An Immunoelectron Microscope Study of the Organization of Proteoglycan Monomer, Link Protein, and Collagen in the Matrix of Articular Cartilage

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ABSTRACT   Monospecific antibodies to bovine cartilage proteoglycan monomer (PG) and link protein (LP) have been used with immunoperoxidase electron microscopy to study the distribution and organization of these molecules in bovine articular cartilage. The following observations were made: (a) The interterritorial matrix of the deep zone contained discrete interfibrillar particulate staining for PG and LP. This particulate staining, which was linked by faint bands of staining (for PG) or filaments (for LP), was spaced at 75- to 80-nm intervals. On collagen fibrils PG was also detected as particulate staining spaced at regular intervals (72 nm), corresponding to the periodicity of collagen cross-banding. The interfibrillar PG staining was often linked to the fibrillar PG staining by the same bands or filaments. The latter were cleaved by a proteinase-free Streptomyces hyaluronidase with the removal of much of the interfibrillar lattice. Since this enzyme has a specificity for hyaluronic acid, the observations indicate that the lattice contains a backbone of hyaluronic acid (which appeared as banded or filamentous staining) to which is attached LP and PG, the latter collapsing when the tissue is fixed, reacted with antibodies, and prepared for electron microscopy. This hyaluronic acid is anchored to collagen fibrils at regular intervals where PG is detected on collagen. PG and LP detected by antibody in the interterritorial zones are essentially fully extractible with 4 M guanidine hydrochloride. These observations indicated that interfibrillar PG and LP is aggregated with HA in this zone. (b) The remainder of the cartilage matrix had a completely different organization of PG and LP. There was no evidence of a similar latticework based on hyaluronic acid. Instead, smaller more closely packed particulate staining for PG was seen everywhere irregularly distributed over and close to collagen fibrils. LP was almost undetectable in the territorial matrix of the deep zone, as observed previously. In the middle and superficial zones, stronger semiparticulate staining for LP was distributed over collagen fibrils. (c) In the superficial zone, reaction product for PG was distributed evenly on collagen fibrils as diffuse staining and also irregularly as particulate staining. LP was observed as semiparticulate staining over collagen fibrils. The diffuse staining for PG remained after extraction with 4 M guanidine hydrochloride. (d) In pericellular matrix, most clearly identified in middle and deep zones, the nature and organization of reaction product for PG and LP were similar to those observed in the territorial matrix, except that LP and PG were more strongly stained and amorphous staining for both components was also observed. (e) This study demonstrates striking regional variations of ultrastructural organization of PG and LP in articular cartilage. These observations are considered to be related to the organization (or lack of it) of these molecules with respect to hyaluronic acid and collagen and regional differences in proteoglycan structure.

Cartilage proteoglycans are composed of proteoglycan monomers whose molecular weights and structures may vary considerably (1, 2), the largest proteoglycan monomers having the highest buoyant densities. High buoyant density proteoglycan monomers can usually bind to hyaluronic acid (3-5) through a part of the protein core called the hyaluronic acid–binding

region (6–8) forming macromolecular aggregates (9). This interaction is stabilized by molecules called link proteins (10, 11) of molecular weights of the order of 44,000 and 48,000 in bovine nasal cartilage (11–13). The attachment to hyaluronic acid of reaggregated proteoglycans has also been demonstrated using electron microscopy of isolated proteoglycans (9, 14). Combined biochemical and morphological studies of isolated aggregates have independently revealed that high buoyant density proteoglycans are attached to hyaluronic acid at regular intervals varying between 25 and 85 nm, depending upon the investigator and the tissue studied (9, 15; J. Buckwalter and L. Rosenberg, unpublished observations). Link protein is thought to be present at the site of attachment of proteoglycan monomer to hyaluronic acid (6–8). Smaller nonaggregating proteoglycan monomers have been described which have structural features different from aggregating monomers (16).

Immunohistochemical studies of proteoglycans and link proteins using immunofluorescence (17, 28, 29) have revealed differences in the distributions of these molecules in articular and other cartilages suggesting differences in the fine structural organization of proteoglycans. Moreover, keratan sulfate concentration increases with increasing depth from the articular surface (21, 23). The lengths of chondroitin sulfate chains may increase with increasing depth (24) or remain unchanged (22). There are believed to be differences in the distribution of keratan sulfate and chondroitin sulfate in territorial and interterritorial domains in articular cartilage (18–20). The concentration of glycosaminoglycans is also significantly lower at the articular surface than elsewhere (18, 19, 21–23): here the proportion of keratan sulfate is probably greater (22). Since the content of proteoglycan monomer core protein is not detectably reduced (17), smaller (22) proteoglycan monomers at the articular surface probably have a lower content of glycosaminoglycan. Some of the proteoglycans at the articular surface are not extractable with 4 M guanidine hydrochloride (17), commonly used to dissociate proteoglycan aggregates, and extract proteoglycan and link protein. These observations together indicate heterogeneity in the organization and content of proteoglycans in cartilage matrix.

Electron microscope studies of cartilage proteoglycans in situ have so far concentrated on the use of metachromatic dyes that bind to the glycosaminoglycans of proteoglycans (25, 27). These reactions which are accompanied by the binding of electron-dense heavy metals have revealed the presence of small ribbons or conglomerates of high electron density. This particulate staining has been provisionally identified as representing collapsed proteoglycans (14). Such observations have been made using tissues in which proteoglycans have been fixed before or at the same time as dye binding. Since metachromatic dyes can bind to anionic species other than glycosaminoglycans and their binding is dependent upon charge density, it is often not possible to detect proteoglycans in reduced concentrations (17) nor is it possible to identify link protein by these methods. Hence we have extended our earlier immunofluorescence studies (17, 28, 29) to the electron microscope level. We now report on the distribution and organizations of proteoglycan monomer, link protein, and hyaluronic acid with respect to collagen in articular cartilage detected with newly developed immunoelectron microscopy methods for the ultrastructural identification of these molecules. Evidence is also presented for the presence of proteoglycan and link protein aggregated with hyaluronic acid in a specific region of cartilage matrix, the interterritorial matrix of the deep zone. A part of this work has been briefly reported in abstract form (30).

MATERIALS AND METHODS

Cartilage

Condylar bovine cartilage from the metacarpal-phalangeal joints of young steers (~10 mo old) was studied as before (17).

Antisera

The primary antibodies used in this study and their preparation for immunolocalization have been described elsewhere (17, 21). They are sheep antibodies (S27) to bovine articular cartilage proteoglycan monomer and rabbit antibodies (R131) to bovine nasal cartilage link protein. The latter were preabsorbed with bovine articular proteoglycan to remove their weak reaction to proteoglycan, rendering them monospecific for link protein. When these antibodies are absorbed with their appropriate purified antigens, they are completely prevented from reacting with tissue antigen in situ (17, 31). Hyperimmune pig antisera to sheep IgG Fab' (17) and to rabbit IgG Fab', and a sheep antiserum (32) to horseradish peroxidase (type III; Sigma Chemical Co., St. Louis, MO) were prepared in the manner described previously (17) using essentially the same immunization schedules.

For proteoglycan monomer localization, a soluble conjugate of horseradish peroxidase and sheep antiperoxidase was prepared according to the method of Sternberger (33), using the sheep antiserum to horseradish peroxidase described above. The protein concentrations of these conjugates were determined, assuming $E_{280}^{1mg/ml} = 1.0$. For the sheep conjugate, the E_{280}/E_{403} ratio is 2.3. A soluble immune complex of rabbit antiperoxidase and horseradish peroxidase was obtained from Sternberger-Meyer (Jarrettsville, MD) with an E_{280}/E_{403} ratio of 2.7.

Hyaluronidase

Streptomyces hyaluronidase specific for hyaluronic acid (34) was obtained from Calbiochem. It was further purified on CM-cellulose as described (35). It was assayed at 37°C using purified hyaluronic acid (human umbilical cord, type III; Sigma Chemical Co.) at a final assay concentration of 1.0 mg/ml in 20 mM acetate buffer, pH 5.0. Conditions of pH and temperature were based on those described elsewhere (36). The reaction was terminated by the addition of 10 μl of 0.8 M potassium tetraborate, pH 9.1, and boiling for 3 min. The appearance of reducing terminal N-acetylglucosamine residues was determined by the method of Reissig et al. (36). To the boiled solution was added 300 μl of dimethylaminobenzaldehyde (1% solution in 12.5 ml of concentrated HCl and 87.5 ml of glacial acetic acid). After 20 min at 37°C, color was read at room temperature at 585 nm against a standard solution of N-acetylglucosamine. 1 U of enzyme activity was expressed as that amount of enzyme which generates 1 μmol equivalent of terminal N-acetylglucosamine residues in 1 h under the conditions of standard assay described above. The purified enzyme had an activity of 0.58 U/ml. The rate of hydrolysis of hyaluronic acid by the enzyme, which is probably a serine glycosidase, was only partly reduced by phenylmethylsulfonyl fluoride, an inhibitor of serine proteinases. To further guard against other contaminant proteinases, this inhibitor was used with other proteinase inhibitors (shown in parentheses) capable of inhibiting the remaining known classes of proteinases, namely metallo (EDTA), aspartic (pepstatin), and cysteine (iodoacetamide) (37). Hyaluronidase remained only partly inhibited. Moreover, in the presence of these inhibitors, pretreatment of proteoglycan monomer with hyaluronidase had no detectable degradative effect on proteoglycan monomer including its ability to interact with hyaluronic acid (Fig. 1). Thus, under these conditions, this hyaluronidase had no detectable proteolytic activity. The enzyme was equally active in 0.15 M Tris at pH 7.3 and in acetate at pH 5.0, and was used at pH 7.3 for treating tissue sections at 37°C. Relatively little activity was observed at room temperature.

