Four classes of cell-associated proteoglycans in suspension cultures of articular-cartilage chondrocytes

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The characteristics of cell-associated proteoglycans were studied and compared with those from the medium in suspension cultures of calf articular-cartilage chondrocytes. By including hyaluronic acid or proteoglycan in the medium during [³⁵S]sulphate labelling the proportion of cell-surface-associated proteoglycans could be decreased from 34% to about 15% of all incorporated label. A pulse-chase experiment indicated that this decrease was probably due to blocking of the reassociation with the cells of proteoglycans exported to the medium. Three peaks of [³⁵S]sulphate-labelled proteoglycans from cell extracts and two from the medium were isolated by gel chromatography on Sephacryl S-500. These were characterized by agarose/polyacrylamide-gel electrophoresis, by SDS/polyacrylamide-gel electrophoresis of core proteins, by glycosaminoglycan composition and chain size as well as by distribution of glycosaminoglycans in proteolytic fragments. The results showed that associated with the cells were (a) large proteoglycans, typical for cartilage, apparently bound to hyaluronic acid at the cell surface, (b) an intermediate-size proteoglycan with chondroitin sulphate side chains (this proteoglycan, which had a large core protein, was only found associated with the cells and is apparently not related to the large proteoglycans), (c) a small proteoglycan with dermatan sulphate side chains with a low degree of epimerization, and (d) a somewhat smaller proteoglycan containing heparan sulphate side chains. The medium contained a large aggregating proteoglycan of similar nature to the large cell-associated proteoglycan and small proteoglycans with dermatan sulphate side chains with a higher degree of epimerization than those of the cells, i.e. containing some 20% iduronic acid.

INTRODUCTION

Chondrocytes in cartilage are surrounded by a predominant extracellular matrix mainly composed of collagen and proteoglycan. Little is known about the interactions of the matrix components with the cell surface, although they are probably important for the regulation of matrix turnover. A receptor for type II collagen has been identified (Mollenhauer & von der Mark, 1983), and proteoglycans can specifically bind to hyaluronic acid at the cell surface (Sommarin & Heinegård, 1983). Furthermore the biosynthetic activity of chondrocytes in culture can be modulated by proteoglycans or hyaluronic acid added to the culture medium (Nevo & Dorfman, 1972; Schwartz & Dorfman, 1975; Handley & Lowther, 1977; Wiebkin & Muir, 1977; Solursh *et al.*, 1980). In studies performed *in vivo* with young rabbits (Thomas, 1956) or in organ culture with chick-embryo cartilage sections (Hardingham *et al.*, 1972), chondrocytes were able to regulate the amount of proteoglycan in the extracellular matrix and replace any such lost upon enzyme treatment. This regulatory function may be mediated via interactions at the cell surface. The structural basis for such a mechanism is, however, not known, in part owing to the fact that the macromolecules present at the chondrocyte cell surface have not been characterized.

In a previous study we used suspension cultures of calf articular-cartilage chondrocytes to study the interaction of added proteoglycans with hyaluronic acid at the chondrocyte cell surface (Sommarin & Heinegård, 1983). In the present study a similar interaction of endogenously

synthesized proteoglycans was studied and proteoglycans bound by other mechanisms at the cell surface were isolated and characterized.

EXPERIMENTAL

Materials

The medium used for spinner culture was Ham's F12 medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 20 μ l of 50 $\times$ essential amino acids (for Eagle's minimum essential medium; Flow Laboratories, Bethesda, MD, U.S.A.)/ml, 20 μ g of dextran sulphate (Pharmacia Fine Chemicals, Uppsala, Sweden)/ml, 50 i.u. each of penicillin and streptomycin (Flow Laboratories)/ml and buffered with 20 mM-Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.), pH 7.4.

Radiochemicals were obtained from Amersham International (Amersham, Bucks., U.K.). Other chemicals were of analytical-reagent grade and obtained from standard commercial sources.

Cell culture

Chondrocytes were prepared from calf articular cartilage from the metacarpophalangeal joint as described previously (Sommarin & Heinegård, 1983). In short, cartilage was digested with collagenase and the liberated cells were washed and transferred to spinner culture in Ham's F12 medium with supplements. The cells were kept in spinner culture to allow reconstitution of their cell surface.

At the time of the experiment, i.e. after 36 h in spinner culture, the cells were transferred to Ham's F12 medium

buffered with Hepes but without other supplements. Radiolabelling was performed at 37 °C in 2 ml polypropylene test tubes (Sarstedt, Numbrecht, W. Germany) with 1 ml of medium containing 5×10^6 cells. The cells were kept in suspension with gentle agitation on a gyrating board (Nutator; Becton and Dickinson Labware, Oxnard, CA, U.S.A.). After labelling, the cells were pelleted by centrifugation in an Eppendorf 3200 centrifuge as described elsewhere (Björnsson & Heinegård, 1981), and the medium was removed. The cells were washed once by suspension in ice-cold medium and pelleted again, the wash medium was discarded and the cells were directly treated with trypsin or kept on ice for no more than 20 min before extraction.

