Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage  

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Summary

Objective: To investigate the effect of long-term exposure to glucosamine or mannosamine on the catabolism of aggrecan by explant cultures of bovine articular cartilage maintained in the presence of retinoic acid.

Design: The kinetics of loss of $^{35}$S-labeled and total aggrecan from explant cultures of bovine articular cartilage maintained in the presence of 1 µM retinoic acid and exposed to varying concentrations of glucosamine or mannosamine was investigated over a 9-day culture period. In other experiments, the reversibility of the inhibition of aggrecan catabolism by glucosamine or mannosamine was investigated in cultures exposed to these amino sugars for the first 5 days of a 15-day culture period. The metabolism of chondrocytes exposed to these amino sugars was evaluated by measurement of lactate production or $^3$H-serine and $^{35}$S-sulfate incorporation into protein and glycosaminoglycans, respectively. The direct effect of these amino sugars on soluble aggrecanase activity was determined from immunoblots of aggrecan digests.

Results: Glucosamine at 5 mM concentration and mannosamine at 2 mM concentration inhibited degradation of radiolabeled and chemical levels of aggrecan. At concentrations of up to 10 mM amino sugars, the metabolism of chondrocytes was not impaired, as determined by lactate production, protein synthesis and the incorporation of $^{35}$S-sulfate into proteoglycans. These amino sugars did not inhibit soluble aggrecanase activity. The exposure of articular cartilage explants to 5 mM glucosamine or mannosamine for 5 days in culture in the presence or absence of retinoic acid did not provide long-term suppression of stimulated aggrecan loss.

Conclusions: This study indicates that continuous presence of amino sugars is required to protect cartilage from stimulated loss of aggrecan.

Key words: Glucosamine, Mannosamine, Aggrecan, Cartilage.

Introduction

The loss of aggrecan from the extracellular matrix of articular cartilage results in a change in biomechanical properties of the tissue, which precede the loss of cartilage from the surface of bones. The degradation of aggrecan in normal and degenerative cartilage and in explant cultures stimulated with retinoic acid or cytokines IL-1, TNF-$\alpha$ has been shown to be primarily due to the action of aggrecanases. In addition, there is a strong correlation between the increase in aggrecanase-derived metabolites and the levels of chemical and radiolabeled glycosaminoglycans present in the medium of retinoic acid-stimulated cultures. The metabolites that can be attributed to other proteinases, such as MMPs, have only been detected, at a late stage of tissue degradation, as minor products. Aggrecanase activity has been attributed to three enzymes, ADAMTS-1, ADAMTS-4 and ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin (TBS) motifs), that cleave aggrecan at specific sites between a glutamate and a hydrophobic residue that are located within the interglobular domain and the chondroitin sulfate attachment regions of the core protein of this proteoglycan. Aggrecanases are secreted metalloproteinases with furin and cysteine switch activation sites and other domains that include one or more TBS type 1 motifs, spacer region, disintegrin and cysteine-rich domains capable of interacting with extracellular matrix components. Such interactions have been proposed to be important in the regulation of activity of these enzymes. The mature enzymes have been reported to be inactive, and the activity is generated by carboxy-terminal truncation of aggrecanase proteins. The aggrecan-degrading activity of truncated aggrecanase proteins has also been reported to depend on the retention of some carboxy-terminal regions, such as a disintegrin and TBS 1 motif in ADAMTS-4. The carboxy-terminal regions in aggrecanases may also have a role in the inhibition of the enzyme activity by highly sulfated polysaccharides, such as heparin, heparan sulfate and calcium pentosan polysulfate. A number of studies have shown that the amino sugars, glucosamine and mannosamine, inhibited IL-1, TNF-$\alpha$ and retinoic acid-stimulated aggrecanase degradation of aggrecan in cartilage and chondrocyte cultures. In addition, glucosamine has been used as a nutritional supplement for treatment of osteoarthritis with positive results relating to narrowing of joint space and symptomatic relief. The effect of glucosamine as an antiarthritic agent is poorly understood, especially the cellular and molecular mechanisms of its action.

The present study investigates the long-term effect of glucosamine and mannosamine on retinoic acid-stimulated degradation of aggrecan in bovine articular cartilage cultures, as well as their impact on the metabolism of articular
cartilage. Also investigated in this study is the potential of these amino sugars to directly affect aggrecanase activity and the ability to protect cartilage from aggrecanase-induced loss of aggrecan.

