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Age-related changes in the structure of proteoglycan link proteins present in normal human articular cartilage

John S. MORT, A. Robin POOLE and Peter J. ROUGHLEY
Joint Diseases Laboratory, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Que. H3G 1A6, Canada, and Department of Surgery, McGill University, Montreal, Que., Canada

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Link proteins were identified immunologicaly in human articular-cartilage protein preparations from various individuals. Irrespective of age, all cartilages contained three link proteins of mol.wts. 48000, 44000 and 41000. However, with increasing age, multiple additional components of mol.wts. 26000–30000 were commonly observed under conditions where disulphide bonds were reduced.

Proteoglycans are essential components of hyaline cartilage in that they endow it with its special property of compressive stiffness (Kempson *et al.*, 1970). Much of the proteoglycan can bind to hyaluronic acid (Hascall, 1977) and thereby form macromolecular aggregates, which are demonstrable *in situ* (Poole *et al.*, 1982) in association with collagen fibrils. The binding of proteoglycan to hyaluronic acid is stabiluzed by a low-molecular-weight component termed 'link protein' (Hardingham & Muir, 1972; Keiser *et al.*, 1972; Gregory, 1973). In the absence of this molecule the proteoglycan aggregate is reported to be less stable at low pH, elevated temperature, high ionic strength or high centrifugal force (Hardingham, 1979; Tang *et al.*, 1979; Franzen *et al.*, 1981). Link proteins have been described in a variety of cartilagenous tissues from various species and are generally characterized as having molecular weights between 40000 and 50000 as determined by SDS/polyacrylamide-gel electrophoresis (Oegema *et al.*, 1975; Baker & Caterson, 1977; Vasan & Lash, 1977; Bonnet *et al.*, 1978). In a previous study (Roughley *et al.*, 1982), human link protein was purified from proteoglycan aggregate prepared from neonatal articular cartilage. Three components, having molecular weights of 48000, 44000 and 41000, were identified by gel electrophoresis, and these were shown to cross-react with bovine nasal-cartilage link protein by using a monospecific antiserum to the above molecules.

Analysis of link protein in all the above work has the preparation of proteoglycan aggregate as a prerequisite. This therefore requires the presence of high-density proteoglycan able to interact with hyaluronic acid and functional link protein. In pathological situations, in particular, it would be desirable to be able to study the total link-protein content of the cartilage irrespective of functional integrity. Furthermore, in some non-cartilagenous tissues where link protein is present it may not be possible to isolate high-density proteoglycan aggregates. The use of the electrophoretic-transfer technique (Towbin *et al.*, 1979), together with a monospecific antiserum to link protein, permits the investigation of link protein in tissue extracts from the upper portion of dissociative CsCl density gradients without the need for further purification. This characterization is therefore dependent solely on the presence of immunoreactive material and does not rely on the functional integrity of the link protein. In this work we use the technique to characterize structural changes that occur in the link protein from human articular cartilage of various ages.

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; $F(ab')_2$, bivalent antigen-binding fragment obtained by pepsin digestion of immunoglobulin G.

Experimental procedures

Materials

Guanidinium chloride, bovine serum albumin, horseradish peroxidase and Tween 20 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CsCl and 3,3′,4,4′-tetraminobiphenyl hydrochloride were from BDH Chemicals (Montreal, Que., Canada), and nitrocellulose sheets were from Bio-Rad Laboratories (Mississauga, Ont., Canada). Rabbit antiserum to bovine nasal-cartilage link proteins (Poole *et al.*, 1980) and pig IgG antibody raised against rabbit $F(ab')_2$ and conjugated with horseradish peroxidase (Champion & Poole, 1981) were prepared as described previously.

