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A comparative analysis of the differential spatial and temporal distributions of the large (aggrecan, versican) and small (decorin, biglycan, fibromodulin) proteoglycans of the intervertebral disc

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ABSTRACT

This study provides a comparative analysis of the temporal and spatial distribution of 5 intervertebral disc (IVD) proteoglycans (PGs) in sheep. The main PGs in the 2 and 10 y old sheep groups were polydisperse chondroitin sulphate and keratan sulphate substituted species. Their proportions did not differ markedly either with spinal level or disc zone. In contrast, the fetal discs contained 2 slow migrating (by composite agarose polyacrylamide gel electrophoresis, CAPAGE), relatively monodisperse chondroitin sulphate-rich aggrecan species which were also identified by monoclonal antibody 7-D-4 to an atypical chondroitin sulphate isomer presentation previously found in chick limb bud, and shark cartilage. The main small PG detectable in the fetal discs was biglycan, whereas decorin predominated in the 2 and 10 y old IVD samples; its levels were highest in the outer annulus fibrosus (AF). Versican was most abundant in the AF of the fetal sheep group; it was significantly less abundant in the 2 and 10 y old groups. Furthermore, versican was immunolocalised between adjacent layers of annular lamellae suggesting that it may have some role in the provision of the viscoelastic properties to this tissue. Versican was also diffusely distributed throughout the nucleus pulposus of fetal IVDs, and its levels were significantly lower in adult IVD specimens. This is the first study to identify versican in ovine IVD tissue sections and confirmed an earlier study which demonstrated that ovine IVD cells synthesised versican in culture (Melrose et al. 2000). The variable distribution of the PGs identified in this study provides further evidence of differences in phenotypic expression of IVD cell populations during growth and development and further demonstrates the complexity of the PGs in this heterogeneous but intricately organised connective tissue.

Key words: Intervertebral disc; proteoglycan heterogeneity; ageing/disc degeneration; 7-D-4 PG epitope; fibromodulin; versican; decorin/biglycan; aggrecan.

INTRODUCTION

The intervertebral disc (IVD) provides flexibility and mechanical stability to the spine during axial compression, flexion and extension (Eyre, 1979; Ghosh, 1990; Buckwalter, 1995). It consists of several specialised connective tissues. These include the hyaline-like cartilage of the cartilaginous end plates which cover the surface of the vertebral bodies; the fibrocartilage of the annulus fibrosus (AF), and the central gelatinous nucleus pulposus (NP) (Eyre, 1979; Ghosh, 1990; Buckwalter, 1995; Urban & Roberts, 1995). Proteoglycans (PGs) and types I, II, III, V, VI, IX, X, XI collagen are variably distributed in these 3 tissue types (Eyre, 1979; Wu et al. 1987; Roberts et al. 1991; Urban & Roberts, 1995; Boos et al. 1997; Nerlich et al. 1997, 1998; Aigner et al. 1998). The disc PGs imbibe water which generates a hydrostatic pressure within the NP and this contributes to the hydrodynamic and viscoelastic properties of the intact structure (Eyre, 1979; Pearce et al. 1987; Ghosh, 1990; Buckwalter, 1995; Urban & Roberts, 1995).

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The IVD undergoes marked compositional changes during development and ageing (Eyre, 1979; Pearce et al. 1987; Ghosh, 1990; Pearce, 1992; Buckwalter, 1995; Antoniou et al. 1996*a*, *b*; Melrose et al. 1997*a*, *b*, 1998). Degeneration of the IVD leads to spondylosis and other spinal disorders which, collectively, are a major cause of morbidity in Western societies (Ghosh, 1990; Pearce, 1992). Mechanical and genetic determinants significantly influence the rate at which degenerative changes occur within the disc (Ghosh, 1990). The degradation and loss of disc aggrecan PGs is considered central to the process of disc degeneration (Pearce et al. 1987; Pearce, 1992; Antoniou et al. 1996*a*, *b*).

While previous studies have identified the qualitative changes which take place in the IVD with ageing and degeneration (Eyre, 1979; Pearce et al. 1987; Ghosh, 1990; Melrose et al. 1992, 1997*a*, *b*, 1998; Pearce, 1992; Buckwalter, 1995; Urban & Roberts, 1995; Antoniou et al. 1996*a*, *b*) there has been limited consideration of the variation of these PG types collectively in different disc regions and spinal levels as a function of ageing. Using sheep as our experimental model we undertook the present study to address this deficiency.

MATERIALS AND METHODS

Guanidine hydrochloride, sodium acetate, Tris (free-base), Tween 20, bovine serum albumin, toluidine blue, avidin alkaline phosphatase conjugate, benzamidine and PMSF were obtained from the Sigma Chemical Co., MO, USA. Stabilised polyacrylamide-bis acrylamide concentrate (40% w/v, 19:1, C = 5%), agarose (low electroendosmosis grade), high purity electrophoresis grade urea, semidry blotting pads, NBT (nitroblue tetrazolium) /BCIP (5-bromo-4-chloro-3-indolyl phosphate) alkaline phosphatase immunoblotting substrate kits and alkaline phosphatase conjugated secondary antibodies were purchased from Bio-rad, N. Ryde, NSW, Australia. Extractigel D affinity columns for the removal of detergents from samples were products of Pierce Chemical Co., RO, USA. Heparitinase and protease free chondroitinase ABC were purchased from Seikegaku Corp., Tokyo, Japan. New Fuchsin and secondary antibodies for immunohistology were purchased from DAKO Australia, Sydney, Australia. Nitrocellulose-extra blotting membranes were purchased from Sartorius AG, Gottingen, Germany. Precast 4–12% polyacrylamide gradient gels and See-blue pre-stained standard proteins were purchased from Novex, San Diego, CA, USA. Monoclonal antibodies to decorin core protein, (clone 6-B-6) (Sobue et al. 1988) were purchased from the Seikagaku Corp., Tokyo, Japan. Antibody LF-96 (antibovine biglycan core protein) was kindly provided by Dr L. Fisher (Fisher et al. 1995), National Institute of Dental Research, National Institutes of Health, Bethesda, MD, USA. Antibodies to versican, fibromodulin and biglycan (Heinegård et al. 1985*b*) were also provided by Prof. D. Heinegård, University of Lund, Sweden. Monoclonal antibody 7-D-4 (Visco et al. 1993) was a gift from Prof. B. Caterson, University of Wales, Cardiff, UK. Specific details of the antibodies used are provided in the Table.