Materials and methods

Dulbecco’s modified Eagles medium (DMEM) containing 1 g/l (5.5 mM) glucose, newborn calf serum (NBCS), non-essential amino acids, penicillin and streptomycin were purchased from CSL (Melbourne, Australia). Mannosamine and chondroitinase ABC were from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Carrier-free $^{35}$S-sulfate in aqueous solution and $[{}^3$H]-L-serine were from DuPont New England Nuclear (Boston, MA, USA). Retinoic acid, glucosamine, keratanase and a lactate assay kit were purchased from Silenus Laboratories (Hawthorn, Victoria, Australia). The monoclonal antibody 5/6/3-B-3 was kindly provided by Professor B. Catterson (School of Molecular and Medical Biosciences, University of Wales at Cardiff, UK). Immobilon-P (polyvinylidene difluoride) membranes were from Millipore (Belford, MA, USA) and PD-10 columns were from Amersham Pharmacia Biotechnology (Uppsala, Sweden).

EXPLANT CULTURES OF BOVINE ARTICULAR CARTILAGE

Articular cartilage, freshly dissected from metacarpophalangeal joints of 1–3 years old cattle was incubated in DMEM containing 20% (v/v) NBCS and 30 µCi/ml of $^{35}$S-sulfate for 6 h at 37°C. The cultures were maintained in the absence of NBCS for the reminder of the culture period in all proteoglycan turnover experiments. In this work, 5.5 mM glucose was present in the culture medium, which reflects the physiological concentration of glucose reported for cartilage. The tissue was washed with DMEM to remove unincorporated $^{35}$S-sulfate. Duplicate cultures containing 100 mg wet-weight tissue/4 ml medium were maintained in either DMEM alone or DMEM containing 1 µM retinoic acid and 0, 2, 5 or 10 mM glucosamine or 0, 1.5, 5 and 10 mM mannosamine for a further 9 days. The medium was changed daily, and the spent medium was collected and stored in the presence of proteinase inhibitors at −22°C. On completion of the experiment, the tissue was extracted with 4.4 ml of 0.5 M NaOH for 24 h at room temperature. In a separate experiment, freshly dissected articular cartilage was incubated with radiolabeled sulfate as described previously, and duplicate cultures of 100 mg wet-weight tissue/4 ml medium were maintained for 5 days in DMEM containing either 5 mM glucosamine or mannosamine in the absence or presence of 1 µM retinoic acid and then returned to culture for another 10 days in DMEM containing retinoic acid. The control cultures were maintained for 15 days in either DMEM or DMEM containing 1 µM retinoic acid or for 5 days in DMEM and then continued for another 10 days in DMEM containing 1 µM retinoic acid.

MEASUREMENT OF TURNOVER OF $^{35}$S-LABELED AGGRECAN

To determine the amount of radiolabeled macromolecules in medium samples and in tissue extracts, the samples were applied to Sephadex G-25 columns (PD-10 columns) equilibrated in a dissociative buffer containing 4 M guanidine hydrochloride. The excluded volume peak was analyzed for radioactivity. The percentage of $^{35}$S-labeled aggrecan remaining in the matrix on each day of culture was determined from the amount of $^{35}$S-labeled macromolecules appearing in the medium on each day of culture and that remaining in the matrix at the end of the culture period, as previously described.

MEASUREMENT OF GLYCOSAMINOGLYCAN LEVELS

The levels of glycosaminoglycans appearing in the medium of explant cultures on each day and present in tissue extracts at the end of culture period were determined using the dimethylmethylene blue (DMB) assay for sulfated glycosaminoglycans, using chondroitin sulfate from bovine nasal septum as standard. The DMB solution was added to diluted samples and standards and appropriate blank solutions prior to absorbance reading at 525 nm.

MEASUREMENT OF LACTATE LEVELS

The levels of lactate released into the medium of explant cultures on days 5 and 9 were determined by the oxidase/peroxidase method using a commercial kit from Sigma.