The In Vitro Binding of Link Protein and Proteoglycan Monomer to Hyaluronic Acid

Purified hyaluronic acid was allowed to react with purified bovine nasal link protein (11) in a 0.6% agarose Tris–boric acid gel buffered at pH 8.3 (11) as follows. 4 μg of hyaluronic acid in 10 μl of water were added to one well of an immunodiffusion pattern and 6.8 μg of link protein in 10 μl of 1 M NaCl or 400 μg of bovine nasal proteoglycan (A1D1) in 10 μl of Tris borate buffer were added to the adjacent well. After diffusion for 24 h at room temperature, the plate was exhaustively washed (11) and treated for 5 h with the appropriate antibody to link protein or proteoglycan (ammonium sulfate concentrated from serum and used at a concentration of 1–2 mg/ml in phosphate-buffered saline. This permitted enhanced sensitivity of subsequent protein staining. The plate was then again washed as before and stained with Coomassie Brilliant Blue R250 (11).
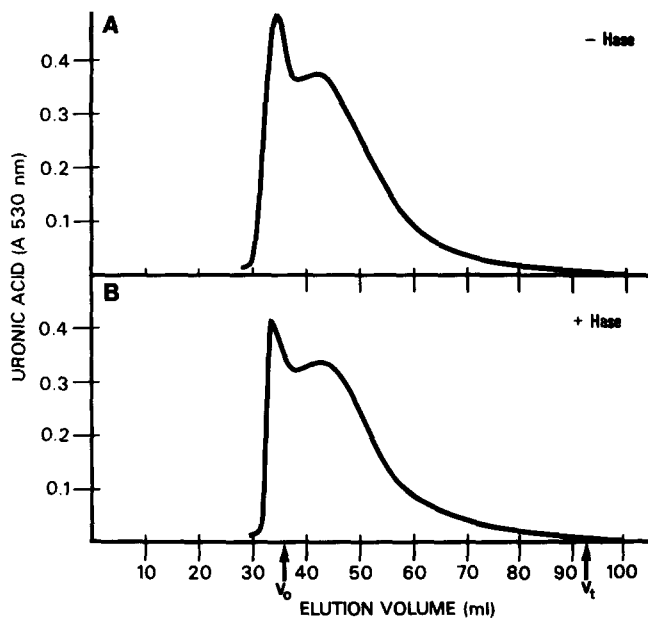
FIGURE 1 The lack of effect of purified hyaluronidase (plus proteinase inhibitors) on proteoglycan monomer and its aggregation with hyaluronic acid. Purified bovine nasal proteoglycan (2 mg) was dissolved in 0.95 ml of 0.1 M Tris-acetate buffer, pH 7.3 and incubated at 37°C with 50 μl of purified hyaluronidase (0.58 U/ml) in the same buffer with pepstatin (1 μg/ml), 1 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. Buffer with no inhibitors was used as the control. After 24 h, aggregation of proteoglycan was induced at room temperature by the addition of 20 μg (1% wt/wt) of hyaluronic acid in 0.1 M Tris-acetate buffer, pH 7.3. After 30 min the samples were immediately chromatographed at room temperature on Sepharose 2B Cl using a 115.5 × 0.8 cm column, with 0.2 M sodium acetate, pH 5.5. Chromatography was performed by downward elution at 4°C with a flow rate of 6.5 ml/h. Fractions (1 ml) were collected and assayed for their uronic acid content using the carbazole reaction (1).

Tissue Preparation, Sectioning, and Enzyme Treatment

Cartilage (extracted with 4 M guanidine hydrochloride where indicated), was frozen, sectioned, fixed, and treated with chondroitinase ABC (Miles Laboratories, Elkhart, IN) as described previously (17) with the following exceptions. For electron microscopy, 4-μm-thick frozen sections where attached to plastic cover slips (10.5 × 22 mm, no. 1½, Thermanox; Lux Scientific Corporation, Newbury Park, CA) immediately after sectioning. Where indicated, some sections were additionally treated at 37°C in a humidified chamber with 50 μl per section of hyaluronidase in 0.15 M Tris, pH 7.3, or buffer only (controls) for 6 h at a concentration of 0.12 U of enzyme/ml in the presence of proteinase inhibitors. These conditions were designed to achieve limited hydrolysis of hyaluronic acid and thus only partial removal of proteoglycan and link protein, as ascertained by immunofluorescence (17).

Localization of Proteoglycan and Link Protein using a Modified Sternberger Method of Antibody Binding

All procedures were at room temperature in humidified containers. Reagent volumes were 50 μl per section. Immediately after fixation and enzyme treatment, sections were initially treated for 1 h with sheep S27 Fab' or nonimmune sheep Fab' (proteoglycan test and control, respectively) or rabbit R131 Fab' or non-immune rabbit Fab' (link protein test and control, respectively), exactly as described (17). After removal of unbound Fab' by washing in phosphate-buffered saline (PBS; [18]) for 30 min, sections were treated for 1 h with an excess of a bridging antibody of either pig antisheep IgG Fab' serum (for proteoglycan) or with a pig anti-rabbit IgG Fab' serum (for link protein). These sera were diluted 1/10 with PBS. Excess binding antibody was removed by subsequent washing in PBS for 1 h. Sections were then treated for 1 h with either a soluble immune complex of sheep antiperoxidase/peroxidase at 5.3 mg/ml (for proteoglycan) or

rabbit antiperoxidase/peroxidase at 0.10 mg/ml (for link protein). After removal of excess peroxidase conjugate by washing in PBS for 30 min, sections were fixed for 30 min at room temperature in 2.5% glutaraldehyde (J. B. EM Services, Pointe Claire, Québec) in PBS. Sections were washed with PBS for 30 min and placed in a solution containing 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (British Drug Houses, Montreal, Québec) in PBS for 10 min and then reacted for peroxidase in the same solution containing in addition 0.005% H_2O_2 for 20 min. The sections were then washed for 10 min in PBS and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 30 min. They were finally rinsed in deionized water, dehydrated for 5-min periods in 60, 80, 90, and 100% acetone and placed in acetone/Spurr resin mixtures at 1:1 and 1:2 each for 15 min. They were finally flat-embedded in Spurr resin at room temperature overnight in a dessicator. Thin sections were cut, stained with lead citrate and uranyl acetate, and examined in a Phillips 400 electron microscope.

RESULTS

A diagram of the regions and zones of articular cartilage is shown in Fig. 2. The diagram is based on the results of the present study, and on earlier work (17). The articular cartilage is divided into superficial, middle, and deep zones. Pericellular, territorial, and interterritorial regions are present in these zones. The structural organization of proteoglycans, link protein, hyaluronic acid, and collagen varies from region to region. The structural organization of the interterritorial matrix of the deep zone is identifiably the most highly developed and elaborate. As described below, proteoglycans, link protein, hyaluronic acid, and collagen in the interterritorial matrix of the deep zone are organized into a highly ordered lattice work not found in other regions.

The Superficial and Middle Zones of Articular Cartilage

The superficial zone has a depth of $\sim$40 μm (Fig. 2). It is characterized by reduced metachromatic dye staining for glycosaminoglycans, a rich content of immunoreactive proteoglycan core protein and link protein, and the presence of proteoglycan that is not extractable with 4 M guanidine hydrochloride (17). Beneath it resides the middle zone, where collagen fibrils are present with diameters similar to those observed in comparable regions of the superficial zone (Table I). Throughout the superficial and middle zones are the pericellular regions up
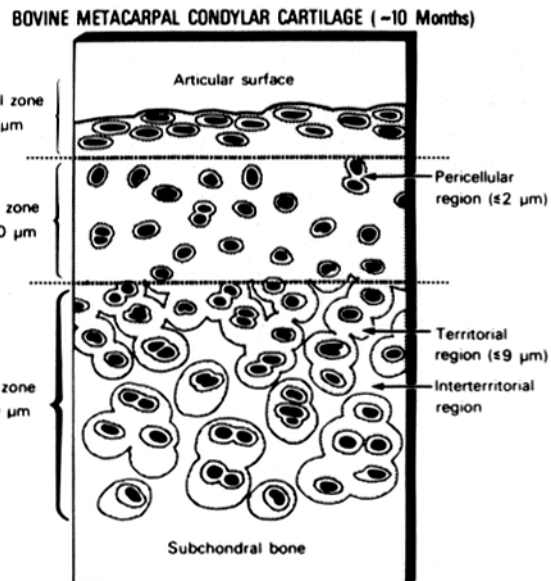

FIGURE 2 Diagrammatic representation of the zones and regions of bovine articular cartilage described in this paper. This information is based on the present study and earlier published work reviewed here, including that by the authors (17).

TABLE I

Collagen Fibril Diameters

Zone/region	Diameter, nm
	mean ± SD (n)
Superficial zone	
1. Pericellular	15.4 ± 8.3 (18)
2. Territorial	29.2 ± 5.4 (22)
Middle zone	
3. Pericellular	21.5 ± 12.7 (18)
4. Territorial	31.8 ± 5.0 (23)
Deep zone	
5. Pericellular	19.0 ± 4.4 (23)
6. Territorial	48.5 ± 7.0 (23)
7. Interterritorial	57.5 ± 7.6 (25)

All measurements were made from one experiment (BC14). Student's t test analyses revealed that the following were significantly different from each other ($P < 0.001$): 1 and 2, 3 and 4, 5 and 6, 6 and 7, 4 and 6, 4 and 7.

to 2 μm in width, which contain significantly narrower collagen fibrils (Table I) and amorphous material. The territorial regions of these zones are structurally similar to the territorial matrix of the deep zone although collagen fibrils are larger deeper in the cartilage.

Territorial Region of Superficial and Middle Zones

Electron microscopy of sections first treated with nonimmune sheep serum IgG-Fab' (control for proteoglycan) revealed only collagen fibrils (Fig. 3d) of relatively narrow diameter (Table I) weakly stained by heavy metals. There was no evidence of the intense black reaction product for peroxidase, namely insoluble oxidized polymerized diaminobenzidine to which electron dense osmium tetroxide, lead, and uranyl are bound. Sections treated to demonstrate proteoglycan by using monospecific antibody Fab' contained much electron dense reaction product in the form of small discrete particles throughout the territorial matrix (Table II and Fig. 3). This particulate staining was mainly associated with collagen fibrils and was completely absent after prior extraction with 4 M guanidine hydrochloride (Fig. 3f). In addition, collagen fibrils in the superficial zone also normally stained diffusely for proteoglycan (Fig. 3). Only in the superficial zone was this staining seen after extraction with 4 M guanidine hydrochloride (Fig. 3f). Similar results were obtained for the middle zone (not shown).

