Isolation and Characterization of Mouse Articular Cartilage Proteoglycans Using Preformed CsCl Density Gradients in the Beckman Airfuge

A RAPID SEMI-MICRO PROCEDURE FOR PROTEOGLYCAN ISOLATION*

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A technique has been developed for the rapid isolation of proteoglycans from small amounts of tissue extracts using the Beckman Airfuge. Aliquots (50 µl) of proteoglycan samples are layered on preformed CsCl density gradients and centrifuged at ~135,000 x g for 4 h. Under both associative and dissociative conditions, proteoglycan sediments to the lower third of the tube (e.g. on a preformed associative gradient, 93% of the uronic acid in a bovine nasal cartilage extract accumulates in this bottom fraction). Gel chromatography indicates predominately aggregative in the bottom third of the associative gradient, and gel electrophoresis of [3H]-labeled fractions reveals that the proteoglycan is well separated from other proteins in the extract. The uronic acid distribution through a dissociative fractionation is similar to that obtained by conventional equilibrium density gradient centrifugation. Gel chromatography of the monomer fraction indicates the absence of hyaluronic acid and a broad included peak typical of proteoglycan monomer from bovine nasal cartilage. Enzymic assay for hyaluronic acid shows the link proteins in the top fraction. Both gel electrophoresis and quantitation by radioimmunoassay show the link proteins in the top fraction.

This rapid fractionation of small sample volumes in the Airfuge is used in conjunction with additional modifications of extraction procedures to isolate and characterize mouse articular cartilage proteoglycan. The modifications, meant to minimize proteolytic degradation of the proteoglycans, include short extraction and dialysis times and maintenance of subzero temperatures. The [3H]-labeled mouse proteoglycan of the aggregate fraction is excluded from Sepharose CL-2B, and the K 얼 of the [35S]-labeled mouse monomer is 0.46. Reacting this [35S]-labeled aggregate with a link-specific antiserum reveals only the smaller link protein 2 in mouse articular cartilage aggregate.

Small animals which possess inborn disease traits are particularly valuable for studies on the biochemical basis of human disease. For example, an inbred mouse strain (STR/IN) has a high spontaneous incidence of osteoarthritis (1).

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Although knee joints of these animals have been studied histologically and by electron microscopy (2, 3), the articular cartilage proteoglycans have not been isolated and characterized biochemically. Indeed, proteoglycans from normal mice have only been partially characterized (4, 5).

To facilitate the study of proteoglycans from mouse articular cartilage there is a need for micromethods of extraction and isolation. Furthermore, there is a need to minimize proteolysis during the isolation procedure (11) even in the presence of 4 M guanidine hydrochloride (6). Especially critical is the rapid separation of extracted proteoglycans from proteins including proteases, which is generally achieved by centrifugation in a CsCl density gradient. The Beckman Airfuge appeared to offer a rapid and simple means of performing this fractionation on a small scale. In the present work, a methodology is described which ensures minimal proteoglycan degradation by rapid low temperature extraction and isolation in the presence of protease inhibitors and uses preformed CsCl density gradients in the Airfuge. Mouse articular cartilage proteoglycans have been extracted and isolated as an example of the use of these microprocedures and as a prelude to the investigation of changes in proteoglycan structure and biosynthesis which might occur in the STR/IN mouse strain.

EXPERIMENTAL PROCEDURES

Materials—Cesium chloride (Analar grade) was purchased from Gallard-Schlesinger Chemical Manufacturing Corp. Sepharose CL-2B was from Pharmacia Fine Chemicals. Guanidine hydrochloride, (grade I), isodecic acid, and phenylmethanesulfonylfluoride were purchased from Sigma. Benzanilide hydrochloride, 6-aminohexanoic acid, and x-ray film (BB-1) were from Eastman. Streptomyces hyalurolyticus hyaluronidase was obtained from Calbiochem-Behring. Carrier-free 125I, in NaOH solution, was from Amersham Corp. Heat-killed and formalin-treated Staphylococcus aureus was prepared according to the method of Goding (7). "Enzymobeads" were from Bio-Rad. A monospecific antiserum was obtained from a rabbit immunized with BNC' link proteins 1 and 2 and has been characterized (9, 10). All other reagents were of analytical reagent grade. Proteoglycan Fractions—Proteoglycans were extracted from bovine nasal cartilage (50 g) in 500 ml of 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 6.5, and protease inhibitors, 5 mM benzamidine hydrochloride, 100 mM 6-aminohexanoic acid, 1 mM iodoacetate, and 1 mM phenylmethanesulfonylfluoride (11). Extraction was done with stirring, at 4 °C, for 24 h. Proteoglycan aggregate (A1), proteoglycan monomer (A1D1), and the link protein-rich fraction

