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Inflammation Research

Evaluation of the suppressive actions of glucosamine on the interleukin-1β-mediated activation of synoviocytes

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Abstract. *Objective:* Recently, we found that administration of glucosamine to adjuvant arthritis, a model for rheumatoid arthritis, suppressed the progression of arthritis in rats. To clarify its anti-inflammatory mechanism, we evaluated the actions of glucosamine on the activation of synoviocytes *in vitro*.
Materials and methods: Synoviocytes isolated from human synovial tissues were stimulated with interleukin (IL)-1β in the presence of 0.01–1 mM glucosamine. IL-8 and prostaglandin (PG) E_2 were measured by ELISA, and nitric oxide was quantitated by Griess assay. IL-8 mRNA was detected by RT-PCR. Furthermore, the effect of glucosamine on the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and the binding of [^{125}I] IL-1β to its receptors were examined using a primary human synovial cell line (CS-ABI-479).
Results: Glucosamine significantly suppressed the IL-1β-induced IL-8 production as well as its mRNA expression ($p < 0.05$) at 1 mM. Furthermore, glucosamine (1 mM) inhibited the IL-1β-induced nitric oxide and PGE_2 production ($p < 0.05$). Moreover, glucosamine suppressed the IL-1β-induced phosphorylation of p38 MAPK ($p < 0.05$ at >0.1 mM) and the IL-1β-binding to its receptors ($p < 0.05$ at 1 mM).
Conclusions: These observations suggest that glucosamine can suppress the IL-1β-mediated activation of synoviocytes (such as IL-8-, nitric oxide- and PGE_2-production, and phosphorylation of p38 MAPK), thereby possibly exhibiting anti-inflammatory actions in arthritis.

Key words: Glucosamine – Synoviocyte – Rheumatoid arthritis

Introduction

Glucosamine, a naturally occurring amino monosaccharide is present in the connective and cartilage tissues as a component of glycosaminoglycans, and contributes to maintaining the strength, flexibility and elasticity of these tissues. For more than two decades in Europe, glucosamine has been clinically used to treat human osteoarthritis, based on its beneficial effect on the reconstruction of joint cartilage [1–4]. Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-modifying effect of glucosamine without apparent adverse events [5–7]. In addition to its chondroprotective actions, glucosamine can exhibit anti-inflammatory actions by suppressing neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis [8]. Moreover, glucosamine has been shown to suppress unprimed T cell response by interfering with antigen presenting cell functions and by inhibiting CD3-induced T cell proliferation [9].

Rheumatoid arthritis (RA) is characterized by the chronic and progressive inflammatory processes with immunological abnormalities, leading to the synovial hyperplasia and destruction of the affected joints. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the pharmacological management of arthritis [10]. Although these medications are effective in relieving pain and limitation of functions, they cannot cure arthritis and cause serious side effects [11]. Previously, we orally administered glucosamine to rats with adjuvant arthritis, and evaluated its effect on the progression of arthritic inflammation. The results indicated that glucosamine significantly suppressed the progression of adjuvant arthritis and also inhibited the production of nitric oxide and PGE_2 in rats [12].

However, the anti-inflammatory mechanism of glucosamine on arthritis is poorly understood. In this study, we evaluated the actions of glucosamine on the activation of human synoviocytes. The results indicated that glucosamine suppressed the mRNA expression and production of IL-8 by IL-1β-activated synoviocytes. Furthermore, glucosamine inhibited the nitric oxide, PGE_2 production and phosphorylation of p38 MAPK by activated synoviocytes.

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Materials and methods

Reagents

IL-1β was purchased from PeproTech EC Ltd. (London, UK). D-Glucosamine hydrochloride was supplied by Koyo Chemical Co., Ltd. (Tokyo, Japan). N-acetyl-D-glucosamine was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). In our preliminary experiments, we confirmed that glucosamine or N-acetylglucosamine (1 mM) per se never stimulated synovial tissue-derived synoviocytes and a primary human synovial cell line to produce the detectable amounts of IL-8, nitric oxide and PGE_2 (as described below), suggesting that the amounts of contaminants, which affect the inflammatory mediator production, are negligible in the glucosamine and N-acetylglucosamine preparations.

Cell culture

Synovial tissue samples were obtained from three patients with RA undergoing total joint replacement at Juntendo University Hospital (Tokyo, Japan). All RA patients were evaluated by the certified rheumatologists and diagnosed as having RA, according to the criteria of the American Collage of Rheumatology [13, 14]. This study was conducted according to the Declaration of Helsinki principles, and the human experiments were approved by the ethical committee of Juntendo University. The synovial tissues were minced into very small pieces, and incubated in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a 5% CO_2 incubator for 96 h. After removing nonadherent cells, and washing with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), adherent cells were recovered with 0.25% trypsin/EDTA (Gibco Invitrogen Corporation, Grand Island, NY) and successively passaged. Purity of the cells was >95% fibroblast-like cells as confirmed by microscopic analysis. All experiments described were performed using the cells between 3 and 10 culture passages.

