions by GLN might mediate suppression of NF-κB signal pathways, and HPSFs seem to have a potential functions as an alternative source of MMPs and chemokines for inducing the degradation of cartilage in OA. J. Cell. Biochem. 104: 38–50, 2008.

Key words: osteoarthritis; matrix metalloproteinases; synovial fibroblasts; glucosamine

Osteoarthritis (OA) is a well-known disease that is part of the aging process, but its exact etiology is far from being completely understood. Clinical observations have indicated that metabolism of glycosaminoglycan together with that of collagen fibers is important in maintaining a matrix of articular cartilage, and that disruption of the balance between synthesis and breakdown causes OA. Interleukin-1 (IL-1) has been revealed to play a central role in the progression of articular cartilage degradation [van de Loo et al., 1995]. It was hypothesized to be produced by mononuclear cells in arthritic synovium [Farahat et al., 1993] and chondrocytes [Moos et al., 1999]. Subsequently, IL-1β stimulates the proliferation of human primary synovial fibroblasts (HPSFs) [Kumkumian et al., 1989] and enhances the production of various chemical mediators such as IL-6 [Guerne et al., 1989], prostaglandin E_2 (PGE_2) [Moos et al., 1999], and nitric oxide (NO) [Stefanovic-Racic et al., 1994] from synoviocytes. IL-1β also stimulates chondrocytes to produce PGE_2 [Campbell et al., 1990], NO [Stadler et al., 1991], and matrix metalloproteases (MMPs) [Tetlow et al., 2001] from chondrocytes. Among them, MMPs including MMP-1, -3, and -13 are important molecules that induce cartilage depletion in osteoarthritis

Hsien-Tsung Lu and Yu-Chih Liang contributed equally to this work.

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(OA) [Okada et al., 1992; Billinghurst et al., 1997]. Activated chondrocytes in OA produce these MMPs and are believed to be responsible for degrading the extracellular matrix (ECM) surrounding them.

However, the disease process affects not only the cartilage, but also the entire joint structure, including the synovial membrane, subchondral bone, ligaments, and periarticular muscles. In the synovium of OA, the inflammatory changes that take place include synovial hypertrophy and hyperplasia with an increased number of lining cells, and also an infiltration of the sublining tissue with a mixed population of inflammatory cells [Fuchs et al., 2004]. Synovial inflammation is clearly reflected in many of the signs and symptoms of OA, including joint swelling and effusion, stiffness, and sometimes redness, particularly at the level of the proximal (PIP) and distal interphalangeal (DIP) joints. Although the presence of synovial inflammation that is often associated with the OA process is recognized to be a secondary phenomenon related to the destruction of cartilage and the release of cartilage breakdown products in the synovial fluid, it is believed that the excessive production of cytokines and growth factors by the inflamed synovium and activated chondrocytes might play an important role in the pathophysiology of OA [Pelletier et al., 2000, 2001]. These factors are closely associated with functional alterations not only in the synovium, but also in the cartilage and subchondral bone. They are suspected of first being produced by the synovial membrane, and then diffusing into the cartilage through the synovial fluid resulting in the activation of chondrocytes, which, in turn, can produce proteases and other catabolic factors such as NO, and are responsible for inducing cartilage catabolism, chondrocyte apoptosis, and other structural changes associated with the disease process.

Furthermore, since cartilage is not innervated, pain signals associated with OA must arise in the synovium, joint capsule, or subchondral bone. Cartilage also lacks a vasculature—the extracellular fluid milieu of chondrocytes arises from synovial fluid, which is sequentially 'pumped' into and out of the cartilage when joints are compressed and relaxed; the synovium might be likened to a 'placenta' for the cartilage. The synovium is therefore the major determinant (in conjunction with the secreted products of the chondrocytes themselves) of the extracellular environment of chondrocytes, and also the likely site of origin of arthritic pain. This suggests that the synovium in OA might be a potential target for therapeutic interventions both from the standpoints of achieving symptomatic relief and preventing or reversing cartilage destruction [Martel-Pelletier et al., 1999]. On the other hand, evidence that implicates fibroblast-like synoviocytes (FLSs) as a central player in the propagation of rheumatoid arthritis (RA) was reviewed by Mor et al. [2005] Since synovial fibroblasts were found to participate in the infiltration and activation of other immune cells in the synovial tissue by secreting cytokines or by expressing adhesion molecules, it was concluded that synovial fibroblasts seem to be crucial regulators of joint inflammation and destruction in RA and OA [McCarty et al., 2000; Mor et al., 2005]. Strong evidence was provided by Fuch et al. to demonstrate a significant impact of synovial-derived MMPs on cartilage destruction in OA, and fibroblasts present in the synovial fluid appeared to play an outstanding role [Chin et al., 1990]. Therefore, HPSFs as an alternative source of MMPs and chemokine production for inducing the degradation of cartilage in OA should not be disregarded.

Glucosamine (GLN) has been used as a supplement for the treatment of OA for decades and has been shown to be effective in relieving the symptoms of OA. Its effects on the production of PGE_2, NO, and MMPs by chondrocytes and HPSFs in OA with IL-1β stimulation have been compared [Nakamura et al., 2004]. In this study, the possible involvement of synovium on cartilage destruction in OA patients was examined by studying the influence of GLN on the regulation of gene expression and protein production of MMPs and chemokines of human primary synovial fibroblasts (HPSFs) stimulated with IL-1β, and the signaling pathway involving in the underlying mechanism was further identified.

MATERIALS AND METHODS

Materials

D-(+)-Glucosamine HCl (GLN) was supplied by Sigma (St. Louis, MO), and IL-1β was purchased from R&D systems (Minneapolis, MN). Anti-mouse IgG-HRP, anti-rabbit IgG-HRP, the phospho-Akt antibody, phospho-ERK antibody, and total-p38 antibody were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

Primary Cell Culture

Human primary synovial fibroblasts (HPSFs) were prepared from surgical specimens obtained during arthroplasty in Taipei Medical University Hospital with signed informed consent. Synovial specimens were minced in 3 ml Dulbecco's modified Eagle's medium (DMEM) to fine pieces and followed by a series of digestions with 0.1% hyaluronidase for 15 min, 0.5% pronase for 30 min, 0.2% collagenase overnight, and with two washings in between with DMEM containing 10% fetal bovine serum (FBS). After setting overnight, the mixture was filtered through a 100-mesh sieve twice and centrifuged at 1,200 rpm for 3 min to obtain cell pellets. Cell residues were suspended with gentle blowing in 5 ml DMEM (containing 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml amphotericin B) and placed in 25 T flasks to culture at 37°C and 5% CO_2 until 80–90% confluence was reached, with the medium being changed every 4 days. HPSFs were passaged by transferring to 75 T flasks and two to five passages were used in this study. In each experiment, cells were rendered quiescent for 24 h by the addition of DMEM without serum and then were stimulated with IL-1β (1–15 ng/ml). Where indicated, cells were preincubated with various concentrations (0.1–1 mg/ml) of GLN for 30 min, and these conditions were maintained during the entire period of incubation, especially for the pH change of the medium after adding GLN.

Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts were prepared as previously described [Gomez-Garre et al., 2001]. After the incubation period, HPSFs were trypsinized and resuspended in buffer A (10 mM HEPES (pH 7.8), 10.0 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF)) and were then homogenized. Nuclear and cytosolic fractions were separated by centrifugation at 14,000 rpm for 5 min. The cytosolic fractions (supernatants) were stored at −20°C. The nuclei (pellets) were washed twice in buffer B (10 mM HEPES (pH 7.8), 10.0 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 1.0 mM PMSF, and 0.1% NP-40) and resuspended in buffer C (20 mM HEPES (pH 7.8), 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1.0 mM PMSF, 0.2 mM EDTA, and 0.5 mM DTT) for 30 min on ice. Samples were centrifuged at 10,000 rpm for 30 min, and supernatants (containing the nuclear proteins) were collected and stored at −20°C. The protein concentration was determined by the Bio-Rad Protein assay method.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as described by Chomczynski and Sacchi [Chomczynski and Sacchi, 1987]. The extracted RNA (2 μg) was reverse-transcribed at 37°C for 1.5 h by adding 5 μM of random hexamer oligonucleotides (Invitrogen Taiwan Ltd., Taipei, Taiwan), 200 units of reverse transcriptase (Takara Bio., Inc., Japan), 2.5 mM deoxyribonucleotide triphosphate (dNTP; Takara Bio., Inc.), and 10 mM dithiothreitol. PCR primers for amplification of MMP-1, MMP-3, MMP-13, IL-8, and GAPDH cDNA were synthesized according to the following oligonucleotide sequences: MMP-1: forward, 5′-GTCAGGGGAGATCATCGG-3′ and reverse, 5′-GCCCAGTAGTTATTCCCT-3′; MMP-3: forward 5′-AGATGATATAAATGGCATTCAG-3′ and reverse, 5′-CTCCAACTGTGAAGATCCAG-3′; MMP-13: forward, 5′-GCTTAGAGGTGACTGGCAA-3′ and reverse, 5′-CCGGTGTAGGTGTAGATAGGAA-3′; IL-8: forward, 5′-AGATATTGCACGGGAGAA-3′ and reverse, 5′-GAAATAAAGGAGAAACCA-3′; and GAPDH: forward, 5′-CTGCCGTCTAGAAAAACC-3′ and reverse, 5′-CCAAATTCGTTGTCATACC-3′. PCR was carried out with 2 μl of template cDNA and 23 μl of PCR mix buffer containing each primer (0.2 μM), dNTP (2.5 mM), and Taq DNA polymerase (1.25 units; Takara Bio., Inc.). After the PCR, 15 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining. The levels of mRNA for MMPs and GAPDH were quantified by scanning densitometry (Image-Pro Plus, Media Cybemetics; Silver Spring, MD).

Western Blot Analysis

HPSFs were extracted from the total protein using ice-cold RIPA lysis buffer (10 mM Tris–HCl (pH 7.6), 158 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1% Triton X-100, 1.0 mg/ml leupeptin, 1.0 mg/ml aprotinin, and 0.5 mM PMSF). The lysates were transferred to Eppendorf tubes and centrifuged at 14,000 rpm for

30 min at 4°C. The supernatants were transferred to fresh tubes, and the protein concentration was determined using the Bio-Rad Protein assay. Similar amounts of protein were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (Gelman, Inc., Ann Arbor, MI) by electroblotting. The membrane was blocked overnight in a 5% milk powder/TBST solution and then further incubated with one of the following antibodies (Abs): MMP-1, -3, or -13 Ab (Merck Taiwan, Inc., Taipei, Taiwan), anti-phospho-p38 mitogen-activated protein (anti-p-MAP; Cell Signaling Technology, Beverly, MA), anti-phospho-c-Jun N-terminal kinase (anti-p-JNK), anti-phospho-extracellular signal-regulated kinase (anti-p-ERK), or anti-phospho-Akt (anti-p-Akt; all three from Santa Cruz Biotechnology) for 2 h. Membranes were washed three times with TBST, then further incubated with the appropriate HRP-labeled secondary Ab in 5% milk powder/TBST, and developed using an ECL system (Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assay (EMSA)

Transcription factor activity was determined as previously described [Gomez-Garre et al., 2001]. Briefly, NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-CGCTTGATGAGTCAGCCGGAA-3′, respectively) were end-labeled with [^{32}P] by T4 polynucleotide kinase (Promega Corporation, Madison, WI). Nuclear extracts (5 μg) were equilibrated for 10 min in a binding buffer (5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 1 μg of poly(dI–dC) (Amersham Biosciences, Piscataway, NJ)), and then the labeled probe (0.35 pmol) was added and incubated for 20 min at room temperature. To establish the specificity of the reaction, negative controls without cell extracts and competition assays with a 100-fold excess of unlabeled oligonucleotides were performed. In the competition assays, the corresponding unlabeled probe was added to the reaction mixture 10 min prior to the addition of the labeled probe. A HeLa cell nuclear extract was used as the positive control of the technique (data not shown).

Enzyme-Linked Immunosorbent Assay (ELISA)

Quantikine® ELISA kits from R&D Systems (Minneapolis, MN) were used for the quantitative determination of IL-8, MCP-1, and RANTES concentrations in the cell culture medium. All reagents, working standards, and sample preparations followed the instructions provided in the product inserts. After adding 200 μl of Substrate Solution to each well and incubating for 30 min at room temperature with protection from light, 50 μl of Stop Solution was added to each well. Determinations of the optical density of each well were performed within 30 min, using a microplate reader set to 450 nm. The average zero standard optical density was subtracted from averaged duplicate readings for each standard, control, and sample. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and the sample concentration was determined by interpolation from the standard curve.

