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ORIGINAL ARTICLE

Disease-modifying Effects of Glucosamine on Interleukin-1β-treated Chondrosarcoma Cells (SW1353) Under Normoxic and Hypoxic Conditions

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Background: With respect to interleukin (IL)-1β concentration, the disease-modifying effects of glucosamine HCl (GLN) on IL-1β-treated chondrocytes followed a dose-dependent manner and were traditionally conducted under normoxic conditions not generally encountered by chondrocytes *in vivo*.
Purposes: To demonstrate the beneficial effects of GLN on IL-1β-induced matrix metalloproteinase (MMP) expression in chondrosarcoma cells in a dose-dependent manner, but at different concentration ranges of IL-1β; additionally, to determine how hypoxia affects GLN's beneficial effects after dose-dependent application.
Methods: Under normoxic and hypoxic conditions, human chondrosarcoma cells (SW1353) induced by high and low IL-1β concentrations were respectively treated with low and high concentration ranges of GLN. mRNA and protein expression for MMPs and glucose transporter protein (GLUT)-1 were examined by reverse transcription–polymerase chain reaction and Western blot analysis.
Results: High concentrations of GLN could reduce mRNA and protein expressions of MMPs induced by low concentration of IL-1β; whereas low concentrations of GLN could reduce the inflammatory response upregulated by high concentrations of IL-1β. The data might substantiate the beneficial effects in clinical application of lower plasma concentrations of GLN achievable by oral administration. Furthermore, the effects were compared under hypoxic conditions to mimic real conditions encountered in human cartilage under normoxia. Upregulation of HIF-1α protein levels under hypoxia was observed. The level of MMP protein expression induced by IL-1β was lower under hypoxic conditions. Instead, inhibition of MMPs by GLN appeared to be more apparent. Finally, GLUT-1 protein expression of SW1353 was upregulated under IL-1β and hypoxia treatment.
Conclusion: Glucose transporter, most likely GLUT-1, inducible by HIF-1α, might play an important role in the therapeutic efficacy of a lower plasma concentration of GLN on osteoarthritis achievable by oral administration.

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1. Introduction

Articular cartilage is an avascular tissue that derives its nutrition and O_2 supply by diffusion from the synovial fluid and subchondral bone.[1] Chondrocytes at the articular surface have been estimated to be exposed to approximately 6–10% O_2, whereas those in the deepest layers of the articular cartilage have access to less.[2,3] In inflamed conditions, such as osteoarthritis (OA), O_2 levels are considerably lower.[4] Hypoxia-inducible factor-1 (HIF-1) has been proven to play a primary role in chondrocyte adaptation to lower O_2 levels (i.e., hypoxia).[5] HIF-1 is a heterodimer consisting of two different subunits, HIF-1α and HIF-1β, which are members of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family of transcription factors.[6,7] HIF-1α is expressed in human normal and OA articular chondrocytes cultured under normoxic conditions, able to be induced or stabilized by hypoxia or treatment with tumor necrosis factor-α (TNF-α).[1]

Several studies have reported that chondrocytes cultured at low O_2 levels demonstrate downregulation of type II collagen, a lack of change in aggrecan mRNA levels, and upregulation of the tissue inhibitor of metalloproteinase-1.[8–11] O_2 levels may also modulate chondrocyte responses to cytokines implicated in joint diseases, including interleukin (IL)-1, TNF-α, and transforming growth factor-β. Hypoxia has been reported to decrease IL-1β- and TNF-α-induced nitric oxide (NO) production in porcine cartilage explants.[12] Furthermore, when chondrocytes are present in hypoxic conditions, the effects of IL-1β on both anabolic (levels of type II collagen and aggrecan core protein messenger RNA decrease) and catabolic [matrix metalloproteinase (MMP)-1 and MMP-3 mRNA increase] processes significantly differ from those in normoxic conditions.[13] It is likely that O_2 levels present in the chondrocyte environment influence cytokine interactions.

Cartilage damage in OA is well known for being mediated by IL-1β, initiating a number of events that lead to cartilage destruction, including the inhibition of the biosynthesis of matrix macromolecules and increase in catabolic pathways. These cause an imbalance between matrix component biosynthesis and degradation, leading to the progressive destruction of tissue and extensive articular damage.[14] Chondroprotective agents, such as glucosamine (GLN), have been shown to be effective in relieving OA symptoms.[15,16] These beneficial effects have been attributed to their ability to counteract IL-1β activity on chondrocytes including: the prevention of marked inhibitory effects on uridine 5′-diphospho-glucuronosyltransferase I (GlcAT-1) mRNA expression,[17] reduction of NO and prostaglandin E_2 (PGE_2) production, complete prevention of the upregulation of stromelysin (MMP-3) mRNA expression, increase in the expression of mRNA encoding type II IL-1β receptor,[18] inhibition of the synthesis of proinflammatory mediators in a dose-dependent manner (10–1000 μg/mL),[19] increase in aggrecan core protein levels in a dose-dependent manner (1.0–150 μM GLN sulfate) by increased aggrecan mRNA levels, and simultaneous inhibition of production and enzymatic activity of matrix-degrading MMP-3.[20] Chan et al reported that physiologically relevant concentrations of GLN (1–20 μg/mL) downregulate IL-1β-induced gene expression of inducible NO synthase and cyclooxygenase-2, repressing cytokine-stimulated microsomal PGE synthase-1 transcription and leading to reduced NO and PGE_2 synthesis in articular cartilage.[21]

Most studies described above were conducted in normoxic conditions not generally encountered by chondrocytes *in vivo* in order to demonstrate that the beneficial effects of GLN occur in a dose-dependent manner, but at different concentration ranges on chondrocytes treated with different concentrations of IL-1β (the activity of which is regulated by O_2 levels as described above). Nevertheless, it would be more appropriate to reexamine GLN's dose-dependent effects on chondrocytes treated with different concentrations of IL-1β under hypoxic conditions.

