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Characterization of link protein(s) from human intervertebral-disc tissues

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Proteoglycan aggregates (A1) were prepared from the anulus fibrosus, nucleus pulposus and cartilage-endplate tissues of postnatal (0–6-month-old)- and young-adult (20-30-year-old)-human intervertebral discs. The A1 fractions from young-adult disc contained a greater proportion of non-aggregating proteoglycans than did postnatal tissues. After dissociative CsCl-density-gradient fractionation of the A1, more than 90% of the uronic acid was found in the postnatal A1D1, whereas only 60–80% of the hexuronate was present in the A1D1 isolated from young-adult disc tissues. These results indicated that more lower-buoyant-density proteoglycans occur in the young-adult disc. Link-protein-rich fractions (A1D3) were subjected to SDS/polyacrylamide-gel electrophoresis and immunolocation analyses using monoclonal antibodies specific for epitopes on link protein or proteoglycan. Under non-reducing conditions, the major link protein present in postnatal disc tissues was link protein 1. By contrast, all three link proteins (1, 2 and 3) were detected in young-adult tissues, with the smaller link protein 3 predominating. Analyses of the A1D3 fractions under reducing conditions also indicated the presence of link-protein-degradation peptides (M_r approx. 26000) from young-adult disc tissues, but not from postnatal tissues. Sequential Sepharose CL-6B and Sephacryl S-300 chromatography in 4 M-guanidinium chloride was employed to separate the link proteins of the A1D3 fraction from protein-rich proteoglycan. Immunolocation analyses indicated that postnatal samples contained no detectable contaminating proteoglycan fragments. However, young-adult link-protein preparations could not be separated from hyaluronic acid-binding region and other proteoglycan fragments by means of these chromatographic procedures. The studies indicate that, compared with hyaline articular cartilage, degraded link protein and proteoglycan accumulate at an early age in young-adult disc tissues. These partially degraded proteoglycan aggregate components may significantly alter the biomechanical properties of disc tissues.

INTRODUCTION

Little is known about the intervertebral disc at the molecular level, although it is one of the major components of the spine. The disc is avascular [1], is relatively acellular [2,3] and thus consists primarily of extracellular matrix macromolecules. The biomechanical properties and physiological function of the tissue are directly related to the relative composition and interactions between the major components of this matrix, collagen and proteoglycan [4].

Several studies have indicated that disc proteoglycans, like those from cartilage, are capable of forming large macromolecular aggregates with hyaluronic acid [5–9]. At least two distinct populations are apparent: proteoglycans which aggregate with hyaluronic acid, and a non-aggregating proteoglycan population [7,9,10]. The relative proportion of aggregating and non-aggregating proteoglycan varies between the anulus fibrosus and the nucleus pulposus, and with the age of the specimen studied [6]. In addition, Buckwalter *et al.* [11] have recently shown that significant changes occur in the overall macromolecular dimensions of the proteoglycan aggregates at an early age in disc tissues. Proteolytic and chemical fragmentation of the aggregating disc proteoglycan monomers indicates that they possess a chondroitin sulphate-attachment region, a keratan sulphate-attachment region and a hyaluronic acid-binding region [9,12], similar to hyaline cartilages. Furthermore, compared with hyaline cartilage, the proteoglycan monomers from disc are smaller and more polydisperse [7–9]. The non-aggregating proteoglycans may not possess a hyaluronic acid-binding region [9] and contain relatively more keratan sulphate [6,10].

In cartilage, the interaction of proteoglycan with hyaluronic acid is stabilized by the link proteins [13–15]. The evidence to date indicates that cartilage neonatal link protein is composed of three subpopulations (LP1, LP2 and LP3), which are glycosylated to various extents (LP1 > LP2 > LP3). Additional heterogeneity can also arise from the proteolytic modification *in situ* of LP1 and LP2, producing LP3 [16]. Tengblad *et al.* [17] have demonstrated that link proteins occur in 60–70-year-old human intervertebral disc, anulus fibrosus and nucleus pulposus. Similar findings have also been observed in this laboratory [18,19]. The aims of the present work were to use biochemical and monoclonal-antibody technologies to further characterize the proteoglycan and link protein from all three intervertebral-disc tissues (anulus fibrosus, nucleus pulposus and cartilage endplate), obtained from postnatal- and young-adult-human spines.

Abbreviations used: GdmCl, guanidinium chloride; PAGE, polyacrylamide-gel electrophoresis.

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MATERIALS AND METHODS

Materials

Sodium hyaluronate (Healon), Sephadex G-25 (PD-10), Sepharose CL-6B and Sephacryl S-300 were purchased from Pharmacia, Uppsala, Sweden. Goat anti-(mouse immunoglobulin) peroxidase-conjugated second antibody was obtained from Southern Biotechnology Associates, Birmingham, AL, U.S.A. Nitrocellulose sheets were acquired from Schleicher and Schuell, Keene, NH, U.S.A. SDS, acrylamide, bisacrylamide, ammonium persulphate and *NNN'N'*-tetramethylethylenediamine were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Guanidinium chloride (GdmCl) was bought from Sigma Chemical Co., St. Louis, MO, U.S.A. $Na^{125}I$ was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. All other reagents were of analytical reagent grade.

Analytical methods

Hexuronate determinations were performed by the method of Bitter & Muir [20]. Protein was determined spectrophotometrically at 280 nm by using the assumption that 1 mg/ml protein solutions in 4 M-GdmCl had an absorbance of 1.0 unit. Scanning densitometry of autoradiograms was performed on a Zeineh model SL-504-XL densitometer.

