Journal of Orthopaedic Research
8:565–571 Raven Press, Ltd., New York

High Susceptibility of Human Articular Cartilage Glycosaminoglycan Synthesis to Changes in Inorganic Sulfate Availability

Peter M. van der Kraan, Elly L. Vitters, Bernard J. de Vries, and Wim B. van den Berg

Department of Rheumatology, Academic Hospital, St. Radboud, Nijmegen, The Netherlands

Summary: The effect of sulfate concentration in the medium on glycosaminoglycan synthesis in articular cartilage of five different species was examined in relation to the physiological serum sulfate concentration in these species. Only the rate of sulfated glycosaminoglycan synthesis in human articular cartilage was sensitive to small deviations from the physiological sulfate concentration. A reduction in the sulfate concentration from 0.3 m*M* (physiological) to 0.2 m*M* resulted in a 33% reduction in glycosaminoglycan synthesis. In addition, we studied the effect of arthritic and "osteoarthritic" alterations in murine cartilage on the dependence of glycosaminoglycan synthesis on low sulfate concentrations. Arthritic and "osteoarthritic" cartilage had a similar dependence on the sulfate concentration in the medium as normal cartilage. Glycosaminoglycan synthesis in human articular cartilage appears to be very sensitive to the potential sulfate-depleting effects of drugs used in the treatment of rheumatoid arthritis and osteoarthritis. **Key Words:** Sulfate—Articular cartilage—Glycosaminoglycans.

Proteoglycans are, with collagen type II, the major structural components of articular cartilage. While collagen type II is responsible for the tensile strength, the proteoglycans are responsible for the pressure-resistant properties of articular cartilage (2). The highly sulfated glycosaminoglycans, which are covalently bound to the core protein of the cartilage proteoglycans, are accountable for these pressure-resistant traits. The negatively charged sulfate esters on the glycosaminoglycans are crucial for the optimal functioning of articular cartilage (2).

Inorganic sulfate is an essential substrate for the synthesis of glycosaminoglycans and the rate of synthesis of these biopolymers depends both in vivo and in vitro on the quantity of sulfate available to the chondrocytes (1,3,4,11,18). Depletion of inorganic sulfate in the serum of mice or rats by salicylate or paracetamol, respectively, can lead to a diminished synthesis of sulfated glycosaminoglycans in vivo (3,4,18). We studied if the physiological serum sulfate concentration in mice, Wistar rats, hamsters, cows, and humans is at a level such that the maximal rate of glycosaminoglycan synthesis would be readily achieved in vivo and if small variations in sulfate concentration would significantly affect the in vivo synthesis rate.

Since there is considerable variation in serum sulfate levels among healthy human individuals [0.08–0.58 m*M* (12,15)], the synthesis of sulfated glycosaminoglycans could also be highly variable as a result of the variation in serum sulfate levels. We wondered if glycosaminoglycan synthesis in cartilage from individuals with a low serum sulfate concentration would be less sensitive to low sulfate concentrations than individuals with higher serum

Received May 15, 1989; accepted December 15, 1989.
Address correspondence and reprint requests to Dr. P. M. van der Kraan at Department of Rheumatology, Academic Hospital St. Radboud, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands.

sulfate concentrations. As a model of this, we investigated the serum sulfate concentration in relation to the susceptibility of glycosaminoglycan synthesis to low sulfate concentrations in several mouse strains and other animal species.

Several drugs, used in the treatment of rheumatoid arthritis and osteoarthritis for their analgesic and/or anti-inflammatory properties, have the potential to reduce the sulfate concentration in the serum of laboratory animals and humans (3,4, 12,18). Depletion of serum sulfate can lead to inhibition of glycosaminoglycan synthesis in vivo (3,4). In addition, we have studied if arthritic cartilage, characterized by a decreased glycosaminoglycan synthesis (16), or arthrotic cartilage, characterized by an increased glycosaminoglycan synthesis (10,11,13), would have an altered sulfate dependence compared with normal cartilage.