2. Materials and Methods

2.1. Materials

D-(+)-glucosamine HCl (GLN) was supplied by Sigma (St. Louis, MO, USA), and IL-1β was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Chondrocyte culture

Human chondrosarcoma cells (SW1353) were obtained from American Type Culture Collection (ATCC HTB-94; Manassas, VA, USA). Human chondrosarcoma cells were grown to >90% confluence in L-15 medium supplemented with 10% fetal calf serum (Invitrogen, Grand Island, NY, USA), 60 U/mL penicillin, 60 μg/mL streptomycin, and 2 mM glutamine at 37°C. In each experiment, cells were rendered quiescent for 24 hours by the addition of L-15 medium without serum, and then stimulated with a low (2 ng/mL) and high (30 ng/mL) concentration of IL-1β for the indicated time period under normoxic ($O_2 \fallingdotseq 21\%$) and hypoxic ($O_2 < 3\%$) conditions. Where indicated, cells were preincubated with low (5–100 μg/mL) and high (0.1–1.0 mg/mL) concentrations of GLN for the indicated time period. These conditions were maintained throughout the entire incubation period, especially during the pH change to the medium after GLN addition. In this study, treatment with 200 μM desferrioxamine was chosen as the positive control for HIF-1α.[22]

2.3. WST-1 cell viability assay

After culturing, 300 μL of a WST-1 diluted solution (1:10) was added to each culture well and incubated for 2 hours at 37°C. The absorbance at 450 nm was recorded using 0.1 mL of the resulting solution sampled from each well.

2.4. Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated as described by Chomczynski and Sacchi.[23] The extracted RNA (2 μg) was reverse-transcribed at 37°C for 1.5 hours by adding 5 μM of random hexamer oligonucleotides (Gibco BRL, Life Technologies, Grand Island, NY, USA), 200 units of reverse transcriptase (Takara Bio Inc., Shiga, 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, aggrecan, and glyceraldehyde 3-phosphate dehydrogenase cDNA were synthesized according to the oligonucleotide sequences shown in Table 1.

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 PCR, 15 μL of the reaction mixture were subjected to electrophoresis on a 1.5% agarose gel, and PCR products were visualized by ethidium bromide staining. The levels of mRNA for MMPs and glyceraldehyde 3-phosphate dehydrogenase were quantified by scanning densitometry (Image-Pro Plus; Media Cybernetics, Bethesda, MD, USA).

2.5. Western blot analysis

Cells 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 ethylenediamine tetraacetic acid, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1.0 mg/mL leupeptin, 1.0 mg/mL aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride]. The lysates were transferred to Eppendorf tubes and centrifuged at 14,000 rpm at 4°C for 30 minutes. The supernatants were then transferred to fresh tubes, and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Similar amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and, via electroblotting, transferred to a nitrocellulose membrane (Gelman, Ann Arbor, MI, USA). The membrane was blocked overnight in a 5% milk powder/TBST (Tris buffered saline with Tween) solution, then further incubated for 2 hours with one of the following antibodies: MMP-1, -3 or -13 antibody (Oncogene, Merck, Taipei, Taiwan). Membranes were washed three times with TBST, further incubated with the appropriate horseradish peroxidase-labeled secondary antibody in 5% milk powder/TBST, and developed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. Statistical analysis

Each value was derived from individual samples; values are expressed as the mean ± standard error of at least three independent determinations. Statistical analysis was performed using one-way analysis of variance at a 0.05 α significance level.

3. Results

3.1. Cell viability of SW1353 cells under normoxic and hypoxic conditions

The viability of SW1353 cells under normoxic and hypoxic conditions was first examined via individual treatment for 24 hours with either IL-1β or GLN at two levels (IL-1β, 2 and 30 ng/mL; GLN, 5 and 1000 μg/mL). Results are shown in Figure 1. Under normoxic conditions, cell viability was not affected when SW1353 cells were treated with IL-1β at either levels and 5 μg/mL GLN. Surprisingly, cell viability discernably increased when SW1353 cells were treated with 1000 μg/mL of GLN alone. Under hypoxic conditions for the same treatment with either IL-1β or GLN, cell viability increased compared to that under normoxic conditions. Treatment with 1000 μg/mL GLN alone seemed to promote cell proliferation under hypoxic conditions.

Table 1 Oligonucleotide sequences used for the PCR primers

	Forward	Reverse
MMP-1	5′-GTCAGGGGAGATCATCGG-3′	5′-GCCCAGTAGTTATTCCCT-3′
MMP-3	5′-AGATGATATAAATGGCATTCAG-3′	5′-CTCCAACTGTGAAGATCCAG-3′
MMP-13	5′-GCTTAGAGGTGACTGGCAA-3′	5′-CCGGTGTAGGTGTAGATAGGAA-3′
IL-8	5′-AGATATTGCACGGGAGAA-3′	5′-GAAATAAAGGAGAAACCA-3′
Aggrecan	5′-TGAGGAGGGCTGGAACAAGTACC-3′	5′-GGAGGTGGTAATTGCAGGGAACA-3′
GAPDH	5′-CTGCCGTCTAGAAAAACC-3′	5′-CCAAATTCGTTGTCATACC-3′

MMP = matrix metalloproteinase; IL = interleukin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

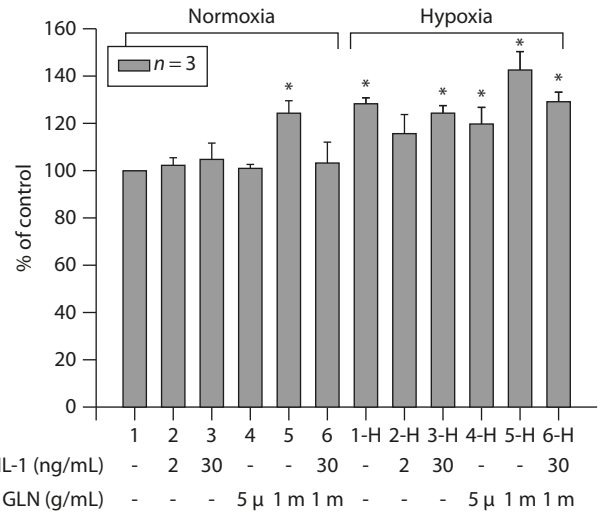

Figure 1 Effects of interleukin (IL)-1β, glucosamine (GLN), and hypoxia on the viability of chondrosarcoma cells. Cells were incubated for 24 hours with various concentrations of IL-1β (2 and 30 ng/mL) and/or GLN (5 μg/mL and 1 mg/mL) under normoxia ($O_2 \fallingdotseq 21\%$) or hypoxia ($O_2 < 3\%$). The extent of cell viability was determined by WST-1 as described in section 2 (Materials and Methods) of this article. y axis = O.D. 450. *$p < 0.05$ *vs.* normoxic control.

