The inability to prepare high-buoyant-density proteoglycan aggregates from extracts of normal adult human articular cartilage

Peter J. ROUGHLEY,*† Robert J. WHITE,* A. Robin POOLE*† and John S. MORT*†

*Joint Diseases Laboratory, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Que. H3G 1A6, Canada, and †Department of Experimental Surgery, McGill University, Montreal, Que. H3A 1A4, Canada

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High-buoyant-density proteoglycan aggregates could not be prepared from extracts of adult human cartilage by associative CsCl-density-gradient centrifugation with a starting density of 1.68 g/ml, even though proteoglycan subunits, hyaluronic acid and link proteins were all present. In contrast, aggregates could be prepared when extracts of neonatal human cartilage or bovine nasal cartilage were subjected to the same procedure. This phenomenon did not appear to be due to a defect within the hyaluronic acid-binding region of the adult proteoglycan subunit, but rather to an interference in the stability of the interaction between the proteoglycan subunit and hyaluronic acid towards centrifugation. The factor responsible for this instability was shown to reside within the low-density cartilage protein preparation obtained by direct dissociative CsCl-density-gradient centrifugation of the adult cartilage extract.

The extracellular matrix of articular cartilage is composed of two major macromolecules, collagen and proteoglycan, with the proteoglycan providing the tissue with its resilience towards compressive loading (Kempson *et al.*, 1976). The cartilage proteoglycan consists of subunit molecules which possess the ability to interact specifically with hyaluronic acid to form large aggregates (Hardingham & Muir, 1972) in which a central hyaluronic acid filament may be in association with upwards of 50 proteoglycan subunits (Hascall, 1977). This aggregate not only is present in isolated extracts of articular cartilage but has also been shown to be an integral part of the cartilage matrix (Poole *et al.*, 1982). In the tissue the hyaluronic acid filaments appear to be anchored between collagen fibrils.

In addition to proteoglycan subunits and hyaluronic acid, the proteoglycan aggregates also contain link proteins, which have been reported to increase the stability of the aggregate towards dissociation by low pH, high temperatures and ionic strength, and centrifugal force (Hardingham, 1979; Tang *et al.*, 1979; Franzen *et al.*, 1981). It has been shown that a single link protein participates in the interaction of each proteoglycan subunit with the hyaluronic acid (Oegema *et al.*, 1977;

Poole *et al.*, 1980), with the link protein being able to interact with both of these molecules (Caterston & Baker, 1978). The interaction of the proteoglycan subunit with both the link protein and the hyaluronic acid takes place via a unique hyaluronic acid-binding region which is located at one terminus of the core protein of the molecule (Heinegård & Hascall, 1974). Unlike the remainder of the core protein, the hyaluronic acid-binding region is reported to be devoid of the chondroitin sulphate and keratan sulphate chains characteristic of cartilage proteoglycans. In addition to these glycosaminoglycan chains, the core protein of the proteoglycan subunits also contain covalently bound *O*-linked and *N*-linked oligosaccharides (Thonar & Sweet, 1979; Sweet *et al.*, 1979; DeLuca *et al.*, 1980; Lohmander *et al.*, 1980). Many of these oligosaccharides are located in the hyaluronic acid-binding region, though it is at present unclear whether they participate directly in the functional properties of the molecule.

The structure of the proteoglycan is not constant throughout life (Bayliss & Ali, 1978*a,b*; Venn, 1978; Roughley & White, 1980; Roughley *et al.*, 1981). The proteoglycan subunits show a higher degree of glycosylation in the juvenile, with chondroitin sulphate being the predominant glycosaminoglycan, whereas in the adult the keratan sulphate content may approach that of chondroitin

Abbreviation used: SDS, sodium dodecyl sulphate.

sulphate. The hyaluronic acid-binding region does, however, appear to be present at all ages. In the human, the link proteins are also subject to age-related changes (Mort *et al.*, 1983), with fragmentation of the molecules occurring progressively with age. The fragmented link proteins are, however, held in a pseudo-native configuration by disulphide bonds. At present it would appear that the ability of the proteoglycan subunits to interact with hyaluronic acid is not impaired during aging, though it has not been conclusively shown whether the interaction of the link protein with either the proteoglycan subunit or the hyaluronic acid is affected by the age-related changes. Any parameter which affects the stability of the proteoglycan aggregate could contribute to an impairment of normal cartilage function.

