Synthesis of Cartilage-Specific Proteoglycan by Suspension Cultures of Adult Chondrocytes

By OLE W. WIEBKIN and HELEN MUIR

*Division of Biochemistry, Kennedy Institute of Rheumatology, Bute Gardens,
Hammersmith, London W6 7DW, U.K.*

(Received 22 December 1976)

Chondrocytes isolated from larynges of adult pigs were cultured as cell suspensions for at least 4 days before use. During continuous-labelling experiments in nutrient medium for 18 h with $^{35}\text{SO}_4^{2-}$ and $[^3\text{H}]\text{glucosamine}$ as precursors, some macromolecular polyanionic material was synthesized which behaved on gel chromatography as proteoglycan. Gel chromatography on Sepharose 2B showed that a proportion of the proteoglycans in the medium appeared to be aggregated, and was dissociated in 4M-guanidinium chloride. Moreover the dissociated proteoglycan interacted with hyaluronic acid. Newly synthesized proteoglycan was larger than the average total cetylpyridinium chloride-precipitable material assayed as uronic acid alone.

Chondrocytes, in cartilages, are sparsely distributed within an avascular matrix. This is a stiff gel into which both the diffusion of large solutes in the body fluids (Maroudas, 1973) and cellular co-operation are limited. Chondrocytes are thus in a peculiar situation and appear to be affected by the conditions of the matrix that surrounds them. For example, the depletion of the matrix of chick-embryonic cartilage in organ culture by the action of degradative enzymes leads to increased production of glycosaminoglycans (Fitton Jackson, 1970) and proteoglycan (Hardingham *et al.*, 1972), whereas in experimental osteoarthritis the synthesis of both collagen and proteoglycan is increased (McDevitt *et al.*, 1975). It is therefore possible that some constituent of the matrix itself may affect cartilage cells directly and regulate the synthesis of macromolecules. Among the various cartilage components that have been reported to affect chondrocyte biosynthesis specifically, hyaluronic acid is the only one that appears to inhibit proteoglycan synthesis (Wiebkin & Muir, 1973*a,b*, 1975*a*; Toole, 1973; Solursh *et al.*, 1974). Hyaluronate also plays a role in early chondrogenesis (Toole *et al.*, 1972).

Handley & Lowther (1976) have shown that xylo-sides will abolish the inhibitory effect of hyaluronate and suggest two alternative ways in which it may be acting. However, the results of Wiebkin & Muir (1975*b*) imply that the initial site of action is at the cell surface, possibly through the specific interaction of hyaluronate with proteoglycan. Cartilage proteoglycans appear to be unique in their ability to interact with hyaluronic acid to form large molecular aggregates described by Hardingham & Muir (1973).

The present results show that isolated chondro-

cytes are capable of synthesizing proteoglycans, which will interact with hyaluronic acid and in so doing become large enough to be excluded on Sepharose 2B.

Experimental

Materials

Foetal calf serum and powdered ingredients of both Hanks balanced salt solution and Leibovitz L-15 nutrient medium were supplied by Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. The ingredients of Tyrode's solution were supplied in powdered form by Difco Laboratories, East Molesey, Surrey, U.K.

Isotopically labelled compounds were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Ovine testicular hyaluronidase (EC 3.2.1.35) and cetylpyridinium chloride were obtained from Fisons Pharmaceutical Division, Loughborough, Leics., U.K. Trypsin (EC 3.4.21.4), crude collagenase of *Clostridium histolyticum* (EC 3.4.24.3) and DNAase* (EC 3.1.4.5) were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sepharose 2B was purchased from Pharmacia (GB) Ltd., Uxbridge Road, London W.5, U.K. Hyaluronic acid from umbilical cord was obtained from British Drug Houses, Poole, Dorset, U.K. Scintillation fluid (toluene/2-methoxyethanol, 3:2, v/v) contained per litre 80g of naphthalene and 4g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen. Trasylol was supplied by Bayer Pharmaceutical, Haywards Heath, Sussex, U.K.

