Extractable proteoglycan from human femoral-head articular cartilage

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Proteoglycans were prepared from human femoral-head articular cartilage by using either guanidinium hydrochloride or MgCl_2 as extractant, followed by density-gradient centrifugation. The proteoglycan subunit had a particle weight of 2.6×10^6 , with a radius of gyration, R_G , of 68.5 nm in 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0. The proteoglycan aggregate had a particle weight of 3.7×10^6 (R_G 84 nm) for guanidinium hydrochloride extracts and 8.7×10^6 (R_G 118 nm) for MgCl_2 extracts in the same buffer. The addition of excess of high-molecular-weight hyaluronate did not significantly alter the particle size of the aggregate. The small increase in size probably reflects a rapid equilibrium between hyaluronate and proteoglycan monomers, and is not due to proteolytic cleavage producing non-aggregating units. Experiments that support the rapid-interaction hypothesis include analytical ultracentrifugation and column chromatography. This interaction does not appear to be pressure-sensitive at 20°C, but is sensitive to temperature variation near the physiological range.

Proteoglycan can be extracted from cartilage with high concentrations of salt, such as 4 M-guanidinium hydrochloride or 3 M- MgCl_2 , and purified by using density-gradient centrifugation (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). Such preparations of proteoglycans and their interaction with hyaluronate have been extensively studied during the last decade (Hardingham & Muir, 1972, 1973, 1974; Heinegård & Hascall, 1974; Bayliss & Ali, 1978). However, these studies have usually been undertaken on non-human and frequently non-articular cartilage. The study presented here concentrates on proteoglycan isolated from healthy human femoral-head articular cartilage, a common site for osteoarthritis in the elderly.

Isolation was by high-molarity salt extraction and density-gradient centrifugation, as in previous studies on animal material. The isolated material was then studied by using gel chromatography, light-scattering and ultracentrifugation techniques. The outcome of these investigations is a physical model of the proteoglycan molecule in human articular cartilage.

Materials and methods

Materials

Reagents were obtained from British Drug Houses, Poole, Dorset, U.K.

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Samples

Samples of normal human femoral head cartilage were obtained at post mortem. One sample consisting of the cartilaginous femoral heads from a cot-death case aged 2 months was also studied.

The finely divided cartilage was extracted at 4°C for 24 h with either unbuffered 3 M- MgCl_2 or 4 M-guanidinium hydrochloride buffered to pH 7.0 with 20 mM-sodium phosphate buffer. After filtration, dissociative proteoglycan samples were prepared from this extract by CsCl-density-gradient centrifugation. Associative proteoglycan preparations were similarly prepared after dialysis of the extract to low-ionic-strength conditions, i.e. 0.5 M- MgCl_2 or 0.4 M-guanidinium hydrochloride. The terms 'associative' and 'dissociative' are therefore used to describe purification by density-gradient centrifugation in the presence of low or high molarity of the appropriate extractant respectively, and all proteoglycan preparations described underwent a one-stage preparative density-gradient centrifugation (Hascall & Sajdera, 1969).

The density gradient was run for 60 h at 5°C and 75 000 g_{av} in an MSE 65 centrifuge with either a 3×25 ml swing-out rotor, r_{av} 9.38 cm (MSE 59590), or an 8×50 ml fixed-angle rotor, r_{av} 7.72 cm (MSE 59595). Solid CsCl was dissolved in the extract to give a loading density of 1.58–1.60 g/ml. The fractions isolated from the gradient were dialysed

against 150 mM-NaCl and then either 150 mM- or 200 mM-NaCl buffered with 20 mM-sodium phosphate to pH 7.0.

Fresh bovine nasal-septum cartilage was treated in an identical manner and used as a control in some of the ultracentrifugation experiments. Proteoglycan subunit (PGS) and aggregated complex (PGA) were isolated from dissociative and associative gradients at densities greater than 1.60 g/ml. These fractions approximately correspond to the D1 and A1 preparations described by Heinegård (1972). The suffixes Gu and Mg are used to denote the original extractant (guanidinium hydrochloride or MgCl_2) and the suffix Bovine or Human denotes the source of material, e.g. PGS (Gu) Bovine and PGA (Mg) Human.

