Fractionation and characterization of proteoglycans isolated from chondrocyte cell cultures

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Chondrocyte cultures were established from foetal bovine tracheal cartilage and maintained in Ham's F12 medium with or without 10% (v/v) foetal calf serum. The proteoglycans were isolated and characterized. (1) The proteoglycans from cultures both with and without serum distributed in associative or dissociative CsCl gradients like proteoglycans from cartilage tissue. (2) The amino acid composition, protein contents and glucosamine/galactosamine ratios were grossly identical with those of the tissue derived proteoglycans. (3) Sedimentation coefficients (s^0) for the monomers were 21.0S and 22.7S from cultures without and with serum respectively. The s^0 values obtained for aggregates were 72.3S and 93.2S respectively. The limiting viscosity numbers $[\eta]$ were 248 ml/g and 298 ml/g respectively. These data corresponded well to those obtained for the tissue-derived proteoglycans. (4) The sizes of the core proteins and chondroitin sulphate chains respectively were the same for both types of cell-culture proteoglycans and similar to those of the tissue proteoglycans. Both the keratan sulphate-rich region and the hyaluronic acid-binding region were identified. The latter, however, was not resistant to limit digestion with trypsin, in contrast with the fragment derived from the bovine nasal cartilage. (5) About 70% of the cell-culture proteoglycans chromatographed in the void volume on a Sepharose 2B column, whereas reduced and alkylated samples (monomers) chromatographed completely included in the column. The two link proteins present in A1 preparations of cartilage proteoglycans were also present in A1 preparations of cell-culture proteoglycans. (6) A minor portion (10%) of the ^{35}S -labelled proteoglycans in the cultures was associated with the cells. Reduced and alkylated samples were larger compared with the monomers in the medium, and chromatographed partly (25%) excluded on the Sepharose 2B column. A larger proportion (50%) of the non-reduced samples chromatographed in the void volume of the column.

The cartilage intercellular matrix contains two predominant macromolecular species: the collagen and the proteoglycan aggregates. The proteoglycan aggregates have a very high molecular weight of 100×10^6 – 200×10^6 . It has been shown (Hascall & Heinegård, 1974*a,b*; Heinegård & Hascall, 1974; Hardingham & Muir, 1972, 1973) that at least three different macromolecular species interact in a specific manner before cartilage proteoglycans form stable aggregates (for review see Hascall, 1977). In order to investigate the biosynthesis of the cartilage proteoglycan a procedure was developed (Björnsson & Heinegård, 1975, 1981) to isolate and culture chondrocytes from bovine cartilage, the source commonly used for isolation of proteoglycans for structural studies. Cells were obtained in quantities

large enough to eliminate the need for proliferation of the cells in culture. Therefore the biosynthesis of proteoglycans can be studied with cells that represent the original population prepared from the cartilage tissue. The cells are maintained either in a medium supplemented with serum or in a chemically defined medium with no protein supplements.

In the study reported in a previous paper (Björnsson & Heinegård, 1981) only a very small amount of [^{35}S]sulphate radioactivity (5% of total) was found associated with the cells. In monolayer cultures of chondrocytes usually a much larger proportion of the proteoglycans is deposited in the cell layer (Lohmander *et al.*, 1976; De Luca *et al.*, 1977; Kimura *et al.*, 1979; Björnsson & Heinegård, 1981). Most of the proteoglycans in the medium or

extracted from the cell layer are of cartilage type (De Luca *et al.*, 1977; Madsen *et al.*, 1978; Kimura *et al.*, 1979).

The present work was undertaken to characterize the proteoglycan prepared from such chondrocyte cultures maintained in a chemically defined medium with or without serum. The proteoglycans were compared with the proteoglycans extracted from bovine nasal or tracheal cartilage.

Materials and methods

Materials

Adult or foetal bovine tracheal cartilages were obtained from the local abattoir.

Ham's F12 medium was obtained from GIBCO (Grand Island, NY, U.S.A.). Guanidinium chloride (practical grade) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All guanidinium chloride solutions were filtered through active carbon and then buffered to pH 5.8 with 50 mM-sodium acetate. Trypsin (diphenylcarbamoyl chloride-treated) and a suspension of crystalline papain (25 mg of protein/ml) was obtained from Sigma Chemical Co. Chondroitinase ABC (*Proteus vulgaris*) was purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Gels for column chromatography and high-molecular-weight hyaluronic acid (Healon) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). [³⁵S]Sulphate was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). All other chemicals were of analytical grade.

