Proteoglycan synthesis in organ cultures from regions of bovine tendon subjected to different mechanical forces

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Synthesis of proteoglycans by morphologically and chemically distinct regions of bovine flexor tendon was investigated in explant cultures. Proximal regions of the flexor tendon which experience only tensile forces and have low contents of proteoglycans initially exhibited relatively low rates of proteoglycan synthesis but high rates of collagen synthesis. The predominant proteoglycan produced by all proximal explants was of small hydrodynamic size and appeared similar to that extracted from proximal tissue. In contrast, explants derived from the distal tendon region, which experiences frictional and compressive forces in addition to tensile forces, and has a high content of proteoglycans, showed relatively high initial rates of proteoglycan synthesis and lower rates of collagen synthesis. These distal explants produced primarily large proteoglycans on the first day in culture. Turnover of newly synthesized proteoglycans was not detectable in proximal tissue, and was low in distal tissue. Loss of unlabelled proteoglycan from proximal and distal explants was not detected during the 12 days of culture. These observations suggest that the increase in specific types of proteoglycans in regions of tendon subjected to frictional and compressive forces is the result of elevated synthesis rates in this tissue. Two alterations in proteoglycan synthesis occurred during the 12-day culture period. (1) The rate of proteoglycan synthesis by all explants increased with time in culture. (2) The proportion of small proteoglycans synthesized by distal explants increased from 32% of the total proteoglycan produced on day 1, to 80% of that produced on day 12. Explants from proximal tendon continued to produce only small proteoglycans throughout the 12 days in culture. This switch in proteoglycan phenotype, resulting in decreased synthesis of large proteoglycans by the distal tissue, may be due to a lack of compressive forces on the cultured explants.

INTRODUCTION

Fibrous skeletal connective tissues which experience compressive loads *in situ* have elevated contents of cartilage-like proteoglycans (Roughley *et al.*, 1981; Vogel & Heinegård, 1985; Koob & Vogel, 1987). These molecules are of large hydrodynamic size, contain glycosaminoglycan chains contributing high negative charge densities, and, if comparable with cartilage proteoglycans, provide the tissue osmotic resistance to compressive loading. The localization of these proteoglycans to sites receiving compressive loads suggests that the differentiating stimulus could be the compressive loads themselves. However, little experimental evidence is available on the biochemical development of the connective-tissue matrices in mechanically specialized fibrocartilaginous tissues to confirm or refute this hypothesis.

We have found that regions of adult bovine flexor tendon which experience frictional and compressive loads in passing through a stiff paratendinous sheath under the sesamoid bones of the foot have a fibrocartilaginous organization, elevated glycosaminoglycan contents and swelling properties similar to those of articular cartilage (Koob & Vogel, 1987). The predominant proteoglycans of this compressive region are large molecules ($M_r = 10^6$) containing abundant chondroitin sulphate and keratan sulphate chains (Vogel & Heinegård, 1985; Vogel & Thonar, 1987). In contrast, the proximal region of the flexor tendon, which experiences only tensional forces, contains only small amounts of proteoglycan. The predominant proteoglycan

population of this region is of small hydrodynamic size ($M_r = 10^5$) and contains dermatan sulphate (Vogel & Heinegård, 1985). The present paper describes proteoglycan synthesis by cultured explants of these morphologically and chemically distinct regions of the deep flexor tendon.

MATERIALS AND METHODS

Materials

[35 S]Sulphate (280–330 mCi/mmol) and L-[2,3- 3 H]-proline (27.2 Ci/mmol) were purchased from New England Nuclear. Minimal Essential Medium (Eagle), penicillin (10 000 units/ml), streptomycin (10 000 μ g/ml) and mycostatin (10 000 units/ml) were obtained from Gibco Laboratories, and heat-inactivated newborn-calf serum was supplied by Sterile Systems. Sephadex G-50 (medium grade) and Sepharose CL-4B and CL-6B were purchased from Pharmacia. DEAE-cellulose DE-52 was obtained from Whatman. Papain was purchased from Sigma Chemical Co., and chondroitin ABC lyase was supplied by Miles Scientific. Screw-cap 20 ml glass vials (Wheaton) were used for culture flasks.

