Changes with age in the glycosaminoglycans of human articular cartilage

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SUMMARY Human articular cartilage was obtained post mortem from the lateral femoral condyles of 30 subjects aged from under 1 to 70 years. Cryostat sections taken 0–100 μm and 900–100 μm deep to the cartilage surface were exhaustively extracted to recover the glycosaminoglycans (GAG). After fractionation by cellulose acetate electrophoresis and enzyme depolymerisation individual GAG were determined by alcian blue 0.05 M MgCl_2 and disaccharide microassay procedures. Changes with age were observed in GAG concentration and in the proportion of individual GAG. Large alterations occurred during the period of skeletal growth (0–16y). At birth GAG formed about 50% of the dry weight of cartilage, a value that decreased to about 15% in adult cartilage. Chondroitin sulphates (ChS) formed the principal GAG of articular cartilage and accounted for almost all of the GAG of the infant material. The ChS decreased with age and were partially replaced by keratan sulphate (KS), so that KS eventually comprised 12% of the GAG. Hyaluronic acid (HA) was identified and was found to increase linearly with age to form 6% by weight of the cartilage GAG by 60y.

Histochemical evidence indicates that the composition of the matrix of human articular cartilage changes during age and in disease (Stockwell and Scott, 1965; Elliott and Gardner 1978a). Biochemical studies of changes in the nature and quantity of cartilage matrix glycosaminoglycans (GAG) during aging have also been obtained in man (Matthews, 1953; Anderson *et al.*, 1964; Rosenberg, *et al.*, 1965; Muir *et al.*, 1970; Stockwell, 1970; Mankin *et al.*, 1971; Hjertquist and Lemperg, 1972; Bjelle *et al.*, 1972; Altman, *et al.* 1973; Greiling and Baumann, 1973; Stuhlsatz, 1973; Venn, 1978) and in the articular cartilage of other vertebrates (Balazs *et al.*, 1966; Mankin and Lipiello, 1969; Lust *et al.*, 1972; Muthiah and Kuhn, 1973; Lemperg *et al.*, 1974; McDevitt and Muir, 1976). Many of these studies were based on hexosamine assays and the age range, and sample numbers have often been limited.

Stockwell and Scott (1967) segregated cartilage into morphologically defined zonal depths prior to analysis. In this investigation thin cartilage sections were prepared tangential to the articular surface from human material with an age range from birth to old age. The GAG following extraction were fractionated

and assayed by recently developed micro-procedures (Elliott and Gardner, 1977, 1978 b, c).

Materials and methods

Collection of articular cartilage. Whole femurs were obtained from the right leg of 14 male and 16 female cadavers within 30 h of death (Table 1). Obese subjects and those with a history of prolonged immobilisation, of trauma, or of other diseases related to the hip or knee joints or of metabolic or endocrine abnormality were avoided.

Sampling of cartilage for analysis. Femurs were orientated by Armstrong and Gardner's (1977b) modification of the method described by Kempson *et al.* (1971) to determine the condylar site at which loading was initially applied in vivo. The marked condyles were removed from the bone shaft with a band saw. The lateral condyle was placed in a wood-worker's vice, and a $2.5 \times 1.5\text{ cm}^2$ rectangle of cartilage, with the initial load bearing point in the centre, was delineated with a surgical scalpel. The cartilage was removed from the condyle with a finely honed wood chisel applied at a 45° angle. Cartilage blocks, trimmed to $20 \times 10\text{ mm}^2$, were placed on the electrically cooled stage of a microtome with the articular cartilage surface face down. The block was trimmed with the microtome knife set at

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Table 1. The glycosaminoglycan composition (as $\mu\text{g}/\text{mg}$ of dry cartilage) of superficial zone (0 to 100 μm depth) human articular cartilage samples aged from 0 to 70 years

