Role of Proteoglycans in Endochondral Ossification: Immunofluorescent Localization of Link Protein and Proteoglycan Monomer in Bovine Fetal Epiphyseal Growth Plate

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ABSTRACT The hypothesis is widely held that, in growth plate during endochondral ossification, proteoglycans in the extracellular matrix of the lower hypertrophic zone are degraded by proteases and removed before mineralization, and that this is the mechanism by which a noncalcifiable matrix is transformed into a calcifiable matrix. We have evaluated this hypothesis by examining the immunofluorescent localization and concentrations of proteoglycan monomer core protein and link protein, and the concentrations of glycosaminoglycans demonstrated by safranin 0 staining, in the different zones of the bovine fetal cartilage growth plate. Monospecific antibodies were prepared to proteoglycan monomer core protein and to link protein. The immunofluorescent localization of these species was examined in decalcified and undecalcified sections containing the zones of proliferating and hypertrophic chondrocytes and in sections containing the zones of proliferating and hypertrophic chondrocytes and the metaphysis, decalcified in 0.5 M EDTA, pH 7.5, in the presence of protease inhibitors. Proteoglycan monomer core protein and link protein are demonstrable without detectable loss throughout the extracellular matrix of the longitudinal septa of the hypertrophic zone and in the calcified cartilage of the metaphysis. In fact, increased staining is observed in the calcifying cartilage. Contrary to the prevailing hypothesis, our results indicate that there is no net loss of proteoglycans during mineralization and that the proteoglycans become entombed in the calcified cartilage which provides a scaffolding on which osteoid and bone are formed. Proteoglycans appear to persist unaltered in the calcified cartilage core of the trabeculae, until at last the entire trabeculae are eroded from their surfaces and removed by osteoclasts, when the primary spongiosa is replaced by the secondary spongiosa.

Hyaline cartilages are specialized connective tissues which consist of relatively few cells distributed throughout an abundant intercellular matrix. The intercellular matrix is composed of collagen fibers, which give the tissue tensile strength (26), and proteoglycans with elastic properties (27). In native cartilages, most of the proteoglycan exists as aggregates (13–15, 34, 42, 43) formed by the noncovalent association of proteoglycan monomers, hyaluronic acid, and link protein (1, 4–6, 12, 13). Link protein appears to bind simultaneously to the hyaluronic acid-binding region of proteoglycan monomer core protein and hyaluronic acid. It stabilizes the binding of proteoglycan monomer to hyaluronate (11, 28, 29, 48) against dissociative forces, thereby maintaining the integrity of proteoglycan aggregates in the intercellular matrix.

Of all the hyaline cartilages, growth-plate cartilage is perhaps the most specialized. Within it cells proliferate and differentiate and extracellular matrix changes in a continuous manner, leading to provisional calcification of the matrix of longitudinal septa and erosion of transverse septa and of dead and dying cells by capillaries invading from the metaphysis. It consists

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first of maturing and then of degenerating cells, surrounded by an intercellular matrix with different properties. Growth-plate cartilage consists of: the zone of resting chondrocytes; the zone of proliferating chondrocytes; the zone of maturation; the zone of hypertrophic cells including the zone of provisional calcification; and the metaphysis, where new bone is formed on the longitudinal septa of partly calcified cartilage. Several crucial processes occur in these regions which result in the longitudinal growth of bone. These processes depend upon the functions of the cells and consequently upon the structure and properties of the intercellular matrix surrounding the cells in a particular region.

In the zone of proliferating chondrocytes, chondrocytes divide at a rapid rate (24, 25, 52). Proteoglycan aggregates of exceptionally large size (34) are present in this region, which contribute to the elasticity and compliance of the intercellular matrix in this region. Isolated proteoglycan aggregates can inhibit mineralization (2) and are thought to prevent calcification in the zone of proliferating chondrocytes and in the zone of maturation and in the upper hypertrophic zone (7, 20–22, 35, 36).