MATERIALS AND METHODS

Cartilage Sources

Articular cartilage from 10-week-old male mice of various strains, male Wistar rats (150–200 g), and male Syrian hamsters (150–200 g) was obtained by careful dissection of the patella with surrounding tissue from the knee joints of these animals, according to the method of van den Berg et al. (17). Bovine metacarpal joints were purchased from a local slaughterhouse within 6 h after killing of the animals. Slices of articular cartilage (3 × 3 × 3 mm) were cut and used for sulfate incorporation studies. Fresh human articular cartilage slices (3 × 3 × 3 mm), from the tibial plateau, were obtained from amputation specimens within 4 h after amputation. The cartilage donors were elderly persons (age range of 60–75 years). All cartilage specimens were placed in sulfate-free RPMI 1640 DM medium (Serva, Heidelberg, F.R.G.) immediately after dissecting.

Induction of Murine "Arthritic" and "Arthrotic" Cartilage

Unilateral arthritis was induced in the right knee joint of 10-week-old male C57Bl10 mice by intra-articular injection of zymosan (60 μg, 6 μl) in this joint. Two days after the zymosan injection, the patellae were carefully dissected from both knee joints and placed in sulfate-free medium. The zymosan arthritis leads to a decreased synthesis of sulfated glycosaminoglycans in the articular cartilage of the patella (17). Articular cartilage with a stimulated synthesis of sulfated glycosaminoglycans, used as a model of "arthrotic cartilage," was obtained by intra-articular injection of the right knee joints of 10-week-old male C57Bl10 mice with a 1% (*w/v*) papain solution (6 μl). The papain solution (type IV, Sigma, St. Louis, MO, U.S.A.) contained 0.03 *M* cysteine (Sigma) to activate the papain. Three days after the papain injection, the patellae from both knee joints were dissected and placed in sulfate-free medium.

Culture of Articular Cartilage

The articular cartilage specimens were washed with sulfate-free RPMI 1640 DM medium for at least 2 h. After the washing procedure, the cartilage specimens were incubated for 2 h in RPMI 1640 DM medium with various magnesium sulfate (Merck, Darmstadt, F.R.G.) concentrations (0.1–1.5 m*M*) at 37°C in a humidified 5% CO_2 atmosphere. In the medium containing less than 1.5 m*M* magnesium sulfate, the deficient ions were replaced by magnesium chloride (Merck). In pilot experiments, a sulfate concentration of 1.5 m*M* appeared to be an optimal concentration for glycosaminoglycan synthesis in all species studied. The RPMI medium was supplemented with 20 μCi of [^{35}S]sulfate (carrier-free, specific activity of 1,200 Ci/mmol, Radiochemical Centre, Amersham, U.K.).

After incubation, the patellae (mouse, rat, and hamster cartilage) were washed twice with physiological saline and subsequently fixed in ethanol (96%, *v/v*, Merck). Additional washing steps did not further reduce the sulfate counts in the patellae. Decalcification of the patellae with 5% formic acid (Merck) was followed by stripping of the patellar cartilage layer (7). Patellar cartilage was digested overnight by lumasolve (Hicol, Oud-Beijerland, The Netherlands) and the amount of incorporated radiolabel was assayed by liquid scintillation analysis. As a measure of glycosaminoglycan synthesis, the total sulfate incorporation per patella was calculated. Hardly any label is incorporated in the underlying bone and bone marrow, as has been shown for the mouse patella (5).

The articular cartilage slices (human and bovine cartilage) were washed twice with physiological saline to remove the free radiolabel. Additional washings did not reduce the quantity of incorporated sulfate counts. Thereafter, the cartilage slices were

rapidly dried with a paper tissue and immediately weighed. The slices were digested by lumasolve and the quantity of incorporated radiolabel was determined by liquid scintillation analysis. The total sulfate incorporation per mg of wet weight was calculated.

