Biochem. J. (1983) **215**, 705–708
Printed in Great Britain

Differences in the rates of aggregation of proteoglycans from human articular cartilage and chondrosarcoma

Michael T. BAYLISS, George D. RIDGWAY and S. Yousuf ALI
Experimental Pathology Unit, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middx. HA7 4LP, U.K.

(*Received 30 August 1983/Accepted 5 October 1983*)

Pieces of adult human articular cartilage and chondrosarcoma were incubated in the presence of [^{35}S]sulphate. After continuous or pulse–change incorporation of radioactivity, proteoglycans were extracted with 4.0M-guanidinium chloride, purified by equilibrium density-gradient centrifugation and fractionated by gel chromatography. A comparison of the results suggests that the formation of stable aggregates occurs at a lower rate in articular cartilage than in chondrosarcoma.

Proteoglycans are major constituents of the extracellular matrix of hyaline cartilage and are largely responsible for the high elasticity and resilience of the tissue (Kempson, 1979). The proteoglycan molecule consists of a central protein core along which variable amounts of chondroitin sulphate, keratan sulphate and oligosaccharides are covalently bound (Heinegård & Axelsson, 1977; Thonar & Sweet, 1979). In the extracellular matrix, proteoglycans are present primarily as large aggregates, where many molecules are bound specifically to hyaluronic acid through an interaction with one end of the protein core, the hyaluronic acid-binding region. This complex is further stabilized by link proteins of small molecular mass that bind to both the hyaluronic acid-binding region and hyaluronic acid. Aggregate structures such as these have been identified in human articular cartilage (Bayliss & Ali, 1978; Bayliss *et al.*, 1983) and in human chondrosarcoma (Pal *et al.*, 1978; Sweet *et al.*, 1979).

Studies of proteoglycan biosynthesis performed with chondrocytes isolated from the Swarm rat chondrosarcoma indicate that newly synthesized molecules are rapidly incorporated into link-stabilized aggregates without going through a detectable non-aggregating form (Kimura *et al.*, 1979). Additional experiments have show that the intracellular pool of core protein, isolated before its substitution with glycosaminoglycan chain, is also capable of forming stable aggregates (Kimura *et al.*, 1981). Oegema (1980), however, has presented evidence for a hyaluronic acid-non-interactive precursor in some diseased specimens of human articular cartilage, but not in normal cartilage. The experiments described in the present paper compare the rates of assembly of proteoglycan aggregates in organ culture of human normal articular cartilage and chondrosarcoma.

Experimental procedures

Materials

Guanidinium chloride, CsCl and human umbilical-cord hyaluronic acid were from Sigma Chemical Co. (Poole, Dorset, U.K.). Eagle's Basal Medium with or without $MgSO_4$ was obtained from Gibco Europe (Glasgow, Scotland, U.K.), and carrier-free [^{35}S]sulphate (25–40Ci/mg) was from Amersham International (Amersham, Bucks., U.K.). Sepharose 2B was from Pharmacia (London W.5, U.K.).

Tissue source

Full-depth plugs of articular cartilage were obtained from the femoral heads of two hindquarter amputation specimens [R. C. (35 years' old) and P. J. (65 years' old). The limbs were amputated because of primary chondrosarcoma in the mid-femur region. The diagnosis was made on the basis of a biopsy sample and confirmed by histological examination of representative areas of the resected tumour. The articular cartilage was macroscopically normal, with no fibrillation in the case of R. C. and only minimal surface disruption around and inferior to the fovea in the case of P. J. Only cartilage with an intact surface was used in the present study. Pieces of tumour with a similar macroscopic appearance (greyish in colour and gelatinous in texture) were dissected from each specimen. The tissues were kept moist with cold saline (0.15M-NaCl) throughout the dissection pro-

cedures, which were completed within 30min of amputation.

