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Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes

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Summary

Objective: The functional integrity of articular cartilage is determined by a balance between chondrocyte biosynthesis of extracellular matrix and its degradation. In osteoarthritis (OA), the balance is disturbed by an increase in matrix degradative enzymes and a decrease in biosynthesis of constitutive extracellular matrix molecules, such as collagen type II and aggrecan. In this study, we examined the effects of the sulfate salt of glucosamine (GS) on the mRNA and protein levels of the proteoglycan aggrecan and on the activity of matrix metalloproteinase (MMP)-3 in cultured human OA articular chondrocytes.

Design: Freshly isolated chondrocytes were obtained from knee cartilage of patients with OA. Levels of aggrecan and MMP-3 were determined in culture media by employing Western blots after incubation with GS at concentrations ranging from 0.2 to 200 μM. Zymography (casein) was performed to confirm that effects observed at the protein level were reflected at the level of enzymatic activity. Northern hybridizations were used to examine effects of GS on levels of aggrecan and MMP-3 mRNA. Glycosaminoglycan (GAG) assays were performed on the cell layers to determine levels of cell-associated GAG component of proteoglycans.

Results: Treatment of OA chondrocytes with GS (1.0–150 μM) resulted in a dose-dependent increase in aggrecan core protein levels, which reached 120% at 150 μM GS. These effects appeared to be due to increased expression of the corresponding gene as indicated by an increase in aggrecan mRNA levels in response to GS. MMP-3 levels decreased (18–65%) as determined by Western blots. Reduction of MMP-3 protein was accompanied by a parallel reduction in enzymatic activity. GS caused a dose-dependent increase (25–140%) in cell-associated GAG content. Chondrocytes obtained from 40% of OA patients failed to respond to GS.

Conclusions: The results indicate that GS can stimulate mRNA and protein levels of aggrecan core protein and, at the same time, inhibit production and enzymatic activity of matrix-degrading MMP-3 in chondrocytes from OA articular cartilage. These results provide a cogent molecular mechanism to support clinical observations suggesting that GS may have a beneficial effect in the prevention of articular cartilage loss in some patients with OA.

Key words: Articular cartilage, Glucosamine, Matrix metalloproteinase, Osteoarthritis, Proteoglycan.

Abbreviations: APMA, *p*-aminophenyl mercuric acetate, BSA, bovine serum albumin, DMEM, Dulbecco's modified Eagle's medium, EDTA, ethylenediamine tetraacetic acid, GAG, glycosaminoglycan, GS, sulfate salt of glucosamine, HRP, horseradish peroxidase, IL, interleukin, MMP, matrix metalloproteinase, OA, osteoarthritis, PBS, phosphate-buffered saline, PG, proteoglycan, SDS, sodium dodecylsulfate, SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis, SSC, standard saline citrate.

Introduction

Osteoarthritis (OA) is a disease of diarthrodial joints characterized by the progressive degradation and loss of articular cartilage. OA affects more than 50 million people in the United States, and its incidence increases with increasing age[1]. There is no cure for this common disease, and the currently used treatments modify only the symptoms of the disease rather than the underlying process. Therefore, development of therapies that modify the pathophysiology of OA is essential. Recently, glucosamine sulfate (GS) has received attention as a putative disease-modifying agent for OA[2,3], and numerous clinical and basic investigations have been or are being conducted to examine the ability of GS to relieve OA symptoms and to modify the disease process[3,4].

Cartilage comprises an extracellular matrix consisting of proteoglycans (PGs), collagens (types II, IX, XI, and others), and water[5,6]. Interaction between PGs and

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collagen provides unique structural and physiological properties for cartilage to function in weight bearing and joint motion. Cartilage PGs consist of a protein core with glycosaminoglycan (GAG) side chains[7]. The GAGs in human cartilage PGs include keratan sulfate, dermatan sulfate, heparan sulfate, and chondroitin sulfate. They are composed of repeating disaccharide units, which are combinations of a hexuronic acid (D-glucuronic acid, L-iduronic acid, or D-galactose) and hexosamine (D-glucosamine or D-galactosamine). D-glucosamine is the hexosamine component of keratan sulfate and heparan sulfate. These GAG components impart high negative-charge density, known as fixed-charge density, to PGs and, in turn, to the matrix. PG have the ability to bind water and cations (Na^+) to form a resilient layer on the epiphyses of long bones that acts in part to cushion and protect the joint and provide cartilage its characteristic resistance to mechanical stress. Loss of this protective layer leads to roughening and fissuring of the cartilage and can eventually result in complete erosion to subchondral bone. Healthy cartilage maintains a dynamic equilibrium between processes that produce and processes that degrade the matrix components[8]. It is thought that in OA, this equilibrium is disturbed, and matrix component degradative processes dominate, leading to increased loss of matrix.

Glucosamine (2-amino-2-deoxy-D-glucose) is one of the two hexosamine sugars. The rationale for using glucosamine for the treatment of OA stems from the metabolic pathway of GAG production and proteoglycan (PG) assembly. Glucosamine in the form of glucosamine 6-phosphate is the basic building block required for the biosynthesis of GAGs and, ultimately, the formation of functional PGs. The putative ability of glucosamine in halting or reversing joint degeneration may be due to its participation as essential substrate for the biosynthesis of the GAG used for the formation of PGs. Glucosamine is also a crucial component of the biosynthetic pathway of hyaluronic acid, a molecule that provides the backbone for assembly of several PG molecules to form the large PG aggregates characteristic of normal articular cartilage. Recent studies using ^{13}C-glucosamine in a cartilage explant culture model have demonstrated a preferential incorporation of glucosamine into the galactosamine moieties of chondroitin sulfate[9].