* Abbreviation: DNAase, deoxyribonuclease.

Methods

Chondrocytes were isolated from adult laryngeal cartilage by sequential enzymic digestion of the matrix with short incubations, each in solutions of hyaluronidase and trypsin, followed by between 6 and 12 h in collagenase solution (125–200 units/ml; 1 unit of collagenase liberates from collagen amino acid equivalent in ninhydrin colour to $1.0 \mu\text{mol}$ of L-leucine in 18 h at pH 7.4 at 37°C). Cartilages from the thyroid plates of bacon pigs were obtained fresh from the slaughterhouse, scraped free of both soft connective tissue and perichondrium and then cut into small pieces ($1 \text{ mm} \times 1 \text{ mm}$). The cartilage from each animal was treated separately. The digestion procedure was essentially that of Green (1971) and described in further detail elsewhere (Wiebkin & Muir, 1973a,b). Cells were washed and suspended in Leibovitz L-15 medium containing 8% foetal calf serum. Such cells remained viable for at least 21 days, as shown by the exclusion of Trypan Blue dye and by the incorporation of $^{35}\text{SO}_4^{2-}$ into material precipitable by cetylpyridinium chloride.

The cells were maintained in culture for a minimum of 4 days before being used in the experiments described here. The cells' ultrastructure was intact (Wiebkin & Muir, 1975a) and neither mitoses nor uptake of ^3H thymidine were noted (Wiebkin & Muir, 1973a). The numbers of cells in suspension were counted in a haemocytometer.

Cells isolated from several cartilages were pooled and incubated at 37°C for 18 h in Leibovitz L-15 medium enriched with 8% foetal calf serum containing either $10 \mu\text{Ci}$ of ^3H glucosamine/ml or $5\text{--}8 \mu\text{Ci}$ of $^{35}\text{SO}_4^{2-}$ /ml. Cells and media were then separated and the cells washed, resuspended in 5–7 ml of Tyrode's solution, and disrupted in the presence of a small amount of kieselguhr by addition of 5% cetylpyridinium chloride in Tyrode's solution, to a final concentration of 1%. Complete rupture of cells was confirmed by microscopical examination.

The medium was similarly treated with kieselguhr, and 5% cetylpyridinium chloride was added in a continuous stream below the surface with gentle stirring to a final concentration of 1%. A mixture of proteinase inhibitors was included throughout both the precipitation and washing procedures, i.e. 1 mg of soya-bean trypsin inhibitor and 0.1 ml of Trasylol (1000 kallikrein inactivator units) per litre of 0.01 M-EDTA/0.1 M-6-aminohexanoic acid/0.005 M-benzamide hydrochloride.

The cetylpyridinium chloride precipitates thus formed were centrifuged at $3640g$ and washed once with 5 ml of 0.1 M- Na_2SO_4 , then dissolved in 2 ml of 1.25 M- MgCl_2 , and the material was reprecipitated with 8 ml of ice-cold ethanol and left at 4°C for 16 h. The flocculent ethanol precipitates were centri-

fuged at $3640g$ for 10 min and redissolved in either 0.5 M-sodium acetate (pH 6.8) or 4 M-guanidinium chloride, adjusted to pH 5.8 with sodium acetate, to give between 70 and 150 g of uronic acid/ml, and applied to a column ($35 \text{ cm} \times 0.9 \text{ cm}$) of Sepharose 2B. The columns were eluted with either 0.5 M-sodium acetate at a flow rate of 12 ml/h, or 4 M-guanidinium chloride at a flow rate of 3 ml/h. Fractions (1 ml) were collected throughout, and each fraction was dialysed separately against water overnight. The radioactivity (Wiebkin & Muir, 1973a) and uronic acid contents of each fraction were then determined, the latter by an automated modification (Heinegård, 1973) of the procedure of Bitter & Muir (1962).