Statistical Analysis

Each value was from individual samples, and values were expressed as the mean ± SE of at least three independent determinations. Statistical analysis was performed using one-way ANOVA at a significant α level of 0.05.

RESULTS

Effect of IL-1β and GLN on mRNA Expression of MMPs and IL-8

The induction effect of IL-1β on the mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 at various concentrations (1–15 ng/ml) was first examined in HPSFs, and this induction effect by IL-1β at 2 ng/ml for 6 h was compared by pretreating HPSFs with various concentrations (0.1–1.0 mg/ml) of GLN for 30 min. Results shown in Figure 1 demonstrate that IL-1β was able to induce the mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 in dose-dependent manners. Figure 2 illustrates that pretreatment with GLN also inhibited the mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 of HPSFs stimulated with 2 ng/ml IL-1β in dose-dependent manners. However, only pretreatment with GLN at a concentration of 1.0 mg/ml was found to statistically significantly inhibit the mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 by HPSFs. The cells viability were also determined when HPSFs treated with various concentrations of GLN (0.1–1.0 mg/ml) for 30 min and then stimulated with 2 ng/ml IL-1β for 24 h. It demonstrates that either treatment with IL-1β alone or

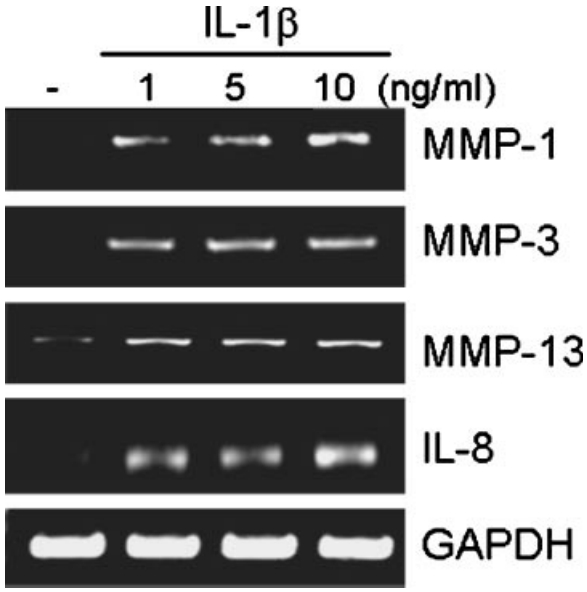

Fig. 1. Effects of IL-1β on MMP and IL-8 gene expressions by human primary synovial fibroblasts. Cells were treated with various concentrations of IL-1β (1, 5, and 10 ng/ml) for 6 h, and gene expressions of MMP-1, MMP-3, MMP-13, and IL-8 were MMP-3 MMP-13 and IL-8 were detected by RT-PCR as described in Materials and Methods Section. Equal loading in each lane was demonstrated by the similar intensities of GAPDH.

co-treatment with various concentrations of GLN had no significant effect on cell viability (data not shown).

Effect of GLN on Protein Production of MMPs and Chemokines

Protein production and secretion of MMPs by HPSFs pretreated with various concentrations (0.1–1.0 mg/ml) of GLN for 30 min and then stimulated with 2 ng/ml IL-1β for 24 h was measured by Western blotting, and results are given in Figures 3 and 4 for the measurement in the culture medium and cell extract, respectively. Results demonstrate that concentrations of MMP-1, -3, and -13 in the culture medium decreased in dose-dependent manners with statistically significant concentrations of GLN being 0.5, 0.25, and 0.1 mg/ml for MMP-1, -3, and -13, respectively. Likely, concentrations in the cell extract decreased as well in dose-dependent manners, but the statistically significant concentrations of GLN were 1.0, 1.0, and 0.5 mg/ml for MMP-1, -3, and -13, respectively.

Protein production of chemokines, including IL-8, MCP-1, and RANTES, by HPSFs pretreated with various concentrations (0.1–1.0 mg/ml) of GLN for 30 min and then stimulated with 2 ng/ml IL-1β for 24 h was measured by ELISA, and results are given in Figure 5 for measurements in the culture medium. Results demonstrate that the concentrations of IL-8, MCP-1, and RANTES in the culture medium decreased in dose-dependent manners with a statistically significant concentration of GLN being 1.0 mg/ml for all of them.

Effects of GLN on Phosphorylation of the MAPK Pathways (JNK, ERK, and P-38) With and Without Activation by IL-1β

Involvement of the MAPK pathways was examined by detecting the respective phosphorylation products in a time-course experiment. Figure 6 illustrates the pretreatment effect of GLN (1 mg/ml) on the time course of promoting phosphorylation of MAPKs (ERK, JNK, and p-38) in HPSFs stimulated with IL-1β. Results illustrate that IL-1β was able to activate the phosphorylation of JNK, ERK, and p38 starting at 15 min, and a burst induction occurred at 30 min. Pretreatment with GLN led to even greater promotion of phosphorylation of JNK, ERK, and p38 stimulated by IL-1β in the initial phase.

Figure 7 shows that treatment with GLN (1 mg/ml) only affected the phosphorylation of ERK, JNK, and p-38) in HPSFs without stimulation by IL-1β. This demonstrates that treatment with GLN only had a significantly direct effect on the phosphorylation of all MAPKs.

Effects of GLN on the Phosphorylation of Akt

Akt may be a downstream effector of phosphatidyl-inositol 3-kinase (PI 3-kinase) in the activation of NF-κB by IL-1β through TRAF6 with Akt being involved in phosphorylation of IKKα which frees up NF-κB for translocation [Reddy et al., 1997; Ozes et al., 1999; Andreozzi et al., 2004]. As demonstrated in Figure 8A, activation of Akt phosphorylation was found to be maintained at a similar level over time in HPSFs after stimulation with IL-1β. Pretreatment with GLN led to the insignificant promotion of phosphorylation of Akt stimulated with IL-1β for all time points examined. Similarly as shown in Figure 8B, treatment with GLN alone led to an insignificant direct effect on the phosphorylation of Akt at 60 min after treatment with GLN.