When the phosphorylation of p38 MAPK and binding of IL-1β (described below) were analyzed using synovial-tissue derived synoviocytes, the data obtained were markedly varied among individuals with RA. In a preliminary experiment, we confirmed that glucosamine inhibited the IL-8 production from IL-1β-activated CS-ABI-479, a primary human synovial cell line (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), as with synovial-tissue derived synoviocytes. Thus, in the experiments for the phosphorylation of p38 MAPK and binding of IL-1β, a synovial cell line CS-ABI-479 was used. CS-ABI-479 cells (4×10^5/well in a 6-well plate) were maintained in CS-C complete medium (4Z3-500; Dainippon Pharmaceutical) containing 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a 5% CO_2 incubator.

Measurement of IL-8

Synovial tissue-derived synoviocytes were plated at 10^5/well in 24-well plates in RPMI 1640 medium containing 10% FCS for 24 h at 37 °C. After washing twice with RPMI 1640 medium (without phenol red), the cells were incubated with 0.01–1 mM glucosamine or 1 mM N-acetylglucosamine, and then stimulated with 4 ng/ml IL-1β in a total volume of 0.5 ml RPMI 1640 medium (without phenol red) for 24 h at 37 °C. In our preliminary experiments, we confirmed the time-dependent production of IL-8 from synoviocytes, and found that IL-8 progressively increased during incubation for 8~72 h after stimulation with 4 ng/ml IL-1β. Thus, we decided to quantitate the IL-8 production using culture supernatants from 4 ng/ml IL-1β-stimulated synoviocytes after 24 h-incubation.

IL-8 in the culture supernatants was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (eBioscience San Diego, CA). Briefly, microtiter plates (Corning, Acton, MA) were coated with anti-IL-8 monoclonal antibody (1:125 dilution; eBioscience) overnight at 4 °C. After blocking with BlockAce (Dainippon Pharmaceutical), the culture supernatants were added, and incubated for 2 h at room temperature. After washing with PBS-0.05% Tween 20, IL-8 was detected by developing with a biotinylated anti-IL-8 polyclonal antibody (1:2500 dilution; eBioscience), streptavidin-conjugated horseradish peroxidase (HRP; 1:2000 dilution) and TMB solution (eBioscience). The detection range of IL-8 was from 25 to 2,000 pg/ml.

Isolation of RNA and evaluation of IL-8 mRNA expression

Synoviocytes (10^6/well) isolated from synovial tissues were incubated in 60 mm dishes in the absence or presence of 1 mM glucosamine or N-acetylglucosamine with 0.1–1 ng/ml IL-1β for 24 h at 37 °C. The total RNA from synoviocytes was isolated using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA).

RT-PCR was performed using a ReverTra Dash RT-PCR (reverse transcription polymerase chain reaction) kit (Toyobo Co., Ltd, Tokyo, Japan) according to the manufacturer's instruction. First-strand cDNA was synthesized in a 20 μl reaction mixture containing 0.4 μg of total RNA with an random primer and ReverTra Ace reverse transcriptase (Toyobo), and the RT reactions were carried out at 42 °C for 20 min, followed by termination at 99 °C for 5 min. The PCR reaction was carried out in a 25 μl reaction mixture containing 0.25 unit of KOD Dash Taq DNA polymerase (Toyobo) and 0.1 μM of each gene-specific primer as shown below. The IL-8 forward primer was 5 -CTGCGCCAACACAGAAATTA-3 (bp 281–300), and the reverse primer was 5 -ATTGCATCTGGCAACCCTAC-3 (bp 518–537) (GeneBank™ accession number Y00787); the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer was 5 -ACCACAGTCCATGCCATCAC-3 , and the reverse primer was 5 -TCCACCACCCTGTTGCTGTA-3 (Toyobo). The reaction was subjected to 35 cycle of PCR with following condition: 98 °C for 10 s, 55 °C for 2 s, and 74 °C for 30 s. The PCR products were analyzed with a 2% (w/v) agarose gel containing 0.5 μg/ml of ethidium bromide. In our preliminary experiments, we tried to semi-quantitatively detect mRNA by using different cycle number of PCR. The results indicated that the amounts of RT-PCR products increased dependently on the cycle number from 30 to 38. Thus, we decided to measure the mRNA levels by RT-PCR with the cycle number of 35; mRNA levels can be semi-quantitatively evaluated under our PCR condition. The detected bands were quantified using Scion Image (Scion Corporation, Frederick, MD).

Quantification of nitrite and PGE_2

Synoviocytes isolated from synovial tissues were plated at 10^5/well in 24-well plates in RPMI 1640 medium containing 10% FCS for 24 h at 37 °C. After washing twice with RPMI 1640 medium (without phenol red), the cells were incubated with 0.01–1 mM glucosamine or 1 mM N-acetylglucosamine, and then stimulated with 4 ng/ml IL-1β in a total volume of 0.5 ml RPMI 1640 medium (without phenol red) for 24 h at 37 °C. In our preliminary experiments, we confirmed the time-dependent production of PGE_2 from synoviocytes, and found that PGE_2 progressively increased during stimulation with 4 ng/ml IL-1β for 12~48 h. Thus, we decided to quantitate the PGE_2 production using culture supernatants from 4 ng/ml IL-1β-stimulated synoviocytes after 24 h-incubation. To measure the optimal production of nitric oxide, synoviocytes were stimulated with different concentrations of IL-1β (6.4 pg/ml~4 ng/ml) for 24 h. However, only 4 ng/ml IL-1β was able to significantly induce nitric oxide production from synoviocytes. Thus, the effect of glucosamine on nitric oxide production was evaluated after stimulation with 4 ng/ml IL-1β for 24 h.

