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Effects of glucosamine on proteoglycan loss by tendon, ligament and joint capsule explant cultures

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Summary

Objective: To investigate the effect of glucosamine on the loss of newly synthesized radiolabeled large and small proteoglycans by bovine tendon, ligament and joint capsule.

Design: The kinetics of loss of ^{35}S-labeled large and small proteoglycans from explant cultures of tendon, ligament and joint capsule treated with 10 mM glucosamine was investigated over a 10-day culture period. The kinetics of loss of ^{35}S-labeled small proteoglycans and the formation of free [^{35}S]sulfate were determined for the last 10 days of a 15-day culture period. The proteoglycan core proteins were analyzed by gel electrophoresis followed by fluorography. The metabolism of tendon, ligament and joint capsule explants exposed to 10 mM glucosamine was evaluated by incorporation of [^{3}H]serine and [^{35}S]sulfate into protein and glycosaminoglycans, respectively.

Results: Glucosamine at 10 mM stimulated the loss of small proteoglycans from ligament explant cultures. This was due to the increased loss of both macromolecular and free [^{35}S]sulfate to the medium indicating that glucosamine affected the release of small proteoglycans as well as their intracellular degradation. The degradation pattern of small proteoglycans in ligament was not affected by glucosamine. In contrast, glucosamine did not have an effect on the loss of large or small proteoglycans from tendon and joint capsule or large proteoglycans from ligament explant cultures. The metabolism of cells in tendon, ligament and joint capsule was not impaired by the presence of 10 mM glucosamine.

Conclusions: Glucosamine stimulated the loss of small proteoglycans from ligament but did not have an effect on small proteoglycan catabolism in joint capsule and tendon or large proteoglycan catabolism in ligament, tendon or synovial capsule. The consequences of glucosamine therapy at clinically relevant concentrations on proteoglycan catabolism in joint fibrous connective tissues need to be further assessed in an animal model.

Key words: Glucosamine, Proteoglycans, Tendon, Ligament, Joint capsule.

Introduction

Fibrous connective tissues of the joint, namely joint capsule, ligaments and tendons together allow for the articulation of the synovial joint. Both joint capsule and ligaments stabilize the joint and tendons transmit the force from the muscle to the bone. Proteoglycans make up less than 3% of fibrous connective tissues of the joint[1] nevertheless these macromolecules contribute to the structural integrity of these tissues. The major proteoglycan in tensile regions of fibrous connective tissues is decorin at $\geq$80% of total proteoglycans present in the tissue. The remainder includes the large aggregating proteoglycans, versican and aggrecan, and other small leucine-rich proteoglycans including biglycan[1–6]. The small leucine-rich proteoglycans are involved in the organization of fibrillar collagen networks and the large aggregating proteoglycans are believed to be involved in the hydration of the tissue and separation of the collagen fibers. Type I collagen is the major component of fibrous connective tissues of the joint[1,3,7]. Furthermore complex interactions between large and small proteoglycans and other collagenous and non-collagenous extracellular matrix components have been documented in fibrous connective tissues *in vivo* and *in vitro*[8,9].

Glucosamine is used as a chondroprotective agent in osteoarthritis to alleviate symptoms and disease progression. There is, however, a controversy about the effectiveness of glucosamine[10] since some long term randomized clinical trials of glucosamine treatment of patients with osteoarthritis reported a reduction in joint space narrowing, osteophyte score and pain which lead to a delay in joint replacement[10–12] and in other trials its effectiveness on joint pain, function and stiffness was reported to be less convincing[13,14]. Some of these discrepancies have been attributed to glucosamine preparations, dosages, trial designs and sponsor bias[10,13]. Glucosamine has been postulated to affect a number of anabolic and catabolic pathways in cartilage including the suppression of matrix degrading enzymes, matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS-4 and -5)[15,16]. Aggrecanases have been implicated in the degradation of aggrecan in cartilage and fibrous connective tissues[2,4,5,17–19]. In fibrous connective tissues aggrecanases and MMPs have been implicated in degradation of versican and may also play a role in degradation of small leucine-rich proteoglycans[2,4,5,20–24].

Previous studies have shown that glucosamine inhibited stimulated aggrecan degradation and loss in explant cultures of cartilage[25–28]. The mechanism by which glucosamine

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inhibits the stimulation of aggrecanase activity in cartilage is not known, however, it has been reported to affect aggrecanase gene expression and activation[15,25,28]. Given that glucosamine is used widely in treatment of osteoarthritis since there is evidence to suggest that it has a potential to inhibit proteoglycan catabolism in cartilage, its effects on joint tissues other than cartilage have not been studied. The proteoglycans contribute to the functional properties of these tissues and as in cartilage their metabolism is finely balanced. A perturbation of this balance as a result of treatment for diseased cartilage may have an adverse impact on the health of joint fibrous connective tissues. At present there are no reports on the effect of glucosamine on the catabolism and loss of proteoglycans in tendon, ligament and joint capsule. This study investigates the effect of glucosamine on the rates of loss of newly synthesized ^{35}S-labeled large aggregating and small leucine-rich proteoglycans from tendon, ligament and joint capsule explant cultures and the metabolism of these tissues.

Materials and methods

Dulbecco's modified Eagles medium (DMEM) containing 1 g/l (5.5 mM) glucose, new born calf serum (NBCS), non-essential amino acids, penicillin and streptomycin were purchased from CSL (Melbourne, Australia). Chondroitinase ABC (protease free from Proteus vulgaris; EC 4.2.2.4) was 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). Glucosamine and keratanase (from *Pseudomonas* sp.; EC 4.2.2.4) were from Sigma Chemical Co. (St. Louis, MO, USA). Amicon® Ultra-4 centrifugal filter devices with molecular weight cut off 10,000 were from Millipore (Belford, MA, USA) and PD-10 columns were from Amersham Biosciences (Sweden).

