The Synthesis of Hyaluronic Acid by Sheep and Rabbit Articular Cartilage *in vitro*

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Standard methods of glycosaminoglycan separation were used to confirm the presence of hyaluronic acid in sheep and rabbit articular cartilage. During incubation of cartilage *in vitro* in the presence of [1-¹⁴C]acetate, cartilage cells synthesize this macromolecule and, under the conditions used, it appears to have a shorter turnover time than chondroitin sulphate in the same tissue. The possible functions of hyaluronic acid in cartilage are discussed.

The major proteoglycans of hyaline cartilage are composed of chondroitin sulphate and keratan sulphate covalently linked to protein. These macromolecules can be isolated from the cartilage by mild methods of extraction, and such preparations contain both proteoglycan complex and proteoglycan subunit (Sajdera & Hascall, 1969). Hyaluronic acid is a minor component of pig laryngeal cartilage (Hardingham & Muir, 1973) and very small quantities of this glycosaminoglycan are able to bring about large increases in the hydrodynamic size of proteoglycan complexes (Hardingham & Muir, 1972). It is reasonable to assume that the hyaluronic acid is synthesized by the cartilage cells, if it is to play a role *in vivo* in the aggregation of proteoglycans, because the matrix would restrict the free diffusion of hyaluronic acid synthesized by other tissues.

Balazs and co-workers (Balazs & Gibbs, 1970; Balazs *et al.*, 1966) have shown that preparations of articular cartilages have a surface layer containing hyaluronic acid which is difficult to remove by washing alone. Hyaluronic acid is, however, a normal constituent of synovial fluid and is present in relatively high concentrations, as high as 0.8 mg/ml in bovine carpal joint fluid (Seppala & Balazs, 1969). It is synthesized by synovial membrane cells of the joint cavity (Baxter *et al.*, 1973). The hyaluronic acid at the surface layer of articular cartilage may therefore be derived from synovial fluid, but cells in the articular cartilage might also synthesize the glycosaminoglycan.

The experiments described here confirm that hyaluronic acid is present in articular cartilage and clearly show that the cartilaginous tissue is able to synthesize it.

Materials and Methods

Articular cartilage was taken from the hind knee joints of 9–15-month-old laboratory rabbits of mixed

breeds and from the hock joints of a 12–15-month-old sheep obtained from the abattoir immediately after slaughter.

Incorporation of [1-¹⁴C]acetate into the glycosaminoglycans of articular cartilage

Incubation of cartilage slices *in vitro* was carried out in Eagle's basal medium (Commonwealth Serum Laboratories, Melbourne, Australia) supplemented with 1 mM-glutamine, 12.5 mM-Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid] and 2 mg of NaHCO₃/ml, pH 7.4. This solution was bubbled with CO₂+O₂ (5:95) for 30 min before use.

Articular-cartilage slices up to 0.5 mm thick were dissected from the underlying bone into 2 ml of the above incubation medium at 20°C. The cartilage was blotted dry between filter papers and 40–50 mg wet wt. was transferred to 1 ml of fresh medium. After preliminary incubation for 20 min at 37°C under CO₂+O₂ (5:95), a further 1 ml of the medium containing 2 mM-sodium [1-¹⁴C]acetate (10 μCi/μmol) was added. Incubations were continued at 37°C under CO₂+O₂ (5:95) for a further 2 h and the reactions stopped by heating the incubation mixtures in a boiling-water bath for 2 min. The medium was decanted, and the cartilage slices were washed with 2×10 ml of water and dried to constant weight at 100°C.

Proteolytic digestion of cartilage samples

Cartilage samples (10–25 mg dry wt.) were suspended in 3 ml of solution containing 0.25 M-potassium acetate, 0.025 M-KH₂PO₄, 0.00125 M-EDTA (sodium salt) and 1 mg of cysteine hydrochloride/ml, at pH 6.3. Papain (twice-crystallized, prepared from papaya latex by the method of Kimmel & Smith, 1957) was added as a suspension in 0.2 M-NaCl to give a final

ratio of cartilage to enzyme between 30:1 and 50:1 (w/w). Digestion was carried out at 65°C for 24h.