Nitric oxide was measured as nitrite accumulation in the culture supernatants by Griess assay (Cayman Chemical Company, Ann Arbor, MI). The detection limit of the assay was > 1 μM. PGE_2 was measured in the culture supernatants by ELISA according to the manufacturer's instructions (Cayman). This assay showed no cross reactivity with other prostanoids, and the detection limit was >15 pg/ml.

Phosphorylation of p38 MAPK

CS-ABI-479 cells (4×10^5/well) were maintained in CS-C complete medium (4Z3-500) containing 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin in collagen-coated plates in a 5% CO_2 incubator. After incubation in CS-C complete medium without FCS for 24 h at 37 °C in collagen-coated 6 well-plates, the cells were treated with 0.01–1 mM glucosamine for 2 h, and then stimulated with 4 ng/ml IL-1β for 5 min at 37 °C. After washing with PBS, the cells were harvested in 0.5 ml of lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate and 1 mM diisopropyl fluorophosphate) containing 1/25 v/v Complete™ (Roche Diagnostic Systems, Mannheim, Germany). After sonication, the lysates were centrifuged at 700 *g* for 5 min. The supernatants were mixed with sodium dodecyl sulfate (SDS)-polyacrylaminde gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.005% bromophenol blue), and boiled. The samples (10 µg protein/lane) were subjected to 10% SDS-PAGE. The separated proteins were electrophoretically transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membranes were blocked in 5% skim milk, and probed with a 5,000-fold dilution of mouse anti-phosphorylated p38 MAPK monoclonal antibody (pT180/pY182; BD Biosciences Pharmingen, San Diego, CA) in 1/10 BlockAce solution. After washing, the membranes were further probed with a 5,000-fold dilution of HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International, Inc., Temecula, CA), and the phosphorylated p38 MAPK was detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The detected bands were quantified by densitometry using LAS-3000 (Fujifilm Corporation, Japan).

Thereafter, the antibodies were stripped from the membranes by incubating in Restore Western Stripping Buffer (Pierce) at 37 °C for 30 min. The p38 MAPK protein contained in each sample were detected by reprobing with 10,000-fold diluted mouse anti-p38 MAPK (p38/SAPK2α; BD Biosciences Pharmingen) monoclonal antibody and HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International). The protein contents were determined with a BCA protein assay kit (Pierce, Rockford, IL).

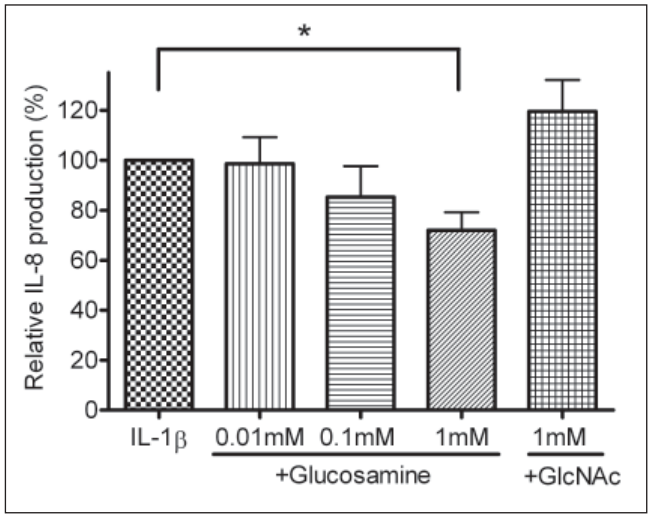

Fig. 1. Effect of glucosamine on IL-8 production by IL-1β-activated synoviocytes.
Synoviocytes isolated from synovial tissues were stimulated with 4 ng/ml IL-1β for 24 h at 37 °C in the absence or presence of 0.01–1 mM glucosamine or 1 mM N-acetylglucosamine (GlcNAc). IL-8 in the culture supernatants was quantified by ELISA. IL-8 production is expressed as a percentage of that (IL-1β; 46 ± 21 ng/ml) obtained with IL-1β-stimulated synoviocytes incubated in the absence of glucosamine or GlcNAc. Data represent the mean ±SD of three separate experiments. Values are compared between IL-1β and +Glucosamine or +GlcNAc. *$p < 0.05$

Binding analysis of IL-1β

CS-ABI-479 cells (2×10^5/well) were incubated in CS-C complete medium (4Z3-500) containing 10% FCS in collagen-coated 6 well-plates in a 5%CO_2 incubator for 3 days, and reached confluence. The cells were incubated with 10 pM of [^{125}I] IL-1β (2,970 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) in the absence or presence of 0.01–1 mM glucosamine for 3 h at 4 °C. After washing 3 times with PBS, the cells were stripped with 0.25% trypsin-EDTA (Gibco), and the radioactivity (Total binding) was measured by scintillation counting. Nonspecific binding was defined as the binding measured in the presence of excess unlabeled IL-1β (10 nM), and specific binding was defined as the difference between total binding and nonspecific binding.

