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Elevated synthesis of biglycan and decorin in an ovine annular lesion model of experimental disc degeneration

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Abstract The aim of this study was to extend our earlier observations on the changes that occur in the proteoglycans (PGs) of discs subjected to experimental injury to the annulus fibrosus (AF). We employed the alginate bead culture method to examine the metabolism of the dermatan sulphate (DS) containing PGs by cells derived from different regions of ovine discs that had been subjected to experimental annular injury. This was compared with the metabolism of the DS-PGs by cells isolated from equivalent regions of normal sham-operated discs. Six months after induction of the annular lesion, AF cells isolated from the lesion produced significantly higher levels of decorin and biglycan in alginate bead culture than did cells from equivalent zones of the controls. Decorin and biglycan were identified in culture media samples by immunoblotting, using specific antibodies (6-B-6, LF-96), and also by positive identification of their de-glycosylated core proteins. The core protein of the DS-PGs has been shown to inhibit type I/II collagen fibrillogenesis, to negatively regulate the action of transforming growth factor-β (TGF-β) and to diminish cellular proliferation in vitro; events which may be detrimental to tissue repair. The findings are therefore consistent with our previous observation the annular lesions in the avascular inner annulus have no capacity to heal.

Key words Decorin and biglycan · Annular lesions · TGF-β · Annular repair

Introduction

The intervertebral disc (IVD) provides a strong bond between adjoining vertebral bodies, is a major contributor to spinal flexibilitiy and acts as a cushion for axially applied spinal loads [5, 55]. The IVD has an outer collagen-rich fibrous annulus fibrosus (AF) composed of concentric intersecting lamellae of fibrocartilage [48, 55]. Collagen (type I and II) fibres in the outer AF insert into adjacent vertebral bodies [5, 32, 36, 48, 55]. Several small proteoglycans (PGs) (fibromodulin, biglycan, decorin, lumican), which are members of a leucine-rich repeat class of proteins, interact with specific regions of the surface of type I and II collagen fibrils. These PGs influence collagen fibrillogenesis and also promote interaction of adjacent collagen fibrils in vitro, and thus may have a role to play in tissue repair and organisation in vivo. The central region of the IVD is the nucleus pulposus (NP), a gelatinous PG-rich tissue in infancy, which becomes progressively less hydrated and more fibrous with age [5, 32, 36, 48, 55]. The NP is enclosed by the AF, but is separated from the vertebral body by a layer of hyaline cartilage, the cartilaginous end-plate [5, 32, 36, 48, 55].

The IVD undergoes profound compositional changes during development and ageing, some of which may diminish its biomechanical competence [5, 32, 36, 55, 57, 58]. Systematic studies of cadavers have shown that, compared with "normal" young lumbar IVDs, discs in older age groups are subject to a range of "pathological" le-

sions. Three types have been classified in humans [15, 35, 58–60]. The rim lesion is a transverse defect at, or close to, the attachment of the AF to the bone of the vertebral body edge; the concentric (circumferential) tear is a separation of the annular lamellae, while the radiating tear results from the propagation of clefts which start in the NP and is associated with age-related degeneration of the IVD [15, 35, 36, 57–60]. Apart from a meagre blood supply to the outer margin of the AF [6, 32, 55], the remainder of the normal IVD is avascular, and despite some vascular ingrowth around annular tears, evidence from human cadaver material indicates that annular tears have a poor healing potential [14, 35, 57–60]. Experimental surgical incision of the AF in sheep [28–30] and pigs [17–20] initiates biochemical changes closely similar to those in human IVD degeneration [5, 36, 57–60]. These include an alteration in the amount and type of collagens synthesised by cells in the lesion site [17, 19, 20], the catabolism of large high-buoyant-density aggrecan-type PGs from the injured disc [18, 28, 29], remodelling of adjacent vertebral bone [30] and a decrease in the biomechanical competence of the injured disc [24].

The aim of our study was to extend earlier observations with the ovine annular lesion model of experimental disc degeneration [28, 29, 34] using alginate bead culture [7, 26, 32] to examine the metabolism of the DS-PGs by cells derived from different ovine disc regions, including the zone of annular injury. These data were compared with the metabolism of the DS-PGs by cultured cells isolated from equivalent regions of control discs.

Materials and methods

Animals

A total of 12 pure-bred merino wethers aged around 2 years at the inception of the study were used in this investigation. All surgical procedures were conducted at the Institute of Medical and Veterinary Sciences, Adelaide, and all procedures were approved by the ethics committee of this institute.