Analysis of competition experiments

In the first two experiments (presented in Table 1 and Fig. 1) the cells were digested with trypsin (0.1%, w/v). To minimize biosynthetic activity a short time (15 min) and a low temperature (4 °C) were used. The cells were then pelleted, and the solubilized material was removed. The cell pellet was dissolved in 1 ml of 0.5 M-NaOH at 65 °C for 6 h. This solution was neutralized by the addition of 10 M-acetic acid. Cell viability after trypsin treatment was above 95% as judged by the Trypan Blue-exclusion test.

Proteoglycans were separated from free radiolabelling compound by cetylpyridinium chloride precipitation. Samples with added carrier chondroitin 6-sulphate (100 µg) were precipitated with 0.5% cetylpyridinium chloride, 0.6 M-NaCl, 20 mM-Na₂SO₄ and 50 mM-sodium acetate overnight at room temperature. The samples were centrifuged and the pellet was washed once with the above buffer. The pellet was solubilized with 4 M-guanidinium chloride, and Instagel (Packard, La Grange, IL, U.S.A.) was added for scintillation counting of radioactivity.

Extraction of cells

In other experiments trypsin digestion of cells was omitted and instead cell-bound material was extracted by a procedure involving the use of detergent. First 500 µl of 8% CHAPS/0.1 M-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors EDTA (20 mM), 6-aminohexanoic acid (0.2 M), benzamidinium chloride (10 mM), *N*-ethylmaleimide (10 mM) and phenylmethanesulphonyl fluoride (2 mM) was added to the cell pellet and the sample was vigorously stirred. After 15 min at 4 °C an equal volume of 8 M-guanidinium chloride was added and extraction was continued for 45 min. The non-extractable material was removed by centrifugation at 105000 *g*_{av} for 1 h at 4 °C in a 70.1 Ti angle rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). The extraction yield with this procedure was about 85% of all [³⁵S]sulphate-labelled material. Increasing the extraction time to 24 h did not increase the extraction yield. The proteolytic activity in these extracts was negligible, since a 24 h incubation of the extracts at room temperature did not change the elution profile of labelled components on Sephacryl S-500.

Analytical Sephacryl S-500 gel chromatography

Cell extracts or medium were chromatographed on a Sephacryl S-500 column (140 cm × 0.6 cm) eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-

sodium acetate buffer, pH 5.8. Fractions (0.7 ml) were collected and assayed for radioactivity.

Preparation of proteoglycan populations

Cells were incubated for 4 h in Ham's F12 medium with 1 mg of hyaluronic acid (human umbilical cord; Sigma Chemical Co.)/ml and 50 µCi of [³⁵S]sulphate/ml. The sulphate concentration was then adjusted to 3 mM, i.e. a 500-fold excess of non-radioactive sulphate was added from a 0.15 M stock solution. The cells were then chased in this medium for 20 min, pelleted and rinsed with fresh medium. They were then extracted with CHAPS and guanidinium chloride as described above. Both extract and medium were centrifuged as above to remove insoluble material and were then kept frozen at -60 °C until analysis.

Samples of extracts were transferred to 6 M-urea/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors listed above by gel filtration on Sephadex G-50 columns (10 ml) eluted with this solution. Samples of medium were adjusted to 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8, before being applied to the column. The void-volume fraction containing the proteoglycans was directly applied to a small ion-exchange column (3 cm × 0.8 cm) of DEAE-cellulose (Whatman DE52) in 6 M-urea/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. The column was eluted with 2 × 3 ml of 6 M-urea/0.5% Triton X-100/0.32 M-sodium acetate buffer, pH 5.8, to remove most of the protein in the samples. Proteoglycans were eluted with 3 ml of 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8.

Purified proteoglycans were fractionated by chromatography on a preparative Sephacryl S-500 column (1.6 cm × 142 cm) eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. Fractions (4 ml) were collected and assayed for radioactivity. The chromatogram was pooled as indicated in Figs. 3(a) and 3(b).

Agarose/polyacrylamide-gel electrophoresis

Fractions were pooled from a Sephacryl S-500 chromatogram of an extract, where the ion-exchange chromatography was omitted. Samples were precipitated with 5 vol. of ethanol overnight at 4 °C, with bovine articular-cartilage proteoglycan monomer (20 µg of an A1 preparation) as carrier. The precipitates were dissolved in 0.5% SDS/10% (w/v) sucrose/1 mM-Na₂SO₄/10 mM-Tris/acetate buffer, pH 6.8, for 4 h at 37 °C and electrophoresed on a slab gel of agarose/polyacrylamide essentially as described by Heinegård *et al.* (1985b). After fixing and staining of the gel with Toluidine Blue the radioactive bands were detected by fluorography (Chamberlain, 1979).