Statistical analysis

Data are expressed as mean ±SD, and analyzed for significant difference by a one-way ANOVA with multiple comparison test (StatView®, Abacus concept, Berkely, CA). Differences were considered statistically significant at $p < 0.05$.

Results

Effect of glucosamine on IL-8 production by synoviocytes

To evaluate the effect of glucosamine on IL-8 production by synoviocytes, we activated synovial tissue-derived synoviocytes with IL-1β in the presence of glucosamine. As shown in Figure 1, glucosamine dose-dependently inhibited the IL-1β-induced IL-8 production by synoviocytes ($p < 0.05$ at 1 mM glucosamine). In contrast, N-acetylglucosamine, an analogue of glucosamine could not inhibit the IL-8 production by synoviocytes even at 1 mM. Thus, glucosamine could suppress the IL-8 production by synoviocytes in response to IL-1β.

Effect of glucosamine on IL-8 mRNA expression by IL-1β-activated synoviocytes

To determine if the suppression of IL-8 protein production is due to the inhibition of mRNA expression by glucosamine, we measured the IL-8 mRNA expression by synoviocytes. As shown in Figure 2, IL-1β stimulation upregulated the expression of IL-8 mRNA by synoviocytes in a dose-dependent manner. Importantly, glucosamine (0.1 and 1 mM) markedly suppressed the IL-1β-induced IL-8 mRNA expression, whereas N-acetylglucosamine could not affect the expression of IL-8 mRNA.

Effects of glucosamine on nitric oxide and PGE_2 production by IL-1β-activated synoviocytes

Nitric oxide and PGE_2 are pleiotropic inflammatory mediators and overproduced in arthritic joints. These substances play a profound role in the pathogenic processes of pannus formation in the RA joints [15, 16]. To examine the effects of glucosamine on the production of these inflammatory mediators, we measured the amounts of nitric oxide and PGE_2 in the supernatants of IL-1β-activated synoviocytes. As shown

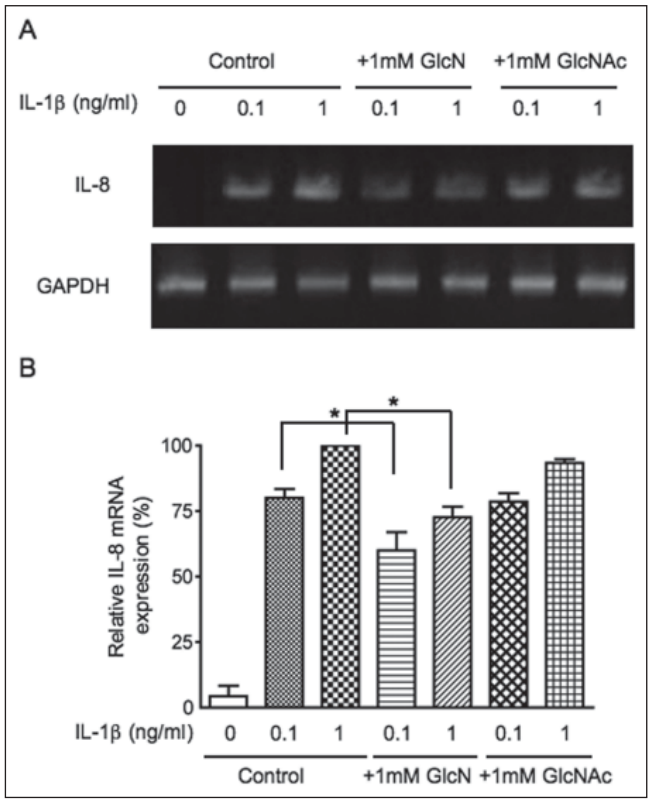

Fig. 2. Effect of glucosamine on IL-8 mRNA expression by IL-1β-activated synoviocytes.
Synoviocytes (10^6/well) isolated from synovial tissues were incubated with 0–1 ng/ml IL-1β in the absence (Control) or presence of 1 mM glucosamine (GlcN) or N-acetylglucosamine (GlcNAc) for 24 h at 37 °C. The total RNA was isolated from synoviocytes, and RT-PCR was performed to detect IL-8 and GAPDH mRNA. Data are the representative of three separate experiments (A). The detected bands were quantified using Scion Image. IL-8 mRNA expression is expressed as a percentage of that obtained with 1 ng/ml IL-1β-stimulated synoviocytes incubated in the absence of glucosamine or GlcNAc. Data represent the mean ±SD of triplicate samples. Values are compared between Control and +Glucosamine or +GlcNAc at the same concentrations of IL-1β (0.1 or 1 ng/ml). *$p < 0.05$