The annular lesion

The lesion group of sheep ($n = 6$) received a controlled 4 mm-deep cut (extending over 10 mm) in the left anterolateral annulus (see Fig. 1) of the L2–L3 and L4–L5 intervertebral discs as previously described [28–30, 34]. The sham-operated control sheep ($n = 6$) received the same retro-peritoneal surgical approach to the annulus, but it was not incised. Animals were sacrificed 6 months after operation. The lumbosacral spine was removed in toto within 1 h of death and transported on ice to the laboratory.

Tissue dissection/digestion/isolation of disc cells/alginate bead culture

Individual L2–L3 and L4–L5 discs ($n = 12$) enclosed by adjacent vertebral body halves were aseptically opened with a single midline incision and dissected into lateral halves of the AF and NP (Fig. 1 B [29]). Disc cells were released from IVD tissues by sequential digestion with pronase and clostridial collagenase [29], resuspended in Hams F12 with 10% fetal calf serum (FCS), and the viable cell count determined by trypan blue exclusion, by use of a haemocytometer. The cells were resuspended in sodium alginate solution (1.2% w/v in 0.15 *M* NaCl) at a density of 1×10^6 cells/ml alginate, so as to give 10,000 cells/calcium alginate bead. The beads ($n = 25$) were cultured in six well 35 mm^2 plates in Hams F12 + 10% v/v FCS buffered with 20 m*M* HEPES pH 7.2 (5 ml) at 37° C, in an atmosphere of 5% CO_2 in air, with 98% humidity. The media was changed daily for 7 days and supplemented with $^{35}SO_4$, (as $H_2{}^{35}SO_4$, 50 µCi/ml, Amersham, Australia) for the last 48 h in culture, to radiolabel the disc PGs.

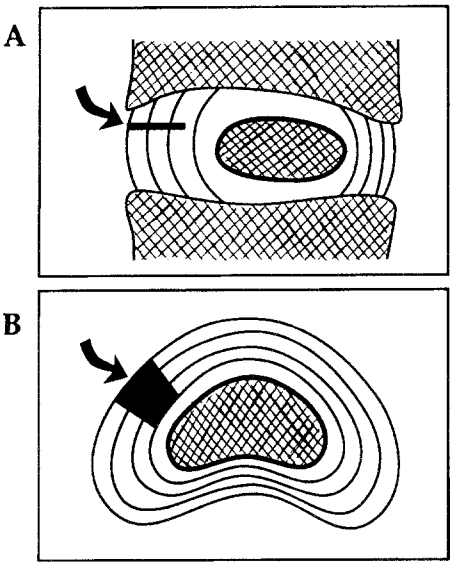

Fig. 1 A, B Diagrammatic representation of the 4-mm deep by 10-mm wide anterolateral annular lesion *(black zone, curved arrow)* used in this study at the L2–L3 and L4–L5 intervertebral disc (IVD) levels. The proximity of the lesion to the annular rim, parallel to the inferior end-plate of the superior vertebral body in sagittal section (**A**) and also its extent in horizontal section (**B**) are indicated schematically. Annulus fibrous (AF) zone 1 corresponded to the contralateral half of the AF opposite AF zone 2, which contained the lesion site

Sephacryl 1000 gel permeation chromatography

Beads were solubilised in 55 m*M* sodium citrate and the ^{35}S-PGs applied to chromatographic columns of Sephadex G25 to remove free $^{35}SO_4$ radiolabel, which is eluted in the included volume. The macromolecular aggregated void volume ^{35}S-PGs were then examined by Sephacryl 1000 (AMRAD Pharmacia Biotech, N. Ryde, NSW, Australia) gel permeation chromatography in the presence of hyaluronan (HA, 20 µg/ml), to assess the hydrodynamic size of, and the degree of, polydispersity of the ^{35}S-IVD PG species. Columns of Sephacryl 1000 (1×60 cm^2) were eluted at flow rates of 20 ml/h using 0.5 *M* sodium acetate buffer pH 6.8 containing 20 m*M* sodium sulphate as eluant. Fractions of 0.5 ml were collected and counted for $^{35}SO^{2-}_4$.

Preparation of media PGs for electrophoresis

Pooled media PG samples from days 1–5 (non-labelled PGs), or days 6 and 7 (^{35}S-PGs), were precipitated overnight at 4° C with

3 vols of cold absolute ethanol saturated in potassium acetate, and the precipitate collected by centrifugation. The pellet was redissolved in 7 *M* (urea 50 m*M* Tris HCl buffer pH 7.0 containing 0.2 *M* NaCl (2 ml) and applied to columns (~1 ml) of DEAE Sepharose CL6B anion exchange equilibrated in the same starting buffer. The columns were washed with starting buffer (6 ml) to remove non-bound material, bound PGs were eluted with 4 *M* GuHCl (2 ml). Under these conditions, serum proteins did not bind to the column, but the sulphated PGs did. Elution with 4 *M* GuHCl was essential to obtain a quantitative recovery of the PGs; these were dialysed against distilled water and freeze dried.