* The abbreviations used are: BNC, bovine nasal cartilage; SDS, sodium dodecyl sulfate. Abbreviations for proteoglycan fractions follow the notation suggested by Henegar (8), except for D1, D2, D3 which denote dissociative gradient fractions isolated directly from a 4 M guanidine hydrochloride extract.
(A1D4) were subsequently isolated from this extract as described previously (12, 13). The link proteins were further purified from the A1D4 fraction as described elsewhere (12).

Similarly, proteoglycans were extracted from rat chondroarcoma, and the aggregate fraction (A1) was isolated (13).

Analytical Methods—Hexuronic acid determinations were done either gravimetrically according to the method of Bitter and Muir (14) or by an automated procedure (15). Hyaluronic acid was determined according to the method described in the Beckman manual. Centrifugations were carried out in a cold room at 4 °C following the Beckman recommendations for cold room installation. In addition, the length of copper tubing used for the cooling coil was increased from 10 feet to 20 feet and was immersed in a salt-ice bath at -15 °C. With this arrangement the effluent was monitored continuously at 206 nm using a UVicord S (LKB, model 2138) and 2-channel recorder (LKB, model 2210). Fractions (0.4 ml) were collected for assay of uronic acid and radionuclides.

Radioiodination—After dialysis to associative conditions, an aliquot (50 μl) of the 4 m guanidine hydrochloride extract from bovine nasal cartilage was iodinated with 125I iodine (1 mCi) using immobilized glucose oxidase and lactoperoxidase ("Enzymobeads") as described by Christner et al. (18). A portion of this iodinated extract, equal to approximately 100,000 cpm (2 μl), was mixed with 300 μl of the unlabeled extract and fractionated on the Airfuge under "associative" conditions to obtain the iodinated A1, A2, A3 fractions.

For analysis of endogenous proteoglycans from mouse articular cartilage an aliquot (50-100 μl) of an A1 fraction was iodinated by the chloramine-T method (19) for 2 min at room temperature with 1 mCi of 125I iodine. After stopping the iodination reaction, carrier BNC A1 (200 μg) was added to diminish nonspecific absorption of mouse 125I-proteoglycan during subsequent gel chromatography. Similarly, for analysis of endogenous proteoglycan monomers from mouse articular cartilage, a D1 fraction was isolated from a 4 m guanidine hydrochloride extract and an aliquot (100 μl) was iodinated with 125I iodine (1 mCi) by chloramine-T for 2 min at room temperature. Carrier BNC A1D1 (400 μg) was added after stopping the iodination reaction. Aliquots (10-20 μl, -50,000 cpm) of both 125I-A1 and 125I-D1 were mixed with carrier BNC A1 and BNC A1D1, respectively, for analysis by gel chromatography.

Isolation and Fractionation of Proteoglycan Aggregate and Its Components Using the Beckman Airfuge—There have been several reports which indicate the feasibility of using the Airfuge to determine molecular weights of proteins (20, 21). In the present study we have investigated the usefulness of this tabletop ultracentrifuge for the rapid fractionation of proteoglycan aggregate from whole tissue extracts and the separation of the aggregate into its components (proteoglycan monomer, hyaluronic acid, and link protein).

The Airfuge was operated according to the general procedure described in the Beckman manual. Centrifugations were carried out in a cold room at 4 °C following the Beckman recommendations for cold room installation. In addition, the length of copper tubing used for the cooling coil was increased from 10 feet to 20 feet and was immersed in a salt-ice bath at -15 °C. With this arrangement the rotor temperature could be maintained at 5-10 °C for the 4-h runs. The A100/30 rotor was preferred because of its capacity for 240-μl tubes, although for most runs, thick walled slightly tapered polyethylene tubes of 100 μl capacity were used. These tubes (Brinkman Instruments micro test tubes, catalog no. 22-36-440-5) were cut to size (2 cm external length). Bothwell et al. (29) suggest that the narrow bore and tapered shape of these tubes may facilitate the transport of macromolecules to the bottom of the tube and minimize convective stirring during deceleration of the rotor. Beckman polyallomer tubes (240 μl) were also found to be satisfactory, but the polycarbonate tubes (240 μl) supplied with the centrifuge did not withstand the 4-h holding periods. All centrifugations were run at maximum air supply (30 psi) which produces a centrifugal force at the bottom of the tube of approximately 160,000 X g. Braking was accomplished with an adjusted automatically timed delay of 3 min (setting 4.5) between turning off the air supply to the driving jets and the application of the mechanical brake.