Isolation of glycosaminoglycans

The papain digests were desalted on a column of Bio-Gel P-4 (28cm × 2cm diameter). The column was eluted at 48ml/h with 0.2M-acetic acid adjusted to pH 6.0 with pyridine, and 9ml fractions were collected.

Fractionation of glycosaminoglycans on ECTEOLA-cellulose

The hexuronate-positive fractions excluded from Bio-Gel P-4 were combined and fractionated on a column (36cm × 2cm diameter) of Whatman Chromedia ET-II [ECTEOLA-cellulose (Cl⁻ form); W. and R. Balston Ltd., Maidstone, Kent, U.K.]. The column was eluted with a linear gradient of NaCl in 0.02M-imidazole buffer, pH 5.7, by the method of Robinson & Hopwood (1973). Columns were run at 60ml/h and 9ml fractions were collected.

Electrophoresis of glycosaminoglycans on cellulose acetate strips

Electrophoresis was performed on cellulose acetate strips (10cm × 2.5cm; Sephaphore III; Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) by the method of Wood *et al.* (1973) at a constant current of 1.25mA/strip. Two samples, each containing 3–5 µg of hexuronic acid, were applied to each strip in bands of 0.5cm width. One sample on each strip contained known standards of hyaluronic acid and chondroitin sulphate; the second was a sample of unknown glycosaminoglycan. Sample and standards were run in duplicate; one strip was stained with aq. 0.5% Toluidine Blue (BDH Ltd., Poole, Dorset, U.K.) and the other was cut in half. The half containing standard glycosaminoglycans was stained as described above and the other half, containing the sample, was cut into 0.25cm sections for radioactivity determinations.

Hexosamine analysis

Samples for hexosamine analysis were hydrolysed in 4M-HCl at 100°C for 8h in tubes lightly sealed with glass marbles. The acid was removed on a Buchler Rotary Evapomix (Buchler Instruments, Fort Lee, N.J., U.S.A.). The hexosamines were fractionated and determined on a Beckman Spinco Amino Acid Auto-Analyser. Correction factors for incomplete hydrolysis of polymer and acidic degradation of free hexosamine were calculated from data obtained when a standard sample of chondroitin sulphate was hydrolysed at 100°C for several measured periods of

time. Extrapolation to zero time showed that a yield of 88% of the total hexosamine was obtained after hydrolysis for 8h.

Preparation of disaccharides from glycosaminoglycans with chondroitin ABC lyase

Chondroitin ABC lyase (EC 4.2.2.4) was prepared from *Proteus vulgaris* and was a gift from Dr. M. Okayama, Department of Biochemistry, Monash University, Melbourne, Australia. The enzyme preparation was stored in 50mM-Tris-HCl buffer, pH 8.0, at 0°C, and released 0.01 µmol of hexuronic acid/min per µl of enzyme solution at 37°C from standard chondroitin sulphate.

The appropriate fractions from ECTEOLA-cellulose were combined and concentrated to 5ml and the solution was adjusted to 0.01M with EDTA (disodium salt). This solution was dialysed against five changes of water (each of 2 litres) with gentle rocking at 4°C over a 72h period. The solution, containing 75–200 µg of hexuronic acid, was evaporated to dryness and the residue dissolved in 0.1ml of 0.1M-Tris adjusted to pH 8.0 with 2M-HCl. Chondroitin ABC lyase (10 µl) was added and the solution incubated at 37°C for 16h. The products were subjected to descending chromatography for 31h on Whatman 3MM paper in isobutyric acid–2M-NH₃ (5:3, v/v). The paper was cut into strips and the material eluted and analysed for hexuronic acid and radioactivity.

