Biochemical changes in progressive osteoarthritis

M. B. E. SWEET, E. J.-M. A. THONAR, A. R. IMMELMAN, AND L. SOLOMON

From the Department of Orthopaedic Surgery, University of the Witwatersrand, Johannesburg, South Africa

SUMMARY Quantitative and qualitative variations in glycosaminoglycan content were studied in fibrillated, intact, and osteophytic cartilage of the human femoral head in osteoarthritis. Total glycosaminoglycan content was reduced in fibrillated, unchanged in intact, and raised in osteophytic cartilage. In fibrillated and osteophytic cartilage the ratio of chondroitin sulphate to keratan sulphate was high and therefore resembled immature cartilage.

Hyaluronic acid was present in reduced amount in all osteoarthrotic material. Proportionally more proteoglycans were extractable by 0.15 M NaCl and 4 M guanidinium chloride from the diseased cartilage than from normal cartilage, and all proteoglycans irrespective of buoyant density were carbohydrate deficient. It is postulated that the changes described are compatible with collagen and matrix disruption due to focal overloading and the general attempt at repair.

Osteoarthritis is characterized by articular cartilage destruction, subchondral bone changes which include cyst formation and sclerosis, and the appearance of new cartilage and osteophytes around the periphery of the articular surface.

Normal articular cartilage provides a smooth bearing and shock absorbing surface for the movable ends of long bones. The special characteristics of cartilage matrix are due to proteoglycans which lie in a fibrillar meshwork of collagen. These macromolecules consist of polyanionic polysaccharide chains of chondroitin sulphate and/or keratan sulphate covalently linked to a protein core. Their high negative charge density establishes a large surrounding electrostatic domain and retards the flow of interstitial water (Maroudas, 1973). These effects may be further magnified by formation of proteoglycan-hyaluronic acid aggregates (Hardingham and Muir, 1974).

In osteoarthrotic cartilage the glycosaminoglycan (GAG) content is reduced, particularly the keratan sulphate moiety (Mankin and Lippiello, 1971). In addition, there appears to be a relative increase in the amount of chondroitin 4-sulphate (Mankin and Lippiello, 1971) and a decrease in the GAG chain length (Bollet and Nance, 1966). Recently, McDevitt and Muir (1976) suggested that the proportion of

proteoglycans present as aggregates is decreased and that the water content is increased in early experimental canine osteoarthritis.

This paper describes the topographical distribution of the GAGs together with the results of sequential extraction and equilibrium density-gradient centrifugation of the proteoglycans in osteoarthritis of the human femoral head.

Methods

MATERIALS

Apart from the following, all chemicals were analytical grade: guanidinium chloride, galactosamine, glucosamine, glucuronolactone, cyanogum, agarose. Acetyl acetone was redistilled. A suspension of 2x crystallized papain was purchased from British Drug Houses, Poole, England. Testicular hyaluronidase was purchased from the same source.

TISSUE

Osteoarthrotic heads of femur of 3 patients aged 52, 56, and 60 years, were obtained soon after removal at operation. The pattern of osteoarthrotic changes was similar in all specimens studied (Fig. 1). Superiorly, on the weight-bearing surface, was an area denuded of cartilage (zone 1). This was surrounded by a ring of obviously fibrillated, but not discoloured cartilage (zone 2). Surrounding the fibrillated cartilage was a large area of unfibrillated but discoloured cartilage (zone 3). Around the edge of the head was heaped white nonfibrillated cartilage

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Correspondence to Dr. M. B. E. Sweet, Department of Orthopaedic Surgery, University of the Witwatersrand, Medical School, Hospital Street, Hillbrow, Johannesburg 2001, South Africa

Fig. 1 Diagrammatic representation of the different zones of articular cartilage of the femoral head in tilt deformity osteoarthritis. Zone 1 = superior loaded surface denuded of cartilage. Zone 2 = area of variable extent consisting of obviously fibrillated but not discoloured cartilage. Zone 3 = ring of unfibrillated but discoloured cartilage; Zone 4 = marginal osteophytes covered by white cartilage.

covering the osteophytes (zone 4). Cartilage from zones 2, 3, and 4 was removed and treated as described below.

Normal human cartilage was obtained from the femoral heads of 2 patients aged 56 and 62 years, after fracture of the femoral neck, and from the femoral condyle of one patient aged 45 years after a comminuted fracture of the knee. Immature articular cartilage was removed from the femoral condyle of calves aged 10–12 weeks immediately after slaughter. Representative slices were removed from the superficial articular cartilage from two areas on the medial femoral condyle: the loaded part of the joint which articulates with the tibia and the unloaded part of the joint adjacent to the patellar groove.

DETERMINATION OF WATER CONTENT

Small slices of cartilage from each zone were weighed immediately after removal and again after being dehydrated in successive changes of absolute ethanol, ethanol: ether (1:1), and ether, and dried *in vacuo* at 60°C.