Some recent studies have demonstrated the effect of glucosamine at a molecular level in a number of chondrocyte-culture systems[10–14]. Glucosamine is capable of stimulating PG synthesis[11], inhibiting the degradation of PGs[12], and stimulating the regeneration of cartilage *in vivo*[15,16]. There is also evidence that glucosamine can affect the biosynthesis of PGs as demonstrated by an increase in the incorporation of radiolabeled sulfur into glucosamine-treated cartilage *in vitro*[11]. Several clinical trials have reported some therapeutic effects when oral glucosamine was administered to patients with OA[2,3]. Evidence from these trials suggests that glucosamine may provide pain relief, reduced tenderness, and improved joint mobility. Furthermore, some studies have suggested that GS may have a chondroprotective effect in patients with OA[3]. The results we report in this article demonstrate that GS stimulates aggrecan mRNA and protein levels and reduces matrix metalloproteinase (MMP)-3 protein and enzymatic activity in cultured human OA articular chondrocytes and, therefore, indicate that GS may arrest the progression of articular cartilage degradation and, at the same time, stimulate production of new cartilage macromolecules.

Methods

CELL CULTURE

Tissue from adult knee cartilage discarded during arthroplastic surgery was obtained from patients with OA. Tissue was obtained and utilized following institutional review board-approved protocols. The average age of the patients from whom tissue was procured was 72 years (range, 58–80 years) and was evenly distributed between males and females; no further demographics were obtained. Chondrocytes were isolated from the articular cartilage of tibial plateaus and femoral chondyles of tissue procured within 6 h of surgery and processed as previously described for isolating chondrocytes from epiphyseal cartilage[17]. Briefly, chondrocytes were isolated from pooled femoral and tibial cartilage from individual patients by an initial 1 h incubation with 1 mg/ml each of trypsin and bacterial collagenase (Worthington Biochemicals Co., Freehold, NJ) followed by an overnight digestion in 0.5 mg/ml bacterial collagenase. The following morning, the isolated chondrocytes were washed three times in Dulbecco's modified Eagle's medium (DMEM), counted, and plated in suspension cultures at $5–10\times10^6$ chondrocytes/60 mm poly-(2-hydroxyethyl methacrylate)-coated culture dishes. Under these conditions, the cartilage-specific phenotype is preserved for ≥6 months in culture[17]. Cultures were maintained in DMEM supplemented with 10% fetal bovine serum, 1% vitamin solution (GIBCO, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml ascorbic acid. A 25 mM stock of GS (the sulfate salt of glucosamine, glucosamine SO_3H; provided by Rotta Research Laboratorium, Monza, Italy) was prepared in distilled/de-ionized H_20 and diluted in DMEM to the appropriate concentrations. The chondrocytes were cultured for 24 h without GS. GS was then added on the second day of culture and every 3 days thereafter. In experiments designed to assess the ability of GS to modify chondrocyte metabolism under the stimulatory effect of interleukin (IL-1β), chondrocyte suspension cultures were established as described earlier, and human recombinant IL-1β (Catalog number 1457756, Roche, Indianapolis, IN) was included at 5 ng/ml for the same incubation period with GS. The concentration of 5 ng/ml was used based on our previous studies[18]. Samples for analysis were harvested following 72-h incubation with GS or under control conditions. The medium was collected and stored frozen with protease inhibitors, and the cell layer was either extracted for RNA analysis as subsequently described or, in some experiments, analyzed for levels of various proteins. In these cases, the cells (and associated matrix) were collected by the addition of a lysis buffer consisting of 0.1 M NaCl and 0.5% Triton-X with proteinase inhibitors.

RNA EXTRACTION

RNA was extracted from cultured chondrocytes (which were harvested by washing with cold Hank's balanced salt solution) by lysis with Trizol (BRL Life Technologies, Gaithersburg, MD). The concentration of RNA was determined by OD_{260}, and its integrity was evaluated by the electrophoretic mobility of 28S and 18S ribosomal RNA in agarose gels containing ethidium bromide. Northern blots were prepared following the electrophoresis of 10 μg of total RNA in denaturing 0.8% agarose gels. Prior to transblotting (by capillary blotting) onto nylon membranes

(Roche), the gels were soaked successively in 0.5 M NaOH, 50 mM Tris, and 10× standard saline citrate (SSC) twice for 15 min.

Probes were prepared by [α-^{32}P]dCTP (Amersham, Piscataway, NJ) random primed labeling of cDNA inserts (Ready-To-Go labeling mix beads, Pharmacia, Summit, NJ). Prehybridization and hybridization were performed at 65°C for 30 and 90 min, respectively, using a Quick-Hybe (Amersham) hybridization buffer. Northern blots were washed three times for 20 min at 55°C with 0.5×, 0.25×, 0.1× SSC, and 0.1% sodium dodecylsulfate (SDS). The membranes were exposed to radiographic film for 6–24 h at –70°C. The resulting images were quantified using computer-integrated densitometry. Variations in gel loading were corrected by densitometric measurement of the ribosomal bands in photographic negatives of the ethidium-stained gels.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