RESULTS

Initial experiments indicated that a satisfactory density gradient (1.3-1.6 g/ml) could not be formed by centrifuging a CsCl solution of uniform density at 30 p.s.i. for as long as 18 h. Therefore, preformed CsCl gradients were made by layering successively into each tube solutions of density 1.65 g/ml (30 μl), 1.40 g/ml (30 μl), and the sample dissolved in a CsCl solution of density 1.20 g/ml (40 μl). Preformed CsCl gradients in CsCl gradient solution of density 1.20 g/ml (40 μl). Preformed CsCl gradients in 4 m guanidine hydrochloride were similarly prepared for "dissociative" fractionation of the proteoglycan aggregate components. After centrifugation, the contents of each tube were removed as 3 approximately equal aliquots by aspiration from the meniscus using a Hamilton syringe. Densities of pooled fractions were measured pycnometrically using a 50-μl constriction pipette.

To determine whether proteoglycan aggregate could be sedimented to the bottom of the CsCl gradient described, an A1 preparation from bovine nasal cartilage (4 mg/ml) was centrifuged under associative conditions for 4 h at maximum speed. The three fractions obtained, A1, A2, and A3 (bottom to top), were analyzed for uronic acid. The results show that 97% of the proteoglycan (measured as uronic acid) accumulated around the bottom of the column.
lates in the bottom fraction, A1 (Table I). Thus, although the preformed gradient shows considerable change after centrifugation, the proteoglycan aggregate migrates from the top and concentrates in the bottom fraction. An aliquot of the A1 fraction was chromatographed on Sepharose CL-2B as described under "Experimental Procedures," and the elution profile, as determined by absorbance at 206 nm and by uronic acid assay, shows that most of the proteoglycan is eluted at \( V_0 \) (Fig. 1a). Therefore, aggregation of the proteoglycans appears unaffected by the centrifugation procedure.

An important step in the isolation of proteoglycans is their separation from proteins, especially degradative enzymes, in the extract. To determine if this separation was achieved in the preformed gradients, extracts of bovine nasal cartilage in 4 M guanidine hydrochloride were prepared, dialyzed to associative conditions, and centrifuged in the Airfuge. The A1, A2, and A3 fractions were analyzed for protein (absorbance at 280 nm) and uronic acid (Table II). The absorbance at 280 nm indicates that some protein remains at the top of the gradient (i.e. fraction A3). The majority of the uronic acid migrates to the bottom fraction, A1. Whether all the protein found in A1 is covalently associated with proteoglycan was determined in a separate experiment. A \(^{125}\text{I}\)-labeled extract of bovine nasal cartilage was centrifuged on a preformed "associative" CsCl gradient, and the A1, A2, and A3 fractions were recovered (see under "Experimental Procedures"). Fractions were dialyzed against deionized water, freeze dried, and redissolved in sample buffer for electrophoresis. Bands were stained with Coomassie blue R.

