Proteoglycans of the Intervertebral Disc

ABSENCE OF DEGRADATION DURING THE ISOLATION OF PROTEOGLYCANS FROM THE INTERVERTEBRAL DISC

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Proteoglycans extracted with 4M-guanidinium chloride from pig intervertebral discs, and purified by equilibrium density-gradient centrifugation in CsCl, were of smaller hydrodynamic size than those extracted and purified in the same way from the laryngeal cartilage of the same animal. Whether this difference in size arose from degradation during the extraction and purification of the proteoglycans of the disc was investigated. Purified proteoglycans labelled either in the chondroitin sulphate chains or in the core protein were obtained from laryngeal cartilage by short-term organ culture. These labelled proteoglycans were added at the beginning of the extraction of the disc proteoglycans, and labelled cartilage and unlabelled disc proteoglycans were isolated and purified together. There was no appreciable loss of radioactivity after density-gradient centrifugation nor decrease in hydrodynamic size of the labelled cartilage proteoglycans on chromatography on Sepharose 2B, when these were present during the extraction of disc proteoglycans. It is concluded that disc proteoglycans are intrinsically of smaller size than cartilage proteoglycans and this difference in size does not arise from degradation during the extraction.

Proteoglycans isolated from pig and human intervertebral discs, as described in the preceding paper (Stevens *et al.*, 1979), were found to be of smaller size than the proteoglycans of pig laryngeal cartilage, when assessed by gel chromatography and analytical ultracentrifugation.

Nevertheless, the proteoglycans of pig intervertebral discs are able to bind to hyaluronic acid and contain a keratan sulphate-rich region and a chondroitin sulphate region (Stevens *et al.*, 1979), as do proteoglycans from pig laryngeal cartilage. However, the lower relative proportions of uronic acid and galactosamine in disc proteoglycans suggests that the chondroitin sulphate region in these macromolecules is smaller than in cartilage proteoglycans, either in length of the chondroitin sulphate chains or in the length of the core protein, and hence in the number of chains in the molecule. This could have arisen from the action of degradative enzymes during the isolation procedure, because disc and hyaline cartilage proteoglycans appear otherwise to be similar in many ways. Proteolytic enzymes which degrade proteoglycans to smaller size have been shown in the Swarm rat chondrosarcoma (Oegema *et al.*, 1975) and in human intervertebral discs (Pearson *et al.*, 1974). Sapolsky *et al.* (1976) have reported on metalloproteinases in human articular

cartilage capable of digesting articular-cartilage proteoglycans to fragments with five to twelve chondroitin sulphate chains, and Roughley & Barrett (1977) have investigated the degradation of proteoglycans of nasal cartilage by six proteinases, including cathepsins B, D and G, and lysosomal elastase. Pearson & Mason (1977) have also studied in considerable detail the stability of cartilage proteoglycans and the importance of adding inhibitors of proteolytic enzymes during their isolation and storage.

To ensure that degradation was avoided, all specimens were kept frozen before analysis, and the extraction was carried out at 4°C. High concentrations of inhibitors of proteolytic enzymes (Oegema *et al.*, 1975) were present throughout the extraction, dialysis and associative density-gradient centrifugation and the pH was not allowed to fall below 5.8, where lysosomal enzymes such as cathepsin D (Bjelle & Osterlin, 1976; Sapolsky *et al.*, 1974) are most active. Moreover, certain lysosomal enzymes such as glucuronidase are inactive in 4M-guanidinium chloride (R. L. Stevens, unpublished work) and 'free' chondroitin sulphate chains were not detected during the isolation procedure. This suggested that the smaller size of disc proteoglycans was intrinsic to these molecules. Whether degradation of exogenous labelled proteoglycans took place during the extraction of disc proteoglycans has been examined here.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials

All reagents were as described in the preceding paper (Stevens *et al.*, 1979), and in addition Eagle's Basal Medium was from Gibco Bio-Cult (Paisley, Scotland, U.K.), and L-[G-³H]leucine and D-[6-³H]glucosamine were from The Radiochemical Centre (Amersham, Bucks., U.K.). Alcian Blue and Coomassie Brilliant Blue were obtained respectively from Hopkin and Williams (Chadwell Heath, Essex, U.K.) and BDH Chemicals (Poole, Dorset, U.K.).