Extraction and isolation of proteoglycan and link protein(s) from intervertebral-disc tissues

Entire postnatal spines from 11-, 60- and 105-day-old infants and mature lumbar spinal regions from 25-, 27- and 29-year-old-human spines were obtained at autopsy within 8–14 h of death and judged non-pathological by radiological and macroscopic examination. Discs were dissected, on ice, into three regions: anulus fibrosus, nucleus pulposus and cartilage endplate. The tissue obtained from each region was then extracted (10 ml of extraction buffer per g wet wt. of tissue) with 4 M-GdmCl [21] in 0.05 M-sodium acetate buffer, pH 6.0, containing proteinase inhibitors [22]. Proteoglycan aggregate (A1), proteoglycan monomer (A1D1) and link-protein-enriched fraction (A1D3) were subsequently isolated from the extract as previously described [23]. Samples (1 ml) of each gradient fraction taken and used for density determination and hexuronate analysis.

Link-protein-enriched fractions (A1D3) obtained from the dissociative gradient were concentrated with a Micro-ProDicon negative-pressure dialysis concentrator, equilibrated with 4 M-GdmCl/0.05 M-sodium acetate buffer, pH 6.4 at 4 °C. Concentrated link-protein samples were stored at −20 °C until further purification.

Proteoglycan and link protein(s) were prepared from 50–70-year-old-human articular cartilage and Swarm rat chondrosarcoma as described above.

Purification of the link protein(s) from protein-rich proteoglycan

Sepharose CL-6B [24] or Sephacryl S-300 [25,26] chromatography under dissociative conditions were previously described as the method for the purification of link protein(s) from protein-rich proteoglycan contaminants. In the present study, sequential chromatography on Sepharose CL-6B and Sephacryl S-300 in 4 M-GdmCl/0.05 M-sodium acetate buffer, pH 6.4, was used in an attempt to separate the link protein(s) from protein-rich proteoglycans. Concentrated link-protein-enriched fractions (A1D3) were chromatographed on a column (1.6 cm × 70 cm) of Sepharose CL-6B, with 4 M-GdmCl in 0.05 M-sodium acetate buffer, pH 6.5, as eluent. Routinely, 1.5–2 ml samples were applied, 2 ml fractions were collected, and the eluate was monitored for absorbance at 280 nm.

The link-protein fractions were pooled (henceforth referred to as 'A1D3-6B/5'), concentrated at 4 °C by using the Pro-DiCon apparatus equilibrated with 4 M-GdmCl in 0.05 M-sodium acetate buffer, pH 6.5, and chromatographed on a column (1.6 cm × 70 cm) of Sephacryl S-300, with 4 M-GdmCl in 0.05 M-sodium acetate buffer, pH 6.5, as eluant. Fractions (2 ml each) were collected and monitored for absorbance at 280 nm. Link-protein fractions were pooled after Sephacryl S-300 chromatography and concentrated as described above.

Monoclonal antibodies

The protocol for immunization, fusion and cloning was described previously [27,28]. Monoclonal antibody 9/30/8-A-4 [28–30] recognizes two epitopes in the *C*-terminal half of the link protein (1, 2 or 3) polypeptide backbone. The epitopes are present on all three link-protein species and are equally detected in immunolocation analyses. The 12/21/1-C-6 [31,32] monoclonal antibody recognizes two epitopes present in the hyaluronic acid-binding globular domains of proteoglycans. Monoclonal antibody 6/1/7-D-1 [33] recognizes an epitope present in the hyaluronic acid-binding region and also the carbohydrate-attachment regions of the proteoglycan monomer. All three antibodies cross-react with epitopes from a wide variety of animal species and humans [31].

SDS/PAGE

SDS/PAGE was performed in 10%- or 12%-(w/v)-polyacrylamide gels in the presence of 0.1% SDS as described by Laemmli [34]. Before electrophoresis, A1D3 fractions in 4 M-GdmCl had to be diluted with distilled water before addition of treatment buffer because of the incompatibility of 4 M-guanidine solvents with SDS buffer solutions. Routinely, the samples (10–200 μg of protein/100 μl) were electrophoresed at 50 mA/gel for 5 min, 35 mA/gel through the stacking gel, and thereafter at 30 mA/gel through the running gel.

Western blotting and immunolocation analyses

After replicate samples were subjected to SDS/PAGE, the fractionated proteins or peptides were electrophoretically transferred to nitrocellulose sheets as described by Burnette [35]. Typically, the electrophoretic transfer was carried out at 0.5 A for 3 h at 4 °C. After electrophoretic transfer, immunochemical detection was performed as described previously [30]. A 1:100 and a 1:500 dilution of the monoclonal-antibody ascites fluid and peroxidase-conjugated goat anti-mouse second antibody respectively were routinely employed. Fresh peroxidase substrate was prepared by adding 5 μl of 30% H_2O_2 to 9 ml of Tris-buffered saline (0.05 M-Tris/0.2 M-NaCl, pH 7.4) and 2 ml of 4-chloro-1-naphthol stock solution (3 mg of 4-chloro-1-naphthol/ml of methanol). In some studies ^{125}I-labelled primary antibody (100000 c.p.m./ml) was used for immunolocation analyses instead of peroxidase-conjugated second antibody [30]. Monoclonal-antibody-9/30/8-A-4 ascites fluid

Table 1. Estimation of degraded link protein by densitometric scanning

Data obtained by densitometric scans of autoradiography films were used to estimate the percentage of degraded link protein from postnatal disc ($n = 4$), young-adult disc ($n = 6$) and pooled 50–70-year-old-human articular cartilage [under non-reducing conditions the percentage of LP3 epitope present was calculated as LP3/(LP1 + LP2 + LP3) × 100; under reducing conditions, the percentage of degradation peptide epitopes was calculated as peptides/(LP1 + LP2 + LP3 + peptides) × 100]. The percentage of epitope present was based on monoclonal antibody 9/30/8-A-4 binding and specificity. Mean values ± S.D. are shown.

