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Enhanced Extractability of Articular Cartilage Proteoglycans in Osteoarthrosis

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Tissue contents of small, easily extracted, proteoglycans, relatively poor in keratan sulphate, were compared in normal and osteoarthrotic cartilage. Although the amounts of small proteoglycans were similar in each tissue, as were the collagen contents, some proteoglycans in the diseased cartilage were much more readily extracted than those in the normal tissue.

Osteoarthrosis remains a disorder of unknown aetiology. The disease is well-defined pathologically, however, and is marked in the early stages by softening of the articular cartilage and later by loss of extracellular ground substance (Sokoloff, 1969). Considerable attention has been focused on changes in cartilage glycosaminoglycans in osteoarthrosis where the chondroitin sulphate and/or keratan sulphate contents have been found to be decreased (Bollet *et al.*, 1963; Mankin & Lippiello, 1971; Bjelle *et al.*, 1972; Lust & Pronsky, 1972) and chondroitin sulphate chains are shorter than those in normal cartilage (Bollet & Nance, 1966; Hjertquist & Wasteson, 1972). The stiffness of cartilage correlates with its content of glycosaminoglycans (Kempson *et al.*, 1970), so that such changes in composition may affect weight-bearing and relate to the pathogenesis of osteoarthrosis.

Glycosaminoglycans do not exist as such in cartilage, but are covalently linked to protein as proteoglycans. The proteoglycans of normal articular cartilage, moreover, are not homogeneous and exist as a family of closely related molecules which vary among themselves in size and composition (Brandt & Muir, 1971*a*). In general, the keratan sulphate and protein contents of cartilage proteoglycans are directly related to their size, as are differences in the ease with which they may be extracted from the tissue. Thus the smallest proteoglycans of articular cartilage contain very little glucosamine and protein and are essentially completely removed from the tissue by brief homogenization in neutral salt (Brandt & Muir, 1971*a*).

Because of the heterogeneity of the proteoglycans in normal articular cartilage, differences in the composition of tissue glycosaminoglycans in osteoarthrosis may reflect selective changes in a particular subpopulation of the proteoglycans. Thus recent reports indicating that proteoglycans of osteoarthrotic cartilage may be more readily extracted than those of normal cartilage (McDevitt *et al.*, 1973), and may contain less keratan sulphate (Mankin & Lippiello, 1971; Bjelle *et al.*, 1972; Lust & Pronsky, 1972), raise the question whether the tissue content of small proteoglycans is increased in osteoarthrosis. The present study was designed to examine this possibility.

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Materials and methods

Tissues. Femurs were obtained from steers 5–8 years of age at the abattoir and stored frozen for up to 10 days. After thawing to 4°C small samples of grossly normal or softened fibrillated cartilage were taken from weight-bearing surfaces of the proximal femurs for histological study and the remainder of the cartilage was removed from the articular surface with a scalpel. After Safranin-O staining the severity of osteoarthrosis of the samples from grossly normal cartilage was grade 0 while that of the samples from grossly diseased areas ranged between grades 6 and 10, according to the criteria of Mankin *et al.* (1971).

Tissue from grossly normal and abnormal areas of nine joints was pooled separately and diced. Portions of approx. 100mg were weighed immediately and placed in acetone for 24h, during which time the acetone was changed twice. The remainder of the diced cartilage was frozen in liquid N_2 and pulverized in a steel die cooled in liquid N_2 before extraction of the proteoglycans.

The weighed samples were removed from the acetone and dried to constant weight *in vacuo* at 80°C. Representative samples of the dried cartilage were employed for determination of hydroxyproline (Brandt & Muir, 1969). Other portions were digested with Pronase (Brandt & Muir, 1969), after which glycosaminoglycans were isolated and purified as described for proteoglycans below, and their uronic acid and hexosamine contents determined.

Table 1. *Sequential extraction of bovine hip cartilage with* 0.15M-*sodium acetate, pH* 6.8

See the text for details.