3.2. mRNA expressions of MMPs and IL-8 by IL-1β-treated SW1353 cells after pretreatment with GLN

mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 by IL-1β-treated SW1353 cells at two levels (2 and 30 ng/mL) for 6 hours were compared to those after pretreatment for 0.5 hours with two concentration ranges of GLN (high concentration range, 0.10, 0.25, 0.50 and 1.00 mg/mL; low concentration range, 5, 10, 25 and 100 μg/mL). Results are illustrated in Figure 2, demonstrating that mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 increased when SW1353 cells were treated at both levels with IL-1β alone. Dose-dependent inhibition was observed with GLN pretreatment on IL-1β-induced mRNA expressions of MMP-1, MMP-3, MMP-13, and IL-8 at both IL-1β levels. This further demonstrates that a higher concentration range of GLN is necessary to dose-dependently inhibit the increase in IL-1β-induced mRNA expression at a lower level of IL-1β treatment; whereas a lower concentration range of GLN is sufficient to dose-dependently inhibit IL-1β-induced mRNA expression at a higher level of IL-1β treatment.

3.3. mRNA expression of aggrecan by IL-1β-treated SW1353 cells pretreated with GLN

mRNA expression of aggrecan by IL-1β-treated SW1353 cells for 6 hours at two levels (2 and 30 ng/mL) was compared to pretreatment for 0.5 hours with two concentration ranges of GLN (high concentration range, 0.10, 0.25, 0.50 and 1.00 mg/mL; low concentration range, 5, 10, 25 and 100 μg/mL). Results are illustrated in Figure 3, demonstrating that mRNA expression of aggrecan was induced when SW1353 cells were treated with IL-1β alone at both levels. However, dose-dependent inhibition was observed with GLN pretreatment on IL-1β-induced mRNA expression of aggrecan at both levels of IL-1β. This further demonstrates that a higher concentration range of GLN is necessary to dose-dependently inhibit the induction of mRNA expression of aggrecan at a lower level of IL-1β treatment; whereas a lower concentration range of GLN is sufficient to dose-dependently inhibit such induction at a higher level of IL-1β treatment.

3.4. Protein expressions of HIF-1α and GLUT-1 by SW1353 cells under hypoxic conditions and treatment with IL-1β under normoxic conditions

Protein expressions of HIF-1α and glucose transporter protein (GLUT)-1 were compared according to incubation of SW1353 cells under hypoxic conditions ($O_2 < 3\%$) for different time periods (0, 3, 6, 12, 24 and 48 hours), and these were also compared to treatment with various concentrations of IL-1β (1, 2, 10, 30 and 50 ng/mL) under normoxic conditions ($O_2 \fallingdotseq 21\%$) for 24 hours. Figure 4A shows that protein levels of both HIF-1α and GLUT-1 respectively increased gradually and sharply after incubation for 12 hours under hypoxic conditions. Figure 4B demonstrates that protein expressions of both HIF-1α and GLUT-1 gradually increased, with IL-1β concentrations increasing up to 2 ng/mL then decreasing thereafter to 50 ng/mL under normoxic conditions.

3.5. Protein expressions of HIF-1α, GLUT-1, and MMPs by SW1353 cells treated with IL-1β under hypoxic and normoxic conditions

Protein expressions of HIF-1α and GLUT-1 were compared by incubation of SW1353 cells with various concentrations of IL-1β (2, 10 and 30 ng/mL) under normoxic and hypoxic conditions for 24 hours. Figure 5A shows that protein expression of HIF-1α did not increase with increasing IL-1β concentration under normoxic conditions, though it was inducible under hypoxic conditions. It was then dose-dependently inhibited by IL-1β. Under normoxic conditions, expression of GLUT-1 by SW1353 cells was induced by a lower O_2 level and increased with increasing IL-1β concentrations, but decreased with increasing IL-1β concentrations under hypoxic conditions.

Protein expressions of MMP-1, MMP-3 and MMP-13 by SW1353 cells treated with various concentrations of IL-1β were compared under hypoxic and normoxic conditions. Figure 5B shows that protein levels of MMP-1, MMP-3 and MMP-13 secreted in the media were induced by IL-1β at a concentration of as low as 2 ng/mL