Source of cartilage

Human articular cartilage was routinely obtained at autopsy (within 16h of death) from the distal femour of individuals in whom there was no macroscopically recognizable superficial cartilage fibrillation. In addition, macroscopically normal adult cartilage was also obtained from surgical specimens after above-knee amputation for vascular insufficiency in the lower limb, and the tissue was processed immediately. All the hyaline cartilage above the subchondral bone was taken. In all cases samples were obtained with the permission of the patient or next-of-kin. Bovine articular cartilage was obtained at the time of slaughter from the metacarpal phalangeal joints of 1-year-old animals, and the Swarm rat chondrosarcoma was obtained from Sprague–Dawley rats which had been injected subcutaneously with a chondrosarcoma-cell suspension. With the exception of surgical specimens, cartilage was routinely stored at −20°C before extraction.

Extraction and isolation of cartilage proteins

All tissues were finely divided then equal wet weights were extracted and fractionated in exactly the same manner with 4M-guanidinium chloride (containing inhibitors of all four proteinase classes) as described previously (Roughley & White, 1980). After 48h the extracts were filtered through glass wool and the density of the filtrate was adjusted to 1.50g/ml by the addition of solid CsCl. Guanidinium chloride was also added to keep its concentration at 4M. The solutions were then subjected to centrifugation at $100000g_{av.}$ for 48h at 10°C, and the resulting dissociative density gradients were fractionated, then monitored for density, uronic acid and A_{280}. Cartilage proteins, including link proteins, were concentrated in fractions at the top of the gradient with densities less than 1.40g/ml. Such fractions were pooled, dialysed against 0.1M-potassium acetate, then exhaustively against water, and freeze-dried.

SDS/polyacrylamide-gel electrophoresis

Cartilage proteins from the top of dissociative density gradients were dissolved at 1mg/ml in 0.125M-Tris/HCl (pH6.8)/2% (w/v) SDS/1% glycerol/0.001% Bromophenol Blue, with or without 5% 2-mercaptoethanol for reduced or non-reduced gels respectively. Samples were immediately placed in a boiling-water bath and left for 3min. All of the cartilage proteins dissolved when this treatment was used. Electrophoresis on 10% (total concentration) polyacrylamide slab gels was as described by King & Laemmli (1971). Protein staining with Coomassie Brilliant Blue R250 was by the method of Fairbanks *et al.* (1971).

Electrophoretic transfer and immunolocalization

Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin *et al.* (1979), and indirect immune staining with rabbit anti-(bovine nasal cartilage link protein) and peroxidase-conjugated pig anti-[rabbit $F(ab')_2$] IgG was used to detect link-protein components as described previously (Roughley *et al.*, 1982). Staining of the acrylamide gel after electrophoretic transfer showed that there was a total removal of proteins from the areas corresponding to link-protein immunolocalization.

Results

Previously we demonstrated that link protein purified from proteoglycan aggregate prepared from human neonatal cartilage is separated into three protein components when analysed by SDS/polyacrylamide-gel electrophoresis (Roughley *et al.*, 1982). In the present study we directly detected link proteins in the protein-rich low-density preparation obtained by direct CsCl density-gradient centrifugation of cartilage extracts, by immunolocalization after electroblotting of protein from SDS/polyacrylamide gels on to nitrocellulose. In foetal and neonatal cartilages the link proteins detected in reducing gels were identical in size with those purified from the neonatal proteoglycan aggregates (Fig. 1*a*). Three similar bands were also observed in specimens from all older cartilages, though in the mature adult the link protein of mol.wt. 48000 was less abundant relative to those of mol.wt. 44000 and 41000 when compared with juvenile specimens. In addition, further smaller link species were identified by the specific antiserum in all specimens. These were only observed under reducing conditions, and formed a cluster of at least five bands of molecular weights between 26000 and 30000, whose abundance tended to increase between the juvenile and the mature adult. Only traces of these low-molecular-weight components were seen in the foetal and neonatal specimens (Fig. 1*a*).