EXPLANT CULTURES OF BOVINE TENDON, LIGAMENT AND JOINT CAPSULE

Bovine deep flexor tendon proximal to bifurcation (tensile region), collateral ligament and joint capsule were dissected from a single metacarpophalangeal joint of a 1–3-year-old bovine. Tensile region of tendon was used in this study since a change in the metabolism of proteoglycans has been suggested to contribute to the altered composition of the extracellular matrix of diseased tendon[29,30]. The synovial membrane and fibrocartilagenous regions were removed from macroscopically normal tissue[3,31]. Tissue pieces were incubated in sulfate-free medium containing 200 μCi/ml of [^{35}S]sulfate for 6 h at 37°C[32]. The tissue was washed with DMEM to remove unincorporated [^{35}S]sulfate, then triplicate cultures containing 100 ± 20 mg wet weight tissue/4 ml medium were maintained in DMEM with or without 10 mM glucosamine for 10 days with daily change of medium. In this work 5.5 mM glucose was present in the culture medium[26,33]. The spent medium was collected and stored in the presence of proteinase inhibitors at −22°C[34]. At the end of the experiment the tissue was extracted with 4 ml 4 M guanidine hydrochloride buffered at pH 5.8 in the presence of proteinase inhibitors for 48 h at 4°C, followed by 4 ml 0.5 M NaOH for 24 h. For the measurements of turnover of large and small proteoglycans (see below), additional tissue cultures were maintained for 0, 2, 4, 6, 8 and 10 days. In a separate experiment, freshly dissected tendon, ligament and joint capsule were incubated with radiolabeled sulfate as described above, and triplicate cultures of 100 ± 20 mg wet weight tissue/4 ml medium were maintained for 5 days in DMEM and then continued in presence or absence of 10 mM glucosamine for another 10 days. The above experiments were performed at least three times with tissue from different animals.

MEASUREMENT OF TURNOVER OF ^{35}S-LABELED PROTEOGLYCANS

Measurement of turnover of total ^{35}S-labeled proteoglycans

The radiolabeled macromolecules present in medium samples and in tissue extracts were isolated on Sephadex G-25 columns (PD-10 columns) and analyzed for radioactivity[32,35]. The percentage ^{35}S-labeled proteoglycans 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[36].

Measurement of turnover of large and small ^{35}S-labeled proteoglycans

The rate of loss of large and small proteoglycan species from tissue with time in culture was determined from the total ^{35}S-radiolabeled macromolecular content of tissue at the time of sampling (days 0, 2, 6, 8 and 10) and the percentages of large or small proteoglycans present in the matrix at those times, determined from gel chromatography of 4 M guanidinium chloride matrix extracts on the column of Sepharose CL-4B. It was expressed as a percentage of each species remaining in the matrix with time in culture relative to that at the commencement of the treatment with glucosamine at day 0[31,32,35].

Measurement of the rate of formation of [^{35}S]sulfate and release of ^{35}S-labeled small proteoglycans

Explant cultures were maintained as triplicate samples in DMEM alone for 5 days to allow time for the loss of ^{35}S-labeled large proteoglycans and unincorporated [^{35}S]sulfate from the tissue prior to treatment with 10 mM glucosamine for further 10 days[31,32,35].The radiolabeled small proteoglycans present in medium samples on each day of treatment and in tissue extracts were isolated on Sephadex G-25 columns (PD-10 columns) and analyzed for radioactivity[32,35]. The data was plotted as cumulative release of ^{35}S-labeled small proteoglycans into culture medium and percentage ^{35}S-labeled small proteoglycans remaining in the matrix against treatment period. In addition the amount of ^{35}S radioactivity which eluted in the total volume on Sephadex G-25 was determined for medium samples for each day of treatment. This data was plotted as cumulative release of free [^{35}S]sulfate into medium during treatment period.

MEASUREMENT OF PROTEIN AND PROTEOGLYCAN SYNTHESIS

Tendon, ligament and joint capsule, dissected from a single metacarpophalangeal joint, were maintained in triplicate cultures (100 mg wet weight tissue/4 ml medium) in presence or absence of 10 mM glucosamine for 7 days with daily change of medium. On day 7 of culture, the triplicate cultures from each treatment were pre-incubated with 2 ml DMEM at 37°C for 1 h. The medium was then replaced with 2 ml DMEM containing 20 μCi [^{35}S]sulfate/ml or 30 μCi [^{3}H]serine/ml that was prepared as batch solution. 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 guanidinium 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 PD-10 columns[32,35]. 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.

ANALYSIS OF PROTEOGLYCAN CORE PROTEINS

Proteoglycans were isolated from pooled spent medium (days 0–5 and 6–10) and tissue (day 5) from ligament, tendon and joint capsule cultures that were maintained in DMEM only for 5 days and then continued with or without 10 mM glucosamine for further 5 days, by ion-exchange chromatography as described previously[37]. Purified samples were concentrated and exchanged into H_2O containing proteinase inhibitors using Amicon® Ultra-4 centrifugal filter devices with molecular weight cut off 10,000 as described by the manufacturer, lyophilized and re-constituted in 0.1 M Tris/0.1 M sodium acetate, pH 7 and digested with chondroitinase ABC (0.025 U) and keratanase (0.05 U) at 37°C for 12 h in the presence of proteinase inhibitors[34]. Digested samples were exchanged into H_2O containing proteinase inhibitors using the filter devices and lyophilized. Samples were subjected to electrophoresis on 4–15% gradient polyacrylamide/SDS slab gels. The gels were fixed and soaked in Amplify (Amersham Pharmacia, Buckinghamshire, UK) for 20 min, dried and exposed to Kodak BioMax Light film at −80°C for 6–8 weeks.

TREATMENT OF DATA

Due to the variation in the absolute metabolic measurements in joint connective tissues from different animals any one experiment was restricted to tissue obtained from a single joint[38]. Triplicate samples of tissue from a single joint were analyzed and the mean ± SD was determined. Statistical significance was determined by Student's *t* test. All experiments were repeated at least three times (kinetics of loss of radiolabeled proteoglycans) or twice (protein and proteoglycans synthesis) with tissue from different animals and showed the same outcome.

Results

GLUCOSAMINE AFFECTED THE LOSS OF PROTEOGLYCANS FROM LIGAMENT BUT NOT FROM TENDON OR JOINT CAPSULE EXPLANT CULTURES

Figure 1 shows the percentage of radiolabeled proteoglycans remaining in the matrix of tendon (A), ligament (C) and joint capsule (E) over the 10-day culture period cultured with or without 10 mM glucosamine. Glucosamine did not affect the proteoglycan loss from the matrix of tendon and joint capsule explants where approximately 35 and 60% of radiolabeled proteoglycans were, respectively, lost from the tissue after 10 days in culture with or without glucosamine. However, glucosamine stimulated the loss of radiolabeled proteoglycans from ligament explants from 41% in control cultures to 60% by day 10 of culture.