Cultures

Feet from freshly slaughtered 1–5-year-old cattle were obtained locally (Karler Packing Co., Albuquerque) and prepared for culture as previously described (Vogel *et al.*, 1986). After excision of the deep flexor tendon together with the surrounding paratendinous sheath, the flexor tendon was dissected free of paratendinous adhesions

Fig. 1. Dissection of bovine flexor tendon for explant culture

Full-thickness cross-sections, indicated by the vertical broken lines, of proximal (tensional) and distal (compressional) regions were further dissected into surface (S), middle (M) and deep (D) layers. The surface layer was taken from the anterior aspect of both proximal and distal segments.

and the distal sheath while immersed in Hanks Balanced Salt Solution. The proximal (tensional) and distal (compressional) tendon segments were separated (1 cm of tissue on either side of the bifurcation being discarded), and placed in Puck's Saline F Solution containing 100 units of penicillin/ml and 100 μ g of streptomycin/ml. Proximal and distal segments were further divided into three surface to deep longitudinal layers, as shown in Fig. 1. The surface layer of the distal segment articulates with the sheath and bone, and the deep layer was taken from the opposite, non-articulating, side. We have previously shown that the anterior surface of this region and extending partially into the tendon is tissue with collagen fibres organized as a random network and accounting for approx. 68% of tissue mass (Koob & Vogel, 1987). This surface layer has glycosaminoglycan hexosamine contents of 1.5–3% of dry weight. The middle tissue contains less hexosamine, 0.5–0.75%, and the deep non-articulating layer opposite the compressed surface contains 0.25–0.4% glycosaminoglycan hexosamine. An identical orientation was applied to layers from the proximal segment, which, in contrast with the distal segment, is homogeneous throughout its thickness. The proximal region of the tendon had collagen contents of 80–85% of dry weight; this region contains only 0.2% glycosaminoglycan hexosamine.

Tendon layers were minced into 1 mm³ explants and washed in Puck's Saline F. Explants from each layer were randomized by thorough mixing, and 15–20 pieces (approx. 300 mg wet wt.) of tissue were placed in sterile 20 ml screw-cap glass vials. Preliminary experiments, varying the amount of tissue per culture flask from 50 to 600 mg, were performed in order to determine the optimal tissue-to-medium ratio for proteoglycan synthesis. The results showed that maximal proteoglycan synthesis was achieved by tissue-to-medium ratios of no more than 400 mg/6 ml. Explants were cultured in 6 ml of Minimal Essential Medium supplemented with 10% (v/v) newborn-calf serum, tryptose/phosphate broth, 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 100 units of mycostatin/ml. The medium was replaced

daily, and cultures were maintained up to 12 days at 37 °C in an atmosphere containing 5% CO₂.

Labelling of cultures

Labelling medium containing 20 μ Ci of [³⁵S]-sulphate/ml or 10 μ Ci of L-[2,3-³H]proline/ml was prepared in sufficient quantity for all flasks of each experiment on the initial labelling day. Labelling medium was Minimal Essential Medium supplemented as described above. Explants were allowed to incorporate labelled compounds for 24 h.

Determination of incorporation rates

Explants labelled with [³⁵S]sulphate were washed with 3 × 20 ml of Hanks solution to remove unincorporated label, and were subsequently freeze-dried. Dry weights were obtained, and the dried tissues were digested with papain (50 μ g/ml) in 0.1 M-sodium acetate/10 mM-EDTA/10 mM-2-mercaptoethanol, pH 5.5, for 24 h at 60 °C. Portions of the papain digests were chromatographed on 8 ml Sephadex G-50 columns equilibrated in 4 M-guanidine hydrochloride/0.05 M-sodium acetate/0.5% Triton X-100, pH 6.0; 0.5 ml fractions were collected and the radioactivity in each fraction was measured. Radioactivity appearing in the macromolecular fractions was totalled and normalized to tissue dry weight. Samples of culture medium from labelled explants were directly chromatographed on 8 ml Sephadex G-50 columns, and the total macromolecular radioactivity was normalized to tissue dry weight. Over 90% of the ³⁵S-labelled macromolecular material produced during the labelling period was associated with the tissue, and all observations reported here relate only to this tissue-associated radioactivity.

Explants labelled with L-[2,3-³H]proline were similarly washed, dried and weighed. Dried tissues were then hydrolysed in 6 M-HCl at 108 °C for 24 h in Teflon-lined screw-cap culture tubes. Hydrolysates were evaporated to dryness, then redissolved in 0.2 M-sodium citrate, pH 2.2, and applied to a 20 cm × 0.9 cm column of Dowex AG-50W (200–400 mesh). The column was eluted with 0.2 M-sodium citrate, pH 3.5; 2 ml fractions were collected. A portion of each fraction was assessed for radioactivity. Preliminary experiments showed that this method sufficiently separated [³H]hydroxyproline from [³H]proline. Total [³H]hydroxyproline was normalized to tissue dry weight.