Culture conditions

The rate of sulphate incorporation by articular cartilage was determined as described by Byers *et al.* (1977). Full-depth plugs of tissue were incubated in 3ml of Eagle's Basal Medium containing 0.8mM-$MgSO_4$, 20mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), 10mM-glutamine, 200 units of penicillin/ml and 10μCi of carrier-free [^{35}S]sulphate/ml. After incubation at 37°C for 4h the cartilage was washed four times with 20ml portions of 0.15M-NaCl at 4°C to remove non-incorporated radioisotope and digested with papain. The incorporated radioisotope was measured in a Nuclear Enterprises liquid-scintillation spectrometer and the rate of sulphate uptake calculated as mmol of sulphate/h per g wet wt. of cartilage.

The radiolabelling of cartilage and tumour before isolation of proteoglycans was carried out in 3ml of the same medium, with a final $MgSO_4$ concentration of 0.05mM, and the [^{35}S]sulphate added was 100μCi/ml. Tissues were incubated at 37°C for various times and the proteoglycans extracted as described below. In pulse–chase experiments cartilage and tumour were incubated with [^{35}S]sulphate as above and then washed with 5ml of warm medium containing 0.8mM-$MgSO_4$. Incubation was then continued for various times of non-radioactive chase in fresh medium containing 0.8mM-$MgSO_4$.

Extraction and isolation of proteoglycans

Articular cartilage was sectioned at 20μm on a cryostat as described by Bayliss *et al.* (1983), and tumour was finely minced with a scalpel on a cooled Petri dish. Proteoglycans were extracted from both tissues with 4M-guanidinium chloride, pH7.0, containing proteinase inhibitors, at 4°C for 24h as described previously (Bayliss *et al.*, 1983). The extracts were filtered through glass-wool and dialysed against 9vol. of 0.1mM-sodium phosphate buffer, pH 7.0, at 4°C. The density of each extract was adjusted to 1.5g/ml with solid CsCl and centrifuged at 100000$g_{av.}$ for 48h at 10°C. Gradients were fractionated into three equal-sized fractions (A_1, A_2 and A_3, in the terminology of Heinegård, 1977). Such fractions were dialysed exhaustively against 0.01M-phosphate buffer, pH7.0, and then against 0.1M-phosphate buffer, pH7.0, containing 0.1M-NaCl.

The high-density A_1 fractions were chromatographed on a column (150cm × 0.75cm) of Sepharose 2B at 3ml/h in 0.1M-phosphate buffer, pH7.0, containing 0.1M-NaCl. Fractions (1ml) were assayed for uronic acid by an automated modification of the Bitter & Muir (1962) method and for radioactivity by mixing 0.5ml fractions with 5ml of Beckman Ready Solv EP scintillation cocktail. The proportion of aggregated proteoglycans in the A_1 fractions was estimated by cutting and weighing a tracing of the uronic acid elution profile (Hardingham & Muir, 1974).

Results and discussion

With the culture system described, the metabolic activity of articular cartilage was constant over the incubation period. The rates of sulphation at 4h and 18h of incubation were 1.86×10^{-6} and 1.94×10^{-6}mmol/h per g wet wt. respectively. Extraction of articular-cartilage 20μm sections with 4M-guanidinium chloride released 85% of the total uronic acid and 95% of the incorporated [^{35}S]sulphate from the tissue (Bayliss *et al.*, 1983). Similar high yields of labelled and non-labelled proteoglycans were obtained from finely diced chondrosarcoma. The A_1 fractions prepared from both tissues accounted for 90–95% of the uronic acid and 75–80% of the [^{35}S]sulphate in the gradients. Although there was no significant change in the total incorporation of radioisotope into articular cartilage during the chase period, the chondrosarcoma specimens did show an increased incorporation over 18h of 40–50%, indicating that additional synthesis had occurred. Gel chromatography of the articular-cartilage and chondrosarcoma A_1 fractions on Sepharose 2B showed that most of the non-labelled proteoglycans were eluted in the column void volume as aggregates (Fig. 1*a*). These could be disaggregated by reduction and alkylation (results not shown). The ^{35}S-labelled proteoglycans in the 4h-pulse samples of chondrosarcoma (P. J.) were also highly aggregated (58%) as were those in the 18h-chase fraction (66%) (Fig. 1*b*). In contrast, those in the 4h-pulse fraction from articular cartilage were predominantly non-aggregated (Fig. 1*c*), but the proportion of radiolabelled aggregate increased from 23% to 47% during the subsequent chase period.