Isolation of proteoglycans from cartilage tissue

Nasal cartilage (NC) samples were extracted with 4 M-guanidinium chloride/50 mM-sodium acetate buffer pH 5.8. Preparations of proteoglycan aggregates (NC-A1 fraction) and subsequently monomers (NC-A1-D1 fraction) were isolated as described previously (Heinegård, 1972; Hascall & Heinegård, 1974a). [The operational abbreviations previously described (Heinegård, 1972; Hascall & Heinegård, 1974a) are used throughout the text.] All preparations were extensively dialysed at 4°C against water and freeze-dried.

Isolation of proteoglycans from chondrocyte cell cultures

Cells were isolated from foetal bovine tracheal cartilage (Björnsson & Heinegård, 1975, 1981). Cultures were maintained in Ham's F12 medium that either contained 10% (v/v) foetal calf serum (F12 FCS medium) or was serum-free (F12 medium) (Björnsson & Heinegård, 1975, 1981). The medium was replaced every sixth day. After withdrawal of the medium from the Petri dishes, cells were sedimented at 280 *g*_{av.} for 10 min. The medium

was then centrifuged at 30 000 *g*_{av.} for 20 min to remove any remaining particulate material. The supernatant was concentrated 10-fold by using an Amicon PM-10 ultrafilter. Guanidinium chloride (7.5 M) was added to the concentrated solution to give a final concentration of 0.4 M and the pH was adjusted to 5.8. Solid CsCl was added to give a density of 1.62–1.64 g/ml. The samples were centrifuged at 100 000 *g* (*r*_{av.} 6.886 cm) for 60 h in an MSE 8 × 25 ml angle rotor at 18°C with 17 ml of solution per tube. The tubes were emptied with the aid of a tube piercer. Contents of proteoglycans in the fractions were determined as the uronic acid contents of the material that could be precipitated from a 50 μ l portion with cetylpyridinium chloride (Antonopoulos *et al.*, 1961). Proteoglycans isolated from serum-containing media are designated ChFCS-A1 fraction and those isolated from serum-free media ChF12-A1 fraction.

Subsequent dissociative centrifugation (Hascall & Heinegård, 1974a) was performed as above but in 4 M-guanidinium chloride/CsCl with a starting density of 1.52 g/ml in an MSE 8 × 25 ml angle rotor at 100 000 *g* (*r*_{av.} 6.886 cm) for 66–72 h at 18°C.

Chemical methods

Hexuronic acid contents of samples were determined by the Bitter & Muir (1962) modification of the carbazole procedure. Column effluents were analysed for contents of uronic acid and protein by automated procedures (Heinegård, 1973). Amino acid and hexosamine contents of samples were determined after hydrolysis as described elsewhere (Heinegård, 1977). Scintillation counting of radioactivity was done with a Packard 2650 instrument, with Instagel (Packard Instrument Co., La Grange, IL, U.S.A.) as the scintillation medium.

Column chromatography

Analytical columns of Sepharose 2B (142 cm × 0.8 cm) and Sephadex G-200 (fine grade) (138 cm × 0.8 cm) were eluted at 18°C with 0.5 M-sodium acetate buffer, pH 7.0. The flow rates were 4.0 and 4.2 ml/h respectively. The effluents were collected in 1.3–1.6 ml fractions.

Reduction and alkylation of proteoglycan aggregates

Aggregates were incubated for 5 h at 37°C in 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-dithiothreitol. Subsequent alkylation was performed with 2.5-fold molar excess of iodoacetamide (Heinegård, 1977).

Viscosimetry

Viscosities of samples were measured with an Ubbelohde dilution viscosimeter (Cannon 100 K 290) at 20.1°C. Stock solutions, with about 7 mg of

material, were dialysed extensively against 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. Dilutions of the dialysis residue with the diffusate were prepared by weight. Determinations of viscosity numbers were performed as described elsewhere (Hascall & Heinegård, 1974a).

Analytical ultracentrifugation

All sedimentation-velocity experiments were done at 20°C in an MSE Centriscan analytical ultracentrifuge, with the modified schlieren optics. The six-place analytical rotor was used with 10 mm single-sector cells equipped with quartz windows. Apparent sedimentation coefficients were determined in 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. The apparent values were extrapolated to s^0 at zero concentration by linear-regression analysis. Rotor speeds were varied from 20000 to 40000 rev./min, depending on the concentration of the sample. Concentrations of stock solutions and weighed dilutions were determined by the carbazole procedure (Bitter & Muir, 1962).