Turnover of ³⁵S-labelled proteoglycans

Explants of proximal and distal surface, middle and deep layers were allowed to incorporate [³⁵S]sulphate (20 μ Ci/ml) during the first 24 h *in vitro*. Explants were then washed twice with medium lacking label and cultured for 2 days in the absence of [³⁵S]sulphate. Preliminary experiments showed this 2-day chase to be necessary in order to eliminate unincorporated label from the tissue. Distal explants retained significant amounts of free [³⁵S]sulphate even after repeated washings in medium, and this resulted in an increase in labelled proteoglycans on days 1 and 2 of the chase. By day 3 after labelling, free [³⁵S]sulphate had been eliminated from distal explants, and therefore this day was chosen as day 0 for measurement of labelled-proteoglycan turnover. Explants were collected on this day and on days 1, 2 and 3 after it. Labelled tissue was washed, dried and weighed, and the amount of

macromolecular [^{35}S]sulphate remaining in the tissue was determined by chromatography on Sephadex G-50. It should be noted that the initial 2-day chase of labelled distal explants obviated detection of any minor pools of rapidly metabolized proteoglycan.

Turnover of tissue proteoglycans

The rate at which the proteoglycans present in the tissue at the initiation of culture were lost in culture was assessed by quantifying glycosaminoglycan hexosamine contents in tissue and medium with time *in vitro*. Explants were collected, washed with Hanks solution and freeze-dried. Dried tissue was digested with papain as previously described. Glycosaminoglycans were partially purified from papain digests by diluting samples of the papain digests 1:1 with water and chromatographing them on 1 ml DEAE-cellulose DE-52 ion-exchange columns equilibrated in water. After sample loading, the columns were flushed with water and the weakly adhering material was eluted with 0.02 M-HCl. Glycosaminoglycans were next eluted into Teflon-lined screw-cap culture tubes with 6 M-HCl and hydrolysed at 100 °C for 5 h. Hexosamine content in dried hydrolysates was quantified by the Elson-Morgan method as adapted by Antonopoulos *et al.* (1974) and normalized to dry weight. Culture medium was assessed for proteoglycan degradation products by quantifying glycosaminoglycan hexosamine contents in dialysed, freeze-dried and hydrolysed medium from days 3, 6, 9 and 12 of culture.

Extraction and analysis of ^{35}S -labelled proteoglycans

Proximal and distal surface, middle and deep explants were allowed to incorporate [^{35}S]sulphate for 24 h on days 1, 5 and 12 of culture. The labelled tissue was washed with Hanks solution and extracted twice at 4 °C for 24 h, first with 10 vol. and then with 5 vol. of 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 6.0, containing the proteinase inhibitors 0.1 M-EDTA, 0.01 M-N-ethylmaleimide, 0.005 M-benzamidine hydrochloride and 0.001 M-phenylmethanesulphonyl fluoride. Extracts were centrifuged at 27000 g for 20 min at 4 °C. Samples of the supernatant were chromatographed directly on Sephadex G-50, and the macromolecular [^{35}S]sulphate was determined. Residues were dried, digested with papain and similarly chromatographed on Sephadex G-50, and the macromolecular [^{35}S]sulphate was quantified. Extraction efficiencies, based on the amounts of labelled proteoglycans in the supernatant and residue, were 56% and 59% for the proximal and distal explants respectively.

Samples of the extract were chromatographed on a 1 cm $\times$ 33 cm Sephadex G-50 column equilibrated with 7 M-urea/0.05 M-sodium acetate/0.1 M-NaCl/0.5% Triton X-100, pH 7.0. The macromolecular fractions were combined and the proteoglycans purified and concentrated by chromatography on a 0.5 ml column of DEAE-cellulose DE-52 equilibrated in the same 7 M-urea buffer. After application of the sample, the column was flushed with 6 ml of this buffer, but containing 0.2 M-NaCl. The proteoglycans were then eluted in 0.6 ml of 4 M-guanidine hydrochloride/0.05 M-sodium acetate/0.5% Triton X-100, pH 6.0.