Similar results were obtained with the second patient (R. C.). Changes in the extracellular environment of chondrocytes may alter their rate of metabolism. Therefore the total incubation time was decreased for this specimen in order to minimize any effect of tissue swelling and leaching of proteoglycans from the chondrosarcoma that might have occurred during longer periods of pulse/chase. The aggregate contents of both the $\frac{1}{2}$h-pulse and 4h-chase fractions of chondrosarcoma (R. C.) were high (56% and 63% respectively) (Fig. 1*d*). Once again a considerable fraction of cartilage ^{35}S-labelled proteoglycan was not aggregated after the $\frac{1}{2}$h-pulse incubation, and the proportion of aggregate increased from 27% to 42% during the non-radioactive chase (Fig. 1*e*). We have also

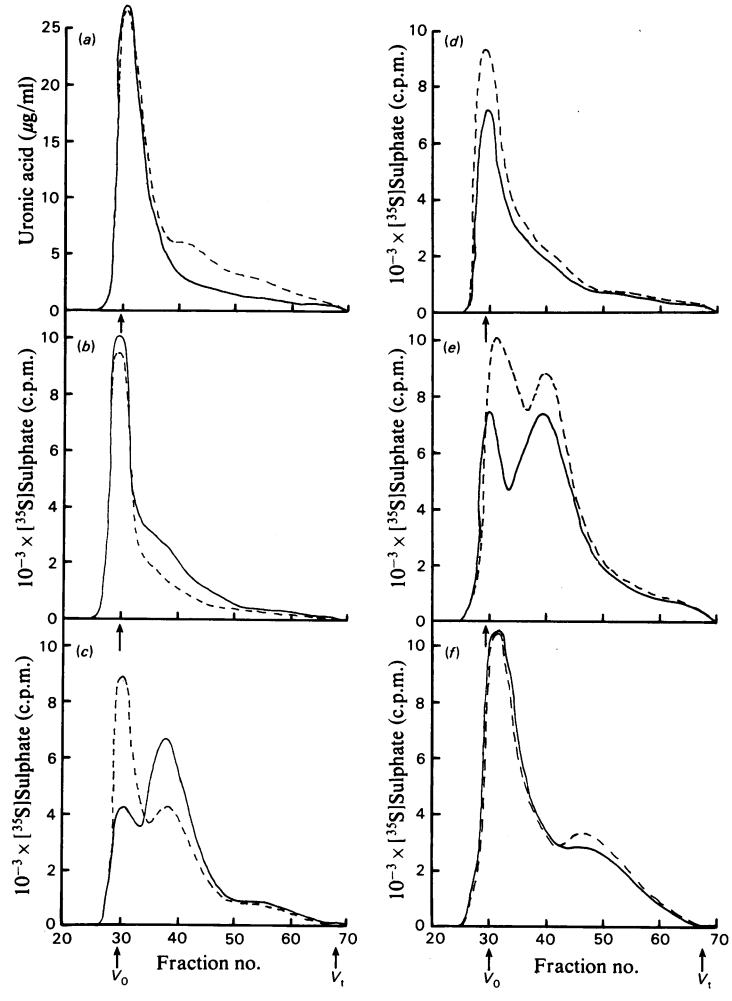

Fig. 1. *Gel chromatography on Sepharose 2B of proteoglycans (A_1) from human articular cartilage and chondrosarcoma*
(*a*) Non-labelled proteoglycans from patient P. J.: chondrosarcoma (——) and articular cartilage (– – –). Fractions (A_1) from patient (R. C.) gave similar profiles. (*b*) and (*c*) [^{35}S]Sulphate-labelled proteoglycans from chondrosarcoma (*b*) and articular cartilage (*c*) from patient P. J.: 4h pulse (——) and 18h chase (– – –). (*d*) and (*e*) [^{35}S]Sulphate-labelled proteoglycans from chondrosarcoma (*d*) and articular cartilage (*e*) from patient R. C.: ½h pulse (——) and 4h chase (– – –). (*f*) [^{35}S]Sulphate-labelled proteoglycans from the ½h-pulse (R. C.) (——) and 4h-pulse (P. J.) (– – –) articular-cartilage preparations in the presence of hyaluronic acid. V_0, Void volume; V_t, total volume of the column.

observed the same changes in aggregate content in normal cartilage samples from femoral-head fractures and normal femoral condyles from above-knee amputations (for vascular insufficiency in the lower limb). It is unlikely, therefore, that the phenomenon is a specific effect of tumour on articular-cartilage metabolism.