The effect of glucosamine on the loss of large and small radiolabeled proteoglycans is shown in Fig. 1 for tendon (B), ligament (D) and joint capsule (F). Over 90% of radiolabeled large proteoglycans was lost from the matrix of all three tissues by day 10 of culture and this loss was not affected by the presence of glucosamine. In ligament cultures glucosamine stimulated the loss of small proteoglycans from 32% in control cultures to 52% by day 10 of culture [Fig. 1(D)]. In contrast glucosamine did not affect the loss of small proteoglycans from tendon and joint capsule explants showing approximately 27% and 51% loss of small proteoglycans from the matrix, respectively, by day 10 of culture [Fig. 1(B and F)].

GLUCOSAMINE STIMULATED INTRACELLULAR DEGRADATION AND THE LOSS OF SMALL PROTEOGLYCANS FROM LIGAMENT

It has been shown previously that two metabolic pathways are responsible for the loss of small proteoglycans from the matrix of fibrous connective tissues. One involves the cellular uptake and intracellular degradation of small proteoglycans that results in the release of free sulfate to the medium of explant cultures and the second one involves

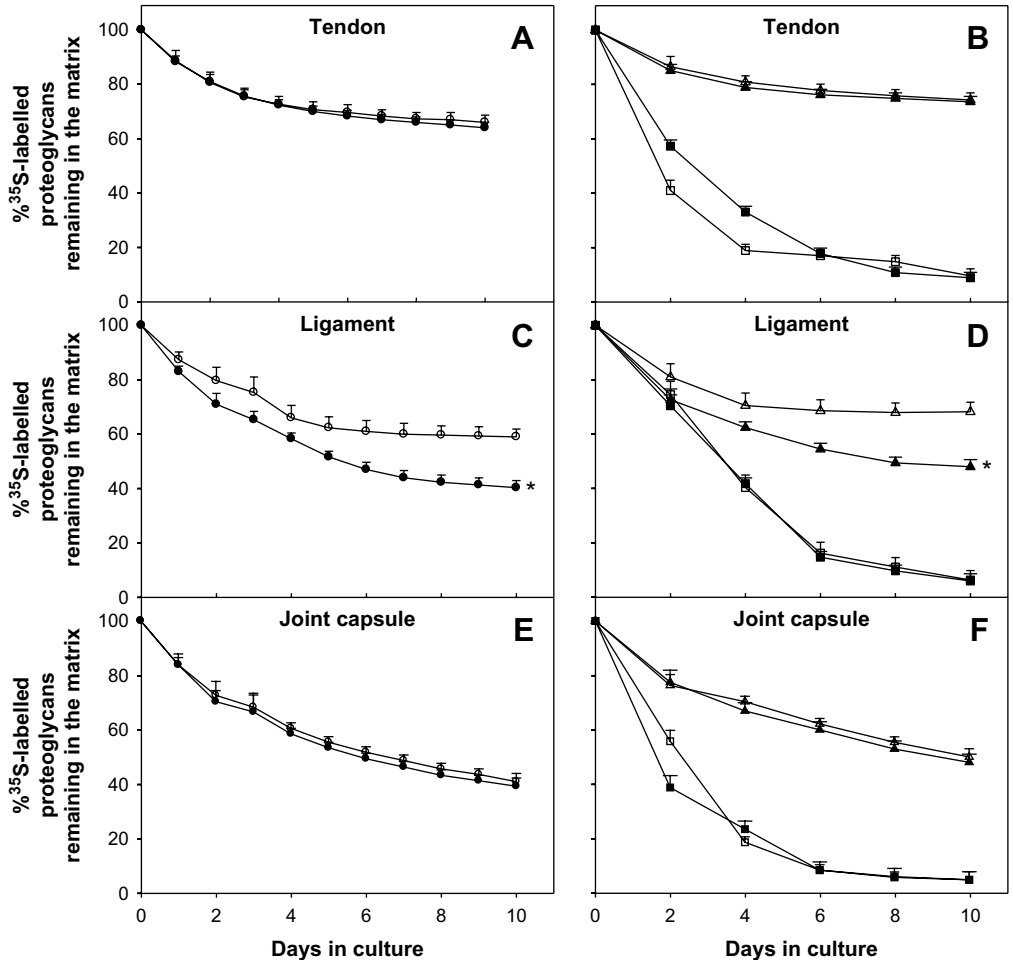

Fig. 1. Glucosamine affects the kinetics of loss of ^{35}S-labeled proteoglycans from the matrix of bovine ligament but not tendon and joint capsule explant cultures. Following the incubation of tissue with [^{35}S]sulfate, the percentage of radiolabeled proteoglycans remaining in the matrix of tendon, ligament or joint capsule maintained in culture for 10 days in the presence or absence of 10 mM glucosamine is plotted against time in culture; (A, C, E) percentage of total (large + small) radiolabeled proteoglycans remaining in the matrix of cultures cultured without (○), or with 10 mM glucosamine (●); (B, D, F) percentage of radiolabeled large and small proteoglycans remaining in the matrix of cultures cultured without (large proteoglycans, □, small proteoglycans, △), or with 10 mM glucosamine (large proteoglycans, ■, small proteoglycans, ▲). Values shown represent the mean of triplicate cultures of tissue from same animal and the SD (positive error bars). *$P < 0.05$ vs DMEM alone by Student's *t* test.

a release of small proteoglycans and their fragments into the medium of explant cultures[32,35,39].

The effect of glucosamine on these two processes is shown in Figs. 2–4 for tendon, ligament and joint capsule explants, respectively, showing cumulative release of free [^{35}S]sulfate into the medium (A), cumulative release of ^{35}S-labeled small proteoglycans into medium (B) and the percentage of ^{35}S-labeled proteoglycans remaining in the matrix (C) with time in culture. In this experiment tissue