			Normal cartilage				Osteoarthrotic cartilage			
	Homogenization		Uronic acid content (%)	% of tissue uronic acid	Glucosamine/ galactosamine molar ratio	% retarded by Sepharose 6B	Uronic acid content (%)	% of tissue uronic acid	Glucosamine/ galactosamine molar ratio	% retarded by Sepharose 6B
Extract	Speed	Duration								
1	None	1h	28.9	0.6	1:2.8	56	29.9	5.3	1:3.0	62
2	Low	5min	29.7	0.7	1:2.7	40	29.2	4.8	1:3.4	45
3	Medium	20min	28.0	25.1	1:2.4	15	28.4	20.4	1:2.7	12
4	High	30min	26.5	38.6	1:1.3	16	29.1	33.1	1:1.8	10
Residual glycosaminoglycans			21.0	26.9	1:1.0	—	24.2	19.2	1:1.4	—

Extraction of proteoglycans. Portions (15g) of pulverized normal or arthrotic cartilage were suspended in 60ml of cold 0.15M-sodium acetate, pH6.8, and stirred gently for 1h at 4°C. The suspension was then filtered and the tissue residue washed twice with fresh sodium acetate. Filtrate and washings were combined and the proteoglycans were isolated by precipitation with 9-aminoacridine hydrochloride and converted into their sodium salts by exchange with Bio-Rad AG-50 (Na^+ form). The proteoglycans were further purified by a second precipitation with 9-aminoacridine and, after removal of the acridine with the resin, washed with ethanol and dried (Tsiganos & Muir, 1969), and designated extract 1 (Table 1). The tissue residue was resuspended in fresh sodium acetate as above and homogenized at low speed for 5min at 4°C in a VirTis homogenizer, after which the suspension was filtered and the residue washed with additional acetate buffer. Filtrate and washings were combined and the proteoglycans isolated and purified exactly as above (extract 2). Two additional homogenizations, yielding extracts 3 and 4, were performed under the conditions shown in Table 1, with the cartilage residue resuspended in fresh sodium acetate each time. All suspensions were cooled in a slush of solid CO_2–acetone so that the temperature during homogenization did not rise above 4°C.

The glycosaminoglycans which were not extracted in sodium acetate were obtained after digestion of the residue with Pronase and isolated as above.

Gel chromatography. A column (40cm×0.8cm) of Sepharose 6B was used. Samples (1.5mg) in 0.5ml of 0.5M-sodium acetate, pH6.5, were dissolved overnight at 4°C before application to the column, which was eluted with the acetate solution at a flow rate of 2ml/h. Fractions (1ml) were collected and the uronic acid content of each fraction was determined. Fractions eluted after the void volume, which had been determined with Blue Dextran (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.), were pooled, dialysed against water at 4°C and freeze-dried.

Analytical methods. Hexuronic acid, molar ratios of glucosamine to galactosamine and dry weights were all determined by standard methods (Brandt & Muir, 1971*a*). Hexosamine contents were determined by the method of Kraan & Muir (1957) after hydrolysis in 8M-HCl for 3h at 95°C, with glucosamine hydrochloride as the standard. Hydroxyproline content was determined on an automated amino acid analyser after hydrolysis in 6M-HCl for 24h at 105°C.

Immunological studies. Double diffusion in agar was performed as described previously with an antiserum specific for small-sized proteoglycans of cartilage (Brandt *et al.*, 1973). When tested against a preparation of small proteoglycans of porcine articular cartilage, which was completely retarded on Sepharose 6B chromatography (uronic acid, 29.9%; molar ratio of glucosamine to galactosamine, 1:70), this antiserum gave a single precipitin line which was not affected by prior digestion of the antigen with hyaluronidase (Brandt *et al.*, 1973). The antiserum had no reactivity against larger proteoglycans, however, unless their chondroitin sulphate chains had been digested.

Results

The normal and diseased cartilage were similar with respect to dry weight, which represented approx. 25% of the total weight, and hydroxyproline content, which represented approx. 8% of the dry weight (Table 2). The arthrotic cartilage, however, contained approx. 10% less uronic acid and hexosamine than the normal. Moreover, its glycosaminoglycans, isolated after proteolytic digestion,

Table 2. *Composition of pooled normal and osteoarthrotic bovine hip cartilage*

Acetone-treated cartilage was dried to constant weight to determine the dry weight. The total glycosaminoglycans were isolated after digestion of the cartilage with Pronase (see the text).

