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Hyaluronic acid in human articular cartilage

Age-related changes in content and size

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Total tissue content and molecular mass of hyaluronic acid was determined in papain digests of human articular cartilage using a sensitive radiosorbent assay [Laurent & Tengblad (1980) Anal. Biochem. **109**, 386–394]. 1) Hyaluronic acid content increased from 0.5 μg/mg wet wt. to 2.5 μg/mg wet wt. between the ages of 2.5 years and 86 years. 2) Hyaluronic acid chain size decreased from M_r 2.0×10^6 to 3.0×10^5 over the same age range. 3) There was no age-related change in the size of newly-synthesized hyaluronic acid, which was of very high molecular mass, in both immature and mature cartilage. The results are consistent with an age-related decrease in proteoglycan aggregate size and suggest that modification of the hyaluronic acid chain may take place in the extracellular matrix.

INTRODUCTION

Hyaline cartilage is a specialized connective tissue, the major function of which depends on its state of hydration and the structural arrangement of a large extracellular matrix. Collagen provides the tensile strength and the proteoglycans are largely responsible for the compressive stiffness of the tissue (Kempson *et al.*, 1970, 1976).

Proteoglycans consist of a protein core to which a large number of chondroitin sulphate, keratan sulphate and *O*- and *N*-linked oligosaccharides are covalently attached. In articular cartilage they exist predominantly in the form of macromolecular aggregates in which many proteoglycan monomers interact in a highly specific way with hyaluronic acid via a globular domain at the *N*-terminus of the proteoglycan protein core (Hardingham & Muir, 1972; Heinegård & Hascall, 1974; Hascall, 1977; Hardingham *et al.*, 1976). In the native aggregate the stability of this interaction is increased by the presence of link protein which binds to both the proteoglycan monomer and the hyaluronic acid chain (Heinegård & Hascall, 1974; Hardingham, 1979; Tang *et al.*, 1979). Although the biological function of aggregation is not fully understood, it is known that the large size of the aggregates immobilizes them very effectively in the collagen network. The size of an aggregate depends partly on the size of the monomeric proteoglycans, but principally on the length of the hyaluronic acid chain, and on the number of monomers attached to it (Hardingham, 1979).

As well as contributing to the structural properties of the extracellular matrix, hyaluronic acid may also have important regulatory functions in cartilage. Very low concentrations of hyaluronic acid inhibit cartilage nodule formation in differentiating chick limb buds *in vivo* (Toole *et al.*, 1972) and addition of hyaluronic acid to the medium of chondrocytes also reduces proteoglycan synthesis (Nevo & Dorfmann, 1972; Wiebkin & Muir, 1973; Solursh *et al.*, 1980; Bansal *et al.*, 1986). The extracellular concentration of hyaluronic acid may therefore have a role in regulating the synthesis of proteoglycans during maturation of articular cartilage and in repair processes such as those that occur in degenerative joint disease.

Considerable progress has been made in understanding the structure and biosynthesis of proteoglycan monomers in human articular cartilage, but very little is known about hyaluronic acid in this tissue (Thonar *et al.*, 1978; Elliot & Gardner, 1979; Bayliss *et al.*, 1987). The aim of the present study was to determine the change in concentration and molecular mass of hyaluronic acid during maturation and ageing of normal human articular cartilage.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade except guanidine hydrochloride (BDH, Poole, Dorset, U.K.) which was purified with activated charcoal (Norit, GSX; Hopkin and Williams) and Celite, grades 505, 545 and hyflo (Koch–Light, Haverhill, Suffolk, U.K.). Papain type 111 (papainase; EC 3.4.22.2), iodoacetic acid, disodium-EDTA, 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, MO, U.S.A.). Sephacryl S-1000 and AH–Sepharose 4B were from Pharmacia (Uppsala, Sweden) and *Streptomyces* hyaluronidase was from Behring Diagnostics (La Jolla, CA, U.S.A.). Dulbecco's Modification of Eagle's Medium was purchased from Flow Laboratories (U.K.), and [6-^{3}H]glucosamine (TRK.398) and sodium [^{125}I]iodide (IMS.30) were purchased from Amersham (U.K.). Iodogen was purchased from Pierce Chemical Company, Rockford, IL, U.S.A. Hyaluronic acid from human umbilical cord (BDH) was used as a standard in the radiosorbent assay and was further purified by density gradient ultracentrifugation essentially as described by Fraser *et al.* (1981).

Source of tissue

Human articular cartilage was obtained from the knee (femoral condyle) at the time of surgery (hindquarter or

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mid-thigh amputation) for chondrosarcoma or osteosarcoma. Only tissue that appeared macroscopically normal was used and specimens where the tumour had penetrated the joint space were discarded. Cartilage that was used for biosynthesis experiments was processed within 1–2 h of surgery.