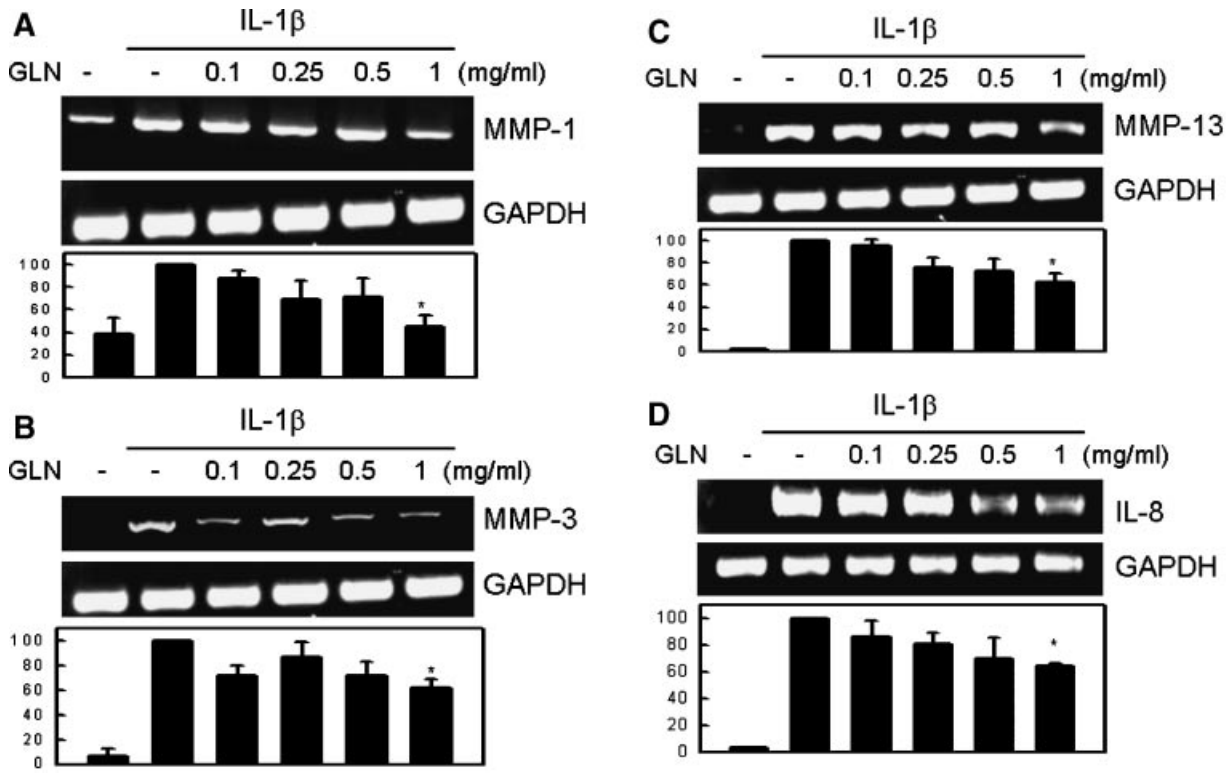

Fig. 2. Effects of glucosamine on IL-1β-induced MMP and IL-8 gene expressions by human primary synovial fibroblasts. Cells were preincubated for 30 min with various concentrations of glucosamine (0.1, 0.25, 0.5, and 1 mg/ml) and then stimulated with 2 ng/ml IL-1β for 6 h, and gene expressions of MMP-1 (**A**), MMP-3 (**B**), MMP-13 (**C**), and IL-8 (**D**) were detected by RT-PCR as described in Materials and Methods Section. Equal loading in each lane was demonstrated by the similar intensities of GAPDH. Data are expressed as the mean ± SE from three independent experiments. $*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

Effects of GLN on IL-1β-Induced Activation of NF-κB and Its Characterization

NF-κB activation occurs following dissociation of an inhibitory subunit, a member of the IκB family, which is degraded by a proteolytic process. Thus, the effect of GLN on the degradation of IκB leading to NF-κB activation induced by IL-1β was examined. As shown in Figure 9A, stimulation of HPSFs with IL-1β led to the phosphorylation of IκB at 15 min, and then the same lower level was maintained for up to 2 h. Preincubation with GLN reduced the phosphorylation of IκB which then remained at the same lower level as that treated with IL-1β alone. On the other hand, stimulation of HPSFs with IL-1β led to the degradation of IκB at 15 min, and then it gradually recovered to the basal level as demonstrated in Figure 9B. However, preincubation with GLN reduced the degradation of IκB in the initial phase.

The translocation of NF-κB subunits (p65) into the nucleus was further examined by comparing its concentrations in the cytosolic and nuclear fractions. Results demonstrated in Figure 10 show a higher concentration of p-65 in the cytosolic fraction and a corresponding lower concentration in the nuclear fraction when pretreated with GLN at 15 min. This clearly indicates that the translocation of one of the NF-κB subunits of p65 into the nucleus might be blocked at as early as 15 min.

DISCUSSION

Degradation of articular cartilage is driven by an imbalance of catabolic and anabolic factors in OA [Goldring, 2000]. It is well established that IL-1β locally increases during the OA process, and induces a large cascade of events that lead to cartilage damage, such as the synthesis of MMPs and ECM proteins that are absent from normal cartilage, the release of other inflammatory mediators, the inhibition of chondrocyte proliferation, and induction of cell death [Lotz, 2001; Vincenti and Brinckerhoff, 2001]. In this study, HPSFs stimulated with IL-1β were found to induce mRNA expression and protein

