Undersulfated Chondroitin Sulfate Does Not Increase in Osteoarthritic Cartilage

MIKKO J. LAMMI, CHENG-JUAN QU, MIKKO S. LAASANEN, SIMO SAARAKKALA, JARNO RIEPPO, JUKKA S. JURVELIN, and JUHA TÖYRÄS

ABSTRACT. ***Objective.*** To test whether there is undersulfation of chondroitin sulfate in osteoarthritic bovine articular cartilage to support the hypothesis that sulfate deficiency is involved with the development of osteoarthritis.

Methods. Cartilage samples from bovine patellae (n = 32) were divided into 3 groups based on their osteoarthritic progression, as assessed by modified Mankin score. Uronic acid contents of the samples were determined. Fragmentation of the proteoglycans due to proteolytic processing was estimated with agarose gel electrophoresis. The molar ratios of chondroitin sulfate isoforms in the extracted proteoglycans were determined with fluorophore-assisted carbohydrate electrophoresis.

Results. Loss of proteoglycans and accumulation of tissue water was evident in groups II and III, and progressive OA increased heterogeneity of aggrecan population in groups II and III. Importantly, the molar ratio of nonsulfated disaccharide was decreased in the osteoarthritic articular cartilage.

Conclusion. The structure of chondroitin sulfate in degenerated bovine cartilage did not support the hypothesis that sulfate depletion is present in osteoarthritic joint. (J Rheumatol 2004;31:2449–53)

Key Indexing Terms:
OSTEOARTHRITIS
CHODROITIN SULFATE
GLYCOSAMINOGLYCANS
ARTICULAR CARTILAGE

Osteoarthritis (OA) is a debilitating musculoskeletal disease common in humans and animals, characterized by progressive degeneration of the articular cartilage[1]. The etiology of the disease is poorly understood, although damage of the cartilage collagen network appears to be one of the key events for the onset and progression of OA[2]. A number of new therapeutic targets have been considered for the design of therapy[3], yet there is a lack of effective treatments for the disease. Glucosamine sulfate has raised interest as a possible disease modifying agent[4-6], although evidence from some recent clinical trials generates doubts about its effectiveness[7-9]. Most trials focused on glucosamine sulfate, while a few have used other forms of glucosamine. A trial with glucosamine hydrochloride showed only a minor positive effect[10].

The mechanism(s) of how glucosamine sulfate might act *in vivo* has remained undetermined. A theory of glucosamine sulfate entering chondrocytes has been proposed[11], although no direct evidence for a cellular transport system for molecular glucosamine sulfate exists. The finding that a single 7.5 g dose of glucosamine sulfate in humans did not increase the concentration of plasma (and probably synovial fluid) glucosamine above the detection limit, 1–3 μg/ml[12], has only rarely been appreciated[13].

An increased level of sulfate in plasma and synovial fluid after oral administration of glucosamine sulfate[14] offers a more likely explanation of how this supplement could manifest its suggested disease modifying abilities. Low sulfate concentration in culture media leads to the synthesis of undersulfated glycosaminoglycans in cartilage explants[15,16]. It is also known that a lack of intracellular sulfate due to mutations in the diastrophic dysplasia sulfate transporter gene causes undersulfation of cartilage proteoglycans[17].

Our hypothesis was that if there is a deficiency of sulfate in the osteoarthritic joint it would generate cartilage proteoglycans that contain increased relative amounts of nonsulfated chondroitin sulfate disaccharide isomer in their glycosaminoglycan chains. The hypothesis was tested by analyzing the disaccharide composition of chondroitin sulfate chains present in the cartilage of normal and diseased bovine patellar articular cartilage.

MATERIALS AND METHODS

Cartilage samples. On the basis of visual evaluation, healthy and degenerated articular cartilage specimens (n = 32) were prepared from the lateral facet of the bovine patellae (animals aged 1–3 years) by drilling osteochondral samples (diameter 19 mm) within 5 h post mortem. Samples are easy to collect from the patella, and although there is no direct weight-bearing, the mechanical forces are transmitted into the cartilage in the patella. The samples were stored at –20°C until thawed for histological and biochemical analyses.