Fractionation of the products of chondroitin ABC lyase digestion on Dowex 1

Dowex 1 (X4; Cl⁻ form; Sigma, St. Louis, Mo., U.S.A.) was converted into the formate form by sequentially washing with 0.5M-NaOH, 2M-sodium formate, 2M-sodium formate–4M-formic acid and 0.01M-formic acid.

To the chondroitin ABC lyase-digested sample was added 5mg of the unsaturated disaccharide derived from chondroitin (3-*O*-β-gluc-4-enuronosyl-*N*-acetyl-galactosamine, a gift from Dr. H. C. Robinson, Department of Biochemistry, Monash University, Melbourne, Australia). The sample was diluted 15-fold with 0.01M-formic acid and loaded on to a column (20cm × 1cm diameter) of Dowex 1 (X4; formate form) previously equilibrated with 0.01M-formic acid. The column was then washed with 20ml of 0.01M-formic acid and the material eluted with a linear gradient generated from 500ml of 0.01M-formic acid and 500ml of 0.8M-formic acid. The flow rate was 60ml/h and 10ml fractions were collected. This fractionation is a modification of the method of Hoevenaars & Robinson (1974).

This procedure was also carried out on a characterized preparation of ¹⁴C-labelled hyaluronic acid (a gift from Dr. R. Fraser, Royal Melbourne Hospital,

Melbourne, Australia), which had been isolated from tissue cultures of human synovial cells.

Hexuronic acid was determined by the method of Dische (1947) as modified by Bitter & Muir (1962).

Radioactivity determinations were carried out with a Packard Tri-Carb liquid-scintillation spectrometer, model 3003. Samples (0.5 ml) were mixed with 10 ml of scintillator mixture prepared by the method of Bruno & Christian (1961). ^{14}C was determined at 76% efficiency. Cellulose acetate strips used in electrophoresis were soluble in the scintillator solution. No corrections were made for quenching.

Results

Glycosaminoglycans were prepared from the articular cartilage of the hind knee joints of three rabbits and the four hock joints of a single sheep after incubation with $[1-^{14}\text{C}]\text{acetate}$ *in vitro*. The material was desalted on Bio-Gel P-4, and was fractionated on ECTEOLA-cellulose. Results are shown in Fig. 1, where they are compared with the separation of a mixture of unlabelled standard hyaluronic acid and chondroitin sulphate. Two peaks of hexuronate-containing material were evident; the first peak (peak I, Fig. 1) was eluted by the same concentration of NaCl in all three samples. This peak from the sheep and rabbit cartilages contained 1.5 and 5% of the total hexuronate respectively. Peak-II material contained the bulk of the hexuronic acid and radioactivity in the sample and corresponded to the major chondroitin sulphate components of the cartilage.

The major hexosamine in peak-I fractions from both sheep and rabbit cartilage was glucosamine, with only a trace of galactosamine present in the sheep material. The molar ratio of glucosamine to galactosamine of the rabbit material was 2.4. The molar ratio of total hexosamine to hexuronic acid was 1.01 in the sheep material and 0.97 in the rabbit. This rules out the presence of significant quantities of keratan sulphate in these peak-I fractions.

Electrophoresis on cellulose acetate strips

Samples of the peak-I material from sheep and rabbits were compared with standard hyaluronic acid and chondroitin sulphate by electrophoresis; the results are shown in Fig. 2. Both samples showed a Toluidine Blue-staining material with a mobility equal to that of hyaluronic acid. Radioactivity determinations along the half of the strip containing articular-cartilage material showed a single peak of radioactivity, which again corresponded to the position of standard hyaluronic acid. Overall recoveries of the radioactivity applied to the strip were calculated to be 102% for the sheep material and 116% for that of the rabbit. It was found, however, that chondroitin, when subjected to this

electrophoretic method, had the same mobility as hyaluronic acid. Nevertheless, these results, together with the results of hexosamine analysis, indicate that