The purpose of the present work was to study the properties of proteoglycan aggregates prepared from normal young and old human articular cartilage by the standard method of CsCl-density-gradient centrifugation after extraction of the cartilage with a high-ionic-strength salt solution. To our surprise, the procedure used to prepare aggregate from neonatal cartilage (Roughley *et al.*, 1982) did not yield aggregate when applied to adult cartilage, and the present paper describes our findings relating to this observation.

Methods

Materials

Guanidium chloride and hyaluronic acid (from human umbilical cord) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and CsCl and 3,3',4,4'-tetra-aminobiphenyl hydrochloride were from BDH Chemicals (Montreal, Que., Canada). The hyaluronic acid was further purified by precipitation with cetylpyridinium chloride by using the procedure described by Cleland & Sherblom (1977). SDS, acrylamide, methylenebisacrylamide, Coomassie Brilliant Blue R250 and nitrocellulose sheets were from Bio-Rad Laboratories (Mississauga, Ont., Canada). Sepharose CL-2B was from Pharmacia Fine Chemicals (Montreal, Que., Canada). Rabbit antiserum to bovine nasal-cartilage link protein (Poole *et al.*, 1980) and pig immunoglobulin-G antibody raised against rabbit F(ab')₂ and conjugated with horseradish peroxidase (Champion & Poole, 1981) were prepared as described previously.

Source of tissue

Human articular cartilage was obtained from the knees of newborns and adults at the time of autopsy. In all cases autopsy was performed within 20 h of death, and only cartilage that appeared macroscopically normal was taken. Bovine nasal

cartilage from 1-year-old animals was obtained immediately after death from the abattoir. Cartilage was stored at -20°C before use.

Extraction of proteoglycan

Cartilage was finely diced with a scalpel to pieces with dimensions less than 1 mm^3 and then extracted with 10 vol. of 4M-guanidinium chloride/0.1M-sodium acetate, pH6.0, at 4°C with continuous stirring for 48 h. The extraction fluid also contained the following proteinase inhibitors: 1 mM each of iodoacetamide, EDTA and phenylmethanesulphonyl fluoride, and $5\mu\text{g}$ of pepstatin/ml. The extract was then separated from cartilage residue by filtration through glass wool.

Density-gradient centrifugation

The cartilage extracts were subjected to CsCl-density-gradient centrifugation under either associative or dissociative conditions (Hascall & Sajdera, 1969). For associative conditions the extracts were dialysed for 24 h at 4°C against 100 vol. of 0.1M-sodium acetate, pH6.0, and then adjusted to a density of 1.68 g/ml by the addition of CsCl (1.2 g/ml). In some cases hyaluronic acid ($40\mu\text{g}/\text{ml}$) was added to the extracts before dialysis. For dissociative conditions CsCl (0.8 g/ml) and guanidinium chloride (0.23 g/ml) were added directly to the extract to give a final density of 1.50 g/ml. Centrifugation under both associative and dissociative conditions was performed at $100000g_{\text{av}}$ for 48 h at 10°C . Gradients were then fractionated for the measurement of density, uronic acid (Bitter & Muir, 1962) and A_{280} , and analysis by SDS/polyacrylamide-gel electrophoresis. Proteoglycan was obtained as either an A1 preparation (from associative conditions), with a density greater than 1.72 g/ml, or as a D1 preparation (from dissociative conditions), with a density greater than 1.54 g/ml. Other cartilage proteins were obtained as a D3 preparation, with a density less than 1.44 g/ml. In all cases, preparations were freeze-dried after conversion into their potassium salts by dialysis against potassium acetate and subsequent exhaustive dialysis against water.