When each culture was harvested, the culture media were precipitated with 9 volumes of ethanol (at –70°C for ≥30 min), and, following centrifugation (l0 min at 12 000×*g*), the resultant pellet was dissolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 5 mM ethylenediamine tetraacetic acid (EDTA). Prior to loading, all samples were heated to 75–80°C for 10 min. Mini-gel electrophoresis and Western blots were prepared and analyzed by standard protocols, as previously described[19], using 6.5% polyacrylamide gels containing 5 mM EDTA. Transfer of proteins to supported nitrocellulose was carried out using methanol-free transfer buffer containing 25 mM Tris and 192 mM glycine, with a pH of 8.3. Western blots were performed as previously described with the exception that the post-transfer membranes were sequentially blocked with 10% non-fat milk in phosphate-buffered saline (PBS) for 1.5 h and 5% bovine serum albumin (BSA) in PBS for 30 min. The method of detection was chemiluminescence based on horseradish peroxidase (HRP; ECL, Amersham). All primary antibodies were monoclonal. The anti-aggrecan monoclonal antibody (BE123) was kindly provided by Dr Tibor Glant, Rush University Medical School, and the anti-MMP-1 and anti-MMP-3 monoclonal antibodies were purchased from Biosource International (Camarillo, CA). A propeptide-specific anti-human collagen type II antibody (SJ443) was generated and characterized by our laboratory[19]. All primary antibodies were diluted to 1/1000 in 2.5% BSA in PBS with 0.1% nonidet and were used more than once. The secondary antibody was an HRP-labeled sheep anti-mouse or rabbit (i.e., for collagen antibody) immunoglobulin (Roche), used at 1:8000 and incubated for 60 min. Detection using the ECL chemiluminescence reagents (Amersham) was performed as outlined by the manufacturer, and exposure times were between 30 and 120 s.

Western blots for collagen were performed using samples of media. An equal volume of each medium sample was precipitated with 9 volumes of alcohol prior to dissolving into sample buffer for SDS-PAGE.

ZYMOGRAPHY

For zymography, 1 µl of 100 µM *p*-aminophenyl mercuric acetate (APMA) was added to 100 µl aliquot of culture media and incubated at 37°C for 30 min. Matching samples were prepared without APMA activation. Proteins in all samples were precipitated in a 2× volume of acetone followed by cooling at –70°C for 1 h. The samples were centrifuged (12 000×*g*, 30 min, 4°C), and the pellets were dried in a vacuum centrifuge. The pellets were resuspended in water, an aliquot was mixed 1:1 with a loading buffer (0.125 M Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 0.05% bromophenol Blue), and they were incubated at room temperature for 10 min. Aliquots were separated in gels containing either 0.1% gelatin or 0.05% casein. The gels were incubated in a renaturing buffer (2.5% Triton-X-100) for 30 min at room temperature followed by another 30 min in a developing buffer (10 mM Tris base, 40 mM Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% (w/v) Brij 35). The gels were then incubated overnight at 37°C in fresh developing buffer, stained with 0.05% Coomassie Blue R250 for 30 min, destained for 15 min in 45% methanol/7% acetic acid solution, and washed twice for 20 min in water before drying. The gels were digitized, and cleared areas of proteinase activity were quantitated.

GAG ASSAY

To determine whether GS had an effect on cell-associated PGs and/or on PGs secreted into the media of the suspension cultures, GAG assays were performed on the clusters of chondrocytes (and their cell-associated matrix) and the media from terminated cultures. Cultures of chondrocytes were incubated with 0–400 µM GS as described in the preceding discussion. In experiments in which chondrocytes were not used for RNA isolation, they were collected by centrifugation and lysed in a homogenization buffer (1 M NaCl, 1.0% Triton X-100). The resultant lysates were assayed prior to and after dialysis, and no significant difference in the results was obtained. Routinely, equal aliquots (100 µl) of the lysates or media were diluted with an equal volume of water and were assayed for GAG content using the dimethyl methylene blue dye-binding assay kit (Blyscan) as described by the manufacturer (BioScan, Belfast, Northern Ireland). For the purposes of standardization, a DNA assay was performed on the cell extracts, and the GAG assay results were normalized to the DNA content in each culture[20].

Results

EFFECTS OF GS ON PG PRODUCTION

To determine the effects of GS on the expression of constitutive matrix and matrix-related proteins by OA articular cartilage chondrocytes, an *in vitro* suspension culture that allows the maintenance of the cartilage-specific phenotype for long periods of time was utilized[17]. In these experiments, isolated chondrocytes were cultured with various concentrations of GS for 3 days to 2 weeks. Analyses were performed on separate media samples from each culture (never pooled). Equal aliquots (100 µl) of media were collected from the cultures, and the amount of PG was determined by Western blot following digestion with chondroitinase-ABC and keratinase. Figure 1 is a representative Western blot of the effects of GS on aggrecan detected in culture media from chondrocytes from a 70-year-old individual with OA. In this experiment, chondrocytes were incubated for 2 weeks with 0, 1.0, 5.0, 25, 100, and 150 µM GS. The antibody used reacts with the chondroitin sulfate stubs that remain associated with the aggrecan core protein. Because aggrecan is more than 250 kDa