At first glance our results would seem to be at variance with those reported by Oegema (1980), who found that in normal cartilage the newly synthesized proteoglycans were just as capable of aggregating at 4h as at 18h. However, in his experimental procedure the ability to form aggregates was assessed by mixing disaggregated proteoglycans with exogenous hyaluronic acid. In the system described in the present paper, re-aggregation was allowed to proceed in the presence of endogenous hyaluronic acid and link-protein. Nevertheless, when the ½h-pulse and 4h-pulse samples from articular cartilage were mixed with additional hyaluronic acid, there was an increase in the fraction eluted in the column void volume, indicating that the

newly synthesized molecules are capable of interacting with hyaluronic acid and therefore do have a functional hyaluronic acid-binding region (Fig. 1*f*),

One possible explanation for this anomalous behaviour is that the newly synthesized proteoglycans cannot initially form as stable an aggregate as the non-radioactive proteoglycans in the extract. The latter, which are in excess, would compete more favourably for binding sites on the endogenous hyaluronic acid. During the subsequent chase period the newly synthesized proteoglycans might then be modified so that they could compete equally for binding sites. Because the labelled proteoglycans in the pulse sample are capable of complexing with hyaluronic acid, it may be that the modification is in their ability to bind link-protein.

The rapid formation of aggregates by newly synthesized proteoglycans in the human chondrosarcoma in in keeping with the results reported by Kimura *et al.* (1979), who isolated aggregated structures in culture media of Swarm rat chondrosarcoma chondrocytes early after synthesis. Whether the conversion into a stable form is faster than in articular cartilage or whether the unstable intermediate is absent from human chondrosarcoma is not known at present. The formation of aggregates by the intracellular core-protein pool from rat chondrosarcoma (Kimura *et al.*, 1981) would, however, argue against the presence of an unstable form.

It would appear, therefore, that adult human articular cartilage synthesizes a hyaluronic acid-interactive precursor that cannot initially form stable aggregates. The absence of this precursor from tumour may be related to the immature type of metabolism, rapid turnover and growth of the tissue.

We are grateful to the Arthritis and Rheumatism Council for financial support and to the surgeons and theatre staff of the Royal National Orthopaedic Hospital for the human specimens.

References

Bayliss, M. T. & Ali, S. Y. (1978) *Biochem. J.* **176**, 683–693

Bayliss, M. T., Venn, M., Maroudas, A. & Ali, S. Y. (1983) *Biochem. J.* **209**, 387–400

Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

Byers, P. D., Maroudas, A., Oztop, F., Stockwell, R. A. & Venn, M. F. (1977) *Connect. Tissue Res.* **5**, 41–49

Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565–581

Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1989

Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979

Kempson, G. E. (1979) in *Adult Articular Cartilage* (Freeman, M. A. R., ed.), 2nd edn., pp. 333–415, Pitman, London

Kimura, J. H., Hardingham, T. E., Hascall, V. C. & Solursh, M. (1979) *J. Biol. Chem.* **254**, 2600–2609

Kimura, J. H., Thonar, E. J.-M. A., Hascall, V. C., Reiner, A. & Poole, A. R. (1981) *J. Biol. Chem.* **256**, 7890–7897

Oegema, T. R. (1980) *Nature (London)* **288**, 583–585

Pal, S., Strider, W., Margolis, R., Gallo, G., Lee-Huang, S. & Rosenberg, L. (1978) *J. Biol. Chem.* **253**, 1279–1289

Sweet, M. B. E., Thonar, E. J.-M. A. & Marsh, J. (1979) *Arch. Biochem. Biophys.* **198**, 439–448

Thonar, E. J.-M. A. & Sweet, M. B. E. (1979) *Biochim. Biophys. Acta* **584**, 353–357