Electrophoresis and Western blotting of media proteoglycan samples

Freeze-dried media PG samples were redissolved in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis application buffer, and heated at 95° C for 10 min to denature the proteins and prepare SDS-protein complexes prior to electrophoresis of these in Novex pre-cast 4–12% polyacrylamide slab gels (Novex, Frenchs Forest, NSW, Australia), using the Tris-glycine buffer system of Laemmli [23]. Novex broad-range globular protein standards were also run to provide a size estimation of resolved PGs; these were: myosin (rabbit muscle, 200 kDa); β-galactosidase (*Escherichia coli*, 116.3 kDa); phosphorylase-b (rabbit muscle, 97.4 kDa); bovine serum albumin (66.3 kDa); glutamic dehydrogenase (bovine liver, 55.4 kDa), lactate dehydrogenase (porcine muscle, 36.5 kDa); carbonic anhydrase (bovine erythrocyte, 31.0 kDa). Novex prestained standard proteins were also electroblotted; these were: myosin, 250 kDa; bovine serum albumin, 98 kDa; glutamic dehydrogenase, 64 kDa; alcohol dehydrogenase, 50 kDa; and carbonic anhydrase, 36 kDa. The identities of the DS-PGs were confirmed by immunoblotting [53] of the native PGs and their de-glycosylated core proteins, using the Abs 6-B-6 [49] (Seikagaku, Tokyo, Japan) and LF-96 [11, 12]. Purified ovine AF decorin and biglycan were also electrophoresed [27]. The LF-96 (obtained from Dr. L. Fisher, National Institute of Dental Research, National Institutes of Health, Maryland, USA) [11, 12] is an affinity-purified rabbit polyclonal antibody raised against the amino terminal peptide sequence LPDLDSLPPTYSC (single-letter code for amino acids) of bovine biglycan. This antibody also cross-reacts with ovine IVD biglycan, but not with decorin (Fig. 4). 6-B-6 [49] is a monoclonal antibody to the core protein of human decorin which cross-reacts with ovine meniscal, tendon and IVD decorin; however, it does not cross-react with ovine biglycan (Fig. 4). Both LF-96 and 6-B-6 react better with the deglycosylated core proteins than with the native forms of biglycan and decorin. Non-specific binding to nitrocellulose membrane blots was prevented by blocking them in 5% non-fat skim milk in 50 mM Tris-HCl pH 7.4 which contained 0.15 *M* NaCl and 0.02% sodium azide; constant shaking was employed for 3 h. LF-96 (1/250 dilution) and 6-B-6 (1/1000 dilution) in 50 m*M* Tris-HCl pH 7.4 500 m*M* NaCl, and 0.05 v/v Tween-20 (TBS-Tween) were then allowed to bind to the membrane for 1 h at room temperature. The membranes were then again washed in TBS-Tween (6 × 3 min) and goat anti-rabbit IgG, or anti-mouse IgG alkaline phosphatase conjugated secondary antibodies (1/1000 dilution in TBS-Tween) were added. After a further 1 h the membranes were washed again, and a solution of nitroblue tetrazolium (NBT)/4-bromo-1-chloro indolyl phosphate (BCIP) substrates in alkaline phosphatase assay buffer was added (20 ml). Colour development was allowed to proceed for 20 min.

Measurement of ^{35}S-decorin and ^{35}S-biglycan synthesis by disc cells in alginate bead culture by Western blotting and phosphor-screen autoradiography

An equivalent number of AF cells (20 beads, 10,000 cells/bead) were cultured from the sham and lesion zones and their ^{35}S-labelled media PGs from days 6 and 7 in culture, were separated on 4–12% polyacrylamide gradient gels to provide a comparison of the relative PG metabolism of each respective cellular population. The gel was dried down and placed in a phosphor-screen cassette for 2 weeks; radio-labelled proteins, ^{35}S-decorin and ^{35}S-biglycan, were visualised by phosphor-screen autoradiography and the image analysed by ImageQuant image software to quantitate individual bands using a Molecular Dynamics PhosphorImager (Sunnyvale, Calif., USA). Integration measurements of the ^{35}S-labelled DS-PGs were made, assuming that biglycan had two glycosaminoglycan (GAG) chains, and therefore incorporated twice as much radiolabel as decorin, and that all biglycan was present in the Mr 200–250 kDa band separate from decorin, a band of Mr 80–110 kDa relative to Amersham rainbow ^{14}C-molecular weight protein standards.