For characterization of the size of newly synthesized proteoglycans, samples of the partially purified labelled proteoglycans were directly applied to a 0.5 cm $\times$ 60 cm Sepharose CL-4B column equilibrated in 4 M-guanidine

hydrochloride, 0.05 M-sodium acetate/0.5% Triton X-100, pH 6.0; 200 μl fractions were collected, and the radioactivity was determined. The relative sizes of glycosaminoglycan chains on small proteoglycans labelled on day 12 were determined by elution from a 0.7 cm $\times$ 88 cm column of Sepharose CL-6B eluted with 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 6.0; 1 ml fractions were collected. Glycosaminoglycan chains were released from the intact proteoglycan by treatment with 0.05 M-NaOH for 18 h at 37 °C.

Both labelled and unlabelled proteoglycans from explants were also characterized by SDS/polyacrylamide-gel electrophoresis. Samples of the proteoglycan preparation from DEAE-cellulose DE-52 chromatography were dialysed, freeze-dried and reconstituted in 50 μl of water. A 10 μl portion of this solution was incubated for 1 h at 37 °C in buffer (0.05 M-Tris/acetate, pH 8.0) with or without addition of 0.01 unit of chondroitinase ABC; samples were applied to a linear 4–20% -acrylamide gradient gel. Overnight electrophoresis was performed and gels were stained with Alcian Blue and Coomassie Blue, as previously described (Fisher *et al.*, 1983). After photography, the stained gels were soaked in 1 M-sodium salicylate/0.002 M-sodium phosphate, pH 7.0, for 1 h, dried on to filter paper, and exposed to pre-flashed film (Kodak X-OMAT) at –70 °C for 5 days in order to detect newly synthesized ^{35}S -labelled components.

RESULTS

Rates of collagen and proteoglycan synthesis in proximal and distal tendon explants

Initial experiments measured the rates of collagen and proteoglycan synthesis in explants prepared from proximal and distal tendon segments on days 1, 2, 3, 4, 8 and 12 of culture. Fig. 2(a) shows collagen synthesis rates based on amounts of [^3H]hydroxyproline produced during the 24 h labelling period per g dry wt. On day 1 of culture, the rate of collagen synthesis in proximal explants was 3-fold higher than that in distal explants. Collagen synthesis declined in proximal explants over the next 2 days, then remained nearly constant for the remaining 10 days. Collagen synthesis remained relatively constant in distal explants for the entire culture period. In contrast, proteoglycan synthesis rates measured as [^{35}S]sulphate incorporation were higher in distal than in proximal explants on day 1 of culture (Fig. 2b). Proteoglycan synthesis remained relatively constant in these distal cultures, increasing somewhat near the end of the culture period. Proximal explants produced relatively little proteoglycan on day 1 in culture, but subsequently increased their rates of proteoglycan synthesis over the next 8 days in culture. The relative rates of collagen and proteoglycan synthesis in these proximal and distal explants during the initial days in culture correlate well with their composition *in situ*.

Proteoglycan synthesis by proximal and distal surface, middle and deep layers

Proximal and distal tendon segments were further dissected into surface, middle and deep layers and cultured for 1, 2 or 3 days. All layers of proximal tendon incorporated relatively low amounts of [^{35}S]sulphate

Fig. 2. Daily rates of collagen (a) and proteoglycan (b) synthesis in explant cultures of proximal (●) and distal (Δ) segments

Full-thickness cross-sections were minced and the explants randomly distributed into 20 ml culture flasks. Duplicate flasks were labelled for 24 h on the indicated days with either L-[2,3- ^3H]proline or [^{35}S]sulphate. [^3H]Hydroxyproline was quantified in acid hydrolysates of dried explant tissue after separation from [^3H]proline on Dowex AG50W-8X. Results are expressed as d.p.m. of [^3H]hydroxyproline/g dry wt. Macromolecular [^{35}S]sulphate was measured in papain digests after separation from unincorporated label on Sephadex G-50, and is expressed as d.p.m./g dry wt.

(Fig. 3a). With time in culture, proteoglycan synthesis rates in proximal layer explants increased about 2-fold. Rates of proteoglycan synthesis in proximal surface, middle and deep layers were not different. Explants derived from the surface layer of distal tendon showed the highest rates of proteoglycan synthesis, incorporating 3–4-fold more [^{35}S]sulphate than did either the middle- or deep-layer explants (Fig. 3b). Distal surface tissue also showed the greatest increase in proteoglycan synthesis rates during the 3 days in culture. [^{35}S]Sulphate incorporation by the middle and deep layers of distal tendon was similar to those of the proximal layer explants. Overall, distal tissue exhibited significantly higher rates of [^{35}S]sulphate incorporation than did proximal tissue (Fig. 3c).