PREPARATION OF GAGS

Known amounts of cartilage from each zone were

digested with papain (1 µg/mg of tissue) in 0.01 M sodium acetate pH 5.8, containing 0.01 M L-cysteine and 0.05 M EDTA for 4 h at 60°C (Scott, 1960). After precipitation by the addition of 3–4 volumes of ethanolic potassium acetate, the GAGs were resuspended in water and used for ion-exchange chromatography on Ecteola cellulose (Antonopoulos *et al.*, 1967), electrophoresis on cellulose acetate (Seno *et al.*, 1970; Larsson *et al.*, 1973), alkaline hydrolysis in 0.5 M KOH for 24 h at 22°C (Hopwood and Robinson, 1974), or further enzymatic digestion by testicular hyaluronidase (100 µg/100 mg hexosamine) in 0.2 M sodium acetate, pH 5.8, for 24 h.

PREPARATION OF PROTEOGLYCAN

Cartilage was subjected to sequential extraction in 0.15 M NaCl at 2°C for 3 h (Hardingham and Muir, 1974) followed by 4 M guanidinium chloride (GnHCl) in 0.05 M sodium acetate (pH 4.5) (Hardingham and Muir, 1974) for 48 h (Sajdera and Hascall, 1969). The unextractable residue was digested with papain as before. The uronic acid contents of each extract were determined.

Alternatively, cartilage from zone 2 or zone 3 was extracted in 4 M GnHCl for 48 h at 2°C with the addition of 0.01 M sodium EDTA, 0.1 M 6-amino-hexanoic acid, and 0.0005 M benzamidinium HCl as enzyme inhibitors (Oegema *et al.*, 1975). The extracts were clarified by centrifugation at 1000×g for 30 minutes. The molarity of the GnHCl was then adjusted to 0.4 by dialysis against 9 vol of 0.05 M sodium acetate, pH 5.8. The proteoglycans were fractionated after equilibrium centrifugation as described elsewhere (Sweet *et al.*, 1976).

CHROMATOGRAPHY ON ECTEOLA CELLULOSE

GAGs were fractionated on columns of Ecteola cellulose as described by Antonopoulos *et al.* (1967). After ethanol precipitation (3–4 vol ethanolic potassium acetate) the fractions were analysed for glucosamine and galactosamine or by electrophoresis on cellulose acetate (Seno *et al.*, 1970; Larsson *et al.*, 1973). Electrophoresis was performed before and after treatment of the fractionated GAGs with hyaluronidase or KOH. Furthermore, the 2.5 M HCOONa+H₂O and 6 M HCl fractions of zone 3 GAGs were isolated, treated with either hyaluronidase or KOH, and refractionated.

In addition, the GAGs prepared by papain digestion of zone 3 and eluted by 2.5 M HCOONa were hydrolysed and analysed for glucosamine and galactosamine before and after precipitation with cetylpyridinium chloride (10%). The GAGs in the supernate were precipitated by alcoholic potassium acetate (3 vol).

ELECTROPHORETIC TECHNIQUES

GAGs were subjected to electrophoresis on cellulose acetate strips by a modification (Larsson *et al.*, 1973) of the method of Seno *et al.* (1970). Samples were applied as an aqueous solution of the GAG (K salt) on the cellulose acetate strips soaked in 0.2 M calcium acetate (pH 7.25), diluted with ethylene glycol (3:2), and run at 250 V and 4–5 mA for 3½ h. The strips were stained in alcian blue and cleared.

Proteoglycans were analysed by polyacrylamide: agarose flat bed gel electrophoresis. A flat bed technique was used in preference to the disc technique described by McDevitt and Muir (1971) in order to compare multiple samples under identical conditions. Agarose, 0.48 g (Hoechst) was dissolved in 45 ml 0.04 M Tris-acetate buffer containing 1 mM sodium sulphate, pH 6.8, by heating (solution A). A second solution containing 0.96 g cyanogum (British Drug Houses) in 9.6 ml 6.4% (v/v in H₂O) dimethyl-amino propionitrile and 19.4 ml Tris buffer was mixed thoroughly (solution B). Solutions A and B were combined at 43°C and 6 ml 3% (w/v) ammonium persulphate (freshly prepared) was added. The solution was stirred quickly and poured into a flat bed gel mould of 150×100×3 mm. The solution was covered with a sheet of perspex and excess solution removed. The gel was left at 2°C for 1 h and then equilibrated in the Tris buffer. Electrophoresis was performed at 2°C for 36 h with constant voltage to produce an average current of 4 mA. Gels were stained in 0.2% toluidine blue in 0.1 M acetic acid for 3 h and left in water until cleared.

ANALYTICAL TECHNIQUES

Uronic acid was determined by an automated modification (M. B. E. Sweet and A. R. Immelman, unpublished) of the method of Bitter and Muir

(1962), using glucuronolactone as a standard. Hexosamine was determined by the method of Cessi and Piliego (1960), using galactosamine HCl and glucosamine HCl as standards. Samples were hydrolysed in 4 M HCl for 4 h at 100°C in sealed tubes that had been flushed with nitrogen. Protein was determined by an automated modification (Sweet and Immelman, unpublished) of the Folin method (Lowry *et al.*, 1951), using standardized human serum as a standard (South African Institute for Medical Research). Glucosamine and galactosamine were estimated on the 15 cm column of a Beckman 116 Automatic Amino Acid analyser after hydrolysis in 6 M HCl for 4 h at 100°C in sealed tubes flushed with nitrogen.