Sections stained with nonimmune rabbit Fab' (control for link protein) contained no staining for peroxidase (Fig. 3a) and only collagen fibrils stained with heavy metals. Sections first treated with monospecific rabbit antibody to link protein contained peroxidase staining of moderate intensity which was again mainly limited to collagen fibrils (Fig. 3b). This was diffuse, banded, and semiparticulate, but of unmeasurable, irregular shape, quite different from the proteoglycan staining and with no recognizable pattern. This staining for link protein on collagen fibrils was almost absent after extraction with 4 M guanidine hydrochloride (Fig. 3c).

The results for the middle zone are not shown but they were the same as those described for the superficial zone.

Pericellular Regions of Superficial and Middle Zones

In pericellular regions, peroxidase staining was again absent

in controls for proteoglycan and link protein: collagen fibrils were lightly stained by heavy metals. In sections treated with antibodies to proteoglycan, particulate and diffuse amorphous staining was observed. The particulate staining was similar in size to that seen in the territorial matrix (Table II) but less common. In the case of sections treated with antibody to link protein, staining was again similar to that described for the territorial region, being associated with narrow collagen fibrils. Amorphous staining for both molecules was observed in interfibrillar sites. Pericellular staining was most clearly defined in the middle zone. There was no detectable staining for proteoglycan or link protein after extraction with 4 M guanidine hydrochloride, except in the superficial zone, where residual diffuse staining for proteoglycan was observed on collagen fibrils as in territorial regions of this zone.

The Deep Zone of Articular Cartilage

The zone is divided up into pericellular, territorial, and interterritorial regions which will be considered separately. In all control sections, collagen fibrils were stained lightly with heavy metals (Figs. 4a and c, 6a, and 8a): there was no identifiable staining for peroxidase.

Territorial Region of Deep Zone

The reaction for proteoglycan was similar in size and distribution to the particulate staining observed in the territorial matrix of the superficial and middle zones (Table II and Fig. 4d). Particulate reaction product (Fig. 4d) was mainly observed in close proximity to the thicker collagen fibrils (Table I) although some was seen in interfibrillar sites. There was no diffuse staining for proteoglycan of the kind seen in the superficial zone. Reaction product for link protein was weak and present mainly on collagen fibrils (Fig. 4b) as observed in the territorial region of the superficial zone. Previously, reduced immunofluorescent staining for link protein was observed in the territorial matrix of this zone (17). Some collagen fibrils stained more strongly for link protein than others (Fig. 5). Between fibrils there was limited particulate reaction product for link protein (Fig. 5 and Table II). This was commonly seen at the well-demarcated junction with the interterritorial zone (Fig. 5). Prior extraction with 4 M GuHCL removed all staining for proteoglycan and link protein in the region.

Pericellular Region of Deep Zone

This region again contained the narrowest collagen fibrils of the zone (Table I). Fibrils stained weakly with heavy metal in control sections. Sections treated with antibodies to proteoglycan or link protein contained reaction product similar to that described for the territorial region of this deep zone. Particulate staining for proteoglycan was similar in size to that observed in pericellular and territorial regions elsewhere (Table II). Sometimes amorphous interfibrillar staining for proteoglycan and link proteins was noted with no recognizable organization. Staining for both components was stronger than in the territorial matrix. Prior extraction with 4 M guanidine hydrochloride removed all staining for proteoglycan and link protein.

These observations indicate that proteoglycan and link proteins are organized in territorial and pericellular regions of the deep zone in a manner similar to that of comparable regions in the superficial and middle zones, with the exception of the nonextractable collagen-associated proteoglycan which was
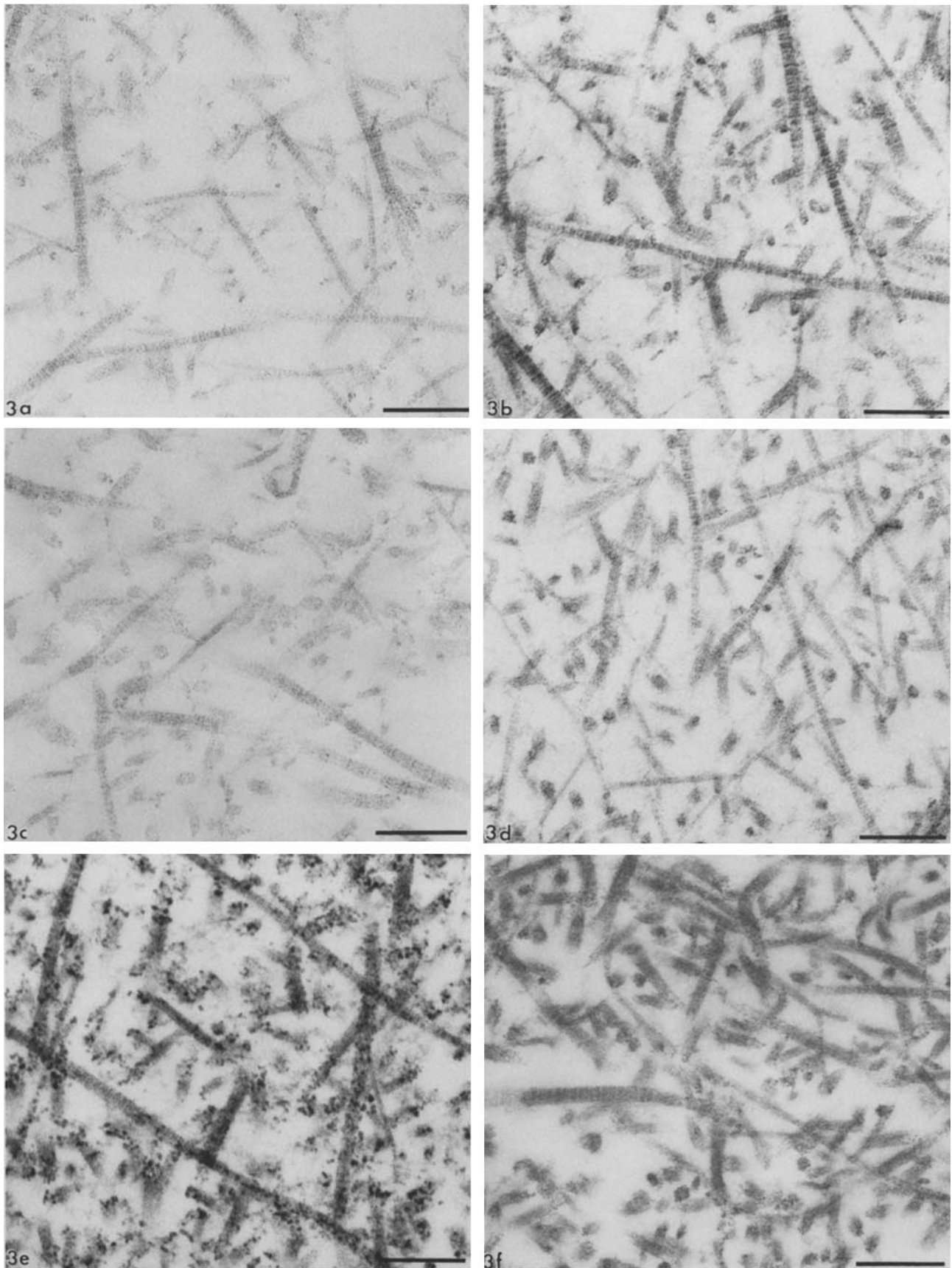
FIGURE 3   Link protein (a–c) and proteoglycan monomer (d–f) in the superficial zone. Control unextracted section (nonimmune rabbit Fab') for link protein (a), and sections treated with rabbit Fab' antibody to link protein before (b) and after (c) extraction with 4 M GuHCL. Control section (nonimmune sheep Fab') for proteoglycan is shown in d. Other sections were also treated with sheep antibody Fab' to proteoglycan, before (e) and after extraction with 4 M guanidine hydrochloride (f). Bars, 200 nm. × 80,000.

Zone/region			Proteoglycan	Link protein
			nm [mean ± SD(n)]	
Superficial and middle zones				
Pericellular			13.3 ± 6.7 (22)	I
Territorial			13.7 ± 3.7 (37)	I
Deep zone				
Pericellular		1.	14.7 ± 8.1 (22)	I
Territorial		2.	12.3 ± 3.6 (32)	7. 21.9 ± 4.7 (26)
Interterritorial				
Interfibrillar	L	3.	27.4 ± 5.2 (21)	8. 18.9 ± 4.4 (22)
	B	4.	21.4 ± 4.6 (21)	9. 13.9 ± 3.7 (22)
Fibrillar	L	5.	29.8 ± 4.2 (7)	Absent
	B	6.	17.7 ± 3.2 (7)	—

All measurements were made from one experiment (BC14). Reaction product was measured only where it was of regular shape. Elsewhere it was irregular (*I*). In some cases where reaction product was more elongated in one dimensions (*L*) than that measured at right angles (*B*) these were recorded independently as the greatest values measurable for each individual reaction product. Student's *t* test analyses revealed that the following were significantly different from each other (*P* < 0.001): *1* and *3*, *1* and *5*, *2* and *7*, *2* and *5*, *3* and *8*.

only found in the superficial zone and the reduced amount of link protein detected in the deep territorial region.

The Interterritorial Region of the Deep Zone

This differed markedly from the rest of the articular cartilage. Control sections for proteoglycan showed no staining (Fig. 6 *a*). Proteoglycan staining was particulate, at its largest size (Table II), and present both on and between collagen fibrils (Figs. 6 *b*, 7, and 11–13). The diameters of collagen fibrils were also maximal in this region (Table I). The proteoglycan staining on collagen fibrils (table II and Figs. 12 and 13) was observed at regular intervals along the fibril at a spacing of 72.5 ± 8.5 nm (mean and standard deviation of 21 determinations). The reaction product was larger than the particulate staining seen in territorial matrix (Table II). Sometimes, one could observe that proteoglycan was situated symmetrically either side of collagen fibrils with faint repetitive double bands of heavy metal staining traversing the collagen fibril between the sites of reaction product (Fig. 7, arrow). In other cases, the cross-fibril metal staining was more clearly defined but single rather than double-banded (Fig. 12). The repetitive heavy metal cross-banded staining of collagen was never clearly seen in these sections, only in controls (Fig. 6 *a*) where peroxidase staining was absent.