Fig. 3. Daily rates of proteoglycan synthesis in proximal (a) and distal (b) surface (S), middle (M) and deep (D) explant cultures

Duplicate flasks of each layer were labelled for 24 h on the indicated days. Total macromolecular [^{35}S]sulphate (from Sephadex G-50 columns) in papain digests of dried explants was normalized to tissue dry weight. Panel (c) shows the means ($\pm$ S.E.M.) of all layers from proximal or distal segments.

Proteoglycan turnover in proximal and distal layer explants

On the basis of sulphate labelling and measurement of hexosamine in tissue and medium, all layers of the proximal tendon exhibited low proteoglycan turnover rates. When normalized to tissue dry weight, the initial amount of radiolabelled glycosaminoglycan in these proximal cultures remained essentially constant throughout the days of chase (Figs. 4a and 4c). The amount of glycosaminoglycan hexosamine per dry weight in proximal tissue samples also showed no significant decline (results not shown). In addition, the absence of detectable amounts of glycosaminoglycan hexosamine in medium from proximal explants cultured for 12 days indicated that little tissue proteoglycan was degraded or leached from the explants.

Fig. 4. Turnover of $[^{35}\text{S}]$ sulphate-labelled proteoglycans in explants of proximal (a) and distal (b) surface (S), middle (M) and deep (D) layers

All explants were labelled during the first 24 h of culture with $[^{35}\text{S}]$ sulphate and then cultured in the absence of label for 2 days. Triplicate explant cultures of each layer were harvested at daily intervals thereafter, and the macromolecular $[^{35}\text{S}]$ sulphate was measured after chromatography of papain digests on Sephadex G-50. Values presented are means $\pm$ S.E.M. Panel (c) shows the means of all layers from proximal or distal segments.

Results from cultures of distal tissue indicated some loss of newly synthesized proteoglycan from the labelled explants. Determination of ^{35}S -labelled macromolecular material in distal explants on days 1, 2 and 3 of chase showed a downward trend when all measurements for distal explants were combined (Fig. 4c). Although these mean values are not sufficiently different to allow precise determination of the turnover rate, the slope of this line suggests a half-life for the newly synthesized proteoglycan of at least 20 days. Even though triplicate cultures were set up from randomized tissue pieces, enough variability was present in replicates that it was difficult to determine turnover rates of the individual distal layers (Fig. 4b). The amount of glycosaminoglycan hexosamine in the distal explants normalized to dry weight showed no significant decline in tissue proteoglycan during the chase period (Fig. 5b). The variability in the measurements of radiolabelled proteoglycan and tissue proteoglycan in

Fig. 5. Turnover of ^{35}S -labelled proteoglycans (a) and tissue proteoglycans (b) in distal surface (S), middle (M) and deep (D) explants

Glycosaminoglycan (GAG) hexosamine contents were measured in papain digests of the labelled explants shown in Fig. 4 and normalized to tissue dry weight (b). Macromolecular $[^{35}\text{S}]$ sulphate of these same explants was normalized to tissue glycosaminoglycan hexosamine content (a).

these distal explants was decreased when the amount of ^{35}S -labelled material was normalized to glycosaminoglycan hexosamine content (Fig. 5a). These data indicate that newly synthesized proteoglycans were not being preferentially degraded or lost from the explants. The absence of measurable amounts of glycosaminoglycan hexosamine in the culture medium from surface, middle and deep distal specimens cultured for 12 days indicated that little tissue proteoglycan was lost from the explants.

Characterization of $[^{35}\text{S}]$ sulphate-labelled proteoglycans

Analysis of labelled macromolecules extracted with 4 M-guanidine hydrochloride by Sepharose CL-4B chromatography demonstrated that the predominant proteoglycan produced by all proximal layer explants on days 1, 5 and 12 in culture was a small proteoglycan, which was eluted at $K_{av} \sim 0.6$ (PS, PM, PD, in Fig. 6). A minor peak of labelled material was eluted in the void

Fig. 6. Sepharose CL-4B elution profiles of [^{35}S]sulphate-labelled proteoglycans produced by proximal and distal surface, middle and deep explants

Explant cultures were labelled for 24 h on days 1 (—), 5 (---) or 12 (.....) of culture. ^{35}S -labelled proteoglycans were extracted with 4 M-guanidine hydrochloride, partially purified by chromatography on Sephadex G-50 and DEAE-cellulose DE-52 ion-exchange columns and then chromatographed on Sepharose CL-4B. Abbreviations: PS, proximal surface; PM, proximal middle; PD, proximal deep; DS, distal surface; DM, distal middle; DD, distal deep.

volume. On day 1 of culture, the small proteoglycan accounted for approx. 90% of the labelled macromolecules in proximal explants. With time in culture, the rate of synthesis of the small proteoglycan increased, so that by day 12 it accounted for over 98% of the proteoglycan synthesized (Fig. 6).