### Table II

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Bovine nasal cartilage</th>
<th>Rat chondrosarcoma</th>
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<tbody>
<tr>
<td></td>
<td>Uronic acid</td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>( \mu g/ml )</td>
<td>( A_{280} )</td>
</tr>
<tr>
<td>A3</td>
<td>8 4</td>
<td>0.097 34</td>
</tr>
<tr>
<td>A2</td>
<td>6 3</td>
<td>0.099 26</td>
</tr>
<tr>
<td>A1</td>
<td>181 93</td>
<td>0.125 39</td>
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To determine whether the components of proteoglycan aggregate could be fractionated on preformed CsCl density gradient in the Airfuge, an A1 preparation from bovine nasal cartilage (4 mg/ml) was dissolved in 4 M guanidine hydrochloride, allowed to dissociate (24 h, 4 °C), and centrifuged under "dissociative" conditions. The three fractions obtained, A1D1, A1D2, and A1D3 (bottom to top), were dialyzed, freeze dried, and analyzed for hyaluronic acid, link protein, and uronic acid (Table I). The A1D1 fraction contains 86% of the uronic acid, with 11% and 3% in the A1D2 and A1D3, respectively. This distribution is similar to that obtained by a conventional dissociative centrifugation fractionation (16, 17). Chromatography of the A1D1 fraction on Sepharose CL-2B gave the broad included profile (K\(_a\) = 0.18), which is typical of bovine nasal cartilage proteoglycan monomer (Fig. 1b). As no excluded material is seen, it may be concluded that hyaluronic acid is absent from this fraction. Analysis of the fractions (A1D1-A1D3) confirmed the absence of hyaluronic acid in the A1D1 fraction (Table I) and indicated that it is predominately in A1D3. Analysis of A1D1, A1D2, and A1D3 by polyacrylamide gel electrophoresis in SDS (Fig. 3) showed that the link proteins are predominately in fraction A1D3, with a trace in A1D2. Quantitation by a radioimmunoassay procedure specific for link protein (23) confirmed these findings and indicated that 95% of the link proteins remained in the A1D3 fraction (Table I). Therefore, a proteoglycan aggregate preparation, A1, layered on a preformed "dissociative" CsCl den-
ural cartilage was identified and removed with the aid of a dissecting microscope. Cartilage (wet weight, ~15 mg) from 6 mice was extracted for 5 h with stirring in 4 M guanidine hydrochloride (300 μl), containing protease inhibitors, at -10 °C. The total dialyzed extract (~300 μl) was layered on preformed CsCl density gradients. After centrifugation for 4 h in the Airfuge, the A1, A2, and A3 fractions were obtained. The resultant A1 fraction was radiolabeled with 125I-iodine as described under "Experimental Procedures." An aliquot (10-20 μl, ~50,000 cpm) of the 125I-A1 was mixed with unlabeled A1 from rat chondrosarcoma (200 μl, 200 μg of proteoglycan, in 0.15 M Na2SO4) and chromatographed on Sepharose CL-2B (Fig. 4a). From the elution profile it is evident that more than 75% of the radiolabeled proteoglycans are aggregated. The included 125I-proteoglycans may have resulted from degradation during extraction and isolation or in the iodination procedure or may truly reflect the proportion of nonaggregated proteoglycans in the endogenous proteoglycan population.

The presence of the link protein(s) in the 125I-labeled A1 from mouse articular cartilage was demonstrated by using specific immunological procedures, polyacrylamide gel electrophoresis, and autoradiography (details are given in the text). a, BNC 125I-A1, Immunoprecipitated; b, BNC 125I-A1, preheated, Immunoprecipitated; c, mouse 125I-A1, Immunoprecipitated; d, mouse 125I-A1, preheated, Immunoprecipitated; e, and f, BNC 125I-A1 and mouse 125I-A1, respectively, not Immunoprecipitated.

Preparative CsCl density gradients. After centrifugation for 4 h in the Airfuge, the A1, A2, and A3 fractions were obtained. The resultant A1 fraction was radiolabeled with 125I-iodine as described under "Experimental Procedures." An aliquot (10-20 μl, ~50,000 cpm) of the 125I-A1 was mixed with unlabeled A1 from rat chondrosarcoma (200 μl, 200 μg of proteoglycan, in 0.15 M Na2SO4) and chromatographed on Sepharose CL-2B (Fig. 4a). From the elution profile it is evident that more than 75% of the radiolabeled proteoglycans are aggregated. The included 125I-proteoglycans may have resulted from degradation during extraction and isolation or in the iodination procedure or may truly reflect the proportion of nonaggregated proteoglycans in the endogenous proteoglycan population.

The presence of the link protein(s) in the 125I-labeled A1 from mouse articular cartilage was demonstrated by using specific immunological procedures, polyacrylamide gel electrophoresis in SDS, and autoradiography. Mouse 125I-A1 (10-20 μl, ~50,000 cpm) and BNC 125I-A1 (10-20 μl, ~50,000 cpm) were each mixed with 0.5% sodium deoxycholate 0.25% Nonidet P-40, 0.01 M sodium phosphate, pH 7.5 (100 μl), heated for 3 min at 100 °C, and cooled to room temperature. Control mixtures were prepared in the same way but not heated. Antiserum (100 μl of a 1/50 dilution) specific for link proteins 1 and 2 from bovine nasal cartilage was added to the heated and unheated samples and incubated at room temperature for 1 h with shaking. S. aureus (100 μl of a 10% w/v suspension in wash buffer (9)) was mixed with the antiserum/antigen mixtures, and the incubation was continued at room temperature for 10 min. The cells were pelleted by centrifugation at ~1000 × g for 10 min, washed twice with wash buffer (1 ml), and then heated in 2% SDS, 5% mercaptoethanol (100 μl) at 100 °C for 3 min. Finally S. aureus was pelleted, and an aliquot (50 μl) of the supernatant was fractionated by polyacrylamide
slab gel electrophoresis in SDS. An autoradiogram of this electrophoretic separation is shown in Fig. 5. Mouse 125I-A1 (Fig. 5f) shows a protein band in the running gel with a mobility similar to that of link protein 2 from BNC 125I-A1 (Fig. 5e). When mouse 125I-A1 was heated with detergents and reacted with the antiserum, only one protein band was eluted from the S. aureus/antibody/antigen complex (Fig. 5d). Again, this protein has the same mobility as that of link protein 2 from BNC A1 which had been treated in a similar manner (Fig. 5b). Thus, the mouse articular cartilage proteoglycan aggregate contains only one link protein (link protein 2). Since other experiments (10) have shown that this antiserum recognizes native undenatured link proteins, the requirement for heating (Fig. 5, a and c) suggests that the link protein(s) is sequestered in the unheated proteoglycan aggregates and unavailable for reaction with the antiserum.

