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OSTEOARTHRITIS and CARTILAGE

Variation in proteoglycan metabolism by articular chondrocytes in different joint regions is determined by post-natal mechanical loading

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Summary

In this study we investigated the hypothesis that cartilage from defined regions of ovine stifle joints, which were subjected to differing mechanical stresses, contained phenotypically distinct chondrocyte populations. Chondrocyte phenotypes were identified by the relative biosynthesis of the proteoglycans (PGs) aggrecan, biglycan and decorin. Articular cartilage (AC) from adult and neonatal ovine stifle joints were examined. Cells were cultured as both full-depth AC explants and in alginate beads after their isolation from the AC matrix. When chondrocytes from the various topographical regions of adult ovine knee joints were cultured as explants they demonstrated a consistent difference with regard to the metabolism of aggrecan and decorin. Significantly, this topographically-dependent phenotypic expression of PGs was preserved when the chondrocytes were cultured in alginate beads. In adult joints, chondrocytes from the central region of the tibial plateau not covered by the meniscus, which is subjected to high mechanical loads in-vivo, synthesized less aggrecan but more decorin than cells from regions covered by the meniscus. When chondrocytes from identical AC regions of neonatal ovine joints were cultured as explants, no topographical difference in aggrecan nor decorin metabolism could be detected The results of this study, in association with the existing literature, lead us to propose that post-natal mechanical loading of AC could select for chondrocyte clones or induce a lasting modulation of chondrocyte phenotypic expression in different joint regions. Such cellular changes could result in the synthesis of PG populations that confer properties to AC most suited to resist the variable mechanical stresses in the different joint regions. This study serves to emphasize the importance of using cartilage from identical joint areas when examining PG metabolism by chondrocytes. Further investigation into the relationship between mechanical loading, regional chondrocyte phenotype selection and the response of these cells to anabolic and catabolic factors may provide important insights into the focal nature of AC degeneration in osteoarthritis.

Key words. Aggrecan, Biglycan, Chondrocytes, Decorin, Mechanical loading

Introduction

Articular cartilage (AC) covers the ends of bones in synovial joints and provides a shear resistant and resilient weight-bearing surface essential for normal joint function. The tissue consists primarily of hydrated proteoglycans (PGs) entrapped within a network of type II collagen fibres. Although the large aggregating chondroitin/keratan sulfate PG (aggrecan) is the predominant species in terms of mass, AC also contains a number of small PGs including two chondroitin/dermatan sulfate PGs, biglycan (DS-PG I) and decorin (DS-PG II) and a keratan sulfate PG fibromodulin [1–3]. These three small PGs are members of a family of insterstitial molecules with leucine rich repeat sequences in their core proteins. The synthesis of type II collagen and aggrecan by chondrocytes is considered to be a marker of the cartilage-specific cellular phenotype [4, 5]. A change from synthesis of type II to types I and III collagen has been used as a definitive marker of chondrocyte dedifferentiation [6]. An alteration in PG metabolism, characterized by decreased synthesis of aggrecan and increased

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synthesis of low molecular weight DS-PGs, has also been demonstrated with chondrocyte dedifferentiation induced by prolonged monolayer culture [4, 7] and in osteoarthritis [8, 9].

Normal AC displays biochemical heterogeneity with regard to both depth from the surface (zone) and topographical location within the joint [10–13]. The aggrecan content and proportion of keratan sulfate chains attached to the aggrecan subunits is increased in deep zone cartilage [14–16], while superficial zone tissue contains a higher proportion of small non-aggregating DS-PGs [17–19]. When full-depth cartilage from different joint regions was evaluated, it was found that cartilage from joint regions subjected to high weight-bearing stress contained an elevated PG content compared with tissue from regions experiencing lesser stress [13]. Furthermore, AC from joint regions subjected to high-contact stress contained elevated levels of DS-PGs compared with other joint locations [20]. The metabolism of PGs by chondrocytes has also been shown to vary with zone and weight-bearing region. Superficial chondrocytes incorporated less $^{35}SO_4^{2-}$ into PGs, synthesized less aggrecan and keratan sulfate [21–23] but more DS-PGs [17, 18] and hyaluronic acid (HA) [24] than deeper-zone cells. Chondrocytes from the full-depth of human femoral head AC regions exposed to high weight-bearing stress incorporated less $^{35}SO_4^{2-}$ into PGs [11, 12] relative to joint areas subjected to lesser compressive loads. We have previously demonstrated in sheep that full-depth cartilage from regions of the tibial plateau not covered by the meniscus and associated with high contact stresses, exhibited not only reduced total synthesis of PGs, but elevated production of DS-PGs in particular decorin [25, 26].

The depth-related variations in PG metabolism in AC have been reported to be associated with phenotypically distinct chondrocyte populations [10]. Maintenance of this zonal PG expression by cells cultured in short-term monolayer or a three dimensional matrix suggested that the cartilage zones may contain distinct clones or populations of chondrocytes [22, 23]. However, depth related differences were progressively lost with time in culture [16, 21, 23] indicating that the distinct zonal chondrocyte phenotype may be modulated by different environmental conditions. It has recently been demonstrated that AC from young calves (8–12 weeks of age) does not show any zonal variation with regard to PG or HA synthesis when compared with adult bovine tissue [27]. This suggests that differentiation of chondrocytes with regard to depth occurs during maturation and is not inherent. The variation in PG metabolism by the full-depth chondrocyte populations from different joint locations, in contrast to cells from different depths, has been attributed to differences in the matrix, most notably the fixed charge density or PG content, surrounding the chondrocytes [11, 12].

In the present study, we investigated whether the patterns of PG metabolism observed in the different weight-bearing regions of ovine joints may be related to the presence of phenotypically distinct populations of chondrocytes rather than differences in the composition of the surrounding matrix. We identified the chondrocyte populations by their proportional synthesis of aggrecan biglycan and decorin using both cartilage explants from distinct joint regions of adult sheep and chondrocytes isolated from these same joint regions and cultured in alginate beads. These data were compared with the results obtained using explant cultures of cartilage from the same topographical joint regions of neonatal animals that had not borne weight.

Materials and methods

SOURCE OF REAGENTS

Ham's F12 culture media was from Cytosystems, Castle Hill, NSW, Australia. Heat inactivated fetal bovine serum (FBS) was from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Tissue culture plates were from Costar, Cambridge, MA, U.S.A. Radio-isotopes were from Du Pont Ltd, North Ryde, NSW, Australia. Dimethyl-methylene blue, bovine tracheal chondroitin sulfate, papain, calf thymus DNA, Hoechst 33258 dye, bacterial collagenase and low-viscosity sodium alginate were from Sigma-Aldrich, Castle Hill, NSW, Australia. Octyl-Sepharose CL-4B, Sephacryl S1000, Sephadex G-25 columns and high molecular weight hyaluronic acid (Hylartil[spr2], HA) were from Pharmacia, North Ryde, NSW, Australia. DEAE-Trysacryl was from IBF Biotechnics, France. Pronase was from Boehringer Mannheim, Castle Hill, NSW, Australia. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were from Novex Australia, Terry Hills, NSW. The PhosphorImager[spr2] and associated computer software were from Molecular Dynamics, Balwyn North, Victoria, Australia. Protease free chondroitinase ABC was from ICN Seven Hills, NSW, Australia.