Exclusion chromatography

Columns (80 cm $\times$ 1 cm) of Sepharose 2B or cross-linked Sepharose 2B were used. They were packed and run in 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0 at 4°C. Column volume was 67 ml. Uronic acid in the column effluent was monitored by the modified H_2SO_4 /carbazole reaction (Bitter & Muir, 1962).

Gilbert technique applied to column chromatography

Gilbert (1955, 1959) derived equations to deal with systems undergoing rapid reversible equilibrium in the ultracentrifuge. These equations have since been applied to the analysis of effluent profiles from column chromatography (Winzor & Scheraga, 1963). The technique is an equilibrium technique in which a volume of material sufficiently large to form a plateau concentration is applied to a chromatographic column. The plateau acts as an undepletable reservoir which feeds the leading and trailing boundaries. An analysis of these boundaries and their first-derivative curves allows distinction between an 'enantiotropic' (non-interacting) and a 'non-enantiotropic' (interacting) system. In an enantiotropic system the leading and trailing edges and their first-derivative curves will be symmetrical, the first-derivative curve characteristically having a diffuse leading and sharp trailing boundary. In a non-enantiotropic system of greater complexity than a dimerization, the trailing edge has more than one point of inflexion and consequently the corresponding first-derivative curve has more than one peak. The presence of more than one peak does not imply the separation of molecular species. Similarly, the presence of two peaks in sedimentation-velocity experiments on systems undergoing rapidly reversible equilibrium does not imply the presence of distinct molecular species (Josephs & Harrington, 1967).

This technique was used to determine if a

proteoglycan preparation behaved as an aggregation system undergoing rapid reversible equilibrium or not. A clarified guanidinium chloride extract of proteoglycan was dialysed against distilled water, centrifuged to remove precipitated collagen and protein and re-dialysed against 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.5, to Gibbs-Donnan equilibrium. The precipitate contained less than 1% of the total uronate in the solution. A column of CGP-10 glass beads, pore size 120 nm ($\pm 10\%$), was equilibrated with buffer. Column volume was 46 ml. Then 57 ml of sample was applied to give a plateau concentration, followed by further buffer until the trailing edge was eluted.

Light-scattering

Measurements were made with a Sofica 42000 photogonioidiffusometer (Société Française d'Instruments de Contrôle et d'Analyses, Le Mesnil-Saint-Denis, France) by using light of wavelength 436 nm over an angular range of 30–150°. Stock solutions were dialysed to Gibbs-Donnan equilibrium against buffer (150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0), and this buffer was used to make further dilutions of the stock solution. Clarification was performed by centrifugation in sealed tubes at 35 000 g_{av} for 1 h. Purified AnalaR benzene was used to calibrate the machine (Coudou, 1960). The specific refractive-index increment was 0.160 ml/g for proteoglycan stock solution against the buffer (measurements were made with a Hilger and Watts MR154 interferometer).

Ultracentrifugation

Sedimentation-velocity measurements were obtained in an MSE Centriscan ultracentrifuge with a six-hole aluminium analytical rotor (MSE 59246). Samples were run in 200 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0, at 40 000 rev./min (116 000 g , r_{av} 6.5 cm) and 20°C unless otherwise indicated.

Results and discussion

Extractability

In 24 h 4 M-guanidinium hydrochloride and 3 M- MgCl_2 extracted approximately half the tissue hexuronate from human articular cartilage and 80–85% of the hexuronate from bovine nasal-septum cartilage used as a control. This suggests that the physical entrapment of proteoglycans within the matrix is more extensive than in bovine nasal-septum cartilage.

Properties of the extracted subunit

Figs. 1 and 2 show the behaviour on Sepharose 2B of the proteoglycan subunit derived from the femoral-head articular cartilage of an 18-year-old

Fig. 1. Sepharose-2B elution profiles of guanidinium chloride-extracted material for proteoglycan subunit (—) and proteoglycan subunit plus 1% (w/w) hyaluronate (----).