Nearby, however, in the lower hypertrophic zone, calcification occurs in the intercellular matrix. In the zone of hypertrophic chondrocytes, matrix vesicles appear in the intercellular matrix. In the lower hypertrophic zone, mineralization occurs within these matrix vesicles. In the region of the lower hypertrophic cells, mineral deposition suddenly spreads from matrix vesicles throughout the intercellular matrix and the intercellular matrix of the cartilage in the metaphysis becomes diffusely and heavily calcified.

While mineralization may begin in the matrix vesicles, mineralization within matrix vesicles does not explain how the intercellular matrix surrounding the matrix vesicles in the zone of the lower hypertrophic cells is suddenly transformed from a noncalcifiable to a calcifiable intercellular matrix. Since proteoglycan aggregates present in the intercellular matrix of the upper growth plate are thought to inhibit mineralization (2, 7, 22, 35), these proteoglycan aggregates must be structurally modified in some way in the lower hypertrophic zone, so that their capacity to inhibit mineralization is abolished, and so that a noncalcifiable matrix is transformed into a calcifiable matrix.

Some investigators have suggested that proteoglycans are removed by degradation by proteases in the lower hypertrophic zone (10, 17, 23, 32) and that proteolytic degradation of proteoglycans results in the transformation of a noncalcifiable matrix into a calcifiable matrix. If this is true, the concentration of proteoglycan should be decreased in the intercellular matrix of calcified cartilage, and the proteoglycans entombed in the calcified cartilage of the metaphysis should consist of fragments of proteoglycan monomers and link protein. One way to examine the above hypothesis would be to use monospecific antibodies to isolated proteoglycan monomer and link protein and localize them in growth plate. If link protein and proteoglycan monomer are removed by proteases in the lower hyper-