	Percentage of link-protein epitope		
	Postnatal	Young-adult	HAC
Non-reduced			
LP1	63.0 ± 11.0	26.5 ± 4.1	45
LP2	26.0 ± 7.8	13.8 ± 2.5	18
LP3	11.0 ± 4.7	59.7 ± 6.5	37
Reduced			
LP1, 2 and 3	99.75 ± 0.5	77.7 ± 6.0	83
Peptides	0.25 ± 0.5	22.3 ± 6.0	17

was iodinated by using the chloramine-T method as previously described [36].

RESULTS AND DISCUSSION

The procedure used for isolating the proteoglycan aggregates (A1), proteoglycan monomer (A1D1) and link-protein-enriched fraction (A1D3) from the anulus fibrosus, nucleus pulposus and cartilage endplate of intervertebral-disc samples was previously described [23]. After associative gradient centrifugation, most of the uronic acid ($\geqslant 90\%$) was found in the A1 fraction, regardless of the disc region or age of the individual. However, distribution of uronic acid in the dissociative CsCl-density-gradient fractions (A1D1–A1D3) suggested that an age-dependent difference occurred. More than 90% of the uronic acid was found in the postnatal A1D1. In contrast, only 60–80% of the uronic acid was found in the A1D1 isolated from young-adult tissues, indicating that a greater proportion of the proteoglycans of lower buoyant density occurs in young-adult lumbar-disc tissues. This suggests that the proteoglycans obtained from young-adult disc tissues contain relatively more protein and/or less carbohydrate than those isolated from postnatal intervertebral disc. An increase in the proportion of low-density protein-rich proteoglycans with age has been observed in dissociative CsCl density gradients from human articular cartilage [37].

The occurrence of link protein(s) in postnatal- and young-adult intervertebral-disc tissues was demonstrated after SDS/PAGE separation of the A1D3 fractions and immunolocation analyses using monoclonal antibody 9/30/8-A-4, which is specific for hyaline-cartilage link proteins. Under non-reducing conditions (Fig. 1*a*), three link proteins (1, 2, and 3) are evident from both postnatal- and young-adult disc tissues. Postnatal disc tissues show a predominance of link protein 1, whereas link protein 3 is the predominant species in the young adult. This

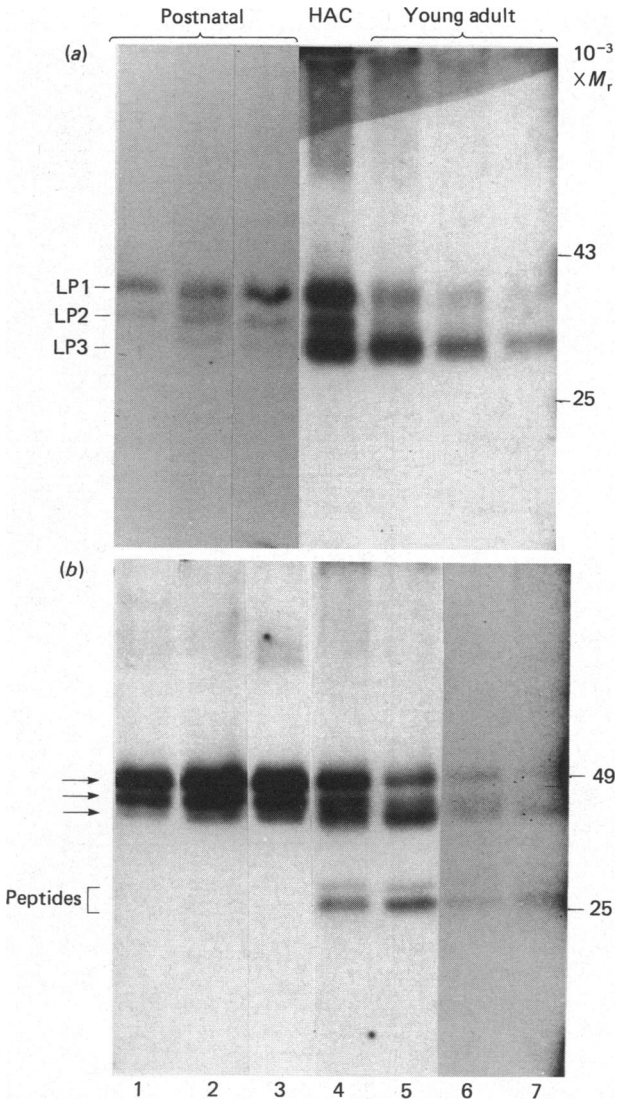


Fig. 1. Analyses of postnatal and young-adult disc link proteins by SDS/PAGE

Immunolocation of the link proteins was performed by using ^{125}I-labelled 9/30/8-A-4 monoclonal antibody. SDS/PAGE was performed under (*a*) non-reducing and (*b*) reducing conditions. Lanes 1 and 5, anulus-fibrosus A1D3 preparations from 60-day-old and 25-year-old individual respectively; lanes 2 and 6, nucleus-pulposus A1D3 preparations from 105-day-old and 25-year-old individuals respectively; lanes 3 and 7, cartilage-endplate A1D3 preparations from 60-day-old and 25-year-old individuals respectively. Link proteins from pooled 50–70-year-old-human articular cartilage (HAC) A1D3 (lane 4) are given for comparison. The same amount of sample (protein) was loaded in the respective wells of both non-reducing and reducing gels. Differences in staining intensity between (*a*) and (*b*) are due to different lengths of exposure time. The electrophoretic mobility of link proteins 1, 2 and 3 from human hyaline cartilage are indicated by LP1, 2 and 3. The M_r values of protein standards are indicated. This Figure illustrates results representative of three individuals from each age group (i.e. postnatal or young adult).