Glycosaminoglycan characterization

Proteoglycans in samples of pooled fractions were precipitated with 5 vol. of ethanol, with 100 µg of chondroitin 6-sulphate as a carrier. After centrifugation, the pellet was dissolved in 0.5 M-NaOH and incubated at 4 °C for 16 h. After neutralization with 10 M-acetic acid, the liberated glycosaminoglycan chains were precipitated with 9 vol. of 95% ethanol overnight at 4 °C. The precipitates were dissolved in 0.1 M-Tris/0.1 M-sodium acetate buffer, pH 7.6. The recovery in this procedure was

better than 90%. Samples were digested with 0.02 nominal unit ($\mu\text{mol}/\text{min}$) of either chondroitinase ACII or chondroitinase ABC at 37 °C for 5 h or subjected to HNO_2 treatment by the method of Shively & Conrad (1976). Treated and untreated control samples were then chromatographed on a Sephadex G-50 column (1 cm $\times$ 6.5 cm) eluted with 4 M-guanidinium chloride/50 mM-sodium acetate, buffer, pH 5.8, to separate disaccharides or short oligosaccharides (close to total volume) formed during enzyme or HNO_2 treatment from large resistant fragment (void volume). Fractions were collected directly into scintillation counting vials and radioactivity was determined.

Size of glycosaminoglycan side chains

Samples were precipitated with ethanol as for glycosaminoglycan analysis and treated with alkaline borohydride as described by Carlsson (1968). The size distribution of the glycosaminoglycan chains was then determined by gel chromatography on a Superose 6B column (0.65 cm $\times$ 92 cm) eluted with 0.2 M- Na_2SO_4 . Fractions (480 μl) were collected and assayed for radioactivity. The column was calibrated with chondroitin sulphate standards of known M_r values, which allowed the calculation of weight-average and number-average M_r values in accordance with Wasteson (1971).

Size of fragments produced by trypsin digestion

Samples were precipitated with ethanol as above but with 0.5 mg of bovine nasal-septum-cartilage proteoglycan monomer as carrier. The precipitates were solubilized in 0.1 M-Tris/acetate buffer, pH 7.6, and treated with 5 μg of trypsin [L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated; Sigma Chemical Co.] for 5 h at 37 °C. The digests were chromatographed on the Superose 6B column used for chain-size determination but were eluted with 0.5 M-sodium acetate buffer, pH 7. Carrier proteoglycan uronic acid was determined by the carbazole reaction, with an automated system (Heinegård, 1973).

Characterization of core proteins by SDS/polyacrylamide-gel electrophoresis

Cells were incubated with 50 μCi of [^3H]leucine and 10 μCi of [^{35}S]sulphate/ml in Ham's F 12 medium containing 1 mg of hyaluronic acid/ml. Proteoglycan populations were prepared essentially as before, but additional purification had been obtained by a second ion-exchange chromatography on DEAE-cellulose eluted with a 0.05–1 M-NaCl linear gradient in 7 M-urea/0.5% Triton/50 mM-sodium acetate buffer, pH 4 (results not shown). Samples were precipitated with 5 vol. of ethanol, and pellets were dissolved in 50 M-Tris/HCl buffer, pH 7.6, and digested with 0.02 unit of chondroitinase ABC and/or 0.02 unit of heparitinase with 5 μg of ovomucoid for 1 h at 37 °C. The samples were then electrophoresed on SDS/polyacrylamide gradient gels (3–10%) with the buffer system of Laemmli (1970). Radioactivity was detected by fluorography as above.

RESULTS

Prevention of binding of newly synthesized proteoglycan to cells by competition with hyaluronic acid or proteoglycan monomer

In control cultures as much as 34% of the newly synthesized labelled proteoglycans were bound at the cell

Table 1. Distribution of newly synthesized glycosaminoglycans among intra-, peri- and extra-cellular pools

Chondrocytes were incubated with 20 μCi of [^{35}S]sulphate/ml for 4 h in medium with 1 mg of hyaluronic acid (HA)/ml, with 3 mg of bovine nasal-septum-cartilage proteoglycan (PG)/ml or without additions as control. Cells were treated with trypsin to separate peri- and intra-cellular pools (see the Experimental section). Glycosaminoglycans were determined as cetylpyridinium chloride-precipitable [^{35}S]sulphate label as described in the Experimental section. The results shown are the means for two cultures. The percentage distribution represents the percentage of label in each pool of the total amount of label in each type of culture.