Statistical methods

Data from the lesion and sham-operated sheep groups were compared, using a one-way analysis of variance. When differences were apparent between groups of data, the raw data were examined using Student's *t*-test. Statistical significance was considered to occur at $P < 0.05$.

Results

Sephacryl 1000 gel exclusion chromatography of the ^{35}S-bead PGs

Comparison of the ability of ^{35}S bead-PG monomers to form macromolecular aggregate structures with HA by Sephacryl 1000 gel permeation chromatography (Fig. 2), showed that of all the samples examined, the PGs derived from the lesion zone cultures of the L2–L3 and L4–L5 discs were least capable of forming aggregates. All lesion group NP bead ^{35}S-PG samples were capable of aggregation to a greater degree than the PGs from corresponding AF zones. However, in the sham-operated group this trend was reversed. Lesion group lesion zone cultures (Fig. 2, L2–L3, L4–L5) contained somewhat smaller hydrodynamic size (~Kav 0.7), and relatively less polydisperse, non-aggregatable PG populations than did other AF samples of the lesion or sham-operated sheep groups. The Kav 0.7 PGs represented the majority of the PGs; only a small proportion of the total ^{35}S-PGs in these cases were capable of aggregation with HA. An earlier study [29] has demonstrated that the non-aggregatable PG species evident in alginate bead culture are a mixture of catabolised aggrecan species, in addition to both decorin and biglycan.

Examination of the media DS-PGs

Electrophoretic examination of media PGs from alginate bead culture on 4–12% polyacrylamide gradient gels demonstrated that 6 months after induction of annular lesions, the ovine annular cells synthesised elevated levels

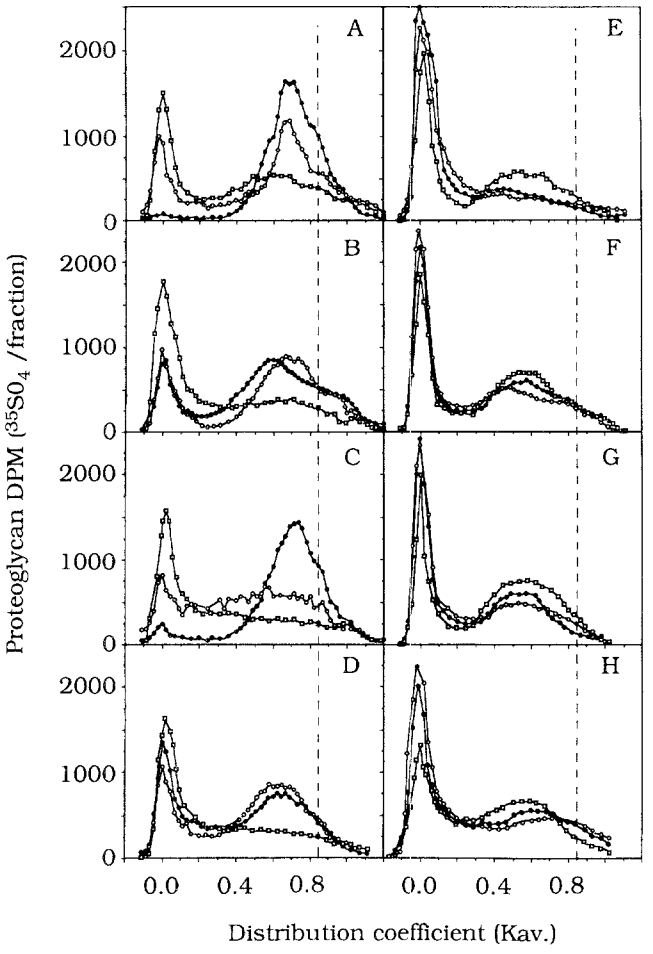

Fig. 2 A–H Sephacryl 1000 chromatograms of ^{35}S-labelled bead PGs synthesised by cells from AF zone 1 (○—○), AF zone 2 (●—●) and the NP zones (□—□) of one individual from each of the lesion (**A–D**) and sham-operated (**E–H**) sheep groups; 50–80,000 disintegrations per minute (DPM) of ^{35}S-labelled PGs were applied per run. ^{35}S-PGs from the L2–L3 (**A, E**); L3–L4 (**B, F**); L4–L5 (**C, G**); and L5–L6 (**D, H**) IVDs were examined. Standard preparations of aggregated ovine disc aggrecan and ovine AF aggrecan monomer, had Kav values of 0 and 0.4–0.6 respectively (Kav = average distribution coefficient; a solute with a Kav of zero corresponds to macromolecular material that elutes in the void volume of the column, while a Kav of 1 corresponds to material that is small enough to pass through the bead pore size and thus be fully included in the column and thus elutes later at Kav = 1). The dotted line at Kav 0.85 depicts the elution position of purified ovine disc decorin