CARTILAGE EXPLANT CULTURE

Cartilage was obtained within 6 h of death from the eight regions (Fig. 1) of both knee (stifle) joints of four skeletally mature (2-year-old) and five neonatal (±24 h of birth) purebred Merino sheep. Stifle joints were opened under sterile conditions and AC explants (3 mm diameter, full thickness) were collected from each joint using a rotary trephine. Four explants were obtained from each region of adult joints and two from the same regions of neonatal joints. The explants were blotted dry and initiated in culture in individual wells of a 48-well plate. Each well contained 0.5 ml Ham's F12 medium pH 7.2, supplemented with 76 mM $NaHCO_3$, 20 mM HEPES buffer, 10% FBS and 50 μg/ml gentamicin sulfate (Ham's/FBS). The explants were cultured at 37°C in an atmosphere of 5% CO_2 in air with 98% humidity for 72 h. The media was changed every 24 h and was supplemented with 5 μCi/ml $^{35}SO_4H_2$ for the final 48 h of culture. Media from each explant was collected and pooled separately over the 72-h culture period and stored at −20°C until analyzed.

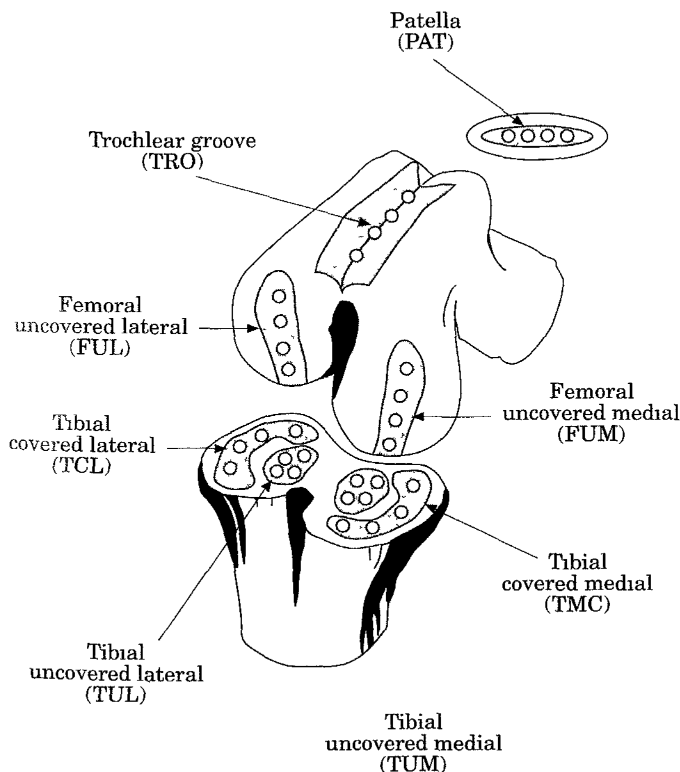

FIG. 1. The cartilage regions sampled for analysis. White circles represent 3 mm diameter full-depth explants collected using a rotary trephine. For alginate bead cultures all the cartilage from the different regions was removed using a scalpel blade (shaded areas) and the cells isolated by enzymatic digestion (see Materials and methods).

ALGINATE BEAD CULTURE

The AC from the same eight joint regions described for explant culture was collected from both stifle joints of four adult sheep. Full-depth regional cartilage (Fig. 1) was obtained using a scalpel blade and placed into separate sterile 15 ml tubes containing 10 ml Ham's/FBS. Chondrocytes were isolated by an initial 3 h digestion in 0.1% (w/v) pronase followed by a 14-h digestion in 0.04% (w/v) collagenase as described previously [28]. All digestions were performed at 37°C in Ham's/FBS. Cells were collected by centrifugation, washed twice in Ham's/FBS, and an aliquot counted on a hemocytometer after vital staining with trypan blue. Cell pellets were mixed with sodium-alginate solution [2% (w/v) Na-alginate in 0.15 M NaCl] to give a final concentration of 3×10^6 cells/ml. Alginate beads were made by extruding the cell suspension dropwise from a 21 gauge needle into 100 mM $CaCl_2$ solution. This resulted in 100 beads/ml with an average cell density of 30 000 cells/bead. Beads were allowed to cure in the $CaCl_2$ for 10 min before washing once with Ham's/FBS. Beads from each region of each joint were cultured separately in 25 ml of Ham's/FBS for 24 h. Four 10 bead aliquots of all samples were then cultured in individual wells of 48-well plates in Ham's/FBS with 5 μCi/ml $^{35}SO_4H_2$ and processed exactly as described for explants. The remaining beads from each region were maintained in bulk cultures for 3 weeks with media changes every 2–3 days. Aliquots of these beads were incubated for 48 h with 5 μCi/ml $^{35}SO_4H_2$ on days 5, 12 and 19.

PG CONTENT, SYNTHESIS AND RELEASE INTO CULTURE MEDIA

PG synthesis in explants and beads was determined by measuring the incorporation of $^{35}SO_4^{2-}$ into macromolecules. After papain digestion of explants or beads and their respective media, unincorporated $^{35}SO_4^{2-}$ was removed by $BaCl_2$ precipitation [29]. Newly synthesized PGs were then quantitated by liquid scintillation photometry of an aliquot of the sample. Incorporation of $^{35}SO_4^{2-}$ into PGs in both the matrix and culture media was expressed per μg DNA present in the explant/bead, measured fluorometrically with Hoechst 33258 dye using calf thymus DNA as a standard [30]. Endogenous PGs in the matrix and culture media were measured as sulfate glycosaminoglycan (GAG) using the dimethyl-methylene blue (DMB) assay [31] with bovine tracheal condroitin sulfate as a standard. This latter analysis was not possible in alginate bead cultures

because alginate interferes with the assay. Total sulfated–GAG in the explant matrix and released into the culture media were also expressed relative to the DNA content of the sample.

SIZE-EXCLUSION CHROMATOGRAPHY

Newly synthesized and endogenous PGs were extracted from the matrix of explants and alginate beads with 10 volumes of 4 M GuHCl, 0.15 M Na Acetate pH 6.3, for 48 h at 4°C in the presence of the proteinase inhibitors EDTA (25 mM), 6 amino-hexanoic acid (25 mM), N-ethyl malemide (10 mM) and benzamidine hydrochloride (5 mM). Aliquots of extracts were applied to Sephadex G-25 columns equilibrated with 0.5 M sodium acetate, pH 6.8 and the void volume containing the PGs collected. These samples were then subjected to size exclusion chromatography in the presence of excess HA (0.1 mg/ml) on a Sephacryl S1000 column (60×0.8 cm) equilibrated with the same buffer. The column was eluted at 20 ml/h and 1 ml fractions collected and monitored for radioactivity (DPM) in both explant and alginate bead cultures and sulfated PGs (DMB positive material) in explant cultures. Culture media samples were precipitated with three volumes of ethanol at 4°C for 12 h. The precipitates were washed twice with 75% ethanol and redissolved in 6 M Urea, 0.15 M $NaCl_2$, 0.02 M Tris pH 7.4. These solutions were applied to 1 ml columns of DEAE Trysacryl equilibrated in the same buffer, washed with three volumes of 0.15 M $NaCl_2$ and the PGs eluted with three volumes of 4 M GuHCl. Aliquots of the PG containing fractions were then desalted and chromatographed exactly as described for the extracts. Recovery of ^{35}S-PGs from ethanol precipitation and anion-exchange chromatography ranged from 95–98% and 93–98% respectively. The void volume of the Sephacryl S-1000 column was determined using aggregated bovine nasal cartilage A1-D1-PGs and the total volume with $^{35}SO_4^{2-}$.