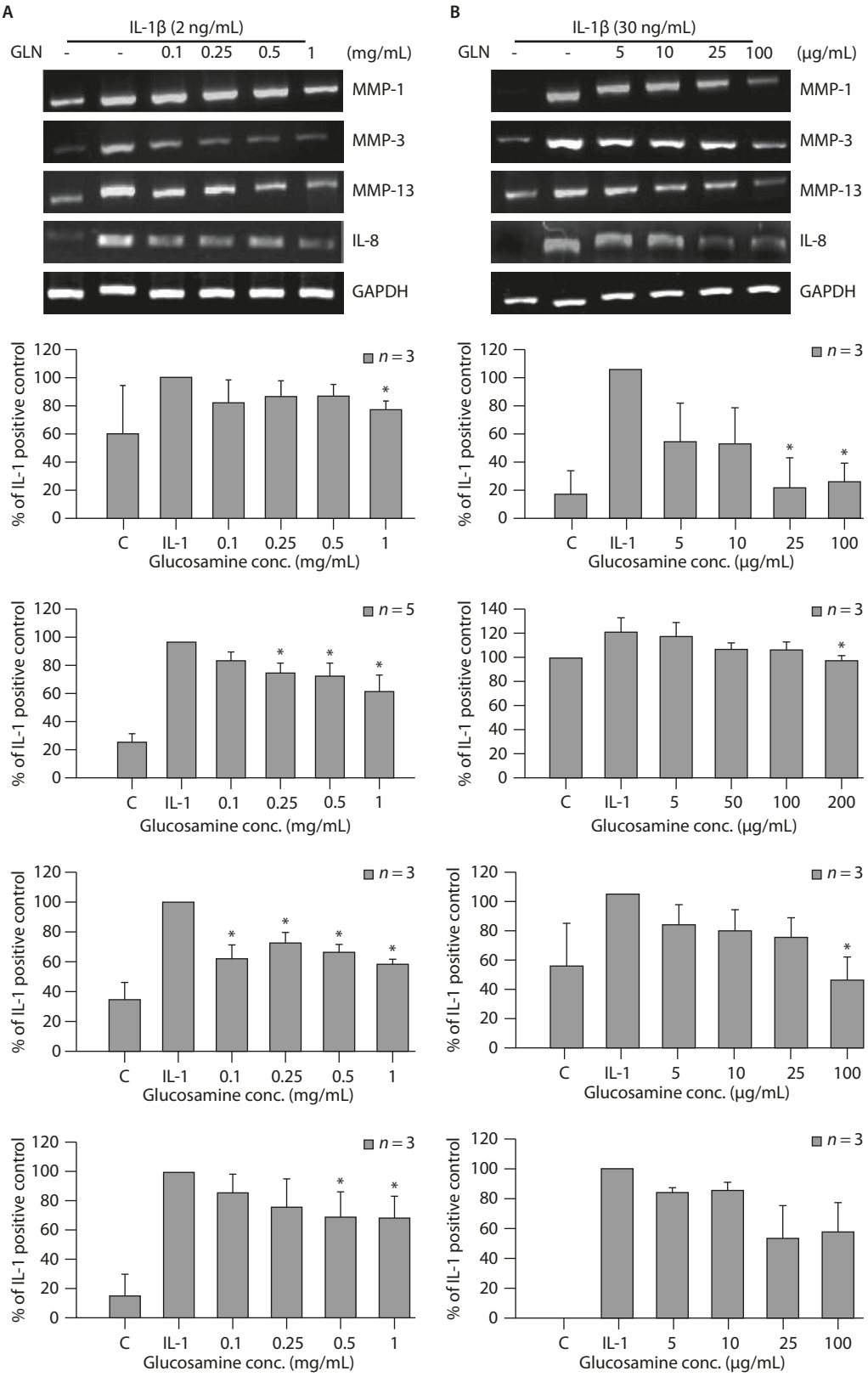

Figure 2 Effects of high concentrations of glucosamine (GLN) on low concentrations of interleukin (IL)-1β-induced matrix metalloproteinases (MMPs) and IL-8 gene expression by chondrosarcoma cells. Cells were preincubated for 30 minutes with various (A) high concentrations of GLN (0.1, 0.25, 0.5 and 1 mg/mL) or (B) low concentrations of GLN (5, 10, 25 and 100 μg/mL), and then stimulated with (A) 2 ng/mL or (B) 30 ng/mL IL-1β for 6 hours. MMP-1, MMP-3, MMP-13 and IL-8 gene expressions were detected by reverse transcription–polymerase chain reaction as described in section 2 (Materials and Methods) of this article.

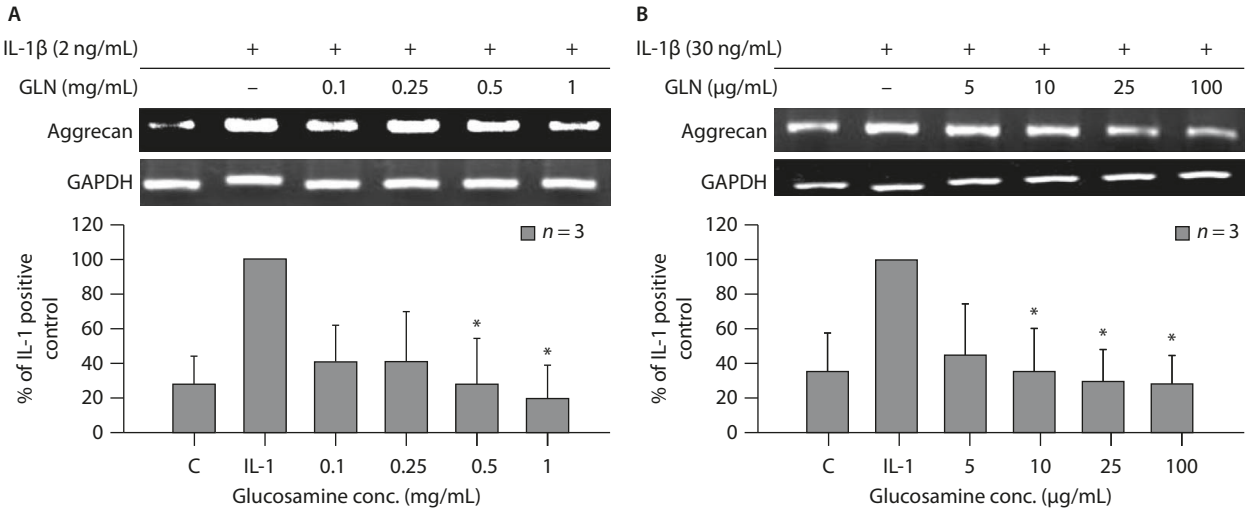

Figure 3 Effects of high and low concentrations of glucosamine (GLN) on low and high concentrations of interleukin (IL)-1β-induced aggrecan gene expression by chondrosarcoma cells. Cells were preincubated for 30 minutes with various (A) high concentrations of GLN (0.1, 0.25, 0.5 and 1 mg/mL) or (B) low concentrations of GLN (5, 10, 25 and 100 μg/mL), and then stimulated with (A) low or (B) high concentrations of IL-1β (2 or 30 ng/mL) for 6 hours. Aggrecan gene expression was detected by reverse transcription–polymerase chain reaction as described in section 2 (Materials and Methods) of this article. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

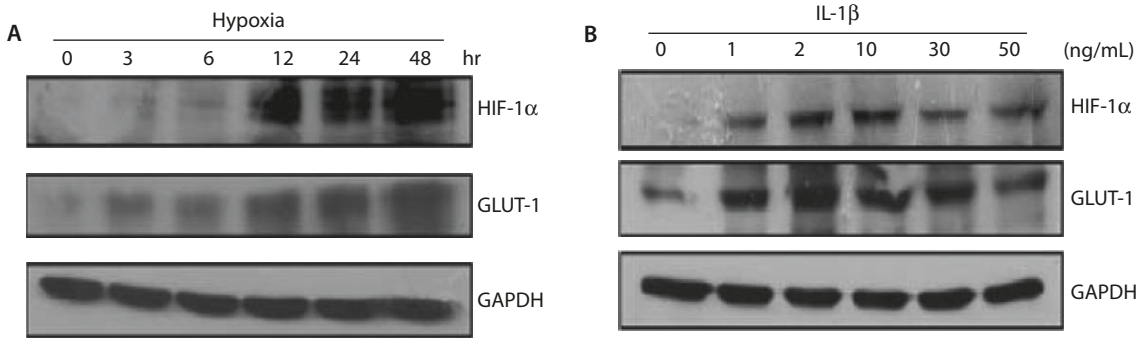

Figure 4 Effects of hypoxia and interleukin (IL)-1β treatment on hypoxia-inducible factor (HIF)-1α and glucose transporter protein (GLUT)-1 expression in chondrosarcoma cells. (A) Cells were incubated under hypoxic conditions for different periods of time (3, 6, 12, 24, 48 hours), and HIF-1α and GLUT-1 protein expression detected by Western blotting. (B) Chondrosarcoma cells were incubated with IL-1β of different doses (1, 2, 10, 30, 50 ng/mL) for 24 hours under normoxia, and HIF-1α and GLUT-1 protein expression detected by Western blotting [as described in section 2 (Materials and Methods) of this article]. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