Histological analyses. The samples were fixed in 4% (w/v) formaldehyde in 0.07 M sodium phosphate buffer, pH 7.0, for 48 h at 4°C. After delcalcification with 10% EDTA in 4% (w/v) phosphate buffered formaldehyde for

From the Departments of Anatomy and Applied Physics, University of Kuopio, Kuopio, Finland.

Supported by University of Kuopio, Jenny and Antti Wihuri Foundation.

M.J. Lammi, PhD; C-J. Qu, MD; J. Rieppo, BMed, Department of Anatomy; M.S. Laasanen, PhD; S. Saarakkala, BSc; J.S. Jurvelin, PhD; J. Töyräs, PhD, Department of Applied Physics, University of Kuopio.

Address reprint requests to M.J. Lammi, Department of Anatomy, University of Kuopio, PO Box 1627, 70211 Kuopio, Finland. E-mail: mikko.lammi@uku.fi

Submitted September 29, 2003; revision accepted June 1, 2004.

12 days, microscopic sections (3 µm) were prepared and cartilage degeneration was histologically graded using the Mankin scoring method[1] adapted to bovine samples and restricted to cartilage and subchondral bone properties. Degeneration was independently determined by 3 authors from blind-coded samples, and the final Mankin score was calculated as a mean of those 3 scores.

Extraction of proteoglycans. The cartilage tissue was detached from the bone, immersed in phosphate buffered saline, and the wet weight was measured. Subsequently, the specimens were extensively freeze-dried to determine the dry weight of the tissue, and the water content was calculated from this information. Proteoglycans were extracted and quantified as described[18].

Agarose gel electrophoresis. The extracted proteoglycans (5 µg of uronic acid) were precipitated in 75% ethanol overnight at 4°C. After dissolving the samples in 100 mM Tris-sodium phosphate buffer, safranin O assay[19] was used to confirm that precipitation had resulted in an equal yield of glycosaminoglycans in the precipitate. Proteoglycan samples were then electrophoresed in horizontal 1.2% agarose gels[20], and the gels were stained with toluidine blue.

Chondroitin sulfate disaccharide isoform analysis. The extracted proteoglycans (5 µg uronic acid) were precipitated in 75% ethanol. After drying, the samples were incubated with chondroitinase ABC and the resulting disaccharides were fluorescently labeled and electrophoresed as described[21]. Different disaccharides generated during enzymic digestion are equivalently labeled during derivatization, and linear behavior on analysis has been described[22]. The optical densities of digitized lanes were analyzed to obtain an estimate of the molar proportions of the chondroitin sulfate disaccharide isoforms.

Statistical analysis. Kruskal-Wallis H and post-hoc tests were used for testing differences in biochemical parameters between the groups. SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Mankin's scoring method was used to divide the samples into 3 different groups according to their stage of degeneration. In 11 samples, the cartilage looked healthy, with smooth, shiny surface and no evidence of superficial degeneration (Group I, Mankin score = 0); in 11 samples minor changes related to integrity of superficial cartilage and matrix stainability were observed (Group II, Mankin score > 0 to 3); whereas 10 specimens were clearly osteoarthritic (Group III, Mankin score > 3). Water content increased and uronic acid content decreased as the osteoarthritic changes became more severe (Table 1). The decrease in uronic acid content was proportionally higher in the extractable portion of glycosaminoglycans (Table 1).

To estimate the extent of proteoglycan fragmentation due to disease, proteoglycan structure was analyzed with agarose gel electrophoresis. The samples in Group I consisted of 2 major aggrecan bands, while heterogeneity of aggrecan increased as Mankin score increased (Figure 1). This in an indication that osteoarthritic changes increased degradation of cartilage proteoglycans, evident in the samples of Groups II and III.