Determination of Serum Sulfate Concentration

To assay the sulfate concentration in the serum of mice, rats, and hamsters, blood samples were taken by orbitapunction under ether anesthesia. Human blood was collected from five healthy volunteers (age range of 25–40 years). Synovial fluid was collected from the bovine metacarpal joints and the sulfate concentration in the fluid was assayed. The sulfate concentrations in serum and synovial fluid are identical (7). The inorganic sulfate concentration in synovial fluid and serum was determined by a modification of the benzidine method of Dogson and Spencer as recently described (7,8).

Statistical Analysis

Statistical analysis of data was performed by two-tailed Student's t test and the Pearson correlation test. A p value <0.05 was considered to be significant. We calculated for the different species the deviation of sulfate concentration from the physiological serum sulfate concentration resulting in a significant inhibition of glycosaminoglycan synthesis. If none of the sulfate concentrations tested was in the range of this sulfate concentration ± SD, we used the concentration just above the physiological sulfate concentration for statistical evaluation.

RESULTS

Figure 1 shows the effect of sulfate concentration in the medium on the synthesis of sulfated glycosaminoglycans in murine patellar cartilage. Major differences in inhibition of glycosaminoglycan synthesis at low sulfate concentrations between the six mouse strains tested were not observed. A significant inhibition of sulfate incorporation was observed at sulfate concentrations below 0.5 mM for all mouse strains. In Table 1, the sulfate concentration in the medium resulting in a 50% inhibition of glycosaminoglycan synthesis together with the serum sulfate concentrations in the mouse strains are presented. The serum sulfate concentration in the Balb/c strain was significantly higher than in the ICR-Br, Swiss, and C57Bl10 strains. There was no correlation between the serum sulfate concentration in the various mouse strains and the susceptibility to low sulfate concentrations of cartilage glycosaminoglycan synthesis in the mouse strains. The sulfate concentration in the serum of the mouse strains was sufficient for an optimal synthesis of glycosaminoglycans.

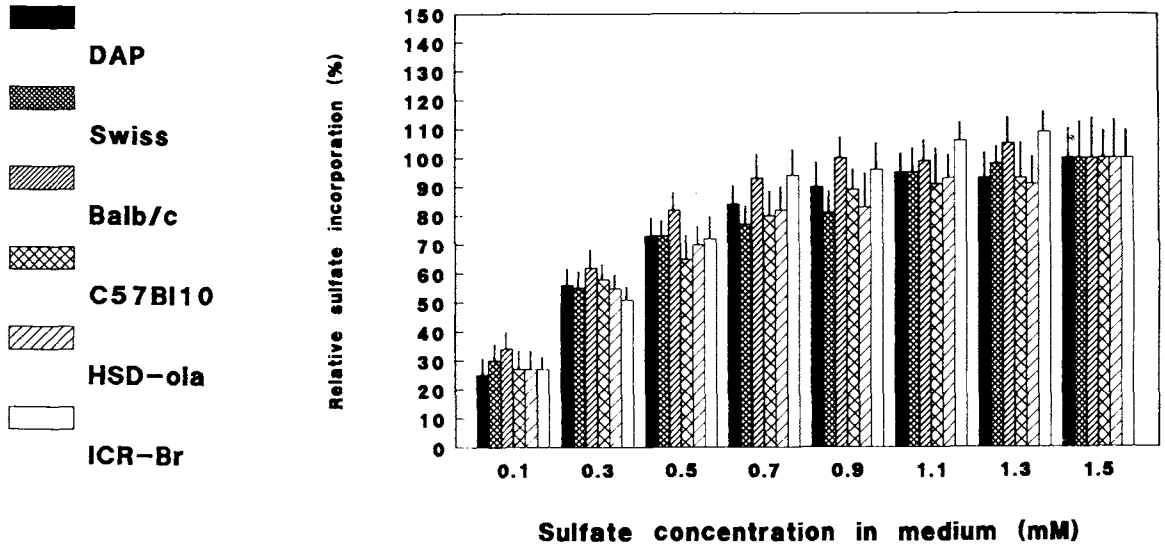

FIG. 1. The effect of sulfate concentration in the medium on the incorporation of sulfate in articular cartilage of various mouse strains. The values are expressed as a percentage of the sulfate incorporation at 1.5 mM sulfate. Each bar shows the mean ± SD of the incorporation of at least five patellae.

