Identification and characterization of glycanated and non-glycanated forms of biglycan and decorin in the human intervertebral disc

Brian JOHNSTONE,*†, Maranthi MARKOPOULOS,* Peter NEAME† and Bruce CATERSON*

*Division of Orthopaedics, Department of Surgery, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, U.S.A., and †Shriners Hospital for Crippled Children, Tampa Unit, Tampa, FL 33612, U.S.A.

Immunological studies revealed the presence of several different forms of biglycan and decorin in human intervertebral-disc tissues (annulus fibrosus, nucleus pulposus and cartilage end-plate). In the young intervertebral disc, glycosaminoglycan-containing (glycanated) forms of both biglycan and decorin represented a greater proportion of the total proteoglycan population present in extracts of annulus fibrosus and cartilage end-plate compared with extracts of nucleus pulposus, in which they were barely detectable. In older discs the glycanated forms of biglycan and decorin represented only a small proportion of the total proteoglycan present. Immunochemical analyses with an antibody to chondroitin/dermatan sulphate isomers indicated differences in the glycosaminoglycans substituted on glycanated forms of small proteoglycans found in different disc tissues. Dermatan sulphate was the predominant glycosaminoglycan present on biglycan and decorin in annulus fibrosus extracts, whereas chondroitin 4-sulphate was present in both small proteoglycans isolated from cartilage end-plate. In addition, immuno-

chemical analyses with antibodies against core protein epitopes identified two non-glycanated forms of both biglycan and decorin. These non-glycanated forms of the small proteoglycans were found in all three regions of the disc. The two non-glycanated forms of biglycan had estimated molecular masses of 37 and 41 kDa and those of decorin were 43 and 45 kDa, respectively. These non-glycanated forms of biglycan and decorin increased in proportion with aging. N-terminal sequence analysis indicated that the larger non-glycanated form of decorin was a degradation product of its glycanated precursor. However, no N-terminal sequence information was obtainable from the other non-glycanated form of decorin or the two non-glycanated forms of biglycan. These data are consistent with the hypothesis that some of the non-glycanated forms of decorin and biglycan are degradation products of native precursors. However, the possibility remains that several different post-translationally modified forms of decorin and biglycan are synthesized by intervertebral-disc tissues.

INTRODUCTION

The proteoglycans of the intervertebral disc are poorly characterized. Although some work has been done on the large proteoglycans of disc tissues, much of their structure is inferred from work on the large proteoglycan of articular cartilage (aggrecan). Very little information has been published about the small proteoglycans of the disc. Biglycan and decorin are dermatan/chondroitin sulphate small proteoglycans that have been well characterized in many connective tissues (for review, see Stanescu, 1990). These small proteoglycans account for only a small proportion of the total mass of proteoglycans present in cartilaginous tissues; however, they may be present in similar molar proportions to the large aggregating proteoglycans (Roughley and White, 1989), and thus may be important in maintaining tissue function. Despite high structural similarity, it appears that biglycan and decorin have different functions. For example, in tendon, skin, sclera and cornea, decorin has been shown to be specifically associated with the *d* band of collagen fibrils (Scott, 1988), and the decorin from tendon has been shown to inhibit the rate of fibrillogenesis of type I and type II collagen *in vitro* (Vogel et al., 1984; Hedbom and Heinegård, 1989). As yet uncharacterized proteoglycans have been histochemically localized at the *d* band of collagen fibrils in the rabbit annulus fibrosus, suggesting that these proteoglycans may also have a role in controlling collagen fibril diameter in the disc (Scott and Haigh, 1986). More recently Bianco et al. (1990) detected decorin

mRNA in the annulus and nucleus of a 14–17-week-old human foetus, using hybridization *in situ*. The expressed protein was also found by immunolocalization. In contrast, no mRNA hybridization or immunolocalization of biglycan was observed.

In addition to the fully glycosylated forms of decorin and biglycan, there is evidence in the literature for forms of decorin that do not contain a glycosaminoglycan chain (Sampaio et al., 1988; Koob and Horoschak, 1990; Quentin et al., 1990; Fleischmajer et al., 1991), and in the case of biglycan contain only one glycosaminoglycan chain (Järveläinen et al., 1991). It has been proposed, but not proven, that the non-glycanated forms of decorin are degradation products of the glycanated precursors (Koob and Horoschak, 1990). The undersubstituted biglycan found in the medium of endothelial-cell cultures (Järveläinen et al., 1991) may be a cleavage product produced during the 22 h radiolabelling period or an artifact of culture *in vitro*. However, Neame et al. (1989) found evidence for undersubstituted biglycan when they performed amino acid sequence analysis of decorin purified from bovine articular cartilage.