of two PG species of 85–110 and 200–250 kDa (Fig. 3). These were identified as decorin and biglycan respectively, since they were immunoreactive with MAbs 6-B-6 [49] and LF-96 [11, 12]. Depolymerisation with chondroitinase ABC resulted in the generation of immunoreactive core proteins of around 45 kDa, for biglycan and around 43 kDa for decorin. Similar to native ovine disc, decorin and biglycan and their chondroitinase ABC generated core proteins [27] (Fig. 3). Immunoblotting demonstrated that qualitatively higher levels of decorin and biglycan were synthesised by cells derived from lesion zone repair sites than by annular cells isolated from comparable regions from a sham-operated sheep group (Fig. 4).

Quantitation of the media ^{35}S-decorin and biglycan levels

Quantitation of ^{35}S-media PGs separated by SDS gradient PAGE by phosphor-screen autoradiography and Image-Quant image analysis indicated that approximately twice as much ^{35}S-labelled decorin and biglycan were present in lesion zone media samples than in media samples from the sham-operated animals (Fig. 5). Measurements of the ^{35}S-labelled DS-PGs were made, assuming that biglycan had two GAG chains and, therefore, incorporated twice as much radiolabel as decorin, and that all biglycan was present in the Mr 200-250 kDA band, and decorin in a band of Mr 80–110 kDa relative to ^{14}C-molecular weight protein standards (Amersham rainbow standards). Furthermore, chondroitinase ABC predigestion removed all ^{35}S label from the biglycan and decorin bands, demonstrating that this label had been incorporated solely into CS or DS chains. Statistical analysis indicated that the lesion zone cells synthesised significantly greater levels of the DS-PGs than did cells from the sham-operated zones ($P < 0.05$). In addition, decorin levels were significantly higher than biglycan levels in all samples ($P < 0.05$). Note that an earlier study which investigated the catabolism of aggrecan in the ovine annular lesion model [29] showed that, although chondroitin-sulphate-containing aggrecan fragments were released into the media during alginate bead culture, these were ≥ 250 kDa in size, and only penetrated the top few millimetres of the stacking gel during gradient SDS PAGE. Thus they would not be confused with the ^{35}S-decorin and biglycan bands, which migrate considerably further into a 4–12% gradient gel under the same electrophoretic conditions.

Discussion

An initial event in the repair of annular lesions involves the deposition of granulation tissue in the defect and recruitment of fibroblasts, which lay down a collagenous repair tissue [13, 48]. Early repair of the outermost AF is also a consistent feature in our ovine annular lesion model. By 4–6 months, the outer margins of ovine annular defects appear by naked eye observation to have repaired appreciably. Polarised light microscopy previously indicated that ordered collagenous structures spanned the original annular defect, but the normal tissue architecture was not restored [28, 34]. As shown in this study, at 6 months, the cells isolated from AF repair tissue from incised discs synthesised significantly higher levels of DS-PGs than did cells from corresponding zones of sham-