In order to isolate proteoglycan monomer, the articular cartilage from 6 mice was extracted with 4 M guanidine hydrochloride as described above. CsCl (253 mg/ml) was added, and the extract was layered on preformed dissociative CsCl density gradients and centrifuged for 4 h in the Airfuge. The D1, D2, and D3 fractions were obtained. The D1 fraction was radiolabeled with 125I-iodine (see under "Experimental Procedures"). An aliquot of the 125I-D1 was mixed with unlabeled A1D1 from bovine nasal cartilage and chromatographed on Sepharose CL-2B (Fig. 4b). From the profiles it can be seen that the mouse articular cartilage monomer ($K_{av} = 0.46$) is smaller than monomer from bovine nasal cartilage ($K_{av} = 3.20$) and also smaller than monomer from sheep articular cartilage ($K_{av} = 0.35$).

**DISCUSSION**

We report here that proteoglycan aggregates and their components can be rapidly isolated using preformed CsCl density gradients in the Beckman Airfuge. In addition, when continuous 206-nm monitoring of gel chromatographic separation is employed, the isolation and characterization of proteoglycans can be accomplished in several days instead of several weeks. In comparison, the microscale technique developed by Pita et al. (22) for CsCl gradient fractionation of proteoglycan requires a 48-h centrifugation to achieve the equilibrium distribution of proteoglycan. Much shorter times have been needed for sedimentation equilibrium studies of proteins in the Airfuge (20, 21). Our study extends the use of the Airfuge to fractionation of proteoglycans in preformed CsCl density gradients in only 4 h. The speed and ease of processing many individual samples in the Airfuge, the improved extraction technique, and the use of specific antibodies as reported in this study make feasible the characterization of mouse articular cartilage proteoglycan from normal and diseased animals at different ages and under various conditions.

Proteoglycans isolated in this manner from mouse articular cartilage have been analyzed for the proportion of aggregate present and the size of the monomer. The A1 fraction from mouse cartilage extract is predominately aggregate as shown by the Sepharose CL-2B profile. The proteoglycan monomer chromatographs with a $K_{av}$ of 0.46, which is greater than that of BNC monomer, but has a similar small average size as other articular cartilage proteoglycans. Presumably, the small extraction volumes, subzero temperatures during extraction and dialysis, and the rapid separation in the Airfuge have all served to prevent degradation of these proteoglycans during isolation. Interestingly, only link protein 2 is found in these aggregates as shown by use of a specific antiserum. This may be a characteristic of rodent hyaline cartilage, since link protein 2 is the only link protein found in the Swarm rat chondrosarcoma (11, 13, 23, 24) and rat epiphyseal growth cartilage (22).

There are many mouse strains that exhibit cartilage abnormalities. Most studies on the characterization of these defects have employed histology and electron microscopy (e.g., Refs. 4 and 5). The studies of brachymorphic mice (bm/bm) by Orkin et al. (4) and of achondroplastic mice (cn/cn) by Kleinman et al. (5) are recent attempts to describe the biochemical defects in these animals. In the case of the bm/bm mice, the cartilage proteoglycans were shown to be undersulfated, whereas in the cn/cn strain no abnormality in the proteoglycan was revealed. The mouse strain STR/IN offers an opportunity to find defects in cartilage biochemistry during the development of osteoarthritis. That the disease is spontaneous in these animals, rather than surgically induced, suggests the possibility of a defect in the physiological control of cartilage metabolism.

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