The fractions eluted with guanidinium chloride were appropriately pooled to represent excluded, partially included and totally included material. The partially included and totally included pooled fractions were reduced to 1 ml by rotary evaporation, and hyaluronic acid was added to each in the proportion of sample to hyaluronic acid of 50:1 based on uronic acid. The resulting mixtures were, in turn, applied to the same Sepharose 2B column, equilibrated and eluted with 0.5 M-sodium acetate as described above.

Fig. 1. Gel chromatography under associative conditions. Material synthesized by chondrocytes during 24 h in nutrient medium containing $^{35}\text{SO}_4^{2-}$ or ^3H glucosamine was applied to a column ($35 \text{ cm} \times 0.9 \text{ cm}$) of Sepharose 2B and eluted with 0.5 M-sodium acetate at pH 6.8. ●, $^{35}\text{SO}_4^{2-}$; □, ^3H glucosamine; ■, uronic acid. Material precipitable with cetylpyridinium chloride in (a) media and (b) cells.

Fractions (1 ml) were again collected, dialysed and the elution profile of radioactivity was determined.

The characteristics of the columns were determined by eluting hyaluronic acid, inorganic $^{35}\text{SO}_4^{2-}$ and standard preparations of proteoglycan extracted from laryngeal cartilage and purified by density-gradient centrifugation under associative and dissociative conditions (Hardingham & Muir, 1974).

Results and Discussion

Products of cell synthesis

That constituents of cartilage matrix may influence chondrocytes can be examined only with cells isolated from matrix, such as those used in the present experiments. Cells isolated from cartilage by the procedures used here were active in synthesizing labelled material from $^{35}\text{SO}_4^{2-}$ or $[^3\text{H}]\text{glucosamine}$, which was precipitable by cetylpyridinium chloride and which appeared to be proteoglycan. Proteoglycans of

cartilage have the unique property of interacting with small amounts of hyaluronate to form very large complexes in which many proteoglycan molecules are bound to one hyaluronic acid chain via a single binding site (Hardingham & Muir, 1972). Natural aggregates of proteoglycan bound to hyaluronic acid exist in cartilage matrix. Gel chromatography on Sepharose 2B of macromolecules synthesized by cultured chondrocytes showed that the labelled material was of large molecular size (Fig. 1) and that a proportion of the labelled material in the medium might consist of proteoglycan aggregates which would be excluded from this gel (Hardingham & Muir, 1974). The elution profiles of $[^3\text{H}]\text{hexosamine}$ and $^{35}\text{SO}_4^{2-}$ in both medium and cells suggest that some hyaluronic acid was present in the excluded material, since this contained more $[^3\text{H}]\text{hexosamine}$ than $^{35}\text{SO}_4^{2-}$. Also, newly synthesized labelled material in the medium was larger than the average

Fig. 2. Gel chromatography under dissociative conditions. Material was synthesized by chondrocytes as in Fig. 1 except that 4M-guanidinium chloride was used for elution at pH 5.8. ●, $^{35}\text{SO}_4^{2-}$; □, $[^3\text{H}]\text{glucosamine}$. Fractions corresponding to retarded material R_1 (hatched areas) were pooled. Material precipitable with cetylpyridinium chloride in (a) media and (b) cells.

Fig. 3. Gel chromatography under associative conditions. Material retarded on a Sepharose 2B column (R_1 ; see Fig. 2) under dissociative conditions was mixed with hyaluronic acid (50:1, w/w, uronic acid) and rechromatographed in 0.5M-sodium acetate at pH 6.8. The hatched area shows the material excluded by Sepharose 2B under these associative conditions. ●, $^{35}\text{SO}_4^{2-}$; □, $[^3\text{H}]\text{glucosamine}$. (a) Material from culture media; (b) material from cells.

total cetylpyridinium chloride-precipitable material assayed as uronic acid alone. This suggestion was further supported by the difference in elution profiles of uronic acid and radioactivity when eluted with 0.5M-sodium acetate (Fig. 1) as compared with 4M-guanidinium chloride (Fig. 2), which dissociates proteoglycan aggregates (Hascall & Sajdera, 1969). Under dissociative conditions there was proportionately more [^3H]hexosamine-labelled material excluded by Sepharose 2B, particularly in the medium. Incubation of chondrocytes in media containing no serum resulted in the appearance of a greater proportion of low-molecular-weight labelled material, possibly due to degradation.