The smallest link-protein components were not seen in similar preparations from 1-year-old bovine articular cartilage nor from Swarm rat chondrosarcoma tissue, though the two major bands of bovine cartilage link protein and the single rat chondrosarcoma link protein were clearly identified (Fig. 2*a*). Since the antiserum used was raised against bovine nasal cartilage link protein, the absence of the low-molecular-weight bands in bovine cartilage extracts supports the notion that the smallest immunoreactive components in adult human cartilage are link-protein-related. This assignment is further strengthened by the loss of the low-molecular-weight bands when electrophoresis was

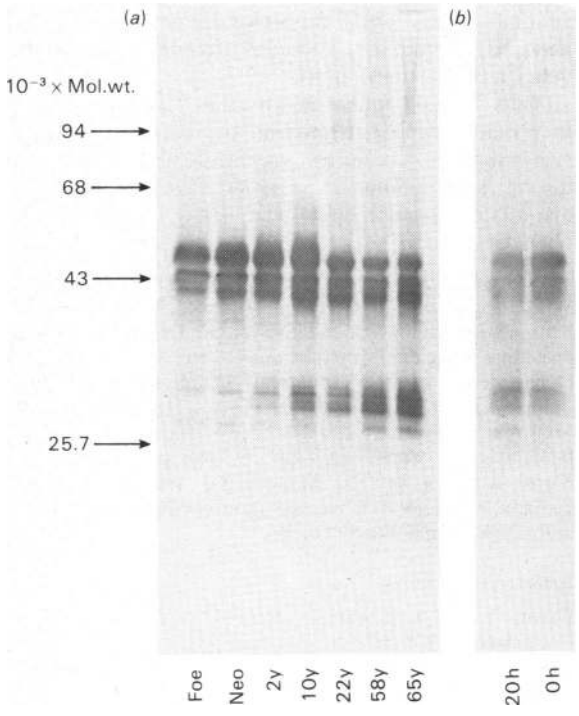

Fig. 1. *Age-related changes in human link protein*
Preparations enriched in cartilage proteins (40 μg) were separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions and transferred to a nitrocellulose sheet. Link-protein components were then localized immunologically. Positions of molecular-weight standards are indicated. (*a*) Samples from foetal (Foe), neonatal (Neo), 2-, 10-, 22-, 58- and 65-year(y)-old human articular cartilage obtained at the time of autopsy; (*b*) samples from 62-year-old human articular cartilage, obtained at the time of surgery, extracted after 20h at room temperature or directly (0h).

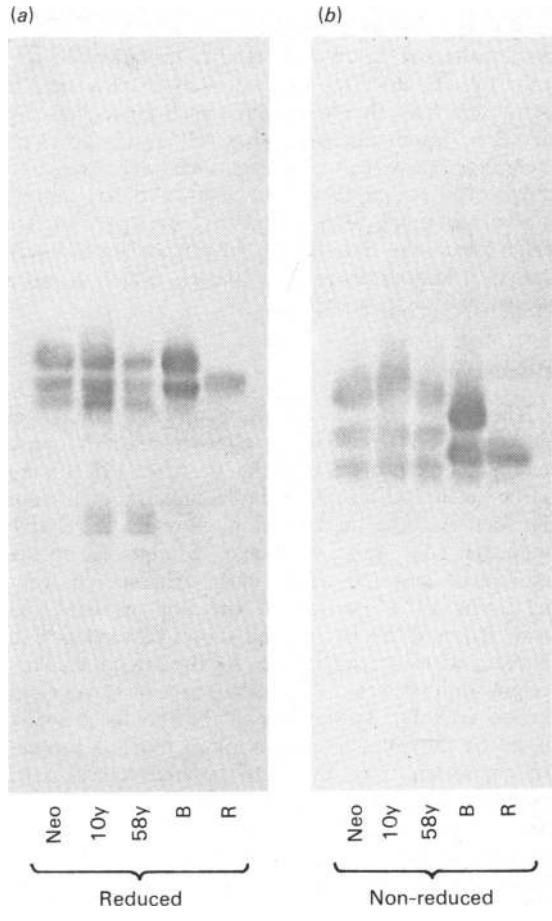

Fig. 2. *Comparison of human, bovine and rat-chondrosarcoma link proteins under reducing and non-reducing conditions*
Cartilage proteins (40 μg) were separated by SDS/polyacrylamide-gel electrophoresis under (*a*) reducing and (*b*) non-reducing conditions then treated as in Fig. 1. Samples are from neonatal (Neo), 10-year(y)- and 58-year-old human articular cartilage, bovine articular cartilage (B) and Swarm rat chondrosarcoma (R).