Results

Water content of articular cartilage from the three different zones of the osteoarthrotic femoral heads varied, but it was always slightly higher than values for normal human cartilage and lower than calf articular cartilage (Table 1). Uronic acid content of cartilage from zone 3 was slightly lower than that of normal articular cartilage, while that of zone 2 was significantly lower. Uronic acid content of zone 4 cartilage was higher than that of normal human cartilage, but similar to that of calf articular cartilage. Hexosamine content of the individual tissues did not run parallel with the uronic acid content in all cases.

The ratios of hexosamine to uronic acid suggested that zone 3 resembled adult human cartilage, while zones 2 and 4 resembled immature cartilage. Further, the differences in the ratio of the two hexosamines indicated qualitative differences in the three pathological areas. For example, osteophytic (zone 4) and fibrillated (zone 2) cartilage contained more galactos-

Table 1 Analysis of the matrix of human osteoarthrotic (OA) and normal articular cartilage, and of calf loaded (L) and unloaded (NL) cartilage. Results were obtained from tissue pooled from the number of individuals or animals indicated. The tissue pools consisted of an equivalent mass of wet tissue from the respective zones of each head of femur or from each normal or animal joint

	Tissue					
	OA zone 2	OA zone 3	OA zone 4	Normal human adult	Calf L	Calf NL
Water content (% of wet weight)	77.1	78.2	77.0	74.0	86.5	82.1
Uronic acid mg/100 mg dry weight	1.45	2.91	7.27	3.92	8.25	8.84
µmol/100 mg dry weight	7.47	14.99	37.45	20.17	42.49	45.53
Hexosamine mg/100 mg dry weight	1.70	6.88	8.80	5.81	9.89	9.08
µmol/100 mg dry weight	9.49	38.40	49.1	32.44	55.19	50.67
Hexosamine/uronic acid molar ratio	1.27	2.56	1.31	1.61	1.30	1.11
Galactosamine/glucosamine molar ratio	4.08	0.75	4.41	1.12	3.86	10.51
n	3	3	3	3	3	3

amine relative to glucosamine than normal adult cartilage. This point was clarified by Ecteola cellulose ion-exchange chromatography.

CHROMATOGRAPHY ON ECTEOLA CELLULOSE

The results of the Ecteola fractionation (Table 2) can be discussed from two points of view; namely, the relative proportions of the hexosamines eluted by each solution, and the absolute amounts of galactosamine and glucosamine as $\mu\text{mol}/100\text{ mg}$ tissue present in each fraction.

(a) Relative proportions of galactosamine and glucosamine in Ecteola fractions

Glycoproteins are eluted from Ecteola columns by H_2O and 0.02 M HCl (Antonopoulos *et al.*, 1967). Less material was eluted in zones 2 and 4 than in the other tissues studied. The proportional amounts of glucosamine eluted by 0.3 M NaCl (hyaluronic acid) were similar in most samples analysed apart from normal human articular cartilage. Glucosamine was slightly raised in zone 2. Some galactosamine was also eluted by 0.3 M NaCl, and this may represent undersulphated and/or short-chain chondroitin sulphate (Bjelle *et al.*, 1972). Less galactosamine was recovered from the osteoarthrotic tissues than from immature cartilage.

The major hexosamine eluted by 2.5 M $\text{HCOONa} + \text{H}_2\text{O}$ was galactosamine, which represents chondroitin sulphate. The proportion of galactosamine was similar in zone 2 (76.4%) and zone 4 (76.2%) and in calf loaded cartilage (74.5%). Only 37.8% was present in zone 3, which therefore resembled normal adult cartilage (47.1%) in which keratan sulphate had become a major GAG at the expense of chondroitin sulphate. An unexpected finding,

however, was the large amount of glucosamine recovered from the 2.5 M $\text{HCOONa} + \text{H}_2\text{O}$ fractions. In zone 3 this accounted for 30.1% of the total hexosamine and in normal articular cartilage for 20.3%. Although proportionately less glucosamine was recovered from zones 2 and 3 and calf loaded cartilage, the percentage was significant (11%–13%). Notably, this glucosamine was consistently absent, or present only in small amounts, in calf unloaded cartilage. Similarly, a mixture of hexosamines was eluted by 6.0 M HCl. Only zone 3 (19%) and normal human cartilage contained significant proportions of glucosamine.

(b) Galactosamine and glucosamine as $\mu\text{mol}/100\text{ mg}$ tissue

The results indicate a general loss of hexosamine from zone 2 cartilage. The concentration of each hexosamine present in each fraction of zone 4 closely resembled the pattern obtained for calf cartilage. The concentration of galactosamine (2.5 M $\text{HCOONa} + \text{H}_2\text{O}$ fraction) of zone 3 was significantly lower than in zone 4, but similar to normal adult cartilage. In addition, more glucosamine (in both 2.5 M HCOONa and H_2O and 6.0 M HCl fractions) was recovered from zone 3 than from any other tissue.

Thus, from (a), it seems that zone 2 and zone 3 bear a qualitative resemblance to immature cartilage and that zone 3 remains adult in nature. However, in (b), zone 2 in particular was hexosamine depleted and zone 3 was glucosamine enriched.