Electrophoretic separation of fluorescently labeled chondroitin sulfate disaccharides was used to measure molar ratios of various disaccharide isoforms and the degree of sulfation in the extracted articular cartilage proteoglycans. Monosulfated Di-6S and Di-4S disaccharides were the most abundant isoforms detected in all of the samples analyzed (Figure 2), while nonsulfated Di-0S isoform formed about 2–8% of the total disaccharide content (arrow, Figure 2). The relative amount of nonsulfated isoform in proteoglycan extracts was significantly lower in Groups II and III, compared with Group I (Table 1). This analysis suggests that the sulfation degree of chondroitin sulfate in osteoarthritic articular cartilage is increased rather than decreased as the dis-

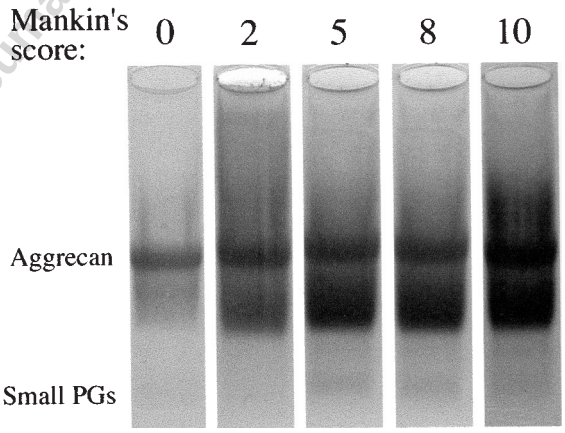

Figure 1. Agarose gel electrophoresis showed increasing heterogeneity of the extracted cartilage proteoglycans with progression of OA, evaluated by Mankin score of cartilage. The mobilities of native aggrecan and small proteoglycans (mainly decorin and biglycan) are marked on the left. PG: proteoglycan.

Table 1. Water content (%, mean ± SD) and concentration of uronic acid (µg/mg wet weight, mean ± SD) in sample groups with different Mankin scores.

Group	Mankin Score	No. of Samples	Water Content	Uronic Acid (extract)	Uronic Acid (tissue residue)	Uronic Acid (total)
I	0	11	79.9 ± 2.4	8.0 ± 2.8	2.2 ± 0.8	10.2 ± 3.5
II	0 < score ≤ 3	11	81.6 ± 1.2	5.2 ± 1.2	1.5 ± 0.4	6.7 ± 1.5
III	score > 3	10	84.2 ± 2.6	2.8 ± 0.8	1.3 ± 0.6	4.1 ± 1.2
I vs II			NS	NS	NS	NS
I vs III			p < 0.05	p < 0.05	p < 0.05	p < 0.05
II vs III			NS	p < 0.05	NS	p < 0.05

Kruskal-Wallis post-hoc test. NS: not significant.