Dissection and extraction of disc tissues

The sheep utilised in this study were pedigree merinos and were obtained from our breeding colony held at our University animal holding facilities, May Farm,

Table. *Reagents used to identify intervertebral disc proteoglycan epitopes by electroblotting*

MAb clone	Antibody class	Dilution used	Epitope(s) recognised	+/− pre-digestion of blot with chondroitinase ABC
5-D-4	IgG 1κ	1/10000	Keratan sulphate I/II	−
3-B-3(+)	IgMκ	1/2000	Δdi-6-S	+
2-B-6(+)	IgG	1/2000	Δdi-4-S	+
6-B-6	IgG1	1/1000	Bovine decorin core protein	+
69, 830916	Rabbit anti-biglycan	1/250	Bovine biglycan core protein	+
A1S1D1D1	Rabbit anti-versican	1/250	Bovine versican core protein	+
74, 831115	Rabbit anti-fibromodulin	1/500	Bovine fibromodulin core protein	−
Biotinylated hyaluronan	Not applicable	2 μg/ml	G1 domain of aggrecan, versican, CD-44, link protein	−
LF-96	Affinity purified rabbit polyclonal	1/250	A specific bovine biglycan core protein peptide sequence LPDLDSPPTYSC	+
7-D-4	IgM	1/1000	An atypical CS isomer present in PGs extracted from limb bud, epiphyseal growth plate and osteoarthritic cartilages	−

Camden (except where indicated). All procedures used in this study received institutional approval from our University Animal Research Ethics Committee. Sheep were euthanised using an overdose of pentobarbitone and lumbar spines removed within 30 min of death. Lumbar IVDs (T13L1 to L7S1) from fetal merinos (1 wk preterm) obtained from our local abbatoir, or 2 y and 10 y old merino wethers were dissected into the inner two thirds and outer one third of the AF, the NP in entirety was sampled separately. Due to the small size of the fetal discs a dissecting microscope was used for these procedures, and in this case disc tissues from 3 animals were pooled to provide sufficient material. Disc tissues were finely diced with scalpels over ice and extracted for 48 h at 4 °C with 4 M GuHCl (10 vol/g tissue) buffered with 100 mM sodium acetate pH 5.8 containing a mixture of proteinase inhibitors (Melrose et al. 1994). The extracted PGs were separated from tissue residue by centrifugation (20 min × 1000 g). Hexuronic acid analyses (Bitter & Muir, 1992) were undertaken of the disc extracts to provide a measure of their PG contents.

Composite polyacrylamide agarose gel electrophoresis (CAPAGE) of IVD proteoglycans

The GuHCl extracted PGs were precipitated with 3 volumes of absolute ethanol, collected by centrifugation (10 min × 800 g), the pellet washed twice with 75% (v/v) ethanol and air-dried. This PG pellet was redissolved overnight in 8 M urea 10 mM Tris-acetate pH 6.3 0.25 mM sodium sulphate and diluted 1:1 with 20 mM Tris-acetate buffer pH 6.3 containing 1 mM sodium sulphate, 60% (w/v) sucrose and 0.01% (w/v) bromophenol blue (CAPAGE sample buffer) immediately prior to CAPAGE (Heinegård et al. 1985*b*; Melrose et al. 1998). PG samples, 0.1–1.0 μg hexuronic acid/10 μl/well (Bitter & Muir, 1992) were electrophoresed at 150 V until the bromophenol blue dye had migrated 2–3 cm. Ovine articular cartilage PG samples (Melrose et al. 1998) were run as reference standards to determine the migration positions of the 2 major aggrecan 1 and 2 populations as well as the more mobile DS substituted PGs (Heinegård et al. 1985*b*). The gels were stained in 0.02% (w/v) toluidine blue in 0.1 M acetic acid for 1 h and destained in 0.5 M acetic acid or were transferred to nitrocellulose membranes by semidry blotting (see below).

SDS PAGE

The 4 M GuHCl PG extracts of the disc tissues were dialysed to remove the guanidine and protease inhibitors of the extraction buffer, then freeze dried. Aliquots (30 μg dry weight/lane/disc zone) of these crude samples were electrophoresed on 4–12% gradient SDS PAGE gels at 125 V for 90 min in 25 mM Tris-192 mM glycine 0.1% SDS buffer pH 8.3. The gels were then transferred to nitrocellulose membranes as indicated below.

Immunoblotting

The CAPAGE gels were electroblotted to nitrocellulose membranes in 25 mM Tris 192 mM glycine transfer buffer pH 8.3 in a Bio-rad transblot semi-dry transfer cell at 5.5 mA/cm^2 constant current for 30 min (Melrose et al. 1998), SDS PAGE gels were transferred to nitrocellulose using a Novex transblot module using 12 mM Tris 96 mM glycine transfer buffer pH 8.2 containing 20% (v/v) methanol at 200 mA constant current for 2 h at 4 °C (Melrose et al. 1997*a*). The blots were blocked 3 h in 4% (w/v) BSA in 50 mM Tris-HCl pH 7.4, 0.5 M NaCl 0.02% (w/v) sodium azide (TBS). Antibodies (see Table 1 for details) were applied in the same buffer (minus BSA) containing 0.05% Tween-20 and allowed to bind for 1 h. The blots were then washed with 20 mM Tris-HCl pH 7.2, 500 mM NaCl, 0.05% (v/v) Tween-20 (TBS-Tween) and then incubated with an appropriate alkaline phosphatase conjugated secondary antibody for 1 h. After washing as above, a solution of NBT/BCIP in alkaline phosphatase assay buffer was added for the visualisation step. Some blots were blocked overnight with 0.1% (v/v) Tween-20 in TBS, then probed with biotinylated hyaluronan as indicated earlier (Melrose et al. 1998).