SDS-PAGE AND WESTERN BLOTTING

Octyl-Sepharose chromatography of the cartilage extracts was performed to partially separate the hydrophobic DS-PGs from aggrecan [32]. We have previously shown that all of the DS-PGs in cartilage extracts can be collected by this technique although a small proportion of aggrecan also binds to the octyl-Sepharose [26]. The cartilage extracts were diluted 1:1 with 0.15 M sodium acetate pH 6.3 to give a final composition of 2 M GuHCl, 0.15 M sodium acetate pH 6.3. These samples were chromatographed on 1 ml columns of octyl-Sepharose CL-4B equilibrated in the same 2 M GuHCl buffer. The columns were washed with three volumes of aforementioned starting buffer to remove nonbound material, and the eluant then changed to 6 M GuHCl, 0.15 M sodium acetate pH 6.3 (three volumes) to remove the octyl-bound PGs. The radioactivity in the bound and nonbound PG fractions was measured by liquid scintillation photometry. In preliminary studies recovery of ^{35}S-PGs from octyl-Sepharose chromatography ranged from 98–100%. The octyl-bound PG samples were precipitated with three volumes of ethanol with overnight shaking at 4°C. The precipitates were collected by centrifugation washed twice with 75% ethanol, redissolved in distilled water and freeze dried.

Aliquots of freeze dried ocytl-bound PGs were dissolved in sample buffer (1% SDS, 1% β-mercaptoethanol, 0.0006% bromophenol blue, 16% glycerine, 80 mM Tris, pH 6.8) at a concentration of 2 mg/ml and heated to 100°C for 5 min. Samples were electrophoresed at 120 V for 2 h on 1.5 mm, 15-well 4–12% gradient gels, as previously described [2, 33]. The gels were fixed in 40% methanol, 8% acetic acid and stained with 0.0015% Coomassie R250, 0.001% alcian blue overnight (no de-staining required). Gels were preserved by washing in 40% methanol, 5% glycerol for 2 h and dried between cellophane. Samples of freeze dried octyl-bound PGs were also dissolved in 100 mM Tris HCl, 0.03 M Na acetate pH 8.0 and digested with chondroitinase ABC (0.01 U/μg) for 12 h before electrophoresis, as described above. The distribution of ^{35}S-PGs in dried SDS-PAGE gels was determined by phosphor screen autoradiography using a PhosphorImager® which has a linear response over a range of five orders of magnitude. The proportion of loaded $^{35}SO_4^{2-}$ in each electrophoretic band was determined by integration of these bands using the Image Quant® software supplied with this equipment.

Octyl-Sepharose-bound PGs were isolated from the cartilage remaining in adult and neonatal ovine joints after explant collection exactly as described above. Aliquots of these PGs were digested for 12 h with chondroitinase ABC. Duplicate aliquots of intact and chondroitinase digested PGs were electrophoretically separated, as described above, and transferred to a nitrocellulose membrane. Western blotting was performed essentially as described by Johnstone *et al.* (1993) [34] except that 0.1% (v/v) Tween 20 was used to block membranes rather than bovine serum albumin [35]. Antisera LF-96 and LF-94 to synthetic peptides corresponding to amino acids 11–24 and 5–17 of bovine biglycan and decorin,

respectively (generously provided by Dr Larry Fisher, NIH, Bethesda, MD, U.S.A.) were used at 1/100 dilution.

STATISTICAL ANALYSIS

Data were normalized by square root or logarithmic transformation. An analysis of variance (ANOVA) of transformed data was used to evaluate the association between joint region and PG content, DNA content, PG synthesis, and release of PGs into the culture media. When the F statistic was significant a Fisher's analysis of least significant difference was used to test for specific inter-regional differences. In order to simplify the presentation of results, and because the variation in loading is best defined for the covered and uncovered tibial plateau, only specific inter-regional differences between these regions are given. Differences were considered significant when $P \leq 0.05$.

Results

CARTILAGE DNA

Total micrograms of DNA/explant (3 mm diameter, full-thickness) from all regions of adult and neonatal joints was measured. Adult cartilage contained 0.97 ± 0.08–1.6 ± 0.27 μg DNA/explant compared with 21.4 ± 0.6–26.5 ± 2.1 μg DNA/explant in neonatal cartilage. There was no association between the amount of DNA/explant and joint region in adult ($P = 0.6$) or neonatal tissue ($P = 0.2$). Neonatal cartilage contained significantly more DNA/explant than adult cartilage in all regions ($P < 0.0001$). Because DNA was constant between regions all subsequent parameters were expressed per microgram DNA.

RESIDENT PG FROM EXPLANT CULTURES OF ADULT AND NEONATAL CARTILAGE

The concentration of PG (measured as sulfated GAG) in the cartilage matrix of different weight-bearing regions of adult and neonatal AC and the extent of release of this material into the culture media is shown in Fig. 2(a) and (b), respectively. There was a significant regional variation in the PG content of adult explants ($P < 0.0001$) with the two regions of the tibial plateau uncovered by the meniscus (TUM, TUL) containing significantly more PG/μg DNA than their covered counterparts (TCM, TCL) ($P = 0.006$ for TUM vs TCM, and $P < 0.0001$ for TUL vs TCL). There was also a significant variation in release of PGs into the

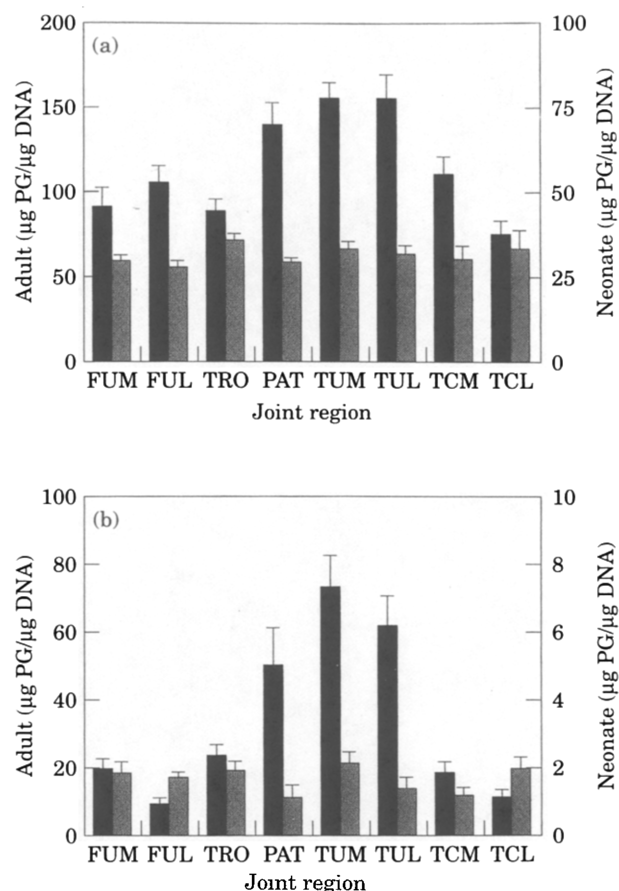

FIG. 2 A comparison of the resident PGs (μg GAG/μg DNA mean ± SEM) from the eight different weight-bearing regions (see Fig 1 for abbreviations) of adult ($N = 8$, black bars) and neonatal ($N = 10$; striped bars) joints. (a) The PG content of cartilage explant matrix (b) PGs released into culture media from explants ■ = Adult explants; ▩ = neonatal explants.

culture media by adult explants ($P < 0.0001$). The uncovered tibial regions released more PGs into the media than their covered counterparts ($P < 0.0001$ for both TUM vs TCM, and TUL vs TCL). In contrast, the AC of neonatal joints showed no regional difference in either PG content or release into culture media. The matrix PG content and release into media were both significantly lower in lambs than adults ($P < 0.0001$).