The 1 mg sample was run at 4°C in 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0. The column effluent was monitored at 530 nm for uronic acid (Bitter & Muir, 1962). The column size was 80 cm × 1 cm, and V_0 and V_t mark the void and total column volume respectively.

Fig. 2. Sepharose-2B elution profiles of $MgCl_2$ -extracted material for proteoglycan subunit (—), proteoglycan subunit plus 1% (w/w) hyaluronate (----) or reduced proteoglycan subunit plus 1% hyaluronate (— · —).

Reduction was accomplished with 10 mM-dithiothreitol for 1 h at 25°C. Conditions were as for Fig. 1, and V_0 and V_t mark the void and the total column volumes respectively.

Fig. 3. Zimm plot for proteoglycan subunit extracted with 4 M-guanidinium chloride

The sample was from an 18-year-old male. Solutions were in Gibbs–Donnan equilibrium with the solvent, which was 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0 at 20°C. The molecular weight (M_w) was 2.63×10^6 ($\pm 0.3 \times 10^6$), with a radius of gyration (R_G) of 68.5 nm and second virial coefficient (A_2) of $8 \times 10^{-5} \text{ cm}^3 \cdot \text{g}^{-2} \cdot \text{mol}^{-1}$.

measurements, shows a particle size of 2.6×10^6 and a radius of gyration (R_G) of 68.5 nm, and the second virial coefficient is small and thermodynamically argues for 'good' solvent conditions. A degree of polydispersity in the material is expressed in the curvature of the $c = 0$ extrapolate, as is also seen in the Sepharose-2B elution profiles in Fig. 1.

Indication of aggregatability

The effect of the addition of 1% (w/w) hyaluronate to the proteoglycan-subunit population is shown in Figs. 1 and 2. The Sepharose-2B elution profiles of proteoglycan extracted with either guanidinium hydrochloride or $MgCl_2$ are compared with the profiles obtained in the presence of hyaluronate. The hyaluronate and proteoglycan preparation were mixed and left at room temperature for $\frac{1}{2}$ h before application to the column. Approx. 12% of the material was originally in the void volume of the Sepharose-2B column. The addition of hyaluronate increased this to 32% for guanidinium chloride-extracted material. With $MgCl_2$ extraction the values were respectively 20% and 58%.

Further experiments yielded similar results. An extended series of ultracentrifugal runs on proteoglycan with and without added hyaluronate (Fig. 4) shows that the guanidinium chloride-extracted material has little change in its sedimentation profiles on addition of hyaluronate (Figs. 4a and 4b, and 4e and 4f). However, the $MgCl_2$ -extracted material changes its profiles markedly when hyaluronate is added (Figs. 4c and 4d, and 4g and 4h), and the

youth. Only a small amount of excluded material is observed in material that has been extracted by the two dissociants. A Zimm plot (Fig. 3) for one of the subunit preparations, derived from light-scattering

Fig. 4. Sedimentation profiles of human and bovine proteoglycan subunit (PGS) preparations in the presence and absence of hyaluronate

The material was extracted by using guanidinium (Gu) or magnesium (Mg) salts. Sedimentation is from left to right, 'M' indicating the air/solution meniscus, at a rotor speed of 40000 rev./min. The solvent was 200 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0. Concentrations varied from 1.0 to 1.8 mg/ml, but each pair of samples, with and without hyaluronate (HA), was at an identical concentration. The ordinate of the schlieren profile reflects the concentration gradient, dc/dr , and the abscissa the radial distance, r .

bimodal distribution of the native subunit isolated from human articular cartilage is replaced by an apparent peak with an intermediate sedimentation coefficient, suggestive of the behaviour observed in interacting systems (Nicol & Winzor, 1972).