The particulate proteoglycan reaction product in the extracellular matrix between collagen fibrils was similar in size to that observed on collagen fibrils in this region and was interconnected by faintly stained fine filaments or narrow pale strands of staining (Figs. 7, 11, and 13). The interfibrillar particulate proteoglycan reaction product was regularly spaced along the connecting filaments or bands at intervals of 85.7 ± 9.3 nm (mean standard deviation, *n* = 23) measured as indicated in Figs. 11 and 14. The net result was a highly ordered latticelike network of fine filaments or bands which connected the regularly spaced proteoglycan particles between collagen fibers to those regularly spaced proteoglycan particles distributed on collagen fibrils. There was no detectable reaction product after extraction with 4 M guanidine hydrochloride (Fig. 6 *c*).

Sections stained with antibody Fab′ to link protein also displayed an almost identical interfibrillar lattice to that for proteoglycan (Figs. 8 *b* and *e*, 9, and 10, and Table II). Although the interfibrillar reaction product for link protein was significantly smaller than that for proteoglycan in this site (Table II), it was also joined (like proteoglycan) by connecting filamentous staining (Figs. 8 *e* and 10) was spaced at intervals of 75.1 ± 8.7 nm (*n* = 19) measured as for proteoglycan and as indicated in Figs. 10 and 14. Compared with the proteoglycan spacings, these were similar but significantly different (*P* < 0.001, according to Student's *t* test). Particulate reaction product for link protein generally was much less commonly observed on collagen fibrils and there was no recognizable regularity to this staining. The overall intensity of link protein staining was much greater in the interterritorial region than in the territorial region of this zone. The transition between the regions was sharply demarcated (Fig. 4).

Pretreatment of sections with hyaluronidase resulted in a gross loss of the lattice formed by either link (Fig. 8 *d*) or proteoglycan (Fig. 6 *d*) staining. The filaments or narrow bands connecting proteoglycan monomer on collagen fibrils to interfibrillar proteoglycan throughout this extracellular matrix largely disappeared. Similar results were observed for the predominantly interfibrillar link protein. Collagen bound proteoglycan was reduced in amount. The residual particulate staining for both proteoglycan and link protein was more intense and clearly defined, probably due to increased permeability of the matrix to immunoreagents.

The in Vitro Binding of Link Protein to Hyaluronic Acid

When hyaluronic acid was allowed to react with link protein in an immunodiffusion gel under associative conditions a precipitin line was formed between the wells (not shown). This indicated that link protein, which forms oligomers under these conditions (11), could cross-link hyaluronic acid and thereby be precipitated. Diffusion of hyaluronic acid against bovine nasal cartilage proteoglycan monomer (A1D1 [11]) produced no precipitin reaction, indicating monovalency for proteoglycan with respect to hyaluronic acid.

DISCUSSION

The detection of proteoglycans within tissues poses many technical problems. The earlier use of metachromatic dyes that bind to glycosaminoglycans in fixed but otherwise untreated tissues has its disadvantages and advantages. Proteoglycans are thought to be precipitated *in situ* particularly by the dye, and loss from the tissue is minimized. The sensitivity of the dye staining method, however, is known to be limited, as is the specificity which is not just for proteoglycans but for molecules with anionic binding sites (see reference 17 for a brief discussion). Yet identification of glycosaminoglycan binding can theoretically be facilitated by pretreatment of tissues with specific glycosidases of well-characterized substrate specificity. The advent of classical and monospecific antibodies to cartilage proteoglycans has permitted the specific identification of not only individual glycosaminoglycans (49, 50) but also proteoglycan core protein-associated antigenic determinants (17, 31). Moreover, antibodies permit the detection of proteoglycans that have structural differences.