	Distribution of ^{35}S label	
	(d.p.m.)	(% of total)
Medium pool		
Control	168 337	63
HA added	269 634	82
PG added	252 191	83
Pericellular pool (trypsin-releasable)		
Control	90 031	34
HA added	52 412	16
PG added	44 945	15
Intracellular pool (trypsin-resistant)		
Control	8 222	3
HA added	6 920	2
PG added	5 591	2

surface and could be released by trypsin digestion (Table 1). Both hyaluronic acid and proteoglycan monomer when added in a large excess competed for binding of a portion of the endogenously synthesized proteoglycans to the cells, i.e. cell-bound proteoglycans were decreased by half to 15% of the total and those in the medium increased correspondingly (Table 1).

Kinetics of proteoglycan pools, with and without competition with hyaluronic acid

A pulse-chase experiment was performed. The cells, with or without added hyaluronic acid in the medium, were labelled with a short pulse of [^{35}S]sulphate and then chased without label for different times. There was no increase in the total amount of ^{35}S -labelled macromolecules during the chase, indicating that the removal of free [^{35}S]sulphate was efficient (Fig. 1a). In control cultures without hyaluronic acid (Fig. 1b) the medium pool had already after 10 min reached a maximum of 68% of the total macromolecular radioactivity, and this then slowly decreased to 39% after 90 min, with a concomitant increase of the pericellular pool. This pattern was reproduced in several experiments, although the relative proportions of label between pools varied somewhat. In other cultures with hyaluronic acid in the medium (Fig. 1c) the medium pool reached a plateau level of about 75% of the proteoglycans after 10 min, while the pericellular pool contained a rather constant amount, 12–19% of the labelled macromolecules, throughout the experiment. It appears that in cultures without added hyaluronic acid most of the proteoglycans are first exported into the medium and that only later a portion binds to the cell surface. This binding was blocked by the addition of hyaluronic acid to the medium. As has been

Fig. 1. Changes in distribution of labelled glycosaminoglycans with time after short labelling in cultures with or without added hyaluronic acid

The cells were incubated with $10 \mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate/ml for 5 min in medium with 1 mg of hyaluronic acid/ml (c) or without addition as control (b). A chase was then performed for the indicated times by a change to non-radioactive medium with or without hyaluronic acid respectively, in both cases containing an excess of $3 \text{ mM-}\text{Na}_2\text{SO}_4$. Analysis was performed as described in the Experimental section. (a) Total amount of $[^{35}\text{S}]$ sulphate incorporated in glycosaminoglycans: —, control without hyaluronic acid in the medium; ----, hyaluronic acid in the medium. (b) Percentage distribution of labelled macromolecules in control cultures: —, medium pool; ----, pericellular pool; ····, intracellular pool. (c) percentage distribution of labelled macromolecules in cultures with hyaluronic acid in the medium: —, medium pool; ----, pericellular pool; ····, intracellular pool.

shown previously (Sommarin & Heinegård, 1983), the added hyaluronic acid competes with that at the cell surface for binding of proteoglycans. Notably, however, the pericellular pool of proteoglycans remains constant and surprisingly large in the presence of hyaluronic acid. The molecules in this pool appear to become directly bound at the cell membrane, possibly being structurally different and intercalated in the cell membrane. We therefore proceeded to isolate and characterize these cell-bound proteoglycans.

Analytical Sephacryl S-500 gel chromatography of cell extracts

Chondrocytes were labelled with $[^{35}\text{S}]$ sulphate and $[^3\text{H}]$ leucine during 4 h and extracted with CHAPS/guani-

Fig. 2. Analytical Sephacryl S-500 gel chromatography of cell extracts

Chondrocytes were incubated with $40 \mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml and $10 \mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate/ml for the times indicated. The cells were washed once and then extracted with a combination of detergent and guanidinium chloride. Cell extracts were chromatographed on a Sephacryl S-500 column eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. —, ^{35}S radioactivity; ····, ^3H radioactivity. Details are given in the Experimental section. (a) 4 h pulse. Bars indicate the fractions chosen for calculation of proportions of the peaks. (b) Hyaluronic acid (1 mg/ml) added, 4 h pulse. (c) Hyaluronic acid (1 mg/ml) added, 4 h pulse followed by 20 min chase by adding Na_2SO_4 to a concentration of 3 mM and leucine to 2 mM. (d) 4 h pulse with 0.1 TRU/ml of *Streptomyces* hyaluronidase and $20 \mu\text{g}$ of ovomucoid/ml (as proteinase inhibitor) present in the medium as described previously (Sommarin & Heinegård, 1983). In this and other Figures V_0 and V_t represent the void volume and total volume respectively.