MEASUREMENT OF PROTEIN AND PROTEOGLYCAN SYNTHESSES

The following experiments were performed using tissue from the same animal. Articular cartilage dissected from a single metacarpophalangeal joint was maintained in culture in 100 mg wet-weight tissue/4 ml medium lots in DMEM in presence or absence of 1 µM retinoic acid and 0, 2, 5 and 10 mM glucosamine or 0, 1.5, 5 and 10 mM mannosamine for 9 days. On days 5 and 10 of culture, the triplicate cultures from each treatment condition were preincubated with 2 ml DMEM at 37°C for 1 h. The medium was then replaced with 2 ml DMEM containing 20 µCi of $^{35}$S-sulfate/ml or 30 µCi $[^3$H]serine/ml, respectively, that were prepared as batch solutions. Following a 2 h incubation period, the $^{35}$S-radiolabeled tissue was extracted with 0.5 M NaOH for 24 h at room temperature and the $[^3$H]serine-radiolabeled tissue was extracted by 4 M guanidine chloride, 0.05 M sodium acetate, pH 6 for 24 h at 4°C, followed by 0.5 M NaOH for 24 h at room temperature. Radiolabeled macromolecules present in tissue extracts were separated from unincorporated radiolabel by gel filtration on Sephadex G-10 (PD-10) columns. The rate of incorporation of $^{35}$S-sulfate into proteoglycans and the rate of incorporation of $[^3$H]serine into protein were expressed as cpm/100 mg wet-weight tissue.

WESTERN BLOT ANALYSIS

Aggrecan monomer (12 µg), purified from bovine articular cartilage, was incubated with conditioned medium (100 µl) obtained from explant culture of joint capsule, in the presence of 0, 2, 5, or 10 mM glucosamine or mannosamine for 24 h at 37°C. We have previously shown that conditioned medium from explant cultures of bovine joint capsule contains aggrecanase activity, and when incubated with aggrecan, results in cleavage of core protein of this proteoglycan at aggrecanase specific sites. The digests were stopped by the addition of proteinase inhibitors.
Aggrecan fragments were deglycosylated using chondroitinase ABC (0.0125 U/mg aggrecan) and keratanase (0.025 U/mg aggrecan) in 1 ml of 0.1 M Tris/0.1 M sodium acetate buffer, pH 7. Samples of deglycosylated aggrecan core protein fragments were subjected to electrophoresis under reducing conditions on a 4–10% gradient polyacrylamide sodium dodecyl sulfate slab gel. The protein bands were electroeluted onto Immobilon-P membranes and probed with antibody 5/6/3-B-3 to terminal unsaturated chondroitin 6-disulfated disaccharides as previously described. These epitopes are present on glycosaminoglycans stubs that remain attached to aggrecan core protein following deglycosylation by chondroitinase ABC. The membrane was then exposed to biotin-conjugated second antibody followed by exposure to horseradish peroxidase-conjugated streptavidin. The protein bands were visualized with 2.8 mM 4-chloro-1-naphthol in a phosphate-buffered saline containing 0.02% (v/v) hydrogen peroxide.

TREATMENT OF DATA

In previous work using articular cartilage in explant cultures, we have reported the variation in the absolute rates of synthesis and catabolism of proteoglycans in articular cartilage from different animals. This variation also extends to other metabolic measurements that include lactate production, protein and proteoglycan syntheses and the amounts of macromolecules present in the extracellular matrix of the tissue. Thus, to circumvent the animal variation, any one experiment is restricted to tissue obtained from a single joint. Consequently, a limited amount of tissue is available in any one experiment and, thus, the number of samples used for the statistical analysis of the data is restricted. In some work presented in this article, duplicate samples containing sufficient amount of tissue necessary to generate accurate and reproducible data, were analyzed, and the mean of the data is given along with the range of values obtained. All experiments were repeated at least three times, and all of these showed the same outcome.

In experiments measuring protein and proteoglycan syntheses, triplicate samples were used to compare the effect of different treatments using the Kruskal–Wallis test (analysis of variance by ranks). Significant differences between the treatment groups were analyzed by the two-tailed Mann–Whitney U-test, and P values of less than 0.05 were taken as significant.

Results

EFFECT OF GLUCOSAMINE AND MANNOSE ON RETINOIC ACID-INDUCED CARTILAGE DEGRADATION

Experiments were performed to establish the effect of varying concentrations of amino sugars on the degradation of aggrecan in long-term cultures of articular cartilage. Bovine articular cartilage was maintained in the presence of retinoic acid and 0–10 mM amino sugars for 9 days in culture, as described in Materials and Methods section. Figure 1(A) shows the percentage of 35S-labeled aggrecan lost from the matrix of cultures with time, for cultures treated with glucosamine. The retinoic acid-stimulated loss of radiolabeled aggrecan from the matrix of tissue was inhibited in a concentration-dependent manner up to 10 mM glucosamine. At the end of culture period (day 9), the retinoic acid-stimulated loss of radiolabeled aggrecan was reduced by 7, 13 and 58% in the presence of 2, 5 and 10 mM glucosamine, respectively. The stimulated loss of aggrecan represents the loss above that observed for cultures maintained in DMEM alone. Figure 1(B) shows the loss of chemical levels of aggrecan from the matrix of cultures in the same experiment. The stimulated loss of chemical levels of aggrecan was also inhibited in a concentration-dependent way by glucosamine [Fig. 1(B)]. Table I shows the chemical levels of aggrecan remaining in the tissue at the end of culture period. There is an increase in chemical levels of aggrecan in retinoic acid-stimulated cultures with increasing concentrations of glucosamine. In tissue treated with 10 mM glucosamine, there was a 53% inhibition of aggrecan loss. It should be noted that both the degradation and the synthesis of aggrecan contribute to the chemical levels of aggrecan present in the tissue during the culture period, while the levels of the radiolabeled aggrecan reflect the loss of aggrecan from a defined pool of aggrecan synthesized at the beginning of culture period.