Sample no.	Age (y)	Electrophoresis AB-0.05 M MgCl_2				Disaccharide assay ChS ($\mu\text{g}/\text{mg}$)
		Total GAG	ChS ($\mu\text{g}/\text{mg}$)	KS	HA	
1	0	256.79	252.17	4.62	<0.5	200.25
2	0.15	393.92	391.16	2.76	<0.5	126.79
3	0.15	416.88	394.48	22.40	<0.5	203.20
4	2	312.35	294.86	17.49	<0.5	188.14
5	3.5	239.01	231.18	6.17	1.66	244.83
6	8	176.58	161.04	15.54	<0.5	107.11
7	10	130.77	116.65	14.12	<0.5	122.35
8	12	82.20	74.64	7.56	<0.5	90.03
9	12	119.33	100.25	17.14	1.94	116.21
10	13	130.72	108.79	19.65	2.28	126.29
11	18	126.30	95.59	26.69	1.02	95.04
12	18	137.79	97.49	37.73	2.57	99.67
13	20	125.21	100.03	23.20	1.98	74.59
14	20	134.16	119.46	12.39	2.31	111.23
15	22	137.06	115.86	19.17	2.03	116.26
16	26	96.07	80.58	10.57	4.92	85.98
17	32	105.25	72.91	30.36	1.98	73.88
18	36	107.51	70.98	34.18	2.35	110.06
19	37	165.13	140.51	20.99	3.63	136.64
20	39	126.62	102.24	18.47	5.91	88.21
21	43	78.28	61.70	9.63	6.95	57.84
22	43	105.89	82.39	16.16	7.34	80.18
23	46	76.90	58.28	14.94	3.68	68.47
24	47	91.82	63.02	25.24	3.56	68.58
25	51	68.91	47.05	17.85	4.01	58.08
26	53	141.34	110.21	28.77	2.36	121.76
27	55	196.46	145.61	47.76	3.09	191.67
28	56	164.21	134.64	21.56	8.01	116.50
29	68	74.26	58.08	11.39	4.79	35.63
30	70	83.30	69.64	10.41	3.25	75.23

The extracted GAGs were fractionated by cellulose acetate electrophoresis (pyridine-formic acid buffer, pH 4.00, 20 mA for 90 min) and stained with 0.2% (w/v) alcian blue in 50 mM acetate buffer, pH 5.8, containing 0.05M MgCl_2 for 60 min. The characteristically stained band of hyaluronic acid and the broad band containing chondroitin sulphates (ChS) and keratan sulphate (KS) (Fig. 2) were measured by densitometry. After removal of ChS by chondroitin ABC lyase (EC 4.2.2.4) the single band of KS was determined. The ChS concentration was obtained (1) from the difference before and after treatment with chondroitin ABC lyase and (2) with the acid periodate—thiobarbituric acid assay (Elliott and Gardner, 1977).

20 μm advancement until a planar surface was obtained. The cartilage block was thawed and then reversed with the surface layer of cartilage now uppermost, refrozen, and 100 μm serial sections made. The first section (0 to 100 μm deep to the surface) and the tenth section (900 to 1000 μm deep to the surface) were freeze-dried and the dry weights determined.

Glycosaminoglycan reference material. Standard GAG were obtained from several commercial suppliers; they frequently contained contaminating material, including other GAG. The GAG preparations were therefore purified by cellulose acetate electrophoresis fractionation (Elliott and Gardner, 1978c). Keratan sulphate (KS II type) was prepared from human nucleus pulposus obtained post mortem from cadavers aged in excess of 70 y.

The KS was extracted by the procedure described below. ChS was removed by treatment with chondroitin ABC lyase EC 4.2.2.4 (Seikagaku Kogyo Co., Tokyo, Japan). Protein was removed with papain (Sigma Chemical Co., London). Analytical grade chemicals were used when available.

Extraction of cartilage glycosaminoglycans. Each dry 100 μm cartilage section was broken into small fragments and transferred to a $5 \times 1 \text{ cm}^2$ test-tube and 2.5 ml of precooled solvent (0.2M NH_4Cl at pH 7.00) added, in a solvent-to-cartilage ratio of 500:1. Capped tubes were placed on a slow speed rotary mixer, transferred to a 4°C cold room, extracted by continuous rotary mixing for 18 h, and then centrifuged at 5000 r.p.m., at 4°C for 30 min. To the cartilage residue a further 2.5 ml of cold solvent was added and the extraction repeated for 21 h. To the supernates and residue 2.5 ml of precooled 0.5N NaOH was added and held at 4°C for 4 h. The residue was centrifuged. All supernates were adjusted to pH 7.00 with acetic acid and transferred to a 250 ml glass-stoppered reagent bottle containing 80 ml ethanol and 12ml 40% w/v sodium acetate, pH 7.6. The residue was extracted with a further 2.5 ml of 0.5 N NaOH for 4 h and the neutralised supernate (fourth extract) added to the pooled extracts. The cartilage extract in acetate saturated ethanol was held at 4°C for 5 days before recovering the finely dispersed GAG precipitate by centrifugation at 15 000 r.p.m. at 4°C for 30 min. The GAG deposit was taken up into 2.0 ml of 5% (w/v) sodium acetate.