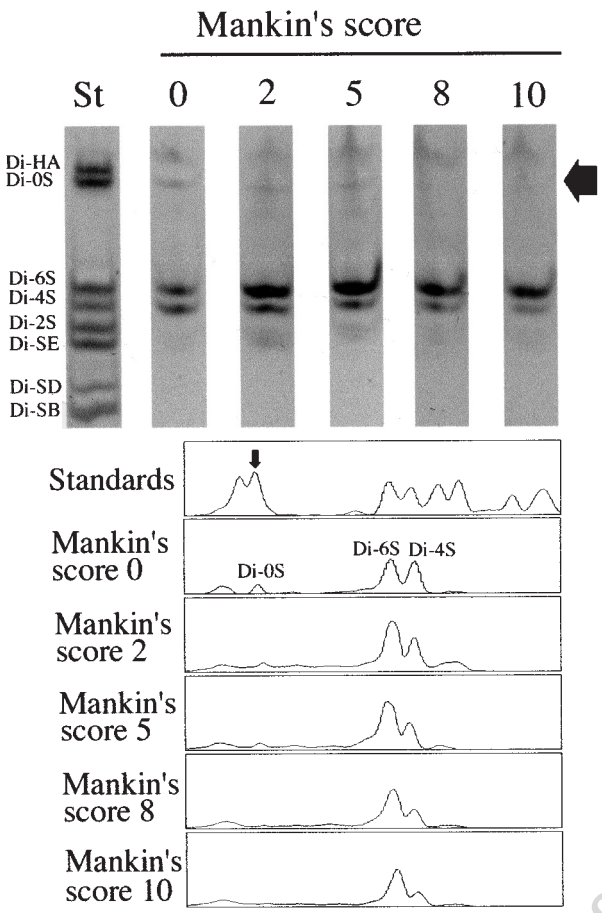

Figure 2. Fluorophore-assisted carbohydrate electrophoresis of chondroitin sulfate disaccharide isoforms indicated that the relative amount of nonsulfated chondroitin sulfate disaccharide of the extractable proteoglycans decreases with progression of OA, evaluated by Mankin score of cartilage. Mobilities of the known standard disaccharides (St) are shown on the left. Migration position of nonsulfated disaccharide Di-0S is indicated by arrows.

ease progresses. Relative increase in chondroitin 6-sulfate was also evident in the samples from Groups II and III compared with Group I (Table 2).

All samples showed a varying amount of a slowly-migrating band that most probably represented glucose. The amounts of di- and tri-sulfated disaccharide isoforms were generally below the detection limit of the method, although many of the samples contained fluorescent material at the migration position close to Di-2S and Di-SE (disulfated isoform). This finding probably represents artefacts due to labeling, causing overestimation of the relative amount of Di-2S and Di-SE. Therefore, Di-2S and Di-SE contents were not used for the calculations presented in Table 2. However, calculations performed with the overestimated values for Di-2S and Di-SE confirmed the same significant changes as presented in Table 2 (data not shown).

DISCUSSION

The present understanding of the initial mechanism of OA is still limited, and there is a lack of effective therapeutic means to treat patients with this progressive disease. Thus there is an urgent need for new therapies before irreversible osteoarthritic changes develop. Glucosamine sulfate is a drug increasingly used for treating osteoarthritic patients[5,6]. It is troublesome that most of the clinical trials on glucosamine sulfate have utilized visual analog scales for pain and/or radiographic imaging of the joint space width, and only a limited number of studies have focused on putative cartilage repair effects in human OA. We lack the evidence based on biochemical analyses of osteoarthritic articular cartilage treated with glucosamine sulfate.

The possible mechanism of action for glucosamine sulfate has not been elucidated. Experiments using intravenous administration of radioactive glucosamine have demonstrated the bioavailability of glucosamine[12], yet the available data do not indicate that oral or intravenous administration of glucosamine sulfate would stimulate proteoglycan synthesis in articular cartilage, as suggested by Piperno, *et al*[23]. Previously, 7.5 g of glucosamine sulfate did not elevate the glucosamine concentration in blood above the detection level, 1–3 µg/ml, or 5–15 µM. *In vitro*, glucosamine sulfate only at the level of 10 µg/ml or higher was reported to increase proteoglycan core protein synthesis significantly[11],

Table 2. Molar proportion of chondroitin sulfate disaccharide isoforms (%, mean ± SD) and percentage of slow-mobility aggrecan of the extracted proteoglycans in sample groups with different Mankin scores.