PG SYNTHESIS AND RELEASE INTO CULTURE MEDIA

The extent of incorporation of ^{35}S-PGs (DPM/μg DNA) into the matrix and their release into the culture media by adult cartilage explants, adult chondrocytes in alginate beads and neonatal explants are shown in Fig. 3(a) and (b). There was a significant regional variation in the incorporation of ^{35}S-PGs into the matrix by adult AC

explants ($P=0.006$). The tibial cartilages not covered by the menisci incorporated less ^{35}S-PGs than those beneath this tissue ($P=0.001$ for both TUM vs TCM and TUL vs TCL). When adult chondrocytes were cultured in alginate beads the overall synthetic activity was lower than in explants, but the topographical variation in $^{35}SO_4^{2-}$ incorporation into the matrix observed for explants was preserved ($P=0.03$). The total ^{35}S-PGs deposited into the matrix of cultured neonatal cartilage was less than for the adult cartilage explants. Most notable was the finding that there was no difference in ^{35}S-PG incorporation into cartilage from the different joint regions ($P=0.9$). There was no regional difference in the release of ^{35}S-PGs into the culture media by either adult explants, alginate beads or neonatal explants, however, there were differences in the ^{35}S-PG release between the three culture systems. Chondrocytes in alginate beads released significantly more ^{35}S-PGs into media, while explants of neonatal cartilage released significantly less ^{35}S-PGs than the corresponding adult explants [Fig. 3(b)].

The regional pattern of total $^{35}SO_4^{2-}$ incorporation into PGs was maintained by the chondrocytes in alginate beads for the 3-week duration of the study although all regions showed a decline over this time (data not shown). The DNA content, used as an index of cell number, doubled in beads from all regions by day 5 of culture and remained constant thereafter (data not shown). Further examination of the PGs synthesized by the chondrocytes in alginate beads was only undertaken for the day 3 bead cultures.

ANALYSIS OF ^{35}S-PGS

Representative size-exclusion chromatograms (under associative conditions in the presence of excess HA) of ^{35}S-PGs extracted from the cartilage–bead matrix of the lateral tibial cartilage covered by the meniscus (TCL) and uncovered by the meniscus (TUL) are shown in Fig. 4(a) and (b), respectively. Size exclusion chromatograms of ^{35}S-PGs released into the culture media from the cartilage/bead cultures of TCL and TUL are shown in Fig. 4(c) and (d), respectively. The ^{35}S-PGs extracted from the matrix of adult and neonatal explants and the alginate beads separated into two pools on Sephacryl S1000 chromatography, one excluded from the column and the other with K_{av} ~0.8 [Fig. 4(a) and (b), pool 1 and 2]. The majority (50–70%) of the PGs extracted from the matrix in all three culture systems formed high molecular weight aggregates with HA (pool 1, K_{av} 0–0.2). In extracts of adult cartilage explants there was an increased proportion of pool 2 PGs in uncovered tibial cartilage compared with other regions. In the alginate beads and neonatal explants the proportion of PGs in pool 2 was decreased in all regions compared with adult explants. Size exclusion chromatograms of the ^{35}S-media-PGs revealed two pools with similar distribution to the extracted PGs [Fig. 4(c) and (d)]. Pool 2 fractions were always better resolved for media from the alginate beads cultures than explant media. There was no major difference between regions in any culture system with regard to the chromatographic profile of the ^{35}S-media-PGs. The distribution of DMB-positive PGs from extracts of adult and neonatal explants followed an identical pattern to

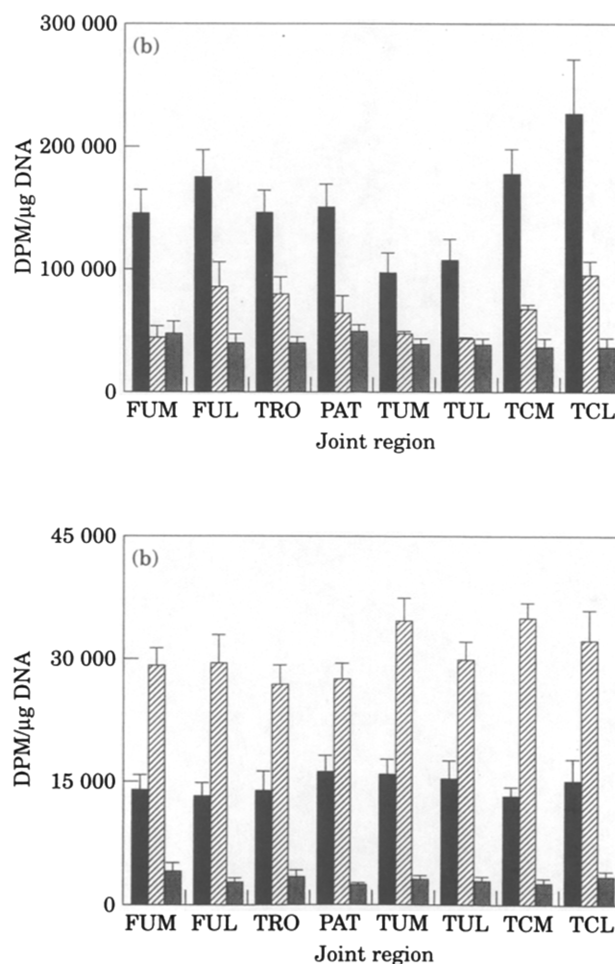

FIG. 3. A comparison of $^{35}SO_4^{2-}$ incorporation into PGs (DPM/μg DNA mean ± SEM) over 48 h of culture from the eight weight-bearing regions of joints (see Fig. 1 for abbreviations): adult explants ($N=8$; black bars), adult chondrocytes in alginate beads ($N=8$; striped bars) and neonatal explants ($N=10$; stippled bars) (a) $^{35}SO_4^{2-}$ incorporated into PGs retained in the matrix. (b) ^{35}S-PGs released into the culture media. ■ = Adult explants; ▨ = alginate beads; ▩ = neonatal explants