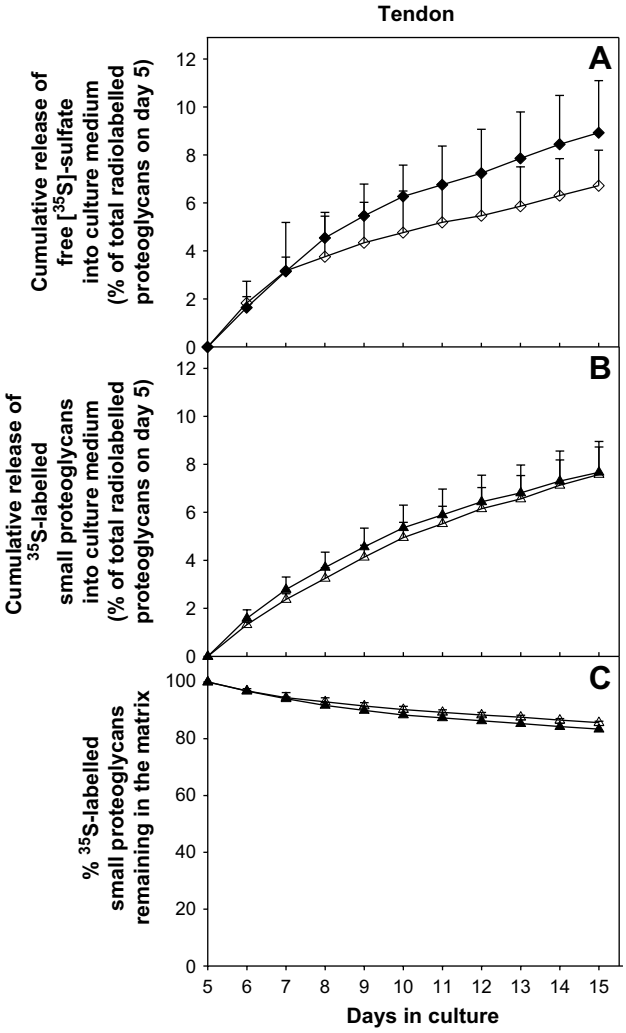

Fig. 2. Glucosamine does not have an effect of the rate of formation of [^{35}S]sulfate and the release of ^{35}S-labeled small proteoglycans from bovine tendon explant cultures. Following the incubation of tissue with [^{35}S]sulfate, tendon was maintained in DMEM for 5 days to allow for the loss of majority of radiolabeled large proteoglycans prior to treatment with 10 mM glucosamine for further 10 days. The culture medium was collected daily and analyzed for the presence of [^{35}S]sulfate and ^{35}S-labeled small proteoglycans; (A) cumulative release of [^{35}S]sulfate into the culture medium for cultures maintained without (◇) or with glucosamine (♦); (B) cumulative release of ^{35}S-labeled small proteoglycans into the culture medium for cultures maintained without (△) or with glucosamine (▲); (C) the percentage of ^{35}S-labeled small proteoglycans remaining in the matrix with time in culture for cultures maintained without (△) or with glucosamine (▲). Values shown represent the mean of triplicate cultures of tissue from same animal and the SD (positive error bars).

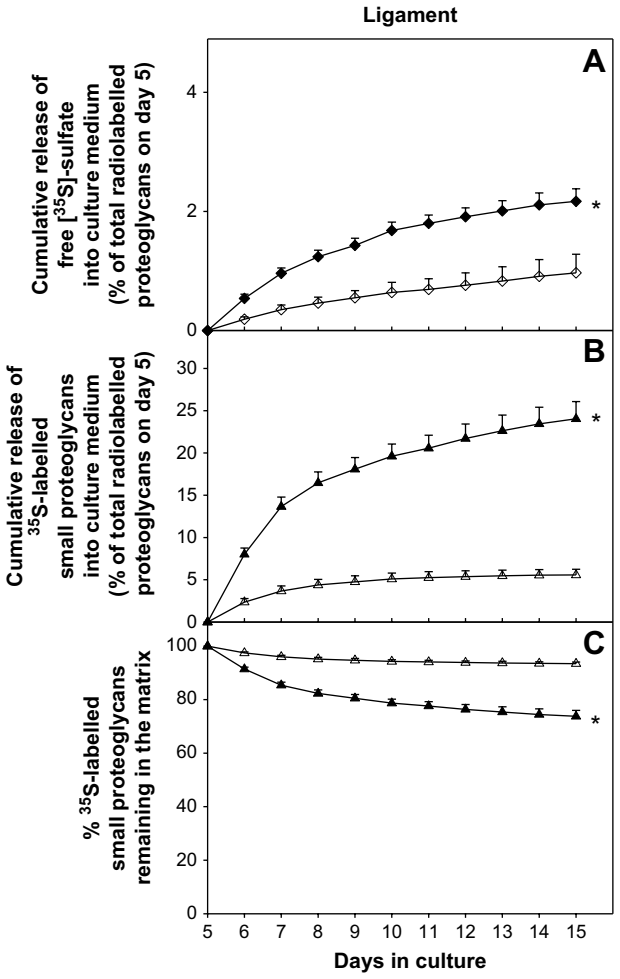

Fig. 3. Glucosamine affects the rate of formation of [^{35}S]sulfate and the release of ^{35}S-labeled small proteoglycans from bovine ligament explant cultures. Following the incubation of tissue with [^{35}S]sulfate, ligament was maintained in DMEM for 5 days to allow for the loss of majority of radiolabeled large proteoglycans prior to treatment with 10 mM glucosamine for further 10 days. The culture medium was collected daily and analyzed for the presence of [^{35}S]sulfate and ^{35}S-labeled small proteoglycans; (A) cumulative release of [^{35}S]sulfate into the culture medium for cultures maintained without (◇) or with glucosamine (♦); (B) cumulative release of ^{35}S-labeled small proteoglycans into the culture medium for cultures maintained without (△) or with glucosamine (▲); (C) the percentage of ^{35}S-labeled small proteoglycans remaining in the matrix with time in culture for cultures maintained without (△) or with glucosamine (▲). Values shown represent the mean of triplicate cultures of tissue from same animal and the SD (positive error bars). *$P < 0.05$ vs DMEM alone by Student's t test.

was maintained in culture for 5 days after incubation with [^{35}S]sulfate to allow for the loss of the majority of radiolabeled large proteoglycans [Fig. 1(B–F)] before the collection and analysis of samples in additional 10-day culture period.

Glucosamine stimulated both pathways of loss of small proteoglycans from ligament with over 100% increase in the release of free [^{35}S]sulfate and over 400% increase in the release of ^{35}S-labeled small proteoglycans into medium [Fig. 3 (A and B)]. The majority (~92%) of total ^{35}S-sulfated molecules released into the medium of cultures in presence of glucosamine were the radiolabeled macromolecules.