Preparation of proteoglycan aggregates (A1)

Full-depth pieces of cartilage were sectioned at 20 μm (Bayliss *et al.*, 1983) and the proteoglycans extracted with 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 6.8, for 65 h at 4 °C with continuous mixing. The extraction buffer contained the following proteinase inhibitors. Disodium-EDTA (10 mM), 6-aminohexanoic acid (100 mM), benzamidine hydrochloride (5 mM), phenylmethanesulphonyl fluoride (0.1 mM) and soya-bean trypsin inhibitor (1 μg/ml). Extracts were filtered through glass wool and the filtrates dialysed against 0.05 M-sodium acetate, pH 6.8, containing the same concentrations of inhibitors to allow reassociation of the proteoglycan aggregates.

Dialysed cartilage extracts were adjusted to a density of 1.5 g/ml with CsCl and centrifuged at 100000 *g* for 48 h at 8 °C. The resultant gradients were fractionated and the bottom third (density > 1.54 g/ml) was designated A1, according to the nomenclature of Heinegård (1972).

Digestion of whole tissue and A1 preparations with papain

Hyaluronic acid was released from whole tissue (20–100 mg wet wt.) and from A1 aggregate preparations by treatment with papain (0.5–1.5 units) in 0.1 M-sodium acetate, pH 6.8, for 24 h at 60 °C. Neither cysteine nor EDTA was added to the digestion mixture as these compounds have the potential to cause a reduction in the size of hyaluronic acid. Cysteine is usually added as a thiol activator of papain whilst EDTA is added as a chelator of heavy metal ions which could inhibit activation. In the absence of these additives, sufficient enzymic activity was present to fully solubilize whole tissue pieces under the conditions described. After complete digestion, iodoacetic acid (10 mM final concentration) was added to inactivate the papain. Also, inhibitors (as above) were added to inhibit endogenous tissue proteinases. The digest was finally heated at 80 °C for 15 min before analysis. When the molecular mass of hyaluronic acid was to be determined, a portion of the digest was taken before heating.

Pulse-labelling of tissue with [6-^{3}H]glucosamine

Full-depth pieces of cartilage (~ 100 mg) were incubated in 3 ml of Dulbecco's Modification of Eagle's Medium containing 15 mM-Hepes, 10 mM-Bes, 10 mM-Tes, 4 mM-glutamine, 3.7 g of sodium bicarbonate/l and [6-^{3}H]glucosamine (100 μCi/ml) for 16 h at 37 °C in an atmosphere of CO_2/air (19:1, v/v). After incubation the tissue pieces were washed free of non-incorporated isotope with phosphate-buffered saline and stored at −20 °C before the determination of wet weight. Uronic acid, hyaluronic acid and hydroxyproline were determined after papain digestion of the tissue. The uronic acid values were verified after ethanol precipitation of the glycosaminoglycans or after dialysis of the papain digests.

Chromatography with Sephacryl S-1000

After treatment of whole tissue with papain, the resultant digests were chromatographed with a column (140 cm × 0.5 cm) of Sephacryl S-1000 and eluted with a flow rate of 3.8 ml/h. Sample size was 0.8 ml containing between 100 and 200 μg of uronic acid. Digests were eluted in 0.15 M-sodium acetate, pH 6.8, and 1.0 ml samples were collected. Fractions were analysed for hyaluronic acid by radiosorbent assay, and for newly synthesized hyaluronic acid, by scintillation counting. In preliminary experiments the elution position of newly synthesized hyaluronic acid was determined by the differences in distribution of radioactivity before and after treatment of the papain digests with *Streptomyces* hyaluronidase. The molecular mass of hyaluronic acid was calculated from the selectivity curve for Sephacryl S-1000 (supplied by the manufacturers) which was verified by the manufacturer using samples of purified hyaluronic acid of known molecular mass.

Digestion of papain-treated cartilage with *Streptomyces* hyaluronidase

Papain digests of articular cartilage (80–100 mg) were incubated at 37 °C for 16 h in 0.1 M-sodium acetate, pH 6.0, containing 0.15 M-NaCl with 5 units of *Streptomyces* hyaluronidase. The resultant digests were chromatographed with Sephacryl S-1000.