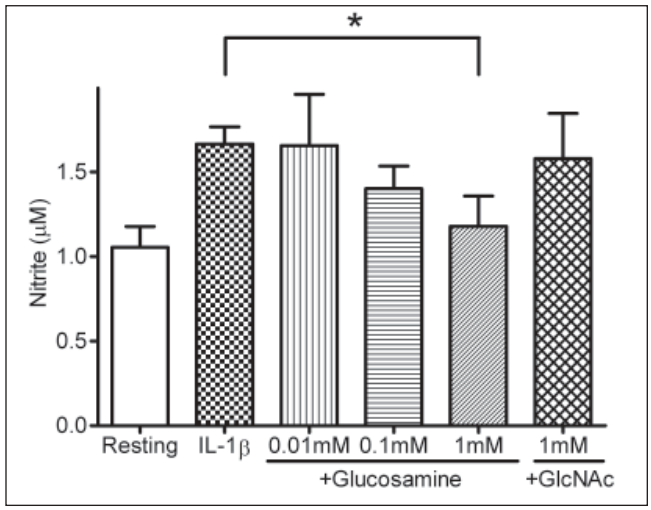

Fig. 3. Effect of glucosamine on nitric oxide production by IL-1β-activated synoviocytes.
Synoviocytes isolated from synovial tissues were incubated without (Resting) or with 4 ng/ml IL-1β in the absence or presence of 0.01–1 mM glucosamine or 1 mM N-acetylglucosamine (GlcNAc) for 24 h at 37 °C. Nitric oxide was measured as nitrite accumulation in the culture supernatants by Griess assay. Data represent the mean ±SD of three separate experiments. Values are compared between IL-1β alone and +Glucosamine or +GlcNAc. *$p < 0.05$

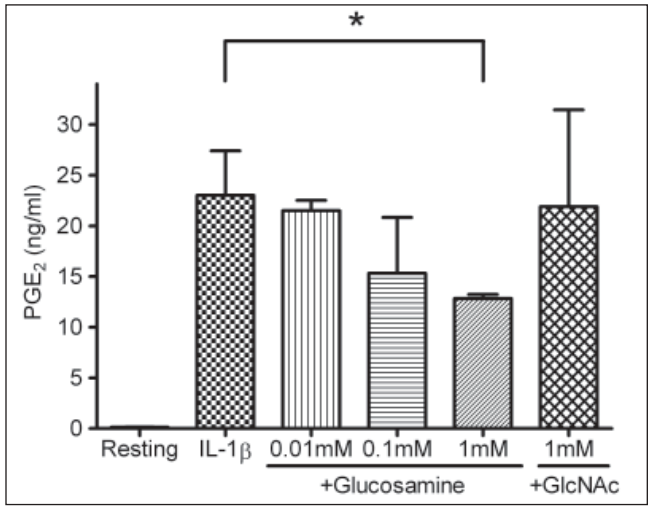

Fig. 4. Effect of glucosamine on PGE_2 production by IL-1β-activated synoviocytes.
Synoviocytes isolated from synovial tissues were incubated without (Resting) or with 4 ng/ml IL-1β in the absence or presence of 0.01–1 mM glucosamine or 1 mM N-acetylglucosamine (GlcNAc) for 24 h at 37 °C. PGE_2 was measured in the culture supernatants by ELISA. Data represent the mean ±SD of three separate experiments. Values are compared between IL-1β alone and +Glucosamine or +GlcNAc. *$p < 0.05$

in Figure 3, glucosamine dose-dependently inhibited the IL-1β-induced production of nitric oxide by synoviocytes ($p < 0.05$ at 1 mM glucosamine). Moreover, glucosamine suppressed the IL-1β-induced production of PGE_2 in a dose-dependent manner ($p < 0.05$ at 1 mM glucosamine) (Fig. 4). In contrast, N-acetylglucosamine inhibited the production of neither nitric oxide nor PGE_2 by synoviocytes even at 1 mM (Figs. 3 and 4).

Effect of glucosamine on the phosphorylation of p38 MAPK

p38 MAPK is demonstrated to be involved in the activation of synovial cells, which leads to the synovial hyperplasia and joint destruction in RA [17–21]. To determine whether the inhibitory action of glucosamine on the synoviocyte activation is mediated by the suppression of p38 MAPK pathway, we evaluated the effect of glucosamine on the phosphorylation of p38 MAPK using IL-1β-activated human primary synoviocytes (CS-ABI-479). As shown in Figure 5, 0.1 and 1 mM glucosamine significantly suppressed the IL-1β-induced phosphorylation of p38 MAPK ($p < 0.05$). We confirmed that almost the same amounts of p38 MAPK protein were analyzed in each sample (a lower panel of Fig. 5A).

Effect of glucosamine on IL-1β-binding to its receptors

IL-1β activates cells via the binding to its receptors. To determine whether the inhibitory action of glucosamine results from the suppression of IL-1β-binding to the receptors, we analyzed the IL-1β-binding to human primary synoviocytes (CS-ABI-479) using ^{125}I-labeled IL-1β as a ligand. As shown in Figure 6, glucosamine moderately but significantly inhibited the binding of IL-1β to its receptors at 1 mM ($p < 0.05$).