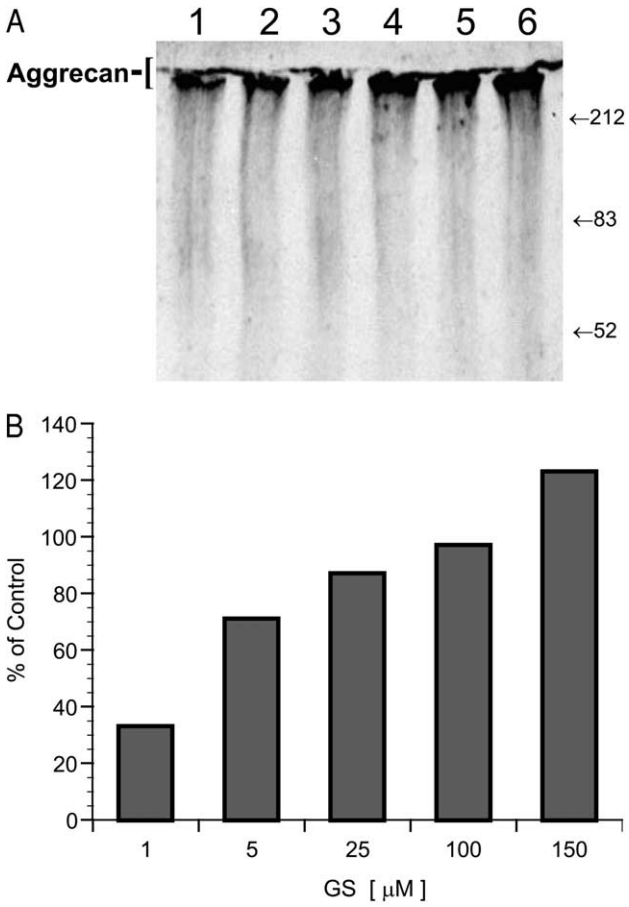

Fig. 1. Aggrecan Western blot. (A) Representative western blot prepared from cultures of chondrocytes from a 70-year-old individual with OA. Cells were cultured as described in 'Methods' section. Samples (100 μl) from the collection of media on day 14 (after the last 72 h with GS) were digested with chondroitinase-ABC and separated on SDS-PAGE (5% gel). Chondrocytes were treated for 2 weeks with 0, 1.0, 5.0, 25, 100, and 150 μM GS (lanes 1–6, respectively). Antibody BE123 was used at 1:1000 dilution, which reacts with the chondroitin sulfate stubs that remain associated with the aggrecan core protein and, as shown, presents as a polydisperse band in polyacrylamide gels. The characteristic band is located at the top of the gel well above the 212 kDa molecular weight standard. (B) Densitometric analysis of the aggrecan protein detected in this Western blot. The data are presented as a percent of control where the control is the untreated culture.

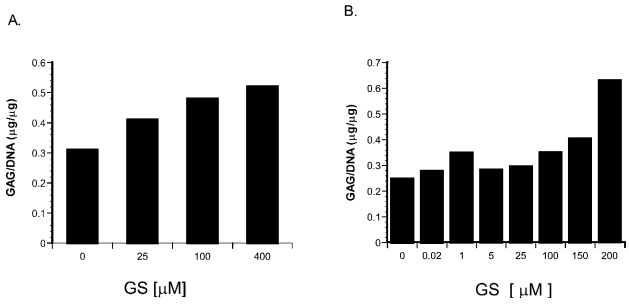

Fig. 2. GAG assay. Culture media and cell lysates from 72-h chondrocyte cultures, from the same patients, from whom samples were analyzed for protein and mRNA, were subjected to a dimethyl methylene blue dye-binding assay. Cell lysate (100 μl each) was tested and the GAG content was determined from a standard curve of known amounts of chondroitin sulfate. Each data point was standardized to the μg of DNA in each lysate. The graphs represent two separate experiments using chondrocytes obtained from two different OA patients. The doses of GS tested range from 25 to 400 μM in panel A and from 0.02 to 200 μM in panel B. Each chondrocyte culture treated with GS shows a greater level of GAG per μg of DNA.

and is likely to contain large numbers of GAG side chains, it is expected that even the lyase-digested PG appears polydisperse in polyacrylamide gels. Figure 1(A) shows the expected PG broad band migrating at the top of a 5% gel above the 212 kDa molecular weight standard. Increasing amounts of aggrecan protein can be detected in these cultures in response to increasing doses of GS. Densitometric analysis of the aggrecan protein detected in this Western blot is shown in Fig. 1(B), which demonstrates that there is a dose-related increase from 30% at 1.0 μM to 120% at 150 μM GS. Each GS-treated culture was compared with the untreated control to determine the percent difference. This experiment is representative of six of the 10 separate patients studied (four of the cases did not respond to any concentration of GS tested).

To confirm these findings, we assayed the cell layers and media from chondrocyte cultures from the same patients for GAG content using the dimethyl methylene blue dye-binding assay. In Fig. 2, we show two separate and representative experiments demonstrating that there was an increase in cell-associated GAG content in the cultures from chondrocytes treated with various concentrations of GS. The increase showed a dose response and was at least 50% greater at the concentrations of GS ranging from 25 to 400 μM compared with untreated controls. Little change was observed in the soluble GAG (media) from these cultures (data not shown). Collectively, the results in Figs. 1(A, B) and 2(A, B) demonstrate that GS increases the expression of aggrecan core protein and that this core protein is post-translationally modified as expected by the attachment of GAG chains.

EFFECTS OF GS ON TYPE II COLLAGEN PRODUCTION

In some cultures, we examined the effect of GS on production of type II procollagen protein by Western blot. As shown in this representative Western blot, there was no measurable change in the levels of this cartilage collagen detected using a propeptide collagen type II-specific antibody (Fig. 3).