Aliquots of cartilage residues remaining after salt and alkali extraction were added to concentrated sulphuric acid and examined for the presence of uronic acid by heating with carbazole. The bulk of each residue was washed with 0.9% w/v NaCl and suspended in citrate buffer, pH 4.2. Drops of the buffered suspensions were placed on carbon coated EM grids and stained with 0.5% w/v uranyl acetate for 10 s. The grids were examined for the presence of noncollagenous material at magnifications up to $\times 190\,000$ in an AEI Model 801 electron microscope at an operating voltage of 60 kV.

Fractionation and measurement of glycosaminoglycans. Total GAG, the HA, and the combined ChS-KS components of the extract samples after fractionation by cellulose acetate electrophoresis were determined after reaction with 0.2% w/v alcian blue in 0.05M MgCl_2 at pH 5.80 (Elliott and Gardner, 1978c). The KS fraction was determined after removal of the ChS with chondroitin ABC lyase. The quantity of ChS was calculated from the difference before and after treatment with chondroitin ABC lyase and as sulphated disaccharides by the periodate-thiobarbituric acid

Fig. 1 Electron micrograph of the cartilage residue remaining after the 4-stage extraction procedure for the recovery of glycosaminoglycans ($\times 40\,000$)

Fig. 2 Cellulose acetate electrophoretic separation of hyaluronic acid from other articular cartilage glycosaminoglycans. Large sample aliquots were used to produce visually discernible articular cartilage hyaluronic acid. Sample (a) is a chondroitin-6-sulphate standard; (b) human articular cartilage, 0–100 μm depth and (c) at 900–1000 μm depth; (d) keratan sulphate standard (impure); (e) human articular cartilage, 0–100 μm depth and (f) at 900–1000 μm depth; (g) hyaluronic acid standard; (h) human articular cartilage, 0–100 μm depth, and (i) at 900–1000 μm depth; (j) chondroitin-4-sulphate standard. The detection agent was 0.2% (w/v) alcian blue—0.05 M MgCl_2 at pH 5.8 for 60 min. Unbound dye was removed in 80% (v/v) ethanol

reaction (Elliott and Gardner, 1977). Purified GAG standards were included with each assay.

Histochemical examination of articular cartilage. Frozen unfixed 5 μm thick cartilage sections were prepared from each sample block and stained with alcian blue (AB)—0.2M MgCl_2 for 8 h. These sections provided supplementary information of cartilage structure and GAG distribution.

Results

The 4-stage extraction process quantitatively recovered the GAG from infant and adult cartilage samples. Uronic acid was not detected in the insoluble residue and electron microscopic examination indicated the absence of non-collagenous material (Fig. 1). A small quantity of hydroxyproline was present in the extracts, most of which was in fine suspension as it could be removed by centrifugation.

The GAG composition of the 60 samples of articular cartilage obtained from 30 femoral condyles in the age range 0 to 70 years are tabulated in Tables 1 and 2. The results, in chronological order, are expressed as μg GAG/mg dry cartilage. The GAG

Table 2 The glycosaminoglycan composition (as $\mu\text{g}/\text{mg}$ of dry cartilage) of deep zone (900 to 1000 μm depth from surface) human articular cartilage samples aged from 0 to 70 years

Sample no.	Age (y)	Electrophoresis AB—0.05 M MgCl_2				Disaccharide assay ChS ($\mu\text{g}/\text{mg}$)
		Total GAG	ChS ($\mu\text{g}/\text{mg}$)	KS	HA	
1	0	522.22	502.33	20.89	<0.5	473.69
2	0.15	476.24	468.52	5.69	2.03	444.15
3	0.15	494.67	460.63	34.04	<0.5	477.89
4	2	281.57	259.32	22.25	<0.5	244.51
5	3.5	174.52	167.36	7.16	<0.5	95.11
6	8	150.67	145.85	4.82	<0.5	79.71
7	10	52.07	45.14	6.93	<0.5	45.11
8	12	108.26	96.02	11.27	0.97	64.99
9	12	186.92	171.90	12.15	2.87	184.70
10	13	88.18	81.66	5.31	1.21	92.83
11	18	101.29	92.65	7.30	1.34	75.27
12	18	226.95	199.54	21.93	5.48	212.04
13	20	68.99	56.62	10.79	1.58	46.42
14	20	177.91	158.64	13.23	6.04	164.02
15	22	196.30	171.28	19.24	5.78	207.45
16	26	454.46	366.60	58.69	29.17	359.42
17	32	83.85	73.02	6.02	4.81	70.41
18	36	238.88	206.78	21.71	10.39	245.70
19	37	180.96	149.04	19.75	12.17	159.35
20	39	169.25	137.64	22.59	9.02	115.61
21	43	122.35	106.87	9.93	5.55	100.53
22	43	127.62	104.63	18.18	4.81	103.37
23	46	208.69	176.82	24.80	7.07	207.62
24	47	105.61	87.19	11.11	7.31	96.99
25	51	181.25	155.59	16.71	8.95	178.20
26	53	187.79	157.61	19.48	10.70	150.80
27	55	195.61	155.47	31.62	8.52	195.44
28	56	147.66	128.26	14.25	5.15	125.03
29	68	137.52	116.13	18.59	2.80	113.45
30	70	120.21	95.58	12.06	12.57	90.69