carried out under non-reducing conditions (Fig. 2*b*). As previously demonstrated, bovine link proteins (Tang *et al.*, 1979) and human link proteins (Roughley *et al.*, 1982) migrate significantly faster on SDS/polyacrylamide-gel electrophoresis under non-reducing conditions as compared with reducing conditions, indicating the presence of intra-chain disulphide bridging in these molecules. It seems likely, therefore, that the low-molecular-weight bands seen on reducing gels represent link-protein molecules that have been partially cleaved but are maintained in a pseudo-native configuration by intra-chain disulphide bonds under non-reducing conditions.

In studies on cartilage obtained at amputation we have shown that the link-protein fragments are not the result of tissue autolysis occurring between death and the time of autopsy (up to 16h later). Collection of adult tissue at the time of surgery and immediate preparation of cartilage proteins still yielded these smaller fragments (Fig. 1*b*). Also, incubation of such tissue for 20h at room temperature (Fig. 1*b*) or 4°C, or storage of the tissue at −20°C for 6 weeks before extraction (results not shown) did not produce any marked change in the electrophoretic patterns. Furthermore, if fragmentation were an autopsy artefact due to autolysis, one might have expected greater evidence of degradation in the more cellular foetal and juvenile tissues, resulting in the opposite trend to that observed. One

could also argue that fragmentation may occur during the preparative procedure, though the presence of inhibitors for each proteinase class in the extraction fluid should preclude such proteolysis. In addition, fragments were observed using the same technique when 40 μm cartilage slices were extracted directly by boiling in the gel-electrophoresis sample buffer containing SDS (results not shown). Thus we believe that the link-protein fragmentation is likely due to a limited proteolysis *in vivo*, which is more prevalent in mature cartilage.

Discussion

The three bands on SDS/polyacrylamide-gel electrophoresis previously characterized in pure neonatal link protein (Roughley *et al.*, 1982) were observed in cartilage from individuals ranging from the foetus to the mature adult, though the relative intensities did vary with age. Moreover, in the adolescent and the adult, clear evidence of fragmentation of a portion of the link protein was observed, and this proportion showed a tendency to increase with increasing age. As the low-molecular-weight components were not seen in 1-year-old bovine articular cartilage or in the rapidly growing tissue of the rat chondrosarcoma, the link-protein fragmentation may therefore be characteristic of mature tissues. It should, however, be pointed out that the appearance of link-protein fragments as a function of age was not absolute. Although fragments were never abundant in the very young, they were not always intense in the adult, though this was the exception rather than the rule. Thus parameters of tissue status, other than just age, may play a role in the process. Although all cartilage studied was macroscopically normal, early degenerative changes may have been present, and such changes would be expected to be more prevalent with age and could contribute to the observed fragmentation.

One might postulate that this fragmentation may have a detrimental effect on the ability of link protein to stabilize proteoglycan aggregates, though under non-reducing conditions a pseudo-native structure appears to be maintained. The inability of some adult human link protein to form stable complexes with high-buoyant-density proteoglycan subunits under the conditions of ultracentrifugation used in proteoglycan preparation has been indicated by the high proportion of link protein sedimenting at the top of associative gradients (Périn *et al.*, 1978). Thus the observed changes in link-protein structure may lead to impaired function and contribute to a decrease in the stability of isolated proteoglycan aggregates from the adult human towards high centrifugal force. However, such an impairment would not necessarily preclude interaction of the link protein with hyaluronic acid and proteoglycan subunits under normal physiological conditions, and hence the formation of stable proteoglycan aggregates in the cartilage matrix.

Thus it would appear that the link proteins present in proteoglycan aggregates in the cartilage matrix are subjected to increased proteolytic cleavage during aging. This may reflect both a normal age-related phenomenon and early degenerative changes.

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