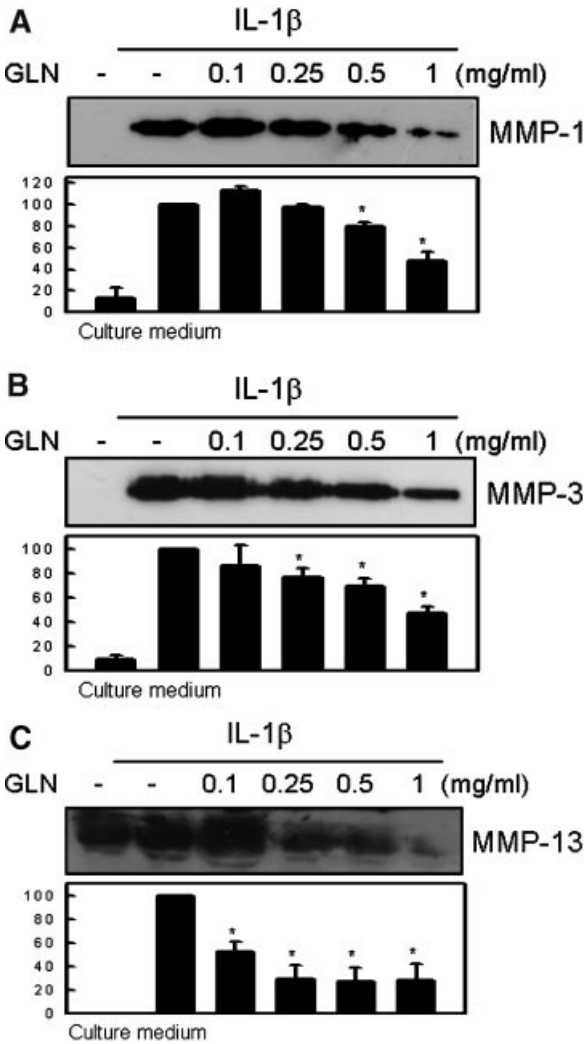

Fig. 3. Effects of glucosamine on IL-1β-induced protein production of MMPs by human primary synovial fibroblasts in culture medium. Cells were preincubated for 30 min with various concentrations of glucosamine (0.1, 0.25, 0.5, and 1 mg/ml) and then stimulated with 2 ng/ml IL-1β for 24 h, and production of MMP-1 (**A**), MMP-3 (**B**), and MMP-13 (**C**) was detected in culture medium by Western blot analysis as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. $*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

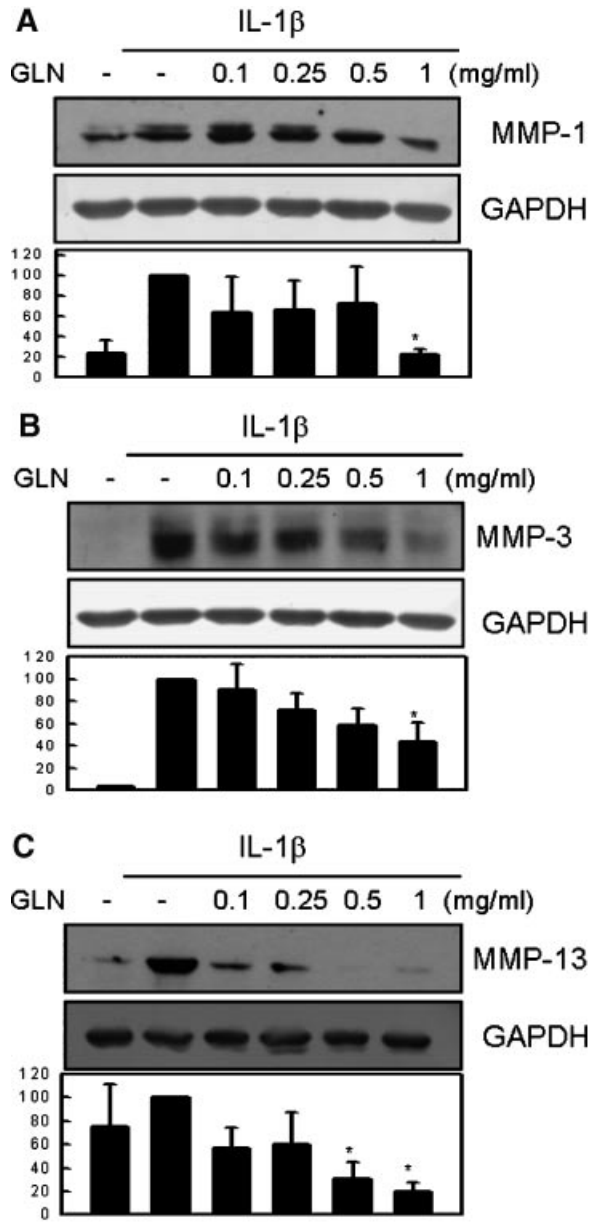

Fig. 4. Effects of glucosamine on IL-1β-induced protein production of MMPs by human primary synovial fibroblasts in cell extracts. Cells were preincubated for 30 min with various concentrations of glucosamine (0.1, 0.25, 0.5, and 1 mg/ml) and then stimulated with 2 ng/ml IL-1β for 24 h, and the production of MMP-1 (**A**), MMP-3 (**B**), and MMP-13 (**C**) was measured in cell extracts by Western blot analysis as described in Materials and Methods Section. Equal loading in each lane was demonstrated by similar intensities of GAPDH. Data are expressed as the mean ± SE from three independent experiments. $*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

production of MMPs (MMP-1, -3, and -13) and chemokines (IL-8, MCP-1, and RANTES) in dose-dependent manners. These inducing effects of IL-1β were suppressed by pretreatment with GLN, but treatment with a higher concentration (mostly >0.5 mg/ml) and GLN alone lacked these inducing effects. Suppression is mediated at the level of transcription involving the transcription factor, NF-κB. Translocation of NF-κB was reduced by GLN as a result of the inhibition of IκB degradation and phosphorylation. Among the MAPK pathways involved in the transcriptional regulation of AP-1 members of the *Jun* and *Fos* families of