Purification of ovine IVD decorin and biglycan and bovine smooth muscle cell versican

Ovine disc decorin and biglycan (Melrose et al. 1998) were isolated as outlined earlier (Melrose et al. 1994) and subjected to preparative electrophoresis on 2.5 mm thick 4–20% gradient SDS PAGE gels. Decorin and biglycan were electroeluted from the gel segments in 25 mM Tris-192 mM glycine buffer pH 8.4 containing 0.1% SDS at 2.5 mA per trap for 2 h in a 'Little blue tank' electroelution device (Isco, Lincoln, USA) (Allington et al. 1978; Bhown et al. 1980), residual SDS was removed by Extractigel affinity chromatography and the samples dialysed against distilled water and freeze dried. Versican was isolated from bovine smooth muscle cell culture media by DEAE sepharose anion exchange chromatography in 50 mM Tris-HCl 0.12 M NaCl pH 7.4, the bound PGs

were eluted with 4 M GuHCl, and the buffer exchanged for 50 mM HEPES pH 7.0 containing 100 mM NaCl and 1 mM $CaCl_2$ by diafiltration over a 200 kDa membrane. Heparitinase (10 mU/mg sulphated glycosaminoglycan), benzamidine (1 mM) and PMSF (2 mM) were then added and the samples were digested overnight at 37 °C then dialysed and freeze dried. Deglycosylated core proteins of versican, decorin and biglycan were prepared by overnight digestion at 37 °C with an excess of protease free chondroitinase ABC (0.02 U/mg dry weight PG) in 20 mM Tris-acetate buffer pH 7.4 containing 2% BSA.

Immunohistochemistry

All soft tissue extraneous to the IVD and the posterior bony elements were removed from lumbar spinal segments and the IVDs of interest isolated by cutting through the superior and inferior vertebral bodies using a bone saw. Entire IVD specimens encompassed by adjacent vertebral body portions were fixed en bloc for 56 h in 10% neutral buffered formalin then decalcified in several changes of 10% formic acid in 5% neutral buffered formalin for 2 wk with constant agitation. The specimens were then dehydrated through graded alcohols by standard histological methods. IVD tissue slices (5 mm thick) were cut and embedded in paraffin wax. Vertical sagittal microtome sections (5 μm) of newborn (2 d old) and 2 and 10 y IVD specimens were cut and mounted on star frost glass slides; these were dried at 75 °C for 10 min followed by 55 °C in an oven overnight. The sections were deparaffinised by washing in xylene (4 changes × 2 min), and rehydrated through graded ethanol washes (100–70% v/v) to water. The immunohistochemistry procedures were performed using a Sequenza cassette and disposable Coverplate immunostaining system (Immunon, Shandon Lipshaw, FSE Pty, Homebush, NSW, Australia). This system permits uniform distribution of fluid over the tissue section and helps to preserve precious antisera since only 100 μl of fluid is required to cover the slide. The sections were predigested with chondroitinase ABC (0.25 U/ml) in 20 mM Tris-acetate buffer pH 8.0 for 1 h at 37 °C then blocked for 1 h in 10% normal swine serum and probed with a rabbit antibovine scleral versican antibody (1/100 diln) for 1 h at 37 °C. Negative control tissue sections were similarly processed, using an irrelevant primary antibody of the same isoptype as the authentic primary antibody. Alkaline phosphatase conjugated goat antirabbit IgG secondary antibodies were used for the colour visualisation step using New fuchsin as chromogen; haematoxylin was used as counterstain. The stained slides were examined by light microscopy and photographed using a Leica MPS 60 photomicroscope system.

RESULTS

Proteoglycans were extracted with GuHCl from each of the tissue zones (outer and inner AF, and NP) of each of the lumbar IVDs (T13L1 to L7S1) of spines from each of the age groups. Preliminary experiments examined these PG samples by CAPAGE employing staining with toluidine blue. Qualitatively similar results were obtained for corresponding tissue zones at each IVD spinal level within each spine (data not shown). Due to the difficulty of electrophoretically processing such a large number of samples (120 per spine), and the limited availability of some of our antibodies, a decision was made to use the PG samples of the L5L6 IVD as representative samples for all subsequent experiments.

Examination of the L5L6 IVD PGs by CAPAGE demonstrated significant differences between the fetal, and the 2 and 10 y old sheep samples. The fetal PGs resolved on CAPAGE as relatively monodisperse species with a mobility similar to that of the aggrecan-1 (Agg-1, Fig. 1 *A*, lane 1) population of ovine articular cartilage (Fig. 1 *A*, lanes 2–4). A further fetal PG species of higher electrophoretic mobility, similar to that of the DS-PGs decorin and biglycan of articular cartilage, was also evident (Fig. 1 *A*, lanes 2–4). In contrast, the other samples contained a high proportion of the Agg-2 population; this population became progressively more polydisperse in the adult 2 and 10 y old IVD PG samples (Fig. 1 *A*, lanes 5–7, 8–10). Further examination of the disc PGs by immunoblotting using the anti-KS monoclonal antibody (MAb 5-D-4) confirmed that the Agg-1 and 2 bands were KS-rich PG species (Fig. 1 *B*). Again, the polydispersity of the Agg-2 PG species was highest in the 10 y old sheep (Fig. 1 *B*, lanes 8–10). All PG samples were loaded on the gels on the basis of their uronic acid contents (a CS marker); thus a significant increase in the relative KS content of IVD PGs compared with CS was also evident in the adult PGs compared with the fetal PG samples (Fig. 1 *B*, compare lanes 2–4 with 5–10). The fetal sheep contained an additional KS substituted aggrecan species which was less mobile than the Agg-1 PG band of ovine AC (Fig. 1 *B*, lanes 2,3 arrow with asterisk), and was also substituted with chondroitin-4-sulphate