Re-aggregation experiments

Various combinations of D1 preparations (1 mg/ml), D3 preparations (1 mg/ml) and hyaluronic acid ($20\mu\text{g}/\text{ml}$) were dissolved in 4M-guanidinium chloride/0.1M-sodium acetate, pH6.0. The mixtures were then dialysed and subjected to CsCl-density-gradient centrifugation under associative conditions as described above to yield A1 preparations.

Sepharose CL-2B chromatography

A1 and D1 preparations were dissolved at 2mg/ml in 0.2M-sodium acetate, pH 5.5. Samples (1ml) of the proteoglycan preparations were analysed by chromatography through a Sepharose CL-2B column (120cm × 1cm) at a flow rate of 6ml/h, with 0.2M-sodium acetate, pH 5.5, as the elution buffer. In some cases 40 µg of hyaluronic acid was added to the proteoglycan samples before chromatography. The resulting fractions (1ml) were assayed for uronic acid content. The void and total volumes of the column were determined by the elution of proteoglycan aggregate (from bovine nasal cartilage) and glucuronolactone respectively.

Viscometry

Proteoglycan samples (1 ml) identical with those used for Sepharose CL-2B chromatography were subjected to viscometry at 25°C in a Cannon-Manning semi-micro viscometer. Specific viscosities were calculated from the difference in flow time between the sample and the buffer divided by the flow time for the buffer.

SDS/polyacrylamide-gel electrophoresis

Samples were analysed by electrophoresis in 10%-polyacrylamide gels by the method of King & Laemmli (1971). Freeze-dried preparations were dissolved at 2mg/ml in 0.125M-Tris/HCl, pH 6.8, containing 0.1% SDS, and fractions from density-gradient centrifugation were dialysed against 400 vol. of the same buffer. Before electrophoresis, samples were mixed with an equal volume of 0.125M-Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 1% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue and 5% (v/v) mercaptoethanol, and heated at 100°C for 3min. After electrophoresis, proteins were either stained with Coomassie Brilliant Blue R250 by the method of Fairbanks *et al.* (1971) or transferred to nitrocellulose sheets for immunoidentification.

Electrophoretic transfer and immunoidentification

Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin *et al.* (1979). Link protein was then identified by indirect immune staining using first a rabbit anti-(bovine nasal cartilage link protein) and then a peroxidase-conjugated pig anti-[rabbit F(ab')₂] immunoglobulin G as described previously (Roughley *et al.*, 1982), and made visible by incubation with tetra-amino-biphenyl hydrochloride and H₂O₂. Staining of the polyacrylamide gel with Coomassie Brilliant Blue after transfer showed that there was a total removal of protein from the areas corresponding to link protein.

Results

A1 preparations from cartilage extracts were analysed by Sepharose CL-2B chromatography to determine the degree of proteoglycan aggregation. The A1 preparations obtained from neonatal human cartilage and bovine nasal cartilage were found to contain a large proportion of proteoglycan aggregate, as indicated by a prominent void-volume peak, compared with the totally included peak observed with the proteoglycan subunits of a D1 preparation from the same tissue (Figs. 1a and 1b). In contrast, the A1 preparations obtained from adult cartilage were found to be devoid of proteo-

Fig. 1. Sepharose CL-2B chromatography of A1 preparations and D1 preparations from cartilage extracts. Extracts from (a) bovine nasal cartilage, (b) neonatal human articular cartilage and (c) adult human articular cartilage were subjected to CsCl-density-gradient centrifugation under associative and dissociative conditions, with starting densities of 1.68g/ml and 1.50g/ml respectively. The resulting A1 preparations (—) and D1 preparations (----) with densities greater than 1.72g/ml and 1.54g/ml respectively were analysed by Sepharose CL-2B chromatography. Abbreviations (and in other Figures): V_0 , void volume; V_t , total volume of column.