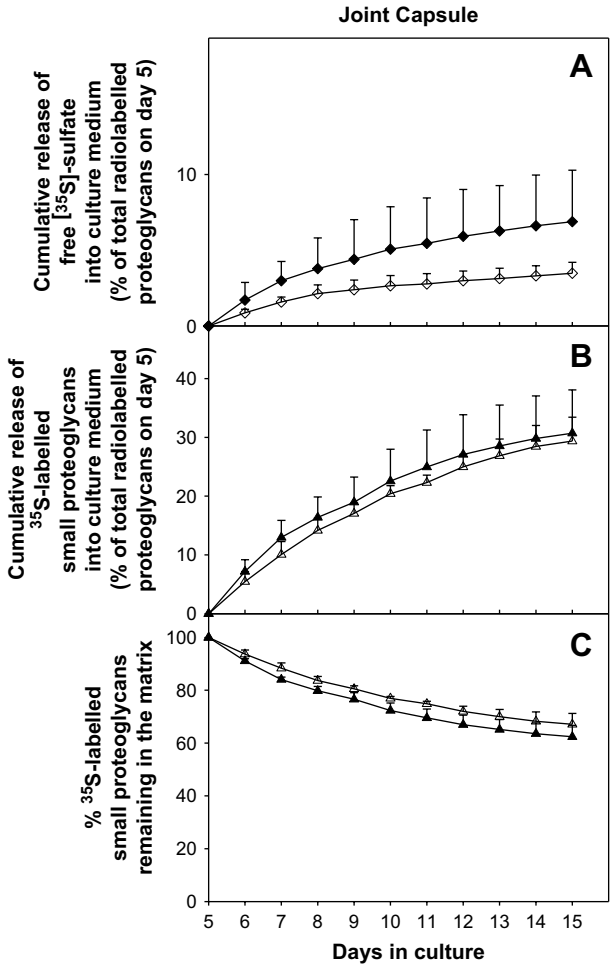

Fig. 4. Glucosamine does not have an effect on the rate of formation of [^{35}S]sulfate and the release of ^{35}S-labeled small proteoglycans from bovine joint capsule explant cultures. Following the incubation of tissue with [^{35}S]sulfate, joint capsule was maintained in DMEM for 5 days to allow for the loss of majority of radiolabeled large proteoglycans prior to treatment with 10 mM glucosamine for further 10 days. The culture medium was collected daily and analyzed for the presence of [^{35}S]sulfate and ^{35}S-labeled small proteoglycans; (A) cumulative release of [^{35}S]sulfate into the culture medium for cultures maintained without (◇) or with glucosamine (♦); (B) cumulative release of ^{35}S-labeled small proteoglycans into the culture medium for cultures maintained without (△) or with glucosamine (▲); (C) the percentage of ^{35}S-labeled small proteoglycans remaining in the matrix with time in culture for cultures maintained without (△) or with glucosamine (▲). Values shown represent the mean of triplicate cultures of tissue from same animal and the SD (positive error bars).

Glucosamine did not affect either metabolic pathway of loss of small proteoglycans in tendon and joint capsule cultures (Figs. 2 and 4).

GLUCOSAMINE DID NOT HAVE AN EFFECT ON THE PROTEOLYTIC PROCESSING OF SMALL PROTEOGLYCANS

The major small proteoglycan present in tendon, ligament and synovial capsule is decorin shown as the major radiolabeled band of ~43 kDa (arrow) present in matrix and medium of explants[1–3,5]. The same degradation pattern of radiolabeled decorin isolated from the matrix of all three

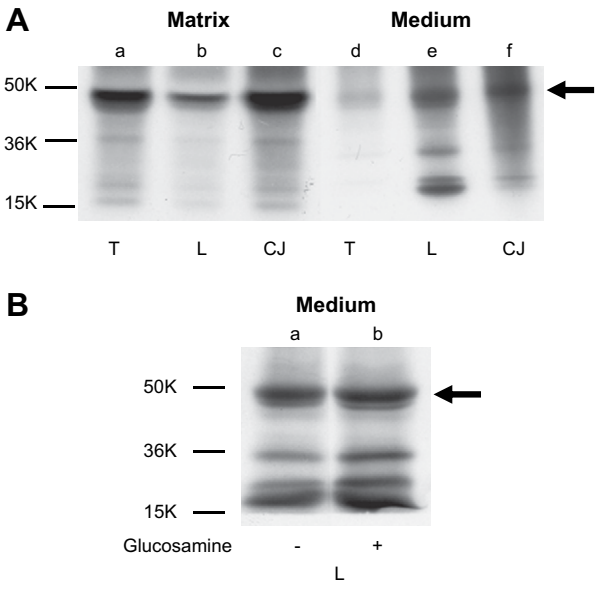

Fig. 5. Glucosamine does not have an effect on the proteolytic processing of ^{35}S-labeled small proteoglycans in ligament, tendon and joint capsule explant cultures. Following the incubation of tissue with [^{35}S]sulfate, ligament, tendon and joint capsule were maintained in DMEM for 5 days to allow for the loss of majority of radiolabeled large proteoglycans prior to treatment with 10 mM glucosamine for further 5 days. A fluorogram of 4–15% polyacrylamide/SDS gels shows radiolabeled small proteoglycan core proteins that were isolated from the matrix of tendon (T), ligament (L) and joint capsule (JC) on day 5 of culture period (a–c) and from the pooled medium from days 6 to 10 of culture period. Lane a: ligament cultured in DMEM only; lanes b, c and d: ligament, tendon and synovial capsule, respectively, cultured in the presence of glucosamine. The weights of tissue (g) corresponding to the amount of sample loaded on the gel were 0.2 g (panel A) and 0.4 g (panel B).

tissues [Fig. 5(A), a–c] after 5 days in culture in DMEM only suggests that the cleavage sites within decorin core protein are identical in these tissues. The analysis of radiolabeled decorin released to the culture medium in the first 5 days of culture, however, shows a higher proportion of decorin fragments relative to intact decori released into the medium of ligament than of tendon and joint capsule cultures [Fig. 5(A), d–f]. The smaller molecular weight bands between 15 and 36 kDa have previously been shown to be decorin fragments that retained decorin amino-terminus in ligament and tendon and resulted from cleavages within the leucine-rich repeats of the core protein[2,5]. The addition of glucosamine did not affect decorin degradation pattern in ligament [Fig. 5(B), a, b] or in tendon or joint capsule (data not shown).

GLUCOSAMINE DID NOT HAVE AN EFFECT ON CELL METABOLISM

The effect of glucosamine on protein and proteoglycan synthesis was investigated in order to determine whether 10 mM glucosamine affected the general metabolism of tendon, ligament and joint capsule.