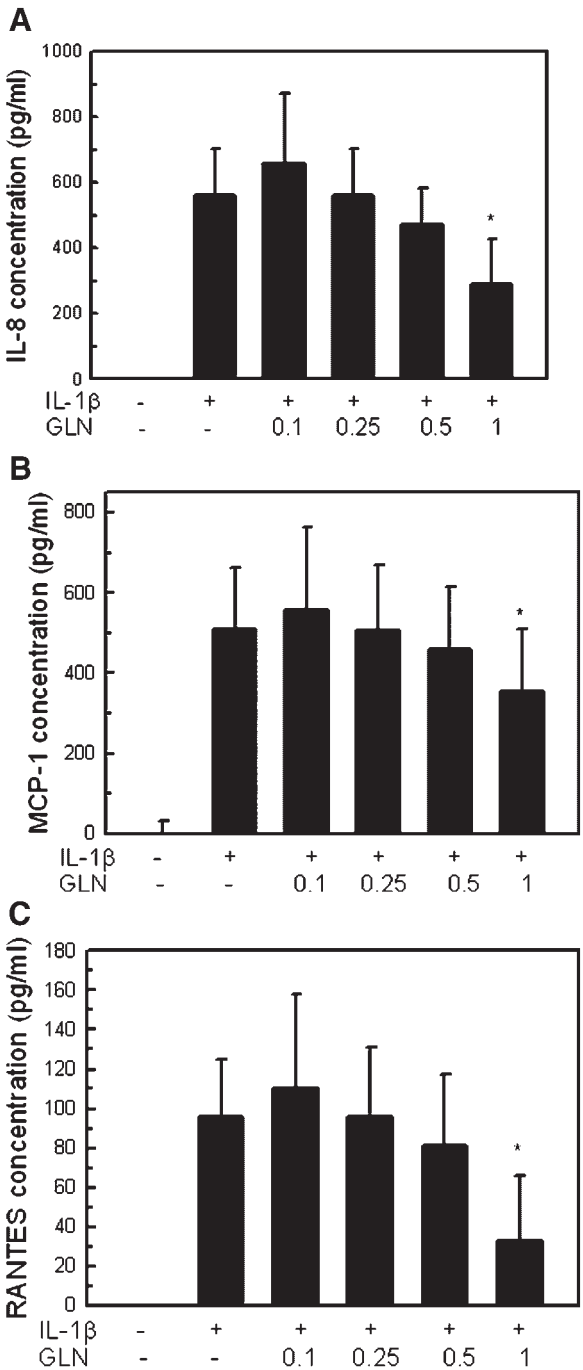

Fig. 5. Effects of glucosamine on IL-1β-induced production of chemokines by human primary synovial fibroblasts. Cells were preincubated for 30 min with various concentrations of glucosamine (0.1, 0.25, 0.5, and 1 mg/ml) and then stimulated with 2 ng/ml IL-1β for 24 h, and the production of IL-8 (**A**), MCP-1 (**B**), and RANTES (**C**) was measured in the culture medium by ELISA as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. $^*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

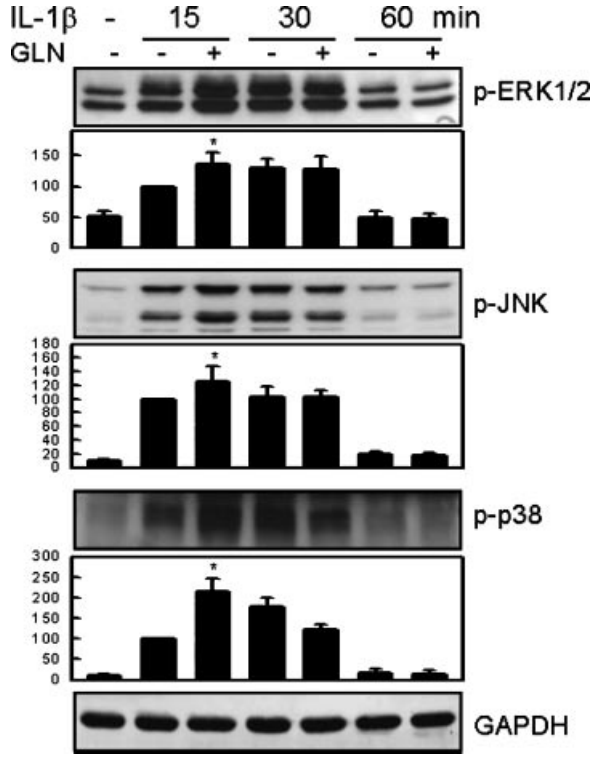

Fig. 6. Effects of glucosamine on IL-1β-induced phosphorylation of ERK, JNK, and p38 in human primary synovial fibroblasts. Cells were preincubated for 30 min with 1 mg/ml glucosamine and then stimulated with 2 ng/ml IL-1β for various time periods (15, 30, and 60 min), and phosphorylated ERK, phosphorylated JNK, and phosphorylated p38 were detected by Western blot analysis as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. $^*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

transcription factors, phosphorylation of JNK, ERK, and p38 was found to be increased in the presence of GLN under IL-1β treatment. It was also found that activation of the PI′3-kinase/Akt pathway was not involved in the suppression mediated by pretreatment with GLN followed by stimulation with IL-1β in HPSFs.

In response to the stimulation of IL-1β at the 5 ng/ml level, the production of MMP-1, -3, and -13 was enhanced in normal and OA chondrocytes, and OA synoviocytes as reported by Nakamura et al. [2004] Overall, OA chondrocytes showed a higher sensitivity to stimulation by IL-1β than did normal chondrocytes and OA synoviocytes. After stimulation with IL-1β, the production of MMP-1, -3, and -13 was partly suppressed by 100 μg/ml GLN in normal chondrocytes. In OA synoviocytes, 500 μg/ml GLN suppressed the production of MMP-1, -3,

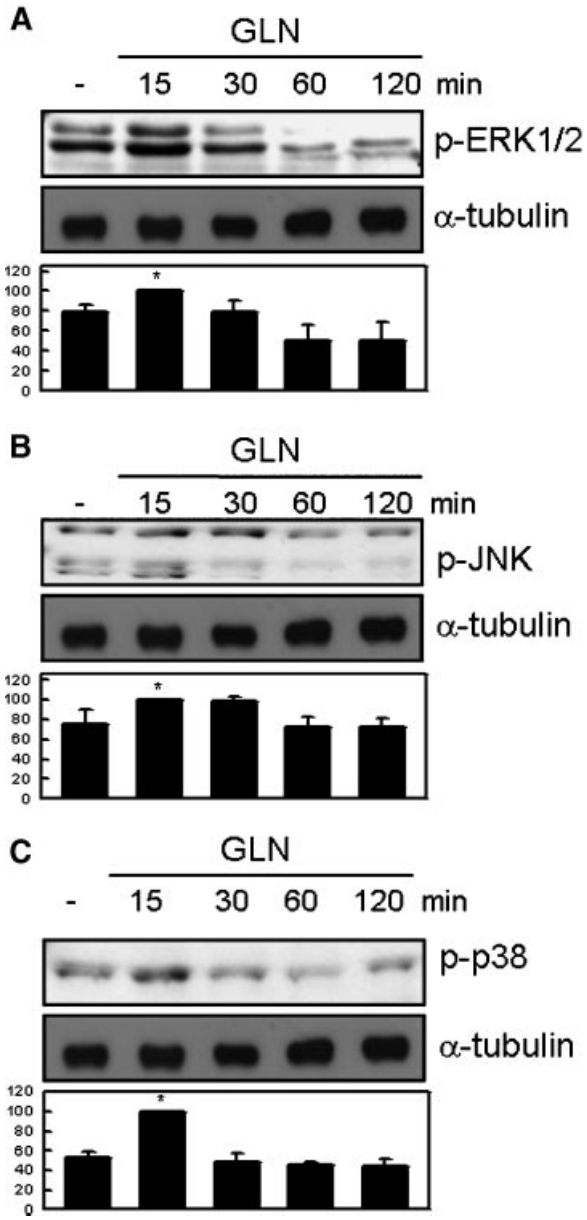

Fig. 7. Effects of glucosamine on the phosphorylation of ERK, JNK, and p38 in human primary synovial fibroblasts. Cells were incubated with 1 mg/ml glucosamine for various time periods and phosphorylated ERK (**A**), phosphorylated JNK (**B**), and phosphorylated p38 (**C**) were detected by Western blot analysis as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. *$P < 0.05$ compared with the control group (**lane 1**).