EFFECTS OF GS ON PG mRNA LEVELS

To ascertain whether the effect of GS on chondrocyte PG biosynthesis was reflected at the level of mRNA, we performed Northern hybridizations of total RNA from the suspension cultures of OA cartilage-derived primary chondrocytes. We examined 10 μg of total RNA from chondrocytes treated for 72 h with 10–150 μM GS and probed as described earlier in the 'Methods' section, with aggrecan cDNA. In Fig. 4(A), we show a representative Northern analysis for steady-state levels of aggrecan mRNA. In this representative experiment, the chondrocytes

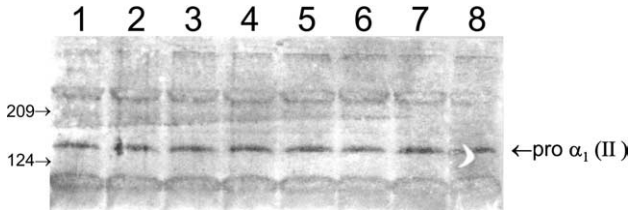

Fig. 3. Type II collagen protein assessment by Western blot. Media collected from chondrocyte cultures treated with or without GS for 72 h were examined by Western blot for changes in production of type II collagen protein. As shown in this representative Western blot, there was no measurable change in the levels of type II cartilage collagen detected using a pro-peptide-specific type II collagen antibody. The GS concentrations tested in this representative experiment were 0, 0.2, 1.0, 5.0, 25, 100, 150, 200 μM. Each concentration is shown in lanes 1–8, respectively.

were treated with 50 and 200 μM GS (lanes 3, 4) in comparison with the levels present in untreated chondrocytes (lane 1). After densitometric analysis and standardization using 28S ribosomal RNA, it can be clearly seen in Fig. 4(B) that the levels of aggrecan mRNA are increased by nearly threefold in response to the 72-h culture with GS (patient 1). Similar results were obtained using chondrocytes from another patient (patient 2). For these analyses, we used the stained ribosomal RNA (28S) for standardization because this is more accurate than relying on 'housekeeping' genes, such as GADPH or actin, that can be altered in response to various cytokines or agents. When Northern blots were incubated with MMP-3 cDNA, no substantial changes in the level of MMP-3 mRNA were observed. The data shown in Fig. 5 were obtained from a representative Northern blot (of six separate experiments) and were standardized using a probe to 7S RNA.

EFFECTS OF GS ON IL-1β-INDUCED CHANGES IN AGGRECAN mRNA

IL-1β is a cytokine that exerts profound effects on chondrocyte metabolism and on extracellular matrix protein and degradative enzyme gene expression. Therefore, we examined the effect of GS on IL-1β-treated OA chondrocyte cultures. In Fig. 4, it can be seen that IL-1β downregulated the expression of aggrecan mRNA (lane 2) and that, when IL-1β-treated cultures were treated with GS, there was a reversal, albeit modest, of the IL-1β effects. As seen in this representative Northern analysis, there was a reproducible increase in the levels of aggrecan mRNA (lanes 5–7) when compared with the levels present in chondrocytes incubated with IL-1β alone.

EFFECTS OF GS ON EXPRESSION OF MMP-1 AND MMP-3

Because OA cartilage pathology is a result, in part, of an imbalance of biosynthesis and degradation resulting in a compromised cartilage structure and function, we performed experiments to examine the effect of GS on MMP-1 and MMP-3 proteins and their activities. We also explored the effects of GS on the expression of MMP-1 and MMP-3 in cultured OA chondrocytes inducted by IL-1β. In Fig. 6, we demonstrate that GS at 1.0–150 μM for 72 h was capable of decreasing the levels of pro-MMP-3 produced by chondrocytes. We also found that there was a modest reduction of the MMP-3 levels in IL-1β-treated chondrocytes (Fig. 7). In Western blots using secreted MMP precipitated from the culture media of chondrocytes after