contrasts with the situation in old (50–70-year-old)-human articular-cartilage link-protein preparations, where near equal amounts of link protein 1 and 3 are evident. Reduction with 2-mercaptoethanol (Fig. 1*b*)

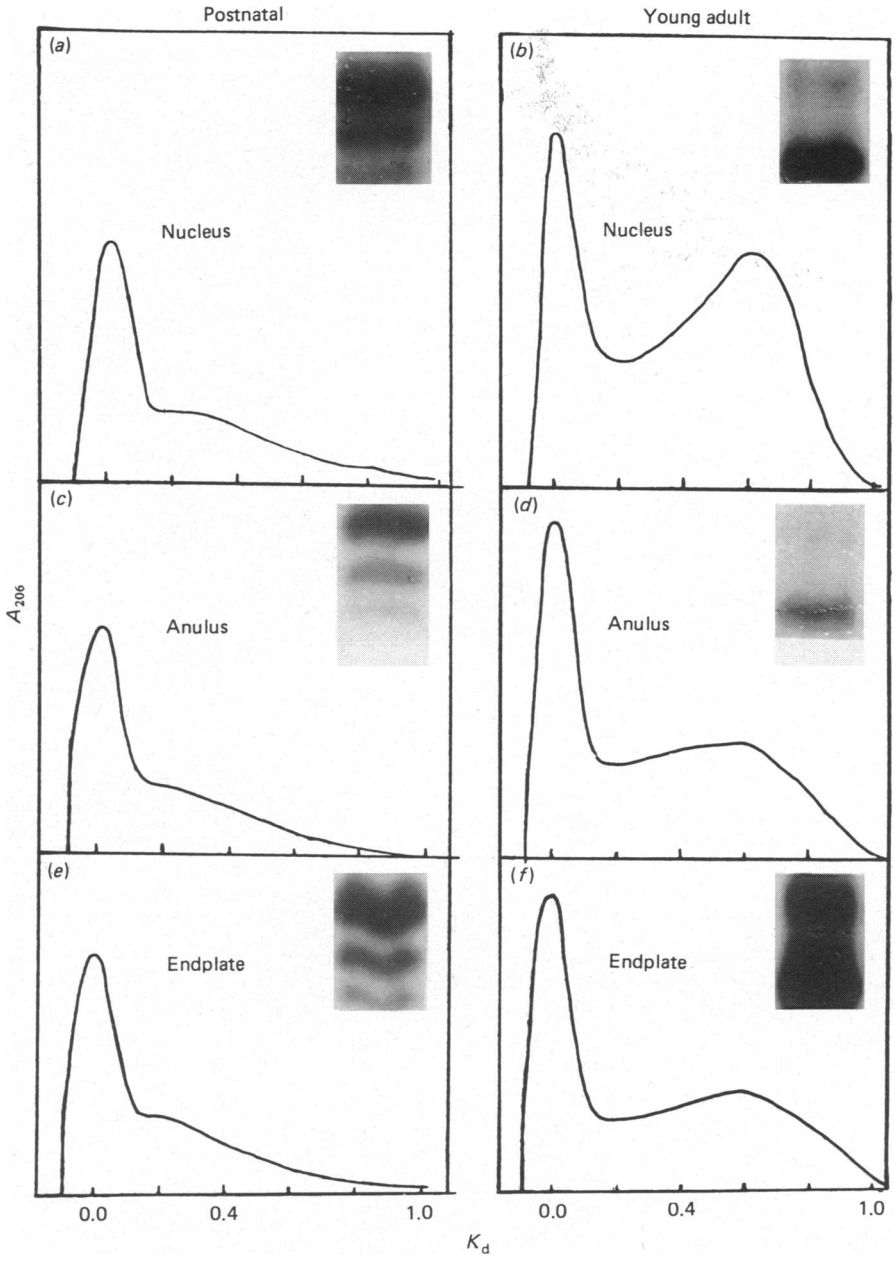


Fig. 2. Sepharose CL-2B analyses of proteoglycans from postnatal and young-adult-human intervertebral disc

Sepharose CL-2B chromatographic profiles of the aggregating and non-aggregating proteoglycans isolated from intervertebral-disc tissues are shown. A1 preparations (1.5–2.0 mg dry wt.) were incubated with exogenous hyaluronic acid (2%, w/w) before application on Sepharose CL-2B in order to separate aggregating from non-aggregating proteoglycans. (*a*), (*c*) and (*e*) are representative (three individuals) chromatographic profiles obtained from the nucleus pulposus (NP), anulus fibrosus (AF) and cartilage endplate (CE) of the postnatal disc respectively. (*b*) (*d*) and (*f*) are representative (three individuals) chromatographic profiles of NP, AF, and CE of young-adult disc respectively. Samples were eluted with 0.15 M-Na_2SO_4, pH 6.5. Absorbance was monitored at 206 nm. Inset: SDS/PAGE patterns for the link proteins associated with each postnatal and young-adult proteoglycan preparation. The distribution coefficient (K_d) is given for comparison.

continues to show the predominance of link protein 1 (M_r 47000) from postnatal, and link protein 3 (M_r 39900) from young-adult, disc tissues. In addition, the occurrence of a series of small peptides of M_r 24500–27000 from all regions of young-adult disc as well as from the old-human articular-cartilage A1D3 preparation are observed under reducing condtions. The finding of degradation peptides from old-human cartilage has been reported [38]. Unlike link protein isolated from young-adult disc tissues, there is no indication of the presence of degradation peptides after reduction of the postnatal link proteins.