For the method of fractionation and assay see Table 1.

content of articular cartilage was highest at birth, deep zone cartilage containing about twice the GAG concentration of surface zone cartilage. The concentration of GAG in both surface and deep cartilage zones declined rapidly during the first 4 years of life. The ChS was found to be the principle GAG with KS forming the second largest component. The presence of HA in human articular cartilage was identified after separation from ChS and KS (Fig. 2).

CHONDROITIN SULPHATE COMPOSITION

The ChS values (Tables 1 and 2) were expressed as a percentage of the total GAG concentration and plotted against specimen age (Fig. 3). At birth ChS accounted for almost all the GAG found in articular cartilage. The ChS as a percentage of cartilage GAG decreased during the first 20 years of life. At growth maturity ChS represented about 85% of the total GAG composition. In deep zone (900 to 1000 μm)

Fig. 3 Age distribution of chondroitin sulphates; from samples obtained at a depth of (○) 0 to 100 μm and (●) 900 to 1000 μm from the cartilage surface. Chondroitin sulphates (ChS) were measured following electrophoretic separation and staining with alcian blue—0.05M MgCl_2 . The ChS is expressed as a percentage of the total glycosaminoglycan concentration of the sample (Table 1)

cartilage there was little further change in the ChS% between 20 and 70 years age; the ChS content remained within $85\% \pm 5\%$ of the total GAG. This small variation in ChS % was maintained, although individual ChS concentrations varied widely (Table 1). Thus specimens 16 and 17 contained 366 and 73 $\mu\text{g}/\text{mg}$ of ChS respectively but differed by $<2\%$ in ChS when expressed as a percentage of total GAG.

After age 20 the proportion of ChS in surface zone cartilage samples was more variable (66–85%) than the deep zone cartilage (Fig. 3). The mean value for ChS was $78 \pm 12\%$; the lowest values were found in samples from specimens aged 30 to 50 years. A high correlation was obtained between the ChS assay procedures, with the exception of samples from 0–2 y aged cartilage (Fig. 4).

KERATAN SULPHATE COMPOSITION

The KS values in Tables 1 and 2 were expressed as a percentage of total GAG and plotted against specimen age (Fig. 5). During the first 20 years of life

Fig. 4 Comparison of the methods used to measure the chondroitin sulphates of articular cartilage. Chondroitin sulphates were determined using (a) alcian blue—0.05M MgCl_2 before and after treatment with chondroitin ABC lyase (0.1 unit enzyme in Tris-acetate buffer, pH 7.90, at 37°C for 2 h) and (b) by acid periodate oxidation (0.02M NaIO_4 at pH 2.20 for 60 min at 37°C) and colour formation with thiobarbituric acid (0.3% w/v at 100°C for 15 min) (□) samples 0 to 2 y, 0 to 100 μm cartilage depth; (●) samples 0 to 2 y, 900 to 1000 μm cartilage depth; (●) samples 3.5 to 70 y at both cartilage depths

Fig. 5 Age-distribution of keratan sulphate; from samples obtained at a depth of (○) 0 to 100 μm and (●) 900 to 1000 μm from the cartilage surface. Cartilage ChS and HA were removed by enzymatic depolymerisation (chondroitin ABC lyase, pH 7.90 at 37°C for 2 h). Keratan sulphate (KS) isolated by cellulose acetate electrophoresis was determined by densitometry of the KS—AB complex. The KS is expressed as a percentage of the total glycosaminoglycan concentration of the sample (Table 1)

KS gradually increased to reach about 15% of the total GAG. This increase in part complemented the decrease in ChS during the growth period. As was observed with ChS, the proportion of KS in deep zone cartilage did not vary widely (12% $\pm$ 4% from 20 to 70 years). The relatively larger quantity of KS in the surface zone cartilage, however, showed greater percentage variation. In several samples from specimens aged 20 to 50 years the KS formed 20–30% of the total GAG.