The present paper describes the detection and characterization of glycanated and non-glycanated forms of decorin and biglycan in human intervertebral-disc tissues. The identification of these two small proteoglycans in the disc was determined by their characteristic mobilities on agarose/polyacrylamide-gel electrophoresis and their immunolocalization with specific antibodies that detect epitopes in their protein cores or glycosaminoglycan chains.

† To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Agarose (type HSC) was purchased from Park Scientific (Northampton, U.K.), and Acrylagel and Bis-acrylagel were from National Diagnostics (Manville, NJ, U.S.A.). Electrophoresis apparatus was purchased from Hoeffer Scientific Instruments (San Francisco, CA, U.S.A.) and the semi-dry electroblotter was from Bio-Rad (Richmond, CA, U.S.A.). Nitrocellulose (BA 83) was purchased from Schleicher and Schuell (Keene, NH, U.S.A.). Chondroitinase ABC and ACII were purchased from Seikagaku America Inc. (Rockville, MD, U.S.A.). Rabbit anti-(mouse IgG) antibody conjugated to alkaline phosphatase and the substrate (Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate) were purchased from Promega (Madison, WI, U.S.A.). Monoclonal anti-(rabbit IgG) antibody conjugated to alkaline phosphatase was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Extraction and fractionation of matrix components from disc tissues

Human lumbar spines were removed within 2–12 h *post mortem* and frozen at -20°C until processed for biochemical analysis. After thawing at 4°C overnight, intervertebral discs from spines of subjects aged 4, 15 and 32 years were dissected out and the annulus fibrosus, nucleus pulposus and cartilage end-plates were separated. All three tissues were finely diced and extracted in 4 M guanidine hydrochloride/0.05 M sodium acetate, pH 6.8, containing protease inhibitors (100 mM 6-aminocaproic acid, 10 mM EDTA, 5 mM benzamidine/HCl, 0.1 mM phenylmethanesulphonyl fluoride) at 4°C for 48 h. Extracts were dialysed into associative conditions and fractionated on CsCl density gradients with a starting density of 1.5 g/ml, at 100000 g_{av} for 36 h at 8°C . After centrifugation the extracts were separated into four fractions (A1–A4) and then dialysed into water. The uronic acid and protein contents of each fraction were determined with a Technicon Autoanalyzer by the methods described by Heinegård (1973).

Sample treatment and electrophoresis methods

Agarose/polyacrylamide gels were made by the method of Carney et al. (1986). Samples of fractions A1 to A3–4 were freeze-dried, dissolved into sample buffer (8 M urea, 0.25 mM Na_2SO_4 , 0.01 M Tris, pH 6.8, containing 0.002% Bromophenol Blue) and electrophoresed into the gel until the Bromophenol Blue had migrated 3 cm. Replicate gels were produced and then either stained with Toluidine Blue or electroblotted on to nitrocellulose by using a semi-dry electroblotter (in 25 mM Tris/0.192 M glycine, pH 8.3, at 5.5 mA/cm² for 30 min) and subjected to immunolocalization analysis (see below). SDS/PAGE utilized 4–20%-acrylamide gradient gels with conditions as described by Rosenberg et al. (1985). After electrophoresis the separated fractions were electroblotted on to nitrocellulose in a tank blotter (in 25 mM Tris/0.192 M glycine, pH 8.3, with 20% methanol at 1 A for 3 h with cooling to 4°C). Purified biglycan from bovine articular cartilage and decorin from bovine skin (generously provided by Dr. Larry Rosenberg) were run as standards for comparison. For some analyses samples were pretreated with 0.01 unit/ml of either chondroitinase ABC in 0.1 M Tris acetate, pH 7.8, or chondroitinase ACII in 0.1 M Tris acetate, pH 6.0, for 2 h.

Immunolocalization analysis

The antibodies used in this study were: monoclonal antibody 2-B-6, which recognizes a 4-sulphated *N*-acetylgalactosamine next to a $\Delta^{4,5}$ -unsaturated uronic acid [this epitope is generated by chondroitinase (ABC or ACII) digestion of chondroitin sulphate glycosaminoglycan chains attached to both large and small proteoglycans (Caterson et al., 1985)]; polyclonal antibodies LF15 and LF30, which recognize peptide sequences near the N-termini of human biglycan and decorin respectively (kindly provided by Dr. Larry Fisher, Bone Research Branch, NIH, Bethesda, MD, U.S.A.); and anti-human biglycan and anti-human decorin polyclonal antibodies, which recognize peptide sequences of the C-termini of human biglycan and decorin respectively (kindly provided by Dr. Peter Roughley, McGill University, Montreal, Canada).