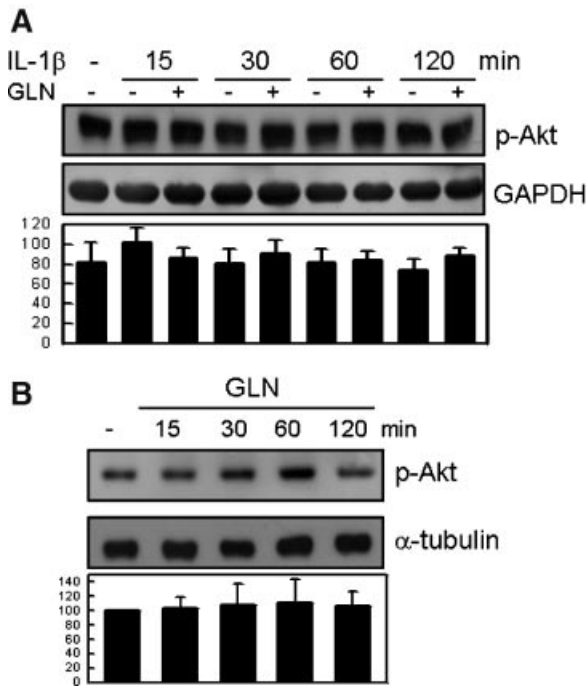

Fig. 8. Effects of glucosamine on phosphorylation and IL-1β-induced phosphorylation of Akt in human primary synovial fibroblasts. Cells were preincubated for 30 min with 1 mg/ml glucosamine and then stimulated with (**A**) or without (**B**) 2 ng/ml IL-1β for various time periods (15, 30, 60, and 120 min), and phosphorylated-Akt was detected by Western blot analysis as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments.

and -13. However, MMP production was not suppressed by GLN at the concentration of 100 μg/ml in OA chondrocytes [Nakamura et al., 2004]. In this study, results consistently showed that stimulation of HPSFs with IL-1β resulted in the enhanced expression and production of MMPs. The stimulating effects of IL-1β at the 2 ng/ml level on the production of MMPs were suppressed by pretreatment with GLN at a higher concentration (mostly >0.5 mg/ml). Therefore, the disease-modifying effects of GLN through inhibition of the production of MMPs from HPSFs seemed to reveal that HPSFs potentially function as an alternative source of MMPs for inducing the degradation of cartilage in OA.

Several chemokines have been shown to be overproduced in arthritic joints, including IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, and Eotaxin-1. The production of chemokines further stimulated expressions of their own receptors and destroyed enzymes of MMPs, which are associated with joint inflammation and cartilage degradation [Hsu et al., 2004]. Ma et al. [2002] reported that GLN is an efficacious agent for suppressing activation of T-cells and dendritic cells, two crucial cells involved in immune responses. By suppressing immune activity, synovial tissue can regenerate unimpeded.

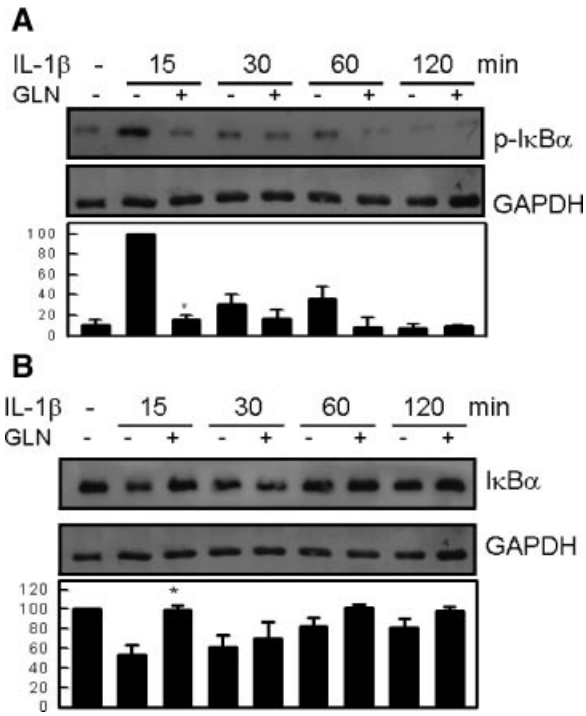

Fig. 9. Effects of glucosamine on IL-1β-induced p-IκBα expression (**A**) and IκBα degradation (**B**) in human primary synovial fibroblasts. Cells were preincubated for 30 min with 1 mg/ml glucosamine and then stimulated with 2 ng/ml IL-1β for various time periods (15, 30, 60, and 120 min), and phosphorylated IκBα and IκBα were detected by Western blot analysis as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. $*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