Densitometric scanning analyses of autoradiograms, such as those shown in Fig. 1, were performed to estimate the relative proportion of link proteins 1, 2 and 3 and the degradation peptides present in young-adult and postnatal disc tissues compared with that found in pooled old-human articular cartilage. These results are

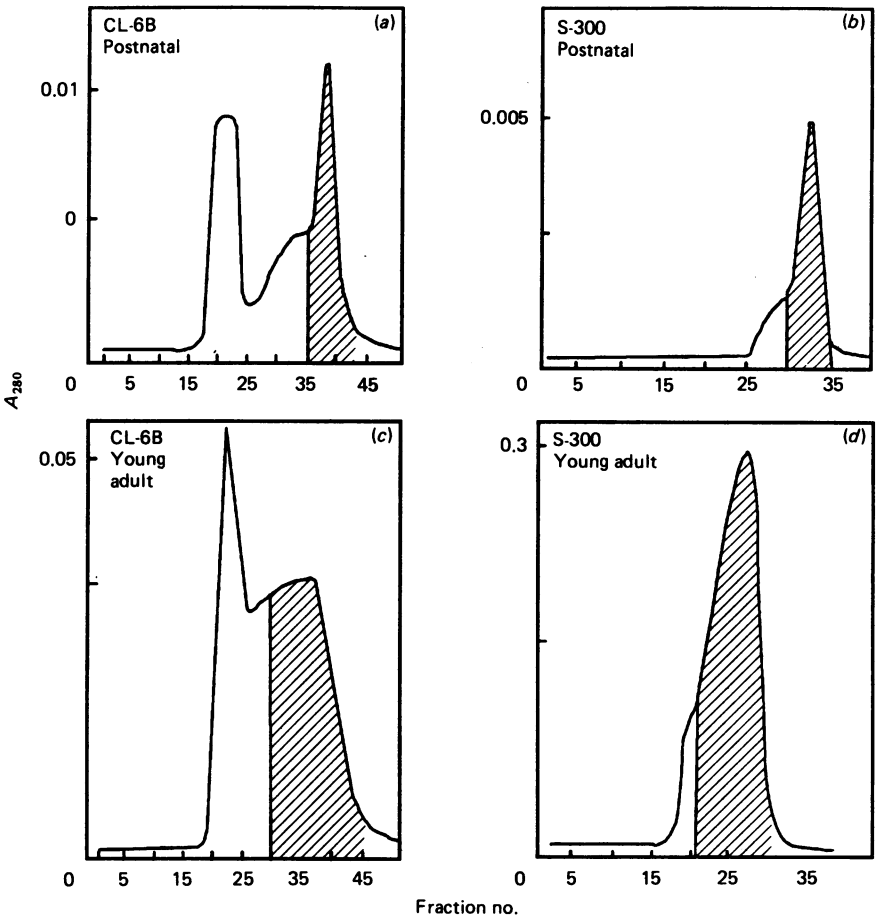


Fig. 3. Sequential Sepharose CL-6B (CL-6B) and Sephacryl S-300 (S-300) chromatography of postnatal and young-adult A1D3

(*a*) and (*b*) are representative profiles obtained from postnatal (60-day-old) A1D3 fractions and (*c*) and (*d*) of young-adult (29-year-old) A1D3 fractions respectively. Samples of up to 30 mg (protein)/2 ml were applied to the columns and eluted with 4 M-GdmCl/0.05 M-sodium acetate buffer, pH 6.5. The flow rate was 6 ml/h, and 2 ml fractions were collected. The eluate was monitored at 280 nm. Sepharose CL-6B link-protein fractions were pooled (hatched area), concentrated, and applied to Sephacryl S-300 in the 4 M-GdmCl buffer described above. The eluate was monitored at 280 nm. The Sephacryl S-300 link-protein fractions were pooled (hatched area) and concentrated. The abscissa unit length is different for (*a*)+(*c*) and (*b*)+(*d*).

shown in Table 1. Under non-reducing conditions link protein 1 was the predominant link-protein species in postnatal disc compared with young adult disc and old-human articular cartilage: 63, 26.5 and 45% respectively. By contrast, link protein 3 was the minor component in postnatal disc tissues (11%) compared with young-adult disc (59.7%) and old human articular cartilage (37%). Under reducing conditions, the link-protein degradation peptides comprised 22.3% of the link-protein epitope of young-adult disc tissues, compared with 17% in the old-human articular-cartilage sample. Degradation peptides were virtually undetectable in the postnatal disc tissues (less than 1%). These data show that the young-adult disc contains almost twice as much link protein 3 and approximately the same percentage of degradation peptides as does the old-human articular cartilage (50–70-year-old tissue). These findings argue that, relative to old articular cartilage [38], the link proteins from the young-adult disc are either undergoing an accelerated rate of degeneration and/or that degraded link protein(s) are accumulating to a larger extent in the disc.

The functionality and size of the proteoglycans from postnatal and young-adult intervertebral-disc tissue was investigated. Exogenous hyaluronic acid was added to the proteoglycan A1 preparations (2%, w/w) to ensure complete aggregate formation of all functional proteoglycan monomers. These hyaluronic acid-supplemented proteoglycans were chromatographed on Sepharose CL-2B under associative conditions to separate aggregating from non-aggregating proteoglycans (Fig. 2). A greater proportion of aggregating proteoglycan was present in postnatal disc tissues (cartilage endplate, 55%; anulus fibrosus, 53%; nucleus pulposus, 53%), compared with young adult (41, 40 and 32% respectively) (Fig. 2). In addition, the included non-aggregating proteoglycan population of the young-adult tissues was smaller in size than the included proteoglycan population of the postnatal tissues (K_d values of 0.6 and 0.2 respectively). Postnatal tissue profiles (Figs. 2*a*, 2*c* and 2*e*) were very similar for all three disc tissues. Young-adult profiles (Figs. 2*b*, 2*d* and 2*f*), however, show tissue-specific differences in the proportion of non-aggregating proteoglycans, the nucleus pulposus having the largest proportion of these smaller non-aggregating proteoglycans.