Nitrocellulose blots of the electrophoretically separated fractions were blocked with 5% BSA in 10 mM Tris (pH 8.0)/150 mM NaCl/0.05% Tween (TBST) for at least 1 h. If required, the nitrocellulose sheets were then treated with 0.01 unit/ml chondroitinase ABC in 10 ml of 0.1 M Tris acetate, pH 7.8, containing 1% BSA for 1 h at 37°C . If pretreated with chondroitinase, the sheets were then washed briefly with TBST and then primary-antibody incubation was carried out in TBST containing 1% BSA for 1 h. This and all subsequent steps were carried out at room temperature. Monoclonal antibody 2-B-6 was used at 1/1000 dilution and polyclonal antibodies LF15 and LF30 were diluted 1/250. The blots were then washed for 3×10 min in TBST and incubated with either rabbit anti-(mouse IgG) antibody or monoclonal anti-(rabbit IgG) antibody conjugated to alkaline phosphatase at 1/7500 dilution for 1 h. After another rinse (3×10 min in TBST) the nitrocellulose sheets were blotted dry and bands were revealed by addition of alkaline phosphatase substrate (Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaCl/5 mM MgCl_2 /0.1 M Tris, pH 9.55) for colour development.

Purification of the non-glycanated small proteoglycans and N-terminal sequence analysis

In order to purify further the non-glycanated small proteoglycans before N-terminal sequencing, samples of A3–4 fractions were applied to a Q-Sepharose Fast Flow column (1 ml bed volume) equilibrated with 7 M urea/0.05 M sodium acetate, pH 7.5, containing 0.1 M NaCl, 0.05 M LiCl, 0.2% CHAPS, 2 mM benzamidine/HCl, 1 mM phenylmethanesulphonyl fluoride and 1 mM *N*-ethylmaleimide. The non-binding proteins were eluted with 3 vol. of the buffer, which were pooled, dialysed into 4 M guanidine/0.1 M Tris, pH 7.5, and subjected to chromatography on a Superose 12 column (HR 10/30). Fractions containing the non-glycanated proteoglycans were pooled, freeze-dried, dissolved in SDS/PAGE sample buffer and separated by SDS/PAGE on 10% gels. Western blots of the separated proteins were immunolocalized with either the LF15 (anti-human biglycan) or LF30 (anti-human decorin) antibodies. Replicate lanes, stained with Coomassie Blue, were used to cut out regions corresponding to the mobilities of the immunoblotted bands before N-terminal sequence analysis. This was carried out on an Applied Biosystems 477A sequencer with on-line analysis of amino acid phenylthiohydantoins on an Applied Biosystems 120A microbore h.p.l.c. The poly(vinylidene difluoride) membrane was placed in a Blott cartridge and was used with the manufacturers' recommended derivatization and cleavage conditions. Norleucine phenylthiohydantoin was used as an internal standard.

RESULTS

Agarose/PAGE of fractionated disc proteoglycans

Extracts of annulus fibrosus, nucleus pulposus and cartilage end-plate were fractionated by density-gradient centrifugation and analysed by separation on agarose/PAGE (Figure 1). Three electrophoretic subpopulations of large proteoglycans were apparent in the A1 fractions of young disc extracts stained with Toluidine Blue. Subpopulations with similar mobility to the faster bands seen in the A1 fraction were present in the A2 and

Figure 1 Agarose/PAGE of CsCl-density-gradient fractions from a 4-year-old disc tissue extract

Extracts of (i) annulus, (ii) nucleus and (iii) end-plate were fractionated on associative density gradients and then subjected to agarose/PAGE. (a) Toluidine Blue staining of the A1, A2 and A3-4 combined fractions; (b) immunolocalization of the fractions with monoclonal antibody 2-B-6 after Western blotting. The three unlabelled arrows mark the migration distances of the three electrophoretic subpopulations of large proteoglycans, and that labelled (S) marks that of the small proteoglycans biglycan and decorin. All samples were loaded at $2 \mu\text{g}$ of uronic acid/ $10 \mu\text{l}$ of sample buffer for Toluidine Blue staining, and at $0.5 \mu\text{g}/10 \mu\text{l}$ for immunolocalization.