Distal tissue showed site related variations in the type of proteoglycans produced (DS, DM, DD, in Fig. 6). On day 1 in culture, distal surface explants produced two populations of ^{35}S -labelled macromolecules, which were eluted from Sepharose CL-4B in the void volume and at $K_{av} \sim 0.6$ (DS, Fig. 6). The large proteoglycan accounted for 68% of the total labelled macromolecules. On day 5 of culture, the amount of both the large and small labelled macromolecules produced by distal surface explants had increased severalfold, but there was little change in their ratio (Fig. 7). However, on day 12 of culture, synthesis of the small proteoglycan had increased, whereas that of the large proteoglycan had decreased. The proportion of small proteoglycan increased from 32% on day 1 to 80% of the total labelled macromolecules produced by distal surface explants on day 12 of culture. This small ^{35}S -labelled material was

eluted somewhat earlier than the small proteoglycan produced on days 1 or 5, or that synthesized by proximal explants. The basis for earlier elution of the 12-day small proteoglycans from distal surface explants was investigated by comparison of elution position from Sepharose CL-6B of the intact molecules and their alkali-released glycosaminoglycan chains (Fig. 8). These profiles indicate that the glycosaminoglycan chains of newly synthesized small proteoglycans from the distal surface are significantly larger ($K_{av} = 0.3$) than those from proteoglycans synthesized in the proximal tissue ($K_{av} = 0.5$).

Two populations of proteoglycans were also synthesized on day 1 in culture by explants derived from middle and deep layers of distal tendon (DM and DD, Fig. 6): 50% (deep) to 60% (middle) of the labelled macromolecules were eluted in the void volume, and 40–50% were eluted at $K_{av} \sim 0.6$. With time in culture, the small proteoglycan became the predominant species synthesized by both middle and deep distal layers. By day 12 of culture, the small proteoglycan accounted for 90% of the labelled proteoglycan produced by middle specimens and 98% of those made by deep explants.

Fig. 7 graphically illustrates this switch in the relative

Fig. 7. Size ratio (small proteoglycan/large proteoglycan) of ^{35}S -sulphate-labelled proteoglycans produced on days 1, 5 and 12 of culture by proximal (a) and distal (b) surface (S), middle (M) and deep (D) explants

The amounts of ^{35}S -labelled proteoglycan in the large and small proteoglycan populations were determined from the Sepharose CL-4B profiles shown in Fig. 6.

synthesis rates of the small and large proteoglycans in distal explants with time in culture. Distal layers produced predominantly large proteoglycans on the initial day in culture; thus the ratio of the small to large populations was low (Fig. 7b). Between days 1 and 5 of culture, the rates of synthesis of both large and small molecules increased, but there was little change in their ratio. By day 12 the ratio of small to large proteoglycans had increased.

Gradient SDS/polyacrylamide-gel electrophoresis of both extracted and radiolabelled proteoglycans from explants harvested on days 5 and 12 of culture is shown in Fig. 9. The proteoglycans present in the explants at the initiation of culture were detected by Alcian Blue and Coomassie Brilliant Blue (upper two panels), and the newly synthesized labelled proteoglycans were identified by fluorography. This method allowed detailed analyses of the small proteoglycans as these molecules penetrate the separating gel, whereas large proteoglycans remain in the stacking gel or at the interface. Both the extracted and radiolabelled small proteoglycans from surface, middle and deep explants from proximal tendon explants migrated as broad, diffuse bands around

Fig. 8. Sepharose CL-6B chromatographic profiles of ^{35}S -labelled small proteoglycans synthesized on day 12 of culture by surface layer explants from proximal (PS) or distal (DS) tendon

The elution positions of intact proteoglycans (—) and their glycosaminoglycans released by treatment with NaOH (----) are shown.