HYALURONIC ACID COMPOSITION

Hyaluronic acid did not exceed 1% of the total GAG until after completion of skeletal growth (Fig. 6). The HA as a percentage increased linearly with age; by 60 years it formed 6% of the total GAG. The HA occurred in both surface and deep zone cartilage (Fig. 2). Deep zone samples contained relatively more HA than surface zone samples (Tables 1 and 2). Expressed as % of total GAG, the proportion of HA in deep zone samples was, however, similar to that in the surface zone (Fig. 6).

In adult articular cartilage the GAG ratio of HA:

Fig. 6 Age distribution of hyaluronic acid; from samples obtained at a depth of (○) 0 to 100 μm and (●) 900 to 1000 μm from the cartilage surface. Articular cartilage hyaluronic acid isolated and detected as shown in Fig. 1 was determined by densitometry and expressed as a percentage of the total glycosaminoglycan concentration of the sample (Table 1)

ChS: KS, by weight, was 3 : 85 : 12. After conversion to μmoles , the HA : ChS : KS ratio became 0.1 : 73 : 27.

Discussion

This study has demonstrated that articular cartilage chondrocytes express ChS in the first few days of human postnatal life. After about 25 months analyses confirmed that KS and HA were also present in the cartilage matrix (Tables 1 and 2). The total content of GAG in cartilage declines rapidly after birth, a decrease attributable to diminished ChS, for although KS and HA increase in amount their smaller absolute quantities do not compensate in full for the loss of ChS. When body growth was complete, the surface zone cartilage contained about 15% (dry weight) as GAG, which decreased during aging to reach about 7% by 70 years.

During aging (20–70 y) the superficial zone of adult cartilage accumulated a higher percentage of KS than deep zone cartilage (Fig. 5). Three explanations are suggested for this observation: (1) that the increase in KS is due to faster turnover of surface zone cartilage GAG caused by wear during the mechanical functioning of the synovial joint; (2) that chondrocyte differentiation and/or feedback control of matrix synthesis is zone dependent, with chondrocytes in the surface cartilage zone synthesising more KS than chondrocytes in the deeper zones. The partial replacement of ChS by KS could significantly alter the structure and size of proteoglycans and decrease cartilage hydration. Alternatively, (3) KS may accumulate coincidentally because of enzymes present in the matrix that degrade ChS and for which KS is not a substrate.

The optimum HA concentrations for the aggregation of PG monomers occurs with HA : PG weight ratios between 1:100 and 10:100. Ratios of <1:100 and >10:100 reduce PG aggregation (Hardingham and Muir, 1974). In this study the ratio of HA to total GAG ($\approx 90\%$ of the weight of PG) ranged from <1:100 to 11:100 (Fig. 6). During the first decade of life little HA could be detected, implying minimal PG aggregation, a hypothesis that could be tested by assessing the ease of PG extraction from infant cartilage. After 10 y the HA content increased to reach 2% by 20 y, 4% by 40 y, and 6% by 60 y. Thus, during the latter half of maturation and the entire aging period covered in this survey the HA: GAG ratio was within the optimum range for PG aggregation as defined by Hardingham and Muir (1974).

The results reported in this paper were derived from material selected on the basis of hospital admission, age, and anatomical location; the samples were further differentiated by cartilage depth. The selection procedures may have failed to reveal subtle pathological changes in cartilage structure not related to aging. Nevertheless, gross abnormalities, including the disorders of overt osteoarthritis, were minimised. On this basis it is considered that changes with age in the patterns of articular cartilage GAG can be identified; these age-associated changes are characteristic for the periods of infancy, growth, and maturation. During adult life a further sequence of changing GAG synthesis can be recognised. Whether any or all of these alterations in cartilage GAG composition are caused by the attributes of senescence, in which sense they could be said to be pathological, is unknown. Aging and degenerative disease may of course be interrelated, but this causal association is not yet known to be the basis of most instances of osteoarthritis. It remains possible that the alterations in molecular composition of articular cartilage described in this paper are

coincidental features of the aging process. Since, however, the molecular composition of articular cartilage and in particular the quality and quantity of the proteoglycans, determine its response to physical stress and its reaction to physiologically induced deformation, it is apparent that the progressive alterations in cartilage composition demonstrated here offer part, if not the entire, explanation for the changed capacity of aging cartilage to act efficiently as a load bearing surface (Armstrong and Gardner 1977a). To obtain more direct evidence for this proposal, and in particular proof that the proteoglycans themselves change with age, must be the object of further investigations that are now in progress.

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