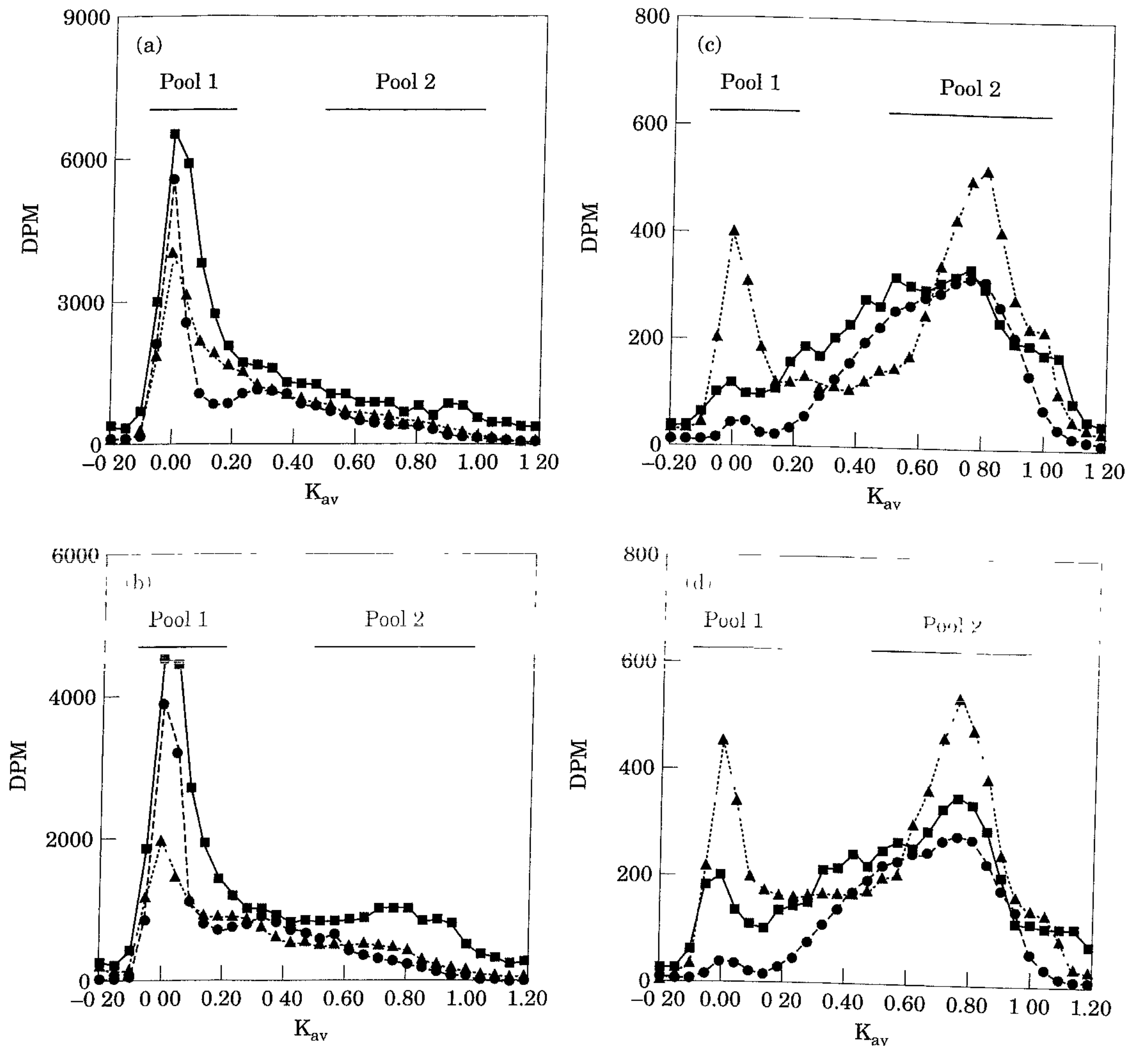

FIG 4 Associative size exclusion chromatograms of ^{35}S-PGs (in the presence of 0.1 mg/ml HA) from the lateral covered tibial cartilage [TCL; (a) and (c)] and lateral uncovered tibial cartilage [TUL; (b) and (d)] of adult cartilage explants, adult chondrocytes in alginate beads and neonatal cartilage explants (a) and (b) ^{35}S-PGs retained in the matrix. (c) and (d) ^{35}S-PGs released into the culture media. ■ = Adult explants; ● = neonatal explants; ▲ = alginate beads.

the ^{35}S-PGs (data not shown). The majority of the DMB-positive PGs released into the culture media were distributed as a broad peak with K_{av} ~0.5 (data not shown) in contrast to the ^{35}S-media-PG distribution shown in Fig. 4(c) and (d).

Representative examples of SDS-PAGE distribution of ^{35}S-PGs extracted from the matrix of uncovered lateral tibial cartilage (TUL) of adult explants, alginate beads and neonatal explants are shown in Fig. 5. The ^{35}S-octyl-bound-PGs extracted from adult explants separated into three broad but distinct bands (lane 1). The slowest migrating band failed to enter the resolving gel consistent with its assignment as aggrecan monomer [33], the second band had an MW ~250 kDa and the fastest migrating broad band had an MW ~116 kDa. A similar pattern of PG separation was evident in the octyl-bound-extract of alginate beads (lane 2) while in neonatal cartilage the PGs separated into the slow and intermediate migrating bands only (lane 4). In all three culture systems, the radioactivity in the intermediate and fast migrating PG bands was mostly eliminated with prior chondroitinase ABC digestion (lane 5) indicating that the $^{35}SO_4^{2-}$ in these bands was incorporated into chondroitin/dermatan sulfate rather than keratan sulfate. After chondroitinase digestion a core protein (MW ~45 kDa), which appeared as a doublet on the original gel, became apparent with Coomassie staining [Fig. 5(b) lane 2]. PGs that did not bind to octyl-Sepharose contained only the slowest migrating band, which failed to enter the resolving gel, confirming the complete removal of the faster migrating species by octyl-Sepharose chromatography [Fig. 5(a), lane 6].

The intermediate and fast migrating ^{35}S-PG

bands were assigned as biglycan and decorin, respectively, based on their interaction with octyl-Sepharose, their migration on SDS-PAGE, the loss of radio-activity after chondroitinase ABC digestion and the appearance of a core protein with a MW of ~45 kDa after chondroitinase ABC digestion [2, 32, 33]. The results of Western blotting using decorin- and biglycan-specific antisera confirmed the identity of these two PGs (Fig. 6). Antibodies to biglycan cross-reacted with PGs in both the intermediate and fast migrating bands (Fig. 6, lane 2), however, chondroitinase digestion before electrophoresis eliminated only the immunoreactivity in the intermediate band (Fig. 6, lane 5). This indicated that the biglycan immunoreactivity in the fast migrating band was not associated with PGs containing chondroitin/dermatan sulfate GAG chains. While not investigated further, this biglycan immunoreactive material may represent cross reactivity of the antibody with the homologous keratan sulfate containing small PG, fibromodulin. Immunoreactivity with anti-decorin was confined to the fast migrating band of intact PGs (Fig. 6, lane 4) and again was eliminated