In view of the heterogeneity of the 2.5 M HCOONa and H_2O Ecteola fraction, the identity of the GAGs present required further elucidation. The

Table 2 Results of fractionation on Ecteola cellulose of glycosaminoglycans prepared from papain digests of osteoarthrotic (OA), normal adult, and calf loaded (L) and unloaded (NL) articular cartilage as in Table 1. Results are expressed as percentages of the total hexosamine content of each digest and, in parentheses as $\mu\text{mol}/100\text{ mg}$ dry tissue. The figures represent the means of at least two determinations

Eluent	Hexosamine	Tissue					
		OA zone 2	OA zone 3	OA zone 4	Normal human adult	Calf L	Calf NL
$\text{H}_2\text{O} + 0.02\text{ M HCl}$	Galactosamine	0.18 (0.02)	0.63 (0.23)	trace	0.62 (0.19)	1.63 (0.89)	1.70 (0.85)
	Glucosamine	0.8 (0.07)	5.73 (2.12)	trace	3.74 (1.17)	5.05 (2.74)	4.27 (2.14)
0.3 M NaCl	Galactosamine	0.71 (0.07)	0.58 (0.21)	0.84 (0.40)	1.86 (0.58)	1.37 (0.74)	1.19 (0.60)
	Glucosamine	2.81 (0.26)	2.18 (0.81)	2.26 (1.08)	13.84 (4.33)	1.97 (1.07)	1.14 (0.57)
2.5 M $\text{HCOONa} + \text{H}_2\text{O}$	Galactosamine	76.44 (7.12)	37.76 (13.97)	76.24 (36.34)	47.05 (14.73)	74.55 (40.51)	86.35 (43.33)
	Glucosamine	10.84 (1.01)	30.13 (11.15)	13.72 (6.54)	20.34 (6.37)	12.94 (7.03)	2.67 (1.34)
6 M HCl	Galactosamine	3.00 (0.28)	3.91 (1.45)	4.41 (2.10)	3.4 (1.07)	1.86 (1.01)	2.07 (1.04)
	Glucosamine	5.22 (0.49)	19.05 (7.05)	2.51 (1.20)	9.15 (2.87)	0.62 (0.34)	0.61 (0.31)

GAGs (zone 2) eluted by 2.5 M HCOONa + H₂O and those eluted by 6 M HCl were precipitated with ethanolic potassium acetate, isolated, digested with testicular hyaluronidase or treated with 0.5 M KOH, and subjected to a second fractionation on Ecteola cellulose (Table 3).

It is clear from the data in Table 3 that a substantial proportion of the galactosamine-containing GAG eluted by 2.5 M HCOONa + H₂O during the first fractionation was hyaluronidase sensitive. The hyaluronidase-sensitive GAG was subsequently eluted by H₂O and 0.02 M HCl as well as by 0.3 M NaCl, probably as chondroitin sulphate oligosaccharides. The glucosamine-containing GAG was largely unaffected by KOH treatment. However, some 30% of the glucosamine-containing GAG originally eluted by 2.5 M HCOONa + H₂O was recovered in the 6 M HCl fraction after treatment with KOH. The galactosamine and glucosamine-containing GAGs in the 6 M HCl fraction were largely unaffected by hyaluronidase or KOH treatment (Table 3).

Table 3 Refractionation of glycosaminoglycans of 2.5 M HCOONa + H₂O and 6 M HCl Ecteola fractions of zone 3 after the glycosaminoglycans had been recovered and treated with testicular hyaluronidase or 0.5 M KOH (see text). Results are expressed as $\mu\text{mol}/100\text{ mg}$ dry tissue and as percentages of the respective untreated hexosamines

Digestion procedure	Hexosamine	2.5 M HCOONa + H ₂ O fraction	6 M HCl fraction
Untreated material	Galactosamine	13.97	1.45
	Glucosamine	11.15	7.05
	molar ratio	1.25	0.21
Hyaluronidase	Galactosamine	6.82 (49)	1.38 (95)
	Glucosamine	10.21 (92)	6.85 (97)
	molar ratio	0.67	0.20
0.5 M KOH	Galactosamine	12.62 (90)	1.32 (91)
	Glucosamine	7.78 (70)	6.63 (94)
	molar ratio	1.62	0.20

These results suggest the presence of some chondroitin sulphate: keratan sulphate doublets in the 2.5 M HCOONa and H₂O fraction (Seno *et al.*, 1965). Chondroitin sulphate is precipitated by cetylpyridinium chloride, whereas free keratan sulphate is not (Scott, 1970). Thus the glucosamine-containing GAG eluted by 2.5 M HCOONa + H₂O that was co-precipitated with galactosamine by cetylpyridinium chloride is likely to have been linked to a common peptide with chondroitin sulphate (Table 4). As expected, nearly all the chondroitin sulphate (90% of the galactosamine eluted) was precipitated, but a small amount was recovered in the supernatant.

A single galactosamine residue forms the linkage region between the repeating sequence of keratan sulphate and the core protein (Hopwood and Robinson, 1974). Thus the galactosamine present in the supernatant was doubtless part of the free keratan sulphate and the galactosamine to glucosamine ratio of 0.17 is consistent with a chain length of about 6 repeating periods. The fact that a similar value was obtained for the material eluted by 6 M HCl indicates a similar average size for keratan sulphate and suggests that the free keratan sulphate eluted at the lower ionic strength was eluted for reasons other than shorter chain length, e.g. lower sulphate content.