The successful use of antibodies is, however, fraught with problems associated with tissue penetration (due to their much
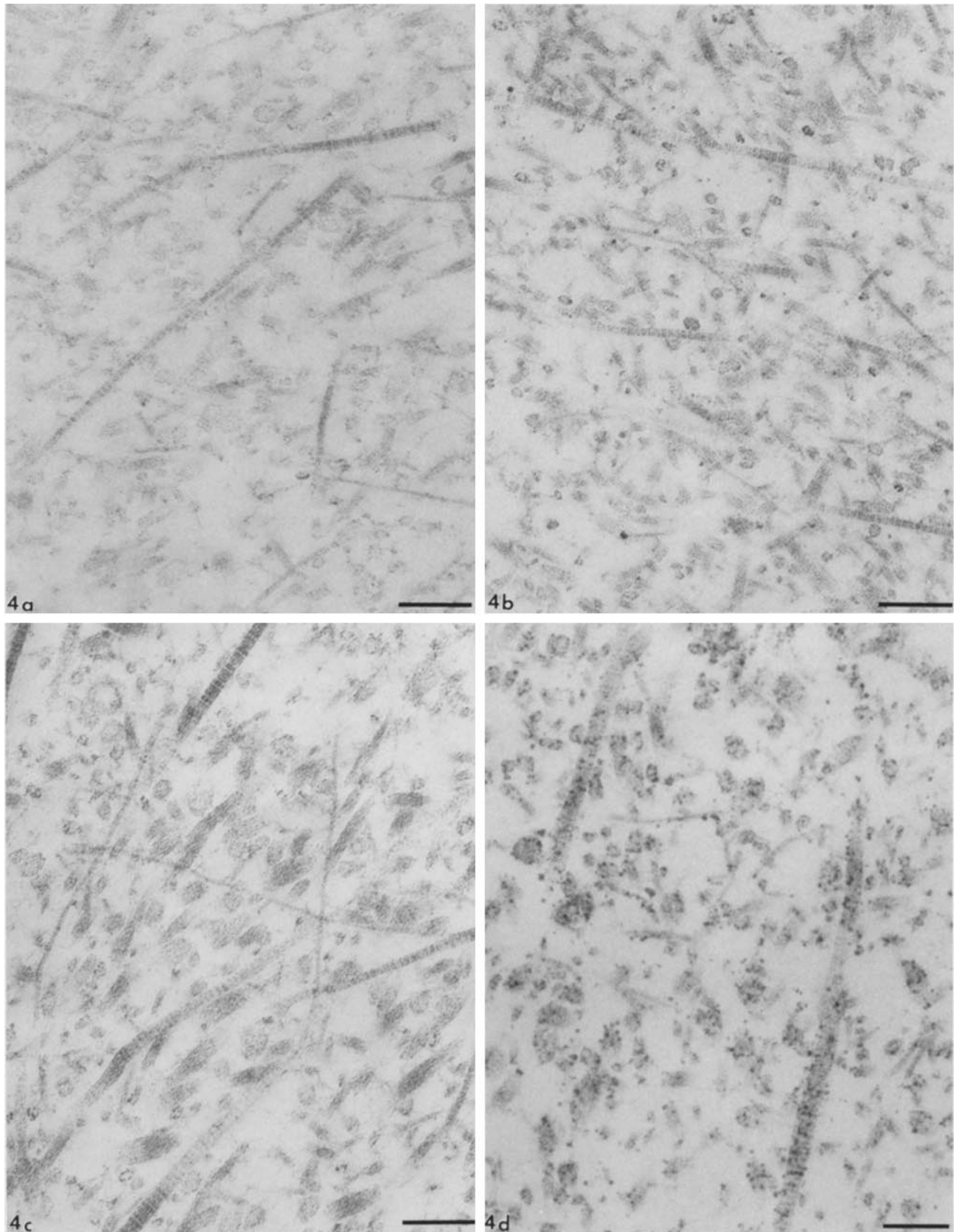
FIGURE 4   Link protein and proteoglycan in the territorial region of the deep zone. Control unextracted sections (as for Fig. 3) for link protein (a) and proteoglycan (c) and unextracted sections treated with antibody to link protein (b) and proteoglycan (d) (as for Fig. 3). Bars, 200 nm. (a, b, and c) × 66,000, (d) × 60,000.
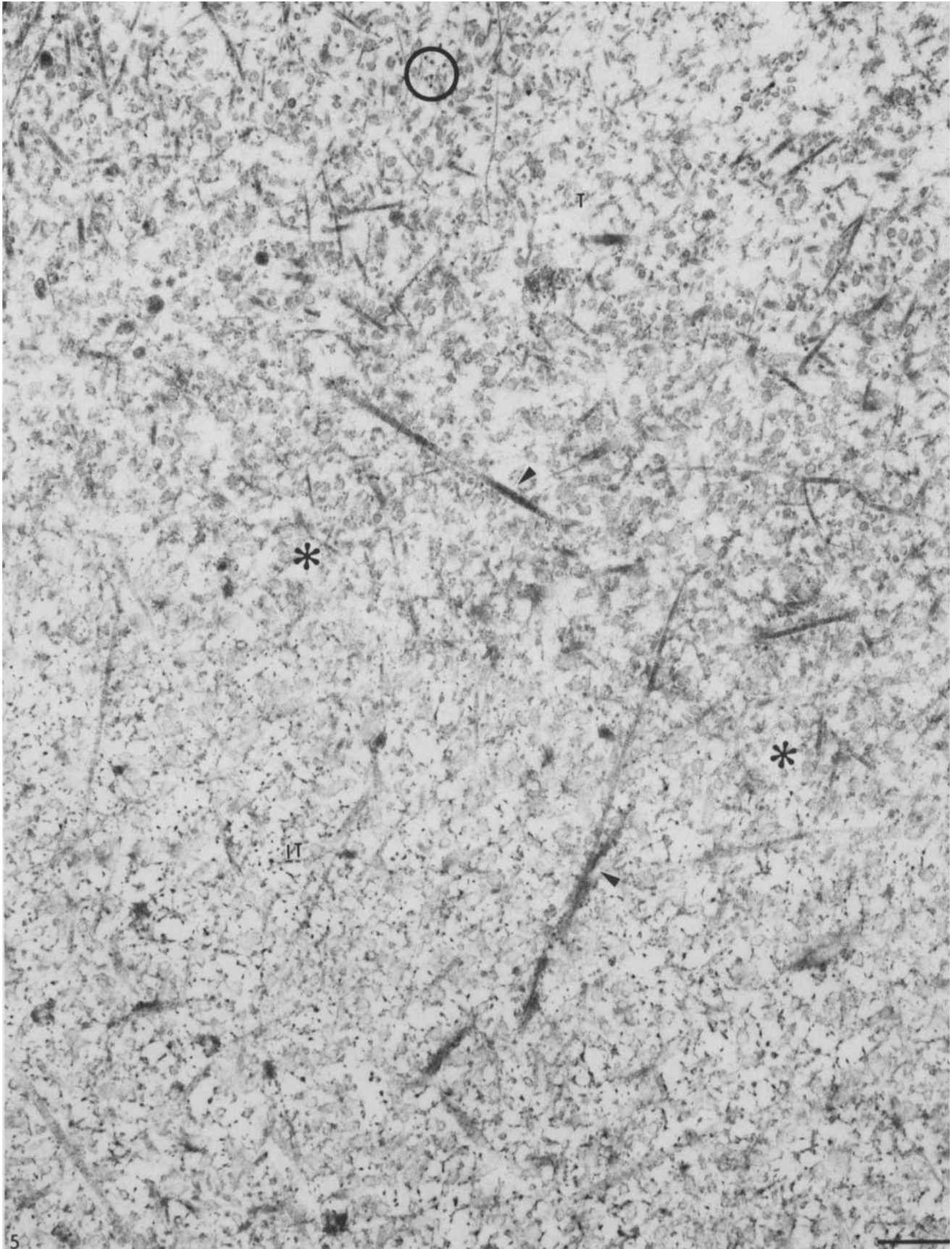
FIGURE 5 Junction (*) between territorial (T) and interterritorial (IT) regions of the deep zone stained for link protein. In the territorial zone, some collagen fibrils stained more intensely for link protein than others (arrowheads). Interfibrillar particulate staining was sometimes observed (within circle). In the interterritorial zone collagen fibrils were generally unstained for link protein with a few exceptions (arrowhead) where fibrils crossed zones. Bar, 500 nm. × 27,600.
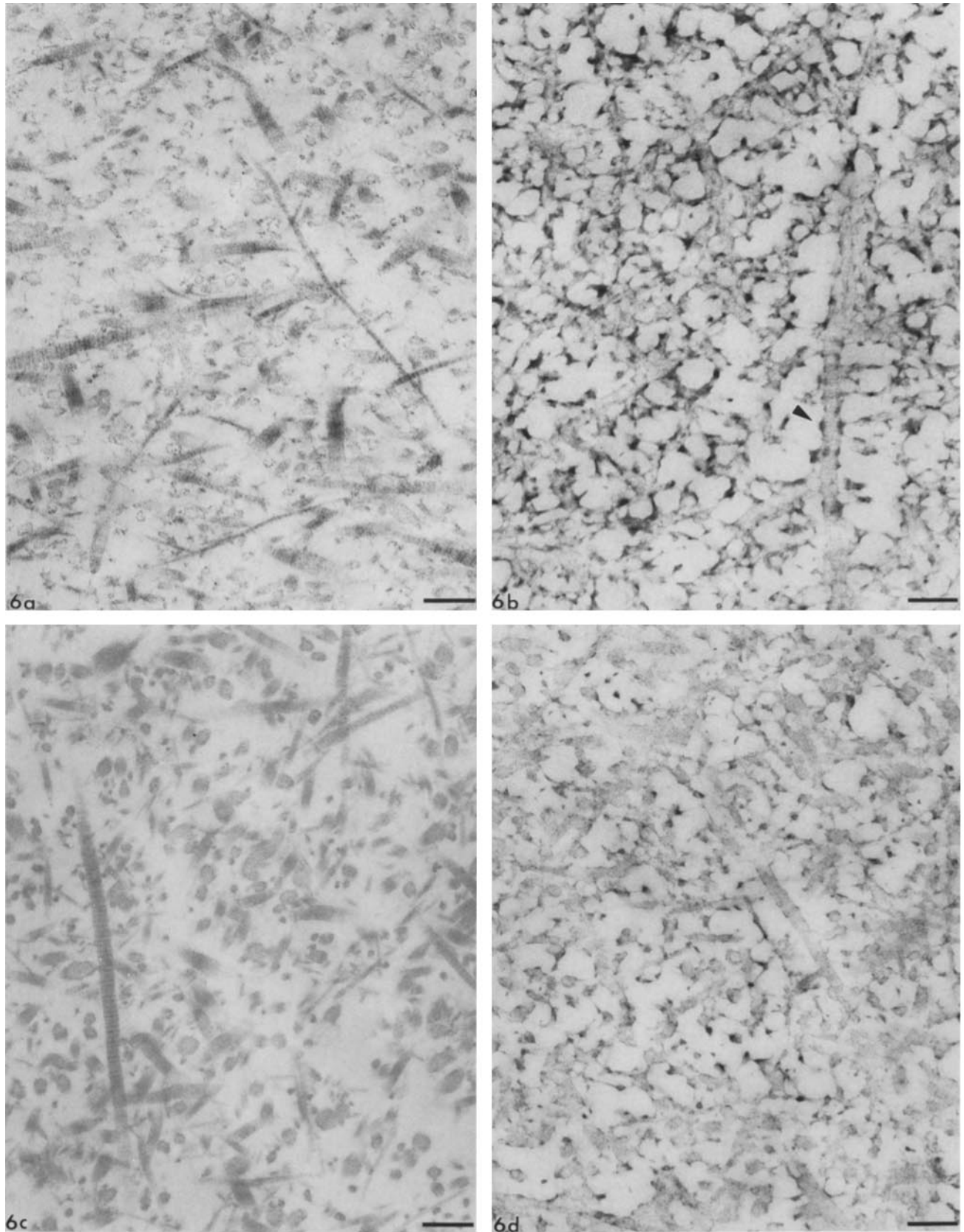
FIGURE 6   Interterritorial region of the deep zone stained for proteoglycan. Control tissue (treated with nonimmune sheep Fab')
(a) and that stained with sheep antibody Fab' to proteoglycan (b). Sections were also pretreated with hyaluronidase (d) or 4 M
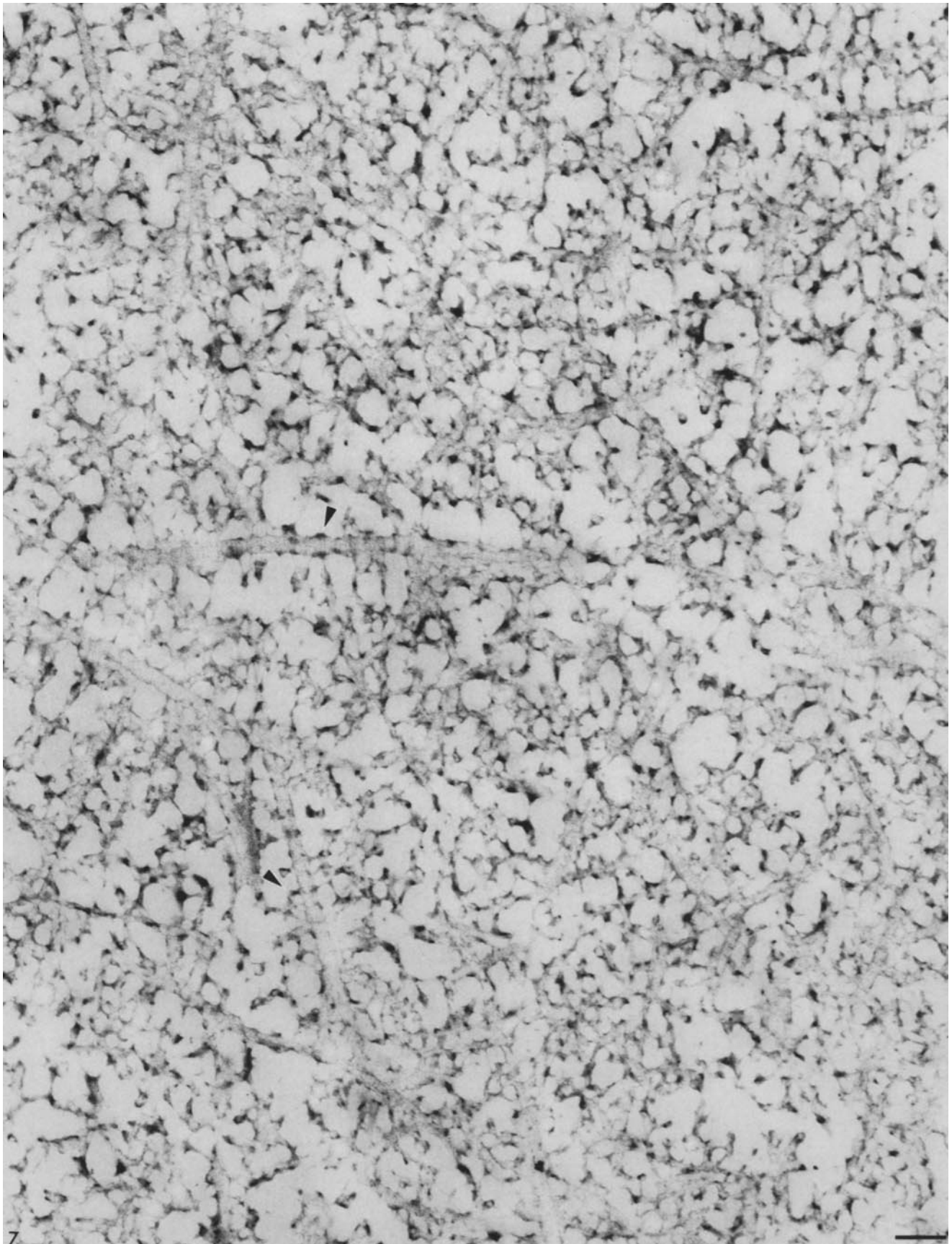guanidine hydrochloride (c) before antibody. Bars, 200 nm. × 45,000.