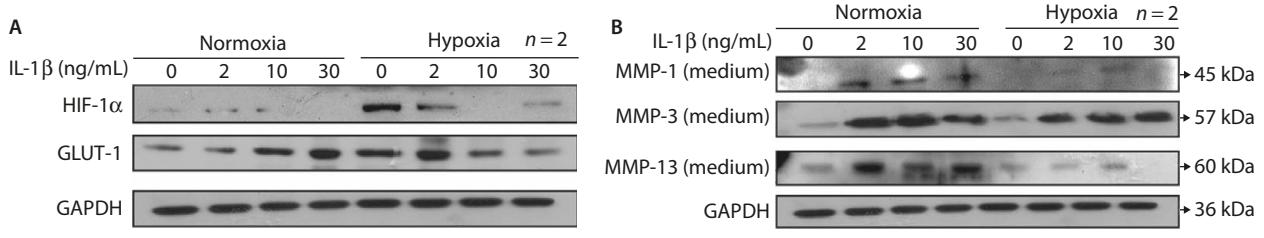

Figure 5 Effects of interleukin (IL)-1β on hypoxia-inducible factor (HIF)-1α, glucose transporter protein (GLUT)-1, and matrix metalloproteinase (MMP) protein expressions under normoxia and hypoxia by chondrosarcoma cells. Cells were treated with various concentrations of IL-1β (2, 10, and 30 ng/mL) for an additional 24 hours under both normoxic and hypoxic conditions. (A) HIF-1α and GLUT-1 and (B) MMP-1, MMP-3 and MMP-13 protein expressions were detected by Western blotting as described in section 2 (Materials and Methods) of this article. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

under normoxic conditions. The protein level of MMP-3 was the highest, followed by MMP-13 and MMP-1. Increasing the IL-1β concentration seemed to maintain the same level of induction of protein secretion of the three MMPs to the medium. Under hypoxic conditions, induction of protein secretion to the medium by IL-1β was enhanced for MMP-3, but decreased for MMP-1 and MMP-13.

3.6. Protein expressions of HIF-1α, GLUT-1 and MMPs by IL-1β-treated SW1353 cells pretreated with GLN under normoxic and hypoxic conditions

Figure 5 shows the protein expressions of HIF-1α, GLUT-1, MMP-1 and MMP-3 by IL-1β-treated SW1353 cells (low concentration of 2 ng/mL for 24 hours) pretreated with two different concentrations of GLN (high concentration range of 0.1 and 1 mg/mL) under normoxic and hypoxic conditions for 0.5 hours. As Figure 6 shows, protein expression of HIF-α by SW1353 cells treated with IL-1β at a concentration of 2 ng/mL are inducible under both hypoxic conditions and IL-1β treatment. However, the induction of HIF-α expression seemed to be inhibited by pretreatment with both GLN concentrations. This inhibitory effect was more apparent under normoxic than hypoxic conditions. The induction of GLUT-1 expression by SW1353 cells was slightly inhibited by treatment with IL-1β at 2 ng/mL under hypoxic conditions. For both O_2 levels, the induction of GLUT-1 expression by SW1353 cells following IL-1β treatment was dose-dependently inhibited by GLN pretreatment. This clearly indicates that under both normoxic and hypoxic conditions, protein levels of MMP-1 and MMP-3 increased in the cell lysate and medium following IL-1β treatment; however, the extent of the cell lysate and medium increases for the latter condition were less than those for the former. With GLN pretreatment, the extent of protein induction of MMP-1 and MMP-3 by IL-1β-treated SW1353 cells significantly decreased at both GLN concentrations under normoxic conditions, but only at 1 mg/mL GLN under hypoxic conditions.

Figure 7 reveals the protein expressions of HIF-1α, GLUT-1, MMP-1 and MMP-3 by IL-1β-treated SW1353 cells (high concentration of 30 ng/mL for 24 hours) pretreated with two different concentrations of GLN (low concentration range of 5 and 100 μg/mL) under normoxic and hypoxic conditions for 0.5 hours. The protein expression of HIF-α by SW1353 cells treated with 30 ng/mL IL-1β was inducible both under hypoxic conditions and treatment with IL-1β. This was similar to treatment with 2 ng/mL IL-1β. Conversely, the induction of HIF-α expression seemed to moderately increase after pretreatment with both GLN concentrations, similarly increasing under both normoxic and hypoxic conditions. The expression of GLUT-1 by SW1353 was slightly induced by treatment with IL-1β at 30 ng/mL under normoxic conditions, but greatly induced under hypoxic conditions; induction of GLUT-1 expression by SW1353 was slightly inhibited by treatment with 30 ng/mL IL-1β under hypoxic conditions. Finally, induction of GLUT-1 expression by SW1353 cells following IL-1β treatment was only slightly affected by pretreatment with GLN under both O_2 levels.

Figure 6 shows similar results: protein levels of MMP-1 and MMP-3 increased in both the cell lysate and medium following IL-1β treatment under normoxic and hypoxic conditions, but the extent of the cell lysate and medium increase for the latter condition were less than those for the former. With GLN pretreatment, the extent of protein induction of MMP-1 and MMP-3 by IL-1β-treated SW1353 cells significantly decreased at both GLN concentrations under normoxic and hypoxic conditions.

4. Discussion

OA is mainly attributed to IL-1β and TNF-α which is secreted by synovial fibroblasts and chondrocytes to initiate a number of events that lead to cartilage destruction, including an inhibition of the biosynthesis of matrix macromolecules (proteoglycan and collagen) and increase in catabolic pathways (by MMPs). This causes an imbalance between the biosynthesis and degradation of matrix components leading to progressive destruction of tissue and extensive articular damage.[14]

In this study, GLN's dose-dependent disease-modifying effects on catabolic and anabolic processes expressed by IL-1β-treated chondrosarcoma (SW1353) cells were evaluated under hypoxic conditions that mimic physiological O_2 levels encountered by chondrocytes and compared to those under normoxic conditions. Results demonstrated that a higher concentration range of GLN (0.1–1.0 mg/mL) was necessary to dose-dependently inhibit the increase of IL-1β-induced mRNA expressions of catabolic enzymes (MMP-1, MMP-3, MMP-13, IL-8) and aggrecan at a lower level (2 ng/mL) of IL-1β treatment. In contrast, under normoxic conditions, a lower concentration range of GLN (5–100 μg/mL) was sufficient to dose-dependently inhibit IL-1β-induced mRNA expression at a higher level (30 ng/mL) of IL-1β treatment. In both the cell lysate and medium following IL-1β treatment with these low and high concentrations, protein levels of MMP-1 and MMP-3 increased under normoxic and hypoxic conditions, but the extent of increase for the latter condition was less than that for the former condition. With GLN pretreatment, the extent of protein induction of MMP-1 and MMP-3 by IL-1β-treated SW1353 cells at the low level of 2 ng/mL significantly decreased only with GLN pretreatment at a concentration of 1 mg/mL, whereas at