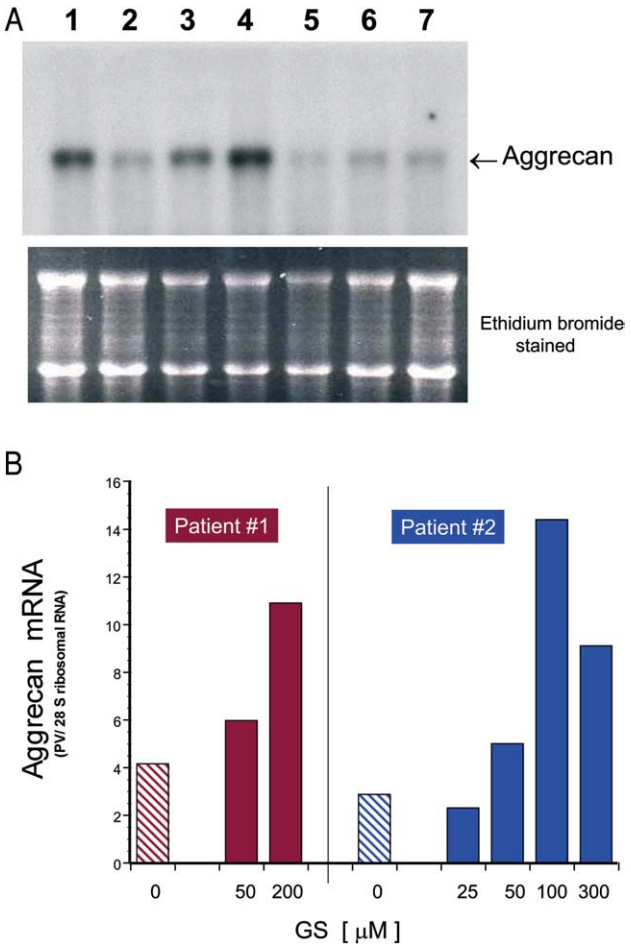

Fig. 4. Assessment of aggrecan mRNA steady-state levels by Northern hybridizations. (A) Total RNA (10 μg) from chondrocyte cultures treated with or without 5 ng/ml IL-1β and various concentrations of GS were analyzed. Shown here is a combined experiment demonstrating the effects of GS alone at 50 and 200 μM (lanes 3 and 4, respectively) on the levels of aggrecan mRNA as compared with mRNA from control cultures (lane 1). The top panel shows the characteristic band for aggrecan mRNA. For quantification, the intensity of ribosomal RNA stained with ethidium bromide (shown in the lower panel) was used. Lanes 2, 5–7 are from samples treated with IL-1β at 5 ng/ml for 72 h with or without GS. Lane 2 is from cultures treated with IL-1β alone, and lanes 5, 6, and 7 were from cultures treated with both IL-1β and GS at 50, 200, and 400 μM, respectively. (B) Graphic representations of the data presented in lanes 1, 3, and 4 along with graphic representation of a separate experiment using chondrocytes from another patient. Both graphs show a dose-dependant upregulation in the steady-state mRNA levels for aggrecan.

24 h treatment with IL-1β, plus various concentrations of GS, it was shown that pro-enzyme levels were decreased by the inclusion of GS in the culture. Figure 7 shows that in IL-1β-treated cultures, MMP-3 levels decreased (18–65%) in cultures treated with GS when compared with non-GS-treated cultures. Levels of MMP-3 were affected to a greater extent than those of MMP-1 (Fig. 8).

The reduction of MMP-3 protein was accompanied by a parallel reduction in enzymatic activity after APMA activation *in vitro*, as determined by casein zymograms (Fig. 9). In these experiments, we tested the levels of MMP-3 enzymatic activity in media from cultures treated for 72 h with or without GS. As shown in this representative casein

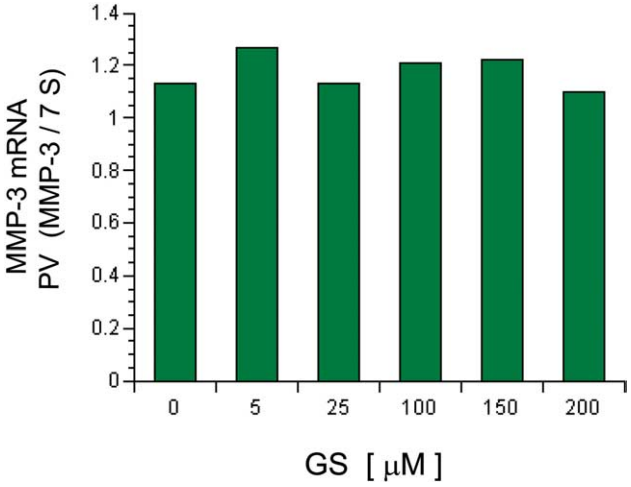

Fig. 5. Assessment of MMP-3 mRNA steady-state levels by Northern hybridizations. The effects of various GS concentrations on the steady-state levels of MMP-3 mRNA were analyzed after a 72-h culture. Shown are data obtained from densitometric analysis from a representative Northern hybridization (representative of six separate experiments). The data are standardized to 7S ribosomal RNA and expressed as pixel volume (PV). No substantial changes in the level of MMP-3 mRNA were detected in response to 5.0, 25, 100, 150, 200 μM of GS when compared with no GS.

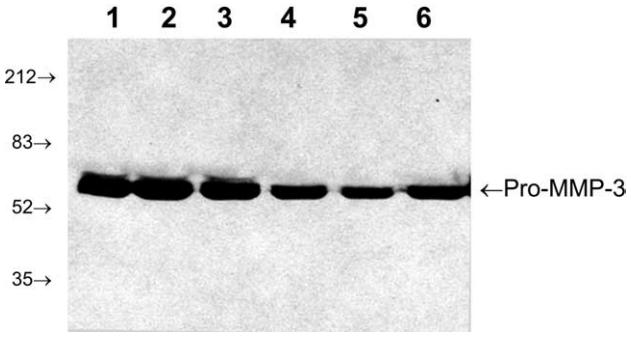

Fig. 6. MMP-3 protein assessment by Western blot. Equal volumes of culture media from each chondrocyte culture were analyzed by Western blots using MMP-3-specific antibodies. The level of pro-MMP-3 detected decreases in a dose-dependant manner in response to GS. Cultures were incubated for 72 h with 0, 1.0, 5.0, 25, 100, and 150 μM GS (lanes 1–6, respectively).

zymogram, a notable decrease in the levels of enzymatic activity was observed in samples from cultures treated with GS at concentrations of 25–150 μM. No active enzyme was detected in these zymograms without activation.