Preparation of binding proteins used in the radiosorbent assay

Binding proteins, used in the radiosorbent assay for hyaluronic acid, were prepared by trypsin digestion of pig laryngeal-cartilage proteoglycan aggregates as described by Bonnet *et al.* (1985). These were reaggregated with an excess of hyaluronic acid, dialysed against water, then lyophilized and stored at −20 °C. The purity of the binding proteins (hyaluronic acid-binding region and link protein) was analysed by gel electrophoresis (Fairbanks *et al.*, 1971; results not shown). Iodination of the aggregated hyaluronic acid-binding region and link protein was carried out by the Iodogen method following the manufacturer's instructions [sodium [^{125}I]iodide (0.5 mCi) with 200 μg of proteins solubilized in 0.05 M-sodium acetate, pH 5.8]. Iodinated binding proteins were dissociated from hyaluronic acid by equilibration in 4 M-guanidine hydrochloride and were then isolated by affinity chromatography under associative conditions and stored bound to hyaluronic acid-coupled AH–Sepharose 4B at 4 °C in 0.05 M-sodium acetate, pH 5.8, containing 1.5 M-NaCl. When required, the proteins were removed from the gel with 4 M-guanidine hydrochloride.

Preparation of hyaluronic acid-coupled AH–Sepharose 4B

Hyaluronic acid (140 mg) was degraded enzymically with testicular hyaluronidase (560 TRU) to a mean average mass of 50000 Da determined viscometrically after determination of the limiting viscometry number (Cleland & Wang, 1970; Wasteson, 1971). This material was allowed to react with 13 g of affinity gel following the recommended carbodi-imide coupling procedure of Pharmacia. The resultant gel contained 0.7 mg of hyaluronic acid/ml of swollen gel.

Analysis of hyaluronic acid by radiosorbent assay

Hyaluronic acid was analysed by a specific radiosorbent assay technique (Laurent & Tengblad, 1980; Tengblad, 1980). The assay was performed in 0.05 M-sodium acetate, pH 5.8, containing 1.5 M-NaCl and 0.1% bovine serum albumin (assay buffer). When whole-tissue digests were analysed, proteinase inhibitors (as above) were also included in the assay buffer to inhibit the papain and the endogenous proteinases. Portions (20–300 μl) were pipetted into 1.5 ml centrifuge tubes (Microtube 3810, Eppendorf, Germany) and diluted to 850 μl with assay buffer. To the samples, 4 μl of hyaluronic acid-substituted affinity gel was added, contained and delivered in a volume of 400 μl. Finally, 100 μl of ^{125}I-binding proteins (15000–20000 c.p.m./sample) in 4 M-guanidine hydrochloride was added and the samples were equilibrated for 20 h at 4 °C with continuous mixing. The tubes were centrifuged and the supernatants removed. The pellets were then washed twice with assay buffer and the radioactivity counted.

Standards ranging from 10–1200 ng of hyaluronic acid were prepared and the standard curve was found to be linear in the range 30–600 ng. All samples were analysed in triplicate.

Analytical methods

Uronic acid was determined by an automated procedure of the modified carbazole reaction (Bitter & Muir, 1962) with glucuronolactone as standard. Hydroxyproline was determined by an automated procedure (Jackson & Cleary, 1967) of the standard method of Stegemann & Stalder (1967). Tritium was determined in a Searle Mark 111 Tracer Analytic LSC using Optiphase 'MP' scintillant (Fisons, UK). [^{125}I]Iodine was determined in an LKB 1282 Compugamma Counter.

RESULTS

Establishment of conditions for the radiosorbent assay

Preliminary studies were carried out to establish the optimum conditions for the radiosorbent assay, as applied to human articular cartilage. It was found that an iodinated mixture of the hyaluronic acid-binding region and link protein, rather than the individual proteins, gave the highest affinity and stability of binding after prolonged equilibration with hyaluronic acid.

A standard equilibration time of 20 h was chosen with samples and standards continuously rolling on a Coulter mixer at 4 °C. These conditions were found to be optimal for maximum binding of the proteins and for reproducibility. Variations of pH and ionic strength were minimal between samples and the inhibitors added, where necessary, were shown to have no effect on the nature or position of the standard curve. The standard curve was found to be highly reproducible with the main variable being only the magnitude of the maximum binding in different gel preparations, which ranged from 50–70%. The use of different iodinated binding protein preparations had no effect on the characteristics of the standard curve. The assay was optimal for detecting hyaluronic acid in the range 30–600 ng (Fig. 1) and the between-sample error was calculated as $\pm 5\%$.

Only hyaluronic acid free in solution can be detected by the radiosorbent technique, therefore all samples were treated with papain to disrupt aggregation. Since even a residual trace of active papain produces large errors in the assay (resulting from the degradation of iodinated hyaluronic acid-binding region and link proteins), iodoacetic acid (10 mM) was added, and the samples were heated at 80 °C for 15 min. Addition of iodoacetic acid, a potent inhibitor of papain, was shown to have no effect on the sensitivity of the assay.

