Effects of glucosamine hydrochloride on the production of prostaglandin E\textsubscript{2}, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis


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Abstract

Objectives
To determine the response of glucosamine hydrochloride on chondrocytes and synoviocytes in terms of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), nitric oxide (NO) and matrix metalloproteases (MMPs).

Methods
Chondrocytes and synoviocytes were prepared from joint specimens of patients who underwent total knee arthroplasty for osteoarthritis (OA). Chondrocytes from patients with femoral neck fracture were served as a normal control. Culture cells were stimulated by 5 ng/ml of IL-1\textbeta and treated with various concentration of glucosamine hydrochloride (from 1 \mu g/ml to 500 \mu g/ml). PGE\textsubscript{2}, NO, MMP-1, MMP-3, and MMP-13 levels were evaluated in the culture supernatant. Further, the expression of COX-2 mRNA was studied by semi-quantitative PCR.

Results
With IL-1\textbeta stimulation, the levels of these mediators increased dramatically, except for NO from synoviocytes. After stimulation, levels of these mediators in OA chondrocytes were higher than synoviocytes and normal chondrocytes, and the level of MMP-3 was higher than those of MMP-1 and MMP-13. Glucosamine hydrochloride at a concentration of 100 \mu g/ml suppressed PGE\textsubscript{2} production, and partly suppressed NO production. It also suppressed the production of MMPs from normal chondrocytes and synoviocytes but not from OA chondrocytes.

Conclusion
Glucosamine modulates the metabolism of chondrocytes and synoviocytes and its mode of action differs between cells and conditions.

Key words
Glucosamine, osteoarthritis, chondrocytes, synovial cells, MMP, PGE2, NO, COX-2.

Glucosamine for PGE₂, NO, MMP production in OA/ H. Nakamura et al.

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Introduction

Glucosamine is aminosugar that is formed from fructose in vivo by glutamin-fructose-6-phosphate amidotransferase and comprises various glycosaminoglycans, the major component of extracellular matrix such as articular cartilage (1). Hyaluronic acid and keratan sulfate are gigantic polymers of the disaccharides found in cartilage, both of which consist of N-acetyl glucosamine and galactose or glucuronic acid. Metabolism of glycosaminoglycans can together with that of collagen fibers is important in maintaining matrix of articular cartilage and the disruption of balance between synthesis and breakdown causes osteoarthritis (OA).

Biological and chemical factors as well as mechanical factors are involved in the pathogenesis of OA. Among various chemical mediators, interleukin-1 (IL-1) has been revealed to play a central role in degradation of articular cartilage (2); it is produced by mononuclear cells in arthritic synovium (3) and chondrocytes (4). IL-1β stimulates the proliferation of synoviocytes (5) and enhances the production of various chemical mediators such as IL-6 (6), prostaglandin E₂ (PGE₂) (4) and nitric oxide (NO) (7) from synoviocytes. IL-1β also stimulates the production of PGE₂ (8), NO (9) and metalloproteases (10) from chondrocytes.

Matrix metalloproteases (MMPs) are important molecules that induce cartilage depletion in OA. MMP-1 and MMP-13 cleave predominantly type II collagen (11), and MMP-3, also known as stromelysin-1, degrades proteoglycans (12). Activated chondrocytes in OA produce these MMPs and degrade extracellular matrix surrounding them. PGE₂, an inflammatory mediator, is involved in the homeostasis of chondrocytes (13) and enhances pain (14). Chondrocyte-derived NO directly breaks down cartilage matrix and induces chondrocyte apoptosis (15,16).

Nutraceutical glucosamine has been used as a supplement for the treatment of osteoarthritis for decades, possibly because the extracellular matrix was believed to wear out in OA cartilage. Many clinical results were reported (reviewed in 17) and some in vitro studies have been published on the effects of glucosamine on chondrocytes or articular cartilage (18-21). Here, we evaluated the effects of glucosamine hydrochloride on cultured cells derived from normal cartilage, OA cartilage and OA synovium in terms of production of PGE₂, NO, MMP-1, MMP-3 and MMP-13.