Fig. 1. Elution from ECTEOLA-cellulose of the glycosaminoglycans from articular cartilages labelled with $[1-^{14}\text{C}]\text{acetate}$ *in vitro*

Cartilages were digested with papain and the products excluded from Bio-Gel P-4 were eluted from a column (36 cm $\times$ 2 cm diameter) of ECTEOLA-cellulose with a linear gradient of NaCl; 9 ml fractions were collected. The glycosaminoglycan profiles of material from sheep (b; 1.7 mg of hexuronic acid) and rabbit (c; 1.8 mg of hexuronic acid) articular cartilages are compared with authentic hyaluronic acid and chondroitin sulphate (a; 1 mg and 3.5 mg of hexuronic acid respectively). The NaCl gradient shown in (a) was the same for all three profiles. Peak I, hyaluronic acid; peak II, chondroitin sulphate. ○, Hexuronic acid; ■, radioactivity; —, NaCl gradient.

Fig. 2. Electrophoresis of glycosaminoglycans on cellulose acetate strips

Electrophoresis was carried out at 1.24mA per strip for 40min in acetic acid-pyridine-water (20:1:179, by vol.) at pH 3.1. (a), Standard hyaluronic acid and chondroitin sulphate stained with aq. 0.5% Toluidine Blue. (bI), Peak-I (Fig. 1) material prepared from sheep articular cartilage and stained with Toluidine Blue. (bII), as for (bI) but radioactivity determined along the strip. (cI), Peak-I (Fig. 1) material prepared from rabbit articular cartilage and stained with Toluidine Blue. (cII), as for (cI) but radioactivity determined along the strip. The arrow indicates the line of application of sample; each sample and standard contained 3–5 μ g of hexuronic acid.

Fig. 3. Fractionation of the chondroitin ABC lyase digestion products of the peak-I materials eluted from ECTEOLA-cellulose on Dowex 1 (X4, formate form)

Samples of the peak-I materials from ECTEOLA-cellulose (Fig. 1; containing 75–200 μ g of hexuronic acid) were digested with chondroitin ABC lyase. Then 5mg of the unsaturated disaccharide derived from chondroitin was added to each and the samples were fractionated on a column (20cm $\times$ 1cm diameter) of Dowex 1 (X4; formate form) with a gradient of formic acid. Fractions (10ml) were collected. Material prepared from sheep (b) and rabbit (c) articular cartilage is compared with the unsaturated disaccharide of hyaluronic acid (a). The formic acid gradient shown in (a) was the same for all three profiles. $\circ$, Hexuronic acid; $\blacksquare$, radioactivity; —, formic acid gradient.

hyaluronic acid is the main component eluted in peak I.

Separation of the products of chondroitin ABC lyase digestion on Dowex 1 (X4; formate form)

After chondroitin ABC lyase digestion of the peak-I material and addition of the unsaturated disaccharide produced by digestion of chondroitin, the mixture was fractionated on Dowex 1 (X4; formate form). Hexuronic acid and radioactivity profiles are shown in Fig. 3, where they are compared with that of a

mixture of the unsaturated disaccharides of chondroitin and of standard ^{14}C -labelled hyaluronic acid (3-*O*- β -gluc-4-enuronosyl-*N*-acetylglucosamine). With each preparation, there was a predominant peak of radioactivity which coincided with a small peak of hexuronic acid. The positions of the peaks of radioactivity in the sheep and rabbit preparations corresponded to the position of the unsaturated disaccharide derived from synovial hyaluronic acid. The major hexuronate peak, corresponding to the added unsaturated disaccharide derived from chondroitin, contained little radioactivity and was separated from the major radioactive peak. Some incorporation of $[1-^{14}\text{C}]$ acetate into chondroitin had taken place in the rabbit cartilage, but little was detected in the sheep cartilage. This result agrees favourably with the hexosamine analysis of the original peak-I material from ECTEOLA-cellulose, where there was measurable galactosamine in the rabbit material but only traces in that of the sheep.