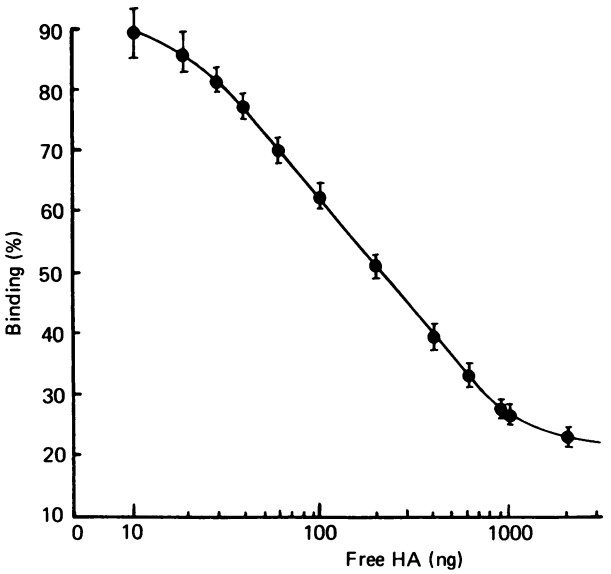

Fig. 1. Standard curve of the radiosorbent assay for hyaluronic acid

Binding of ^{125}I-binding proteins to HA–Sepharose gel in the presence of competing free hyaluronic acid (HA) was carried out as described in the text. The background counts of non-specific binding (1–2% of added counts) have been subtracted in calculating percent binding. The magnitude of protein binding is given as a percent of the maximum binding achieved in the absence of added hyaluronic acid.

Analysis of hyaluronic acid in aggregate preparations (A1) and whole tissue

A1 Fractions were prepared from cartilage that was sectioned at 20 μm before extraction; a technique which optimizes the extraction of hyaluronic acid from human cartilage (Bayliss *et al.*, 1983). The amount of hyaluronic acid in the aggregate preparations was calculated as a percentage of the total uronic acid content (Fig. 2).

It is clear from Fig. 2 that the proportion of hyaluronic acid in the A1 fractions increases with age. It is also apparent that there is a large scatter in the values obtained from aged tissue. This may be a normal age-related phenomenon or might reflect pathological changes within the cartilage that are not detected from a macroscopical examination of the joint.

More detailed analyses of cartilage hyaluronic acid were made possible by the availability of whole tissue specimens, enabling both the amount and size of hyaluronic acid to be investigated. Since preliminary studies showed that trace amounts of endogenous tissue enzymes degraded the ^{125}I-hyaluronic acid-binding region and ^{125}I-link protein, additional inhibitors were included in the buffer system of the radiosorbent assay for these studies.

Whole tissue pieces were diced and digested with

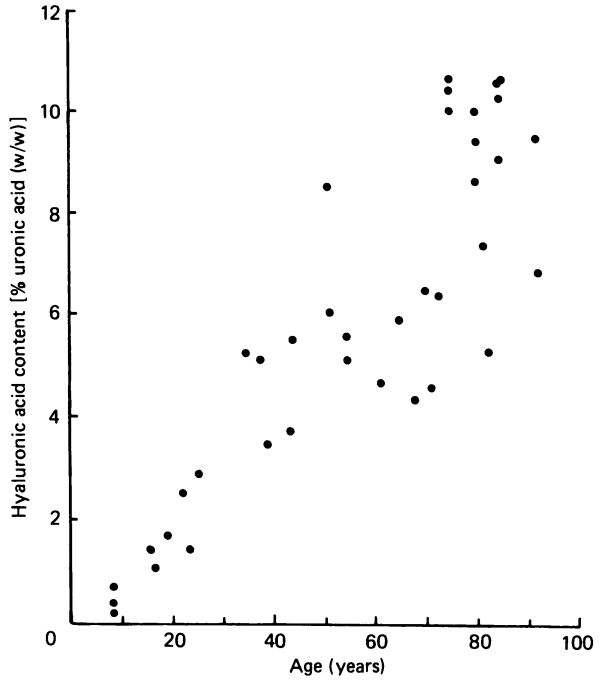

Fig. 2. Hyaluronic acid content in proteoglycan aggregate preparations (A1)

Samples (38) of A1 preparations from cartilage of different ages were digested with papain and their uronic acid contents determined. Portions of the samples containing between 30 and 600 ng of hyaluronic acid were diluted in 0.05 M-sodium acetate, pH 5.8, containing 1.5 M-NaCl for the determination of hyaluronic acid in the radiosorbent assay. The points represent the average of triplicate measurements and the proportion of uronic acid from hyaluronic acid is expressed as a percentage of the total uronic acid.