Methods

Analytical methods

Uronic acid was measured as described in the preceding paper (Stevens *et al.*, 1979). Radioactivity (d.p.m.) was measured in a Searle mark III liquid-scintillation spectrometer. Samples (3 ml) were placed in 5 ml of Luma Gel scintillation fluid (Lumac Systems, Basel, Switzerland) and a correction for background corresponding to the buffer alone was subtracted from the observed radioactivity counts of each sample.

The presence of protein contaminants such as link protein in proteoglycan fractions was investigated by SDS/polyacrylamide-gel electrophoresis (Fairbanks *et al.*, 1971). A running gel of 7.5% (w/v) polyacrylamide and a stacking gel of 4% (w/v) polyacrylamide were employed. Samples were dissolved in 0.01 M-Tris/HCl, pH 8.0, containing 0.001 M-EDTA, 0.1% (w/v) SDS and 10% (w/v) sucrose, at a concentration of 2 mg dry wt./0.1 ml. Electrophoresis was carried out in 0.2 M-sodium acetate/0.04 M-Tris/HCl, pH 7.4, containing 0.1% (w/v) SDS and 0.002 M-EDTA at a constant current of 2.5 mA/tube. Gels were stained with either 0.05% (w/v) Coomassie Brilliant Blue in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid or 0.5% (w/v) Alcian Blue in 3% (v/v) acetic acid. Gels were destained in 10% propan-2-ol containing 10% acetic acid.

Incubation in vitro

Laryngeal cartilage of a single pig was prepared and cut into small pieces and incubated in supplemented Eagle's Basal Medium as previously described (Dondi & Muir, 1976), except that approx. 5.0 g wet wt. of tissue was placed in a sterile screw-top 100 ml jar (Richardson's of Leicester Ltd., Leicester, U.K.).

The tissue was incubated at 37°C for 30 min in a Gallenkamp shaking water bath before D-[6-³H]glucosamine hydrochloride or L-[G-³H]leucine was added to the medium (50 μ Ci/ml). After 4 h the incubation was stopped by cooling in ice, removing the medium and washing the tissue with ice-cold water.

Preparative methods

Cartilage proteoglycans. Labelled proteoglycans were extracted from cartilage in the same way as from intervertebral disc, as described in the preceding paper (Stevens *et al.*, 1979). The suspension was filtered through glass wool and the retained cartilage washed with a small volume of fresh extracting solution. Extract and washings were combined and dialysed for 2 days against 7 vol. of 0.05 M-sodium acetate containing the same four proteinase inhibitors at the same concentrations as above. To the dialysis residue, which now contained 0.5 M-guanidinium chloride, solid CsCl was added and the proteoglycans were purified by associative and dissociative equilibrium density-gradient centrifugation as described in the preceding paper (Stevens *et al.*, 1979), except that higher starting densities of 1.6 g/ml and 1.5 g/ml respectively were used. After dialysis the bottom fraction from the second centrifugation (A1-D1) was divided into several portions for analysis and for assessment of hydrodynamic size by gel chromatography on Sepharose 2B.

Extraction of disc proteoglycans in the presence of labelled cartilage proteoglycans. Four nuclei pulposi were pooled, extracted and purified by associative and dissociative density-gradient centrifugation as described in the preceding paper (Stevens *et al.*, 1979), except that pig laryngeal-cartilage proteoglycan labelled with [³H]glucosamine (1.8 mg of uronic acid, approx. 7.2 mg of proteoglycan) or with [³H]leucine (10 mg of uronic acid, approx. 40 mg of proteoglycan) was added to the extracting solution (14 ml volume). The radioactivity (d.p.m.) was measured after dialysis of the extract to associative conditions and in all fractions from the density gradients. Efficiency of extraction from nucleus pulposus is high (>90%; Stevens *et al.*, 1979) in the absence of added proteoglycan, but was not measured in this instance.