Figure 2 Agarose/PAGE of low-buoyant-density (A3-4) CsCl-density-gradient fractions of disc tissue extracts from a 15- and a 32-year-old

Agarose/PAGE separated A3-4 combined fractions of (i) annulus, (ii) nucleus and (iii) end-plate were either (a) stained with Toluidine Blue or (b) immunolocalized with monoclonal antibody 2-B-6 after Western blotting as described in the Experimental section. Unlabelled arrows mark migration distance of large proteoglycan subpopulations, and that labelled (S) indicates the migration distance of small proteoglycans. Samples were loaded at $2 \mu\text{g}$ of uronic acid/ $10 \mu\text{l}$ of sample buffer for both Toluidine Blue staining and immunolocalization.

A3-4 fractions, but there was also a faster-migrating band with a mobility similar to that of biglycan and/or decorin in the young annulus and end-plate extracts (Figure 1a). Additional analysis of the different electrophoretic proteoglycan subpopulations was done by Western blotting them on to nitrocellulose and immunolocalization with monoclonal antibody 2-B-6 (anti-chondroitin 4-sulphate). Figure 1(b) shows the 2-B-6 immunolocalization analysis of proteoglycans present in CsCl-density-gradient fractions, indicating the presence of this epitope on large proteoglycan subpopulations in all fractions of the three disc tissues. However, the 2-B-6 epitope was only detectable on small proteoglycans (decorin or biglycan) present in the low-buoyant-density fractions from the annulus and end-plate extracts.

Agarose/PAGE separated A3-4 fractions of later ages (15- and 32-year-old shown in Figure 2) showed little detectable staining with Toluidine Blue at the expected migration position of the small proteoglycans. However, immunostaining with antibody 2-B-6 indicated that glycanated small proteoglycan was present in the annulus and cartilage end-plate samples. There was only weak staining of the large proteoglycan bands with antibody 2-B-6 in the annulus extracts of 4-, 15- and 32-year-old discs (Figures 1b and 2b). In contrast, strong 2-B-6 immunoreactivity was found with the large proteoglycans in 4- and 15-year-old nucleus extracts and at all ages in cartilage end-plate samples. These data indicate that the relative abundance of chondroitin 4-sulphate is varied in different proteoglycan subpopulations, between different disc tissues and with aging. Immunolocalization with monoclonal antibodies 3-B-3 (anti-chondroitin 6-sulphate) and 5-D-4 (anti-keratan sulphate) produced strong staining with the large proteoglycan subpopulations in all tissue at all ages (results not shown). These data demonstrate the occurrence of different patterns of glycosaminoglycan post-translational modification in different disc tissues.

Identification and characterization of small proteoglycans after SDS/PAGE

Samples of A3-4 fractions from the annulus and end-plate of a 4-year-old disc were separated on 4-20% gradient polyacrylamide gels under the conditions described by Rosenberg et al. (1985) that allow the self-association of biglycan, and thus its electrophoretic separation from decorin (Figure 3). Western blotting and immunolocalization with antibody 2-B-6 after chondroitinase ABC digestion confirmed the presence of proteoglycan subpopulations in the annulus and end-plate fractions with an electrophoretic mobility characteristic of biglycan and decorin. No such staining was evident in the nucleus fraction (results not shown). If the blots were digested with chondroitinase ACII before immunolocalization with antibody 2-B-6, no staining was seen in the annulus fraction, indicating that the small proteoglycans of the annulus contain dermatan sulphate glycosaminoglycan chains. In contrast, positive immunostaining was seen on the chondroitinase-ACII-digested blots from the end-plate small proteoglycans, indicating that chondroitin sulphate chains are substituted on biglycan and decorin from this tissue.

Detection of non-glycanated small proteoglycan core proteins in disc tissue extracts

Samples of the A3-4 fractions of annulus, nucleus and end-plate were separated by gradient gel electrophoresis and Western-blotted on to nitrocellulose as described above, then immunolocalized with either LF15 or LF30 polyclonal antibodies directed against protein core epitopes in biglycan and decorin respectively

Figure 3 Biochemical characterization of the glycosaminoglycan chains present on the small proteoglycans isolated from disc tissues

Immunolocalization of glycanated biglycan and decorin in extracts from a 4-year-old annulus (a, b) and cartilage end-plate (c, d) with monoclonal antibody 2-B-6 after pretreatment with either chondroitinase ABC (a, c) or chondroitinase ACII (b, d). Proteoglycan subpopulations were separated on 4–20% gradient SDS/polyacrylamide gels, Western-blotted on to nitrocellulose and pretreated with chondroitinase ABC or ACII before immunolocalization analysis. Lanes (e) and (f) are purified bovine articular-cartilage biglycan (B) and bovine skin decorin (D) respectively, digested with chondroitinase ABC before immunolocalization. The slower-migrating material in lane (f) is either a biglycan contaminant in the skin decorin preparation or aggregated decorin. The molecular masses (kDa) of protein standards are shown.