Material from both cells and medium was eluted under dissociative conditions in 4M-guanidinium chloride. A proportion of the retarded labelled fraction of R_1 (Figs. 2a and 2b), when mixed with hyaluronic acid in the ratio 50:1 (w/w, uronic acid), became excluded from the gel on elution under associative conditions in 0.5M-sodium acetate (Figs. 3a and 3b). The amount of excluded labelled material, particularly $^{35}\text{SO}_4^{2-}$, was greater in the medium than in the cells.

In the absence of proteinase inhibitors, with the 0.5M-sodium acetate very little material from the cells was excluded. It is assumed that liberation of lysosomal enzymes caused this effect.

Toole *et al.* (1972) have suggested that, by interacting with the cell surface, hyaluronate may regulate cell-surface activity, whereas attachment of small amounts of [^3H]- and [^{14}C]-hyaluronate to the surfaces of cultured chondrocytes inhibited incorporation of $^{35}\text{SO}_4^{2-}$ into macromolecular material by 20% (Wiebkin & Muir, 1975b). Delay in export of newly formed proteoglycans was noted, such that the distribution between cells, cell surface and medium was altered. This initial delay in export may consequently lead to the decrease in proteoglycan synthesis, at least during the 2h incubation of a standard test previously described (Wiebkin & Muir, 1975a, b). The present data, therefore, are in keeping with the suggestion that the material at the chondrocyte surface which binds hyaluronate has some of the

properties of cartilage proteoglycans that interact specifically with hyaluronate.

We thank Miss Glynis Oliver for technical assistance the Medical Research Council for the support of O.W.W., the Arthritis and Rheumatism Council for general support and T. Wall and Son Ltd., Southall, Middx., U.K., for supplying larynges.

References

- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
- Fitton Jackson, S. (1970) *Proc. R. Soc. London Ser. B* **175**, 405–453
- Green, W. T. (1971) *Clin. Orthop.* **75**, 248–260
- Handley, C. J. & Lowther, D. A. (1976) *Biochim. Biophys. Acta* **444**, 69–74
- Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
- Hardingham, T. E. & Muir, H. (1973) *Biochem. Soc. Trans.* **1**, 282–284
- Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565–581
- Hardingham, T. E., Fitton Jackson, S. & Muir, H. (1972) *Biochem. J.* **129**, 101–112
- Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396
- Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201
- Maroudas, A. (1973) in *Adult Articular Cartilage* (Freeman, M. A. R., ed.), pp. 131–170, Pitman Medical, London
- McDevitt, C. A., Eyre, D. R. & Muir, H. (1975) *Scand. J. Rheumatol.* **4**, Suppl. **8**, O3–O8
- Solursh, M., Vaerewyck, S. A. & Reiter, R. S. (1974) *Dev. Biol.* **41**, 233–244
- Toole, B. P. (1973) *Am. Zool.* **13**, 1061–1065
- Toole, B. P., Jackson, G. & Gross, J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1384–1386
- Wiebkin, O. W. & Muir, H. (1973a) *FEBS Lett.* **37**, 42–46
- Wiebkin, O. W. & Muir, H. (1973b) in *Biology of Fibroblast* (Kulonen, E. & Pikkariainen, J., eds.), pp. 231–236, Academic Press, New York
- Wiebkin, O. W. & Muir, H. (1975a) *Philos. Trans. R. Soc. London Ser. B* **271**, 283–291
- Wiebkin, O. W. & Muir, H. (1975b) in *Extracellular Matrix Influences on Gene Expression* (Slavkin, H. & Greulich, R., eds.), pp. 209–223, Academic Press, New York