Fig. 3. Preparative Sephacryl S-500 gel chromatography of all extract and medium

Chondrocytes were labelled with [^{35}S]sulphate in medium containing hyaluronic acid (1 mg/ml) for 4 h followed by a chase in fresh medium without radiolabel for 20 min. Proteoglycans in cell extracts and medium were purified by DEAE-cellulose ion-exchange chromatography and chromatographed on a Sephacryl S-500 column eluted with the same buffer as before. (a) Cell extract. (b) Medium. Bars indicate pooled fractions.

dinium chloride as described in the Experimental section. Samples of extracts were chromatographed on Sephacryl S-500. Those from the culture where no hyaluronic acid had been added to the medium (Fig. 2a) contained a predominating peak of ^{35}S label eluting at the same position as large cartilage proteoglycans. This peak represented 60% of the [^{35}S]sulphate-labelled macromolecules (the amount of radioactivity in the total volume of the column was not included in the calculation of percentage distribution since it contained some free [^{35}S]sulphate). Two smaller, more-retarded, peaks represented 19% each of ^{35}S label. When hyaluronic acid was included in the medium, the total amount [^{35}S]sulphate-labelled material recovered from the cells was reduced by about half (53%), owing to a decrease in the first peak (Fig. 2b). In a control experiment, the 4 h pulse was followed by a 20 min chase to allow export of newly synthesized intracellular [^{35}S]sulphate-labelled material (Fig. 2c). The total amount of [^{35}S]sulphate-labelled

Fig. 4. Fluorography of agarose/polyacrylamide-gel electrophoresis of pooled fractions

Samples were precipitated with ethanol and electrophoresed on agarose/polyacrylamide gels as described in the Experimental section. Lane a, pool ChM1; lane b, pool ChC1; lane c, pool ChC2; lane d, pool ChC3; lane e, pool ChC4; lane f, pool ChM1; lane g, pool ChM2; lane h, pool ChC3. The two outer lanes show the carrier bovine articular-cartilage proteoglycans.

macromolecules at the cell surface, however, was almost the same with and without the chase (6% reduction only) (Figs 2b and 2c). Consequently most of the [^{35}S]sulphate-labelled material, identified in the cell extracts, was bound at the cell surface. The cell-associated [^{35}S]sulphate label was decreased by 56% when binding of the large proteoglycans appearing in the first peak was prevented by digestion of hyaluronic acid at the cell surface by *Streptomyces* hyaluronidase (Fig. 2d). No changes were seen in [^3H]leucine-labelled material, which probably largely represents proteins.

Preparation of [^{35}S]sulphate-labelled proteoglycans from cell extracts and medium

Cells were pulse-labelled in medium with added hyaluronic acid and [^{35}S]sulphate for 4 h followed by a 20 min chase to allow newly sulphated molecules to be exported. Proteoglycans bound to the cells were extracted with CHAPS/guanidinium chloride. An initial ion-exchange chromatography on DEAE-cellulose eluted with solutions containing 6 M-urea removed approx. 90% of the protein material in the cell extracts. Also, the proteoglycans in the medium were purified by using DEAE-cellulose chromatography. The proteoglycan fractions were then chromatographed on Sephacryl S-500 (Fig. 3). The peaks were pooled and are referred to by the numbers indicated in Fig. 3.

Agarose/polyacrylamide-gel electrophoresis

On agarose/polyacrylamide-gel electrophoresis of pooled fractions (Fig. 4) the large components from the cell extract, ChC1, and medium, ChM1, both migrated as the slowest band of the carrier bovine articular-cartilage proteoglycan. Interestingly these cultured chondrocytes appear to synthesize only one type of large aggregating proteoglycan, whereas two have been identified in extracts of cartilage (for references see Heinegård *et al.*,

Fig. 5. Sephacryl S-500 chromatography of dermatan sulphate proteoglycans and heparan sulphate proteoglycans in fraction ChC3

Samples of fraction ChC3 was prepared for gel chromatography by dialysis against 0.1 M-Tris/0.1 M-sodium acetate buffer, pH 8. One half was digested with 0.05 unit of chondroitinase ABC for 4 h at 37 °C, and the other half was incubated in parallel without enzyme, as a control. Gel chromatography was performed on a Sephacryl S-500 column (145 cm × 0.6 cm) eluted as indicated in Fig. 2 legend. —, Control; ----, chondroitinase ABC-digested sample representing heparan sulphate proteoglycan;, curve representing the dermatan sulphate proteoglycan, constructed by subtracting the chondroitinase ABC-resistant material from the control (negative values for fractions 56–65 omitted).

1985a). The cells used in the present study, however, were derived from calf articular cartilage, and it has been observed that cartilage from foetus and calf contains only the slowly migrating proteoglycan (Inerot & Heinegård, 1983).