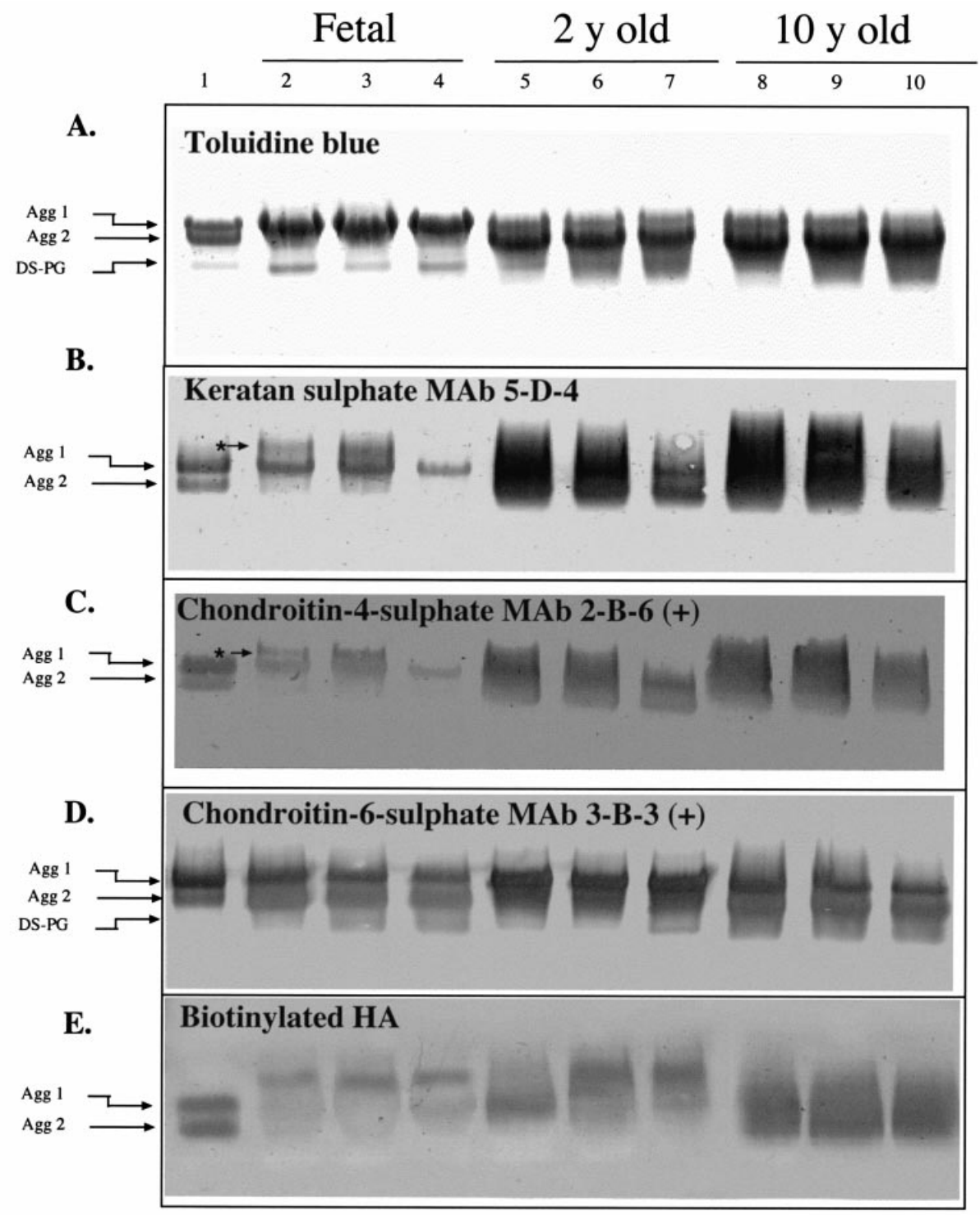

Fig. 1. Composite agarose polyacrylamide gel electrophoretograms (*A*) and semidry electroblots (*B–E*) of L5L6 IVD PG extracts from the outer (lanes 2, 5, 8) and inner (lanes 3, 6, 9) AF and the NP (lanes 4, 7, 10) of the fetal (lanes 2–4); 2 year old (lanes 5–7) and 10 y old (lanes 8–10) sheep. A sample of a 3-y-old ovine articular cartilage crude PG extract is also presented in lane 1; the arrows at the left-hand side of the figure (Agg-1 and 2, DS-PGs) depict the migration positions of the 2 major CS- and KS-rich articular cartilage aggrecan populations respectively and also the DS-rich PGs. The (+) notation after the Mab used indicates that the blot was predigested with chondroitinase ABC prior to addition of primary antibody. Alkaline phosphatase conjugated secondary reagents and the NBT/BCIP substrate system were used for visualisation. Sample loadings used were 1 μg hexuronic acid/lane in *A* and *E* and 0.1 μg hexuronic acid in *B*, *C* and *D*. The electrophoretic migration direction was towards the anode (bottom of figure).