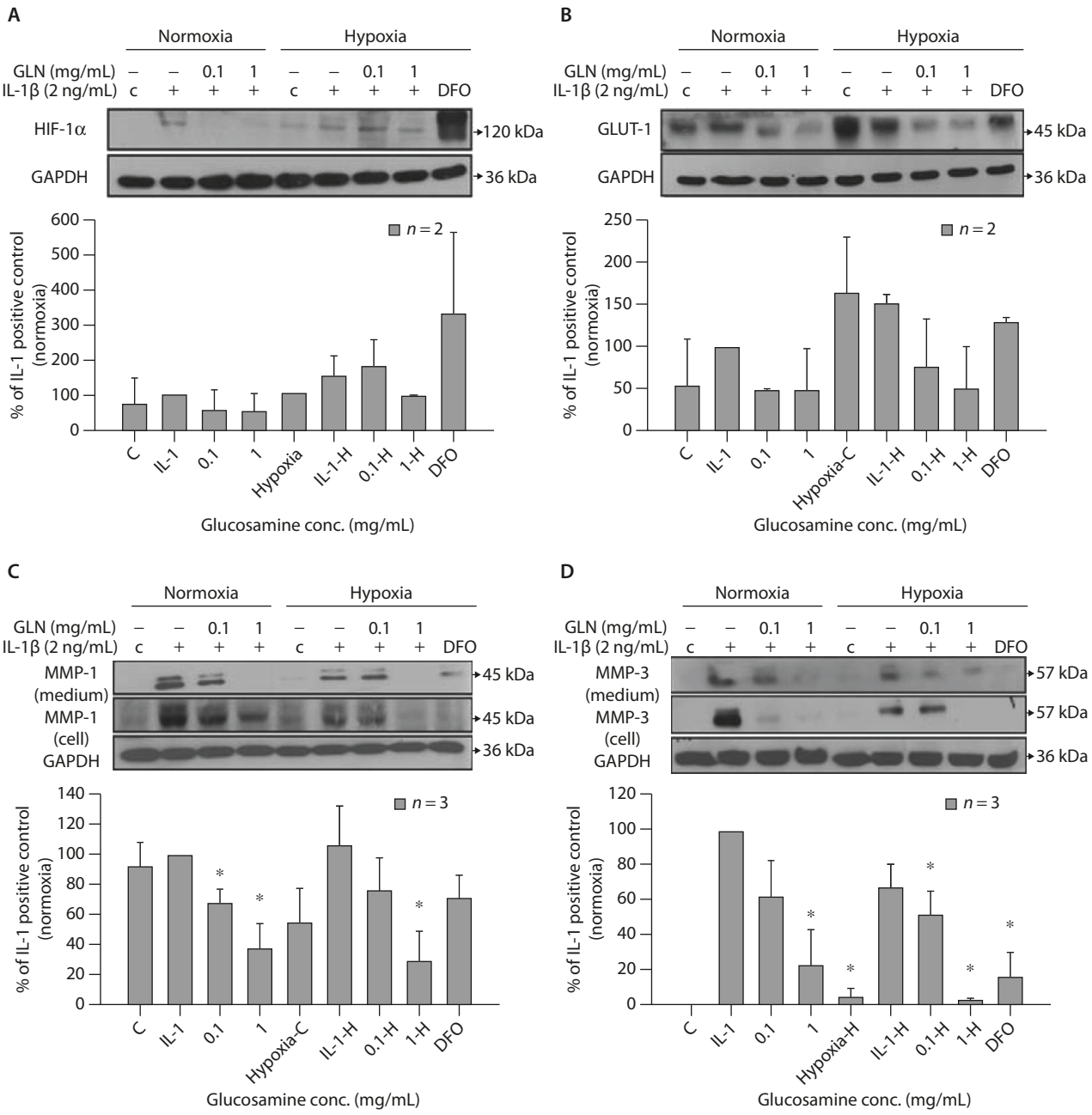

Figure 6 Effects of high concentrations of glucosamine (GLN) on low concentrations of interleukin (IL)-1β-induced hypoxia-inducible factor (HIF)-1α, glucose transporter protein (GLUT)-1, and matrix metalloproteinase (MMP) protein expressions under normoxia or hypoxia by chondrosarcoma cells. Cells were preincubated for 30 minutes with high concentrations of GLN (0.1 and 1 mg/mL) and then stimulated with 2 ng/mL IL-1β for 24 hours under normoxic or hypoxic conditions. (A) HIF-1α, (B) GLUT-1, (C) MMP-1, and (D) MMP-3 protein expressions were detected by Western blotting as described in section 2 (Materials and Methods) of this article. Cells treated with desferrioxamine (DFO) 200 μM served as the positive control for HIF-1α. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

30 ng/mL, the level significantly decreased at both concentrations (5 and 100 μg/mL).

Nakamura et al reported that after stimulation of 5 ng/mL IL-1β, GLN at a concentration of 100 μg/mL suppressed PGE_2 production and partly suppressed NO production.[24] Additionally, it suppressed the production of MMPs from normal chondrocytes and synoviocytes, but not from OA chondrocytes.[24] This is consistent with observations that the suppression of mRNA and protein expressions of MMP-1, MMP-3 and MMP-13 by GLN at a similar concentration range (0.1–1.0 mg/mL) were found for SW1353 cells treated at an even lower IL-1β concentration of 2 ng/mL. Chan et al reported that with stimulation at an IL-1β concentration of 50 ng/mL, GLN at a physiological concentration (5 μg/mL) was able to regulate gene expression and NO and PGE_2