SDS/PAGE analyses of the link-protein subpopu-

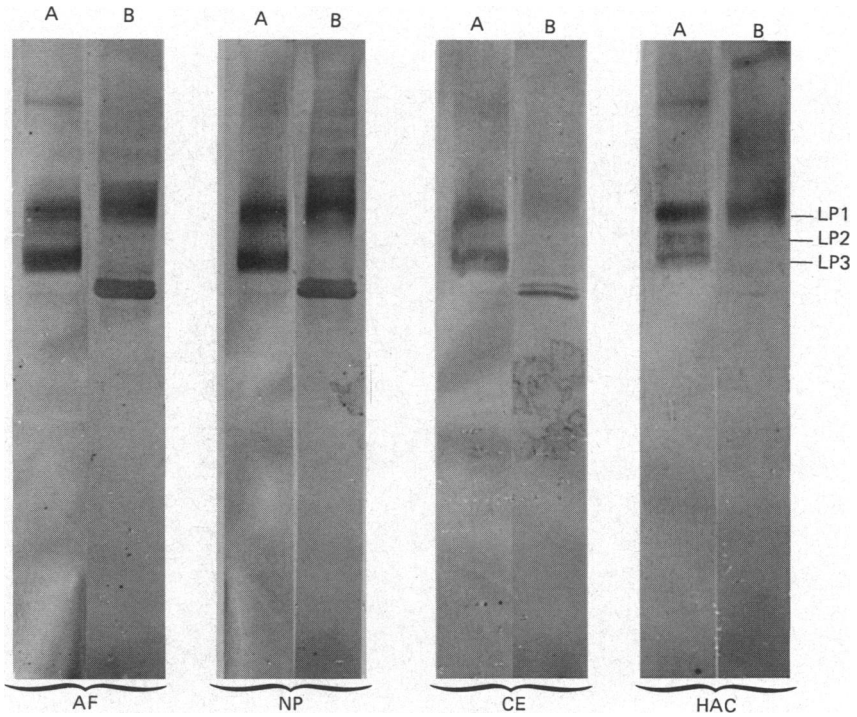


Fig. 4. Immunochemical analyses of young-adult disc Sephacryl S-300 samples

Unreduced SDS/12%-(w/v)-polyacrylamide-gels were electrophoretically transferred to nitrocellulose and immunolocated with either 9/30/8-A-4 (*A*) or a mixture of 12/21/1-C-6 and 6/1/7-D-1 (*B*). A1D3-6B/5-300 samples were from the anulus fibrosus (AF), nucleus pulposus (NP) or cartilage endplate (CE) of a 29-year-old and pooled old-human articular cartilage (HAC). For each tissue analysed, equal amounts of protein were loaded in *A* and *B*. Monoclonal antibody 9/30/8-A-4 detected an oligomer of link protein (M_r 79000) from all tissues analysed.

lations obtained from the proteoglycan aggregates of the corresponding tissues of postnatal and young-adult intervertebral discs are shown as insets in Fig. 2. A relatively higher proportion of link protein 3 is found in young-adult disc tissues that have the greatest proportion of non-aggregating proteoglycan. This result suggests that the relative abundance of link protein 3 degraded *in vivo* correlates with the increased proportion of non-aggregating proteoglycans present. Correspondingly, in postnatal tissues that contain fewer non-aggregating proteoglycans, link protein 3 and evidence for degradation peptides are less prevalent (Figs. 1 and 2).

Link protein(s) were purified from A1D3 fractions obtained from young-adult and postnatal disc tissues after sequential chromatography on Sepharose CL-6B and Sephacryl S-300 eluted with 4 M-GdmCl (Fig. 3). The Sepharose CL-6B elution profile obtained for postnatal disc tissues (Fig. 3*a*) was similar to that reported from rat chondrosarcoma and bovine nasal cartilage A1D3/4 fractions [39,40]. The presumptive link-protein peak (hatched area in Fig. 3*a*) was pooled and chromatographed on Sephacryl S-300 to purify further the link proteins from contaminating proteoglycan fragments. Young-adult disc A1D3 fractions were similarly chromatographed on Sepharose CL-6B and Sephacryl S-300 (Figs. 3*c* and 3*d* respectively). The Sepharose CL-6B elution profile indicated that there was a larger proportion of protein-rich proteoglycans present in the young-adult A1D3 fractions. Fractions corresponding to the approximate elution position for link proteins were pooled and chromatographed on Sephacryl S-300. However, this fractionation, or re-fractionation on Sephacryl S-300, did not satisfactorily resolve the link protein(s) from contaminating proteoglycan fragments.

SDS/PAGE of the young-adult disc A1D3-6B/5-300 preparations under non-reducing conditions, and subsequent Amido Black protein staining, indicated a complex protein pattern (results not shown). Under reducing conditions these preparations continued to exhibit a heterogeneous protein population. It should be noted that the link-protein degradation peptides which were detected using ^{125}I-labelled 9/30/8-A-4 monoclonal antibody (Fig. 1*b*) are not detected by the Amido-Black-staining procedure.