In a separate experiment, it was shown that mannosamine inhibited the retinoic acid-induced loss of radio-
TABLE I
Amount of glycosaminoglycans remaining in the matrix of bovine articular cartilage explant cultures maintained in medium alone, medium containing retinoic acid or medium containing retinoic acid and varying concentrations of glucosamine or mannosamine for 9 days.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Glycosaminoglycan concentration in matrix (µg CS/mg wet-weight tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>10.7±0.2</td>
</tr>
<tr>
<td>Medium+retinoic acid</td>
<td>5.8±0.2</td>
</tr>
<tr>
<td>Medium+retinoic acid+2 mM glucosamine</td>
<td>6.1±1.6 (6)</td>
</tr>
<tr>
<td>Medium+retinoic acid+5 mM glucosamine</td>
<td>6.3±0.5 (10)</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM glucosamine</td>
<td>8.4±0.8 (53)</td>
</tr>
<tr>
<td>Medium+retinoic acid+2 mM mannosamine</td>
<td>6.6±1.5 (16)</td>
</tr>
<tr>
<td>Medium+retinoic acid+5 mM mannosamine</td>
<td>8.4±0.1 (53)</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM mannosamine</td>
<td>10.0±0.5 (86)</td>
</tr>
</tbody>
</table>

Values are the mean±range of duplicate determinations. Values in parentheses show the percentage inhibition of the retinoic acid-stimulated loss of glycosaminoglycans from the matrix. The stimulated loss of glycosaminoglycans represents the loss above that observed for cultures maintained in DMEM alone. Articular cartilage from different animals was used in glucosamine and mannosamine experiments.

EFFECTS OF GLUCOSAMINE AND MANNOSAMINE ON CHONDROCYTE METABOLISM

The effects of glucosamine and mannosamine on lactate production, protein and proteoglycan syntheses were investigated in order to determine whether concentrations of amino sugars observed to inhibit aggrecan loss in explant cultures of cartilage affected the general metabolism of the tissue.

The lactate concentrations present in the medium of explant cultures, presented in Figs. 1 and 2, were determined on days 5 and 9 (Table II). Higher lactate production was observed in cultures maintained in the presence of retinoic acid than in the non-stimulated cultures. This has been observed by other researchers for cartilage cultures exposed to retinoic acid14–23,24. For cultures exposed to retinoic acid and in the presence of 5 and 10 mM of either of the amino sugars, there was a trend showing a decrease in lactate production with increasing concentration of amino sugar (Table II). Bryson et al.14 also reported an increase in lactate production in IL-1α-treated cultures of bovine nasal cartilage, and this effect was abrogated in the presence of 5 mM mannosamine. However, Table II shows that lactate production in the presence of 5 and 10 mM of either of the amino sugars was 80–95% of levels observed for tissue cultured in DMEM alone, indicating a significant lactate production throughout the culture period.

The rate of incorporation of 3H-serine into protein was measured on days 5 and 9 of culture in articular cartilage cultures maintained in the presence of 5 or 10 mM glucosamine or mannosamine. There were no significant differences between different culture conditions for the incorporation of 3H-serine into protein for cultures maintained in the presence of glucosamine or mannosamine (Table III).

The rates of incorporation of 35S-sulfate into proteoglycans were measured on days 5 and 9 of culture in cartilage explant cultures maintained in presence of glucosamine or mannosamine. The levels of proteoglycan synthesis in cultures maintained in the presence of retinoic acid were statistically different between treatment groups. This was indicated by a 23% decrease in the proteoglycan synthesis in cultures the presence of 10 mM glucosamine [Table III(A)] and less than 20% in cultures supplemented with 5 and 10 mM mannosamine [Table III(B)]. Similar trends were also observed in duplicate experiments. Table III also shows that exposure of cartilage to retinoic acid resulted in the suppression of the incorporation of 35S-sulfate into proteoglycans [Table III(A)]. This is in agreement with a previous report showing reduction in proteoglycan synthesis in articular cartilage cultures in the presence of 1 µM retinoic acid25. It is apparent that the
addition of glucosamine or mannosamine to the culture medium containing retinoic acid did not affect the proteoglycan synthesis [Table III(A)]. Overall, the above-mentioned data indicate that the metabolic activity of the chondrocytes was not compromised in presence of the amino sugars over the culture period.