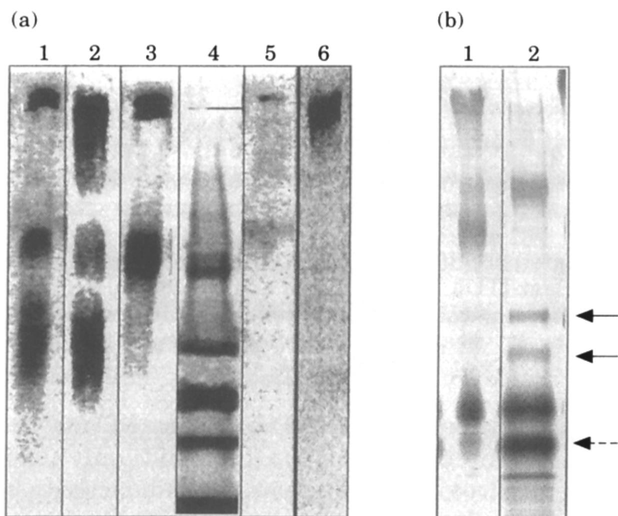

FIG 5. (a) PhosphorImage of representative samples demonstrating the 4–12% SDS-PAGE distribution of ^{35}S-PGs extracted from the matrix of lateral uncovered tibial cartilage cultures (lanes 1–5 are PGs that bound to octyl-Sepharose and lane 6 is PGs that did not bind to octyl-Sepharose): lane (1) adult explants, lane (2) adult chondrocytes in alginate beads, lane (3) neonatal explants, lane (4) ^{14}C-labeled protein standards (200, 97.4, 69, 46 and 30 kDa from top to bottom, respectively), lane (5) adult explant PGs digested with chondroitinase ABC before electrophoresis, lane (6) adult explants. (b) Coomassie stained gel of adult explant PGs as in 5(a) lane 1: (1) without and (2) after chondroitinase ABC digestion. Digestion with chondroitinase ABC produced a protein band with a MW ~45–50 kDa (open arrow). Solid arrows indicate protein bands present in the chondroitinase ABC preparation.

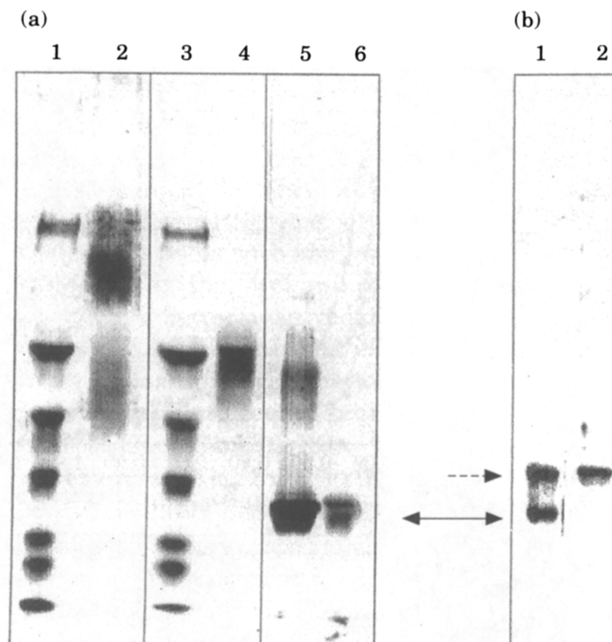

FIG 6. Western blot of octyl-Sepharose bound PGs. Lanes 1–6 = adult sheep and lanes 7 and 8 = neonatal sheep. Lanes 1 and 3 are pre-stained globular protein standards (250, 98, 64, 50, 36, 16 and 6 kDa from top to bottom, respectively). Lanes 2, 5 and 7 blotted with antibody LF-96 against a segment of bovine biglycan core protein. Lanes 4, 6 and 8 blotted with antibody LF-94 against a segment of bovine decorin core protein. Lanes 5, 6, 7 and 8 were digested with chondroitinase ABC before electrophoresis Solid arrow indicates immunoreactive biglycan and decorin core proteins present after chondroitinase digestion. Open arrow indicates the unidentified ~55 kDa protein present in neonatal samples which reacted with antisera to both biglycan and decorin.

with prior chondroitinase ABC digestion (Fig. 6, lane 6). Chondroitinase ABC digestion of adult octyl-bound samples resulted in the appearance of core protein bands with an MW ~45 kDa which immunoreacted with both DS-PG antibodies (Fig. 6, lanes 5 and 6, solid arrow). In chondroitinase ABC digested neonatal but not adult octyl-bound samples, a protein with an MW ~55 kDa, which reacted with both biglycan and decorin anti-sera, was detected (Fig. 6, lanes 7 and 8, open arrow). The identity of this band was not investigated further. Specific immunoreactivity with the anti-biglycan sera was evident in the chondroitinase digested neonatal samples (MW ~45 kDa), however, immunoreactive decorin core protein was not detected.

It was concluded from these studies that although material that immunoreacted with biglycan was detected in both the intermediate and fast migrating SDS-PAGE bands, the ^{35}S-containing biglycan was confined to the intermediate band.

Thus quantitation of the $^{35}SO_4^{2-}$ in the intermediate and fast migrating bands, which unlike the immunoreactivity could be eliminated with prior chondroitinase ABC digestion, would enable measurement of the synthesis of glycosylated biglycan and decorin, respectively.

DS-PG QUANTITATION

The proportion of $^{35}SO_4^{2-}$ in the matrix that was incorporated into the DS-PG electrophoretic bands (biglycan plus decorin) in the three culture systems was calculated from the PhosphorImage scans and is shown in Fig. 7. There was a significant regional variation in incorporation of ^{35}S-DS-PGs into the matrix of adult explants ($P=0.001$). The two regions of the tibial plateau not covered by the meniscus incorporated significantly more ^{35}S-DS-PGs into their matrix than their covered counterparts ($P=0.002$ for both TUM vs TCM and TUL vs TCL). This significant regional difference was maintained by the chondrocytes when cultured in alginate beads ($P < 0.0001$). In neonatal explants the incorporation of ^{35}S-DS-PGs into the matrix was lower than adults and there was no significant difference between regions.

Whilst a subtle regional variation in biglycan synthesis may exist, there was no significant difference in the regional incorporation of ^{35}S-biglycan into adult cartilage matrix, alginate bead matrix or neonatal cartilage matrix [Fig. 8(a)]. It is possible that use of a larger number of animals may have enabled differences in biglycan synthesis to be detected, however, the numbers were sufficient for the incorporation of newly synthesized decorin into the matrix of adult explants and alginate beads to show a significant regional variation [$P < 0.001$ for both adult explants and beads. Fig. 8(b)]. The uncovered tibial cartilages incorporated more ^{35}S-decorin into their matrices than their covered counterparts. In neonatal cartilage the synthesis of ^{35}S-decorin was barely detectable.