ELECTROPHORESIS

Ecteola fractions 1 and 2 (H₂O and 0.02 M HCl) Glycoproteins could not be detected by cellulose acetate electrophoresis in calcium acetate: ethylene glycol, pH 7.25.

Ecteola fraction 3 (0.3 M NaCl)

A band with the electrophoretic mobility of hyaluronate was present in all extracts electrophoresed. The intensity of the stained band was proportional to the amount of glucosamine eluted by 0.3 M NaCl. A second slower moving band was occasionally seen and probably represented undersulphated

Table 4 Cetylpyridinium chloride (CPC) and ethanolic potassium acetate precipitation of the glycosaminoglycans eluted by 2.5 M HCOONa + H₂O from a papain digest of zone 3 cartilage. Results are expressed as $\mu\text{mol}/100\text{ mg}$ dry tissue in the original fraction, the respective precipitates and supernatants as the respective percentages of each hexosamine recovered

	Galactosamine ($\mu\text{mol}/100\text{ mg}$ dry tissue)	Glucosamine ($\mu\text{mol}/100\text{ mg}$ dry tissue)	% total hexosamine	% original galactosamine	% original glucosamine	Galactosamine/glucosamine molar ratio
Untreated	13.97	11.15	100	100	100	1.25
CPC precipitate	12.56	3.58	64.2	89.9	32.1	3.51
CPC supernate	1.26	7.44	34.6	9.02	66.7	0.17
Ethanolic potassium acetate precipitate	13.82	11.00	98.8	98.9	98.7	1.26
Ethanolic potassium acetate supernatant	0.03	0.06	0.36	0.21	0.54	0.50

chondroitin sulphate. Both GAGs were hyaluronidase sensitive but were not affected by KOH treatment.

Ecteola fractions 4 and 5 (2.5 M HCOONa and H₂O) GAGs eluted by 2.5 M HCOONa migrated as one band. The relative mobility of this band differed from tissue to tissue. In zone 3 the band had a mobility similar to that in normal adult cartilage, slightly faster than chondroitin 6-sulphate, but slower than keratan sulphate. In zones 2 and 4 the GAGs migrated more slowly, with the same mobility as calf articular cartilage or chondroitin 4-sulphate. In calf cartilage this band was only just detectable after treatment with hyaluronidase. In the osteoarthrotic and normal adult fractions, however, this band was less sensitive to hyaluronidase and its relative mobility was unaffected. KOH treatment affected neither the staining intensity nor the mobility of GAGs eluted by 2.5 M HCOONa.

GAGs of zone 3 were precipitated by 10% cetylpyridinium chloride; GAGs in the supernate were then precipitated by ethanolic potassium acetate. Both precipitates were subjected to electrophoresis. It is clear from Fig. 2 that the total GAGs eluted by 2.5 M HCOONa + H₂O had an electrophoretic mobility minimally faster than standard chondroitin 6-sulphate. The band was also broader and some trailing was observed. The cetylpyridinium chloride precipitate, however, moved as a far more discrete band, definitely faster than standard chondroitin 6-sulphate but not quite as fast as standard keratan sulphate. GAGs remaining in the cetylpyridinium chloride supernate moved slightly faster than the total GAGs present in the fraction and showed similar trailing.

Ecteola fraction 6 (6.0 M HCl)

In normal articular cartilage a band with the electrophoretic mobility of keratan sulphate was detected. In osteoarthrotic cartilage, on the other hand, the mobility was variable and slightly slower, suggesting that in the osteoarthrotic extracts the keratan

Fig. 2 Electrophoresis of glycosaminoglycans (GAG_{tot}) prepared from the 2.5 M HCOONa + H₂O fraction (zone 3) and the cetylpyridinium chloride precipitate (CPC_p) and supernatant thereof (CPC_s). International reference standards of chondroitin 4-sulphate (CS₄), chondroitin 6-sulphate (CS₆), hyaluronic acid (HA), and keratan sulphate (KS) were a gift from Dr. Martin B. Mathews.

sulphate eluted by 6 M HCl was slightly under-sulphated. Too little keratan sulphate was present in calf immature articular cartilage to be detected on the cellulose acetate strips. Neither hyaluronidase nor KOH treatment affected either staining intensity or mobility of the GAG present.

SEQUENTIAL EXTRACTION

The proportion and absolute amounts of uronic acid present in each of the sequential extracts are set out in Table 5. Although only a small proportion of the

material was present in the 0.15 M NaCl extract of zone 2 and zone 3, the percentage of 0.15 M NaCl extractable uronic acid was significantly lower in the other tissues. Generally, more uronic acid was extractable from osteoarthrotic cartilage with 4 M GnHCl than from normal adult cartilage. The results, however, were variable (Table 5): zone 4 cartilage was the most extractable and zone 2 the least extractable of the pathological material.

PROTEOGLYCANS

Purified (A1) (Hascall and Heinegard, 1974) proteoglycans which had been extracted with 4 M GnHCl from normal and zone 2 and zone 3 cartilage were fractionated on a dissociative density gradient. The distribution of uronic acid throughout the gradient at equilibrium was similar (Table 6), but the ratio of uronic acid to protein differed in each case (Fig. 3). All proteoglycans of osteoarthrotic cartilage had a lower ratio of uronic acid to protein than that of the proteoglycans of normal cartilage of equivalent buoyant density. Proteoglycans of zone 2 were poorer in carbohydrate than those of zone 3, suggesting that all proteoglycans of osteoarthrotic cartilage are carbohydrate depleted.