### Table II

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Lactate concentration in medium (mg lactate/100 mg wet-weight tissue/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Medium alone</td>
<td>1.01±0.06</td>
</tr>
<tr>
<td>Medium+retinoic acid</td>
<td>1.40±0.12</td>
</tr>
<tr>
<td>Medium+retinoic acid+5 mM glucosamine</td>
<td>0.95±0.05 (68)</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM glucosamine</td>
<td>0.89±0.10 (64)</td>
</tr>
<tr>
<td>Medium alone</td>
<td>0.99±0.07</td>
</tr>
<tr>
<td>Medium+retinoic acid</td>
<td>1.31±0.01</td>
</tr>
<tr>
<td>Medium+retinoic acid+5 mM mannosamine</td>
<td>0.94±0.02 (72)</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM mannosamine</td>
<td>0.81±0.08 (61)</td>
</tr>
</tbody>
</table>

Values are the mean±range of duplicate determinations. Values in parentheses are percentage of values observed for cultures maintained in medium containing retinoic acid.

### Table III

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Days in culture</th>
<th>$[^3]H$serine incorporation (cpm/100 mg wet-weight tissue/2 h)</th>
<th>$[^35]S$sulfate incorporation (cpm/100 mg wet-weight tissue/2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Glucosamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium alone</td>
<td>5</td>
<td>24 075±5745*</td>
<td>54 107±831*</td>
</tr>
<tr>
<td>Medium+5 mM glucosamine</td>
<td>5</td>
<td>27 945±2767* (113)</td>
<td>48 084±3265* (89)</td>
</tr>
<tr>
<td>Medium+10 mM glucosamine</td>
<td>5</td>
<td>25 737±6848* (104)</td>
<td>41 685±1361* (77)</td>
</tr>
<tr>
<td>Medium alone</td>
<td>9</td>
<td>21 298±2990*</td>
<td>53 322±1431* (88)</td>
</tr>
<tr>
<td>Medium+5 mM glucosamine</td>
<td>9</td>
<td>19 981±1455* (94)</td>
<td>46 712±877* (88)</td>
</tr>
<tr>
<td>Medium+10 mM glucosamine</td>
<td>9</td>
<td>18 232±748* (86)</td>
<td>43 687±518* (82)</td>
</tr>
<tr>
<td>Medium+retinoic acid</td>
<td>5</td>
<td>22 009±1454*</td>
<td>37 292±932*</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM glucosamine</td>
<td>5</td>
<td>17 154±2504* (78)</td>
<td>42 111±113* (113)</td>
</tr>
<tr>
<td>Medium+retinoic acid</td>
<td>9</td>
<td>18 354±3082* (92)</td>
<td>36 675±466* (109)</td>
</tr>
<tr>
<td>Medium+retinoic acid+5 mM glucosamine</td>
<td>9</td>
<td>16 909±835* (92)</td>
<td>36 675±466* (109)</td>
</tr>
<tr>
<td>Medium+retinoic acid+10 mM glucosamine</td>
<td>9</td>
<td>15 131±1645* (82)</td>
<td>35 998±204* (107)</td>
</tr>
</tbody>
</table>

| (B) Mannosamine    |                 |                                                             |                                                             |
| Medium alone       | 5               | 32 221±964*                                               | 54 102±831*                                                 |
| Medium+5 mM mannosamine | 5             | 24 832±1986* (83)                                        | 44 693±1301* (83)                                         |
| Medium+10 mM mannosamine | 5             | 26 857±1769* (84)                                        | 47 198±661* (87)                                         |
| Medium alone       | 9               | 21 298±2999*                                              | 53 322±1431* (88)                                         |
| Medium+5 mM mannosamine | 9             | 19 891±1896* (93)                                         | 46 777±3181* (88)                                         |
| Medium+10 mM mannosamine | 9             | 22 130±1761* (104)                                        | 43 052±591* (81)                                         |
| Medium+retinoic acid | 5             | 22 009±1454*                                              | 37 292±932*                                                 |
| Medium+retinoic acid+5 mM mannosamine | 5     | 20 682±900* (94)                                        | 39 747±357* (107)                                         |
| Medium+retinoic acid+10 mM mannosamine | 5     | 18 983±1771* (86)                                        | 34 133±911* (92)                                         |
| Medium+retinoic acid | 9             | 18 354±3082* (92)                                         | 33 520±364* (107)                                         |
| Medium+retinoic acid+5 mM mannosamine | 9     | 14 882±2465* (81)                                        | 35 569±950* (106)                                         |
| Medium+retinoic acid+10 mM mannosamine | 9     | 16 712±1660* (91)                                        | 35 122±29* (105)                                         |