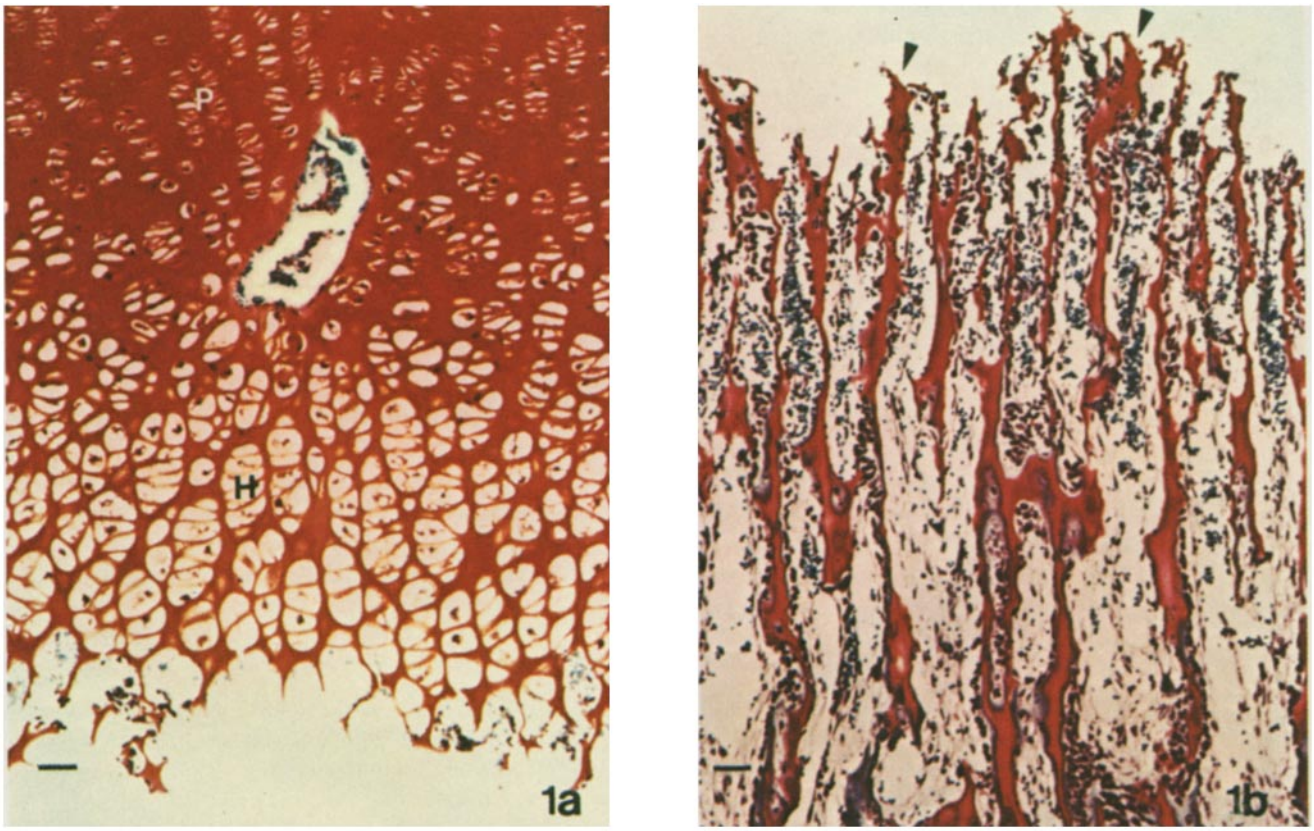


FIGURE 1 Sections of (*a*) growth plate and (*b*) metaphysis stained with safranin O and fast green. *a* was prepared without decalcification. One can see the proliferating (*P*) and hypertrophic (*H*) chondrocytes down to the level of the transverse septa of the last hypertrophic chondrocytes at their junction with the metaphysis. There is strong red staining with safranin O everywhere in the extracellular matrix. In *b* the trabeculae (spicules) of the metaphysis have been decalcified to show their origin from the longitudinal septa of the hypertrophic zone: their fracture face is shown (arrows) at the top of the figure. These trabeculae stain strongly with safranin O. They are surrounded by osteoblasts which are depositing osteoid and bone. Bars, 50 μm. × 90.