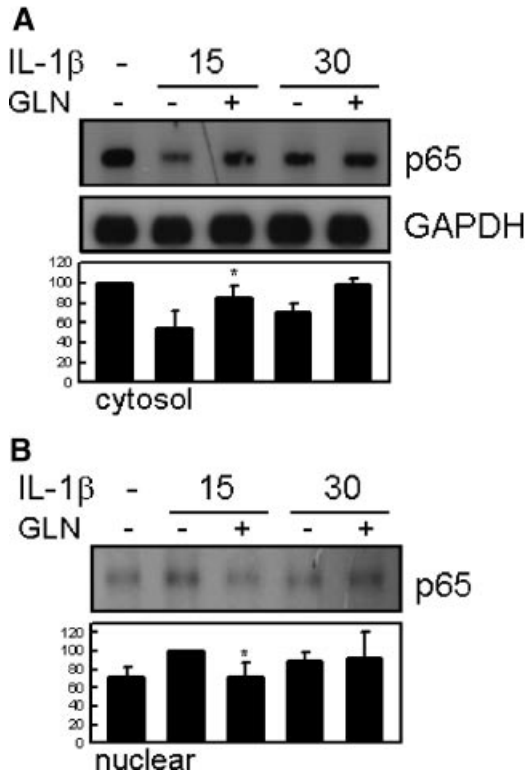

Fig. 10. Effects of glucosamine on IL-1β-induced p65 translocation in human primary synovial fibroblasts. Cells were preincubated for 30 min with 1 mg/ml glucosamine and then stimulated with 2 ng/ml IL-1β for two time periods (15 and 30 min), and p65 in the cytosol (**A**) and nuclear (**B**) fractions were detected by EMSA as described in Materials and Methods Section. Data are expressed as the mean ± SE from three independent experiments. $*P < 0.05$ compared with the positive control group with IL-1β treatment (**lane 2**).

The absence of soluble mediators of inflammation and pain generated by immune cells at sites of cartilage erosion may provide patients with an increased sense of well being that might or might not result in any remarkable long-term therapeutic effect. In this study, the results consistently showed that the production of IL-8, MCP-1, and RANTES stimulated by IL-1β from HPSFs significantly increased, and this effect was suppressed at a GLN level of 1.0 mg/ml. Therefore, the beneficial effects of GLN on the progression of OA disease through its suppression of the stimulated expression and production of IL-8 and the production of MCP-1 and RANTES from HPSFs might provide evidence to reveal that HPSFs might potentially be involved in the induction of the degradation of cartilage in OA.

The molecular mechanisms through which IL-1 increases the expression of MMPs in chondrocytes of various sources have been elucidated. Mengshol et al. [2000] reported that IL-1 induction of MMP-13 requires p38 activity, c-Jun N-terminal kinase (JNK) activity, and NF-κB translocation in SW1353 chondrosarcoma cells and rabbit articular chondrocytes. In chondrosarcoma cells, MMP-1 induction depends on p38 and ERK and does not require JNK or NF-κB. In articular chondrocytes, inhibition of ERK had no effect, while inhibition of p38 produced variable results. Liacini et al. [2002] further demonstrated the involvement of MAPKs, AP-1, and NF-κB transcription factors in IL-1's induction of MMPs in primary cultures of human and bovine chondrocytes. Regarding synovial fibroblasts, studies by Vincenti et al. and Barchowsky et al. both demonstrated that the NF-κB pathway is required for activation of MMP-1 transcription in rabbit primary synovial fibroblasts (RSFs). Stimulation of the ERK and AP-1 pathways is an essential component of MMP-1 transcriptional activation, and

cooperation between the MAPK and NF-κB signaling pathways for IL-1-dependent MMP-1 transcription is necessary [Barchowsky et al., 2000; Berenbaum, 2004]. In this study on HPSFs, suppression by GLN of the activation of MMP-1, -3, and -13 by IL-1β was mainly found to similarly mediate the reductions in the translocation of NF-κB as a result of the inhibition of IκB degradation and phosphorylation. Since phosphorylation of JNK, ERK, and p38 was enhanced in HPSFs in the presence of GLN, it might be concluded that cooperation between the MAPK and NF-κB signaling pathways for IL-1-dependent MMP transcription in HPSFs is essential.

The concentration of GLN in synovial fluid of a large animal model in which the GLN was administered by nasogastric intubation and intravenous injection of 20 mg/kg body weight was found to be 0.05–0.112 and 1.5–2.5 μg/ml, respectively, which are lower than that found in serum (6–300 μg/ml) and even much lower than that used in those studies described above (100–1,000 μg/ml) [Laverty et al., 2005]. Determining how to substantiate the resultant serum or synovial fluid concentrations after administering clinically relevant doses of GLN several orders of magnitude lower than those reported to modify chondrocyte anabolic and catabolic activities in tissue and cell culture experiments is controversial. This controversy might inevitably exist for the disease-modifying effect of GLN on HPSFs since the most effective concentration of GLN was around 100–1,000 μg/ml as revealed above. Perhaps, it might be speculated that IL-1β at a concentration of 50 ng/ml, which is much higher than that used in those studies described above and most studies reported in the literature (<5 ng/ml), was used to treat cartilage explants in Chan's study, which reported that the physiologically relevant concentrations (1–20 μg/ml) of GLN that can downregulate IL-1β-induced gene expression of iNOS and COX-2 and repress cytokine-stimulated mPGEs1 transcription lead to a reduction in the synthesis of NO and PGE_2 on cartilage explants [Chan et al., 2005]. Furthermore, it was reported that IL-1β induces increased expression of the glucose transporter (GLUT)1 mRNA and protein, and GLUT9 mRNA which facilitates glucose transport in human articular chondrocytes [Shikhman et al., 2001]. If the uptake of GLN by HPSFs follows the same receptors as glucose or a specific receptor exists for transporting GLN, then the facilitated transport of GLN in IL-1β-treated chondrocytes or HPSFs would be able to explain why physiologically relevant concentrations of GLN on the gene expression of several mediators need to be effective at a higher concentration of IL-1β, whereas a much higher concentration than physiologically relevant concentrations needs to be used so that GLN can express its effects at lower concentrations of IL-1β. Whether the GLUT in HPSFs plays an important role as a specific receptor for GLN in initiating signaling cascades remains to be elucidated.

On the other hand, since chondrocytes are embedded in cartilage that lacks a vasculature, the synovial fluid should be the only source supplying chondrocytes with effective mediators including GLN. Unless those mechanisms described above are operable, concentrations of GLN around those targeted cells of chondrocytes should be similar to or even less than those in synovial fluid, which is too low to modify catabolic and anabolic activities on chondrocytes. However, synoviocytes are located in the intimal lining of the synovium, its membrane directly contacts the synovial fluid, and its sublining region contains blood vessels. Therefore, concentrations of GLN in synoviocytes can be comprised of that from the synovial fluid and that from the blood vessels in the sublining region leading to the situation where a GLN concentration able to modify synoviocytes is more-readily achievable than in chondrocytes. From this point of view, the disease-modifying effects of GLN on the clinically observed efficacy of OA highly substantiates that the involvement of synoviocytes in the disease progression of OA should be possible.

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