Proteoglycans in fraction ChC2 migrated much more slowly than any of the others. This fraction also contained a less-pronounced faster-moving component at a position corresponding to the smaller proteoglycans in fraction ChC3, probably from admixture of small proteoglycans of the type found in fraction ChC3. The third pool from the cell extract, ChC3, appeared as a broad band, at the position of small cartilage proteoglycans (Heinegård *et al.*, 1985b). The small proteoglycan from the medium, ChM2, produced a distinct band of mobility similar to that of ChC3. The smallest component in the cell extract, ChC4, could not be detected, possibly because of loss of these molecules during immersion of the gel in the aqueous scintillator.

Size of different proteoglycans in fraction ChC3

Since fraction ChC3 appears to contain at least one heparan sulphate-containing and one dermatan sulphate-containing proteoglycan (see below), information on their size distributions was studied by chromatography of chondroitinase ABC-treated fraction ChC3. The resistant heparan sulphate-containing proteoglycan was eluted somewhat more retarded on Sephacryl S-500 (Fig. 5,

Table 2. Glycosaminoglycan composition [³⁵S]sulphate-labelled material in proteoglycan fractions

Free glycosaminoglycan chains from the pooled fractions were tested for the presence of dermatan sulphate, chondroitin sulphate and heparan sulphate by degradation with chondroitinase AC, chondroitinase ABC and HNO₂ respectively. Details are given in the Experimental section.

Pooled fraction	Percentage degraded by treatment with		
	Chondroitinase AC	Chondroitinase ABC	HNO ₂
ChC1	96.8	97.1	0.1
ChC2	92.3	95.9	0.9
ChC3	60.7	69.0	23.2
ChC4	47.7	57.2	36.2
ChM1	98.0	98.2	0.5
ChM2	73.0	94.9	4.0

broken line) than the dermatan sulphate proteoglycans (Fig. 5, dotted line).

Glycosaminoglycan composition of pooled fractions

Glycosaminoglycan chains were liberated from the proteoglycans by alkaline treatment and analysed for the presence of chondroitin sulphate and heparan sulphate by susceptibility to degradation with chondroitinase AC, chondroitinase ABC and HNO₂ respectively (Table 2). Glycosaminoglycans present in fractions ChC1, ChC2 and ChM1 were almost completely depolymerized by chondroitinase AC treatment, indicating that they contained only chondroitin sulphate. The two fractions of the cell extract containing smaller molecules, i.e. ChC3 and ChC4, were only partially depolymerized, i.e. 60–70% and 47–57% respectively of the label was recovered as disaccharides. They were depolymerized to a somewhat larger extent by chondroitinase ABC, although depolymerization was not complete. The difference between the results with the two enzymes indicates that the materials in these two fractions contain a form of dermatan sulphate with a low degree of epimerization, with up to 10% of the disaccharides containing iduronic acid. A portion (25–35%) of the total material in both fractions, corresponding to that resistant to chondroitinase ABC, could be depolymerized by treatment with HNO₂. This material represents heparan sulphate. Digestion of fraction ChM2 with chondroitinase AC gave 73% depolymerization, whereas chondroitinase ABC gave almost complete depolymerization, indicating this fraction contains dermatan sulphate, probably with an iduronic acid content of some 25%.

Size of glycosaminoglycan chains

The size of free glycosaminoglycan chains from the different preparations were determined by gel chromatography on a Superose 6B column calibrated with chondroitin sulphate chains of known *M_r* values (Fig. 6). Comparison of glycosaminoglycan chains from fractions ChC1 and ChM1 shows that they are of similar size. The chondroitin sulphate chains from fraction ChC2 are larger than those from fractions ChC1 and ChM1, indicating the different nature of these proteoglycans. The largest chains were from the small proteoglycan in the

Fig. 6. Size determination of labelled glycosaminoglycan chains by gel chromatography on Superose 6B

Free glycosaminoglycan chains were prepared from pooled fractions and chromatographed on a Superose column calibrated with chondroitin sulphate standards. Details are given in the Experimental section. Hatched areas indicate fraction chosen for calculation of M_w (weight-average M_r) and M_n (number-average M_r). (a) Pool ChC1; (b) pool ChC2; (c) pool ChC3; (d) pool ChC3 treated with chondroitinase ABC leaving heparan sulphate chains intact; (e) pool ChC3 treated with HNO_2 , leaving dermatan sulphate chains intact; (f) pool ChC4; (g) pool ChM1; (h) pool ChM2.

medium, ChM2, which also were very polydisperse, shown both by the chromatogram and by the difference between weight-average and number-average M_r values. The material in pool ChC3 gave a profile with two peaks

Fig. 7. Size of trypsin-digest fragments of proteoglycans determined by Superose 6B gel chromatography

Samples of pooled fractions were treated with trypsin and chromatographed on a Superose 6B column. Details are given in the Experimental section. (a) pool ChC1; (b) pool ChC2; (c) pool ChC3; (d) pool ChM1; (e) pool ChM2. —, ^{35}S radioactivity; ----, A_{530} .