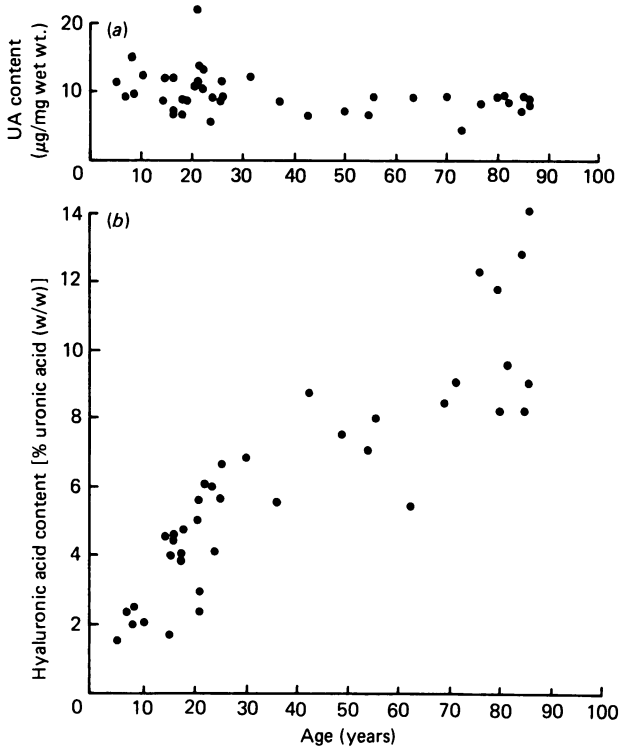

Fig. 3. Total uronic acid and hyaluronic acid content in papain digests of whole tissue

Samples (39) of whole tissue of varying ages were finely-diced, washed in phosphate-buffered saline, and their wet weights recorded. The tissues were digested with papain (1.5 units) and their total uronic acid contents determined (*a*). Portions were taken and diluted in 0.05 M-sodium acetate, pH 5.8, containing 1.5 M-NaCl with iodoacetic acid (10 mM) and proteinase inhibitors (see the Materials and methods section) (*b*). Each point represents the average of triplicate measurements of the proportion of uronic acid (UA) from hyaluronic acid, expressed as a percentage of the total tissue uronic acid.

papain as described in the Materials and methods section. The amount of uronic acid was determined in each case, calculated as a proportion of the wet weight of tissue, and was found to be constant throughout the age-range studied (Fig. 3*a*). In Fig. 3(*b*) an increase in the proportion of hyaluronic acid relative to other glycosaminoglycans is shown, which is similar in magnitude to the change observed with isolated A1 fractions over the same age range. Also, in accordance with the findings from the A1 fractions, there was a larger scatter of values obtained from the older age samples than was found for the younger specimens. A similar age-related change was observed with hyaluronic acid, expressed as a proportion of tissue wet wt. (Fig. 4). However, since it is known that the water content of cartilage decreases with age (Venn, 1978) it was pertinent also to compare the proportion of hyaluronic acid with the total content of hydroxyproline which was constant throughout the age range (Fig. 5*a*). The same age-related change in hyaluronic acid was observed (Fig. 5*b*).

Measurement of hyaluronic acid chain size in tissue digests

In order to investigate the size distribution of newly synthesized hyaluronic acid compared with the endogenous hyaluronic acid in the tissue, several of the specimens of intact tissue were incubated for 16 h with [6-^{3}H]glucosamine (100 μCi/ml) before digestion with papain. In Figs. 6(*a*) to 6(*e*) the size distribution of endogenous hyaluronic acid from whole tissue is compared with the distribution found for newly synthesized material from a representative range of ages. The ^{3}H-labelled material (newly synthesized hyaluronic acid) was consistently of a high molecular size range, eluting at the void volume with Sephacryl S-1000, whilst endogenous material (as detected by the radiosorbent assay) was always smaller in size. Furthermore, the endogenous hyaluronic acid showed a further decrease in average size with increasing age and became less heterogeneous. This trend was consistent for all samples studied.

Verification that the high molecular mass material was indeed hyaluronic acid was confirmed by treatment of pulse-labelled tissue digests with *Streptomyces* hyaluronidase (5 units) which is highly-specific for hyaluronic acid. Chromatography of the digests with Sephacryl S-1000 revealed a complete absence of radioactivity at the void volume in enzyme-treated samples whereas untreated control samples retained the high molecular mass material (Fig. 7).