TABLE 1. *Sulfate concentration in the medium resulting in a 50% inhibition of maximal in vitro sulfate incorporation in murine articular cartilage and the corresponding physiological sulfate concentration*

Mouse strain	Sulfate concentration, 50% inhibition (m*M*)	Sulfate concentration in serum (m*M*)
ICR-Br	0.25 (0.23–0.27)	0.95 ± 0.13 (n = 10)
HSD-ola	0.22 (0.20–0.25)	1.15 ± 0.14 (n = 10)
DAP	0.26 (0.23–0.30)	1.18 ± 0.19 (n = 10)
Swiss	0.26 (0.25–0.27)	0.99 ± 0.13 (n = 10)
C57Bl10	0.25 (0.22–0.29)	1.03 ± 0.09 (n = 10)
Balb/c	0.22 (0.20–0.25)	1.27 ± 0.14 (n = 10)

The sulfate concentration of 1.5 m*M* is considered to be optimal under the culture conditions used and the sulfate incorporation at this concentration is set at 100%. The sulfate concentrations resulting in a 50% inhibition are expressed as the mean values (range) of at least three experiments. The values of the physiological sulfate concentration are expressed as the mean ± SD.

For five different species, the physiological serum sulfate concentration and the sulfate concentration resulting in 50% inhibition of sulfated glycosaminoglycan synthesis are expressed in Table 2. Figure 2 shows the sulfate dependence of glycosaminoglycan synthesis in relation to the physiological sulfate concentration in the different species. The serum sulfate concentration in human serum was significantly lower than the concentration in the serum of the other species and the physiological sulfate concentration in cows and mice was significantly higher than this level in rats and hamsters. There was no correlation between the serum sulfate concentration in the serum or synovial fluid of the species studied and the susceptibility of articular cartilage to low sulfate concentrations.

TABLE 2. *Sulfate concentration in the medium resulting in a 50% inhibition of maximal in vitro sulfate incorporation in articular cartilage and the corresponding physiological sulfate concentration in five different species*

Species concentration	Sulfate concentration, 50% inhibition (m*M*)	Sulfate in serum (m*M*)
Mouse	0.24 (0.20–0.30)	1.10 ± 0.13 (n = 60)
Hamster	0.39 (0.36–0.43)	0.80 ± 0.07 (n = 10)
Rat	0.23 (0.20–0.25)	0.83 ± 0.08 (n = 10)
Cow	0.49 (0.45–0.54)	0.98 ± 0.08 (n = 3)
Human	0.25 (0.22–0.27)	0.30 ± 0.03 (n = 5)

The mouse values are mean values of Table 1. The sulfate concentration of 1.5 m*M* is considered to be optimal under the culture conditions used and the sulfate incorporation at this point is set at 100%. The sulfate concentrations resulting in a 50% inhibition are expressed as the mean values (range) of at least three experiments. The values of the physiological sulfate concentration are expressed as the mean ± SD.

We calculated the deviation of sulfate concentration in the medium from the physiological sulfate level resulting in a significant suppression of glycosaminoglycan synthesis for the different species. A change in sulfate concentration in the medium from 0.3 to 0.2 m*M* led to a significant inhibition of glycosaminoglycan synthesis (33%) in human cartilage. A decrease in sulfate concentration from 0.9 to 0.5 m*M* led to a significantly reduced rate of synthesis in hamsters and rats. Mouse and bovine cartilage appeared to be least sensitive to alterations in sulfate concentration. Reduction from 1.1 to 0.5 m*M* sulfate resulted in a significantly diminished glycosaminoglycan synthesis in these species. Human articular cartilage was far more sensitive to changes in sulfate availability than the other species tested.