Table I shows that the exposure to glucosamine for 7 days did not have a significant effect on proteoglycan or protein synthesis in tendon, ligament or joint capsule cultures where only a small reduction (2–10%) in [^{35}S]sulfate and [^{3}H]serine incorporation into macromolecules was observed. Thus 10 mM glucosamine did not impair the

Table I
Incorporation of [^{35}S]sulphate and [^{3}H]serine into macromolecules by bovine tendon, ligament and joint capsule explant cultures maintained in DMEM in the absence or presence of glucosamine

Culture condition	[^{35}S]Sulphate incorporation (cpm/100 mg wet weight of tissue/2 h)	[^{35}H]Serine incorporation (cpm/100 mg wet weight of tissue/2 h)
	Day 7	Day 7
Tendon		
DMEM alone	62,947 ± 5043*	143,449 ± 7240*
DMEM + 10 mM glucosamine	56,956 ± 5251 (90)	136,170 ± 7320 (95)
Ligament		
DMEM alone	70,896 ± 6125*	250,456 ± 9889*
DMEM + 10 mM glucosamine	69,814 ± 4450 (98)	225,880 ± 54181 (90)
Joint capsule		
DMEM alone	61,459 ± 5513*	281,874 ± 9497*
DMEM + 10 mM glucosamine	58,649 ± 1055 (95)	274,867 ± 3156 (98)

Values shown represent the mean of triplicate cultures of tissue from same animal and the SD. Values in parentheses are expressed as a percentage of values observed for cultures maintained in DMEM alone.

*Not significant vs DMEM alone by Student's *t* test.

metabolic activity of fibroblasts in joint fibrous tissues and this is in line with results reported for cartilage[26].

Discussion

This work shows that unlike in cartilage where glucosamine acts on the pathways responsible for generating stimulated aggrecanase activity at either transcriptional and/or activation levels, glucosamine at 10 mM, did not affect the loss of large aggregating proteoglycans in explant cultures of ligament, tendon or synovial capsule [Fig. 1(B,D,F)][25–28]. In addition there was no effect on the proteolytic processing of large proteoglycans (data not shown). ADAMTS-4 and-5 are expressed in joint fibrous connective tissues and one or both of these proteinases may be responsible for the degradation of large aggregating proteoglycans in these tissues[2,4,18,20,40–43]. The lack of effect of glucosamine treatment in joint fibrous connective tissue explants is likely to be due to the difference in the regulation of aggrecanase activity in these tissues compared to cartilage. This difference in the regulation of aggrecanase activity is indicated by: (1) the rates of loss of radiolabeled large proteoglycans from tendon, ligament and joint capsule in explant cultures cultured in the absence of cytokines are higher ($t_{1/2} \sim 1.5$ days[31,32,35]) than those reported for cartilage explants ($t_{1/2} > 8$ days[37,44]), (2) under the same conditions, the high rates of loss of radiolabeled and total large proteoglycans are associated with extensive proteolysis of the core proteins unlike in cartilage where the extensive proteolysis of the core protein occurs in the presence of cytokines such as interleukin-1, tumour necrosis factor α or retinoic acid[2,4,5,31,32,37,45], and (3) the catabolic stimulators do not appear to affect large proteoglycan degradation and loss from explants of joint fibrous connective tissues[2,4,20].

Furthermore a higher proportion of degraded large aggregating proteoglycans is observed *in vivo* in normal joint fibrous connective tissues then in cartilage from animals of similar age[2,4,5,18,41,46]. This may be a result of relatively higher large proteoglycan degrading activity in joint fibrous connective tissues compared to cartilage and, unlike cartilage these tissues retain large proteoglycan fragments that lack the hyaluronan binding G1 domain. Aggrecan degradation and loss in normal human cartilage is low since $t_{1/2}$ for aggrecan has been estimated to be in excess of 3 years and that for G1 domain 25 years[47]. The large proteoglycans turnover rates *in vivo* in joint fibrous connective tissues are not known, however, proteolytic processing of large proteoglycans in these tissues appear to be important for their normal function and, therefore, any treatment that aims to inhibit aggrecanase activity in diseased cartilage may have an adverse effect in joint fibrous connective tissues where the studies of tendon pathology indicate that large aggregating proteoglycans accumulate in the extracellular matrix of diseased tissue[29,30,48]. In addition, studies have shown that the inhibition of matrix degrading enzymes caused joint stiffness and promoted changes in the composition of joint fibrous connective tissues[49,50]. In this context, this study suggests that the benefit of the use of glucosamine for treatment of osteoarthritis is that it is not likely to affect the catabolism and loss of large aggregating proteoglycans from fibrous connective tissues of the synovial joint.

Decorin is the major proteoglycan present in the extracellular matrix of joint fibrous connective tissues and represents $\geq$80% of newly synthesized ^{35}S-labeled macromolecules in these tissues[3,31,32]. Unlike large aggregating proteoglycans, radiolabeled decorin was lost from the matrix of explants of joint fibrous connective tissues at a much slower rate, $t_{1/2} \geq 10$ days [Fig. 1(B,D,F)]. Similar rates of decorin loss have been previously reported for joint fibrous connective tissues and cartilage[31,32,35,51]. In this study the comparison of radiolabeled decorin appearing in the medium of tendon, ligament and joint capsule showed that mainly intact decorin was lost in tendon and synovial capsule explant cultures while significant levels of decorin fragments were present in the medium of ligament explants. Since previous studies have reported that in tendon and ligament explant cultures over 50% of total decorin lost to the medium was fragmented it appears that in ligament but not in tendon explant cultures the newly synthesized radiolabeled decorin follows a similar pattern of release to that of total decorin[2,5]. The treatment with glucosamine did not inhibit degradation of radiolabeled decorin in ligament cultures (Fig. 5) even though glucosamine has the potential to suppress the expression and activation of MMPs including gelatinases which are reported to be the most likely enzymes involved in degradation of decorin[21,23,52,53]. On the contrary glucosamine increased the rate of loss of radiolabeled decorin in ligament cultures [$t_{1/2} = 6$ days, Fig. 1(D)]. It appears that glucosamine has acted at multiple pathways since it affected both mechanisms of loss of radiolabeled decorin in ligament explants, the intracellular degradation and the release of decorin from the matrix. The intracellular degradation pathway involves endocytosis through specific decorin receptors, transfer to- and degradation within- lysosomes[39,54,55]. The enhanced release by glucosamine of decorin into the medium of ligament cultures is likely to involve the disruption of interactions between decorin and other macromolecular components in the extracellular matrix of this tissue. The intermolecular interactions would have been further weakened for radiolabeled decorin fragments that have been generated at a higher rate in ligament than in tendon and joint capsule cultures.