Discussion

The search for potential chondroprotective agents is an intensely pursued area of research with as many approaches as there are the aspects of OA pathogenesis suitable for modulation. Numerous studies have described a variety of agents that may be capable of modifying the structural alterations occurring in articular cartilage during the development of OA. One such putative chondroprotective agent that has generated a large amount of recent attention is glucosamine, either as glucosamine-SO_4 (GS) or glucosamine-HCl. Glucosamine is a molecule that is an

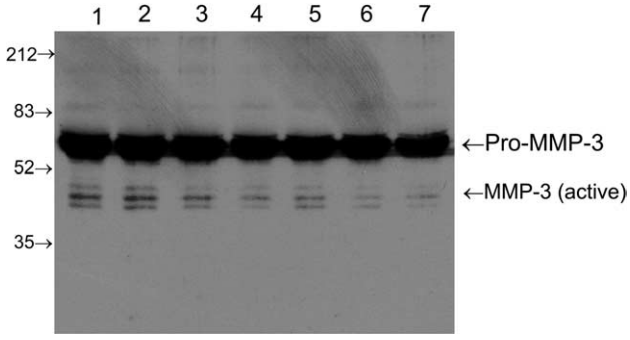

Fig. 7. MMP-3 protein assessment by Western blot in samples cultured with IL-1β and GS. Samples from a different patient than those from the patient shown in Fig. 6 were examined for the effects of GS on the levels of active MMP-3 in cultures incubated in the presence or absence of IL-1β. MMP-3 detected in media from untreated control (lane 1) chondrocyte cultures and those that had been treated for 72 h with IL-1β alone (lane 2), GS alone at 50 μM (lane 3), or GS alone at 200 μM (lane 4) is shown. To ascertain whether the MMP-3 expressed in cultures treated with IL-1β could be downregulated, GS at 50, 200, and 400 μM was added to cultures incubated with IL-1β at 5 ng/ml (lanes 5–7, respectively). The results show that GS caused a modest reduction in IL-1β-stimulated active MMP-3 levels.

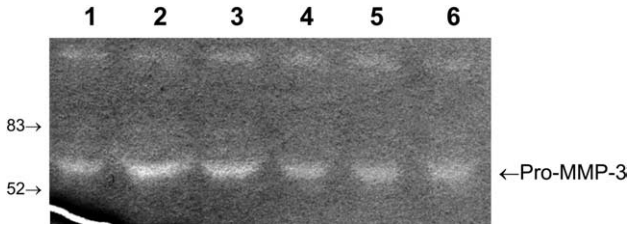

Fig. 9. Functional assay of MMP-3 activity by zymography. Cultures were examined for the presence of MMP-3 by a functional assay employing casein zymography. In this representative figure (of six experiments), the media were activated with APMA, and equal volumes of culture media were electrophoresed into casein gels. Chondrocytes were incubated for 72 h without GS (lane 1) or with 1.0, 5.0, 25, 100, 150 μM GS (lanes 2–6, respectively). Note the reduction in MMP-3 activity induced by incubation with GS.

example of an agent that was the subject of numerous poorly controlled and anecdotal clinical studies before it was studied in more rigorous basic science or controlled clinical research studies. Although there is still substantial controversy regarding the beneficial effects of glucosamine

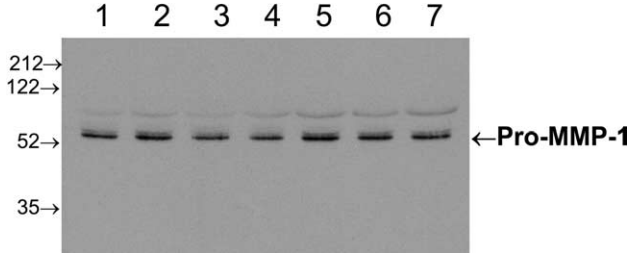

Fig. 8. MMP-1 protein assessment by Western blot. Parallel samples to the ones studied in Fig. 7 were prepared for Western blot and probed with antibodies to MMP-1. The order of each lane is identical to that of Fig. 7. It is clear that MMP-1 is not decreased in the same manner as is MMP-3.

in the treatment of OA[2,4,21], several studies have provided evidence suggestive of both symptomatic as well as chondroprotective effects[3]. The mechanisms responsible for these putative beneficial effects of glucosamine have not been completely elucidated to date. In this report, we explored the effect of GS on the expression of molecules representative of the biosynthetic and catabolic aspects of articular cartilage. We studied aggrecan and the protease MMP-3 that uses aggrecan as substrate as representative paradigms of each of these pathways. The results of these studies provide a biochemical and molecular foundation to explain, at least in part, the recently described effects of glucosamine in improving cartilage loss in patients with OA. We employed an *in vitro* model of freshly isolated human OA chondrocytes in a short-term suspension culture that allows the preservation of the cartilage-specific phenotype. We found that physiologically relevant doses of GS consistently increased the levels of aggrecan core protein mRNA from 30 to 120% when compared with untreated control cultures.

We believe that these results are important because they indicate that GS causes an increase in aggrecan gene expression. Although it is possible that this increase results from changes in aggrecan transcript stability, it is more likely that it results from increased transcriptional activity of the gene promoter. In this regard, several recent studies have shown that components of the hexosamine pathway, of which glucosamine is an important member, are capable of potent modulation of the transcriptional activity of several genes including those encoding fibronectin[22] and plasminogen activator inhibitor-1[23] in glomerular mesangial and endothelial cells, and leptin in adipocytes[24]. Furthermore, it has been demonstrated that these effects appear to be mediated by the transcription factor Sp1[23,25]. Other studies in support of these findings have demonstrated that hexosamines, including glucosamine, increased the activity of a construct containing the Sp1 transcriptional activation domain fused to a reporter gene DNA-binding domain[23]. Notably, the human aggrecan gene promoter contains several Sp1-binding sites that may be important in the regulation of its expression[26]. Therefore, it is possible that glucosamine-induced stimulation of aggrecan mRNA levels observed in our studies may be the result of increased transcriptional activity of the aggrecan gene promoter mediated by Sp1. This putative mechanism is currently being explored in our laboratories.