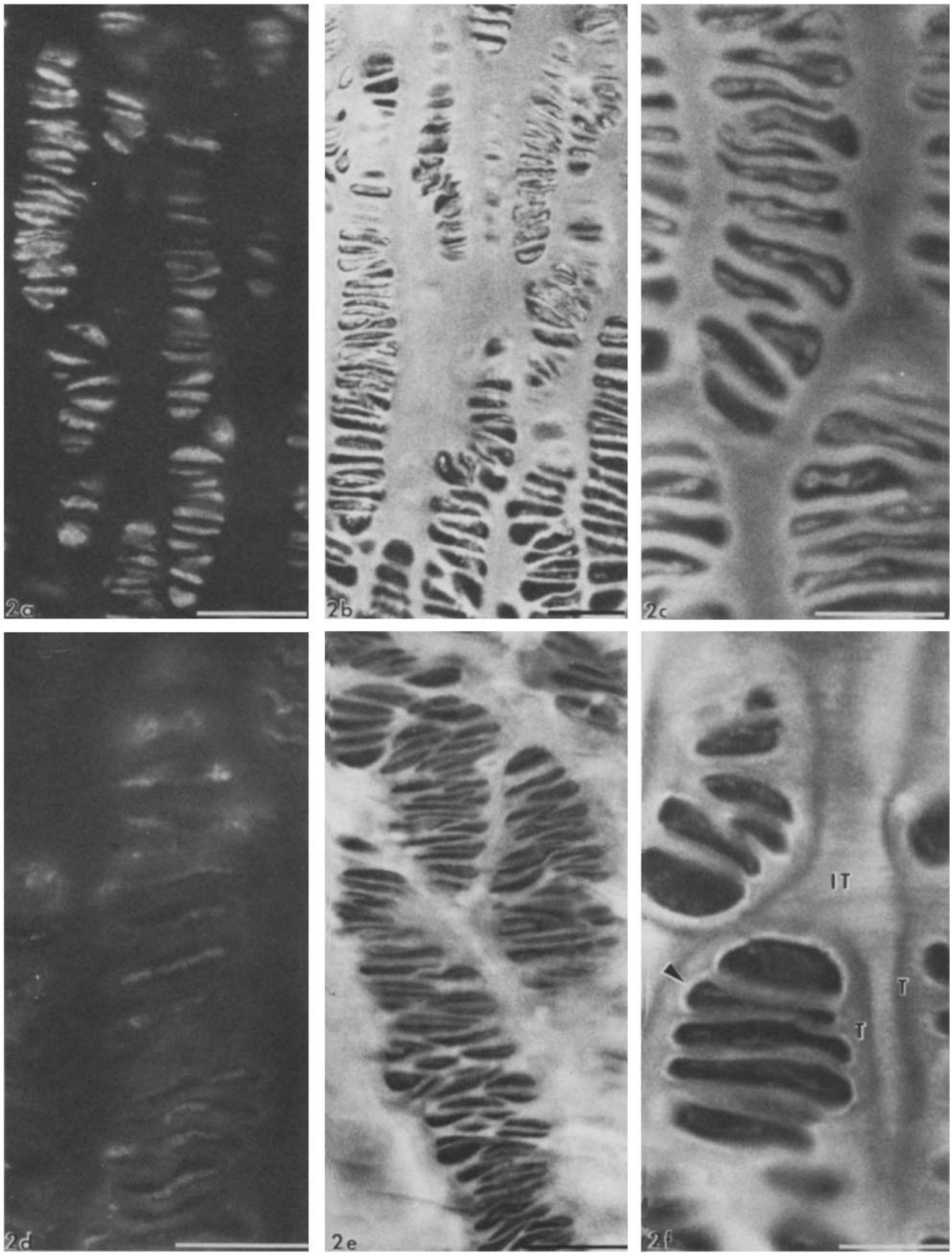
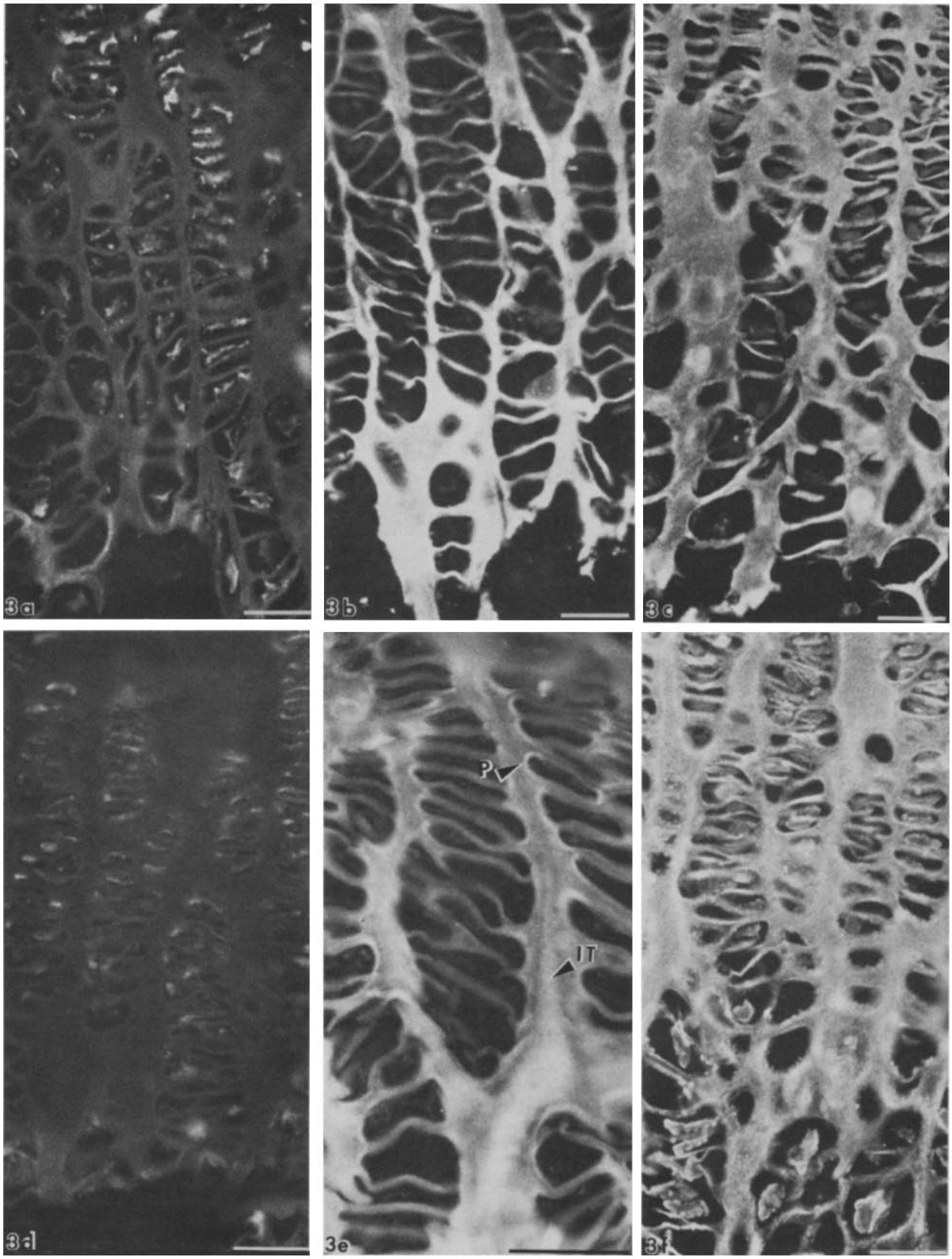