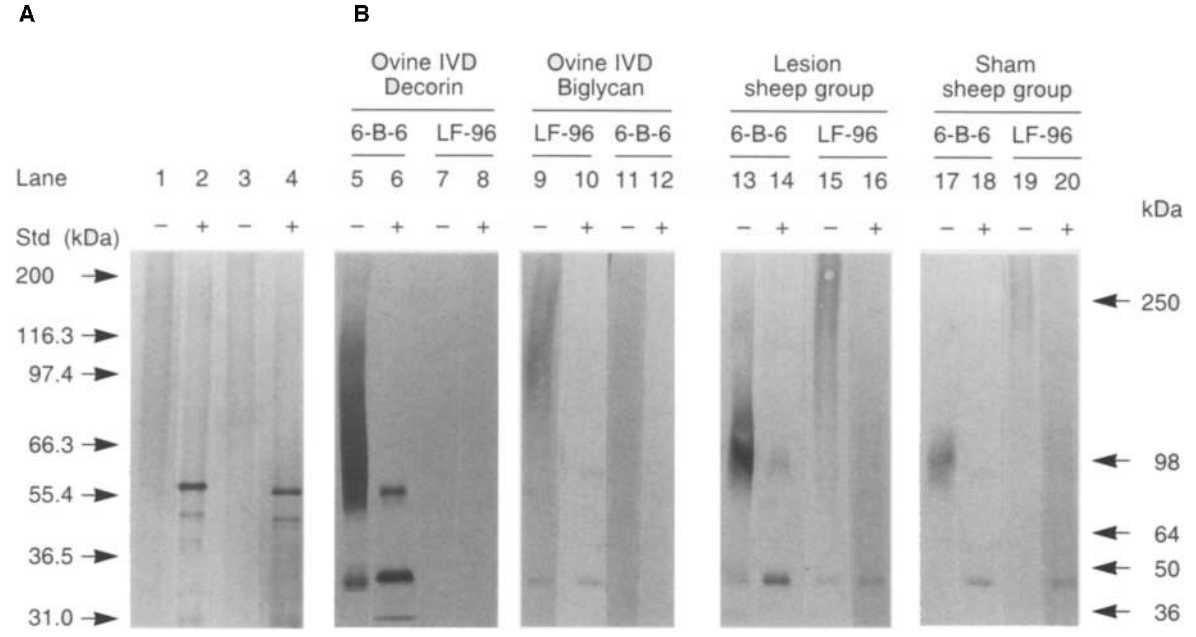

Fig. 3 A, B Electrophoretic examination on 4–12% gradient SDS PAGE and immunoblotting using the MAbs 6-B-6 [49] and LF-96 [11, 12] of purified ovine AF biglycan and decorin [27] and PGs secreted into alginate bead culture media samples by ovine annular fibrochondrocytes. **A** Lanes 1 and 3 depict samples of purified native ovine AF biglycan and decorin [27] and lanes 2 and 4 their respective de-glycosylated core proteins prepared by overnight digestion with protease-free chondroitinase ABC (Seikigaku, Tokyo). Lanes 1–4 were stained with ammoniacal silver (Novex); the major band at 55.4 kDa is derived from the chondroitinase preparation, Novex broad-range globular protein standards are indicated at the left-hand side of the figure. **B** Duplicate samples to those run in lanes 1–4 were also run in lanes 5–12, media PG samples from alginate bead culture of cells isolated from the control sham-operated sheep group (lanes 13–16) and the lesion sheep group (lanes 17–20) were electrophoresed with (+) and without (–) predigestion with chondroitinase ABC prior to immunoblotting. Novex prestained standard proteins were electroblotted as indicated at the right-hand side of the figure

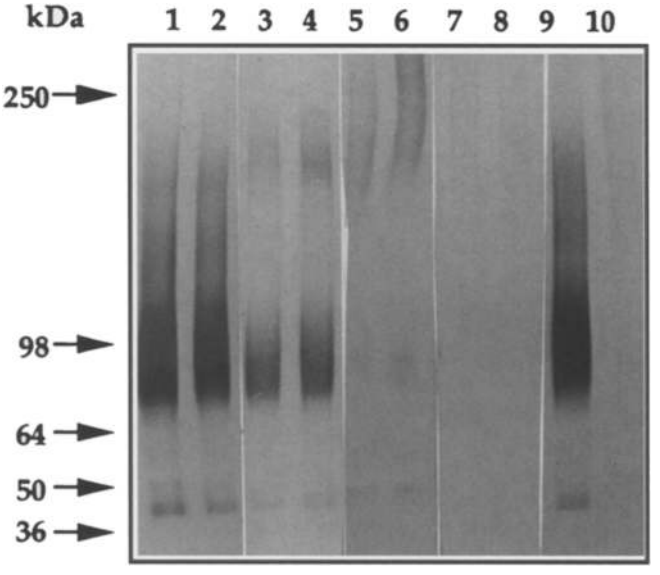

Fig. 4 Demonstration of the elevated synthesis of decorin (lanes 1–4) and biglycan (lanes 5–8) in alginate bead culture by ovine annular cells of the lesion (lanes 1, 2, 5, 6) and sham-operated (lanes 3, 4, 7, 8) sheep groups; L2–L3 IVD (lanes 1, 3) and L4–L5 (lanes 2, 4). The media PGs were separated by 4–12% polyacrylamide gradient SDS PAGE; electroblotted to nitrocellulose, and decorin and biglycan were identified using the MAbs 6-B-6 [49] and LF-96 [11, 12]. PGs derived from an equivalent number of AF cells in culture from the sham and lesion groups were run in each lane. Lanes 9 and 10 are standard preparations of ovine AF decorin and biglycan [27] detected using the MAbs 6-B-6 and LF-96 respectively. Novex prestained protein standards are indicated at the left-hand side of the figure

operated control IVD samples (Figs. 2–4). This strongly suggests that the DS-PGs were synthesised by disc cells in response to the injury and that they could be involved in the repair process.