Gel chromatography

Sephadex G-200 chromatography of glycosaminoglycans. Free chondroitin sulphate chains were obtained from cartilage proteoglycans labelled with [³H]glucosamine by treatment with alkali and NaB³H₄ as described in the preceding paper (Stevens *et al.*, 1979). After neutralization the solution was divided in approximately half. One half was applied directly to a Sephadex G-200 column (140 cm $\times$ 1.0 cm) and eluted with 0.2 M-NaCl/0.05 M-Tris/HCl, pH 7.0, at a flow rate of 4 ml/h. The other half was exhaustively dialysed against water, freeze-dried, redissolved in 0.5 ml of 0.05 M-sodium acetate/0.05 M-Tris/HCl, pH 8.0, with 0.01% (w/v) bovine serum albumin, digested with 1 unit of chondroitinase ABC for 1 h at 37°C by the method of Saito *et al.* (1968), and applied to the same Sephadex G-200 column. Fractions

(1.5 ml) were collected and analysed for radioactivity and uronic acid content.

Proteoglycans obtained from dissociative density-gradient centrifugation

Molecular size assessed by gel chromatography. Samples of proteoglycans obtained from the bottom fraction of the dissociative density-gradient centrifugation (A1-D1) were dissolved in 4.0 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, at a concentration of 1.0–1.5 mg of uronic acid/ml, and 1 ml of the solution was applied to a column (165 cm × 1.4 cm) of Sepharose 2B. The column was eluted at 4°C with the same buffer at 2 ml/h. Each fraction (2.4 ml) was analysed for radioactivity and after exhaustive dialysis against water for uronic acid content.

Interaction with hyaluronic acid. Proteoglycan solutions were also prepared in 0.5 M-sodium acetate, pH 5.8, at concentrations of 1–1.5 mg of uronic acid/ml and samples (1 ml) mixed with 15 µl of a solution of hyaluronic acid in the same buffer containing 2 mg of hyaluronic acid/ml. The mixed solution was applied to a column (160 cm × 1.6 cm) of Sepharose 2B, which was eluted at 4°C with the same buffer at a rate of 7 ml/h by using a peristaltic pump (Watson Marlow, Falmouth, Cornwall, U.K.). Fractions (4 ml) were collected and their radioactivity and uronic acid contents determined.

Results

Isolation and characterization of radioactively labelled laryngeal-cartilage proteoglycans

After dissociative density-gradient centrifugation, 18 mg of uronic acid of laryngeal-cartilage proteoglycan was obtained from each preparation. The relative specific radioactivity was 110 000 d.p.m./mg of uronic acid when [³H]glucosamine was the precursor and 13 900 d.p.m./mg of uronic acid when [³H]leucine was the precursor.

As judged by SDS/polyacrylamide-gel electrophoresis (Fig. 1), the proteoglycan preparations were free from contaminating glycoproteins, which would also have been labelled. No protein of lower molecular weight that should have migrated into the running gel was detected, and all protein that also stained with Alcian Blue remained in the top stacking gel.

When proteoglycans labelled with [³H]glucosamine as the precursor were subjected to alkaline β-elimination, the ³H was found mainly in chondroitin sulphate chains, since, firstly, on chromatography on Sephadex G-200, the majority of the radioactivity was eluted in the same position as the chondroitin sulphate chains assayed as uronic acid (Fig. 2a), and, secondly, after digestion with chondroitinase ABC, the majority of the radioactivity was eluted with the

Fig. 1. SDS/polyacrylamide-gel electrophoresis of ³H-labelled laryngeal proteoglycans at different stages of purification

Gels: (a), 4 M-guanidinium chloride extract; (b), fraction A1 after associative equilibrium density-gradient centrifugation; (c) and (d), fraction A1-D1 after dissociative equilibrium density-gradient centrifugation of fraction A1. Gels (a), (b) and (c) were stained with Coomassie Brilliant Blue, and gel (d) with Alcian Blue. Arrows mark the junction of the running gel and the stacking gel.