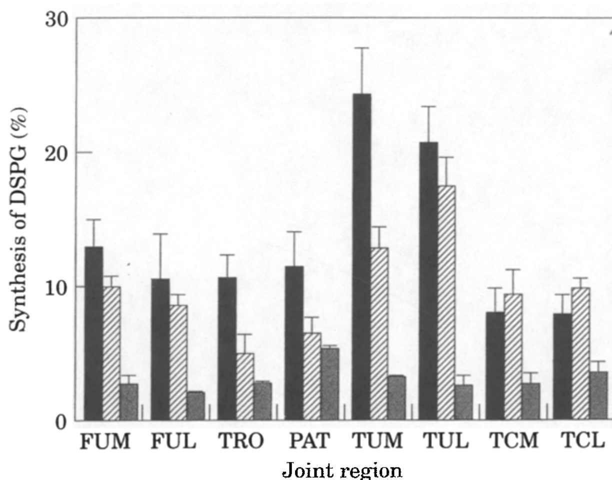

FIG. 7. The percentage of matrix $^{35}SO_4^{2-}$ that was incorporated into DS-PGs (DPM/µg DNA mean ± SEM) from different joint regions in adult explants ($N=8$; black bars), adult chondrocytes in alginate beads ($N=8$; striped bars) and neonatal explants ($N=10$; stippled bars) as determined from PhosphorImage scans (see Fig. 5). ■ = Adult explants; ▨ = alginate beads; ▩ = neonatal explants

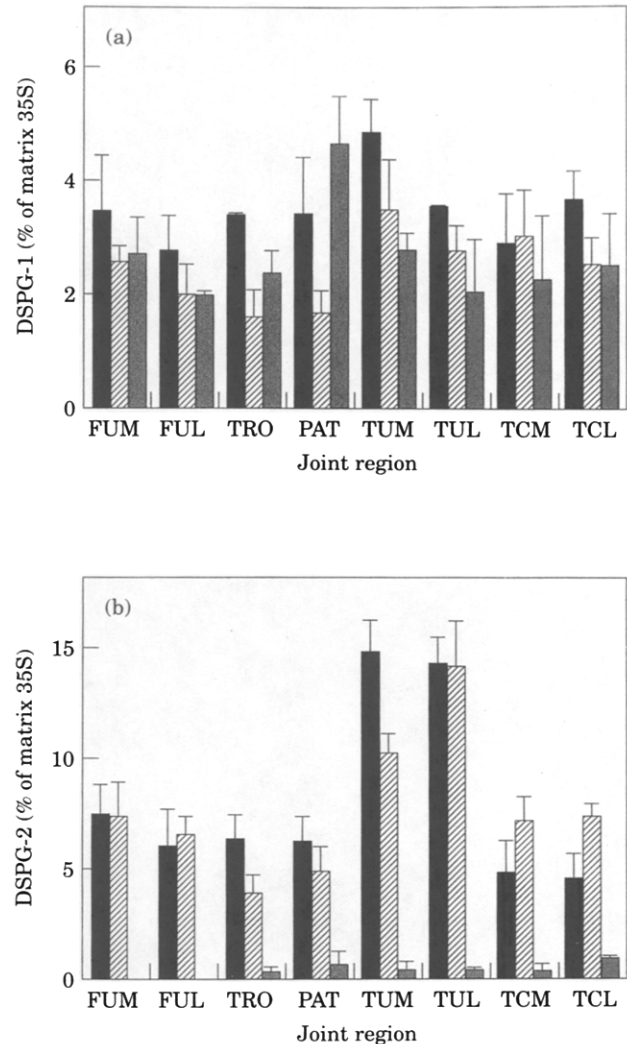

FIG. 8 The percentage of matrix $^{35}SO_4^{2-}$ that was incorporated into (a) biglycan and (b) decorin (DPM/µg DNA mean ± SEM) from different joint regions in adult explants ($N=8$; black bars), adult chondrocytes in alginate beads ($N=8$; striped bars) and neonatal explants ($N=10$; stippled bars) as determined from PhosphorImage scans (see Fig. 5). ■ = Adult explants; ▨ = alginate beads, ▩ = neonatal explants.

Discussion

In adult ovine knee joints, as in humans, the tibial AC uncovered by the meniscus is subject to high compressive loads compared with the regions under the meniscus [36, 37]. The relative mechanical stresses experienced by the other joint regions evaluated in the present study have not been well defined. Nevertheless basic principles would indicate that as the femoral condyles rotate across the stationary tibial plateaux, the stresses borne by the femoral cartilages would be distributed over a greater surface area than the opposing tibial cartilages. Similarly, as the patella moves along the trochlea groove during joint flexion, the loads on the trochlea cartilage are distributed over the length of the groove. Accordingly, cartilage on the femoral condyles are subjected to less focal stress than the contact area on the corresponding uncovered tibial plateau. Likewise, the trochlea groove cartilage experiences less contact stress than the patella cartilage. In the present study the cartilage areas subjected to high contact stresses (TUL and TUM compared with TCL, TCM, FUM and FUL; PAT compared with TRO) were found to contain more PG but incorporated less $^{35}SO_4^{2-}$ into PGs per microgram DNA than low stress areas. These findings were consistent with previous reports which advocated a primary role of the matrix PG content (as fixed charge density) in modulating chondrocyte PG synthesis [11, 12].

Articular chondrocytes [4, 5], as well as meniscal fibrochondrocytes [38], have been shown to maintain their normal biosynthetic phenotype when cultured in alginate beads. Our experiments have demonstrated that chondrocytes from different weight-bearing regions of ovine joints, when cultured in alginate beads with identical cell densities and conditions, preserved the topographical differences in total $^{35}SO_4^{2-}$ incorporation/μg DNA observed for explants derived from the same regions. This topographically dependent chondrocyte biosynthetic expression, and thus phenotype, was observed for up to 3 weeks in culture, despite cell replication within the alginate beads. When the newly synthesized PGs that were retained in the explant matrices were separated into different types by SDS-PAGE it was found that the AC regions exposed to high-contact stress *in-vivo* synthesized a greater proportion of small non-aggregating ^{35}S-DS-PGs, in particular decorin, than less stressed regions. This regional difference in DS-PG synthesis observed for adult explant cultures was maintained when the chondrocytes were cultured in alginate beads. These data lead us to suggest that AC from joint regions exposed to different mechanical loads contains phenotypically distinct chondrocyte populations.

In order to investigate whether the regional variations observed in adult explant and alginate bead cultures were associated with inherently distinct cell populations, the metabolism of PGs by explants from the same joint regions of neonatal sheep that had not borne weight was investigated. In these experiments we could not demonstrate a topographical pattern in total PG synthesis nor in the proportions of PG types that were synthesized by neonatal explants. These data were consistent with the hypothesis that the regional chondrocyte phenotype observed in both explant and bead culture for adult cartilage resulted from environmental factors imposed on the joint after birth, the most notable being weight bearing and articulation.

The cell density of AC has been shown to decline post-natally up to the time of skeletal maturity after which it remains relatively stable [39, 40]. This post-natal decline in chondrocyte numbers involves a selective loss of cells from the superficial zone [39, 40]. Furthermore, the distinct zonal populations of chondrocytes do not become apparent until maturity [27]. It would appear plausible, therefore, that the post-natal mechanical stresses imposed on cartilage of different joint regions as a consequence of weight bearing, may select for different clones of cells in those regions. This interpretation is compatible with the studies of others. Mechanical stress has been shown to modulate the maturation and differentiation of chondroblasts in the AC of the rat mandibular condyle during maturation [41]. Increased mechanical loading has been reported to delay maturation and differentiation of mesenchymal cells into chondrocytes [42]. Basdra *et al.* [42] have suggested that this effect may be mediated by altered phosphorylation of intracellular proteins.