In mature bovine articular cartilage cultures glucosamine concentrations of between 5 and 10 mM were needed to suppress stimulated aggrecan catabolism without affecting cell metabolism[26]. In the work described in this paper using ligament cultures, 5 mM glucosamine did not have a significant effect on proteoglycan loss from the tissue (data not shown). Persiani *et al.* showed in humans with osteoarthritis that a therapeutic dose of glucosamine of 1500 mg/day leads to a significant increase in plasma (range 3.35–22.7 μM) and synovial fluid (range 3.22–18.1 μM) concentrations of this amino sugar from the baseline of 0.25 μM and 0.21 μM, respectively[56]. It should be emphasized that this work did not take into account any glucosamine that was associated with plasma proteins. At present it is not known what glucosamine concentration can be achieved within cartilage or joint fibrous connective tissues *in vivo*. Animal studies using radiolabeled glucosamine have shown that the radioactivity was taken up by articular cartilage[57] and that the radiolabeled glucosamine could originate from two distinct pools. The first was free glucosamine present in plasma that had a fast clearance rate. The second radiolabeled pool of glucosamine appeared in plasma 20 min after the animals were administered glucosamine and the radioactivity was associated with the globulin protein fraction and had a long clearance rate. Indeed, after repeated administration of radiolabeled glucosamine 90% of the total radioactivity present in plasma was associated with the protein fraction[57]. It has been suggested that the radioactivity associated with the globulin protein may represent a possible delivery pathway of glucosamine to cells where it is released to cells by their metabolic activity. However, it must be emphasized that the nature of the radioactivity associated with the globulins has yet to be characterized. The pool of glucosamine associated with globulin proteins present in plasma was not taken into account in the studies of Persiani *et al.*[56]. Furthermore, high levels of glucosamine in joint tissues may be achieved by derivatives of glucosamine, such as cell permeable lipophilic derivatives of glucosamine that are under investigation as potential chondroprotective agents[58].

In conclusion, glucosamine use as a therapeutic agent for cartilage pathology is not likely to affect proteoglycan catabolism in joint fibrous connective tissues, with the exception of ligament where it may up-regulate the loss of decorin from the matrix and thus affect the biomechanical properties of this tissue. In recent paper it has also been reported that glucosamine in conjunction with chondroitin sulfate stimulates collagen synthesis in tendon but more so in ligament cells[59]. Further studies using *in vivo* animal models are needed to confirm these effects.

Conflicts of interest

All authors have no conflicts of interest.

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References

1. Hey NJ, Handley CJ, Ng CK, Oakes BW. Characterization and synthesis of macromolecules by adult collateral ligament. Biochim Biophys Acta 1990;1034:73–80.
2. Ilic MZ, Carter P, Tyndall A, Dudhia J, Handley CJ. Proteoglycans and catabolic products of proteoglycans present in ligament. Biochem J 2005;385:381–8.
3. Kleftogiannis F, Handley CJ, Campbell MA. Characterization of extracellular matrix macromolecules from bovine synovial capsule. J Orthop Res 1994;12:365–74.
4. Rees SG, Flannery CR, Little CB, Hughes CE, Caterson B, Dent CM. Catabolism of aggrecan, decorin and biglycan in tendon. Biochem J 2000;350(Pt 1):181–8.
5. Samiric T, Ilic MZ, Handley CJ. Characterisation of proteoglycans and their catabolic products in tendon and explant cultures of tendon. Matrix Biol 2004;23:127–40.
6. Vogel KG, Heinegard D. Characterization of proteoglycans from adult bovine tendon. J Biol Chem 1985;260:9298–306.
7. Benjamin M, Ralphs JR. Tendons and ligaments – an overview. Histol Histopathol 1997;12:1135–44.
8. Melrose J, Smith SM, Appleyard RC, Little CB. Aggrecan, versican and type VI collagen are components of annular translamellar crossbridges in the intervertebral disc. Eur Spine J 2007.
9. Wiberg C, Klatt AR, Wagener R, Paulsson M, Bateman JF, Heinegard D, *et al.* Complexes of matrilin-1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan. J Biol Chem 2003;278:37698–704.
10. Reginster JY. The efficacy of glucosamine sulfate in osteoarthritis: financial and nonfinancial conflict of interest. Arthritis Rheum 2007;56:2105–10.
11. Bruyere O, Pavelka K, Rovati LC, Gatterova J, Giacovelli G, Olejarova M, *et al.* Total joint replacement after glucosamine sulphate treatment in knee osteoarthritis: results of a mean 8-year observation of patients from two previous 3-year, randomised, placebo-controlled trials. Osteoarthritis Cartilage 2007 [OARS, Osteoarthritis Research Society].
12. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001;357:251–6.
13. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795–808.
14. Towheed TE, Maxwell L, Anastassiades TP, Shea B, Houpt J, Robinson V, *et al.* Glucosamine therapy for treating osteoarthritis. Cochrane Database Syst Rev 2005;CD002946.
15. Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, *et al.* Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage 2006;14:250–7 [OARS, Osteoarthritis Research Society].
16. Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. Glucosamine modulates chondrocyte proliferation, matrix synthesis, gene expression. Osteoarthritis Cartilage 2007;15:59–68 [OARS, Osteoarthritis Research Society].
17. Abbaszade I, Liu RQ, Yang F, Rosenfeld SA, Ross OH, Link JR, *et al.* Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J Biol Chem 1999;274:23443–50.
18. Rees SG, Waggett AD, Dent CM, Caterson B. Inhibition of aggrecan turnover in short-term explant cultures of bovine tendon. Matrix Biol 2007.
19. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, *et al.* Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 1999;284:1664–6.
20. Ilic MZ, Vankemmelbeke MN, Holen I, Buttle DJ, Clem Robinson H, Handley CJ. Bovine joint capsule and fibroblasts derived from joint capsule express aggrecanase activity. Matrix Biol 2000;19:257–65.
21. Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y. Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochem J 1997;322(Pt 3):809–14.
22. Kashiwagi M, Enghild JJ, Gendron C, Hughes C, Caterson B, Itoh Y, *et al.* Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing. J Biol Chem 2004;279:10109–19.
23. Monfort J, Tardif G, Reboul P, Mineau F, Roughley P, Pelletier JP, *et al.* Degradation of small leucine-rich repeat proteoglycans by matrix metalloprotease-13: identification of a new biglycan cleavage site. Arthritis Res Ther 2006;8:R26.
24. Sandy JD, Westling J, Kenagy RD, Iruela-Arispe ML, Verscharen C, Rodriguez-Mazaneque JC, *et al.* Versican V1 proteolysis in human aorta *in vivo* occurs at the Glu441–Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. J Biol Chem 2001;276:13372–8.
25. Gao G, Westling J, Thompson VP, Howell TD, Gottschall PE, Sandy JD. Activation of the proteolytic activity of ADAMTS4 (aggrecanase-1) by C-terminal truncation. J Biol Chem 2002;277:11034–41.
26. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular

cartilage. Osteoarthritis Cartilage 2003;11:613–22 [OARS, Osteoarthritis Research Society].
27. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335(Pt 1):59–66.
28. Sandy JD, Thompson V, Verscharen C, Gamett D. Chondrocyte-mediated catabolism of aggrecan: evidence for a glycosylphosphatidylinositol-linked protein in the aggrecanase response to interleukin-1 or retinoic acid. Arch Biochem Biophys 1999;367:258–64.
29. Riley G. The pathogenesis of tendinopathy. A molecular perspective. Rheumatology 2004;43:131–42.
30. Scott A, Lian O, Roberts CR, Cook JL, Handley CJ, Bahr R, *et al*. Increased versican content is associated with tendinosis pathology in the patellar tendon of athletes with jumper's knee. Scand J Med Sci Sports 2007.
31. Campbell MA, Winter AD, Ilic MZ, Handley CJ. Catabolism and loss of proteoglycans from cultures of bovine collateral ligament. Arch Biochem Biophys 1996;328:64–72.
32. Samiric T, Ilic MZ, Handley CJ. Large aggregating and small leucine-rich proteoglycans are degraded by different pathways and at different rates in tendon. Eur J Biochem 2004;271:3612–20.
33. Handley CJ, Lowther DA. Extracellular matrix metabolism by chondrocytes. III. Modulation of proteoglycan synthesis by extracellular levels of proteoglycan in cartilage cells in culture. Biochim Biophys Acta 1977;500:132–9.
34. Hascall VC, Kimura JH. Proteoglycans: isolation and characterization. Meth Enzymol 1982;82(Pt A):769–800.
35. Winter AD, Campbell MA, Robinson HC, Handley CJ. Catabolism of newly synthesized decorin by explant cultures of bovine ligament. Matrix Biol 2000;19:129–38.
36. Handley CJ, Campbell MA. Catabolism and turnover of proteoglycans. Meth Enzymol 1987;144:412–9.
37. Ilic MZ, Handley CJ, Robinson HC, Mok MT. Mechanism of catabolism of aggrecan by articular cartilage. Arch Biochem Biophys 1992;294: 115–22.
38. Munteanu SE, Ilic MZ, Handley CJ. Highly sulfated glycosaminoglycans inhibit aggrecanase degradation of aggrecan by bovine articular cartilage explant cultures. Matrix Biol 2002;21:429–40.
39. Gotte M, Kresse H, Hausser H. Endocytosis of decorin by bovine aortic endothelial cells. Eur J Cell Biol 1995;66:226–33.
40. Jones GC, Corps AN, Pennington CJ, Clark IM, Edwards DR, Bradley MM, *et al*. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. Arthritis Rheum 2006;54:832–42.
41. Rees SG, Curtis CL, Dent CM, Caterson B. Catabolism of aggrecan proteoglycan aggregate components in short-term explant cultures of tendon. Matrix Biol 2005;24:219–31.
42. Vankemmelbeke MN, Holen I, Wilson AG, Ilic MZ, Handley CJ, Kelner GS, *et al*. Expression and activity of ADAMTS-5 in synovium. Eur J Biochem 2001;268:1259–68.
43. Vankemmelbeke MN, Ilic MZ, Handley CJ, Knight CG, Buttle DJ. Coincubation of bovine synovial or capsular tissue with cartilage generates a soluble ''aggrecanase'' activity. Biochem Biophys Res Commun 1999;255:686–91.
44. Campbell MA, Handley CJ, Hascall VC, Campbell RA, Lowther DA. Turnover of proteoglycans in cultures of bovine articular cartilage. Arch Biochem Biophys 1984;234:275–89.
45. Ilic MZ, Robinson HC, Handley CJ. Characterization of aggrecan retained and lost from the extracellular matrix of articular cartilage. Involvement of carboxyl-terminal processing in the catabolism of aggrecan. J Biol Chem 1998;273:17451–8.
46. Vogel KG, Sandy JD, Pogany G, Robbins JR. Aggrecan in bovine tendon. Matrix Biol 1994;14:171–9.
47. Maroudas A, Bayliss MT, Uchitel-Kaushansky N, Schneiderman R, Gilav E. Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a marker of molecular age. Arch Biochem Biophys 1998;350:61–71.
48. Lohmander LS, Neame PJ, Sandy JD. The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, osteoarthritis. Arthritis Rheum 1993;36:1214–22.
49. Hutchinson JW, Tierney GM, Parsons SL, Davis TR. Dupuytren's disease and frozen shoulder induced by treatment with a matrix metalloproteinase inhibitor. J Bone Joint Surg 1998;80:907–8.
50. Renkiewicz R, Qiu L, Lesch C, Sun X, Devalaraja R, Cody T, *et al*. Broad-spectrum matrix metalloproteinase inhibitor marimastat-induced musculoskeletal side effects in rats. Arthritis Rheum 2003; 48:1742–9.
51. Burton-Wurster N, Liu W, Matthews GL, Lust G, Roughley PJ, Glant TT, *et al*. TGF beta 1 and biglycan, decorin, fibromodulin metabolism in canine cartilage. Osteoarthritis Cartilage 2003;11:167–76 [OARS, Osteoarthritis Research Society].
52. Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, *et al*. Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. Arthritis Res Ther 2006;8:R173.
53. Rajapakse N, Mendis E, Kim MM, Kim SK. Sulfated glucosamine inhibits MMP-2 and MMP-9 expressions in human fibrosarcoma cells. Bioorg Med Chem 2007;15:4891–6.
54. Hausser H, Ober B, Quentin-Hoffmann E, Schmidt B, Kresse H. Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. J Biol Chem 1992;267:11559–64.
55. Yanagishita M, Hascall VC. Metabolism of proteoglycans in rat ovarian granulosa cell culture. Multiple intracellular degradative pathways and the effect of chloroquine. J Biol Chem 1984;259:10270–83.
56. Persiani S, Rotini R, Trisolino G, Rovati LC, Locatelli M, Paganini D, *et al*. Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose. Osteoarthritis Cartilage 2007;15:764–72 [OARS, Osteoarthritis Research Society].
57. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51: 699–725.
58. Huser CA, Davies ME. Effect of a glucosamine derivative on impact-induced chondrocyte apoptosis *in vitro*. A preliminary report. Osteoarthritis Cartilage 2008;16:125–8 [OARS, Osteoarthritis Research Society].
59. Lippiello L. Collagen synthesis in tenocytes, ligament cells and chondrocytes exposed to a combination of glucosamine HCl and chondroitin sulfate. Evid Based Complement Alternat Med 2006;4:219–24.