Fig. 2. Gel chromatography on Sephadex G-200 of [³H]glucosamine-labelled cartilage proteoglycans

Proteoglycans were treated with (a) 0.5 M-NaOH and (b) chondroitinase ABC. The total uronic acid (—) content and radioactivity (---) of each fraction were determined. V_0 and V_t mark the void volume and the total volume of the column respectively.

uronic acid in the position of chondroitin sulphate disaccharides (Fig. 2b).

Fig. 3. Gel chromatography on Sepharose 2B of dissociated monomeric proteoglycans

(a) [^3H]Glucosamine-labelled laryngeal-cartilage proteoglycans; (b) after co-extraction with excess disc proteoglycans. (c) [^3H]Leucine-labelled laryngeal-cartilage proteoglycans; (d) after co-extraction with excess disc proteoglycans. The total uronic acid (—) content and radioactivity (---) of each fraction were determined. V_0 and V_t mark the void volume and the total volume of the column respectively.

Effect of the extraction procedure on ^3H -labelled laryngeal-cartilage proteoglycans

Size. Proteoglycans synthesized by short-term organ culture of cartilage with either [^3H]glucosamine (Fig. 3a) or [^3H]leucine (Fig. 3c) as precursors were eluted in the same position irrespective of the precursor used and were of large molecular size, similar to those pre-existing in the cartilage as judged by chromatography on Sepharose 2B (Figs. 3a and 3b). The elution profile of radioactivity, however, did not

Table 1. Recovery after associative and dissociative equilibrium density-gradient centrifugation of proteoglycans labelled with either [^3H]glucosamine or [^3H]leucine
For details see the text.

	Radioactivity	
	(d.p.m.)	(% of total)
[^3H]Glucosamine-labelled proteoglycans		
Associative: A2 (top $\frac{1}{4}$)	4200	5.9
Dissociative: A1-D1 (bottom $\frac{1}{4}$)	137500	96.6
A1-D2 (top $\frac{1}{4}$)	600	0.4
[^3H]Leucine-labelled proteoglycans		
Associative: A2 (top $\frac{1}{4}$)	6200	5.9
Dissociative: A1-D1 (bottom $\frac{1}{4}$)	97500	93.0
A1-D2 (top $\frac{1}{4}$)	1100	1.1

coincide completely with the elution profile of uronic acid and suggested that these newly formed molecules were, if anything, of slightly larger size and therefore distinct from those in the disc.

When cartilage proteoglycans labelled with [^3H]glucosamine or [^3H]leucine were present throughout the extraction of unlabelled disc proteoglycans with 4M-guanidinium chloride, 99.7 and 99.3% respectively of the total radioactivity was recovered in the non-diffusible material after dialysis to a concentration of 0.5M-guanidinium chloride. The extracts were centrifuged under associative conditions and the radioactivity of each fraction was determined. The bottom fraction (A1) was then centrifuged under dissociative conditions and the radioactivity determined in the lower and upper fractions. The bottom fractions (A1-D1), which contained the majority of proteoglycans (Table 1), contained almost all the radioactivity. Thus there was no appreciable degradation of the glycosaminoglycans or of the protein core to fragments small enough either to be lost on dialysis or to appear in fractions of lower density.

When the mixture of proteoglycan from discs and cartilage was chromatographed on Sepharose 2B a considerable difference in hydrodynamic size was seen (Fig. 3). Because the disc proteoglycans were in 10-fold excess over the glucosamine-labelled cartilage proteoglycans, the uronic acid elution profile mainly represents the disc proteoglycans, whereas the radioactivity profile represents cartilage proteoglycans which were eluted earlier than disc proteoglycans (Fig. 3b). The position of elution was the same whether the glycosaminoglycans or protein core were labelled. It is notable that there was no change in elution and hence in hydrodynamic size of leucine-labelled cartilage proteoglycans when these had been present throughout the extraction of disc proteoglycans (Figs. 3b, 3d).