We also studied the effects of a decreased glycosaminoglycan synthesis and an enhanced glycosaminoglycan synthesis in articular cartilage on the susceptibility to low sulfate concentrations. Arthritic cartilage, with a decreased glycosaminoglycan synthesis, as well as "arthrotic cartilage," with an elevated glycosaminoglycan synthesis, was induced in the right knee joints of C57Bl10 mice. The incorporation of sulfate in the arthritic joints was 70% and in the athrotic joints was 155% of the sulfate incorporation in the contralateral control knee joints. As can be seen in Fig. 3, both arthritic cartilage and arthrotic cartilage showed no significantly different susceptibility to low sulfate concentration compared to normal cartilage.

DISCUSSION

Inorganic sulfate is an essential substrate in the synthesis of cartilage glycosaminoglycans and this and other studies show that sulfate availability can be a rate-limiting factor in the synthesis of these glycosaminoglycans (1,3,4,11,18). The incorporation of sulfate is a reflection of the rate of synthesis of sulfated glycosaminoglycans in cartilage. The reduced incorporation of sulfate, seen at low sulfate concentrations, is not the result of the synthesis of undersulfated glycosaminoglycans or of glycosaminoglycans of shorter chain length (20). Undersulfation of glycosaminoglycans occurs only at very low sulfate concentrations in the medium but not in the sulfate concentration range we tested in the above-described experiments (19,20). These results suggest that low sulfate availability will result in the synthesis of proteoglycan cores with a reduced