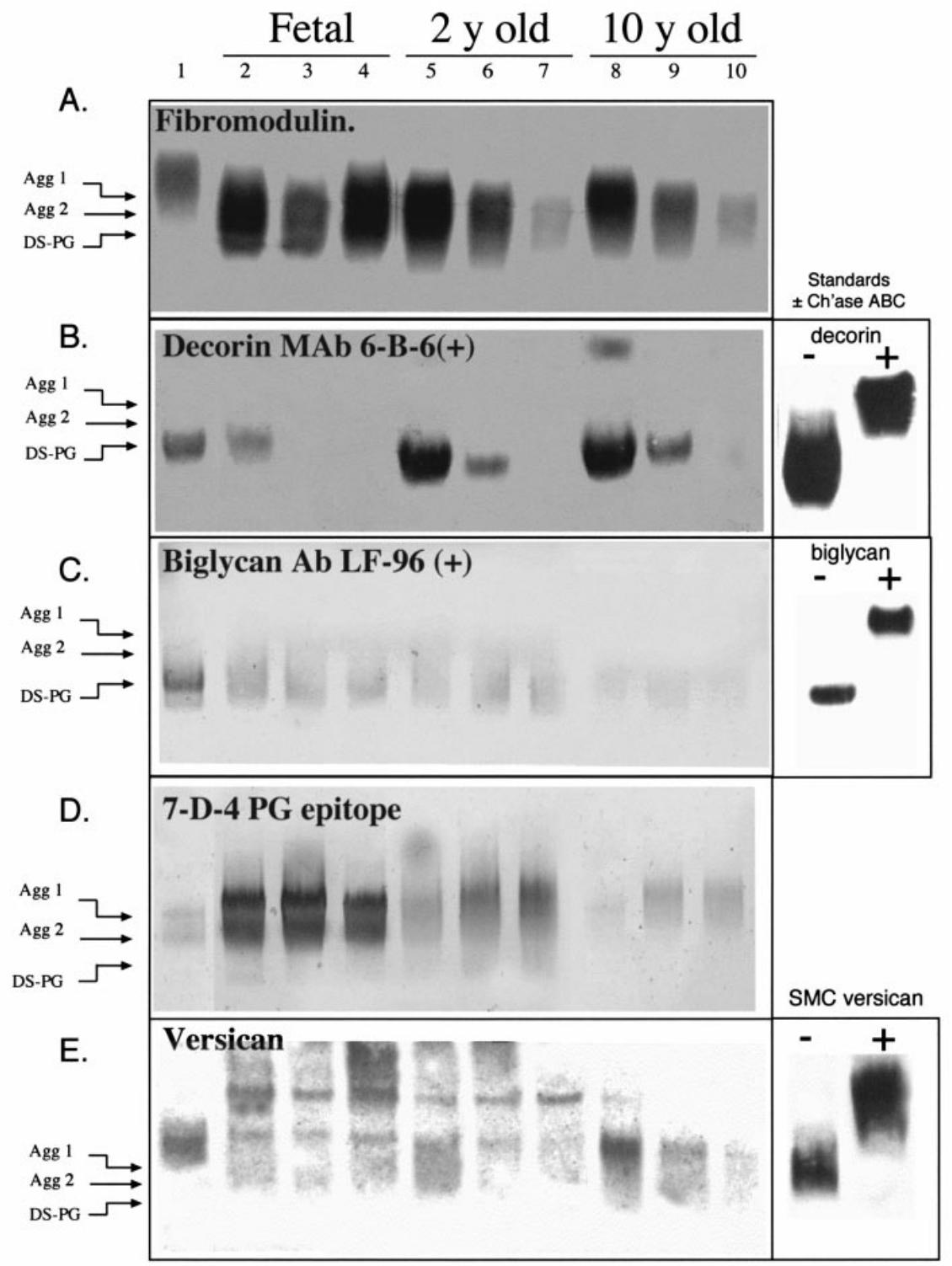

Fig. 2. Immunoblots of ovine IVD proteoglycan samples separated by CAPAGE. The arrangement of samples and the notation of symbols are as indicated in the legend to Fig. 1. Standard preparations of ovine AF decorin and biglycan and smooth muscle cell versican which had been predigested with protease free chondroitinase ABC (+) or otherwise (−) were also run in the same separation system in a separate run, these are indicated at the right hand side of relevant photo-segments. Sample loadings used were 0.1 μg hexuronic acid for all lanes in *A–D* and 1 μg hexuronic acid/lane for segment *E*.

(Fig. 1*C*, lanes 2,3). Three PG species were demonstrable using MAb 3-B-3 indicating regions in their GAG side chains terminating in the chondroitinase ABC generated Δ di C-6-S stub epitope recognised by this MAb and also that the ovine IVD DS-PGs contained this substitution pattern (Fig. 1*D*). Affinity

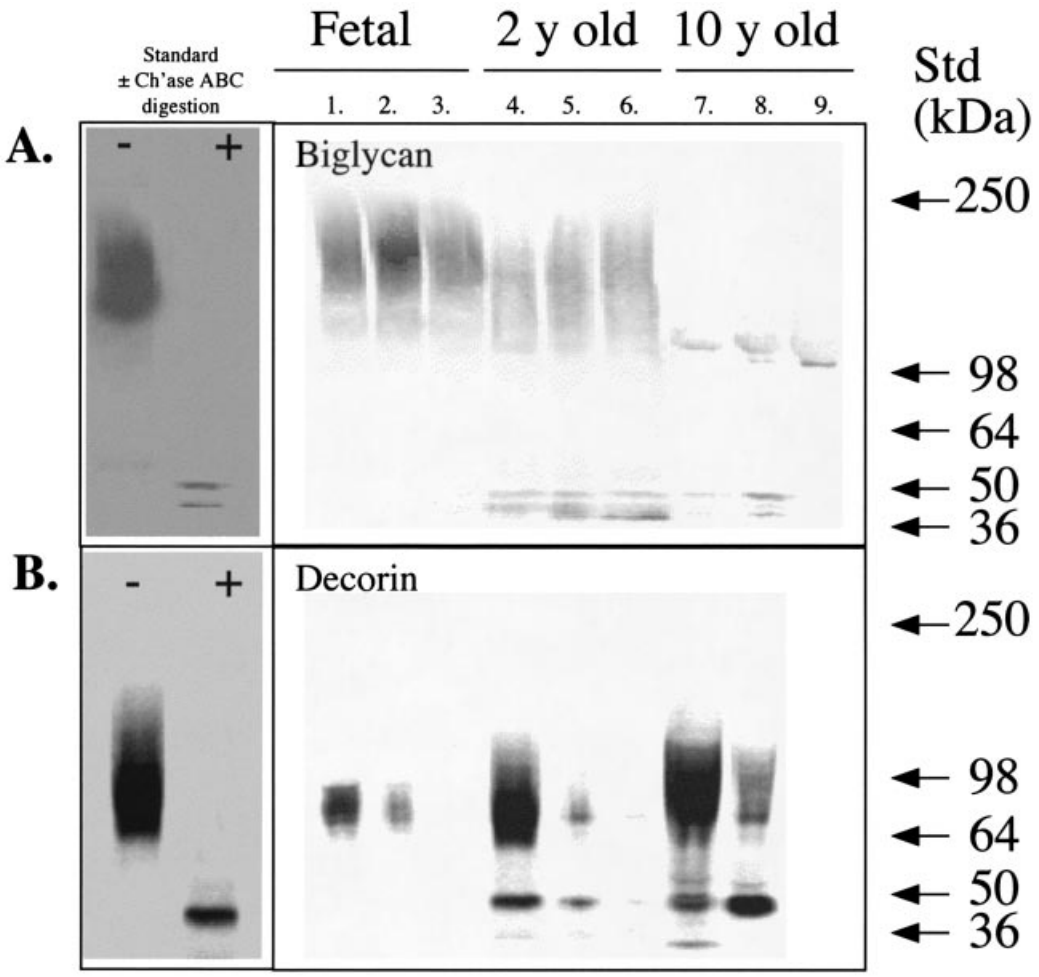

Fig. 3. Demonstration of free coreprotein forms of biglycan (*A*) and decorin (*B*) separated by 4–12 % gradient SDS PAGE followed by transfer to nitrocellulose membranes and detection of PG species by immunoblotting using the antidecorin MAb 6-B-6 and rabbit antibovine biglycan LF-96 (see Table 1 for details). The samples examined were PGs from the outer AF (lanes 1, 4, 7), inner AF (lanes 2, 5, 8) and NP (lanes 3, 6, 9). Dialysed and freeze dried samples of crude 4 M GuHCl disc extracts (30 μg dry weight of crude PG) were examined in each lane for each zone. Colour visualisation of the blots was with the NBT/BCIP alkaline phosphatase detection system. The migration positions of the Novex seeblue prestained standards used are indicated on the right-hand side of the figure with arrows; these included myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa) and carbonic anhydrase (36 kDa). The lanes at the lefthand side of the figure contained standard reference preparations of ovine disc decorin and biglycan which had been predigested with protease free chondroitinase ABC to remove their GAG side chains (indicated with a + symbol), while the corresponding nondigested samples have a (−) sign above them.