Immunolocation analyses under non-reducing conditions with either 9/30/8-A-4 (Fig. 4, lanes A) or a combination of two anti-proteoglycan antibodies, 6/1/7-D-1 and 12/21/1-C-6 (lanes B), was employed to identify and characterize further the complex protein pattern detected in the purified young-adult disc preparations. Link proteins 1, 2 and 3 are evident in all disc preparations [Fig. 4; lanes A of AF (anulus fibrosus), NP (nucleus pulposus) and CE (cartilage endplate)] and the old-human articular-cartilage (HAC) sample (lane A of HAC). By using the anti-proteoglycan antibody mix, a heterogeneous population of contaminating proteoglycan fragments was detected in all cases (lanes B of AF, NP, CE and HAC). These results show that the disc and articular-cartilage preparations both contain a range of proteoglycan fragments (M_r 44500–200000). Young-adult disc fractions also contain a lower-M_r doublet (M_r 29000 and 27500) that is not detected in the articular-cartilage fraction. This doublet of proteoglycan fragments was best resolved by using SDS/PAGE

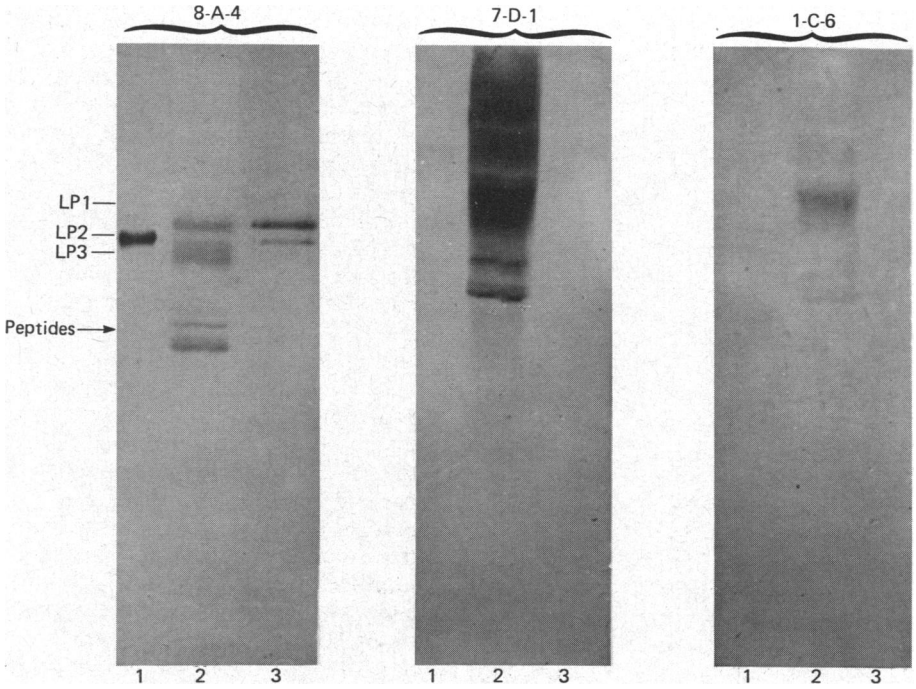


Fig. 5. Immunochemical analyses of Sephacryl S-300 purified young-adult and postnatal disc samples

Reducing SDS/12%-(w/v)-polyacrylamide gels were electrophoretically transferred to nitrocellulose and immunolocated separately with 9/30/8-A-4, 6/1/7-D-1 and 12/21/1-C-6. Lanes 1, 2 and 3 represent A1D3-6B/5-300 preparations of rat chondrosarcoma, young adult disc (29 years old) and postnatal disc (11 days old), respectively. The same amount of protein was loaded in identically labelled lanes. Abbreviation: LP, link protein.

under non-reducing conditions. These results suggest that alternate pathways of proteoglycan degradation (metabolism) occur in the intervertebral disc relative to articular cartilage.

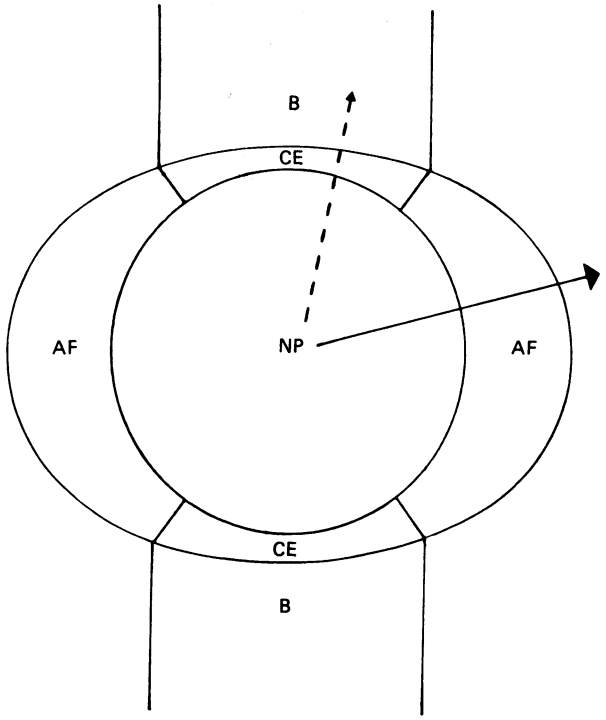
Further experiments were performed to determine and to characterize better the contaminating proteoglycan fragments in the purified young-adult disc link-protein preparations. After fractionation in SDS/polyacrylamide gels under reducing conditions, rat chondrosarcoma (Fig. 5, lane 1), young-adult disc (lane 2) and postnatal disc (lane 3) A1D3-6B/5-300 preparations were immunolocated separately by using 9/30/8-A-4, 12/1/1-C-6 and 6/1/7-D-1 monoclonal antibodies. Immunolocation with 9/30/8-A-4 indicates the presence of link proteins 1, 2 and 3 and degradation peptides (M_r range 24000–28500) from the young-adult disc link-protein preparation (lane 2). The postnatal disc link-protein preparation (lane 3) contains predominantly link protein 1 (48500) and some link protein 2, but no degradation peptides were detected. Similar findings were shown above in Fig. 1. For comparison, the rat chondrosarcoma preparation shows link protein 2 having an M_r of 45000 (lane 1).