Light-scattering studies show a small increase in weight-average molecular weight for proteoglycan-aggregate preparations (Figs. 5 and 6) compared with proteoglycan subunit preparations even if additional hyaluronate is added (Fig. 7). Polydispersity is decreased, and the second virial coefficient (A_2) is almost unchanged for material

Fig. 5. Zimm plot for proteoglycan aggregate extracted with 4 M-guanidinium chloride

The sample was from a 64-year-old male and was obtained within 4 h of death. The solvent and other conditions were as in Fig. 3. The molecular weight (M_w) was 3.74×10^6 ($\pm 0.5 \times 10^6$), with a radius of gyration (R_G) of 84 nm and second virial coefficient (A_2) of $1.25 \times 10^{-4} \text{ cm}^3 \cdot \text{g}^{-2} \cdot \text{mol}^{-1}$.

Fig. 6. Zimm plot for proteoglycan aggregate extracted with 3 M-MgCl₂

The sample was from a 63-year-old female and was obtained within 8 h of death. The solvent and other conditions were as in Fig. 3. The molecular weight (M_w) was 8.7×10^6 ($\pm 1.0 \times 10^6$), with a radius of gyration (R_G) of 118 nm and second virial coefficient (A_2) of $2 \times 10^{-4} \text{ cm}^3 \cdot \text{g}^{-2} \cdot \text{mol}^{-1}$.

prepared with guanidinium hydrochloride as extractant (Fig. 5).

We conclude from these experiments that hyaluronate does interact with proteoglycan monomers from human femoral articular cartilage.

Effect of temperature on the aggregation process

The distribution of proteoglycan subunits and aggregates in MgCl₂-extracted femoral-head cartilage varies with temperature, as shown in Fig. 8. At 37°C (Fig. 8b) less aggregate is apparently present

Fig. 7. Zimm plot for proteoglycan aggregate plus 1% (w/w) hyaluronate

The material was extracted with 4M-guanidinium chloride from the cartilaginous femoral heads of a cot-death case aged 2 months. The solvent and other conditions were as in Fig. 3. The molecular weight (M_w) was 3.7×10^6 ($\pm 0.5 \times 10^6$), with a radius of gyration (R_G) of 74 nm and second virial coefficient (A_2) of $1.6 \times 10^{-4} \text{ cm}^3 \cdot \text{g}^{-2} \cdot \text{mol}^{-1}$.

Fig. 8. Sedimentation profiles of MgCl_2 -extracted human proteoglycan subunit (1 mg/ml) with and without hyaluronate at 25°C and 37°C

Sedimentation coefficients at 37°C have been corrected to 25°C. The solvent and other conditions were as in Fig. 4. Figs. 8(a) and 8(b) represent PGS(Mg) Human (1 mg/ml) at 25°C and 37°C respectively. Figs. 8(c) and 8(d) are the same preparation with the addition of 0.7% (w/w) hyaluronate at 25°C and 37°C respectively.

when the profile is compared with that at 25°C (Fig. 8a). Moreover, the effect of adding hyaluronate (0.7%, w/w) not only increases the proportion of faster-moving component, but is more effective at the lower temperature (Fig. 8c) than at the higher (Fig. 8d). The effect was reversible, as could be seen when material originally run at 37°C was cooled, remixed and re-centrifuged at 25°C. The aggre-

gation process under study is therefore a reversible temperature-dependent event.

Effect of pressure on the aggregation process

When various runs were performed at a series of speeds between 20000 rev./min (28000g, r_{av} 6.5 cm) and 54000 rev./min (216000g, r_{av} 6.5 cm) at 20°C there was no change in the corrected value of the sedimentation coefficient, suggesting that the system is not sensitive to pressure under these conditions.

Effect of hyaluronate size on the aggregation process

When hyaluronate from rooster comb (mol.wt. 5×10^6) was used in aggregating experiments, no change in sedimentation profile was observed when compared with preparations in which degraded hyaluronate from umbilical cord (mol.wt. 5×10^4) was used. However, when oligosaccharides prepared from hyaluronate by ascorbate-mediated depolymerization (Pigman & Rizvi, 1959) followed by Sephadex G-100 chromatography were added to the hyaluronate-proteoglycan aggregate mixture, the effect shown in Fig. 9 was observed. The unimodal aggregated species seen with high-molecular-weight hyaluronate (Fig. 9a) gives rise to a bimodal distribution in which more rapidly sedimenting species are observed (Fig. 9b).