blotting with biotinylated HA is a recently introduced technique which can identify whether electrophoretically separated PG populations can interact with hyaluronan, a function which has functional implications for the respective PG within the IVD matrix (Melrose et al. 1998). This technique demonstrated 2 major aggregatable IVD PG populations, although their respective distributions differed with age (Fig. 1*E*). The least mobile fetal IVD aggrecan sample was the major HA binding fetal PG species (Fig. 1*E*, lanes 2–4) however in the 10 y old IVD PG samples the Agg-2 population was the major HA binding aggrecan species (Fig. 1*E* , lanes 8–10). Using purified Agg-1 as a model substrate and a collection of samples taken over a time course-digestion using matrix metalloproteinases 2, 3 or 9, it has been demonstrated using CAPAGE that a progressive increase in the Agg-2 population occurs at the expense of the Agg-1 population (unpublished data). The shift in distributions of the Agg-1 and 2 populations with ageing evident in the present study (Fig. 1*A–E*) therefore appears to reflect the extent of endogenous proteolytic processing which each aggrecan subpopulation had received within the tissue.

Examination of the small disc PGs by CAPAGE/immunoblotting also identified site specific and age dependant changes in their distributions. Fibromodulin resolved somewhat diffusely in CAPAGE with an average mobility slightly less than the Agg-1 population of ovine articular cartilage (Fig. 2*A*).

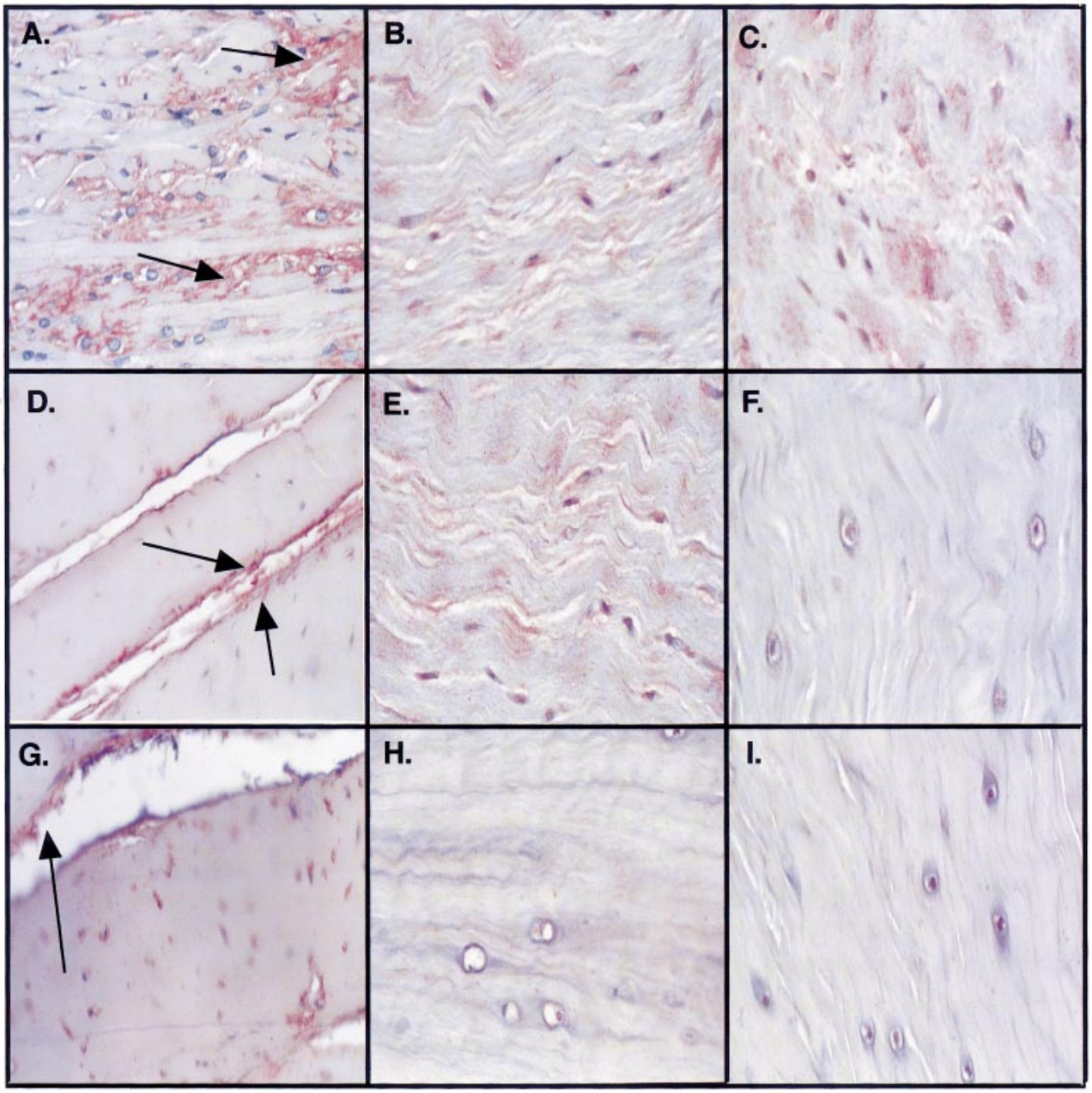

Fig. 4. Immunolocalisation of versican in vertical midsagittal sections of outer AF, inner AF and the NP of new-born discs (*A–C*); outer AF, inner AF and NP of young adult (2 y old) (*D–F*) and outer AF, inner AF and NP of old adult (10 y old) (*G–I*) lumbar sheep IVDs. Final magnification ×400, the chromogen used was New Fuchsin (red), Harris haematoxylin (blue) was used as counterstain. The arrows indicate the presence of versican between adjoining annular lamellae which have partially separated during the histology fixation/dehydration/rehydration steps.