Matrix PGs such as biglycan and decorin have been implicated in the control of several growth factors involved in tissue remodelling, including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) [38, 44, 45]. Osteonectin is a non-PG member of the leucine-rich repeat group of proteins to which biglycan and decorin also belong [10]. Osteonectin is expressed in high levels by fibroblasts and macrophages in tissues undergoing remodelling. It is also associated with the α-granules of platelets, and thus is released with PDGF during platelet degranulation [37]. The effects of the cytokines PDGF and basic fibroblast growth factor (bFGF) are abrogated by osteonectin [41]; thus, it may regulate the activity or distribution of PDGF in situ in a similar manner to that suggested for TGF-β by decorin [63]. IVD cells are responsive to TGF-β, PDGF and bFGF [52]. These cytokines induce cellular proliferation and extracel-

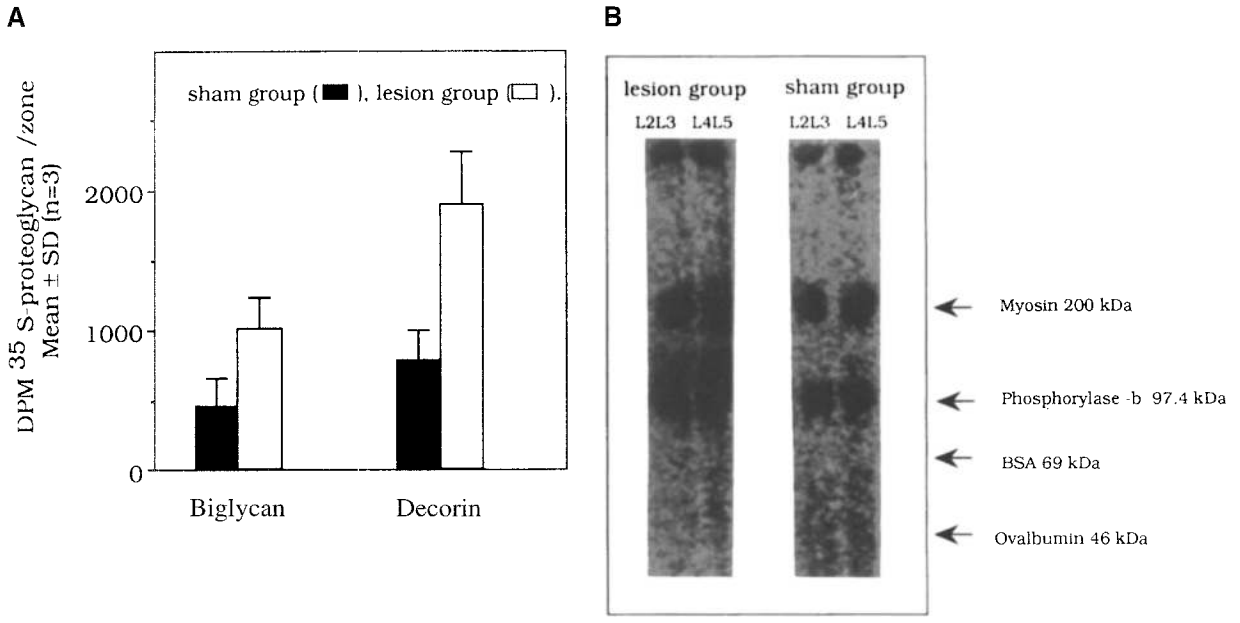

Fig. 5 A, B Comparison of ^{35}S-decorin and ^{35}S-biglycan biosynthesis in alginate bead culture by annular cells derived from AF zone 2 (see legend to Fig. 1) of the lesion and sham-operated sheep groups. An equivalent number of AF cells (20 beads, 10,000 cells/bead) were cultured and the ^{35}S-labelled media PGs from days 6 and 7 were electrophoresed as in Figs. 4 and 5. The gel was then dried down and the ^{35}S-PGs visualised by phosphor-screen autoradiography. Separated PG bands were quantitated using the ImageQuant software (histogram) provided by the manufacturer (*DPM* disintegrations per minute)

lular matrix production, events that are essential for the repair of annular lesions [25, 52].