Group	Mankin Score	No. of Samples	Di-OS	Di-6S	Di-4S	Di-6S/Di-4S	PG-I (%)
I	0	11	8.1 ± 3.4	53.0 ± 10.8	38.9 ± 8.2	1.5 ± 0.6	65.8 ± 7.1
II	0 < score ≤ 3	11	2.9 ± 2.9	68.7 ± 6.6	28.2 ± 4.3	2.5 ± 0.6	48.8 ± 7.7
III	score > 3	10	1.5 ± 0.8	72.6 ± 3.5	25.8 ± 3.0	2.9 ± 0.5	46.1 ± 4.6
I vs II			$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
I vs III			$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
II vs III			NS	NS	NS	NS	NS

Kruskal-Wallis post-hoc test. NS: not significant. PG-I: the percentage of slow-mobility aggrecan, low values indicating an increased proteolytic processing of the aggrecan molecules due to OA.

and many biological effects of glucosamine (sulfate) *in vitro* have been shown to require at least 50 µM concentrations[23,24]. Recently, experiments using [^{3}H]glucosamine for metabolic labeling of chondroitin sulfate showed that exogenous glucosamine did not stimulate chondroitin sulfate synthesis at concentrations likely present in the joints, even after oral administration of glucosamine sulfate[25]. It is also notable that glucosamine is not an essential source for biosynthesis of glycosaminoglycans, and cannot be used for glycosaminoglycan synthesis without UDP derivatization[26].

Clearly, the possible mechanism of action of glucosamine sulfate can be debated. The increase of sulfate concentration in blood and synovial fluid after oral administration of glucosamine sulfate[14] has so far offered the best explanation how glucosamine sulfate may be beneficial for the health of osteoarthritic joints. However, it is not fully evident that deficiency of sulfate is associated with osteoarthritic changes, although it certainly is a possible condition. Therefore, we investigated the degree of sulfation of bovine knee samples showing a variable degree of osteoarthritic progression, assuming that sulfate deficiency should be reflected as an increased proportion of nonsulfated chondroitin sulfate over the sulfated ones. Importantly, the hypothesis that the osteoarthritic joint would have a low sulfate concentration, leading to increased molar proportion of nonsulfated chondroitin sulfate disaccharide moiety, could not be confirmed — instead the results suggested the opposite phenomenon.

We are aware that comparing human and bovine OA must be done with caution. We could not obtain the exact ages (1–3 years) of the individual animals included in this study. It may be that the increased ratio of Di-6S to Di-4S in this study partially reflects the possibility that animals with more advanced osteoarthrosis were also older than those having normal articular cartilage. Aging alone probably cannot explain our results, since the degenerative changes were so clearly evident in Groups II and III. It may also be speculated that preferential loss of superficial proteoglycans, richer in nonsulfated chondroitin sulfate disaccharide in bovine cartilage[27], during development of OA could explain the decrease in nonsulfated disaccharide observed in this study. However, the percentage of nonsulfated disaccharide in the deep zone decreased only by 50% from the superficial zone[27], clearly less than the change observed in this study between Groups I and III. Therefore, loss of superficial zone proteoglycans does not explain the decrease of nonsulfated chondroitin sulfate disaccharide in progressive degeneration in bovine samples. More important, there was no indication that proteoglycans were undersulfated in the osteoarthritic cartilage.

Our study did not confirm the hypothesis that there is a deficiency of sulfate in osteoarthritic joints. However, access to cartilage material, preferably from humans, of known donor age would help to avoid certain limitations of this study. We are confident that precise information on the sulfation pattern in osteoarthritic cartilage will be necessary to evaluate the importance of glucosamine sulfate therapy for the production of cartilage proteoglycans with normal content and biochemical structure, warranting further investigations.

ACKNOWLEDGMENT

The authors acknowledge Eija Rahunen for technical assistance in the preparation of histological sections and Päivi Perttula for help in disaccharide analyses, and Prof. Markku Tammi for discussions concerning the analysis of chondroitin sulfate disaccharides.