Digestion with chondroitinase ABC and trypsin

Fractions obtained as described above were dissolved in 0.1 M-Tris/HCl/0.1 M-sodium acetate buffer, pH 7.3, and digested with 0.01 unit (nominal) of chondroitinase ABC/mg of proteoglycan for 4 h at 37°C. The samples were extensively dialysed against distilled water and freeze-dried. For a following digestion with trypsin the samples were dissolved in 1% (w/v) NH_4HCO_3 and digested with 1 μg of trypsin (diphenylcarbamoyl chloride-treated)/mg of proteoglycan for 4 h at 37°C. The samples were then freeze-dried directly to remove the NH_4HCO_3 .

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Samples were electrophoresed on 8% polyacrylamide gels in the presence of 1% (w/v) sodium dodecyl sulphate as described by Neville (1971). The gels were stained with Coomassie Brilliant Blue and scanned at 605 nm with a Gilford spectrophotometer equipped with a gel-transporting device.

Digestion with papain

Samples (approx. 1 mg) were incubated at 50°C for 5 h with 50 μg of crystalline papain in 1 ml of 0.1 M-sodium phosphate/10 mM-cysteine hydrochloride/10 mM-EDTA buffer, pH 6.5. Digests were chromatographed on the analytical Sephadex G-200 column.

Extraction of cell-associated proteoglycans with 4 M-guanidinium chloride

Cultures of chondrocytes in F12 medium with and without foetal calf serum were established as described elsewhere (Björnsson & Heinegård, 1975,

1981). [^{35}S]Sulphate (5 $\mu\text{Ci/ml}$) was added and the cultures were left for 6 days. The culture medium was withdrawn and the cells remaining in suspension were collected by centrifugation. The Petri dishes and the cell pellets were rinsed with fresh medium and the rinse was pooled with the supernatant. The cells were suspended in 5 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and added back to the Petri dishes. Extraction was performed at 4°C for 24 h. The extracts were centrifuged at 105000 g_{av} for 1 h at 4°C. The supernatants were decanted and the pellets carefully rinsed with 4 M-guanidinium chloride. The pellets were suspended in 5 ml of 4 M-guanidinium chloride and sonicated for 4×15 s with a Branson sonicator, and again centrifuged as above to produce an extract and a residue. Four fractions were prepared from each culture: The medium (ChF12-M and ChFCS-M fractions), the guanidinium chloride extracts (ChF12-Gu and ChFCS-Gu fractions), the supernatant from the sonication (ChF12-Gu-Son and ChFCS-Gu-Son fractions) and the residues remaining after sonication (ChF12-R and ChFCS-R fractions). Samples of all the extracts were dialysed to associative conditions and centrifuged associatively in CsCl essentially as described above. Other samples of the extracts and of the residues were digested with papain, and the glycosaminoglycans were isolated by using precipitation with cetylpyridinium chloride essentially as described by Antonopoulos *et al.* (1961).

Results and discussion

Isolation of proteoglycans from chondrocyte cell cultures

Proteoglycan aggregates were prepared by centrifugation in CsCl density gradients after the medium had been concentrated by ultrafiltration (Amicon PM-10). Media from the first and second weeks of culture were used with the same result. The proteoglycans isolated were not exposed to dissociating agents, and represent molecules or complexes originally assembled in the culture. The distribution of uronic acid after associative CsCl-density-gradient centrifugation of ChF12 and ChFCS fractions are shown in Figs. 1(a) and 1(c). When cultures were pulse-labelled with [^{35}S]sulphate, more than 90% of the macromolecular [^{35}S]sulphate of the medium was recovered from the bottom third of the associative gradient (not shown in the Figure). To avoid interference by Phenol Red from the culture medium with the carbazole procedure, a cetylpyridinium chloride precipitation step had to be introduced, as described in the Materials and methods section. Phenol Red absorbed u.v. light, but no correction could be made since its

Fig. 1. Distribution of uronic acid and protein in the centrifuge tube from density-gradient centrifugations of proteoglycan fractions