Values are the mean±SD of triplicate determinations. Values in parentheses are percentage of values observed for cultures maintained in medium alone or medium containing retinoic acid.

* Differences between treatment groups within a set determined by Kruskal–Wallis test were not statistically significant ($P>0.05$).
† Differences between treatment groups within a set determined by Kruskal–Wallis test were statistically significant ($P<0.05$).
EFFECTS OF GLUCOSAMINE AND MANNOSAMINE ON SOLUBLE AGGREGANASE ACTIVITY

These experiments were undertaken to determine whether the inhibitory effects of glucosamine and mannosamine on the catabolism of aggrecan in articular cartilage explant culture could be attributed to the direct inhibition of aggrecanase activity. Aggrecan monomer was digested with aggrecanase activity present in conditioned medium from explant culture of joint capsule in the presence of varying concentrations of glucosamine or mannosamine. Figure 3 shows immunoblots of aggrecan core protein fragments using the monoclonal antibody 5/6/3-B-3. Three typical bands representing aggrecanase degradation products of aggrecan with &sim;Mr 200 000 (band C), 170 000 (band D) and 130 000 (band E) were observed in all digests. In a number of studies, we have analyzed the bands of the same electrophoretic mobility by the amino-terminal amino acid analysis and have shown that these bands represent aggrecanase-generated aggrecan fragments resulting from cleavages of aggrecan within the interglobular domain between residues E373–A374 (band C), and between residues E1480–G1481, E1666–G1667 and E1771–A1772 (bands C, D and E, respectively) within the chondroitin sulfate attachment domains. Bands of approximately equal intensity were observed in the absence (lane b) or presence of 2, 5 and 10 mM glucosamine (lanes c–e) or 2, 5 and 10 mM mannosamine (lanes f–h), indicating that glucosamine and mannosamine do not have a direct effect on aggrecanase activity.

REVERSIBILITY OF SUPPRESSION OF AGGREGAN DEGRADATION BY GLUCOSAMINE AND MANNOSAMINE

The section, showed effects of Glucosamine and Mannosamine on Soluble Aggrecanase Activity has shown that glucosamine and mannosamine do not directly inhibit aggrecanase activity. These experiments were performed to investigate if aggrecanase activity remains suppressed after the withdrawal of glucosamine and mannosamine from explant cultures of articular cartilage exposed to retinoic acid. Freshly dissected articular cartilage was incubated with [35S]sulfate in DMEM containing 20% (v/v) NBCS for 6 h and returned to culture. (A) The cultures were maintained for 5 days in DMEM containing 5 mM glucosamine in the absence of or presence of 1 µM retinoic acid and continued for another 10 days in DMEM containing 1 µM retinoic acid. (B) The cultures were maintained for 5 days in DMEM containing 5 mM mannosamine in the absence or presence of 1 µM retinoic acid and continued for another 10 days in DMEM containing 1 µM retinoic acid. The control cultures (A and B) were maintained for 15 days in DMEM or DMEM containing 1 µM retinoic acid or 5 days in DMEM and continued for another 10 days in DMEM containing 1 µM retinoic acid. A and B show cumulative loss of 35S-labeled aggrecan from the matrix of explant cultures with time. It is expressed as percentage of total radiolabeled aggrecan for each time point. Values represent the mean of duplicate cultures and the range over the 15 days (error bars).
aggreccan continued, and by day 11 of culture period, approached the level of loss similar to that for tissue stimulated with retinoic acid, but not exposed to glucosamine. When the tissue was cultured in DMEM in the presence or absence of 5 mM glucosamine for 5 days, similar rates of loss of radiolabeled aggrecan were observed for the two culture conditions [Fig. 4(A)]. On removal of glucosamine and exposure of these cultures to retinoic acid, similar enhanced rates of aggrecan loss were observed from day 6 of culture period onwards. By day 11 of culture period, the levels of loss of radiolabeled aggrecan reached values similar to those observed for control cultures maintained in the presence of retinoic acid for entire culture period. At this point, an inhibition of stimulated aggrecan loss of 26% would be expected to be achieved in a culture maintained in presence of 5 mM glucosamine (Fig. 1). Hence, the stimulation of catabolism of radiolabeled aggrecan was achieved between 24 and 48 h, following exposure of cultures to retinoic acid, and was then maintained throughout the culture period regardless of pretreatment with glucosamine. Taken together, it appears that exposure of cartilage to glucosamine either in the presence or absence of retinoic acid does not provide continuing protection from aggrecan loss from tissue wherein the catabolism of aggrecan is stimulated with retinoic acid.