Polyacrylamide: agarose gel electrophoresis of

Table 5 Sequential extraction of proteoglycans of osteoarthrotic, normal human adult, and pooled calf articular cartilage. Results are expressed as percentages of the total uronic acid in each tissue (in parentheses) and as $\mu\text{mol/g}$ wet tissue

Extracting solution	Tissue				
	OA zone 2	OA zone 3	OA zone 4	Normal human adult	Calf L + NL pool
0.15 M NaCl	0.82 (5)	0.78 (2.4)	2.43 (2.9)	0.41 (0.8)	1.06 (1.5)
4 M GnHCl	8.66 (52.7)	20.45 (63.0)	69.08 (82.5)	25.61 (49.6)	59.98 (85.2)
Residue	6.95 (42.3)	11.23 (34.6)	12.23 (14.6)	25.61 (49.6)	9.36 (13.3)

Table 6 Percentages of uronic acid and protein distributed through a dissociative density gradient at equilibrium. Proteoglycans from normal and zone 2 and zone 3 osteoarthrotic cartilage were analysed as described in the text

	Normal		OA zone 2		OA zone 3	
	Uronic acid	Protein	Uronic acid	Protein	Uronic acid	Protein
P > 1.57 g/ml	79.17	63.40	78.77	40.64	77.63	54.40
P = 1.43 - 1.57 g/ml	16.25	25.12	18.29	36.27	20.18	31.37
P < 1.43 g/ml	4.58	11.48	2.94	23.09	2.19	14.23

Fig. 3 Equilibrium density-gradient centrifugation of proteoglycans in 4 M GnHCl, CsCl, $\rho_0 = 1.50$ g/ml after purification in an associative gradient. Ratios of uronic acid to protein of each fraction are given for zone 2 ●, zone 3 ○, and normal adult cartilage ▲.

zone 2 and zone 3 type proteoglycan monomers (A1–D1) (Hascall and Heinegard, 1974) showed that the former migrated faster than the latter. Both monomers migrated faster and displayed greater heterogeneity than normal articular cartilage monomer. This suggests that the reduction in the ratio of uronic acid to protein is accompanied by a diminution in hydrodynamic size.

Discussion

The clinical condition of osteoarthritis is characterized by progressive pain and limitation of movement in a joint. Initially the pathological changes are focal. Cartilage destruction and fibrillation spread to the remaining apparently normal cartilage, and at the margin of the joint cartilage-covered osteophytes appear. There is loss of GAG but no corresponding loss of collagen. The precise pathogenesis of the disease is not known, but a large body of biochemical evidence supports the hypothesis that proteolysis by lysosomal enzymes is partly responsible for the loss of proteoglycan from cartilage (Woessner, 1973; Ali and Evans, 1973; Erhlich *et al.*, 1973; Sapolsky *et al.*, 1973). This loss appears to be proportional to the severity of the disease (Mankin *et al.*, 1971). On the other hand, Mankin and Lippiello (1970) have shown that the rate of proteoglycan synthesis in osteoarthritis, with the exception of very severe lesions, is significantly higher than in normal adult cartilage, and that this increased rate of synthesis is also proportional to the severity of the disease. Only in very severe lesions does chondrocyte failure occur. It has become apparent too that the type of GAG synthesized in osteoarthritis is different, and that an immature form of matrix rich in chondroitin 4-sulphate and poor in keratan sulphate is produced (Mankin and Lippiello, 1971). Variations in the type of collagen synthesized have also been reported (Nimni and Deshmukh, 1973).

Recently, McDevitt and Muir (1976) studied the matrix components of experimental canine osteoarthritis, and showed by sequential extraction that proteoglycans are more readily extractable by isotonic salt solutions. They suggested that this might be the consequence of a relative inability of osteoarthrotic proteoglycans to form aggregates with hyaluronic acid. They also showed, by comparing the opposite normal knee joint, that the water content of the intact and minimally fibrillated cartilage was about 6% higher than normal. Experimentally induced osteoarthritis has been criticized because the lesions produced are not similar to those of true osteoarthritis and because the methods are not comparable to the breakdown of articular cartilage in osteoarthritis (Bentley, 1975). However, the

results of the experimental study of McDevitt and Muir (1976) are in general agreement with the results obtained for natural canine osteoarthritis and with some of our results. Therefore the model is likely to prove valuable and the results provide a useful basis for comparison.

The changes reported by McDevitt and Muir (1976) occurred without any obvious change in the structure of the surface of the articular cartilage, and increased extractability of proteoglycans was found in the absence of fibrillation. They proposed that an initial chondrocyte malfunction might precede the changes in the interfibrillar matrix and lead to fibrillation. On the other hand, Freeman and Meachim (1973) suggested that fibrillation may occur in the absence of GAG depletion, and that it is due to fatigue failure of collagen in cartilage subjected to high local pressure. This may then lead to simple diffusion of proteoglycan out of cartilage and subsequent extension of the disease. However, not all areas of fibrillation proceed to osteoarthritis and these nonprogressive lesions may be a manifestation of aging (Byers *et al.*, 1970).