Experimental details are given in the text. (a) ChF12 medium centrifuged for 66h in 0.4M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.62g/ml. (b) ChF12-A1 fraction centrifuged for 66h in 4M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.52g/ml. (c) ChFCS medium centrifuged for 66h in 0.4M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.62g/ml. (d) ChFCS-A1 fraction centrifuged for 66h in 4M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.52g/ml. □, Uronic acid; ▬, protein; —, density.

distribution in the gradient is uneven. To isolate the proteoglycan monomer, fractions ChF12-A1 and ChFCS-A1 were centrifuged in dissociative CsCl density gradients (Figs. 1b and 1d). The major proportion of the uronic acid (85%), representing the proteoglycan monomers, was recovered in the bottom fraction of the gradients, and the proteins distributed more to the top. The results are similar to those obtained with extracts of bovine cartilage (Heinegård, 1972).

Gel chromatography of proteoglycans on Sepharose 2B

Sepharose 2B chromatograms of fractions ChF12-A1 and ChFCS-A1 show that 75% and 67% respectively of the uronic acid were eluted in the void volume (Figs. 2a and 2c). In contrast, other samples that were reduced and alkylated, to abolish interaction with hyaluronic acid, chromatographed completely included, with K_{av} 0.24 (Figs. 2b and 2d). Similar chromatograms were obtained when bovine nasal cartilage A1 fraction was chromatographed under the same conditions (not shown in the Figure). Proteoglycans prepared from cell cultures by CsCl-density-gradient centrifugation aggregate with hyaluronic acid, as do proteoglycans from cartilage (Hascall & Heinegård, 1974a).

Medium from serum-free cultures pulse-labelled with [35 S]sulphate was subjected to associative centrifugation in CsCl/0.4M-guanidinium chloride. The gradient was divided in three fractions, A1, A2 and A3, containing 94%, 4% and 2% of the [35 S]sulphate respectively. The size of the proteoglycan monomers, as determined by Sepharose 2B chromatography (not shown) of reduced and alkylated samples, increased with density (K_{av} 0.17, 0.28 and 0.53 respectively). Large proteoglycan monomers distribute at a higher density than do small monomers (Heinegård, 1977).

Cells cultured for 6 days were labelled with [35 S]sulphate 4h before being harvested. The proteoglycan A1 fraction was isolated as described above and chromatographed on Sepharose 2B. The radioactivity representing the newly synthesized proteoglycans chromatographed identically with the uronic acid representing the total population of proteoglycan synthesized during 6 days of culture (results not shown).

The results indicate that the cells maintain their phenotype during the culture period of 6 days and that degradation of extracellular material is negligible.

Viscosimetric studies of fractions ChF12-A1 and ChFCS-A1

ChF12-A1 fraction had lower viscosity numbers for all concentrations and showed a somewhat lower concentration-dependence than did ChFCS-A1 fraction (Fig. 3). The limiting viscosity numbers $[\eta]$ for fractions ChF12-A1 and ChFCS-A1 were 248 ml/g and 298 ml/g respectively. The corresponding values for tracheal A1 fraction and nasal A1 fraction are 220 ml/g and 295 ml/g respectively (Hascall & Heinegård, 1974a).

The fact that ChFCS-A1 fraction has a higher limiting viscosity number than that of ChF12-A1 fraction suggests that the former contains larger aggregates and/or a greater percentage of aggre-

Fig. 2. Gel chromatography of proteoglycan fractions on a Sepharose 2B column

Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.26 ml fractions were collected and analysed for uronic acid. (a) ChF12-A1 fraction. (b) ChF12-A1 fraction, reduced and alkylated. (c) ChFCS-A1 fraction. (d) ChFCS-A1 fraction, reduced and alkylated. V_0 , Void volume; V_t , total volume.

Fig. 3. Viscosity of proteoglycan fractions at different concentrations in 0.5 M-guanidinium chloride

Experimental details are given in the text. Arrows indicate the limiting viscosity numbers obtained. —, ChFCS-A1 fraction; ----, ChF12-A1 fraction.

gates. Since both the ratio of aggregates to monomers and the size of the monomers were similar for the two preparations, as discussed below, it appears that the higher viscosity number indicates a larger molecular size of the aggregates from ChFCS fraction.