Similar patterns of aggrecan loss were observed in the experiment with mannosamine. In presence of 5 mM mannosamine, the stimulated loss of radiolabeled aggrecan was inhibited in cultures maintained in the presence of retinoic acid by 54% after 5 days in culture [Fig. 4(B)]. On removal of mannosamine from culture medium, the enhanced loss of radiolabeled aggrecan reached the level of loss similar to that shown for tissue stimulated with retinoic acid in the absence of mannosamine by day 11 of culture period. At this point, an inhibition of stimulated aggrecan loss of 60% would be expected to be achieved and maintained in a culture maintained in presence of 5 mM mannosamine (Fig. 2).

When the tissue was cultured in DMEM in the presence of 5 mM mannosamine, inhibition of basal loss of radiolabeled aggrecan was apparent on days 5 and 6 of culture period [Fig. 4(B)]. Removal of mannosamine and introduction of retinoic acid resulted in an enhanced rate of aggrecan loss. Similar results were observed for cultures that were not treated with mannosamine, but were introduced to retinoic acid at the same time. By day 11 of culture period, the levels of loss of radiolabeled aggrecan reached the values similar to those observed in control cultures maintained in the presence of retinoic acid for entire culture period. Hence, like glucosamine, mannosamine did not provide a long-term suppression of aggrecan loss from cultures wherein aggrecan catabolism was stimulated with retinoic acid.

**Discussion**

This study shows that glucosamine and mannosamine at concentrations of up to 10 mM inhibited aggrecanase-mediated degradation of radiolabeled and chemical aggrecan in dose-dependent manner in explant cultures of bovine articular cartilage stimulated with retinoic acid over a 9-day culture period. Mannosamine was found to have a greater effect on inhibiting aggrecan degradation than glucosamine. This is in agreement with previous studies that used retinoic acid and cytokines IL-1 and TNF-α to promote catabolism in cartilage and aggrecanase cultures in presence of these amino sugars. These researchers reported above 80% of inhibition of aggrecan degradation by retinoic acid-stimulated bovine articular cartilage explants at lower mannosamine concentrations, at 1 and 1.5 mM, and at 5 mM glucosamine. The difference with the present study may be due to the variation in culture protocols. Calf cartilage was used in one of the studies, and in another study, glucose was absent from the culture medium.

It was important to determine that the effects of glucosamine and mannosamine on the catabolism of aggrecan over a 9-day culture period was not due to the impaired metabolic activity of the chondrocytes. It has been suggested that cellular energy levels may be compromised due to reduced glucose uptake in presence of glucosamine and mannosamine, since they are internalized by a common transport system. Furthermore, ATP levels could be depleted as the result of enhanced phosphorylation of glucosamine and mannosamine and reduced regeneration of ATP from oxidation of the glucose. However, we observed that concentrations of up to 10 mM glucosamine or mannosamine did not have an adverse effect on the metabolism of chondrocytes, as measured by lactate production even after 9 days of exposure to the respective amino sugar. It is surprising that mannosamine at concentrations of up to 10 mM did not have a major effect on the cell metabolism, since Sandy et al. report a significant inhibition of protein synthesis (40%) in IL-1β-stimulated rat chondrocytes cultures maintained in presence of 5 mM mannosamine. In addition, Bryson et al. reported a significant suppression of protein synthesis in bovine nasal cartilage maintained in presence of 1 mM mannosamine; however, in IL-1α-stimulated cultures, the presence of 1 mM mannosamine did not have an effect. It is possible that the disparity between this work and others was due to differences between cell and explant cultures and between articular and nasal cartilage cultures. Therefore, the inhibition of aggrecan degradation by glucosamine and mannosamine observed in this study was unlikely to be the outcome of decreased metabolic activity of articular cartilage. This agrees with a recent study wherein chondrocyte viability was shown not to be compromised by exposure of cartilage to concentrations of glucosamine of up to 11 mM (2.5 mg/ml) for 24 h. However, when incubated with concentrations of glucosamine of 30 mM (6.5 mg/ml) and above, significant changes in cell structure and reduced cell viability were observed.