Most workers who have studied GAG content of articular cartilage in relation to aging or to osteoarthritis have used pooled cartilage (Bollet and Nance, 1966; Stockwell and Scott, 1967; Mankin and Lippiello, 1971). Maroudas *et al.* (1973), however, found that the topographical variation in fixed charge density (and therefore GAG content) over the femoral head correlates closely with fibrillation. Fibrillated areas always have a lower fixed charge density than normal. In support of the hypothesis of Freeman and Meachim (1973), Maroudas *et al.* (1973) found that degeneration in one area has no effect on fixed charge density elsewhere on the femoral head.

TOPOGRAPHICAL DISTRIBUTION OF CHONDROITIN SULPHATE AND KERATAN SULPHATE

Fibrillated articular cartilage (zone 2) was the only tissue which was severely GAG-depleted. The GAG content of zone 3 was comparable to that of normal adult cartilage, while the cartilage covering the osteophytes had a high GAG content similar to that of immature articular cartilage. The ratio of chondroitin sulphate to keratan sulphate was similar in zones 2 and 4, resembling immature cartilage. Zone 3 was rich in keratan sulphate and therefore resembled normal adult cartilage. The small increase in the absolute amount of keratan sulphate in zone 3 as compared to normal adult articular cartilage may indicate a higher local synthesis of keratan sulphate. Normally there is a time-related shift from the synthesis of chondroitin 4-sulphate to chondroitin 6-sulphate and keratan sulphate (Mathews and

Glagov, 1966). With aging, chondroitin 4-sulphate is gradually replaced by chondroitin 6-sulphate. Infrared analysis of GAGs was not possible in this study but the electrophoretic data suggested that chondroitin 4-sulphate was the predominant isomer present in both zone 2 and zone 4. Thus it seems that chondroitin 4-sulphate is produced by chondrocytes not only in severely GAG-depleted cartilage but also in cartilage covering the marginal osteophytes. In addition, the presence of chondroitin 4-sulphate was associated with a low keratan sulphate content. This altered synthesis may represent an attempt at repair. The fact that cartilage rich in the 4-sulphate isomer overlies the osteophytes is not surprising in view of the association between this isomer and ossification (Mathews, 1975).

Clearly the keratan sulphate of osteoarthrotic cartilage was heterogeneous and present in different forms. About 40% was eluted by 6 M HCl, and although of average short chain, was possibly normally sulphated. Another 40% was eluted by 2.5 M HCOONa unassociated with chondroitin sulphate, and was of similar average short chain, had a slower electrophoretic mobility than normal, and was likely to be undersulphated. A third form of keratan sulphate (20%) was associated with chondroitin sulphate as a keratan sulphate-peptide-chondroitin sulphate *mélange* and was probably normally sulphated owing to its elution by 6 M HCl after treatment with 0.5 M KOH. The glycosaminoglycans (2.5 M HCOONa + H₂O) of zones 2 and 4 were not investigated in the same detail, but it is notable that much less glucosamine was recovered from these respective fractions. Bollet and Nance (1966) reported a fall in keratan sulphate in 'advanced' lesions, but a normal content in 'mild' lesions. In addition, more keratan sulphate (2.5 M HCOONa + H₂O fraction) was recovered from tissue rich in chondroitin 6-sulphate (zone 3) than from tissues rich in the 4-sulphate isomer (zones 2 and 4, calf loaded and unloaded cartilage). This agrees with the work of Seno *et al.* (1965).

HYALURONIC ACID AND PROTEOGLYCAN EXTRACTABILITY

As a result of the work of Hardingham and Muir (1974), Gregory (1973), and Hascall and Heinegard (1974), hyaluronic acid has been ascribed an important role in cartilage, namely that of an aggregating factor with proteoglycan monomer. Non-aggregated proteoglycans are readily extractable by iso-osmotic NaCl while dissociative solvents such as 4 M GnHCl and 2 M CaCl₂ are necessary for aggregate extraction. Some proteoglycans resist extraction and are evidently firmly bound to collagen. The proportion of proteoglycans extracted by 0.15

M NaCl was slightly higher in osteoarthrotic material than in normal adult or calf articular cartilage. However, the amounts extracted were comparatively small and not as high as the results obtained by McDevitt and Muir (1976) in experimental canine osteoarthritis. The osteoarthrotic joints studied here were invariably the site of a synovial effusion. Chondroitin sulphate has been detected in bovine synovial fluid (Silpananta *et al.*, 1967) and in human osteoarthrotic synovial fluid (Sweet, unpublished): although some of the chondroitin sulphate was probably synthesized by synovial membrane cells, it is possible that a dilute synovial fluid can extract nonaggregated proteoglycan and cause an apparent decrease in the content of this molecular species in cartilage. In addition, both zones 2 and 3 were more extractable by 4 M GnHCl and had less proteoglycan in the unextractable residue than normal adult cartilage. Not surprisingly, the extractability profile of zone 4 closely resembled immature calf cartilage. The sequential extraction procedure of McDevitt and Muir (1976) differed from ours after the first step, and they were able to show a general decrease in the proportion of proteoglycan monomers forming aggregates.