FIGURE 7   Interterritorial region of the deep zone stained for proteoglycan to reveal more clearly the latticework of particulate staining associated with collagen (arrowheads) and present in interfibrillar sites. Bars, 200 nm. × 47,600.
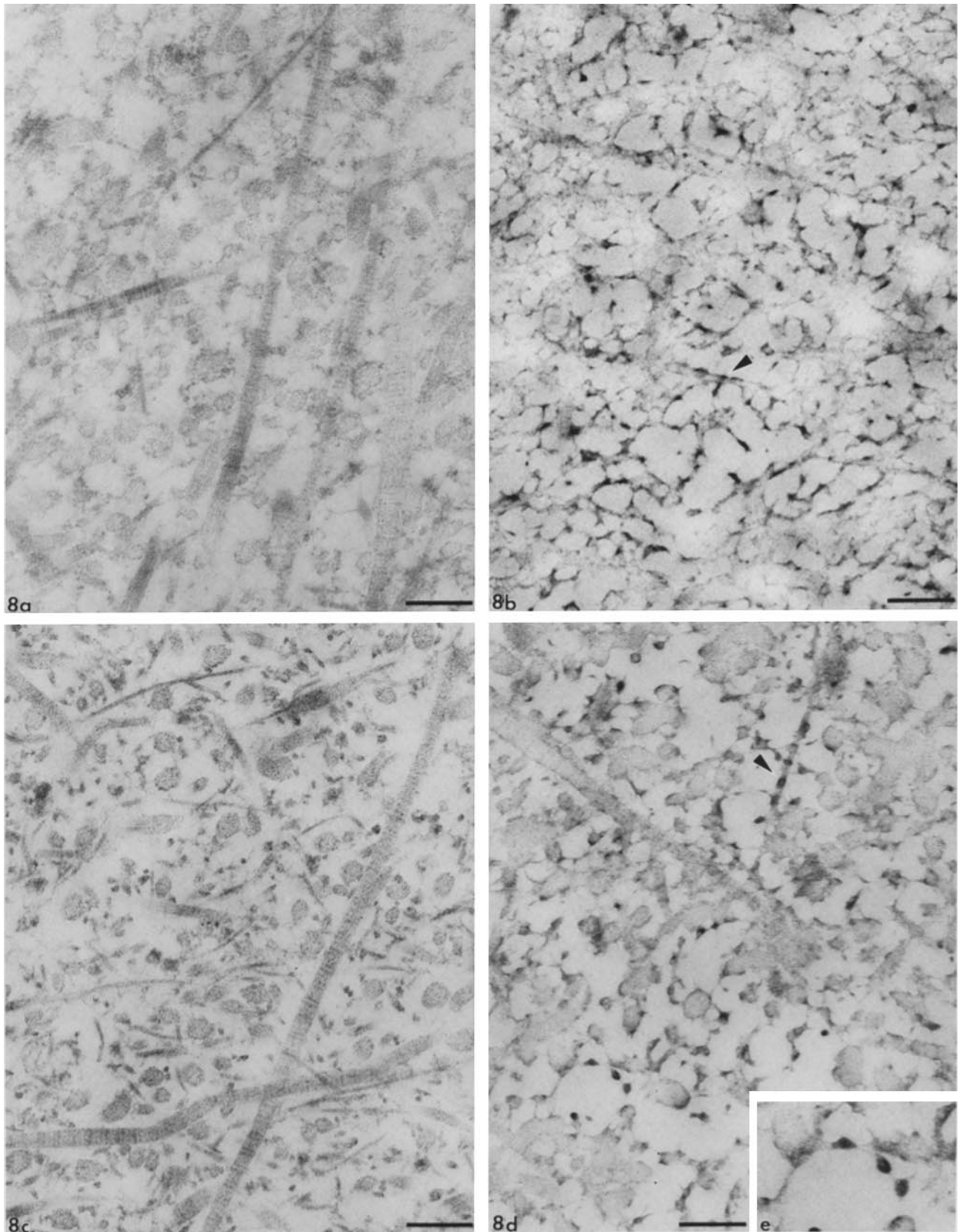
FIGURE 8 Interterritorial region of the deep zone stained for link protein. Control (nonimmune rabbit Fab') (a) and rabbit antibody Fab' to link protein (b) associated with narrow collagen fibrils (arrowhead). Other staining between fibrils was seen in the form of a latticework. Some sections were pretreated with hyaluronidase (d and e) or 4 M guanidine hydrochloride (c) before antibody Fab'. Particulate interfibrillar stain linked by filamatous material is shown in (e). Bars, 200 nm. × 60,000, except e, which is an enlargement of a portion of d shown to the left of e. × 100,000.
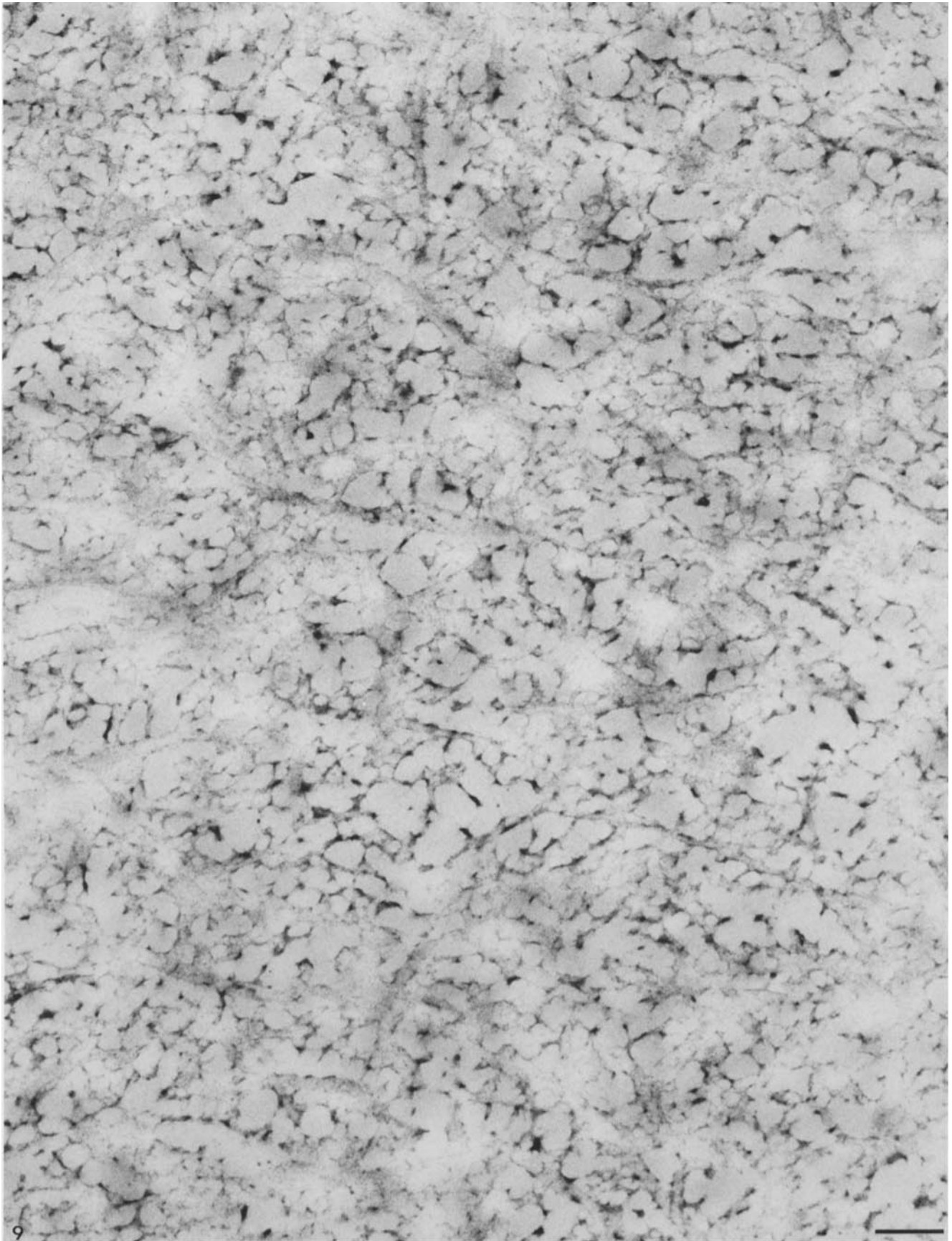
FIGURE 9   Interterritorial region of the deep zone stained for link protein to reveal more clearly the latticework of particulate staining, which is very similar to that seen in this region for proteoglycan (Fig. 7). Bar, 200 nm. × 61,600.
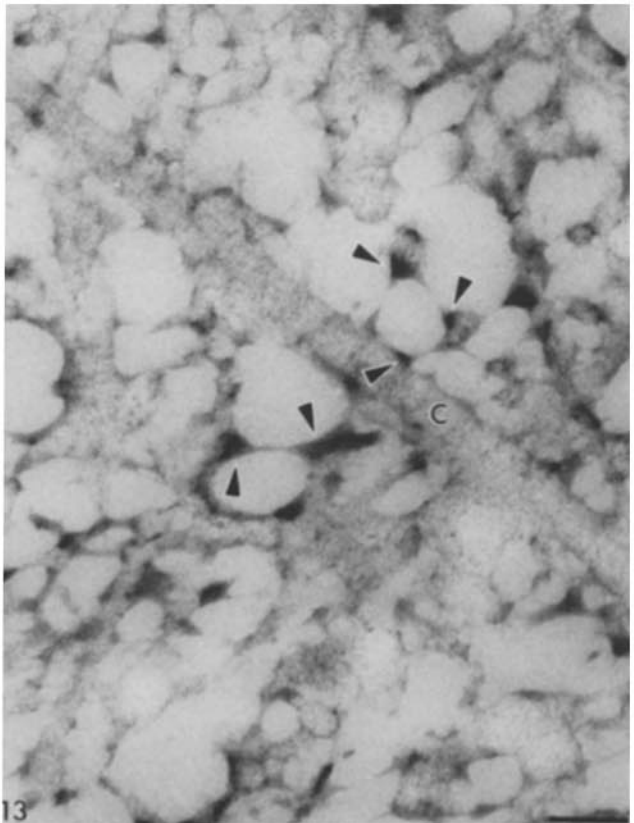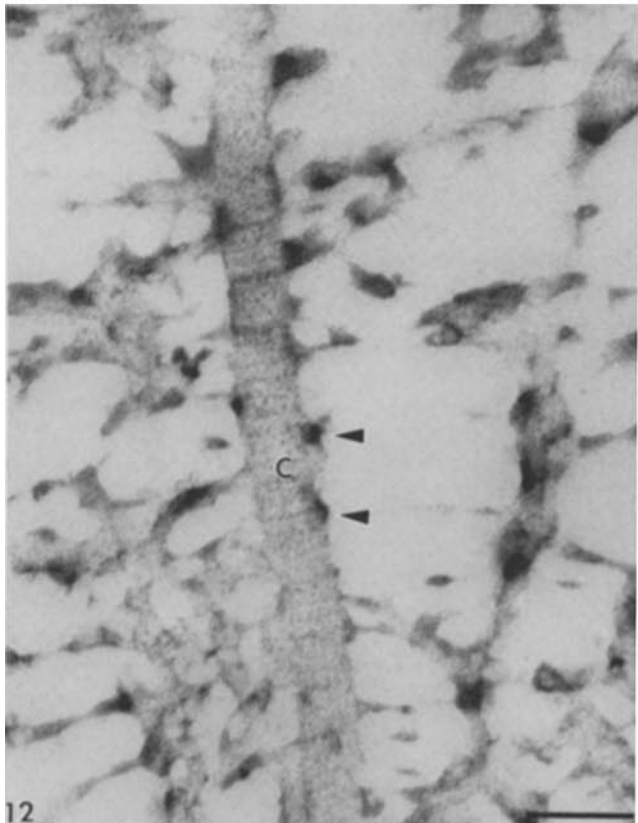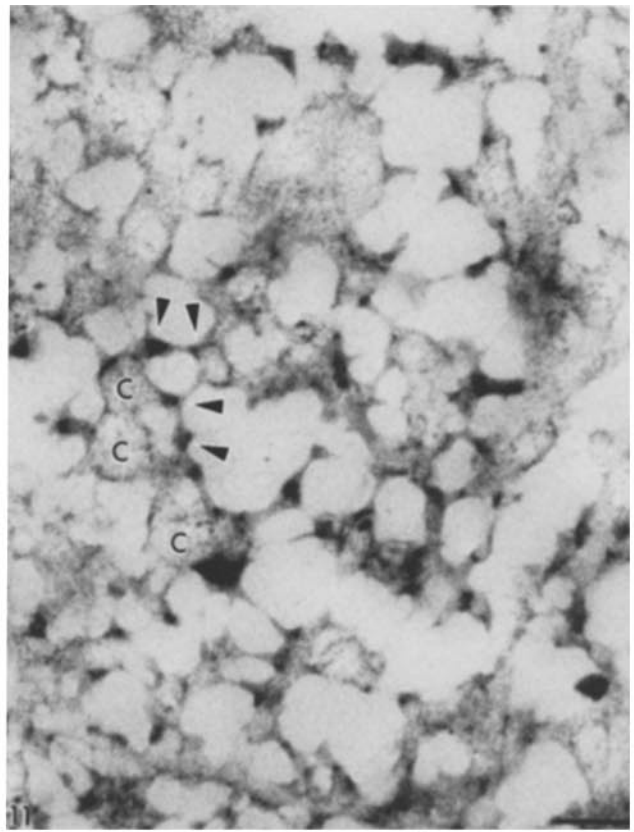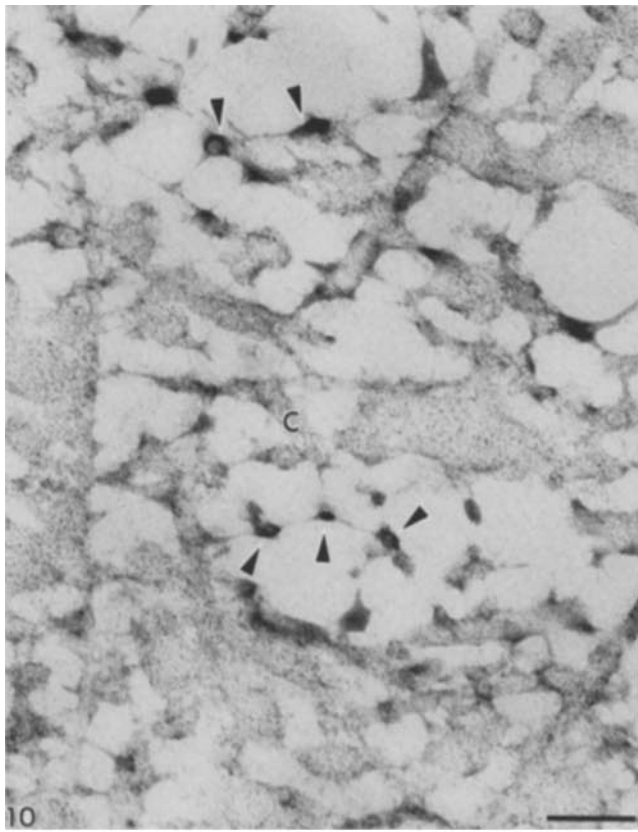
FIGURE 10   Interterritorial region of deep zone stained for link protein to show adjacent interfibrillar particulate staining (arrowheads) interconnected by fine filaments. Measurements of spacings between such particulate staining, indicated by arrows, are reported in the text. Bar, 100 nm. × 115,000.