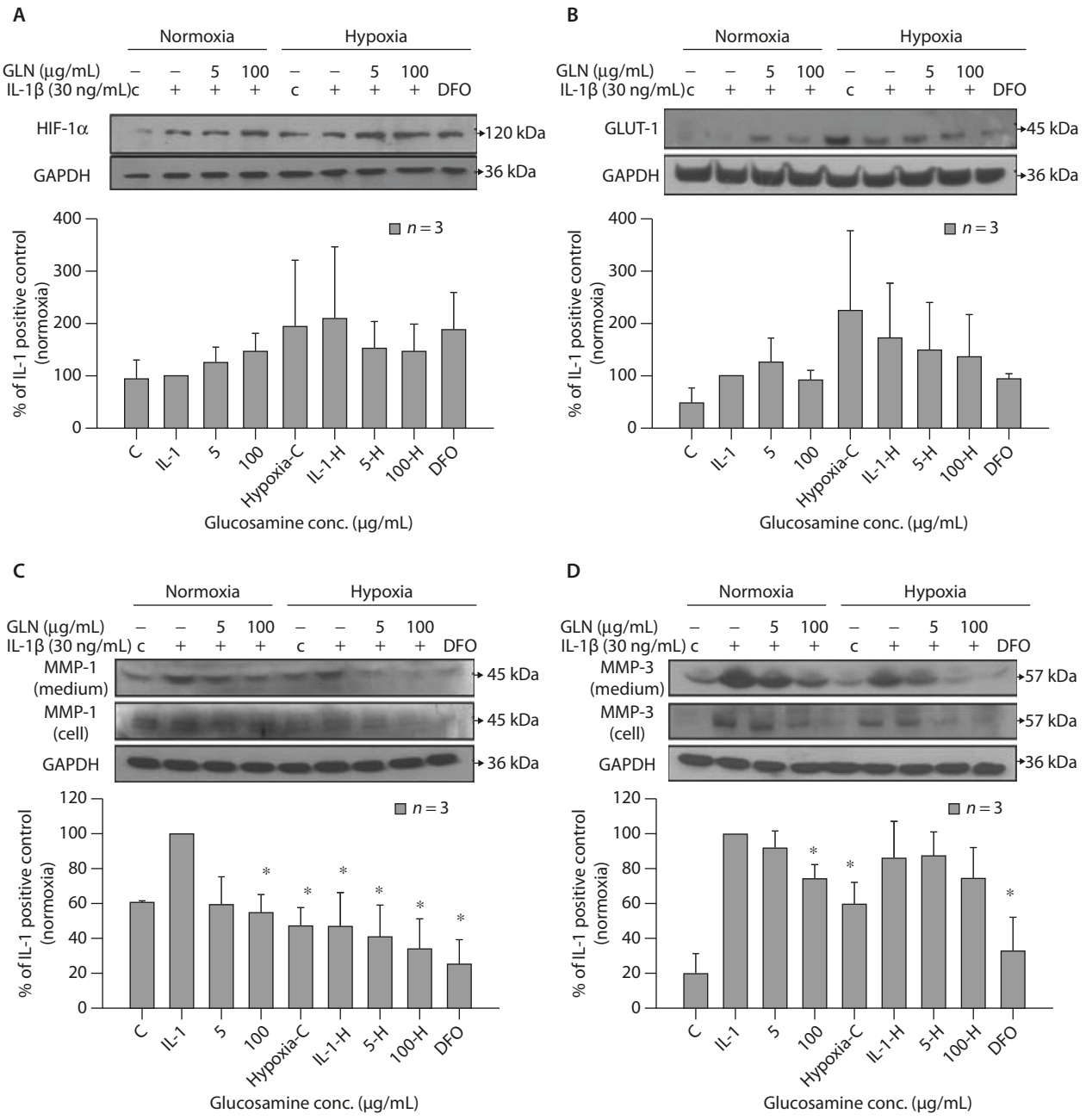

Figure 7 Effects of low concentrations of glucosamine (GLN) on high concentrations of interleukin (IL)-1β-induced hypoxia-inducible factor (HIF)-1α, glucose transporter protein (GLUT)-1, and matrix metalloproteinase (MMP) protein expressions under normoxic or hypoxic conditions by chondrosarcoma cells. Cells were preincubated for 30 minutes with high concentrations of GLN (5 and 100 μg/mL) and then stimulated with 30 ng/mL IL-1β for 24 hours under normoxic or hypoxic conditions. (A) HIF-1α, (B) GLUT-1, (C) MMP-1, and (D) MMP-3 protein expressions were detected by Western blotting as described in section 2 (Materials and Methods) of this article. Cells treated with desferrioxamine (DFO) 200 μM served as the positive control for HIF-1α. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

syntheses.[21] Their level was consistent with the one revealed in this study.

de Mattei et al[25] reported that pharmacological doses of GLN (0.25, 2.5, 6.5 and 25 mg/mL), which induce a broad impairment of the metabolic activity of bovine chondrocytes leading to cell death and inhibition of IL-1β-induced catabolic effects (50 ng/mL), are related to GLN toxicity. Since IL-1β treatment was at a high concentration of 50 ng/mL, suppression of IL-1β-induced catabolic effects on chondrocytes might be observed with the elimination of GLN toxicity if a lower GLN concentration range similar to that of this study (5–100 μg/mL) was present in the culture. Similarly, a clinically relevant dose of GLN (20 mg/kg) in a large

monogastric animal model resulted in serum (6.1 μM: 1.0 μg/mL) and synovial fluid (0.6 μM: 0.1 μg/mL) concentrations that were at least 500-fold lower than those reported to modify chondrocyte anabolic and catabolic activities in tissue and cell culture experiments.[26] This study demonstrated that the induction of IL-1β's catabolic effects (MMPs) on SW1353 cells with a low concentration of 2 ng/mL was only suppressed by GLN pretreatment at high concentrations (0.1–1.0 mg/mL), whereas low concentrations of 25–100 μg/mL (similar to levels based on oral administration) suppressed the induction of catabolic effects after treatment with a high concentration of IL-1β. This implies that a concentration of IL-1β >30 ng/mL might be encountered by chondrocytes during OA disease. Therefore, an even lower concentration of GLN, close to the level that results after oral administration in OA patients, might effectively suppress the catabolic effects induced by IL-1β and lead to improvements in clinical symptoms.