(Figure 4). These antibodies were raised against peptides with sequences beginning with the amino acid glycine that is C-terminal to the glycosaminoglycan-substituted serine in both molecules (the second glycosaminoglycan-substituted serine in biglycan). Neither antibody reacted well with the two glycanated proteoglycan subpopulations seen with 2-B-6 in Figure 3, indicating that the amino acid sequence epitopes for these antibodies, which are immediately adjacent to glycosaminoglycan attachment sites, were sterically blocked by glycosaminoglycan substitution on the protein core of these molecules. In addition, glycanated forms of biglycan and decorin are very heterogeneous, thus the epitopes for these antibodies are not concentrated into discrete bands and staining is diffuse. However, both antibodies reacted with non-glycanated proteins that migrate with electrophoretic mobilities similar to the core proteins of chondroitinase-treated biglycan and decorin. The LF15 epitope was located in two bands of estimated molecular masses 37 and 41 kDa (Figure 4a), and reactivity with LF30 was seen in bands of 43 and 45 kDa (Figure 4b) in extracts of both the 4- and 32-year-old discs. The proportions of these non-glycanated forms of biglycan and decorin increased with aging.

The occurrence of these faster-migrating forms of biglycan and decorin may have resulted from the removal of N- and possibly also C-terminal peptides in the catabolism of the glycanated forms. In order to test this hypothesis, these molecules were examined for their immunoreactivity with polyclonal antibodies raised against peptide epitope sequences from the C-termini of human biglycan and decorin (Figure 5). The peptide antigens to which these antibodies were raised have sequences beginning at the last cysteine of the molecules to the most C-terminal amino acid (P. Roughley, personal communication). The data shown in Figure 5 indicate that the C-terminal domains of decorin and biglycan were intact in these faster-migrating (non-glycanated) forms of these molecules.

N-terminal sequence analysis of the non-glycanated forms of biglycan and decorin

After further purification of the non-glycanated forms of biglycan and decorin, they were subjected to N-terminal amino acid

Figure 4 Detection of non-glycanated forms of biglycan and decorin in disc tissues

Immunolocalization with polyclonal antibodies (a) LF15 (anti-human biglycan core protein) and (b) LF30 (anti-human decorin core protein) of A3–4 combined fractions from the (a) annulus, (b) nucleus and (c) end-plate extracts of (i) 4-year-old and (ii) 32-year-old discs. Samples were separated on 4–20% gradient SDS/polyacrylamide gels and Western-blotted before immunolocalization. (1) and (2) mark the proteins specifically immunolocalized with LF15 and (1') and (2') the proteins specifically immunolocalized with LF30. Bands not marked are stained non-specifically with the secondary antibody. The molecular masses (kDa) of protein standards are shown.

sequence analysis in order to investigate whether the non-glycanated forms of biglycan and decorin resulted from N-terminal cleavages. Sequence information was only obtainable from the larger (45 kDa) non-glycanated form of decorin. No sequence could be obtained for either the 43 kDa non-glycanated form of decorin or the two non-glycanated forms of biglycan. The deduced sequence for the 45 kDa form of decorin was Xaa-Ile-Gly-Pro-Glu-Val-Pro, indicating that an N-terminal peptide had been cleaved from the glycanated form of decorin. This sequence, beginning with a glycine, is located immediately after the serine attachment point for the glycosaminoglycan chain (underlined) in the sequence of human articular cartilage decorin: Asp-Glu-Ala-Ser-Gly-Ile-Gly-Pro-Glu-Val-Pro ... (Roughley and White, 1989). The first residue of this sequence was difficult to determine. The first cycle of sequencing often has higher backgrounds, particularly from glycine. Subsequent cycles were readily interpreted within the limitations of Edman chemistry.