(Fig. 6c), probably representing dermatan sulphate and heparan sulphate. This material was treated with chondroitinase ABC (Fig. 6d) or with HNO_2 (Fig. 6e) before gel chromatography to depolymerize selectively dermatan sulphate and heparan sulphate respectively. The heparan sulphate chains (Fig. 6d) were small, of average M_r only 7500. The dermatan sulphate chains (Fig. 6e) were smaller than those from the small proteoglycan in the medium. They were also polydisperse, with similar differences in weight-average and number-average M_r values as those of fraction ChM2. The pool with the smallest material, ChC4, contained only low- M_r material, which did not change elution position after chondroitinase ABC treatment (results not shown).

Some caution should be exerted when interpreting the

Fig. 8. Detection of proteoglycan core protein by SDS/polyacrylamide-gel electrophoresis of [^3H]leucine/[^{35}S]sulphate-labelled proteoglycan populations

Samples were prepared and electrophoresed on polyacrylamide gradient gels (3–10%) as described in the Experimental section. (a) Pool ChC1: lane 1, untreated; lane 2, chondroitinase ABC-digested. (b) Pool ChM1: lanes as in (a). (c) Pool ChC2: lanes as in (a). (d) Pool ChC3: lane 1, untreated; lane 2, heparitinase-digested; lane 3, heparitinase- and chondroitinase ABC-digested; lane 4, chondroitinase ABC-digested. (e) Pool ChM2: lanes as in (a). Arrowheads indicate position of core protein. M_r values of reference proteins are indicated.

absolute values for the M_r values, since the values are all related to chondroitin sulphate standards, and dermatan sulphate and heparan sulphate may behave somewhat differently on the gel-permeation column.

Size of fragments produced by trypsin digestion

The chondroitin sulphate chains in the major, large, proteoglycans from cartilage appear to be clustered along the core protein. Trypsin treatment yields peptide fragments, probably corresponding to these clusters, each containing several chains (Heinegård & Hascall, 1974). It is possible that the enzyme has access only to sites surrounding the clustered chondroitin sulphate chains and that the size of the fragments produced will give information on the distribution of glycosaminoglycans along a proteoglycan protein core. The pooled fractions were therefore treated with trypsin and chromatographed on Superose 6B to reveal any differences in the distribution of glycosaminoglycans between proteoglycans (Fig. 7). On comparing profiles with that of the added bovine nasal-septum-cartilage proteoglycan carrier, only fractions ChC1 and ChM1 appear to have similarly clustered chains (Figs. 7a and 7d). The fragments, however, are somewhat smaller than those from the carrier, perhaps depending on the smaller chondroitin sulphate chains (Heinegård *et al.*, 1985a). The second pool from the cell extracts, ChC2, showed a predominant peak of size similar to that of the free chains in this fraction. It also contained a smaller leading shoulder with molecules of the size similar to that of the trypsin fragments from fractions ChC1 and ChC3. This material

probably represents admixture of these fractions. The other pools, ChC3 and ChM2, gave fragments rather similar in size to the free chains (Figs. 7c and 7e). The last pool from cell extracts was not analysed.

Identification of core proteins

An attempt was made to identify core proteins of the different proteoglycans by SDS/polyacrylamide-gel electrophoresis. Proteoglycan preparations from cells labelled with both [^3H]leucine and [^{35}S]sulphate were used. Although the preparations contained some contaminating proteins, core proteins could be identified after enzymic removal of glycosaminoglycan chains (Fig. 8). The large proteoglycans, ChC1 and ChM1, appeared to have large core proteins of similar size (Fig. 8a, lane 2, and Fig. 8b, lane 2). Fraction ChC2 contained a smaller core protein (Fig. 8c, lane 2) with an apparent M_r of about 300 000. In both fractions ChC3 and ChM2 major core proteins of M_r about 50 000 were found after removal of dermatan sulphate chains (Fig. 8d, lane 4, and Fig. 8e, lane 2). This is in the same range as the core protein of small proteoglycans from cartilage (Heinegård *et al.*, 1981). Fraction ChC3, however, appears to contain more than one core protein of similar size, as indicated by the broad band seen in Fig. 8(d), lane 4. A heparan sulphate proteoglycan core protein of M_r 40 000 was identified after heparitinase treatment (Fig. 8d, lane 2). It was only partially resolved from contaminating proteins, but is distinctly smaller than the core protein of the small dermatan sulphate proteoglycan. Since the results discussed above indicate that the separation between

fractions ChC2 and ChC3 was only partial, it is not surprising to find some core protein of the small dermatan sulphate proteoglycan type in fraction ChC2 and some of the large type in fraction ChC3 (Fig. 8c, lane 2, and Fig. 8d, lane 4). Fraction ChC4 was not studied.