Nature of the aggregate

Reference to Figs. 5 and 6, where the light-scattering-generated Zimm plots of aggregated complex from guanidinium chloride- and MgCl_2 -extracted material are seen, shows that there is a very small change in particle weight from the monomer size. This is not due to large numbers of non-aggregating molecules being present, as can be seen from the low curvature of the angular dependence of light-scattering of the extrapolated zero-concentration line (Kratochvil, 1965). Nor had proteolytic degradation taken place, for the following reasons.

(1) Material isolated in the presence of proteolytic inhibitors behaved in an identical manner.

(2) Proteoglycan has been isolated from the articular cartilage of dogs under conditions similar to those used here without degradation (Inerot *et al.*, 1978).

(3) Incubation of human material with bovine-nasal-septum-derived proteoglycans gave no change in the sedimentation profiles of the latter species, as would have resulted if proteolysis had occurred.

Rapid reversible nature of the interaction

Experiments discussed above hint at a rapid reversible equilibrium between hyaluronate and proteoglycan monomers.

Fig. 9. Sedimentation profiles of MgCl_2 -extracted human proteoglycan aggregate plus excess hyaluronate with and without hyaluronate fragments

Fig. 9(a) is the sedimentation profile of PGA (Mg) at 1 mg/ml plus 4% (w/w) hyaluronate. Fig. 9(b) shows the effect of preincubating the preparation used in Fig. 9(a) with 2% hyaluronate fragments at 25°C for 30 min. The temperature was 25°C and rotor speed 40000 rev./min. The solvent was 200 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0.

The ultracentrifugation experiments, where the bimodal distribution is changed on addition of hyaluronate to a unimodal one with intermediate s value, is suggestive of such a process (Figs. 4g and 4h), and additional support for this view is seen in the temperature-dependence of this process (Fig. 8).

The experiments utilizing the Gilbert frontal-elution technique for analytical chromatography also support the presence of a rapid reversible equilibrium. The differential elution profile obtained (Fig. 10) has a sharp leading peak and diffuse trailing boundary. The latter has more than one point of inflexion, suggesting the presence of an interacting system of greater complexity than a dimerization. Control experiments using the same technique for large polydisperse material which does not form an interacting system (Commercial Blue

Fig. 10. Elution profile (—) and first-derivative curve (---) of proteoglycan preparation by the Gilbert technique

Sample (57 ml) was applied to a column of volume 46 ml (31 cm $\times$ 1.4 cm) to provide a plateau concentration. The column was of CPG-10, pore size 120 nm ($\pm 10\%$), in 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.5.

Dextran on Sepharose-6B) did not give similar effects.

General discussion

The experiments reported in this paper are directed to an examination of the physical behaviour of cartilage proteoglycans extracted from a human load-bearing tissue.

The data suggest that human femoral-head cartilage proteoglycan subunits have a particle weight of 2.6×10^6 and a radius of gyration (R_G) of 68.5 nm in 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.0. The size of proteoglycan aggregate was 3.7×10^6 (R_G 84 nm) with guanidinium chloride extracts and 8.7×10^6 (R_G 118 nm) with MgCl_2 extracts in the same buffer. The addition of excess high-molecular-weight hyaluronate did not alter the particle size of the aggregate.

These are not the weights observed for the aggregation process seen with material derived from bovine nasal septum, a non-load-bearing tissue, notwithstanding the fact that the human material readily interacted with hyaluronate. That the proteoglycans are in fact in rapid reversible equilibrium with hyaluronate is favoured because:

(a) the temperature-dependence seen in the ultracentrifugal experiments when a mixture of proteoglycan subunits and hyaluronate is observed sedimenting suggests a substantial shift in population from a more to a less aggregated state at higher temperatures;

(b) the marked change in sedimentation profile on adding hyaluronate fragments to proteoglycan is in agreement with the concept of rapid reversible equilibrium, or else no effect should be seen;

(c) use of frontal chromatography and an

analysis of the leading and trailing boundaries so generated indicates an interacting system.

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