Fig. 4. Gel chromatography on Sepharose 2B of dissociated monomeric proteoglycan in the presence of 1% (w/w) hyaluronic acid

(a) [^3H]Glucosamine-labelled laryngeal proteoglycans alone; (b) after co-extraction with excess disc proteoglycans; (c) [^3H]leucine-labelled laryngeal proteoglycans after co-extraction with excess disc proteoglycans. The total uronic acid content (—) and radioactivity (---) of each fraction was determined. V_0 and V_t mark the void volume and the total volume of the column respectively.

Interaction with hyaluronic acid. In the presence of hyaluronic acid under non-dissociating conditions, the majority of (monomeric) proteoglycans of cartilage became excluded from Sepharose 2B (Fig. 4a), and, since the radioactivity was co-eluted with the uronic acid, newly formed proteoglycans were also able to interact with hyaluronate. This ability was essentially unaffected when the cartilage proteoglycans were co-extracted with disc proteoglycans (Fig. 4b), despite the effects of four density-gradient centrifugation runs and the consequent extensive handling. Similar results were obtained with [^3H]leucine-labelled cartilage proteoglycans (Fig. 4c).

Discussion

Proteoglycans of the disc differ from laryngeal-cartilage proteoglycans principally in being of much smaller hydrodynamic size and in containing less chondroitin sulphate. Proteoglycans of various non-cartilaginous tissues have now been reported, such as a heparan sulphate proteoglycan in brain (White & Hardy, 1977; Margolis *et al.*, 1976), a chondroitin sulphate proteoglycan in aorta (Eisenstein *et al.*, 1975; Radhakrishnamurthy *et al.*, 1977) and a keratan sulphate proteoglycan in cornea (Axelsson & Heinegård, 1978). The sizes of none of these so far appear to be comparable with those of proteoglycans of hyaline cartilage. However, because of difficulties inherent in extracting proteoglycans from different types of connective tissues, the procedures used are not necessarily the same, nor are the factors that might cause degradation of these macromolecules during their purification. Because the structure of proteoglycans renders them inherently vulnerable to attack by lysosomal and neutral proteinases, stringent controls are necessary to ensure that such degradation is minimized.

The possibility that disc proteoglycans were smaller because they had undergone proteolytic degradation of either the chondroitin sulphate-rich region (Heinegård & Axelsson, 1977) or the hyaluronate-binding region (Heinegård & Hascall, 1974) during their preparation appeared unlikely, because labelled cartilage proteoglycans when present throughout the extraction procedure suffered no change in molecular size, and the ability to interact with hyaluronate was retained. Further, there was little evidence of degradation of the carbohydrate chains of the proteoglycans, because no significant amounts of free or labelled chondroitin sulphate chains were detected in other density-gradient fractions. This suggests that *in vivo* any partially degraded molecules and glycosaminoglycan fragments are normally rapidly removed from the tissue.

Whether proteoglycans, like collagen (Bellamy & Bornstein, 1971) or elastin (Foster *et al.*, 1976), are first synthesized as larger polypeptides which then undergo specific proteolytic cleavages before deposition in connective tissue is not known. The smaller size of human disc proteoglycans might arise from more extensive cleavage of the chondroitin sulphate-bearing region during synthesis. Although the size of newly formed disc proteoglycans was not determined, the amino acid sequences around the regions where glycosaminoglycans are attached (Isemura & Ikenaka, 1975; Hopwood & Robinson, 1974) suggest that disc and cartilage proteoglycans are homologous, but not identical. From the present results it would appear that, *in vivo*, disc proteoglycans are intrinsically of smaller size.

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