glycan aggregates by this technique (Fig. 1c). As it has been previously reported that hyaluronic acid is difficult to extract from adult human articular cartilage (Bayliss *et al.*, 1983), it was possible that the absence of aggregate was due to a lack of hyaluronic acid in the extract. Hyaluronic acid was therefore added to the cartilage extracts before dialysis and centrifugation. Although this resulted in the neonatal A1 preparation being eluted almost entirely as proteoglycan aggregates (Fig. 2a), there was little change in the composition of the adult A1 preparation, with proteoglycan subunits being by far the predominant species present (Fig. 2b).

It has also been reported that the proteoglycan-subunit/hyaluronic acid complex exhibits decreased stability towards high centrifugal force in the absence of link protein (Hardingham, 1979; Tang *et al.*, 1979). The CsCl gradients obtained after centrifugation of the cartilage extracts were therefore analysed for the presence of link protein by SDS/polyacrylamide-gel electrophoresis and subsequent transfer of proteins to nitrocellulose for immunoidentification. After centrifugation of the neonatal extract, nearly all the link protein was

present in the high-density fractions (Fig. 3a), compatible with the A1 preparation being link-protein-stabilized proteoglycan aggregate. In contrast, after centrifugation of the adult extract the link protein was found mainly at low density (Fig. 3b), indicating that under the conditions of centri-

Fig. 2. Sepharose CL-2B chromatography of A1 preparations from cartilage extracts containing exogenous hyaluronic acid

Extracts from (a) neonatal and (b) adult human articular cartilage were supplemented with exogenous hyaluronic acid and then subjected to CsCl-density-gradient centrifugation under associative conditions with a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of fractions from associative CsCl-density-gradient centrifugation of cartilage extracts

Fractions from associative CsCl-density-gradient centrifugation of (a) neonatal and (b) adult human articular cartilage extracts supplemented with exogenous hyaluronic acid were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions. Link proteins were detected by immune staining after electrophoretic transfer of protein from the polyacrylamide gel to nitrocellulose sheets.

fugation the link protein does not bind to the high-density proteoglycan subunits present in the A1 preparation. The link-protein distribution was the same whether or not exogenous hyaluronic acid was added to the extracts before centrifugation. Thus the inability to prepare proteoglycan aggregates from adult cartilage extracts is not due to the absence of either link protein or hyaluronic acid.

Another possible explanation for the lack of aggregate in the adult A1 preparation could be the absence of a functional hyaluronic acid-binding region on the proteoglycan subunit. However, when proteoglycan subunits (D1 preparations) from either the neonatal or the adult cartilage extracts were subjected to Sepharose CL-2B chromatography in the presence of hyaluronic acid, the proteoglycans in both preparations were eluted predominantly at the void volume (Figs. 4a and 4b). Furthermore, viscosity measurements showed that the proteoglycan subunits present in the adult A1 preparations were able to interact with hyaluronic acid to the same degree as those purified by direct dissociative-density-gradient centrifugation. Although this indicates that the hyaluronic acid-binding region is present and functional in the adult, it is still possible that the functional integrity is diminished towards high centrifugal force. However, when mixtures of proteoglycan subunits (D1 preparations) and hyaluronic acid were subjected to centrifugation under associative conditions, the resulting A1 prepara-

tions were found to contain a high proportion of proteoglycan aggregates for both neonatal and adult preparations (Figs. 5a and 5b). This also indicates that, under the conditions of centrifugation used in this work, link proteins are not essential for the formation of a stable aggregate. Stability of the proteoglycan subunit/hyaluronic acid interaction towards centrifugation in the absence of link protein has been reported previously by Swann *et al.* (1976). It thus appears that the defects in aggregation reside not in the proteoglycan subunits themselves, but in another component of the adult cartilage extract which interferes or competes with the binding of the proteoglycan subunits to hyaluronic acid.