Evidence that further supported the above findings

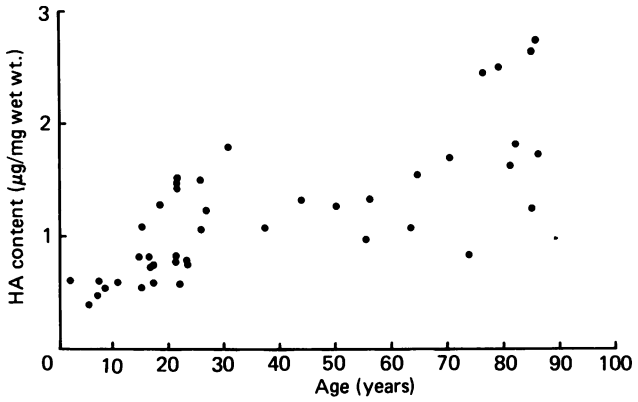

Fig. 4. Hyaluronic acid content as a proportion of total wet wt. in papain digests of whole tissue

Samples (43) of whole tissue were equilibrated in phosphate-buffered saline and their wet weights recorded. The tissues were digested with papain and their hyaluronic acid (HA) contents were determined by the radiosorbent method, expressed as a proportion of the tissue wet weight.

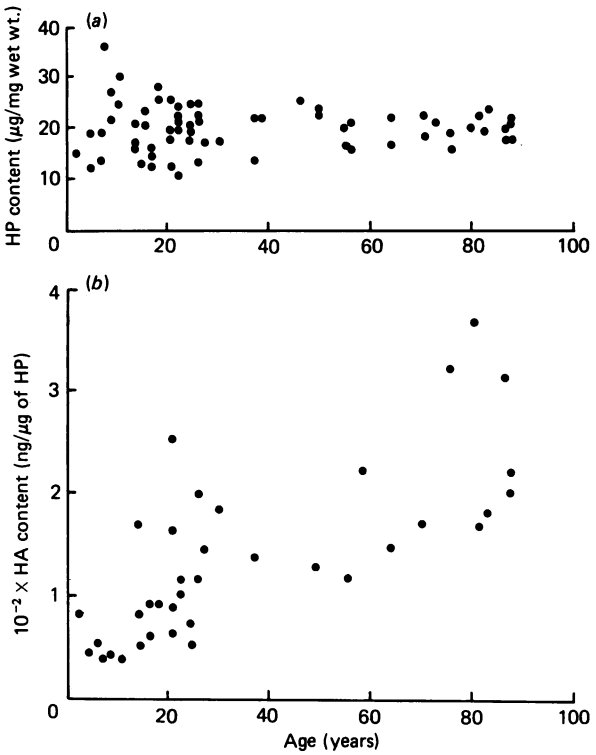

Fig. 5. Hyaluronic acid and hydroxyproline contents in papain digests of whole tissue

Samples (63) of whole tissue of varying ages were digested with papain and the amount of hydroxyproline (HP) determined (*a*). Portions were taken from 37 of the samples for measurement of hyaluronic acid (HA), as described for Fig. 3. The points represent the average of triplicate measurements (*b*).

was provided by studies of human chondrosarcoma tissue. Hyaluronic acid was isolated from this tissue and chromatographed with Sephacryl S-1000. Detection of hyaluronic acid by the radiosorbent assay showed a size range similar to that observed for newly synthesized

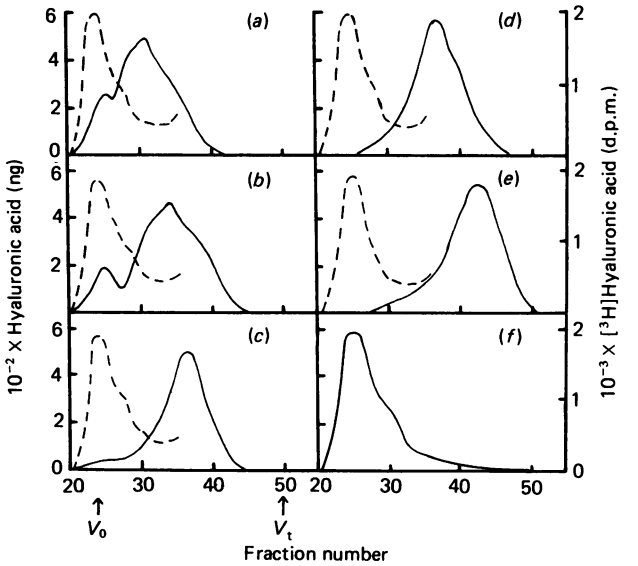

Fig. 6. Chromatography of tissue digests with Sephacryl S-1000

Full-depth pieces of cartilage (20–100 mg wet wt.) labelled in culture with [6-^{3}H]glucosamine (100 μCi/ml) were digested with papain (1.0–1.5 units) and chromatographed with a column of Sephacryl S-1000 (140 cm × 0.5 cm) equilibrated in 0.15 M-sodium acetate, pH 6.8. Elution profiles are shown that compare the size distribution of newly synthesized (----) with endogenous (———) hyaluronic acid for a selection of ages: (*a*) 2.5 years; (*b*) 5 years; (*c*) 15 years; (*d*) 38 years; (*e*) 86 years. The size distribution of endogenous hyaluronic acid from human chondrosarcoma tissue is also shown (*f*). Endogenous hyaluronic acid was detected by the radiosorbent assay and newly synthesized material was detected by identification of ^{3}H-labelled hyaluronic acid. All comparisons were performed on the same chromatographic elutions from single samples. The radioactivity is shown for only part of the chromatogram and the glycosaminoglycans emerging later have been omitted.