Sedimentation-velocity centrifugation of fractions ChF12-A1 and ChFCS-A1

Proteoglycan A1 fractions were prepared from the medium of cultures grown in the presence of F12 and F12 FCS media and analytically centrifuged at concentrations from 0.35 to 4.27 mg/ml (Table 1). The sedimentation-velocity runs were performed in 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, to minimize the charge effects exhibited by proteoglycans without dissociating the proteoglycan aggregates. At the highest concentration used (4 mg/ml) only one sedimenting peak was observed. At lower concentrations, however, three sedimenting peaks were observed and used for calculation of s^0 values. The apparent sedimentation coefficients obtained at the different concentrations fitted well to a straight line when plotted against their concentration, those of aggregates being markedly

Table 1. *Sedimentation coefficients of proteoglycan fractions*

Values were obtained at the concentrations indicated and extrapolated to zero concentration. Experimental details are given in the text. Abbreviation: GuHCl, guanidinium chloride.

Fraction	Peak	s^0 (S)		
		P ₁	P ₂	P ₃
CHF12-A1				
4.27 mg/ml		4.6	—	—
2.12 mg/ml		10.9	11.9	15.1
0.70 mg/ml		19.0	36.9	54.1
0.35 mg/ml		19.8	53.6	62.4
ChFCS-A1				
2.01 mg/ml		18.8	18.8	18.8
1.00 mg/ml		20.7	32.6	53.6
0.67 mg/ml		21.5	41.8	70.0
ChF12-A1				
s_{20}^0 0.5 M-GuHCl		21.0	57.2	72.3
ChFCS-A1				
s_{20}^0 0.5 M-GuHCl		22.7	51.1	93.2

more concentration-dependent than those of the monomers (Table 1). The slowest-sedimenting peak (P₁) had an s^0 value of 21.0 S (ChF12-A1 fraction) or 22.7 S (ChFCS-A1 fraction), correlating closely with the s^0 values obtained for proteoglycan monomers extracted from tracheal cartilage with 4 M-guanidinium chloride (Hascall & Heinegård, 1974a). The fastest-sedimenting peak (P₃) had an s^0 value of 72.3 S (ChF12-A1 fraction) or 93.2 S (ChFCS-A1 fraction), consistent with the viscosity data and indicating that the aggregates in ChFCS-A1 fraction are larger. Between the slowest-sedimenting and the fastest-sedimenting peaks another peak sedimenting with an intermediate velocity (P₂) appeared. The behaviour of peak P₂ varied with the speed and/or the concentration. It is possible that the aggregates are not completely stable under the conditions of centrifugation and that peak P₂ represents decomposing aggregates.

Characterization of the proteoglycan monomers

Amino acid and hexosamine composition. The

Table 2. *Amino acid and hexosamine compositions of proteoglycan fractions obtained from density-gradient centrifugations in 4 M-guanidinium chloride/CsCl*

Experimental details are given in the text.

Amino acid	Amino acid composition (residues/1000 residues)	
	ChFCS-A1-D1 fraction	ChF12-A1-D1 fraction
Aspartic acid	69	67
Threonine	58	59
Serine	142	151
Glutamic acid	152	144
Proline	75	82
Glycine	138	146
Alanine	75	71
Cysteine	—*	13
Valine	63	55
Methionine	—*	—*
Isoleucine	30	32
Leucine	66	70
Tyrosine	15	12
Phenylalanine	45	25
Lysine	34	16
Histidine	16	34
Arginine	23	24
Hexosamines		
$\frac{\text{Glucosamine}}{\text{Hexosamine}} \times 100$	7.9	7.7
Glucosamine/protein (w/w)	0.274	0.316
Galactosamine/protein (w/w)	3.483	3.780
Hexosamine/protein (w/w)	3.757	4.096
Protein (% of dry wt.)	7.3	7.3

* Too low to be calculated.

amino acid compositions of fractions ChF12-A1-D1 and ChFCS-A1-D1 are nearly identical, as shown in Table 2. The results are also in good agreement with those obtained with nasal and tracheal A1-D1 fraction (Hascall & Heinegård, 1974a; Heinegård, 1972).

The galactosamine/glucosamine ratios are also similar, being 12.0 (ChF12-A1-D1 fraction) and 12.7 (ChFCS-A1-D1 fraction) for proteoglycans from the cultures and from bovine cartilage respectively.

Core protein. A sample of [35 S]sulphate-labelled ChF12-A1-D1 fraction was mixed with 5 mg of carrier bovine nasal cartilage A1-D1 fraction and digested with chondroitinase.