Further evidence in support of this view was provided from re-aggregation experiments, in which combinations of proteoglycan subunits (D1 preparations), cartilage proteins (D3 preparations) and hyaluronic acid were mixed in 4M-guanidinium chloride, dialysed to associative conditions and then subjected to density-gradient centrifugation to yield A1 preparations. As expected, when neonatal D1 and D3 preparations were used, the resulting A1 preparation contained predominantly proteoglycan aggregates (Fig. 6a), and when

Fig. 4. Sepharose CL-2B chromatography of D1 preparations in the presence and absence of hyaluronic acid. D1 preparations prepared by direct dissociative CsCl-density-gradient centrifugation of (a) neonatal and (b) adult human articular cartilage extracts were analysed by Sepharose CL-2B chromatography in the absence (---) or presence (—) of 2% (w/w) hyaluronic acid.

Fig. 5. Sepharose CL-2B chromatography of A1 preparations obtained by associative CsCl-gradient centrifugation of D1 preparations in the presence of hyaluronic acid. D1 preparations from (a) neonatal and (b) adult human articular cartilage were mixed with hyaluronic acid in 4M-guanidinium chloride, and were then dialysed to associative conditions before CsCl-density-gradient centrifugation at a starting density of 1.68g/ml. The resulting A1 preparations with densities greater than 1.72g/ml were analysed by Sepharose CL-2B chromatography.

Fig. 6. Sepharose CL-2B chromatography of A1 preparations obtained by associative CsCl-density-gradient centrifugation of mixtures containing D1 preparations, D3 preparations and hyaluronic acid

Mixtures in 4M-guanidinium chloride containing hyaluronic acid and (a) neonatal D1 and neonatal D3, (b) adult D1 and neonatal D3, (c) neonatal D1 and adult D3 and (d) adult D1 and adult D3 preparations were dialysed to associative conditions and then subjected to CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

adult D1 and D3 preparations were used the resulting A1 preparations contained mainly proteoglycan subunits (Fig. 6d). However, it was noteworthy that, when adult D1 preparations were

Fig. 7. SDS/polyacrylamide-gel electrophoresis of A1 preparations obtained by associative CsCl-density-gradient centrifugation of mixtures containing D1 preparations, D3 preparations and hyaluronic acid

A1 preparations obtained from associative CsCl-density-gradient centrifugation of mixtures containing hyaluronic acid (a) neonatal D1 and neonatal D3, (b) neonatal D1 and adult D3, (c) adult D1 and neonatal D3 and (d) adult D1 and adult D3 preparations were analysed by SDS/polyacrylamide-gel electrophoresis. Link proteins were detected by immune staining after electrophoretic transfer of protein from the polyacrylamide gel to nitrocellulose sheets.

used in conjunction with neonatal D3 preparations, the resulting A1 preparation contained a large proportion of aggregates (Fig. 6b), whereas when neonatal D1 preparations were used with adult D3 preparations the A1 preparation contained mainly subunits (Fig. 6c). The A1 preparations from the re-aggregation experiments were also analysed for the presence of link protein.

Fig. 8. Sepharose CL-2B chromatography of a mixture containing hyaluronic acid and adult D1 and D3 preparations

A mixture of an adult D1 preparation, an adult D3 preparation and hyaluronic acid in 4M-guanidinium chloride was dialysed to associative conditions and then analysed by Sepharose CL-2B chromatography without prior centrifugation.

Whenever neonatal D3 preparations were used, the resulting A1 preparation always contained link protein in association with proteoglycan aggregates, irrespective of whether neonatal or adult D1 preparations were used (Figs. 7a and 7c), whereas whenever adult D3 preparations were used the resulting A1 preparations were always devoid of link protein (Figs. 7b and 7d).