Discussion

Glucosamine, a principal substrate for the biosynthesis of glycosaminoglycans, has been widely used as a therapeutic agent for osteoarthritis. Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-modifying effects of glucosamine and its good safety profile [6, 7]. As a mechanism for the chondroprotective action, glucosamine is assumed to increase proteoglycan synthesis, thereby exhibiting the therapeutic efficacy in osteoarthritis [22]. In addition to its chondroprotective action, glucosamine is expected to exert anti-inflammatory actions by suppressing neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis [8]. Recently, we have evaluated the effects of oral administration of glucosamine on adjuvant arthritis, a model of RA. The results indicated that glucosamine administration significantly suppressed the production of nitric oxide and PGE_2, and the progression of adjuvant arthritis in rats [12]. Furthermore, the administration of glucosamine to the patients with RA for 12 weeks has been found to significantly reduce pain and swelling of arthritis joints, compared with placebo [23]. However, the anti-inflammatory mechanism of glucosamine on RA is poorly understood. In this study, we examined the effects of glucosamine on the activation of human synoviocytes isolated from synovial tissues and a primary human synovial cell line (CS-ABI-479). The results indicated that glucosamine suppressed the mRNA expression and production of IL-8 by IL-1β-activated synoviocytes. Furthermore, glucosamine inhibited nitric oxide and PGE_2 production, and phosphorylation of p38 MAPK by IL-1β-activated synoviocytes.

RA is a typical inflammatory disease with the permanent synthesis of inflammatory mediators and cytokines, and characterized by the chronic and progressive inflammatory reactions, which result in the synovial hyperplasia and destruction of the affected joints. It is known that nitric oxide

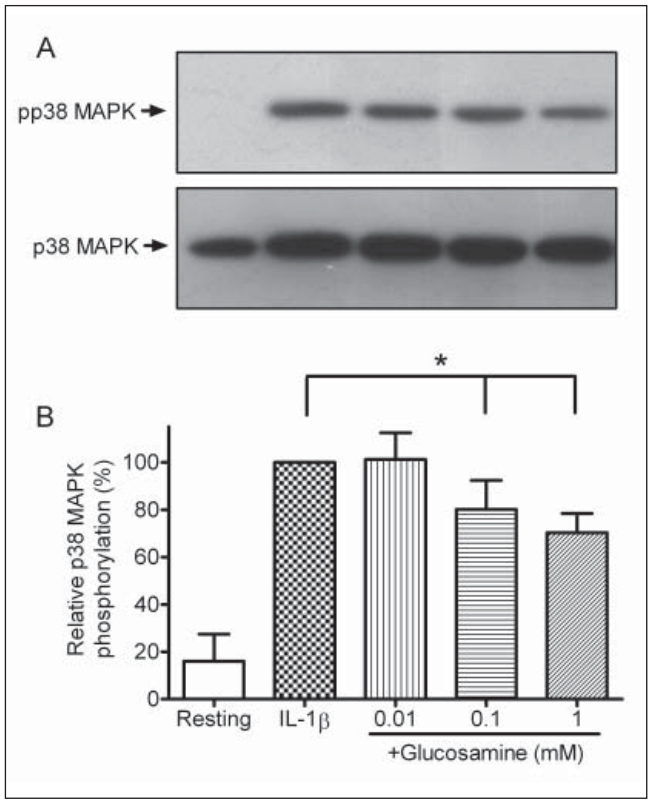

Fig. 5. Effect of glucosamine on the phosphorylation of p38 MAPK. CS-ABI-479, a human primary synoviocyte cell line was preincubated in the absence or presence of 0.01–1 mM glucosamine for 2 h at 37 °C, and further incubated without (Resting) or with 4 ng/ml IL-1β for 5 min. After the incubation, the cell lysates were prepared and subjected to 10 % SDS-PAGE. Phosphorylated p38 MAPK (pp38MAPK) was detected by probing with mouse anti-phosphorylated p38 MAPK (pT180/pY182) monoclonal antibody and HRP-conjugated goat anti-mouse IgG/IgM. To confirm that equal amounts of proteins were analyzed, the blots were stripped, and p38 MAPK protein (p38MAPK) contained in each sample were detected by reprobing with mouse anti-p38 MAPK (p38/SAPK2α) monoclonal antibody and HRP-conjugated goat anti-mouse IgG/IgM (A). The detected bands were quantified by densitometry, and the phosphorylation of p38 MAPK was expressed as the percentage of that of IL-1β-stimulated cells without glucosamine (IL-1β) (B). Data represent the mean ±SD of three separate experiments. Values are compared between IL-1β alone and +Glucosamine. *$p < 0.05$