Critical to the functional integrity of aggrecan is the fact that the core protein requires post-translational modification with the covalent addition of GAG side chains. To ascertain whether the aggrecan core protein molecule contained GAG, we measured the level of GAG and demonstrated that in parallel with the increase in core protein mRNA, there was an increase in GAG content in cultures treated with GS.

Collectively, the aggrecan mRNA data presented are representative of results observed with chondrocyte samples from separate patients. Whereas the data varied from experiment to experiment in absolute amounts, the results indicated that an increase in aggrecan biosynthesis was observed in 60% of cases. The remaining 40% of patients did not respond in the same manner to GS, failing to show any differences from untreated control cultures. The cartilage used in these studies was freshly obtained from OA patients undergoing arthroplasty, and, because the chondrocytes were studied within the first 3 days following their isolation from the tissue, it would be expected that the cultures would closely parallel the *in vivo* situation. This, for example, would presumably explain the high baseline MMP levels detected in the cultures and would also place emphasis on the importance of quantitatively small changes in a situation in which the chondrocytes have been already maximally stimulated *in vivo*. Generally, the patients' condition or therapy prior to surgery was not known. However, we observed that one patient whose chondrocytes consistently demonstrated the highest response to GS was not taking any medication prior to surgery. Moreover, this particular patient received neither intraarticular steroid injections nor non-steroidal anti-inflammatory medications for several weeks prior to surgery.

The upregulation of the expression of a critical extracellular matrix molecule such as aggrecan is likely to cause a profound impact on the overall well-being of articular cartilage, particularly if the degradative activities present in the OA joint are favorably affected as well. In this regard, we studied whether there was any effect of GS on the expression of MMP-3, one of the proteases for which aggrecan is a substrate. We examined the protein levels by Western blot and demonstrated that the levels of proenzyme were reduced by as much as 65% at the doses of GS tested. In addition, we confirmed the effect of GS on MMP activity by performing zymography, an assay that also showed a parallel decrease in MMP-3 enzymatic activity. In contrast with the ability of GS to reduce the levels of MMP-3, no changes were observed in the levels of MMP-1 (data not shown). Even though the level and activity of MMP-3 are high in OA cartilage and presumably also in the OA chondrocytes used in these studies, we tested whether IL-1β could further stimulate *in vitro* MMP-3 and evaluated the effects of GS. As shown in the 'Results' section, we found that the addition of GS to IL-1β-stimulated chondrocytes caused a reversal, albeit modest, of MMP-3 protein levels detected by Western blot. Recently, it has been shown that glucosamine inhibits the aggrecanase-mediated response to IL-1β and retinoic acid, and, moreover, that a similar effect is also attained with other hexosamines[27]. Another study showed that glucosamine reversed the decrease in PG synthesis and in UDP-glucuronosyl-transferase I mRNA induced by IL-1β in rat chondrocytes[28]. Further work by the same group suggested that these effects may be due to both interference by glucosamine of ligand binding to its cognate membrane receptors as well as to inhibition of post-receptor signaling[29]. Indeed, glucosamine has been shown to cause a potent inhibition of activation of NFκβ, an important transcription factor involved in many of the downstream effects of the cytokine[30]. These observations suggest that in addition to modifying the expression of aggrecan by affecting the pool of building-block sugars for GAG, glucosamine may exert a direct or indirect modulation effect on the biosynthesis of the protease MMP-3. The mechanism for this effect is not clearly established, although one mechanism suggested is that glucosamine and other amino sugars inhibit the production of glycosylphosphatidylinositol (GPI)-linked proteins, which are necessary for the response of chondrocytes to IL-1β[31]. Other studies have shown that GS affects aggrecan biosynthesis by affecting a reduction in protein kinase C and phospholipase A2[14].

The recent clinical human studies showing that glucosamine or GS are capable of modifying the symptoms of OA and improving the cartilage alterations characteristic of the disease may find biochemical support in the studies we describe in this article. Our studies show that GS is capable of acting on chondrocyte function *in vitro*. However, it has

not been demonstrated whether similar effects can be attained *in vivo*. For *in vivo* effects, it is required that glucosamine of GS traverses the gut and reaches joint tissues to have a direct effect on cartilage chondrocytes. Although some earlier data have provided support for this possibility, recent evidence using ^{13}C-glucosamine in our own laboratory conclusively demonstrated that ingested glucosamine can be traced to articular cartilage where it was found at high concentrations (unpublished observations). This study employed an animal model and the administration of GS doses equivalent to those used in humans. The results support the notion that one of the mechanisms involved in the improvement of cartilage composition or characteristics caused by GS is through an increase in the available glucosamine in relevant tissues such as articular cartilage. Thus, results presented in this article provide a cogent molecular mechanism to support the clinical observations and the results of recent studies suggesting that GS may have a beneficial effect on articular cartilage chondrocytes by improving their biosynthetic activity and preventing articular cartilage loss in some patients with OA.

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