Similar conclusions were drawn concerning the incorporation of label into hyaluronate when chondroitin ABC lyase digests were subjected to paper chromatography. However, this method results in poor separation of chondroitin and hyaluronate products.

Discussion

The results of these experiments demonstrate that hyaluronic acid is present in articular cartilage and can be synthesized by the cartilage itself. The quantities of hyaluronic acid are larger than those present in pig laryngeal cartilage (Hardingham & Muir, 1973), but the articular-cartilage samples are undoubtedly contaminated with synovial-fluid hyaluronate. In both rabbit and sheep cartilage, the specific radioactivity (d.p.m./mg of hexuronate) of the hyaluronic acid in peak I (Fig. 1) was higher than that of the chondroitin sulphate in peak II, even though the hyaluronate was only a minor component of the total cartilage glycosaminoglycan. These results suggest that, in our experimental system, hyaluronate has a considerably shorter turnover time than chondroitin sulphate, but they should be considered in the light of observations that the rate of synthesis of hyaluronate by embryonic chicken cartilage in culture is altered if glucosamine is added to the culture medium (Kim & Conrad, 1974).

Hyaluronic acid in articular cartilage could function in several ways. It might lead to aggregation of proteoglycan subunits (Hardingham & Muir,

1972; Gregory, 1973). Hyaluronic acid synthesized by the cartilage may also form the layer of hyaluronic acid and protein on the surface of articular cartilage, and this could serve to protect the underlying cartilage from mechanical damage (Balazs & Gibbs, 1970). Further, blood cells and fibroblasts do not adhere to a film of hyaluronic acid, which also inhibits the mobility of these cells (Balazs, 1972). By preventing the close proximity of cartilage and any cells which may be present in the synovial fluid, the cartilage matrix may be protected from attack by enzymes present in such cells which are known to be able to degrade proteoglycans (Ziff *et al.*, 1960). Finally, hyaluronic acid could serve as a lubricant for the articulating surfaces during movement (Walker *et al.*, 1970).

References

- Balazs, E. A. (1972) *Newsletter of Aging of Connective Tissue Group*, May Issue, pp. 3–22
- Balazs, E. A. & Gibbs, D. A. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 3, pp. 1241–1254, Academic Press, London
- Balazs, E. A., Bloom, G. D. & Swann, D. A. (1966) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **25**, 1813–1816
- Baxter, E., Fraser, J. R. E. & Harris, G. S. (1973) *Ann. Rheum. Dis.* **32**, 35–40
- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
- Bruno, G. A. & Christian, J. E. (1961) *Anal. Chem.* **33**, 1216–1218
- Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198
- Gregory, J. D. (1973) *Biochem. J.* **133**, 383–386
- Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
- Hardingham, T. E. & Muir, H. (1973) *Biochem. Soc. Trans.* **1**, 282–284
- Hoevenaars, R. & Robinson, H. C. (1974) *Proc. Aust. Biochem. Soc.* **7**, 32
- Kim, J. J. & Conrad, H. E. (1974) *J. Biol. Chem.* **249**, 3091–3097
- Kimmel, J. R. & Smith, E. L. (1957) *Methods Enzymol.* **19**, 267–335
- Robinson, H. C. & Hopwood, J. J. (1973) *Biochem. J.* **133**, 457–470
- Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
- Seppala, P. & Balazs, E. A. (1969) *J. Gerontol.* **24**, 309–314
- Walker, P. S., Unsworth, A., Dawson, P., Sikowski, J. & Weight, V. (1970) *Ann. Rheum. Dis.* **29**, 591–602
- Wood, K. M., Wusteman, F. S. & Curtis, C. G. (1973) *Biochem. J.* **134**, 1009–1013
- Ziff, M., Gribetz, J. & Lospalluto, J. (1960) *J. Clin. Invest.* **39**, 405–412