Subsequent gel chromatography of the chondroitinase digests was performed on Sepharose 6B

(Fig. 4a). Removal of chondroitin sulphate chains with chondroitinase ABC leaves the proteoglycan protein core still containing the keratan sulphate chains. Therefore the size distribution on gel chromatography is dependent both on the size distribution of the protein cores as well as on their degree of substitution with keratan sulphate. Part of the material was excluded from the column, representing the largest-size core population. Thus 39% of the radioactivity (chondrocyte culture material) and 37% of the uronic acid (tissue material), not taking the material in the total volume into account, was found in the void volume. The proportions of the total sample chromatographing in the total volume were 97.6% and 51.6% respectively. This material represents chondroitin sulphate oligosaccharide liberated by chondroitinase. The relatively high proportion of radioactivity in the total volume may be explained if the remaining disaccharides adjacent to the link region have a lower degree of sulphation than those removed by the enzyme, as suggested by other studies (De Luca *et al.*, 1977).

Sequential digestions with chondroitinase and trypsin

Identification of the keratan sulphate-enriched region. A sample of [35 S]sulphate-labelled ChF12-A1 fraction was mixed with 10 mg of bovine nasal cartilage A1-D1 fraction. After sequential chondroitinase and trypsin digestion the material was chromatographed on Sepharose 6B (Fig. 4b). Three peaks were observed: two with a well-included position and one eluted later in the total volume. The position of the radioactivity peaks representing the material synthesized *in vitro* coincide with the uronic acid peaks representing the tissue material. In a previous investigation (Heinegård & Axelsson, 1977) the first peak was identified as the keratan sulphate-rich region and the second peak as fragments of the chondroitin sulphate-rich region with remaining stubs of chondroitin sulphate linkage regions and a few keratan sulphate chains. The first peak contains 48.5% of the radioactivity and 33% of the uronic acid, and the second peak contains 51.5% of the radioactivity and 67% of the uronic acid in peptide-bound material. The relatively larger proportion of radioactivity in the first peak (keratan sulphate-rich region) compared with the second peak (chondroitin sulphate-rich region) may be explained by lower sulphation of the remaining chondroitin sulphate stubs of the chondroitin sulphate-rich-region fragments in the second peak as discussed above.

Identification of link protein and hyaluronic acid-binding region. Samples of nasal cartilage fractions A1, ChF12-A1 and ChFCS-A1 were separately digested either with chondroitinase only or with chondroitinase and trypsin in sequence. The

Fig. 4. Gel chromatography of enzymically digested proteoglycan fractions on a Sepharose 6B column. Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.6 ml fractions were collected. (a) ChF12-A1 fraction digested with chondroitinase. (b) ChF12-A1 fraction sequentially digested with chondroitinase and trypsin. —, [35 S]Sulphate radioactivity; ----, uronic acid; - · - · -, protein. V_0 , Void volume; V_t , total volume.

digests were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in order to demonstrate the presence of link proteins (Keiser *et al.*, 1972; Baker & Caterson, 1977) and liberated hyaluronic acid-binding region fragments (Heinegård & Hascall, 1974). Samples digested with chondroitinase only contained two components with the mobility of link proteins (Figs. 5*a*–5*c*). Com-

parable amounts (about 150 μ g of proteoglycan) of the different samples were applied on the gels, but the intensity of the staining of the two link proteins derived from chondrocyte cultures was much lower than that of tissue-derived proteoglycans, indicating a lower recovery of these proteins during isolation or enzyme treatment. In all cases the larger link protein seems to be predominant. Samples digested with

Fig. 5. Densitometric scans of sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of enzymically digested proteoglycan fractions

Experimental details are given in the text. S, Starting point; M, position of the tracking dye. (a) NC-A1 fraction digested with chondroitinase. (b) ChF12-A1 fraction digested with chondroitinase. (c) ChFCS-A1 fraction digested with chondroitinase. Arrows indicate the positions of the link proteins.

Fig. 6. Densitometric scans of sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of enzymically digested proteoglycan fractions

Experimental details are given in the text. S, Starting point; M, position of the tracking dye. (a) NC-A1 fraction digested with chondroitinase and trypsin. (b) ChF12-A1 fraction digested with chondroitinase and trypsin. (c) ChFCS-A1 fraction digested with chondroitinase and trypsin.