material (Fig. 6*f*). This was not an unexpected result since chondrosarcoma tissue is metabolically very active, grows rapidly, and lacks the limitations of the rigid organized extracellular matrix inherent in cartilage hence the molecular organization is more characteristic of an immature tissue.

The weight-average change with age for hyaluronic acid in all samples analysed is shown in Fig. 8. The greatest decrease in size occurred in the youngest age groups with the chain size continuing to decrease between 5 and 86 years of age.

DISCUSSION

Many age-related changes in the structure and composition of human articular cartilage proteoglycans have been described. These studies indicate that there is an increase in the heterogeneity of proteoglycan monomers (Bayliss & Ali, 1978, 1981; Roughley & White, 1980) and an overall decrease in monomer size calculated to be M_r 1.80×10^6 for human fetal and M_r 1.17×10^6 for human adult monomer (Ohno *et al.*, 1986). Furthermore, because of the asymmetric distribution of glycosaminoglycan chains in cartilage proteoglycans within

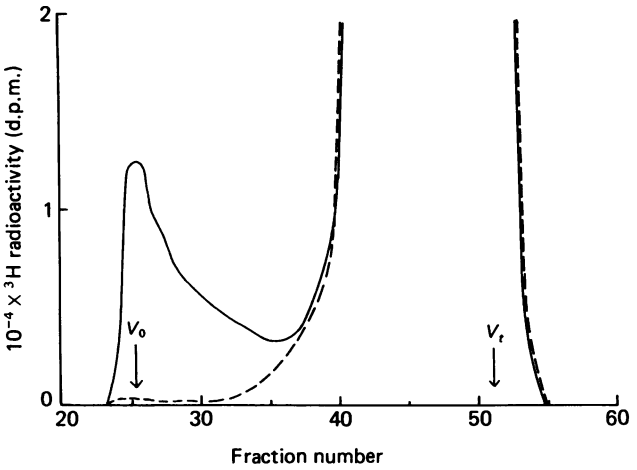

Fig. 7. *Streptomyces* hyaluronidase treatment of a [6-^{3}H]-glucosamine-labelled tissue digest

A full-depth piece of cartilage (207 mg wet wt.) from a 38 year femoral condyle was labelled with [6-^{3}H]glucosamine (100 μCi/ml) and digested with papain (1.5 units). After digestion the sample was boiled for 10 min and proteinase inhibitors were added as described in the text. To half the sample *Streptomyces* hyaluronidase (5 units) was added in 0.1 M-sodium acetate, pH 6.0, containing 0.15 M-NaCl. The remainder of the sample (control) was not treated with enzyme. Both the enzyme-treated and untreated samples were incubated at 37 °C for 16 h. Both the control (———) and enzyme-treated (– – – –) samples were chromatographed with Sephacryl S-1000 and the fractions monitored for radioactivity.

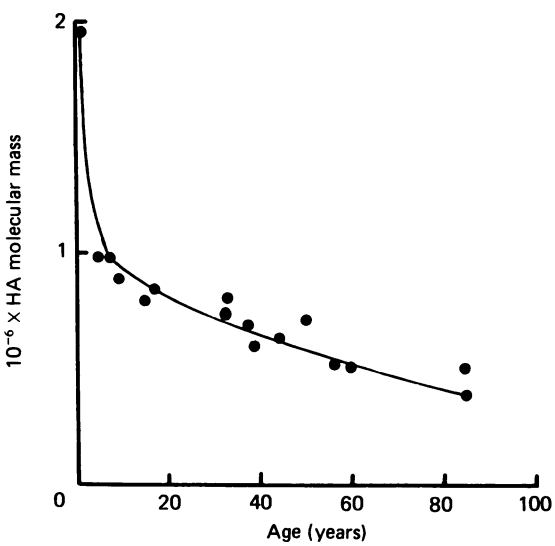

Fig. 8. Mass-average variation of hyaluronic acid (HA) with age

Samples (16) of the 39 analysed for total hyaluronic acid content (Fig. 3) were chromatographed with Sephacryl S-1000 (see Fig. 6 for representative profiles) and the mean average-molecular-mass variation with age was determined. The values were calculated from the $K_{av.}$ values at which 50% of the total hyaluronic acid was recovered after chromatography.