REFERENCES

1. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 1971;53:523-37.
2. Panula HE, Hyttinen MM, Arokoski JP, et al. Articular cartilage superficial zone collagen birefringence reduced and cartilage thickness increased before surface fibrillation in experimental osteoarthritis. Ann Rheum Dis 1998;57:237-45.
3. Pelletier JP, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum 2001;44:1237-47.
4. McAlindon TE, LaValley MP, Gulin JP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA 2000;283:1469-75.
5. Reginster JY, Deroisy R, Rovati LC, et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001;357:251-6.
6. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
7. Rindone JP, Hiller D, Collacott E, Nordhaugen N, Arriola G. Randomized, controlled trial of glucosamine for treating osteoarthritis of the knee. West J Med 2000;172:91-4.
8. Hughes R, Carr A. A randomized, double-blind, placebo-controlled trial of glucosamine sulphate as an analgesic in osteoarthritis of the knee. Rheumatology 2002;41:279-84.
9. McAlindon T. Why are clinical trials of glucosamine no longer uniformly positive? Rheum Dis Clin North Am 2003;29:789-801.
10. Houpt JB, McMillan R, Wein C, Paget-Dellio SD. Effect of glucosamine hydrochloride in the treatment of pain of osteoarthritis of the knee. J Rheumatol 1999;26:2423-30.
11. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
12. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51:699-725.
13. Brandt KD. Disease-modifying drugs for OA. In: Diagnosis and nonsurgical management of osteoarthritis. Caddo, OK: Professional Communications; 2000:259-73.
14. Hoffer LJ, Kaplan LN, Hamadeh MJ, Grigoriu AC, Baron M. Sulfate could mediate the therapeutic effect of glucosamine sulfate. Metabolism 2001;50:767-70.
15. van der Kraan PM, Vitters EL, de Vries BJ, van den Berg WB, van de Putte LB. Synthesis of aberrant glycosaminoglycans during cartilage culture in 'sulfate free' medium. J Biochem Biophys Methods 1988;15:273-7.

16. Brand HS, van Kampen GP, van de Stadt RJ, Kuijer R, van der Korst JK. Effect of sulfate concentration on glycosaminoglycan synthesis in explant cultures of bovine articular cartilage. Cell Biol Int Rep 1989;13:153-62.
17. Rossi A, Bonaventure J, Delezoide AL, Cetta G, Superti-Furga A. Undersulfation of proteoglycans synthesized by chondrocytes from a patient with achondrogenesis type 1B homozygous for an L483P substitution in the diastrophic dysplasia sulfate transporter. J Biol Chem 1996;271:18456-64.
18. Lammi MJ, Häkkinen TP, Parkkinen JJ, et al. Adaptation of canine femoral head articular cartilage to long distance running exercise in young beagles. Ann Rheum Dis 1993;52:369-77.
19. Lammi M, Tammi M. Densitometric assay of nanogram quantities of proteoglycans precipitated on nitrocellulose membrane with Safranin O. Anal Biochem 1988;168:352-7.
20. Saamanen AM, Tammi M, Kiviranta I, Helminen HJ. Running exercise as a modulatory of proteoglycan matrix in the articular cartilage of young rabbit. Int J Sports Med 1988;9:127-33.
21. Inkinen RI, Lammi MJ, Ågren U, Tammi R, Puustjärvi K, Tammi MI. Hyaluronan distribution in the human and canine intervertebral disc and cartilage endplate. Histochem J 1999;31:579-87.
22. Calabro A, Benavides M, Tammi M, Hascall VC, Midura RJ. Microanalysis of enzyme digests of hyaluronan and chondroitin/dermatan sulfate by fluorophore-assisted carbohydrate electrophoresis (FACE). Glycobiology 2000;10:273-81.
23. Piperno M, Reboul P, Hellio Le Graverand MP, et al. Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2000;8:207-12.
24. Gouze JN, Bianchi A, Becuwe P, et al. Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
25. Mroz PJ, Silbert JE. Effects of [^{3}H]glucosamine on [^{3}H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
26. Silbert JE, Sugumaran G. A starting place for the road to function. Glycoconj J 2002;19:227-37.
27. Korver GHV, van de Stadt RJ, van Kampen GPJ, van der Korst JK. Composition of proteoglycans in different layers of cultured anatomically intact articular cartilage. Matrix 1990;10:394-401.