Materials and methods

Chondrocyte and synoviocyte culture

Chondrocytes and synovial cells were prepared from surgical specimens obtained during arthroplasty for OA and femoral neck fracture. As articular cartilage from the latter samples was macroscopically and microscopically normal, they were used as the normal controls. OA patients (n=7) had an average age of 70.1 (range 50 - 77 yrs) and were diagnosed as having primary OA. Articular changes were advanced and the patients were being treated with analgesics. Fracture patients (n=6) had an average age of 76.6 yrs (range 70-83). Cartilage was carefully cut from the subchondral bone.

Fragments of cartilage and synovial tissue were minced to fine pieces followed by digestion in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, NY) with 1 mg/ml of bacterial collagenase (Sigma, MO). The cells were filtered, washed, and seeded into 75 cm² flasks (Becton Dickinson Labware, Bedford, UK). The cells were cultured in DMEM containing 10% fetal calf serum (FCS; Gibco BRL, NY), 100 units/ml penicillin (Gibco BRL), and 100 µg/ml streptomycin (Gibco BRL) and cells from the primary cultures were used for the experiments.

After the cells became confluent in 24-well plates, medium was changed to 2.5% FCS/DMEM and the cells were stimulated with 5 ng/ml of IL-1β (Rosch, Mannheim, Germany) in the presence of 1, 10 or 100 µg/ml of glucosamine hydrochloride (Yaizu Marine Products, Japan) for 24 hours. For synoviocytes, 5, 50 and 500 µg/ml of glucosamine hydrochloride were used as they were considered to be less responsive to glucosamine. Supernatants were then collected to measure the levels of PGE₂, NO, MMP-1, -3, and -13. The cells were harvested to detect COX-2 by semi-quantitative RT-PCR and Western blot.
Glucosamine for PGE\(_2\), NO, MMP-1, MMP-3 and MMP-13

PGE\(_2\), and NO were assessed by a PGE\(_2\) ELISA kit (Cayman Chemical Co., MI) and a Nitrite colorimetric assay kit (COXIS International, Inc., OR), respectively. MMP-1, MMP-3, and MMP-13 were determined by ELISA or EIA kits (MMP-1 and MMP-13; Amersham Pharmacia Biotech, UK, MMP-3; Daiichi Kagaku Yakuhin, Japan). Precursor and active MMP as well as active complex with tissue inhibitor of metalloprotease-1 (TIMP-1) are measured with these systems.

Preparation of mRNA and semi-quantitative PCR

COX-2 mRNA was quantified using a Gene Specific Relative RT-PCR kit (Ambion, Inc, UK) following the manufacturer's instruction. Briefly, mRNA was extracted from 10\(^6\) chondrocytes using Isogen (Nippon Gene, Japan). cDNA was synthesized in a 20 \(\mu\)l reaction mixture containing 5 \(\mu\)g of total RNA, 2.5 mM of each dNTP, 1 mM of random hexamer primers, 40 units of ribonuclease inhibitor (RNasin; Toyobo, Japan), and 200 units of Superscript II RT (Gibco BRL) at 42°C for 2 hours. The resulting cDNAs were amplified in 50 \(\mu\)l reaction mixture containing COX-2 and 18S primer pairs (included in the kit), 2.5 mM of each dNTP and 0.3 \(\mu\)l of Taq polymerase (Takara, Japan) at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds for 24 cycles in a PCR thermal cycler (Perkin-Elmer, CT). PCR products were electrophoretically analyzed on 2% agarose gels, stained with ethidium bromide (10 mg/ml), and the intensity of the bands were quantified by a fluoro-image analyzer, FLA-2000 (Fuji Film, Japan). The result was expressed as the intensity of COX-2 PCR products against those of 18S.

Western blot for COX-2

Normal chondrocytes in 24-well dish were stimulated by 5 ng/ml of IL-1\(\beta\), in the presence of 100 \(\mu\)g/ml glucosamine hydrochloride or 1 mM diclofenac. After 24 hours, cells were dissolved in lysis buffer (20 mM Tris, 250 mM NaCl, 10% NP-40, 1mM DTT, 10 mM NaF, 2mM Na\(_2\)VO\(_4\), 10 mM sodium pyrophosphate) and sonicated for 3 seconds on ice following centrifugation at 3000 rpm for 5 minutes. Supernatant was dissolved in the same amount of sample buffer (0.1M Tris, 4% SDS, 12 mM DTT, 20% glycerol, 1% BPB) and boiled for 5 minutes. Electrophoretically analyzed proteins (15 \(\mu\)g/lane) on 12.5% SDS-PAGE gel were transferred onto Nitrocellulose Membrane (Bio-RAD, CA). The membrane was blocked in 5% skim milk for 1 hour at room temperature and subsequently immunoblotted with anti-COX-2 antibody (Cayman Chemical Co.) at a 1000 dilution and then incubated with anti-rabbit-IgG antibody conjugated with HRP (Nichirei, Japan) at a 10,000 dilution for 1 hour. The membrane was washed 3 times between each step. Then the membrane was developed using an ECLsystem (Amersham, IL).