Thus it appears that the hyaluronic acid-binding regions of both the neonatal and adult proteoglycan subunits function normally, and that a component of the adult cartilage protein preparations is responsible for the inability to isolate proteoglycan aggregates from adult cartilage extracts. It does not, however, appear that this component totally abolishes the interaction between the proteoglycan subunits and hyaluronic acid, since, when the dialysed mixture of adult D1 and D3 preparations and hyaluronic acid described above was subjected directly to Sepharose CL-2B chromatography before centrifugation, a prominent void-volume peak was observed (Fig. 8). It is therefore likely that interference is only reflected after centrifugation as a lack of recovery of high-buoyant-density proteoglycan aggregate.

Discussion

We have shown that, if one subjects a 4M-guanidinium chloride extract of adult human articular cartilage to CsCl-density-gradient centrifugation under associative conditions with a starting density of 1.68g/ml, one obtains an A1 preparation that contains only proteoglycan subunits rather than the expected proteoglycan

aggregates. In contrast, identical conditions do yield aggregates when applied to extracts of neonatal human articular cartilage or bovine nasal cartilage. This apparent anomaly in the adult human is not due to an absence of either hyaluronic acid or link protein from the extract, nor is it due to an abnormality in the ability of the hyaluronic acid-binding region of the proteoglycan subunits to interact with hyaluronic acid. Instead, it appears that the problem resides with the molecules isolated at low buoyant density by direct dissociative-density-gradient centrifugation of the cartilage extracts. Most of this material consists of protein-rich proteoglycans and proteins, including link proteins. Since the link proteins of adult human articular cartilage differ from the link proteins described in other cartilage, in that they can occur as fragmented molecules held in a pseudo-native conformation by disulphide bonds (Mort *et al.*, 1983), one could speculate that such link proteins might interfere with the binding of the proteoglycan subunits to hyaluronic acid rather than stabilize the aggregates. Alternatively, an as yet unidentified component abundant in the adult cartilage could cause such interference. For example, if excessive amounts of hyaluronic acid-binding-region-like molecules were present in the adult, they could compete with and displace the intact proteoglycan subunits from re-forming aggregates.

It is noteworthy that other workers have reported the isolation of proteoglycan aggregates from normal adult human articular cartilage by density-gradient centrifugation, and some have used these preparations to prepare the adult link proteins (Pal *et al.*, 1978; Bayliss & Ali, 1978b; Perin *et al.*, 1978; Bayliss *et al.*, 1983). At first this appears to contradict the observations reported in the present paper. However, all those workers used a starting density lower than that used in the present work (often as low as 1.5g/ml) for the associative gradients. It has been shown previously (Mashburn *et al.*, 1974) that in associative CsCl density gradients hyaluronic acid has a buoyant density of about 1.65g/ml and hence might be expected to be present in the A1 preparations obtained by those workers, whether or not it was in association with the proteoglycan subunits. Our centrifugation and fractionation conditions were chosen so that 'free' hyaluronic acid would not be present in the A1 preparation. Furthermore, preliminary observations by ourselves indicate that the link protein even in the adult will interact and hence co-migrate with the hyaluronic acid under associative centrifugation conditions. Hence A1 preparations containing 'free' hyaluronic acid would also contain link proteins. Thus proteoglycan aggregates can be obtained from adult

human cartilage by performing the initial associative-density-gradient centrifugation at a low starting density, where all components sediment to the bottom of the gradient, though not necessarily in association with one another.

At present we cannot comment as to the age at which this phenomenon begins. It does, however, appear to be a common property of those adults, aged over 40 years, that we have studied in this work. Irrespective of the time or mode of origin, it appears that the problem is an instability of adult proteoglycan aggregate towards the conditions of centrifugation, rather than an inability of the components of the aggregate to undergo association under normal conditions. It is therefore quite feasible that proteoglycan aggregates exist within the normal adult cartilage matrix, though one cannot comment on the stability of these molecules towards adverse conditions such as those that may be encountered in pathological situations.

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