100 000 Da. The overall shape and position of these small proteoglycan bands were similar for all proximal layers from the day-5 and day-12 cultures. The newly synthesized proteoglycan appeared virtually identical with that in the tissue as assessed by this method. In addition, the core proteins of the small tissue proteoglycans resulting from chondroitinase ABC digestion migrated to the same position (Fig. 9, Coomassie Blue stain). In every case a doublet was observed, with the major band at 45000 Da and a minor band which migrated slightly faster. These results confirm the Sepharose CL-4B analyses of the newly synthesized small proteoglycan produced by all proximal layers.

Electrophoretic analysis of radiolabelled proteoglycans from distal explants also confirmed the increase in synthesis of the small molecule, especially in the surface and middle explants (Fig. 9). Relatively little small proteoglycan was detected in fluorograms of newly synthesized proteoglycans from the day-5 surface and middle distal layers. This result correlated with the

Fig. 9. Linear 4–20%-gradient SDS/polyacrylamide-gel electrophoresis of proteoglycans from tendon explants on days 5 and 12 of culture

Samples (the same as shown in Fig. 6) were digested with chondroitinase (C'ase) ABC. Control (–) and chondroitinase ABC-treated (+) unlabelled components stained with Alcian Blue and Coomassie Brilliant Blue (top) can be compared with control and digested ^{35}S -labelled proteoglycans (fluorography, below). Abbreviations: S, surface; M, middle; D, distal; 94 K, 67 K and 43 K indicate the migration positions of molecular mass markers of 94, 67 and 43 kDa.

relative lack of small proteoglycan appearing on the Alcian-Blue-stained gel and the small amount of core protein in these samples after chondroitinase ABC treatment. By day 12 of culture, the surface and middle explants produced substantially more of this small proteoglycan. Fluorograms of the day-12 material from the surface and middle explants indicated an amount of labelling comparable with that of the deep distal layer and all proximal layers.

Fig. 9 also demonstrates the presence of significant quantities of chondroitinase-ABC-resistant material that is present in distal surface and middle samples, but completely lacking in proximal samples. After chondroitinase ABC digestion this material migrated as a broad band near the top of the gel. This component was not detected by fluorography, indicating that it was not synthesized *de novo* during the labelling period on day 5 or 12 of culture. This chondroitinase-resistant Alcian-Blue-positive material represents keratan sulphate chains that are part of large proteoglycans found in the compressional region of bovine flexor tendon (Vogel & Thonar, 1987).

DISCUSSION

Proteoglycan synthesis by these tendon explants on day 1 in culture was quantitatively and qualitatively similar to the proteoglycan composition *in situ* of the explant tissue. Proximal tendon contains low amounts

(<1% of tissue dry wt.) of proteoglycans of small hydrodynamic size, which are evenly distributed in surface, middle and deep layers (Koob & Vogel, 1987). In culture these proximal layers had relatively low proteoglycan-synthesis rates, and these were predominantly small proteoglycans. Distal tendon contains elevated amounts of large proteoglycans, which are enriched in the surface layer, which receives direct frictional and compressive loads (Koob & Vogel, 1987). During day 1 in culture, explants derived from this surface layer exhibited relatively high proteoglycan-synthesis rates. Distal surface explants produced primarily large proteoglycans. This high rate of synthesis of large proteoglycan may account for the elevated proteoglycan content *in situ* of this region.

An increasing rate of proteoglycan synthesis was observed for all tendon explants during the first few days in culture. Proximal layer explants showed a significant increase in the small proteoglycan, whereas distal layer explants showed increased synthesis of both the large and the small proteoglycans. These increased rates of synthesis could have resulted from the presence of serum in the culture medium. Hascall *et al.* (1983a,b) showed that proteoglycan synthesis was enhanced 2–3-fold in adult bovine articular cartilage when it was cultured in the presence of fetal-calf serum. Rabbit articular cartilage has also been shown to increase proteoglycan synthesis when cultured in medium supplemented with serum (Sandy *et al.*, 1980; Lane & Brighton, 1974). We

cannot, however, rule out other possibilities for the increase in proteoglycan-synthesis rates, such as trauma incurred from the extensive dissection of tendon into explants, rapid cell proliferation or removal of mechanical forces, as discussed below. In addition, the turnover of proteoglycans in bovine articular cartilage was lowered in the presence of fetal-calf serum (Hascall *et al.*, 1983b; Campbell *et al.*, 1984). The addition of serum to our cultures may also be the explanation for the observed low proteoglycan turnover rates in these tendon explants. The important point here is that this tendon tissue remains viable and synthetically active for at least 12 days in culture. The present investigation augments previous studies demonstrating that adult tendons from various animal species have an intrinsic metabolic capacity that could be directed toward tendon healing and repair (Manske & Lesker, 1984).