There are a number of reports in which the biosynthesis of DS-PGs has been examined in relation to tissue injury or disease states [22, 31, 33, 40, 50, 51, 56, 62, 64]. Yeo et al. [64] observed an increase in decorin biosynthesis in healing wounds, and elevated DS-PG levels have been noted in normal and hypertrophic scar tissues [51], and also in an experimental model of rat brain injury [50]. Biglycan expression is chronically elevated in glomerulonephritis [33], glomerulosclerosis [31], pulmonary and hepatic fibrosis [22, 56, 62], and in atherosclerosis [40]. The manner by which DS-PGs influence these pathobiological processes is unclear, but it is known that they can operate at three functional levels. They modulate collagen fibril size and organisation; inhibit cellular adhesion to matrix proteins (and growth factors); and down-regulate cellular proliferation. Decorin has been the most extensively studied of the DS-PGs. It is associated with the 'd' and 'e' bands of collagen fibrils, while biglycan generally has a more pericellular distribution [4]. Biglycan displays a spatial and temporal regulatory pattern distinct from that of decorin. Decorin is mainly expressed by connective tissue cells (chondrocytes, fibroblasts), whereas biglycan is synthesised by endothelial cells [2, 17, 39], by cells that possess a myogenic phenotype such as vascular smooth muscle cells [1, 16, 54] and myocytes [2], by renal mesangial cells [3] and peritubular myofibroblasts of the human testis [54] and even by epithelial cells such as keratinocytes [2] and glomerular cells of the kidney [31]. Fetal ovine IVD tissues contain relatively high levels of DS-PG. This is almost exclusively biglycan, whereas the mature ovine IVD contains mainly decorin, but little biglycan (unpublished data). Biglycan synthesis has been shown to be decreased and decorin synthesis elevated in mature connective tissues relative to immature tissues [12, 42, 46]. Decorin and biglycan expression is generally differentially influenced by TGF-β in a range of cell types [21, 22, 41, 61].

In this study, ^{35}S-DS-PGs were quantitated in alginate bead media samples (Fig. 5). The media ^{35}S-PGs represented 25–30% of the total radio-labelled PGs [29]. It was not possible with phosphor-screen autoradiography to quantitate the proportion of the DS-PGs retained within the alginate beads, although Sephacryl S1000 gel chromatography indicated that a mixture of aggrecan monomer and fragments were also present in addition to the DS-PGs (Fig. 2). The sodium alginate of the solubilised bead is a copolymer of guluronic and mannuronic acids, and has a size of around 120,000 Da. It migrates similarly to the DS-PGs in SDS PAGE, and due to its relative abundance in the solubilised bead samples, it distorts the electrophoretic separation of decorin and biglycan. Furthermore, while it is technically possible to separate sodium alginate from the DS-PGs by anion exchange chromatography, the variable losses of the DS-PGs that occur in this process make it an unacceptable alternative. Also, under the culture conditions employed, it is unlikely that the extracellular matrix deposited in the beads would be sufficient to retain the DS-PGs elaborated by the cells. Notwithstand-

ing these reservations, our experiments indicate that annular fibrochondrocytes derived from the lesion sheep group synthesised (and released into the media) significantly higher levels of DS-PGs than did AF cells isolated from the sham-operated animals cultured under the same conditions (Figs. 3–5).

The variable synthesis of the DS-PGs by ovine annular cells identified in this study may reflect phenotypic differences in cellular populations resident in the AF, plus their respective capacities to respond to injury. Peripheral regions of the injured annulus have been noted to heal both by cellular proliferation within the lesion site and by migration of cells into the wound from its outer margins [13, 14, 48]. This involves the localised up-regulation of integrin expression in the outer AF [48], which facilitates cellular migration. This process, in turn, is modulated by the leucine-rich repeat proteins, further demonstrating their pivotal role in matrix remodelling [44, 55, 63]. The fibrochondrocytes of the inner and outer AF are derived from different cell lineages. The AF develops from the cellular condensation of the anlage disc with the outer part, initially fibrous tissue, and the inner AF arising from embryonic cartilage [5, 6, 55]. Recent evidence indicates that fibroblasts acquire a functional heterogeneity from the microenvironment in which they develop [47]. This observation may explain, at least in part, the variable healing capabilities of cells derived from the inner and outer AF. This is certainly consistent with the healing pattern so far observed for annular lesions [14, 35], and in animal models of experimental disc degeneration [14, 17–20, 28, 29, 34, 48]. The cells within the fibrocartilaginous knee joint meniscus, which also synthesize DS-PGs [43] and have other characteristics closely similar to those of the AF fibrochondrocytes, also display a similar inability to effect repair of tears or incisions that extend into the dense, avascular mid-substance of this structure [8, 9].

The results presented in this study indicate that the DS-PGs would appear to have an important role to play in the annular repair process. A greater understanding of this repair process in terms of the stimulatory growth factors and regulatory proteins that orchestrate tissue repair by endogenous IVD cell populations may in the future facilitate the formulation of more effective treatment strategies for annular lesions of the type simulated in this study. This could also shed light on why dense and relatively avascular fibrocartilaginous connective tissues such as the AF and meniscus have such a poor and incomplete healing capability.

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