As mentioned above (Fig. 4), immunolocation with 6/1/7-D-1 shows an extensive contamination of proteoglycan fragments (M_r range 29500–200000) in the young-adult disc link-protein preparation (lane 2), whereas no contaminating proteoglycan fragments were detected in the postnatal disc preparation (lane 3). Immunolocation with 12/1/1-C-6 indicates the presence of contaminating hyaluronic acid-binding region (M_r 53000) and smaller fragments (M_r approx. 30000) in the young-adult preparation (lane 2). However, no contaminating hyaluronic acid-binding region or hyaluronic acid-binding-region fragments were detected in the postnatal disc (lane 3) or rat chondrosarcoma (lane 1) link-protein samples prepared in a similar manner. The data would suggest, therefore, that the presence of protein-rich proteoglycans in the young-adult disc link-protein preparations correlates with normal tissue maturation.

General discussion

Immunolocation analyses with monoclonal antibody 9/30/8-A-4 indicated that, whereas link protein 1 is the major link-protein species in postnatal disc tissues, link protein 3 is the predominant species in young-adult tissues. Link protein 3, the smallest link protein, is thought to be a degradation product *in vivo* of link protein 1 or 2 [40]. Link protein 3 can also be generated by trypsin or clostripain treatment from either link-protein species [41]. Tengblad *et al.* [17], using a polyclonal antiserum, reported the existence of link proteins 1, 2 and 3 in human intervertebral-disc tissues. Monoclonal antibody 9/30/8-A-4 has been shown to recognize two peptide epitopes located on the *C*-terminal half of the link protein [42], and hence orients the molecule with respect to fragmentation. All parental link proteins (1, 2 and 3) contain two epitopes per molecule of link protein and thus facilitate better quantitative estimations of link protein(s) in immunolocation analysis.

Our results suggest that a subpopulation of the parental young-adult link protein (1, 2 and 3) has been proteolytically cleaved in the polypeptide backbone. Under non-reducing conditions, these cleaved link-protein fragments would remain intact (and functional),



Scheme 1. Potential diffusion pathways in the intervertebral disc

This Scheme illustrates the possible diffusion pathways in postnatal disc (continuous and broken arrows) or young-adult disc (continuous arrow). Anatomical regions are indicated as follows: AF, anulus fibrosus; B, bone; CE, cartilage endplate; NP, nucleus pulposus.

owing to the presence of intra-chain disulphide bridges. Reduction of the disulphide bonds reveals the occurrence of degradation peptides from this fragmented link-protein subpopulation. The immunochemical detection of these link-protein-degradation peptides (M_r 24500–27000) by 9/30/8-A-4 indicate that they are derived from the *C*-terminal half of the parental link protein. In addition, there is a smaller *N*-terminal link-protein peptide (M_r about 15000) that is not recognized by this monoclonal antibody and therefore was not detected. The sharpness of the bands representing the degradation peptides from young-adult disc link proteins indicates a degree of specificity in the cleavage site on the link proteins. Such specificity would most likely be due to the action of enzyme(s), although the generation of similar degradation peptides from intact human articular-cartilage link proteins has been accomplished with H_2O_2 [43].

We have shown that chromatographically purified young-adult disc link-protein preparations contain a heterogeneous population of proteoglycan fragments (hyaluronic acid-binding region as well as other regions of the proteoglycan molecule). This finding is consistent with the observation of several unidentified proteins reported by Tengblad and co-workers [17] in their disc-link-protein-enriched fractions. In addition, the absence of these proteoglycan fragments from postnatal disc tissues and their presence in young-adult disc tissues correlates well with a similar observation in human articular cartilage [44].

The present data indicate that the proteins in human intervertebral disc undergo considerable degradation at a young age. The evidence for this is: (1) the predominance of link protein 3 from young-adult tissues; by contrast, link protein 1 is the major link-protein species in postnatal tissues; (2) the occurrence of link-protein-degradation peptides from the disc tissues of the young adult; (3) the presence of proteoglycan fragments in the purified link-protein samples from young adult which are not detected in postnatal link-protein samples; and (4) the presence of greater proportions of both lower-buoyant-density proteoglycans in CsCl density gradients and non-aggregating proteoglycan after gel-permeation chromatography from young-adult disc tissues.

The increased degenerative state of the disc proteoglycan and link protein may be the result of an increased accumulation of catabolic products. Thus, in the disc, the highly collagenous anulus fibrosus may prevent the diffusion of partially degraded link protein and proteoglycan from the nucleus pulposus. At skeletal maturity, the closing of the growth plate limits the number of routes by which fragmented products could leave the tissue (Scheme 1). Similar conclusions were obtained by Oegema *et al.* [45] from their investigations of proteoglycan biosynthesis in human nucleus pulposus. These degraded components of the proteoglycan aggregate would form smaller and more heterogeneous aggregate structures, as has been shown by Buckwalter *et al.* [11]. Studies [46,47] have indicated that cartilage depleted of matrix proteoglycan responds by increasing biosynthesis *de novo* of proteoglycan. This synthesis is halted when the proteoglycan levels return to normal. In the disc tissues, accumulation of these catabolic products may prevent the chondrocytes from responding to feedback signals that stimulate the renewed synthesis of proteoglycan and link protein. The resulting situation may markedly influence the ability of the disc tissues to respond to everyday biomechanical stresses.

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