The small proteoglycans synthesized by explants in culture appear similar to those extracted from the tissue. It is of particular interest that similar diffuse bands were generated by both extracted and radiolabelled small proteoglycans in SDS/polyacrylamide gels. The diffuse nature of this pattern on electrophoretic gels was presumed to result from heterogeneity of glycosaminoglycan chain lengths (Vogel & Heinegård, 1985) and could have been a post-synthetic modification of the molecules. The pattern for newly synthesized molecules indicates that the heterogeneity appears within 24 h, however. The significance of longer glycosaminoglycan chains on radiolabelled small proteoglycans from the distal surface is not clear, because the proteoglycans of this region have not yet been thoroughly characterized.

The presence of a major proteoglycan population in the compressional region of tendon that was not detectable by [³⁵S]sulphate incorporation indicates that not all proteoglycan populations present *in situ* were produced in culture. It is possible that synthesis of these proteoglycans has been shut down, owing to the artificial conditions of culture. On the other hand, these proteoglycans may represent a product that accumulates very slowly in the tissue, possibly owing to a low synthesis rate or to protracted modification of another large proteoglycan population. The two populations of large proteoglycans extracted from compressional regions of tendon (Vogel & Heinegård, 1985; Vogel & Thonar, 1987) resemble the two populations of aggregating proteoglycans that have been described from bovine nasal cartilage (Heinegård *et al.*, 1985).

With time in culture, distal explants switched from producing predominantly large proteoglycans to mostly small proteoglycans. We have previously observed that cells placed into culture from this region of tendon also show diminished synthesis of large proteoglycans after a period of time (Vogel *et al.*, 1986). This switch in proteoglycan phenotype may indicate that distal tissue receives a stimulus *in situ* which maintains synthesis of the large proteoglycan. In culture this stimulus is absent, and thus synthesis of the large proteoglycan becomes a smaller proportion of the total proteoglycan produced. Since it appears that distal tissue adapts to compressive forces by accumulating elevated amounts of large proteoglycans (Koob & Vogel, 1987), we speculate that the lack of compressive loads in culture results in the decline in synthesis of the large proteoglycan. This explant system can now be used to examine the role of mechanical loads in regulating both small- and large-

proteoglycan synthesis by proximal and distal regions of tendon.

Mechanical forces have been shown experimentally to influence tendon composition. Gillard *et al.* (1979) altered the mechanical forces acting on the rabbit flexor digitorum profundus tendon *in vivo* by anterior translocation of specialized tendon regions. The original pressure-bearing region showed a rapid loss of glycosaminoglycan after translocation to an area where it no longer received compressive loads. This region, which contained high concentrations of glycosaminoglycans, gradually attained low glycosaminoglycan contents similar to those of the tension-bearing region. Replacement of the translocated region to its normal position, where it again experienced compressive loads, resulted in a slow accumulation of glycosaminoglycans. Although we did not detect any loss of proteoglycan from our distal tendon explants, the response of distal tissue to long-term culture was a significant decline in the proportion of large proteoglycan produced. Thus both the rabbit flexor digitorum profundus tendon *in vivo* and the distal bovine flexor tendon in culture lose their differentiated proteoglycan phenotype in the absence of compressive forces. These results are consistent with the hypothesis that compressive loads stimulate and maintain synthesis of large proteoglycans in specialized regions of tendon.

Tensional forces may also play a regulatory role in determining the type of proteoglycan produced by tendon. Our explants were cultured in the absence of tensile forces as well as compressive forces. Since both proximal and distal explants increased synthesis of the small proteoglycan, it may be that tension is necessary for maintaining low amounts of the small proteoglycan. Gillard *et al.* (1979) showed that glycosaminoglycan contents increased in the tension-bearing regions of the rabbit flexor tendon after translocation to regions where they no longer experience tensile forces. On the other hand, evidence that tensile forces can also stimulate proteoglycan synthesis was provided by experiments which showed that a tensile load applied to embryonic-chicken tendons in organ culture caused an increase in glycosaminoglycan synthesis (Slack *et al.*, 1984). The role of tensional loads in directing proteoglycan synthesis in the bovine flexor tendon remains uncertain.

The technical assistance of Mrs. Kathleen Campbell and Mr. Ali Gargoum is gratefully acknowledged. Support was provided through grant AM36110 from the National Institutes of Health.

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Received 14 January 1987/7 April 1987, accepted 28 May 1987