Fibromodulin was detected in all IVD samples examined, its levels being lowest in extracts of the 2 and 10 y old NP samples (Fig. 2*A*, lanes 7, 10); however it was equally abundant in all IVD tissue zones of the fetal sheep (Fig. 2*A*, lanes 2–4). Immunoblotting using antidecorin (6-B-6) and biglycan (LF-96) core protein antibodies indicated that the fetal IVD extracts contained very little decorin (Fig. 2*B*, lane 2) but it was readily detected in the AF of the 2 and 10 y old IVD samples (Fig. 2*B*, lanes 5, 6, 8, 9). A free core protein form of decorin of relatively low mobility in the CAPAGE system was also detectable in the oldest IVD group (Fig. 2*B*, lane 8). Biglycan was also detectable in the fetal and 2 y old samples in all IVD zones; it was less abundant in the other specimens (Fig. 2*C*, lanes 2–7 and 8–10). The identities of the free core protein and glycosylated forms of decorin and biglycan were confirmed by comparison with purified standards (Fig. 2*B*, *C*). Collectively the small IVD PG data indicated a

progressive loss of fibromodulin and biglycan from ovine disc tissues with ageing, although the distribution of decorin followed an opposite trend.

Immunoblotting using MAb 7-D-4 identified reactivity within 2 PG populations which were of similar mobility to the Agg-2 band and a less mobile fetal PG population (Fig. 2*E*, lanes 2–4). The 7-D-4 PG epitope was significantly less abundant in the 2 and 10 y disc PG samples (Fig. 2*E*, lanes 5–10). This is the first study to identify individual IVD PG populations which display the 7-D-4 PG epitope. This PG epitope has formerly been identified in shark cranial, epiphyseal growth plate and chick limb bud cartilages (Caterson et al. 1990). It is believed to be associated with skeletal development which is consistent with the relatively high expression of 7-D-4 epitope in the neonatal IVD PGs and its diminished levels in IVDs from skeletally mature sheep (Fig. 2*D*, lanes 2–4).

Several species of versican were identified by CAPAGE/immunoblotting using an antibody to bovine versican core protein (Fig. 2*E*). These PG species had different migration characteristics to aggrecan in the CAPAGE separation system; the antiversican antibody did not cross-react with aggrecan in this study. Smooth muscle cell versican was also examined in this study; its deglycosylated core protein was of similar mobility to some of the lower mobility ovine disc versican species identified in this study, further confirming the specificity of the antiversican antibody used (Fig. 2*E*). Versican levels appeared to decrease with the onset of IVD age in this study (Fig. 2*E*, lanes 8–10).

Gradient SDS PAGE/immunoblotting confirmed the presence of native and free core protein forms of decorin and biglycan in the ovine IVD PG samples (Fig. 3). The fetal samples, however, did not contain free core proteins while these were evident in the 2 and 10 y PG samples (Fig. 3*A*, *B*). Decorin expression was confined to the inner/outer AF and its levels apparently increased with ageing (Fig. 3*B*), biglycan however was present in all disc zones sampled and its levels decreased with ageing (Fig. 3*A*).

Immunolocalisation studies confirmed the presence of versican in ovine IVD tissues. It was readily demonstrated between the developing annular lamellae in the outer and inner AF of the newborn sheep group but had a somewhat diffuse distribution in the NP (Fig. 4). Versican was less evident in IVD sections of the 2 and 10 y old sheep where it was diffusely present mainly in the outer AF; again it was most prominent between adjacent annular lamellae (Fig. 4). These data are consistent with the relative distributions of versican identified by immunoblotting at each respective age group (Fig. 2*E*). We have also colocalised elastin fibres and versican in interlamellar regions of the adult ovine AF (data not shown) in regions of human and canine IVDs previously reported to contain elastin (Johnson et al. 1982, 1984). Versican has also been colocalised with elastic networks in a range of other connective tissues which is consistent with the findings of our study (Sakai et al. 1986; Kumuratilake et al. 1989; Zimmermann et al. 1994; Roark et al. 1995).

DISCUSSION

Fibromodulin, decorin and biglycan are members of a leucine-rich repeat class of proteins (Heinegård & Oldberg, 1993), which interact with specific regions on the surface of type I and II collagen fibrils (Rosenberg et al. 1986; Brown & Vogel, 1989; Rosenberg, 1992; Heinegård & Oldberg, 1993; Harper et al. 1994; Schönherr et al. 1995). The highly ordered arrangement of the collagen fibres of the annular lamellae is critical to the biomechanical properties of the IVD. From the cited studies it would be anticipated that, collectively, the leucine-rich repeat PGs have an important organisational role to play in the co-ordination of assembly, transport and incorporation of collagen fibrils into discrete areas of the extracellular matrix of the IVD during growth and maturation and also during repair processes.

The variable distribution of the leucine-rich repeat PGs observed in this study may reflect phenotypic differences in cellular populations in different regions of the IVD. Cells of the AF range in morphology from 'chondrocyte-like' in the inner AF to 'fibroblast-like' in the outer AF, while NP cells are more typically 'chondrocytic' (Buckwalter, 1995; Chelberg et al. 1995; Rufai et al. 1995; Urban & Roberts, 1995). This morphology correlates with a switch in collagen production from type II collagen in the NP to type I collagen in the outer AF (Eyre, 1979; Buckwalter, 1995; Urban & Roberts, 1995). The AF develops from the cellular condensation of the disc anlage with the outer part initially fibrous tissue although the cells of the inner AF and NP arise from embryonic hyaline cartilage (Buckwalter, 1995; Chelberg et al. 1995; Rufai et al. 1995; Urban & Roberts, 1995). In the present study, the AF contained the highest levels of decorin and versican. These PGs are generally synthesised by cells which express a fibroblastic phenotype (Bianco et al. 1990; Götz et al. 1997). In contrast, biglycan was equally abundant in all zones of the fetal IVD, but was less abundant in the adult AF specimens. These data are consistent with the

reported distribution of biglycan and decorin in connective tissues (Bianco et al. 1990; Götz et al. 1997). Biglycan is considered to be a coordinator of matrix synthesis by proliferating cells, it has a pericellular distribution and its levels are elevated in proliferating cartilage. In contrast, decorin and fibromodulin are collagen fibril-associated in the interstitial matrix (Heinegård & Oldberg, 1993; Johnstone et al. 1993; Markopolous et al. 1994; Götz et al. 1997; Inkinen et al. 1997; Sztrolovics et al. 1999). The demonstrated predominance of biglycan in the ovine fetal IVD and decorin in mature IVD tissues is in keeping with this pattern of events.