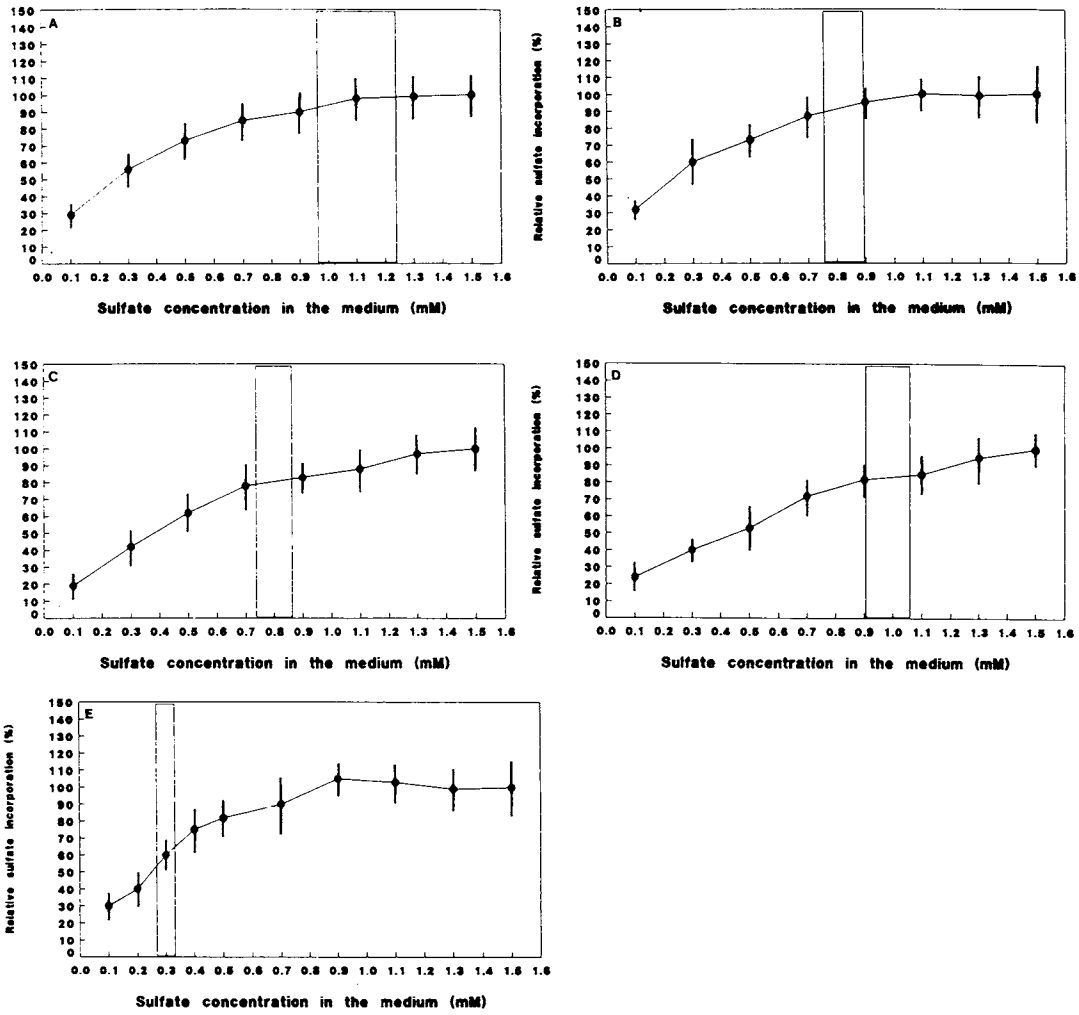

FIG. 2. The effect of sulfate concentration in the medium on sulfate incorporation in articular cartilage of five different species. The values are expressed as a percentage of the sulfate incorporation at 1.5 m*M* sulfate. Each point shows the mean ± SD of at least five patellae or cartilage slices. The bars represent the physiological sulfate concentration ± SD in the corresponding species [(**A**) mouse, (**B**), rat, (**C**) hamster, (**D**) cow, (**E**) human].

quantity of normal glycosaminoglycan chains. However, a diminished synthesis of proteoglycan core proteins, although unlikely, can not be excluded.

Of all species studied, only the sulfate concentration in human serum was significantly suboptimal for the incorporation of sulfate in articular cartilage while the sulfate concentration in the serum of the other species was sufficient to support maximal sulfate incorporation, at least in vitro (Fig. 2). Moreover, already small variations in serum sulfate concentrations in humans will result in an altered rate of glycosaminoglycan synthesis in articular cartilage while the rate of glycosaminoglycan synthesis in the other species was relatively insensitive to concentration changes. Therefore, glycosaminoglycan synthesis in human cartilage will be very sensitive to the potential sulfate-depleting effects of drugs used in the treatment of rheumatoid arthritis and osteoarthritis (3,4,12,18). Although the incorporation of sulfate into glycosaminoglycans was determined in vitro in this study, decreased synthesis of glycosaminoglycan is also shown to occur in vivo after paracetamol-induced reduction in sulfate availability in rats (18).

There was neither a correlation between the sulfate concentration in the serum of the mouse strains and the susceptibility of glycosaminoglycan synthesis in murine articular cartilage nor a correlation between serum sulfate concentration in the different species and the sensitivity of glycosaminoglycan synthesis to low sulfate concentrations in these