chondroitinase and trypsin contained one sharp peak with a high mobility corresponding to that of the small link protein and one broader peak with a lower mobility, similar to that of the hyaluronic acid-binding-region fragment (Figs. 6a–6c). The samples derived from the chondrocyte cultures again showed less intense staining of both the hyaluronic acid-binding region fragment and the link protein compared with the material derived from the cartilage. The proportion between the two peaks is different in that the digest of ChFCS-A1 fraction contained much less of the link protein in relation to the hyaluronic acid-binding-region fragment than did the others. Whether these types of cultures actually synthesize less link protein relative to core protein or if the recovery of link protein is not as good is not known. It should be emphasized, however, that when trypsin digestion was extended the hyaluronic acid-binding-region fragments of both ChF12-A1 fraction and ChFCS-A1 fraction were degraded to small peptides not capable of binding to hyaluronic acid. In contrast, the hyaluronic acid-binding-region fragment of proteoglycan aggregates prepared from cartilage is resistant even to limit digestion with trypsin. The lower recovery of fragments from trypsin digests of culture proteoglycans, as discussed above, is probably due to an increased sensitivity to proteinases of their hyaluronic acid-binding region.

Digestion with papain

Single chondroitin sulphate chains were prepared from nasal cartilage fractions A1, ChF12-A1 and ChFCS-A1 by extensive digestion with papain. The elution position on Sephacryl S-200 of the chondroitin sulphate chains as indicated by the uronic acid profiles was essentially the same for all three samples (results not shown), as would be expected from the similarity in sizes of the monomers.

Extraction of cell-associated proteoglycans with 4M-guanidinium chloride

The distribution of radioactivity in the papain digests of the different extracts and the residues is

Table 3. Distribution of cetylpyridinium chloride-precipitable [³⁵S]sulphate-labelled material in the culture medium and guanidinium chloride extracts of cells cultured in the presence and in the absence of serum
Experimental details are given in the text.

Fraction	Distribution (%)	
	F12 cultures	F12 FCS cultures
Medium	96.0	92.1
Guanidinium chloride extract	2.8	6.9
Guanidinium chloride sonicate	1.1	1.0
Residues	0.04	0.02

Fig. 7. Gel chromatography of reduced and alkylated proteoglycan fractions on a Sepharose 2B column
Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.26 ml fractions were collected and analysed for radioactivity. (a) ChF12Gu-A1 fraction. (b) ChF12Gu-A1 fraction, reduced and alkylated. (c) ChFCSGu-A1 fraction. (d) ChFCSGu-A1 fraction, reduced and alkylated. V_0 , Void volume; V_t , total volume.

shown in Table 3. Preliminary experiments indicated that the presence of proteinase inhibitors decreased the proportion of the cell-associated glycosaminoglycans that was extracted with 4M-guanidinium chloride (S. Björnsson & D. Heinegård, unpublished work). Similar proportions of these glycosaminoglycans were removed by trypsin digestion (2mg/ml, 10min at 37°C) or extracted with 4M-guanidinium chloride. A portion of the glycosaminoglycans not released by trypsin, however, was extractable with 4M-guanidinium chloride (not containing proteinase inhibitors), indicating that the residues after trypsin digestion and guanidinium chloride extraction respectively differ in some respects. The major portion (90%) of the cell-associated ³⁵S label at day 6 represents the pericellular pool, removable by trypsin, rather than the intracellular pool (Björnsson & Heinegård, 1981).

The papain digests of the medium, the extracts prepared with and without sonication and the residues were chromatographed on Sephacryl S-200. In all cases the radioactivity was eluted as free chondroitin sulphate chains of similar size distribution (results not shown).

The A1 fractions of the guanidinium chloride extracts of the cells (ChF12-Gu and ChFCS-Gu fractions) were chromatographed on Sepharose 2B. About half of the radioactivity (50.0% and 52.5% respectively) was in both cases eluted with the void volume (Figs. 7a and 7c). The same samples were reduced and alkylated and chromatographed on the same column to give the monomer size distribution (Figs. 7b and 7d). Still a proportion (25.5% of ChF12-Gu-A1 fraction and 26.6% of ChFCS-Gu-A1 fraction) was eluted with the void volume, although the average size of molecules had decreased. In contrast, monomers prepared from the culture medium were included in the column, as discussed above, indicating that the monomers of the cell-associated proteoglycans were larger than those prepared from the culture medium of the same cultures. Although a proportion of the reduced and alkylated proteoglycan monomers was excluded from the column, the elution profile probably represents a continuous distribution of molecules. It appears, then, that the A1 fractions of cell extracts contain

monomers as well as hyaluronic acid and that aggregates are formed on dialysis of the extract.

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