It was observed that the mRNA level of aggrecan tended to increase after 6 hours of treatment with IL-1β alone, and this effect, induced by two different levels of IL-1β, was dose-dependently suppressed by GLN pretreatment at different concentration ranges for 0.5 hours. This seems to conflict with most studies reporting that IL-1β is responsible for matrix degradation of cartilage by inhibiting aggrecan synthesis, and GLN is able to protect cartilage from degradation by increasing aggrecan synthesis.[27] A study conducted by Goodstone and Hardingham revealed that in inhibiting aggrecan synthesis in primary porcine articular chondrocytes, IL-1β and TNF-α at concentrations lower than 150 pg/mL had similar potencies.[28] Gouze et al proved in a probing study of RNA microarray chips that GLN alone had no observable stimulatory effect on the transcription of primary cartilage matrix genes, e.g., aggrecan and collagen type II, but was proven to be a potent, broad-spectrum inhibitor of IL-1β.[29]

This study further compared the catabolic effects of IL-1β and the beneficial effects of GLN under hypoxic conditions since microelectrode studies have shown that an O_2 gradient exists in cartilage, with the superficial layers, middle layers, and deep zones respectively featuring O_2 levels of 6–7%, 3–4%, and <1%.[30] It was found that productions of MMP-1, MMP-3 and MMP-13 under normoxic conditions, as indicated by protein levels secreted to the medium, were enhanced—but to different extents—by IL-1β at the three concentrations examined (2, 10 and 30 ng/mL). Under hypoxic conditions, protein productions of MMP-1, MMP-3 and MMP-13 were enhanced by IL-1β treatment at the three concentrations examined; however, that for MMP-3 did not significantly differ, while those for MMP-1 and MMP-13 decreased in comparison to those under normoxic conditions at respective IL-1β concentration levels. This is consistent with findings reported by Martin et al[13] that in both normoxic and hypoxic conditions, MMP-1 and MMP-3 mRNAs increase with IL-1β treatment of chondrocytes, although the difference between the two O_2 conditions is not statistically significant.

Survival and proper function of articular chondrocytes crucially depend on their ability to adapt to hostile microenvironmental conditions characterized by extremely low O_2 levels. Like most mammalian cells, this adaptive response is controlled and organized most prominently by HIF-1. HIF-1α, one of its subunits, was found to be expressed in human normal and OA articular chondrocytes cultured under normoxic conditions, and further induced or stabilized by hypoxia in articular chondrocytes.[1] Studies have shown that articular chondrocytes derive up to 75% of their ATP requirements from anaerobic glycolysis. Although glycolysis is less efficient than oxidative phosphorylation for generating ATP, in the presence of sufficient glucose, glycolysis can sustain ATP production due to GLUT-1 induction (perhaps also GLUT-3) and increases in glycolytic enzyme activity (11 kinds), both of which target HIF-1 genes.[31,32] Figure 4A demonstrates that after incubation for 12 hours under hypoxic conditions, protein levels of both HIF-1α and GLUT-1 consistently and gradually increased and sharply increased, respectively. It was also observed for the first time by Hernvann and coworkers that accelerated glucose uptake in chondrocytes and synoviocytes occurred through enhanced expression and plasma membrane incorporation of the highly glycosylated GLUT-1 in response to IL-1β stimulation.[33,34] Using a study of the expression patterns of HIF-1α and its target genes, GLUT-1 and phosphoglycerate kinase-1, in normal and OA cartilage, Pfander et al suggested that increased synthesis of glucose transporter and glycolytic enzymes might result from accumulated HIF-1α, possibly caused by reduced O_2 levels during OA progression.[35] Besides hypoxia, increased IL-1β levels and mechanical forces are candidate factors for inducing transcription factor, HIF-1α, in OA cartilage. This study confirmed (Figure 4B) that not only protein expressions of GLUT-1 but also HIF-1α gradually increased with increasing IL-1β concentration up to 2 ng/mL, but then decreased thereafter to 50 ng/mL under normoxic conditions.

Since glucose serves as a major energy source[36,37] and principal precursor for glycosaminoglycan synthesis,[38,39] the increased glucose uptake observed in chondrocytes in response to adaptation to hypoxia (more likely due to anabolic factors, insulin-like growth factor-I and insulin) and cytokine stimulation may be due to the mutual dependency of both anabolic and catabolic pathways on regulated glucose transport. Chondrocytes adaptively respond to hypoxia (or stimulation by anabolic factors like insulin-like growth factor-I or insulin) by upregulating GLUTs (GLUT-1) to accelerate glucose accumulation and divert accumulated glucose into metabolic and structural pools. The

accelerated glucose transport observed following catabolic IL-1β/TNF-α stimulation leads to the accumulation of intracellular glucose, although accumulated substrate is likely to be channeled mainly into metabolic pools in order to generate the ATP required by chondrocytes to degrade the extracellular matrix.[40] Some evidence has shown that in order to serve as efficient precursors of glycosaminoglycan synthesis,[41] GLUTs also engage in the transport of GLN and N-acetylglucosamine into chondrocytes.[42,43]

It was consistently observed that the protein expressions of HIF-1α and GLUT-1 increased with IL-1β treatment under normoxic conditions, while further increasing during hypoxia. Nevertheless, protein expressions of MMP-1 and MMP-3, as indicated by the secreted levels in the medium induced by IL-1β under normoxic conditions, were found to be less than those under hypoxia. Under normoxic conditions, pretreatment with GLN was observed to have an inhibitory effect on IL-1β-induced protein expressions of MMP-1 and MMP-3, with even greater inhibition under hypoxic conditions, which might be attributed to either the lesser extent of protein induction under hypoxia or the greater uptake of GLN by chondrocytes as a result of dose-dependent increases in GLUT-1 expression by IL-1β and hypoxia.

In both normal and OA chondrocytes, accumulated HIF-1α caused by reduced O_2 levels and IL-1β stimulation leads to an increase in GLUT-1 synthesis, its target gene, thus accelerating the uptake of glucose and GLN into chondrocytes as metabolic and structural sources. Therefore, through stimulation with a low IL-1β concentration, the induction extent of GLUT-1 might be lower since a higher concentration range of GLN in the culture had a significant effect on the inhibition of events following IL-1β treatment; whereas a low GLN concentration range was enough to have anti-IL-1β effects because a higher extent of GLUT-1 was induced when chondrocytes were treated with a high IL-1β concentration level.

Furthermore, Gouze et al reported that GLN 4.5 g/L presented with only IL-1β 25 or 250 U/mL in a culture of rat chondrocytes could lead to dose-dependent increases in the mRNA expression of IL-1 receptor type II (IL-1RII) in chondrocytes, a decoy receptor that interacts with cytokines and is unable to generate signaling.[18] This indicates that at the same GLN concentration, IL-1RII mRNA expression increases with increasing IL-1β concentrations. Therefore, by trapping part of the IL-1β on IL-1RII, a low concentration range of GLN might be enough to reduce the binding of IL-1β on IL-1RI and therefore modulate its effects dose-dependently in a manner similar to this study.

In conclusion, the present work demonstrates the disease-modifying effects of GLN on IL-1β-treated chondrosarcoma SW1353 cells dose-dependently with respect to IL-1β concentrations under normoxic and hypoxic conditions. Because the physiological level of GLN reached by oral administration effectively treats OA, GLUT-1 induced by both hypoxia and IL-1β as well as IL-1RII expressions regulated by GLN and IL-1β might potentially contribute to this dose-dependent effect.

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