Statistical analysis

Each value was from individual samples. Values were expressed as the mean ± SE. Statistical analysis included the Wilcoxon signed rank test for comparisons of paired values, and the Mann-Whitney U test for inter-group comparisons.

Results

IL-1\(\beta\) stimulated production of PGE\(_2\), NO, MMP-1, MMP-3, and MMP-13

With IL-1\(\beta\) stimulation, the level of all mediators from chondrocytes and synoviocytes increased dramatically, except for NO from synoviocytes. These levels and the response to IL-1\(\beta\) were higher in OA chondrocytes than in normal chondrocytes and synoviocytes. It is worth noting that the net level of MMP-3 in stimulated OA chondrocytes was extremely high compared to those of MMP-1 and MMP-13 (Fig. 1).

Effects of glucosamine on PGE\(_2\) production

Prior to assay of the effects of glucosamine hydrochloride on the production of various mediators, its effect on cell viability was evaluated by MTT assay (22). Cell viability was not affected at
concentrations ranging from 1 to 500 µg/ml (data not shown). PGE₂ production was enhanced by IL-1 stimulation in normal chondrocytes and OA synoviocytes as well as OA chondrocytes. The PGE₂ level in IL-1 stimulated OA chondrocyte cultures was 10 times higher than that in unstimulated ones. PGE₂ production was suppressed by 100 µg/ml glucosamine in normal and OA chondrocytes, whereas dexamethasone and diclofenac, which were tested as positive controls, almost completely inhibited stimulated PGE₂ production. IL-1β enhanced the expression of COX-2 mRNA, but glucosamine had no effect on this response. Dexamethasone suppressed the expression of COX-2 mRNA, whereas diclofenac enhanced its expression in OA chondrocytes, which may reflect a feedback regulation caused by diclofenac-mediated COX-2 inhibition (Fig. 2). This result was also supported by Western blot analysis which showed that COX-2 expression was enhanced by IL-1β, and that addition of 100 µg/ml glucosamine had no effect whereas the addition of diclofenac slightly increased the expression (Fig. 3).

Effects of glucosamine on NO production
Glucosamine slightly suppressed the production of NO from IL-1 treated normal chondrocytes, whereas it did not affect OA chondrocytes and OA synoviocytes (Fig. 4).

Effects of glucosamine on MMP production
The production of MMP-1, MMP-3 and MMP-13 was measured in the supernatants of chondrocytes and synoviocytes. The net level of MMP-3 was the highest among the MMPs tested and the level of MMP13 was the lowest. All MMP levels were enhanced by IL-1β. After stimulation with IL-1β, the production of MMP-1, -3 and -13 was partly suppressed by 100 µg/ml glucosamine in normal chondrocytes. In OA synoviocytes, 500 µg/ml glucosamine suppressed the production of MMP-1, -3 and -13. Constant suppression by dexamethasone was shown in all cell types. MMP production was not suppressed by glucosamine at the concentrations to 100 µg/ml in OA chondrocytes (Fig. 5).