Cartilage	Dry weight (% of wet weight)	% of dry weight			Glucosamine/galactosamine molar ratio of total glycosaminoglycans
		Uronic acid	Hydroxyproline	Hexosamine	
Normal	28	3.0	7.9	2.9	1:1.1
Osteoarthrotic	25	2.7	8.1	2.6	1:1.6

contained somewhat less glucosamine relative to galactosamine than those of the normal cartilage (glucosamine/galactosamine, 1:1.1 and 1:1.6 respectively).

Approx. 80–90% of the total uronic acid in both the normal and arthrotic cartilage was accounted for in the various sodium acetate extracts and the residual glycosaminoglycans (Table 1). Essentially all the uronic acid in every extract was precipitated by 9-aminoacridine, since none was detectable in any of the supernatants after addition of this reagent.

The proteoglycans of the diseased cartilage were more readily extracted than those of the normal cartilage and, whereas extracts 1 and 2 of the normal cartilage contained 0.6 and 0.7% respectively of the tissue uronic acid, the yields from the abnormal cartilage were 7 to 9 times as great. On the other hand, with lengthier and much more vigorous homogenization markedly more proteoglycans were extracted. Extracts 3 and 4 together contained 63.7% of the uronic acid of the normal cartilage and 55.5% of that in the diseased cartilage (Table 1).

No significant difference in the elution profiles of uronic acid was apparent when the results of Sepharose 6B chromatography of the four extracts of normal cartilage were compared with those of the analogous extracts of the diseased cartilage. Approx. 40–60% of the proteoglycans in extracts 1 and 2 of both were small enough in hydrodynamic size to be retarded by Sepharose 6B whereas extracts 3 and 4 were composed to a much greater extent of larger proteoglycans.

The proteoglycans in each extract contained similar amounts of uronic acid. However, with increasing resistance to extraction from the matrix, glucosamine represented an increasing proportion of the total hexosamine in proteoglycans of both the normal and diseased cartilage.

Insufficient material was available for full analysis of the proteoglycans retarded by Sepharose 6B. However, hexosamine molar ratios of the fractions of extracts 1 and 2 of the diseased cartilage and of extract 2 of the normal, which was eluted after the void volume of the Sepharose 6B column, showed that these contained only about one-third as much glucosamine, relative to galactosamine, as the unfractionated extracts.

Extracts 1 and 2 of the diseased cartilage and extract 2 of the normal cartilage formed a single precipitin line with anti-(small proteoglycan) serum whether or not they had been digested with hyaluronidase before diffusion, and all three gave a reaction of complete immunological identity with small proteoglycans from porcine articular cartilage. Extracts 3 and 4 of both the normal and diseased cartilage, on the other hand, did not react with the antiserum unless digested with hyaluronidase before diffusion.

Discussion

Although the results of Safranin-O staining suggested a decrease in glycosaminoglycans in the diseased cartilage, uronic acid and hexosamine determinations indicated that its glycosaminoglycan content was approx. 90% of that of the normal (Table 2). From the molar ratios of the hexosamines and hexosamine contents of the isolated glycosaminoglycans, galactosamine constituted similar proportions of the dry weight of normal and arthrotic cartilage (1.5 and 1.6% respectively). However, although glucosamine accounted for 1.4% of the dry weight of normal cartilage it represented only 1.0%, or approx. 30% less, of the dry weight of diseased cartilage, which is consistent with the finding that osteoarthrotic cartilage may be deficient in keratan sulphate (Mankin & Lippiello, 1971).

Approx. 10% of the proteoglycans in the osteoarthrotic tissue could be extracted merely by stirring in iso-osmotic sodium acetate, followed by very brief low-speed homogenization of the cartilage. This treatment, however, solubilized only 1.3% of the proteoglycans of normal cartilage (Table 1). Although collagen may entrap proteoglycans in cartilage matrix and interfere with their extraction (Brandt & Muir, 1971*b*), the differences in extractability noted above cannot be attributed to differences between the amounts of collagen in the normal and diseased cartilage, since their hydroxyproline contents were essentially the same.