chondroitin sulphate-rich and keratan sulphate-rich regions, the age-related increase in keratan sulphate content suggests that the molar concentration of proteoglycan monomer probably increases, contrary to what would be expected from the chondroitin sulphate contents. The capacity of proteoglycans for interacting with hyaluronic acid and thereby forming aggregates, however, remains rather constant with age (Bayliss & Ali, 1978; Roughley & White, 1980). These results suggest that an increase in hyaluronic acid concentration would be required to maintain a constant level of fixed charge density in an aggregated form. It is significant, therefore, that the present study showed a 4–5-fold increase in total tissue hyaluronic acid content. Preliminary studies (Bayliss *et al.*, 1987) have shown, however, that the hyaluronic acid concentration in adult cartilage is not sufficient to accommodate all of the hyaluronic acid-binding region and link protein that also accumulates in the tissue with increasing age. Consequently, it is possible that there is a pool of proteoglycan monomer and/or hyaluronic acid-binding region in adult cartilage (Roughley *et al.*, 1985; Bayliss *et al.*, 1987) that is not aggregated, or only weakly complexed, with hyaluronic acid and recent studies (M. W. A. Holmes & M. T. Bayliss, unpublished work) have shown that this is the case.

Given a constant hyaluronic acid chain size, a decrease in monomer size alone would result in a smaller aggregate. However, Figs. 6 and 7 show that the molecular mass of hyaluronic acid is not constant and that it decreases considerably (~ 7-fold) during maturation and ageing of the tissue. Thus, at least two factors regulate the size of proteoglycan aggregates in normal human articular cartilage.

Comparison of the newly synthesized and non-labelled hyaluronic acid from samples of different ages (Fig. 6) confirms that there is extracellular modification of hyaluronic acid that increases somewhat with increasing age. Physical breakdown of hyaluronic acid and proteoglycan aggregates has been observed in solution under conditions of high shear (Hascall & Sajdera, 1969), but the necessary conditions for this mechanism are unlikely to occur in cartilage. Free radicals generated by a number of chemical and enzymic reactions can degrade proteoglycan aggregates and hyaluronic acid in solution (Greenwald & Moy, 1980; Wong *et al.*, 1981; Bates *et al.*, 1984; McNeil *et al.*, 1985; Roughley *et al.*, 1987). It is also well established that free radicals can be produced by phagocytic cells and may have a role during inflammatory episodes within the joint (McCord, 1974). Whether such an oxygen-derived free-radical flux can be generated normally within the cartilage matrix, however, is open to question. Enzymic degradation of hyaluronic acid by hyaluronidase, an endoglycosidase, would be the most likely mechanism, but this enzyme has not been detected so far in articular cartilage. Leaback (1974) suggested that the reason for this may be the relative insensitivity of the assays for hyaluronidase, which are 1000-fold less sensitive than the fluorimetric assays for exoglycosidases. Whatever the reason, it is clear that very little enzymic cleavage of the chain would be necessary to reduce its size considerably.

It is useful to consider what effect these changes might have on the physicochemical properties of cartilage. Firstly, the increase in hyaluronic acid content maintains the overall aggregation of proteoglycan monomers and, as a consequence, keeps the fixed charge density and thus

the contribution of proteoglycans to the compressive properties of cartilage constant. Although some mechanical properties of articular cartilage, such as tensile strength and stiffness, also undergo age-related changes, the magnitude of these is far smaller than the dramatic change observed for aggregate size ($\sim$ 14-fold). These properties are more likely to be influenced by changes in the collagen network, although the influence of interactions between proteoglycan and collagen cannot be ruled out. Furthermore, it has been shown that the osmotic pressure of cartilage proteoglycan is not measurably affected by aggregation (Urban *et al.*, 1979) and is, therefore, also unlikely to be influenced to any great extent by aggregate size. The percentage of proteoglycan aggregate in solution has been shown to affect the shear-rate-dependent viscosity, but a conclusion of these studies was that this change would not be expected to influence the shear behaviour of cartilage (Hardingham *et al.*, 1987). The permeability of large solutes is very sensitive to pore size distribution (Maroudas, 1979). Even though aggregation does not affect the concentration of proteoglycans, it will make the network more random and hence affect pore size distribution and possibly solute permeability.

It therefore seems reasonable to assume that if aggregate size influences the physical properties of cartilage, it is more likely to be an indirect or second-order effect, and that the higher levels of hyaluronic acid primarily maintain the fixed charge density in an aggregated form.

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