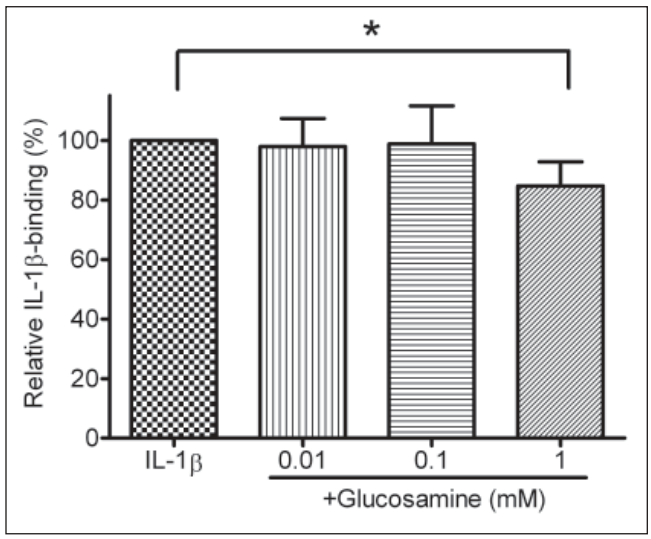

Fig. 6. Effect of glucosamine on the binding of IL-1β to the receptors. CS-ABI-479 cells were incubated with 10 pM of [^{125}I] IL-1β in the absence or presence of 0.01–1 mM glucosamine without or with excess unlabeled IL-1β (10 nM) for 3 h at 4 °C. After washing with PBS, the cells were stripped with 0.25 % trypsin-EDTA, and the radioactivity was measured by scintillation counting. The specific binding of IL-1β is expressed as a percentage of that (IL-1β; 169 ± 59 cpm) obtained with CS-ABI-479 cells incubated in the absence of glucosamine. Data represent the mean ±SD of three separate experiments. Values are compared between IL-1β alone and +Glucosamine. *$p < 0.05$

produced by cartilage and synovial cells is implicated in the pathogenesis of RA [16, 24], and PGE_2 is also an important inflammatory mediator in RA [25]. Moreover, IL-8 is acknowledged to participates in the induction of neutrophil-mediated cartilage damage in RA [26, 27] and assume to be involved in the monocyte accumulation in rheumatoid synovium [28]. We previously found that glucosamine administration reduced the nitric oxide and PGE_2 levels in the plasma, and the inflammatory cell accumulation (mononuclear cells and neutrophils) and synovial hyperplasia in the synovium of rat adjuvant arthritis [12]. In this study, we further revealed that glucosamine significantly suppressed the nitric oxide, PGE_2 and IL-8 production by IL-1β-activated synoviocytes *in vitro*. These observations suggest that the anti-inflammatory action of glucosamine on RA may be associated with its suppressive effect on the inflammatory mediator and cytokine production by synoviocytes.

p38 MAPK is a member of serine/threonine protein kinases, which allow the transduction of extracellular stress signals to the nucleus [20, 21]. Activation of p38 MAPK is considered to be a key step in the signaling of inflammation [20, 21]. In RA, p38 MAPK activation has been demonstrated to be involved in the process of synovial inflammation, which lead to the development of synovial hyperplasia and joint destruction [17–21]. In this study, we revealed that phosphorylation of p38 MAPK, as well as the production of IL-8, nitric oxide and PGE_2 was suppressed by glucosamine. Thus, glucosamine is likely to suppress the synoviocyte activation via its inhibitory effect on p38 MAPK.

IL-1β is an important proinflammatory cytokine that can be detected in the synovial tissue and joint fluids of patients with RA. The present study has shown that IL-1β stimulates the production of chemokine and inflammatory mediators such as IL-8, nitric oxide and PGE_2 that are involved in articular inflammation. It is known that the binding of IL-1β to its cell surface receptors causes a cascade of signaling events including the phosphorylation of p38 MAPK [29, 30]. In this study, we revealed that glucosamine moderately but significantly inhibited the IL-1β–binding to the receptors at 1 mM (Fig. 6). However, the suppressive effect of glucosamine on the IL-1β binding was marginal; glucosamine inhibited the IL-1β binding by only ~20 % even at 1 mM but could never affect the IL-1β binding at 0.1 mM. In contrast, the IL-8 production, IL-8 mRNA expression, nitrite production, PGE_2 production and p38MAPK phosphorylation were suppressed by 0.1~1 mM glucosamine. Thus, the suppressive effects of glucosamine on the IL-1β-induced synoviocyte activation can not be solely explained by its effect, if any, on the ligand (IL-1β) binding. The inhibitory actions of glucosamine on the synoviocyte activation possibly involve other mechanisms such as suppression of intracellular signaling and transcription (as observed for p38MAPK phosphorylation and IL-8 mRNA expression, respectively).

It is now recognized that the addition of O-linked N-acetylglucosamine (O-GlcNAc) to target proteins could modulate cellular functions, such as nuclear transport, transcription, translation, cell signaling, apoptosis and cell shape [31, 32]. In this context, it has been recently found that glucosamine treatment protects rat heart from ischemia-reperfusion injury, accompanied with the increased O-GlcNAc levels and attenuated phosphorylation of p38 MAPK [33]. Of note, we preliminarily tried to detect O-GlcNAc in the synoviocytes by western blotting using anti-O-GlcNAc monoclonal antibody (Covance Research Products, Inc., Princeton, NJ) and revealed that glucosamine but not N-acetylglucosamine increased the O-GlcNAc levels in human synoviocytes at >0.1 mM (data not shown); the effect of glucosamine on the O-GlcNAc-modification was negatively correlated with that on the synoviocyte activation (the phosphorylation of p38 MAPK, and the production of IL-8, nitric oxide and PGE_2). Thus, it is possible that glucosamine suppresses the synoviocyte activation via the modification of target proteins with O-GlcNAc.

In conclusion, the present study has revealed that glucosamine could suppress the activation of synoviocytes (such as nitric oxide-, PGE_2- and IL-8-production, and phosphorylation of p38 MAPK), thereby possibly exhibiting anti-inflammatory actions in arthritis.