FIGURES 11–13   Interterritorial region of deep zone stained for proteoglycan to show interfibrillar particulate staining (arrowheads) interconnected by fine filaments or paler bands of staining (Figs. 11 and 13). Measurements of spacings between such particulate staining (arrowhead pairs) (Fig. 12) are indicated in the text. The particulate staining on collagen (C) fibrils (arrowheads) is shown (Figs. 12 and 13). Bars, 100 nm. Fig. 11, × 115,000; Fig. 12, × 139,000; and Fig. 13, × 107,300.
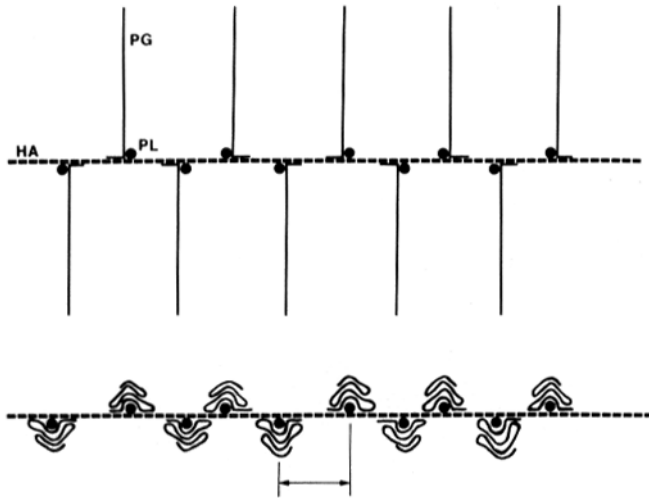
FIGURE 14 Diagrammatic representation of the attachment points of proteoglycan core protein monomer (*PG*) and link protein (*LP*) to hyaluronic acid (*HA*) in the interterritorial matrix. In the upper half of the figure is shown the arrangement under hydrated conditions. In the lower half is shown theoretically the collapsed proteoglycan monomer after fixation, treatment with antibody Fab′, and dehydration. The spacing indicated (between centers of particulate interfibrillar reaction product) was measured on electron micrographs to determine the spacings reported in the text. The glycosaminoglycan chains of proteoglycan have been omitted for simplicity.

larger sizes) and nonspecificity of immunoglobulin binding, usually through the Fc piece of the immunoglobulin G molecule. This has been circumvented in part by the use of Fab′ antibody fragments from which the Fc piece has been removed (51). Penetration of cartilage matrix can still be limited due to the high concentration of glycosaminoglycan, particularly chondroitin sulfate. Thus we have used thin sections of fixed tissues (to control fixation damage) and have treated these fixed sections with chondroitinase ABC to remove chondroitin sulfate (17) to increase tissue permeability. Inevitably these treatments must lead to some loss of proteoglycan and other matrix components from the cartilage matrix, and this is the subject of further study. We have, however, observed that after these treatments and reaction with antibody there is substantial staining left in the cartilage matrix. This is the subject of the present study.

Evidence for Proteoglycan and Link Protein Aggregated with Hyaluronic Acid in the Deep Zone

Electron microscope studies of cartilage proteoglycans using metachromatic dyes have revealed particulate, often short ribbonlike staining, indicating the presence of collapsed proteoglycans (due to dye binding, fixation, and dehydration). It has been suggested that this individual particulate staining represents either aggregated proteoglycans (38, 52) or monomer proteoglycans (15, 25). The results of the present study, using monospecific antibodies to proteoglycan monomer and to link protein, support the latter interpretation. The sizes of the reaction products observed in the present study are similar to those reported for dye-stained proteoglycans previously (15, 25, 38). The fine filamentous threads associated with these reaction products were thought by some to represent hyaluronic acid (15, 25, 38). The present study indicates that in the

interterritorial matrix of the deep zone, proteoglycan monomers and link proteins, particularly in interfibrillar sites, are indeed associated with hyaluronic acid. These are the three components of proteoglycan aggregate as extracted from cartilage (4, 6). These three components may not be associated together at the same attachment points, although our present biochemical understanding would suggest this (7, 8). The demonstration that fine filamentous staining (for link protein) or fine banded staining (for proteoglycan) between stained particles in the interterritorial matrix is much less commonly seen after cleavage with purified hyaluronidase, would indicate that it represents hyaluronic acid. This visualization of hyaluronic acid may result from the tendency of proteoglycans to collapse on a backbone of hyaluronic acid (Fig. 14), revealing its presence. Also, reaction product may bind nonspecifically to hyaluronic acid, either directly or indirectly via proteoglycan. The spacings of reaction product for link protein and proteoglycan monomer were similar (but not the same) in the interterritorial matrix in interfibrillar regions. They were also of an order similar to those reported for the spacing of proteoglycan monomers attached to hyaluronic acid in isolated proteoglycan aggregates examined by electron microscopy (9, 15, 39) and biochemically (5, 9, 40). The spacings of reaction product were, significantly, approximately twice the lengths of extended side chains of cartilage proteoglycans (14, 15). Thus, it is concluded that these individual proteoglycan particulate reaction products represent collapsed proteoglycan monomers that in interterritorial regions are bound to hyaluronic acid in the presence of link protein. Earlier electron microscope studies of cartilage using *Streptomyces* hyaluronidase to treat cartilage sections revealed that metal-stained particles, considered to represent proteoglycans, were removed by treatment with this enzyme (38). However, although these authors used proteinase inhibitors, their results indicated that their enzyme preparation degraded proteoglycan, rendering their observations inconclusive.