The large hydrodynamic size, space-filling, hydrophilic, aggrecan-like PGs of the IVD also have an important functional role to play, namely in the provision of the IVD with its unique hydrodynamic weight bearing properties (Buckwalter, 1995; Urban & Roberts, 1995). The majority of the large disc PGs resemble cartilage aggrecan (Johnstone & Bayliss, 1995), a PG which is characteristic of the chondrocytic phenotype (Buckwalter, 1995; Urban & Roberts, 1995). Versican has also been identified in the human and porcine IVD, human meniscus and articular cartilage (Johnstone et al. 1993*b*; Markopolous et al. 1995; McAlinden et al. 1998; Roughley et al. 1998). Versican was also identified in the ovine disc extracts examined in this study and was immunolocalised to discrete regions of the IVD (Figs 2, 4). Ovine IVD disc cells have also recently been shown to synthesise versican in alginate bead culture (Melrose et al. 2000). Several versican isoforms have been detected in human articular cartilage which is consistent with the multiple forms of versican identified in this study (Roughley et al. 1998). The role of versican in the IVD is presently not known, however based on its known structure, and the tissue localisation demonstrated in this study, it is unlikely that it has a weight or space filling role similar to that of aggrecan. Versican may however help to attach adjacent annular lamellae to one another via elastic fibres or networks. Fibulin-1, a glycoprotein associated with fibrillar scaffolds around elastin fibrils, has recently been shown to have a high affinity for the C-lectin carboxyl terminal G3 domain of versican (Aspberg et al. 1999). Fibulin-1 can also interact with fibrillin, a component glycoprotein of elastic fibres which are known to be present between adjacent annular lamellae (Johnson et al. 1982, 1984). This may provide a means whereby interlamellar elastin fibrils can anchor to versican via its G3 carboxyl domain, while versican is anchored to hyaluronan in the interstitial matrix via its G1 domain. Hyaluronan has also been localised to interlamellar regions of the human and canine IVD where it may contribute towards the lubrication of adjacent lamellae and thus facilitate movement between adjacent lamellae during weight bearing (Inkinen et al. 1999). Such an interrelationship would be envisaged to be important for the viscoelastic properties of the AF. Versican has also been shown to be closely associated with the elastic fibres and laminae in a range of elastic tissues (Zimmermann et al. 1994; Bode-Lesniewska et al. 1996). Furthermore, its distribution in these tissues closely follows that of elastin, fibulin-1, microfibril associated glycoproteins and fibrillin-1 (Sakai et al. 1986; Kumaratilake et al. 1989; Roark et al. 1995).

Immunoblotting experiments employing monoclonal antibody 7-D-4, which detects an abnormal CS-isomer presentation (Caterson et al. 1990), yielded some interesting results in this study. This PG epitope was strongly expressed in 2 aggrecan populations in the fetal PG samples (Fig. 2*D*) but its expression became progressively less in the 2 and 10 y old IVD PG samples. This indicated that the CS glycosylation patterns of these aggrecan populations underwent time dependant alterations. While this demonstrated that disc PGs in aged IVDs were synthesised with subtle alterations in their GAG side chains, the relative importance of this observation with regard to the modulation of the functional properties in these PGs within the tissue has yet to be addressed. Elevated levels of the 7-D-4 PG epitope have previously been found associated with cartilaginous matrices of developing tissues (Caterson et al. 1990). This epitope is present at basal levels within skeletally mature tissues which is consistent with our findings in the present study. The 7-D-4 PG epitope is also associated with the onset of osteoarthritis in mature cartilaginous tissues (Visco et al. 1993; Hazell et al. 1995). The glycosylation patterns of the GAG side chains of human IVD decorin and biglycan also undergo alterations with the onset of disc degeneration; these subtle changes in GAG substructure have been detected using antibody 7-D-4 (Inkinen et al. 1997).

This study demonstrated a progressive time dependant conversion of the Agg-1 (CS rich) aggrecan population to the Agg-2 (KS rich) population. Time-course digestion experiments employing purified Agg-1 as substrate and matrix metalloproteinases 2, 3 and 9 have shown that the Aggrecan-2 population can be generated from the Aggrecan-1 population in vitro suggesting that the endogenous disc matrix metalloproteases are responsible for at least some of the aggrecan species we have identified in this study (J. Melrose, unpublished data). Furthermore, radioisotope pulse-chase metabolic labelling studies with

human disc explants have shown that the initially synthesised Agg-1 population is gradually converted over time into the Agg-2 population (Johnstone & Bayliss, 1995). The Agg-2 population which predominates with ageing of the IVD is more heterogeneous than the Agg-1 population, it is also more highly substituted with KS rather than CS. Presently it is not known what impact these changes in glycosylation patterns of aggrecan have on the viscoelastic and hydrodynamic properties of aged disc tissues. However, since the GAG side chains of aggrecan are important for the imbibition of water and thus for the generation of intradiscal hydrostatic pressure, it is logical to deduce that changes in the composition of the GAG side chains of aggrecan is liable to have some effect on the weight bearing properties of the IVD. This may partly explain why older IVDs are less capable weight bearing structures compared with their juvenile counterparts. As already mentioned, the functional role of versican in IVD tissues is currently unknown; data from this study however indicated that its core protein was more extensively processed over time than was that of aggrecan. Further studies will be required to ascertain the relative importance of how these changes to this PG alter its functional properties within the IVD.

In conclusion, this study has identified differences in the spatial and temporal distribution of several large and small PGs in the ovine IVD which presumably reflect phenotypic differences in cell populations in different regions of the IVD. Free core protein forms of these PGs were also variably distributed in IVD tissues. Versican was immunolocalised to interlamellar regions in areas containing elastin fibrils suggesting that it may help to contribute towards the elastic recoil properties of the AF, a feature which is essential for its viscoelastic biomechanical properties and also for the weight bearing properties of the composite disc structure. This study further serves to demonstrate the complex nature of the PG species present in the IVD during development, tissue maturation and ageing and the important role they are likely to have with regard to the functional properties of this heterogeneous but intricately organised connective tissue.

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