Figure 5 Immunolocalization of non-glycanated biglycan and decorin with polyclonal antibodies against peptide epitopes in the C-termini of their core proteins

Samples of the annulus A3–4 fraction of a 15-year-old disc extract were separated on a 4–20% SDS/polyacrylamide gel, Western-blotted and immunolocalized with polyclonal antibodies (a) anti-human biglycan and (b) anti-human decorin. The molecular masses (kDa) of protein standards are shown. Abbreviations B, D, 1, 2, 1', 2' are explained in the legends of Figures 3 and 4.

Sequence data were not obtained for the other non-glycanated form of decorin or those of biglycan, because of high backgrounds and/or multiple amino acids occurring with each sequence cycle. Further purification may eliminate these problems, but it is also possible that some or all of the other non-glycanated small proteoglycans have blocked N-termini.

DISCUSSION

Several different forms of the small proteoglycans biglycan and decorin were identified in A2 and A3–4 fractions of dissociatively extracted human intervertebral-disc tissues. These different forms fell into two categories: (i) glycosaminoglycan-containing (glycanated) forms and (ii) non-glycanated species. Biochemical and immunochemical characterization of these small proteoglycans revealed differences between their relative amounts and occurrence in the different disc tissues. The glycosaminoglycan chains linked to the glycanated forms of biglycan and decorin in the annulus fibrosus appear to contain dermatan sulphate, as shown by their resistance to digestion with chondroitinase ACII. The presence of dermatan sulphate in intervertebral-disc proteoglycans is not consistent within the published literature. Dermatan sulphate has been detected in dog discs (approx. 2–8 %, depending on region), but the proteoglycan species to which it was attached was not identified (Cole et al., 1985). Scott and Haigh (1986) described the small proteoglycan that they detected histochemically in the rabbit annulus as containing chondroitin sulphate. Inerot and Axelsson (1991) reported that dermatan sulphate was not present (represented less than 1 % of the total glycosaminoglycan) in proteoglycans isolated from human discs from a single 69-year-old spine, which was in agreement with unpublished observations reported in a review of disc biochemistry by Eyre (1979). Our results indicate that dermatan sulphate-containing small proteoglycans are pres-

ent in the annulus fibrosus of young human intervertebral discs and that there is an age-related decrease in the glycanated forms of both biglycan and decorin. Therefore it is not surprising that others have found that the dermatan sulphate content of proteoglycans from old human discs is very low.

The constitution of extracellular matrix of the intervertebral-disc cartilage end-plate has been presumed to be like that articular cartilage (Eyre, 1979; Ayad and Weiss, 1987). However, morphological studies have reported structural differences between the two tissues, e.g. the collagen fibres of the end-plate join the annulus fibrosus, and it can be seen that the cell arrangement is very different from that of articular cartilage in that no change in cell size, shape or formation into columns is evident towards the bone (Roberts et al., 1989). Furthermore, biochemical studies indicate that both the large proteoglycans (Bishop and Pearce, 1988; Jahnke and Caterson, 1986) and the collagens (Roberts et al., 1991) are different from those of articular cartilage. Dermatan sulphate-containing small proteoglycans are the prevalent form of decorin and biglycan found in articular cartilage (Rosenberg et al., 1985). However, data in the present study indicate that those of the disc cartilage end-plate contain only chondroitin sulphate. Collectively these data emphasize that the cartilage end-plate should be considered a separate connective tissue and not described as articular cartilage in either its biochemistry or morphology.

Toluidine Blue staining indicated that the glycanated forms of biglycan and decorin decreased as a proportion of the total proteoglycan content with increasing age. In addition, two non-glycanated forms of both biglycan (37 and 41 kDa) and decorin (43 and 45 kDa) were found in human intervertebral-disc tissues. The non-glycanated forms of both biglycan and decorin increased in proportion with aging. Taken together these data suggest that there may be a conversion of the glycanated forms into the non-glycanated forms. Amino acid sequence analysis indicated that catabolic cleavage of native decorin was responsible for the generation of one of the two non-glycanated forms of decorin found in disc extracts. The occurrence of the 45 kDa form of decorin involved cleavage of the N-terminal region of the glycanated decorin between the serine (residue 4) that is the glycosaminoglycan attachment site and glycine (residue 5). We were unable to ascertain whether or not the other non-glycanated forms of decorin and biglycan in intervertebral-disc tissue were also catabolic products from their glycanated precursors.