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References

[1] Crolle G, D'Este E. Glucosamine sulphate for the management of arthrosis: a controlled clinical investigation. Curr Med Res Opin 1980; 7: 104–9.
[2] Drovanti A, Bignamini AA, Rovati AL. Therapeutic activity of oral glucosamine sulfate in osteoarthrosis: a placebo-controlled double-blind investigation. Clin Ther 1980; 3: 260–72.
[3] Tapadinhas MJ, Rivera IC, Bignamini AA. Oral glucosamine sulphate in the management of arthrosis: report on a multi-centre open investigation in Portugal. Pharmatherapeutica 1982; 3: 157–68.
[4] Lopes Vaz A. Double-blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthrosis of the knee in out-patients. Curr Med Res Opin 1982; 8: 145–9.
[5] Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002; 162: 2113–23.
[6] McAlindon TE, LaValley MP, Gulin JP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA 2000; 283: 1469–75.
[7] Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357: 251–6.
[8] Hua J, Sakamoto K, Nagaoka I. Inhibitory actions of glucosamine, a therapeutic agent for osteoarthritis, on the functions of neutrophils. J Leukoc Biol 2002; 71: 632–40.
[9] Forchhammer L, Thorn M, Met O, Gad M, Weidner MS, Claesson MH. Immunobiological effects of glucosamine in vitro. Scand J Immunol 2003; 58: 404–11.
[10] Santana-Sabagun E, Weisman MH. Nonsteroidal anti-inflammatory drugs. In: Ruddy S, Harris JED, Sledge CB, Budd RC, Sergent JS, editors. Kelly's textbook of rheumatology. Vol. 1. Philadelphia: W. B. Saunders Company, 2001: 799–822.
[11] Gabriel SE, Jaakkimainen L, Bombardier C. Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. Ann Intern Med 1991; 115: 787–96.
[12] Hua J, Suguro S, Hirano S, Sakamoto K, Nagaoka I. Preventive actions of a high dose of glucosamine on adjuvant arthritis in rats. Inflamm Res 2005; 54: 127–32.

[13] Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; 31: 315–24.
[14] Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 1986; 29: 1039–49.
[15] Amin AR, Attur M, Abramson SB. Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. Curr Opin Rheumatol 1999; 11: 202–9.
[16] Jang D, Murrell GA. Nitric oxide in arthritis. Free Radic Biol Med 1998; 24: 1511–9.
[17] Suzuki M, Tetsuka T, Yoshida S, Watanabe N, Kobayashi M, Matsui N et al. The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-α- or IL-1β-stimulated rheumatoid synovial fibroblasts. FEBS Lett 2000; 465: 23–7.
[18] Meyer LH, Pap T. MAPK signalling in rheumatoid joint destruction: can we unravel the puzzle? Arthritis Res Ther 2005; 7: 177–8.
[19] Schett G, Tohidast-Akrad M, Steiner G, Smolen J. The stressed synovium. Arthritis Res 2001; 3: 80–6.
[20] Qi M, Elion EA. MAP kinase pathways. J Cell Sci 2005; 118: 3569–72.
[21] Cuschieri J, Maier RV. Mitogen-activated protein kinase (MAPK). Crit Care Med 2005; 33: S417–9.
[22] Reginster JY, Bruyere O, Fraikin G, Henrotin Y. Current concepts in the therapeutic management of osteoarthritis with glucosamine. Bull Hosp Jt Dis 2005; 63: 31–6.
[23] Nakamura H, Masuko K, Yudoh K, Kato T, Kamada T, Kawahara T. Effects of glucosamine administration on patients with rheumatoid arthritis. Rheumatol Int 2007; 27: 213–8.
[24] McInnes IB, Leung BP, Field M, Wei XQ, Huang FP, Sturrock RD et al. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J Exp Med 1996; 184: 1519–24.
[25] Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR et al. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids. J Clin Invest 1994; 93: 1095–101.
[26] Elford PR, Cooper PH. Induction of neutrophil-mediated cartilage degradation by interleukin-8. Arthritis Rheum 1991; 34: 325–32.
[27] Peichl P, Ceska M, Effenberger F, Haberhauer G, Broell H, Lindley IJ. Presence of NAP-1/IL-8 in synovial fluids indicates a possible pathogenic role in rheumatoid arthritis. Scand J Immunol 1991; 34: 333–9.
[28] Hayashida K, Nanki T, Girschick H, Yavuz S, Ochi T, Lipsky PE. Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8. Arthritis Res 2001; 3: 118–26.
[29] Abramson SB, Amin A. Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. Rheumatology 2002; 41: 972–80.
[30] Christodoulou C, Choy EH. Joint inflammation and cytokine inhibition in rheumatoid arthritis. Clin Exp Med 2006; 6: 13–9.
[31] Zachara NE, Hart GW. Cell signaling, the essential role of O-GlcNAc! Biochim Biophys Acta 2006; 1761: 599–617.
[32] Hanover JA. Glycan-dependent signaling: O-linked N-acetylglucosamine. FASEB J 2001; 15: 1865–76.
[33] Fulop N, Zhang Z, Marchase RB, Chatham JC. Glucosamine cardioprotection in perfused rat heart associated with increased O-linked N-acetylglucosamine protein modification and altered p38 activation. Am J Physiol Heart Circ Physiol 2007; 292: H2227-36.