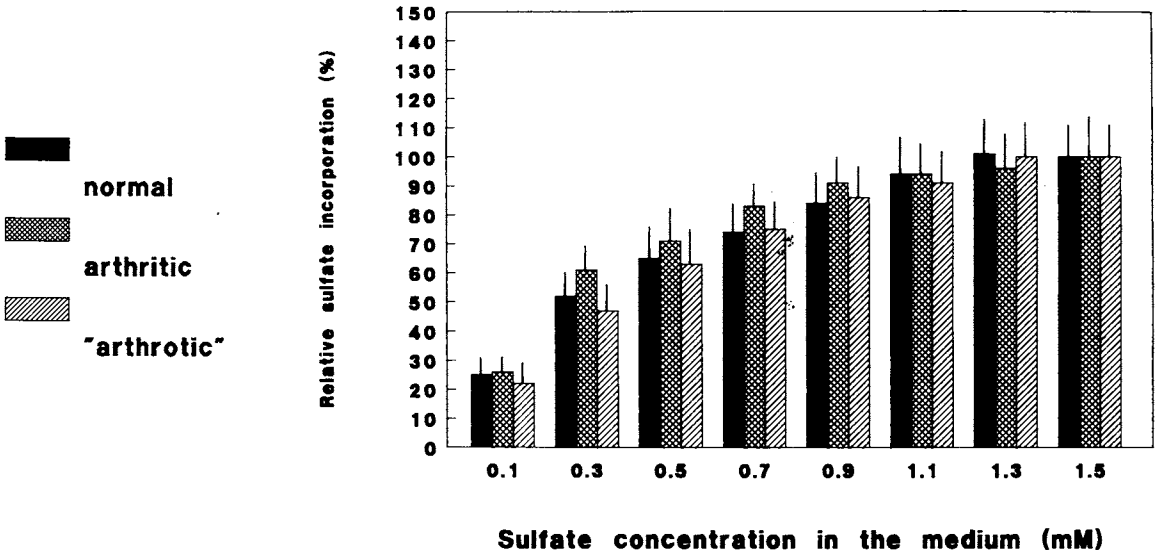

FIG. 3. The effect of sulfate concentration in the medium on sulfate incorporation in arthritic, arthrotic, and normal murine cartilage. The values are expressed as a percentage of sulfate incorporation at 1.5 m*M* sulfate. Each bar shows the mean ± SD of at least five patellae. The incorporation of sulfate in the arthritic joints was 70% and in the arthrotic joints was 155% of sulfate incorporation in the contralateral knee joints.

species. Therefore, species and strains with low serum sulfate concentration do not appear to be less susceptible to lowering of the sulfate concentrations than species with a high serum sulfate concentration. When these results can be extrapolated to human individuals, the rate of glycosaminoglycan synthesis will be highly variable among humans as a consequence of the observed variation in serum sulfate concentrations (15). That variation in sulfate availability and as a consequence the variation in glycosaminoglycan synthesis might not lead to cartilage inferiority under normal physiological conditions might be explained by the following. Sandy et al. demonstrated that of the proteoglycans synthesized by rabbit chondrocytes, only about 50% remained in the cartilage matrix for longer than 168 h (14). Chondrocytes appear to synthesize a pool of proteoglycan monomers that does not change from the low-affinity hyaluronate binding monomers to high-affinity monomers. This pool will diffuse through the extracellular matrix without "finding" an available hyaluronate binding site. This pool might function as a buffer for the variation in glycosaminoglycan synthesis due to variation in serum sulfate concentrations in different individuals.

One of the first events in the pathogenesis of osteoarthritis is a reduced content of glycosaminoglycans in the affected articular cartilage (9,10). Individuals with a low serum sulfate concentration might have a lower capacity of glycosaminoglycan synthesis and as a result also a more limited potential of matrix repair than individuals with a high serum sulfate concentration. The low-affinity monomer pool appears to be insufficient to overcome the matrix depletion in osteoarthritic cartilage. As a consequence, individuals with a low serum sulfate concentration might be more vulnerable to the development of osteoarthritis than those with a higher serum sulfate level.

The dependence of glycosaminoglycan synthesis on the sulfate concentration in the medium was similar for arthritic, arthrotic, and normal cartilage. Arthritic and arthrotic cartilage was not more or less vulnerable to low sulfate concentrations than normal cartilage. Variation in serum sulfate concentrations, potentially induced by antirheumatic medication (3,4,6,18) will result in similar changes in the rate of glycosaminoglycan synthesis in normal and arthritic or arthrotic cartilage.

The results of this study show that already a slight alteration in sulfate availability will result in a significant change in glycosaminoglycan synthesis in human cartilage, in contrast to the other species tested. As a consequence, the rate of glycosaminoglycan synthesis in humans will be sensitive to drug-induced depletion of serum sulfate (12) and this sensitivity might play a role in the susceptibility of certain individuals to osteoarthritis.