Non-glycanated forms of decorin have been detected in extracts of human and pig articular cartilage (Sampaio et al., 1988) and adult human skin (Fleischmajer et al., 1991). Koob and Horoschak (1990) also found a non-glycanated form of decorin in several bovine connective tissues and suggested that this was a degradation product of the glycanated form. They also reported a second minor immunoreactive non-glycanated form, but did not characterize it further. A survey of the literature reveals that these non-glycanated forms may be present in many (or all) tissues. However, their presence has either been missed or overlooked, because most researchers use an ion-exchange-chromatography step in their purification procedures to separate biglycan and decorin (containing polyanionic glycosaminoglycans) from other non-glycanated proteins. Non-glycanated forms of small proteoglycans would be missed by using these isolation procedures. Our data confirm the hypothesis of Koob and Horoschak (1990) that the major non-glycanated form of decorin is a degradation product. It is of interest that the site of cleavage is the glycosaminoglycan attachment region, since this would be expected to be a sterically hindered site for proteolytic cleavage.

The results of histochemical (Scott and Orford, 1981; Orford

and Gardner, 1984; Meek et al., 1985; Scott and Haigh, 1986; Scott, 1988) and immunohistochemical studies (Fleischmajer et al., 1991) led to the proposal that small proteoglycans interact with fibrillar collagens. Studies *in vitro* support this hypothesis (Vogel and Trotter, 1987; Brown and Vogel, 1989; Koob and Vogel, 1989; Scott et al., 1989). Vogel and Trotter (1987) reported that bovine tendon decorin decreased the average diameter of collagen fibrils formed *in vitro*. However, decorin from uterine cervix (Uldbjerg and Danielsen, 1988) and skin (Scott et al., 1989) seemed to have little effect on fibril diameter. The decorin from skin produced different effects to that of the temporomandibular disc in a fibrillogenesis assay (Scott et al., 1989). Koob and Horoschak (1990) found that both glycanated and non-glycanated forms of decorin were extractable under the same conditions, indicating that they were both associated with collagen fibrils, a plausible finding since the glycosaminoglycan chain is not required for collagen binding (Vogel et al., 1984, 1987; Uldbjerg and Danielsen, 1988; Brown and Vogel, 1989). Collectively, these results suggest that functional differences may be related to the distinct glycosylation patterns of the decorin from different tissues. If this is so, then it may be that the removal of the glycosaminoglycan-chain-bearing portion of decorin will alter its function in collagen organization and thereby alter the physiological function of the tissue. The occurrence of decorin in the highly organized matrix of the annulus fibrosus is consistent with the notion that this proteoglycan is involved in the regulation of collagen organization. The finding of little detectable glycanated or non-glycanated decorin in the nucleus pulposus is accordant with this idea, since the nucleus contains a random array of collagen fibrils. However, since the nucleus contains much less collagen than the annulus (Eyre, 1979), it may also have proportionately less decorin. Thus the molar ratio of decorin to collagen may be similar to that for the annulus.

It still remains to be determined whether or not the two non-glycanated forms of biglycan are degradation products or are synthesized as such. There is some evidence supporting the notion of the synthesis of undersubstituted forms of biglycan: during N-terminal peptide sequencing of biglycan isolated from bovine articular cartilage, Neame et al. (1989) found that approx. 10% of the serine at both of the glycosaminoglycan attachment positions was unsubstituted, indicating that biglycan could exist in a form that has only one glycosaminoglycan chain. Furthermore, Järveläinen et al. (1991) immunoprecipitated two populations of glycanated biglycan from cultures of bovine aortic and human umbilical-vein endothelial cells. One of these had the mobility predicted for biglycan with a single glycosaminoglycan chain. Thus it is possible that a non-glycanated form of biglycan could also be produced. Non-glycanated forms of other proteoglycans have been found, e.g. variants of CD44 (Brown et al., 1991). Of interest is the finding that a non-glycanated form of decorin has been isolated from a patient with a progeroid syndrome (Quentin et al., 1990),

although this was found to be caused by a deficiency in galactosyltransferase I, which is involved in the second glycosyl transfer reaction in the assembly of the dermatan sulphate chain.

We thank Dr. Larry Rosenberg for providing the purified biglycan and decorin and Dr. Larry Fisher and Dr. Peter Roughley for providing the polyclonal antibodies described in the text. This work was supported by a Fellowship grant from the Arthritis Foundation (to B.J.) and NIH grants AR32666 and AR40364. We also thank Barbara Nail for typing the manuscript.