Whether link protein is bound at the attachment site of proteoglycan monomer to hyaluronic acid in interfibrillar sites in the interterritorial matrix remains to be determined, although our observations do clearly indicate that the spacings of reaction product for both these molecules on hyaluronic acid were of the same order. Biochemical studies of isolated aggregates extracted under associative conditions (7, 8) indicated that proteoglycan and link protein are present at the same attachment sites, probably side by side. It is likely that wherever we detect interfibrillar proteoglycan in the interterritorial zone, then link protein is present at the same site. A theoretical representation of the arrangement of these molecules before and after fixation, antibody reaction, and dehydration is shown in Fig. 14.

Based on these and our other observations, a model for the interfibrillar organization of proteoglycan, link protein, hyaluronic acid, and collagen in the interterritorial zone is proposed in Fig. 15. Although current concepts of proteoglycan aggregates indicate individual points of attachment of proteoglycan monomer to hyaluronic acid (5, 8), a close inspection of electron micrographs of proteoglycan aggregates spread on cytochrome *c* monolayers (9, 14, 39) reveals frequent sites on hyaluronic acid at which more than one proteoglycan monomer is attached. The possibility of binding of more than one pair of molecules of link protein and proteoglycan at the same site is thus also shown in Fig. 15. Moreover, as our results have indicated, these aggregated proteoglycans are apparently anchored by hyaluronic acid to thick collagen fibrils at regular
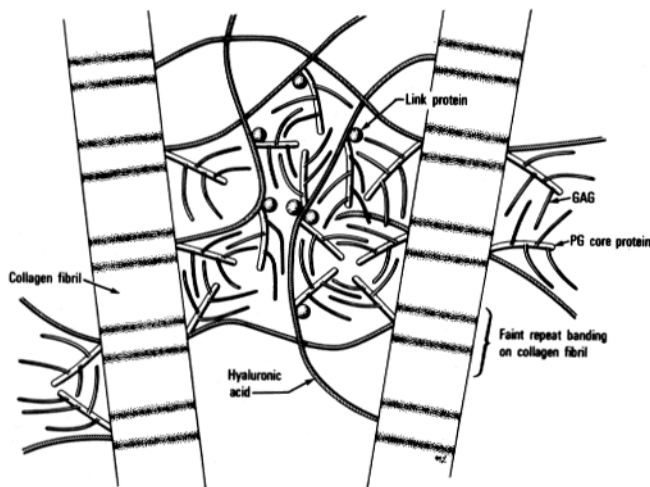
FIGURE 15 A diagrammatic representation of the proposed organization of proteoglycan monomer, link protein, hyaluronic acid, and collagen observed in section in the hydrated interritorial matrix. This is based on a summary of the experimental results obtained in this investigation. The attachment points of link protein and proteoglycan to hyaluronic acid and of proteoglycan to collagen fibrils and the diameters of collagen fibrils have been drawn to scale. It is not known whether more than one proteoglycan monomer and link protein can be present at the same attachment point to hyaluronic acid (see Discussion), and both possibilities are shown. Proteoglycan monomer and hyaluronic acid were found to be associated with collagen fibrils at the same regular sites in the absence of link protein. GAG represents glycosaminoglycan; chondroitin sulphate chains can be up to 40 nm in length and are not drawn to scale. Proteoglycan core protein has also been sectioned along its length and is shown in part only; it can reach a length of 400 nm (9). Only those parts of the monomers are shown which include the binding region for hyaluronic acid and a possible attachment region for collagen. Hyaluronic acid molecules may reach a length of 2,000 nm and several portions of these molecules are shown interlacing with each other to form a weave encompassing the collagen fibrils and interfibrillar space.

attachment points, where proteoglycan is also present (Fig. 15). Thus, a highly organized weave of hyaluronic acid (of which molecules may be between 200 and 2,000 nm in length [9, 39]) between collagen fibrils forms the basis of an ordered arrangement of proteoglycans and link proteins in the interterritorial matrix.

The observed branching of hyaluronic acid from sites where link protein and proteoglycan are present is similar to that reported for proteoglycan from dye binding studies where fine filaments were seen to originate from single stained particles (25). Under nondissociative conditions, isolated link protein can form oligomers, including tetramers (11). Thus, link protein may act through self-association as a linkage protein joining hyaluronic acid molecules together between collagen fibrils. This is supported by our observations that link protein, but not proteoglycan monomer, can precipitate hyaluronic acid under associative conditions in vitro. We do not yet know whether link protein can self-associate when bound to these molecules, although this is proposed in Fig. 15.

Link Protein Associated with Collagen

The presence of link protein on collagen fibrils with a staining pattern different from that seen for proteoglycan (in the territorial zone) raises the possibility that link protein performs functions other than stabilizing proteoglycan aggregates. Alternatively, if this difference of link protein on collagen fibrils (compared with that of proteoglycan) is artifactual, this would indicate a looser association of link protein with matrix molecules, such as proteoglycan, than is seen elsewhere.

The previously observed relative lack of staining for link protein in the territorial matrix of the deep zone compared with the interterritorial matrix (17) has been confirmed. It may be related to differences in organization, which may limit antibody reactivity with link protein. The reasons for the relative absence of link protein in the deep territorial matrix compared with that in the superficial and middle zones where it is also present on collagen fibrils are not known, but would favor as observed, a different organization of proteoglycan in such sites, possibly independent of hyaluronic acid, or a reversible interaction with hyaluronic acid, since link protein can prevent reversible binding (5, 10, 11).

Heterogeneity of Proteoglycan Structure

Elsewhere in cartilage matrix, outside the interterritorial zone, the organization of proteoglycan as well as link protein is strikingly different. Smaller particulate reaction product for proteoglycan was observed in close association with collagen fibrils, in both territorial and pericellular matrix. Extracts of articular cartilage (in 4 M guanidine hydrochloride) contain a variety of proteoglycans of varying buoyant density and hydrodynamic size (2, 41). If the size of the reaction product is related to size of the proteoglycan, determined in part by the length of the core protein where antigenic sites reside (31), the largest proteoglycans may be represented by those mainly found in the interterritorial matrix. The smallest and intermediate-sized proteoglycans would likewise be expected to be concentrated in the pericellular and territorial matrix, particularly in superficial and middle zones. These conclusions are supported by recent biochemical studies of bovine hip cartilage which indicated that the superficial and middle zones lack the larger proteoglycans found deeper in the cartilage (22).

Proteoglycan that is bound to collagen in the superficial zone and resists extraction with 4 M guanidine hydrochloride may be intimately entrapped in collagen fibrils as they form; it may even be covalently bound. This tight association may be very important for the function of collagen fibrils in this site where the glycosaminoglycan content is much lower (18, 19, 21, 23) and where fibrils are often arrayed parallel to the articular surface (42). Collagen is responsible for the tensile strength of cartilage (43) and the added association of proteoglycan in this zone may strengthen by interfibrillar cross-linking fibrils in this region which is very exposed to the shearing stresses of articulation. In the growth plate, Eisenstein et al. (25) also detected, with dye staining, proteoglycan bound to collagen which was unextractible with 3 M guanidine hydrochloride.

The association of proteoglycan with collagen fibrils in the deep interterritorial matrix was more ordered. Large discrete reaction products were observed at regular 72-nm intervals along collagen fibrils. Similar regular arrays of proteoglycan on cartilage collagen were reported earlier by workers using bismuth (44) and ruthenium red (45) and by Scott et al. (46, 47) using a phthalocyanin-like dye to study the very differently structured, dermatan sulfate–rich proteoglycans of rat tail tendon. The ~67-nm axial periodicity of types I, II, and III collagens which is commonly seen by electron microscopy is strikingly similar to that observed in situ for the distribution of

proteoglycan on collagen in interterritorial zones. The proteoglycan binding was related to the faint double- and single-banded staining detected by metal staining of collagen fibrils (Fig. 7). Similar correlations of proteoglycan spacing on collagen with its periodicity have recently been reported (47). These interactions of interfibrillar and fibrillar proteoglycans are shown diagrammatically in Fig. 15. Although hyaluronic acid has been seen to interact with interterritorial proteoglycan on collagen fibrils, this may not be a direct or stable interaction, particularly since link protein was not normally detected here. Thus proteglycan bound to collagen may here represent the nonaggregating proteoglycan that has been more commonly detected biochemically deep in bovine hip articular cartilage (22).

Proteoglycans are generally considered to be responsible for the comprehensive stiffness of articular cartilage, through their ability to reversibly bind water (48). The ability of cartilage to withstand reversible compression on articulation probably resides mainly with aggregated proteoglycans stabilized by link protein. Link protein stabilizes against physical stresses particularly those produced artificially by ultracentrifugation (11) and naturally by articulation. In the interterritorial region the collagen fibrils are well-spaced and at their widest, and proteoglycans are probably link-stabilized and at their largest. Thus, the major features of the organization of the interterritorial cartilage suggest that its properties may endow it with the greatest compressibility found in articular cartilage.

In conclusion, we have provided evidence for a diversity of organization of proteoglycan and link protein in bovine articular cartilage. The techniques used to study this inevitably may result in some loss or denaturation of these components in the cartilage matrix and a change in the natural organization. However, the many new questions raised by our observations and the indications that proteoglycan organization is diverse and that aggregates exist *in situ* draw attention to the complexity of organization of articular cartilage and a future need to understand its functional significance.

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