REFERENCES

1. Bayliss MT, Urban JPG, Johnstone B, Holm S: In vitro method for measuring synthesis rates in the intervertebral disc. *J Orthop Res* 4:10–17, 1986
2. Carney SL, Muir H: The structure and function of cartilage proteoglycans. *Physiol Rev* 68:858–910, 1988
3. De Vries BJ, van den Berg WB, van de Putte LBA: Salicylate-induced depletion of endogenous inorganic sulfate. *Arthritis Rheum* 28:922–929, 1985
4. De Vries BJ, van den Berg WB, Vitters E, van de Putte LBA: The effect of salicylate on anatomically intact articular cartilage is influenced by sulfate and serum in the culture medium. *J Rheumatol* 13:686–693, 1986
5. De Vries BJ, van den Berg WB, Vitters E, van de Putte LBA: Quantitation of glycosaminoglycan metabolism in anatomically intact articular cartilage of the mouse patella: in vitro and in vivo studies with ^{35}S-sulfate, ^{3}H-glucosamine, and ^{3}H-acetate. *Rheumatol Int* 6:273–281, 1986
6. De Vries BJ, van der Kraan PM, van den Berg WB: Decrease of inorganic blood sulfate following treatment with selected antirheumatic drugs, potential consequence for articular cartilage. *Agents Actions* 29:224–231, 1990
7. De Vries BJ, Vitters E, van den Berg WB, Schram D, van de Putte LBA: Determination of small quantities of sulfate (0–12 nmol) in serum, urine, and cartilage of the mouse. *Anal Biochem* 163:408–417, 1987
8. Dogson KS, Spencer B: Studies on sulphatases. 5. The determination of inorganic sulphate in the study of sulphatases. *Biochem J* 55:436–440, 1953
9. Mankin HJ, Dorfman H, Lippiello L, Zarins A: Biochemical and metabolical abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolical data. *J Bone Joint Surg [Am]* 53:523–537, 1971
10. Mankin HJ, Johnson ME, Lippiello L: Biochemical and metabolical abnormalities in articular cartilage from osteoarthritic human hips. III. Distribution and metabolism of aminosugar-containing macromolecules. *J Bone Joint Surg [Am]* 63:131–139, 1981
11. Maroudas A, Evans H: Sulphate diffusion and incorporation into human articular cartilage. *Biochim Biophys Acta* 338: 265–279, 1974
12. Morris ME, Levy G: Serum concentration and renal excretion by normal adults of inorganic sulfate after acetaminophen, ascorbic acid, or sodium sulfate. *Clin Pharmacol Ther* 33:529–536, 1983
13. Sandy JD, Adams ME, Billingham MEJ, Plaas A, Muir H: In vivo and in vitro stimulation of chondrocyte biosynthetic activity in early experimental osteoarthritis. *Arthritis Rheum* 27:388–397, 1984
14. Sandy JD, O'Neill JR, Ratzlaff LC: Acquisition of hyaluronate-binding affinity in vivo by newly synthesized cartilage proteoglycans. *Biochem J* 258:875–880, 1989
15. Tallgren LG: Inorganic sulphates in relation to the serum thyroxine level and renal failure. *Acta Med Scand* suppl 640:34–95, 1980
16. Van den Berg WB, Kruysen MWM, van de Putte LBA, van Beusekom HJ, van der Sluis M, Zwarts WA: Antigen-induced and zymosan-induced arthritis in mice: studies on in vivo cartilage proteoglycan synthesis and chondrocyte death. *Br J Exp Pathol* 62:308–316, 1981
17. Van den Berg WB, Kruysen MWM, van de Putte LBA: The mouse patella assay: an easy method of quantitating articular cartilage chondrocyte function in vivo and in vitro. *Rheumatol Int* 1:165–169, 1982
18. Van der Kraan PM, de Vries BJ, Vitters EL, van den Berg WB, van de Putte LBA: Inhibition of glycosaminoglycan synthesis in anatomically intact rat patellar cartilage by paracetamol-induced serum sulfate depletion. *Biochem Pharmacol* 37:3683–3690, 1988
19. Van der Kraan PM, Vitters EL, de Vries BJ, van den Berg WB, van de Putte LBA: Synthesis of aberrant glycosaminoglycans during cartilage culture in "sulfate free" medium. *J Biochem Biophys Methods* 15:273–278, 1988
20. Van der Kraan PM, Vitters EL, de Vries BJ, van den Berg BJ, van de Putte LBA: The effect of low sulfate concentrations on the glycosaminoglycan synthesis in anatomically intact articular cartilage of the mouse. *J Orthop Res* 7:645–653, 1989