Although the smallest proteoglycans tend to be

those most readily extracted by sodium acetate (Brandt & Muir, 1971*a*), in the present study differences in hydrodynamic size also did not appear to account for the greater yields in the earlier extracts of the osteoarthrotic cartilage. Hence, 40–60% of the proteoglycans in extracts 1 and 2 of the normal, as well as of the diseased, cartilage were retarded by Sepharose 6B. On the other hand, small proteoglycans accounted for little of the uronic acid in the later extracts, confirming earlier results showing that nearly all are extracted by brief homogenization in iso-osmotic sodium acetate (Brandt & Muir, 1971*a*). Assuming that only minor amounts of the small proteoglycans remained in the tissue after the sodium acetate extractions, from the percentage of the total tissue uronic acid in the various extracts and the elution profiles of each on gel chromatography, it could be estimated that proteoglycans retarded on Sepharose 6B accounted for approx. 7% of the uronic acid in the normal cartilage and 11% of that in the osteoarthrotic cartilage.

In the above proteoglycans, galactosamine and glucosamine serve as markers for chondroitin sulphate and keratan sulphate respectively (Brandt & Muir, 1971*a*). Since glucosamine represented a high proportion of the total hexosamine, the proteoglycans in each of the extracts were notably rich in keratan sulphate. Proteoglycans wholly retarded by Sepharose 6B have been found to contain very little keratan sulphate in comparison with larger proteoglycans of the same tissue. No attempt was made in the present study to separate further the smaller proteoglycans from the larger, e.g. by preparative gel chromatography (Brandt & Muir, 1969), and the smaller proteoglycans contained some glucosamine. Nonetheless, glucosamine accounted for a lower proportion of the hexosamine in the proteoglycans retarded by Sepharose 6B than it did in the whole extracts. Moreover, the small proteoglycans of both the normal and arthrotic cartilage were immunologically identical with those having a glucosamine to galactosamine molar ratio of only 1:70. They differed, therefore, from the small proteoglycans extracted from articular cartilage of lame pigs (Šimůnek & Muir, 1972), whose chemical composition was the same as that of larger proteoglycans of the tissue.

In conclusion, although normal and osteoarthrotic cartilage appeared similar with respect to collagen content and glycosaminoglycans, a proportion of the proteoglycans of the diseased cartilage were less firmly held in the matrix than those of the normal. Both normal and arthrotic cartilage contained some proteoglycans, sufficiently small so as to be retarded on Sepharose 6B chromatography, which were relatively poor in keratan sulphate. The tissue content of the small proteoglycans of normal and diseased cartilage was similar, however, indicating that a factor other than hydrodynamic size was responsible for the enhanced extractability of proteoglycans of osteoarthrotic cartilage.

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Bjelle, A. O., Antonopoulos, C. A., Engfeldt, B. & Hjertquist, S. O. (1972) *Calcif. Tissue Res.* **8**, 237–246
Bollet, A. J. & Nance, J. L. (1966) *J. Clin. Invest.* **45**, 1170–1177
Bollet, A. J., Handy, J. R. & Sturgill, B. C. (1963) *J. Clin. Invest.* **42**, 853–859
Brandt, K. & Muir, H. (1969) *Biochem. J.* **114**, 871–876
Brandt, K. & Muir, H. (1971*a*) *Biochem. J.* **121**, 261–270
Brandt, K. & Muir, H. (1971*b*) *Biochem. J.* **123**, 747–755
Brandt, K. D., Tsiganos, C. P. & Muir, H. (1973) *Biochim. Biophys. Acta* **320**, 453–468
Hjertquist, S. O. & Wasteson, A. (1972) *Calcif. Tissue Res.* **10**, 31–37
Kempson, G. E., Muir, H., Swanson, S. A. V. & Freeman, M. A. R. (1970) *Biochim. Biophys. Acta* **215**, 70–77
Kraan, J. & Muir, H. (1957) *Biochem. J.* **66**, 55P
Lust, G. & Pronsky, W. (1972) *Clin. Chim. Acta* **39**, 281–286
Mankin, H. J. & Lippiello, L. (1971) *J. Clin. Invest.* **50**, 1712–1719
Mankin, H. J., Dorfman, H., Lippiello, L. & Zarins, A. (1971) *J. Bone Joint Surg. Amer. Vol.* **53**, 523–537
McDevitt, C. A., Muir, H. & Pond, M. J. (1973) *Biochem. Soc. Trans.* **1**, 287–289
Šimůnek, Z. & Muir, H. (1972) *Biochem. J.* **130**, 181–187
Sokoloff, L. (1969) *The Biology of Degenerative Joint Disease*, University of Chicago Press, Chicago
Tsiganos, C. P. & Muir, H. (1969) *Biochem. J.* **113**, 885–894