Because it is well accepted that chondrocytes from the superficial cartilage zone are phenotypically distinct from deeper cells, the question arises as to whether the differences in chondrocyte phenotype observed in the present study were simply related to variations in the proportion of superficial versus deep cells in the various joint regions. The percentage of chondrocytes with superficial zone morphology has been found to vary topographically within joints, being related to the magnitude of stress to which the AC had been exposed during weight bearing [43, 44]. Cartilage from high weight-bearing regions such as the tibial plateau not covered by the meniscus, has been shown to contain fewer superficial type cells than tissue from low stress areas despite

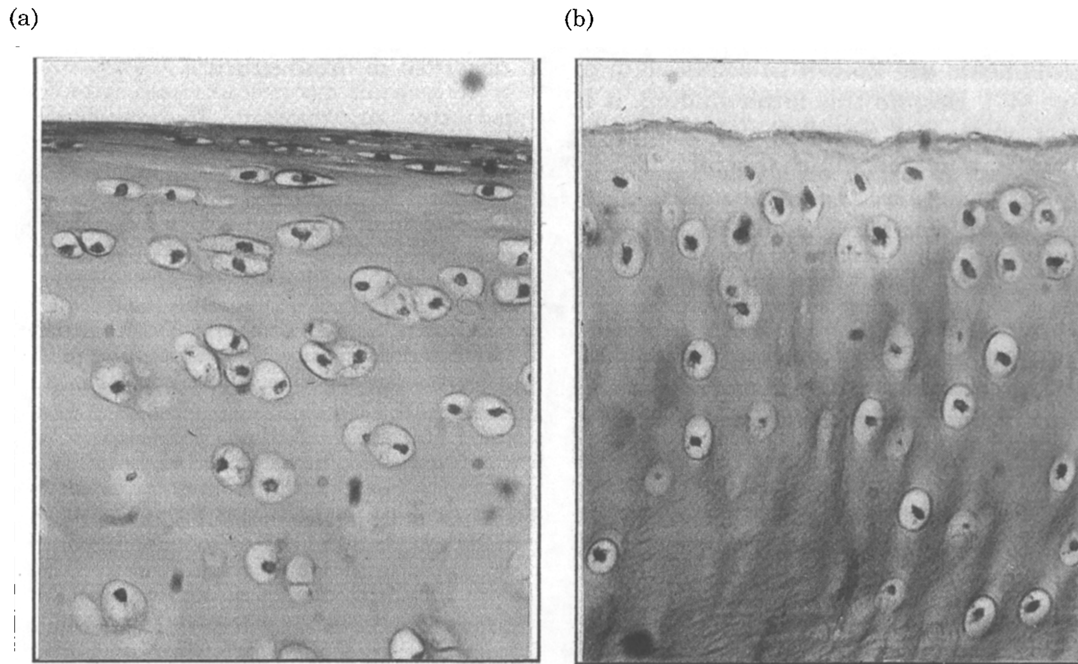

FIG 9. Histological appearance of the superficial cartilage zone of 2-year-old sheep. (a) Tibial plateau covered by the meniscus and (b) tibial plateau uncovered by the meniscus. Note the lack of flattened cells in the uncovered tibial plateau (Toluidine blue/fast green. Original magnification ×40.)

similar total cell numbers [42, 44]. Histological examination revealed that an identical situation exists in the 2-year-old sheep examined in the present study (Fig. 9). Such differences in chondrocyte populations, however, would not account for the regional variations in PG metabolism observed in the present study. Given the biosynthetic profile of superficial compared with deep zone chondrocytes, a reduced proportion of superficial zone cells in high weight-bearing cartilage regions, such as the tibial plateau not covered by the meniscus, would be expected to result in elevated total $^{35}SO_4^{2-}$ incorporation and decreased synthesis off DS-PGs relative to tissue containing more superficial zone cells (low weight-bearing regions). Such a metabolic profile was not observed in the present experiments.

In the present study the chondrocytes were all cultured in the presence of 10% FBS. The metabolic differences observed between chondrocytes from distinct joint regions under these conditions could be related to a variable response to growth factors present in the culture media. In this regard it should be noted that we have previously demonstrated that the chondrocytes from the tibial plateau uncovered by the meniscus showed a significantly greater increase in PG synthesis in response to exogenous transforming growth factor-β (TGF-β) (1 ng/ml) than cells cultured from the tibial cartilage covered by the meniscus [45]. We consider that this finding supports the concept of regional chondrocyte phenotypic heterogeneity in articulating joints.

Differences in the DS-PG species synthesized by neonatal and adult cartilage explants were demonstrated in the present study. Neonatal explants synthesized and incorporated predominantly ^{35}S-biglycan into the cartilage matrix while only negligible amounts of ^{35}S-decorin could be detected. While explants from adult joints incorporated the same amount of ^{35}S-biglycan into their matrices as neonatal tissue, the levels of ^{35}S-decorin present were higher than those for biglycan. This difference in the relative synthesis biglycan and decorin was consistent with reports on the maturation related change in DS-PG metabolism by human chondrocytes [1, 46]. However, we found that biglycan synthesis remained constant with maturation rather than undergoing a decline as reported by Melching and Roughly [1]. Decorin synthesis increased with maturation in our studies and eventually superseded that of biglycan. This latter finding was in agreement with previous reports [1, 46]. It should be noted that in both the present study and that by Melching and Roughley [1], only ^{35}S-labeled DS-PGs incorporated into the matrix were quantitated. Inadequate SDS-PAGE separation of the ^{35}S-PGs released into the culture media from explants precluded their accurate quantitation in

the present study. GAG-free DS-PG core proteins and their fragments are known to accumulate in AC with age [47]. Despite this latter finding, it is evident from studies of maturational change in DS-PG synthesis in human AC, that similar results were obtained whether the concentration of DS-PGs was quantitated by immunologic means [46] or by measuring the ^{35}S-labeled DS-PGs retained in the matrix [1].

The increased incorporation of decorin into the matrix of mature cartilage relative to neonatal tissue is compatible with the suggested role of this PG in collagen matrix organization [9, 48]. With the onset of weight bearing, the structural organization of the cartilage collagen network would be critical for the optimal mechanical functions required of the tissue [49]. Bullough *et al.* [43] have demonstrated that the collagen fibres in the uncovered tibial cartilage (high-stress region) were more uniform in diameter, thinner and arranged in parallel bundles as compared with collagen fibres in the cartilage region covered by the meniscus (low-stress region). This regional difference in collagen architecture is consistent with the described effect of decorin on collagen fibrillogenesis *in-vitro* [48]. The enhanced incorporation of decorin into the matrix of highly stressed cartilage regions in the adult ovine (uncovered tibial cartilage) is again supportive of its role in the mechanical integrity of the tissue. The functional role of biglycan in AC is presently unknown but significantly in the present study we could not demonstrate any association between biglycan synthesis and joint region and hence mechanical loading of AC. This finding is in agreement with the work of Visser *et al.* [50] who demonstrated that mature bovine chondrocytes retained in the AC matrix responded to *in-vitro* cyclic mechanical loading by increasing decorin synthesis while biglycan synthesis remained at steady-state levels.

In conclusion these studies have demonstrated a topographical variation in PG biosynthesis by chondrocytes in adult ovine AC. This regional difference in PG synthesis was not demonstrable in cultures of neonatal cartilage that had not been exposed to weight-bearing stress. The extracellular matrix was not found to be the primary determinant in controlling PG biosynthesis by chondrocytes, as has been hitherto postulated [11, 12], because adult chondrocytes cultured in alginate beads retained their regional phenotype with respect to PG metabolism. The presence of distinct chondrocyte populations in different joint regions, and their variable response to catabolic and anabolic stimuli may be important in understanding the focal nature of cartilage degeneration observed in osteoarthritis.

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