REFERENCES

- Ayad, S. and Weiss, J. B. (1987) in *The Lumbar Spine and Back Pain* (Jayson, M. I. V., ed.), pp. 100–137. Churchill–Livingstone, Edinburgh.
- Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D. and Gheron Robey, P. (1990) *J. Histochem. Cytochem.* **38**, 1549–1563.
- Bishop, P. B. and Pearce, R. H. (1988) *Trans. Orthop. Res. Soc.* **13**, 152.
- Brown, D. C. and Vogel, K. G. (1989) *Matrix* **9**, 468–478.
- Brown, T. A., Bouchard, T., St. John, T., Wayner, E. and Carter, W. G. (1991) *J. Cell Biol.* **113**, 207–221.
- Carney, S. L., Bayliss, M. T., Collier, J. M. and Muir, H. (1986) *Anal. Biochem.* **156**, 38–44.
- Caterson, B. C., Christner, J. E., Baker, J. R. and Couchman, J. R. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 386–393.
- Cole, T.-C., Burkhardt, D., Frost, L. and Ghosh, P. (1985) *Biochim. Biophys. Acta* **839**, 127–138.
- Eyre, D. R. (1979) *Int. Rev. Connect. Tissue Res.* **8**, 227–291.
- Fleischmajer, R., Fisher, L. W., MacDonald, E. D., Jacobs, L., Perlisch, J. S. and Termine, J. D. (1991) *J. Struct. Biol.* **106**, 82–90.
- Hedborn, E. and Heinegård, D. (1989) *J. Biol. Chem.* **264**, 6898–6905.
- Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201.
- Inerot, S. and Axelsson, I. (1991) *Connect. Tissue Res.* **26**, 47–63.
- Jahnke, M. R. and Caterson, B. C. (1986) *Trans. Orthop. Res. Soc.* **11**, 419.
- Järveläinen, H. J., Kinsella, M. G., Wight, T. N. and Sandell, L. J. (1991) *J. Biol. Chem.* **266**, 23274–23281.
- Koob, T. J. and Horoschak, S. A. (1990) *Trans. Orthop. Res. Soc.* **15**, 38.
- Koob, T. J. and Vogel, K. G. (1989) *Trans. Orthop. Res. Soc.* **14**, 36.
- Meek, K. M., Scott, J. E. and Nave, C. (1985) *J. Microsc. (Oxford)* **139**, 205–219.
- Neame, P. J., Choi, H. U. and Rosenberg, L. C. (1989) *J. Biol. Chem.* **264**, 8653–8661.
- Orford, C. R. and Gardner, D. L. (1984) *Connect. Tissue Res.* **12**, 345–348.
- Quentin, E., Gladen, A., Rodén, L. and Kresse, H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1342–1346.
- Roberts, S., Menage, J. and Urban, J. P. G. (1989) *Spine* **14**, 166–174.
- Roberts, S., Menage, J., Duance, V., Wotton, S. and Ayad, S. (1991) *Spine* **16**, 1030–1038.
- Rosenberg, L. C., Choi, H. U., Tang, L.-H., Johnson, T. L., Pal, S., Webber, C., Reiner, A. and Poole, A. R. (1985) *J. Biol. Chem.* **260**, 6304–6313.
- Roughley, P. R. and White, R. J. (1989) *Biochem. J.* **262**, 823–827.
- Sampaio, L. O., Bayliss, M. T., Hardingham, T. E. and Muir, H. (1988) *Biochem. J.* **254**, 757–764.
- Scott, J. E. (1988) *Biochem. J.* **252**, 313–323.
- Scott, J. E. and Haigh, M. (1986) *Biosci. Rep.* **6**, 879–888.
- Scott, J. E. and Orford, C. R. (1981) *Biochem. J.* **197**, 213–216.
- Scott, J. E., Nakano, T., Dodd, C. M., Pringle, G. A. and Kuc, I. M. (1989) *Matrix* **9**, 284–292.
- Stanescu, V. (1990) *Semin. Arthritis Rheum.* **20** (Suppl. 1), 51–64.
- Uldbjerg, N. and Danielsen, C. C. (1988) *Biochem. J.* **251**, 643–648.
- Vogel, K. G. and Trotter, J. A. (1987) *Collagen Relat. Res.* **7**, 105–114.
- Vogel, K. G., Paulsson, M. and Heinegård, D. (1984) *Biochem. J.* **223**, 587–597.
- Vogel, K. G., Koob, T. J. and Fisher, L. W. (1987) *Biochem. Biophys. Res. Commun.* **148**, 658–663.