Discussion
Degradation of articular cartilage is driven by an imbalance of catabolic and anabolic factors in OA(23). The former include proinflammatory cytokines such as IL-1 and TNF, proteases such as MMPs and cathepsins, and NO that induces chondrocyte apoptosis as well as matrix damage. IL-1 also enhanced the production of PGE₂. In this experiment, the level of MMP-3 in the supernatant of stimulated OA chondrocytes was not suppressed by glucosamine at the concentrations to 100 µg/ml in OA chondrocytes (Fig. 5).
µg/ml whereas its concentration in OA synovial fluid has been reported to be a little higher (from 13 µg/ml to 15 µg/ml) (24, 25). On the contrary, MMP-1 and MMP-13 levels in the supernatant of OA chondrocytes (551.6 ng/ml and 12.8 ng/ml, respectively) were higher than those in OA synovial fluid (356.1 ng/ml and 5.27 ng/ml, respectively) (25, 26). Overall, OA chondrocytes showed a higher sensitivity to the stimulation of MMP, PGE$_2$ and NO by IL-1 than normal chondrocytes did. This could be attributed to the fact that OA chondrocytes had a higher (about 2-fold) density of IL-1 receptor compared with normal chondrocytes (27).

As the cartilage samples of OA were derived from an advanced stage of the disease in which arthroplasty is needed, the chondrocytes are supposed to have been exposed to the specific condition in the OA joint for a long time, which may have altered their characteristics.

PGE$_2$ synthesis is regulated by the coordination of phospholipase A$_2$ (PLA$_2$), COX and PGE synthase (PGES) activities (31,32). Nonsteroidal anti-inflammatory drugs inhibit COX activity, whereas glucocorticoids suppress its expression at the transcriptional level. Shikhman et al. reported that 10 mM N-acetyl glucosamine suppressed COX-1 and COX-2, but did not observe suppressive effects on the expression of

![Fig. 4. Production of NO in normal chondrocytes (n = 6), OAchondrocytes (n = 7) and OAsynoviocytes (n = 5). Cells were stimulated with L-1β (5 ng/ml) and treated with glucosamine hydrochloride (1-100 µg/ml) or dexamethasone (0.1 mM). Glucosamine (100 µg/ml) suppressed the production of NO only in normal chondrocytes. Statistical analysis was performed using the Wilcoxon signed-rank test. *p < 0.05. N: normal chondrocytes, OA: OA chondrocytes, S: OA synoviocytes. Gluco: Glucosamine, Dex: Dexamethasone.](image)

![Fig. 5. MMP-1, -3 and -13 levels in the normal chondrocytes (n = 6), OAchondrocytes (n = 7) and OAsynoviocytes (n = 5). After stimulation with IL-1β (5 ng/ml), cells were treated with various concentration (1-100 µg/ml) of glucosamine hydrochloride or dexamethasone (0.1 mM). Glucosamine (100 µg/ml) showed a suppressive effect on MMP production from normal chondrocytes and OA synoviocytes. The Wilcoxon signed-rank test was used for the statistical analysis. *p < 0.05. N: normal chondrocytes, OA: OA chondrocytes, S: OA synoviocytes. Gluco: Glucosamine, Dex: Dexamethasone.](image)
MAP kinases or translocation of NFκB (21). On the other hand, Largo et al. demonstrated that glucosamine sulfate inhibited IL-1-induced NFκB activity in a dose-dependent manner and suppressed COX-2 expression at the concentration of 1 μg/ml (33). In these reports, authors used different glucosamine derivatives with a higher concentration than those used in this study. Glucosamine hydrochloride did not affect the expression of COX-2 mRNA and protein at 100 μg/ml, thus the suppressive effect of glucosamine hydrochloride on PGE₂ in this study should be investigated further. Phospholipase activity might be involved in this process as Piperno et al. clearly showed that glucosamine suppressed PLA₂ activity (18).

Recently, Dodge et al. reported that 25 μM glucosamine sulfate suppressed MMP-3 from IL-1 stimulated OA chondrocytes (34) and Byron et al. demonstrated that glucosamine suppressed MMP-1, -3 and -13 from LPS stimulated horse chondrocytes (35). In this study, glucosamine was shown to suppress MMP-1, -3 and -13 in normal chondrocytes and OA synoviocytes. However, OA chondrocytes showed no response to glucosamine at 100 μg/ml. As OA chondrocytes intensely express IL-1 receptor and are sensitive to IL-1 stimulation (27), they are supposed to be less responsive to counteracting glucosamine.

Nitric oxide is also a catabolic factor for extracellular matrix in articular cartilage and glucosamine hydrochloride slightly suppressed IL-1 induced NO, while N-acetyl glucosamine dramati-


34. DODGE GR, JIMENEZ SA: Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritic articular chondrocytes. *Osteoarthritis Cartilage* 2003; 11: 424-32.