The decreased hyaluronic acid content in all osteoarthrotic cartilage in our study suggests that there may be insufficient hyaluronic acid to interact with all the protein core present. The effect of the low hyaluronic acid content may be further aggravated by the fact that the reduction in carbohydrate content of cartilage is not accompanied by a corresponding fall in protein content (Fig. 3), assuming the hyaluronic acid binding region of the protein core remains intact. However, this section of the 'core' protein may be deficient in fibrillated cartilage (Palmoski and Brandt, 1976). The presence of fewer aggregates and more monomeric proteoglycans in osteoarthrotic cartilage will result in an increased osmotic pressure which may partly account for the slightly higher water content. Maroudas (1976) has shown that degenerate cartilage swells more easily than normal cartilage because of a failure in the elastic restraint of the collagen network. The cartilage of zone 4 was also overhydrated and it is possible, therefore, that both factors play a part in increasing the water content. However, the water content of the pathological tissue was not as high as that of the immature cartilage in which the hyaluronic acid content was also low but where the GAG content was much higher.

The type of osteoarthritis we are considering is thought to result from failure of normal cartilage under abnormal load. The initial step might well be collagen disruption and/or fracture. This is accompanied, even at an early stage, by

cartilage repair. It is postulated that the balance between destruction and repair determines whether the changes are progressive or not. In zone 2 destruction outruns repair; in zone 3 these processes are more or less in equilibrium. If zone 4 represents a state of repair without any corresponding destruction, then the pattern of GAGs found in osteophytic cartilage must represent the newly synthesized 'repair' GAGs. The relative depletion of hyaluronic acid was noted in all zones of osteoarthrotic cartilage. Immature articular cartilage had a similar low hyaluronic acid content. Thus a reduction in the hyaluronic acid content of zone 3 might represent an early repair phenomenon rather than indicate selective enzymatic activity. Free hyaluronic acid has been shown to depress the incorporation of ^{35}S -sulphate into chondrocyte suspensions (Wiebkin *et al.* 1975; Wiebkin and Muir, 1976). A low hyaluronic acid content may be associated with more rapid proteoglycan synthesis and, therefore, repair.

PROTEOGLYCANS

The proteoglycans of osteoarthrotic cartilage were all, irrespective of buoyant density, carbohydrate deficient. This agrees with the findings of Altman *et al.* (1973). In addition, the proteoglycans were smaller than those of normal cartilage. On polyacrylamide: agarose electrophoresis proteoglycans of zone 2, and to a lesser extent zone 3, migrated faster than those of normal cartilage. McDevitt *et al.* (1973) reported changes in hydrodynamic size of proteoglycans in experimental canine osteoarthritis. Furthermore, the chain length of chondroitin sulphate in osteoarthritis is short (Bollet and Nance, 1966). On the other hand, proteoglycans extracted from fibrillated and nonfibrillated areas of bovine knee joints are of similar hydrodynamic size (Brandt, 1974), but these are likely to have been nonprogressive lesions. We suggest that where repair fails to keep step with loss or degradation, qualitatively inferior proteoglycans are produced to a greater or lesser extent. As the low hyaluronic acid content throughout osteoarthrotic cartilage suggests a general change in synthesis it may be that the differences noted in the proteoglycans represent to a certain extent similar attempts at repair.

The changes described in osteoarthritis of the femoral head may be summarized as follows: (i) GAG depletion of zone 2; (ii) carbohydrate-deficient proteoglycans; (iii) increased ratio of keratan sulphate to chondroitin sulphate in zone 3; (iv) fewer aggregates (McDevitt and Muir, 1976); (v) decreased hyaluronic acid content; (vi) decreased ratio of keratan sulphate to chondroitin sulphate in zones 2 and 4; (vii) increased total GAG content in zone 4; (viii) synthesis of chondroitin 4-sulphate, unassoci-

ated with keratan sulphate as a *mélange* in zones 2 and 4.

Items *i*, *ii*, and *iii* may be the result of enzymatic degradation but this is uncertain. For example, the severe fibrillation of zone 2 may result in excessive 'leakage' of proteoglycan (Freeman and Meacham, 1973). The small size of the proteoglycans may be associated with the predominance of chondroitin 4-sulphate (Lohmander, 1975). Items *iv* and *v* are intermediate and could be the result of the altered synthesis of repair or degradation. Items *vi*, *vii*, and *viii* are almost certainly the result of altered synthesis. The newly synthesized matrix may be inappropriate in the adult, be unable to bear load adequately and, moreover be more susceptible to proteolytic digestion. The immature matrix of zone 4 appeared to be intact, but then it is loaded only minimally or not at all. Many lysosomal systems have been proposed in osteoarthritis (Ali and Evans, 1973; Sapolsky *et al.*, 1973). More recently, a system (the hyaluronidase, β -glucuronidase, and β -N-acetylhexosaminidase) has been shown in synovial fluid of osteoarthritis (Stephens *et al.*, 1975) which could have produced some of the changes attributable to enzymatic degradation. However, no enzymatic degradation of zone 4 seems to have occurred.

In the light of our findings and the arguments outlined above we conclude that enzymatic activity is unlikely to be the sole or even the major factor in the pathogenesis of progressive osteoarthritis and that the biochemical changes are much more compatible with collagen and matrix disruption due to focal overloading in one part of the joint and attempted repair elsewhere.

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