DISCUSSION

In a previous study proteoglycans added to the medium of cultured chondrocytes were shown to bind to hyaluronic acid at the cell surface in a process similar to proteoglycan aggregate formation (Sommarin & Heinegård, 1983). In the present work, however, only about half of the cell-surface-associated endogenous [³⁵S]sulphate-labelled proteoglycans appeared to bind to hyaluronic acid. This suggested the existence of proteoglycans at the cell surface that were structurally different from the large aggregating proteoglycans present in cartilage matrix.

In support of this, extracts of labelled chondrocytes, when chromatographed on Sephacryl S-500, showed three distinct peaks of [³⁵S]sulphate-labelled proteoglycans. One peak, ChC1, contains high- M_r proteoglycans of the type considered typical for cartilage. The large cell-bound proteoglycans appeared to be identical with the large proteoglycans in the medium. A portion of the large proteoglycans only was removed from the cells by competition with hyaluronic acid or treatment with *Streptomyces* hyaluronidase. Therefore it is possible that some of these large proteoglycans were bound by some other mechanism or that they preferentially bound to hyaluronic acid at the cell surface.

In cultures with hyaluronic acid added to the medium, the cell-associated [³⁵S]sulphate label represents about 15% of the total in the culture. The large cell-bound proteoglycan represents 6%, whereas the large one in the medium represents 76% of all incorporated [³⁵S]sulphate. Thus the major biosynthetic product of articular-cartilage chondrocytes are large aggregating proteoglycans exported into the medium.

The other proteoglycans isolated from these cultures appeared to be different. The second peak, ChC2, contained proteoglycans of a different nature from any previously described cartilage proteoglycan. This novel proteoglycan represent some 4% of the total labelled proteoglycans. It migrated more slowly than the large proteoglycans on agarose/polyacrylamide-gel electrophoresis, the chondroitin sulphate chains were not arranged in 'clusters' and the core protein was of high M_r , although smaller than those from the largest proteoglycans. On CsCl-density-gradient centrifugation it has a low buoyant density (results not shown), indicating that it has a high relative content of protein and few side chains. It is similar in size and low buoyant density to the collagenase-sensitive and disulphide-bonded proteoglycan from chick-embryo cartilage described by Noro *et al.* (1983). The proteoglycan reported here, however, was not sensitive even to prolonged collagenase treatment, and did not change position on Sephacryl S-500 chromatography after reduction and alkylation (results not shown). Another proteoglycan of similar character has been described as a specific melanoma-cell antigen (Ross *et al.*, 1983; Bumol *et al.*, 1984) and from rat ovarian granulosa-cell cultures (Yanagashita & Hascall, 1983). Interestingly, the amount of fraction ChC2 showed the largest variation between different cell preparations,

perhaps reflecting a response of the chondrocytes to environmental factors.

The third peak in the cell extracts, ChC3, contained at least two different kinds of proteoglycans. One, representing 3% of all [³⁵S]sulphate-labelled material, contained dermatan sulphate chains with a low degree of epimerization. The other one contained heparan sulphate chains and represented only about 1% of all the labelled material. Heparan sulphate proteoglycans showing considerable structural diversity have been isolated from a variety of sources. Examples are small (75000- M_r) proteoglycans from rat liver membranes (Oldberg *et al.*, 1979), large (300000-400000- M_r) dimeric disulphide-bonded proteoglycans from cultured fibroblasts (Cöster *et al.*, 1983) and both small (130000- M_r) and large (400000- M_r) proteoglycans from a basement-membrane-producing tumour (Fujiwara *et al.*, 1984). The chondrocyte proteoglycan appears to be similar with regard to both size of intact proteoglycan and size of core protein to the small proteoglycan from basement membrane and that from rat liver membranes. Much caution should be exerted when comparing the data, however, since different techniques have been used and the proteoglycans behave non-ideally.

The cell extract also contained low- M_r material, representing only 1% of the total label, probably a pool of intracellular material representing molecules being degraded.

The second major biosynthetic product was the small dermatan sulphate proteoglycan in the medium, representing about 9% of the labelled material. Indeed, the presence of small dermatan sulphate-containing proteoglycans in bovine articular cartilage has been shown by the studies made by Rosenberg *et al.* (1985). The small proteoglycan in the medium may be related to the small dermatan sulphate proteoglycan at the cell surface. Even though their glycosaminoglycan compositions and chain sizes differ, the proteoglycans are of approximately the same size as are their core proteins.

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