This work shows that glucosamine or mannosamine did not directly inhibit aggrecanase activity, since the presence of these amino sugars did not inhibit soluble aggrecanase activity (Fig. 3). In addition, it appeared that mannosamine was able to inhibit the rate of aggrecan degradation in cultures maintained in the absence of retinoic acid (Fig. 4). The loss of aggrecan in non-stimulated cultures is likely to be due to both the passive loss of aggrecan and aggrecanase activity, since the amino-terminal amino acid sequence analysis of aggrecan metabolites in cartilage cultures maintained in DMEM in the absence of retinoic acid indicates that the proteolytic cleavage of aggrecan core protein occurs specifically at aggrecanase sites (data not shown and reference 34). The results in this study indicate that glucosamine and mannosamine are capable of modulating the regulation of aggrecanase activity under condition of catabolic stimulation. In recent studies, ‘aggrecanase activity’ has been attributed to an increasing number of aggrecanase proteinases; and so far,
ADAMTS-1, ADAMTS-4 and ADAMTS-5 have been detected in cartilage. There is ongoing work investigating which ADAMTS proteinases are constitutively expressed and/or induced in cartilage. It is possible that the amino sugars are mediators of these processes, or they may also play a role in the activation of the latent aggrecanase proteinases and their distribution within the extracellular matrix. Furthermore, this ability of amino sugars to modulate the regulation of aggrecanase activity under conditions of catabolic stimulation is reversible. This is shown in Fig. 4, wherein the removal of glucosamine and mannosamine from retinoic acid-stimulated cultures results in an immediate loss of suppression of aggrecanase activity in metabolically active cultures.

The diverse metabolic pathways mediated by amino sugars have been proposed to have an impact on the generation of aggrecanase activity. Since mannansamine, in particular, and also glucosamine have been shown to inhibit glycosylphosphatidylinositol (GPI) anchor formation, it has been suggested that the regulation of aggrecanase activity by these amino sugars is exerted at a posttranslational level, since aggrecanase proteinases do not belong to GPI family of proteins. A direct involvement of GPI-anchored proteins in posttranslational processing of aggrecanase protein(s) is yet to be confirmed. The impediment of lysosomal function, necessary for the posttranslational activation of aggrecanases, due to depletion of ATP levels has also been proposed as a possible cause of suppression of aggrecanase activity by amino sugars. The levels of lactate production observed in this study may provide argument against this suggestion.

A possible role of amino sugars and their biosynthetic derivatives in signaling pathways and consequent gene regulation that may affect the expression, secretion and/or activation of aggrecanases is yet to be explored. There is emerging evidence that a biosynthetic derivative of glucosamine, N-acetylglucosamine, has a prominent role in the regulation of signaling pathways wherein many intracellular proteins, such as nuclear factors, nuclear pore proteins, kinases and cytoskeletal macromolecules are dynamically modified with the addition of O-linked N-acetylglucosamine. The amino sugars have been shown to modulate the expression of several genes in a number of cell types. Such modulation of proteins involved in signaling pathways may impact the catabolic processes. In chondrocytes, glucosamine has been reported to suppress MMP-3 expression in presence of IL-1.

In conclusion, continuous exposure to 5–10 mM glucosamine and 2–10 mM mannosamine were required for suppression of aggrecanase catabolism of aggrecan in long-term cultures of articular cartilage stimulated with retinoic acid. At these concentrations of amino sugars, the metabolism of chondrocytes was not compromised. This effect was reversible, since the removal of the amino sugars from the culture medium led to the increased rate of loss of aggrecan from cartilage. This study suggests that the long-term protection of cartilage to stimulated aggrecan loss in osteoarthritis relies on continual presence of glucosamine in plasma, and this is supported by clinical studies suggesting that the long-term treatment with glucosamine produces beneficial results in the treatment of osteoarthritis. In these studies, glucosamine was administered orally at a daily dose of 1.5 g. Glucosamine is rapidly utilized in the biosynthetic processes in body, but little is known about its bioavailability or pharmacokinetics. In man, it has been shown that there is a rapid absorption of glucosamine close to 90% of the oral dose and has a bioavailability of 26%. Furthermore, the studies in dogs indicated that glucosamine accumulates in tissues that includes cartilage. Based on the current literature, the long-term daily administration of 1.5 g glucosamine in humans is likely to maintain levels of glucosamine in cartilage comparable with the lowest concentrations of the amino sugar used in this study.

References
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