FIGURE 2 Decalcified sections of the growth plate to show the zone of proliferating chondrocytes. In *b* and *c*, sections were first stained with sheep antibody Fab' against cartilage proteoglycan; this produced a fairly even staining of the cartilage matrix in longitudinal and transverse septa with some more intense pericellular staining as seen in *c* at a higher magnification. Sections initially treated with a nonimmune sheep serum Fab' showed no matrix staining but some weak cellular staining (*a*). Similar observations were made for link protein and for proteoglycan. Thus, sections initially treated with rabbit antibody Fab' to link protein contained bright matrix staining (*e* and *f*). This was most concentrated in pericellular sites (arrowhead) and in interterritorial sites (*IT*). The territorial matrix (*T*) was weakly stained. Although link staining was stronger in transverse septa in the zone of very flattened cells, transverse septa stained less strongly nearer the hypertrophic zone. Sections initially treated with nonimmune rabbit Fab' displayed no matrix or cellular staining (*d*). Bars, 50 μm. *a*, *e*, and *f*, × 400. *b*, × 260. *c* and *d*, × 460.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trophic zone during endochrondral ossification, one would expect to see a decrease in the concentration of these species just before the onset of mineralization. We have therefore developed methods for the immunofluorescent localization of link protein and proteoglycan monomer core protein (38) and examined the distribution of these species in the zones of proliferating chondrocytes, hypertrophic cells, and in the calcified cartilage of the metaphysis of growth-plate cartilage. We describe the results here.

MATERIALS AND METHODS

Materials

GdmCl "absolute grade" and CsCl "O.D. grade" were obtained from Research Plus Laboratories, Inc. (Bayonne, N. J.). 2-(N-morpholino)ethanesulfonic acid (MES) was obtained from Calbiochem-Behring Corp., (American Hoechst Corp., San Diego, Calif.), and iodoacetamide and phenylmethylsulfonyl fluoride from Sigma Chemical Co. (St. Louis, Mo.). Benzamidine hydrochloride was purchased from Aldrich Chemical Co. (Metuchen, N. J.), gelatin was from British Drug Houses (Montreal), and Eriochrome black from Difco Laboratories (Detroit, Mich.). Chondroitinase ABC was obtained from Miles Laboratories Inc. (Research Products Div., Elkhart, Ind.).

Antisera and Immunoreagents

A full description of the antisera we used has already been given (37, 38). Briefly, we prepared a specific sheep antiserum (S27) against native high buoyant density bovine articular cartilage proteoglycan monomer AIDI according to the nomenclature of Heinegård (16). A rabbit antiserum to bovine nasal cartilage link protein (R131) was shown to be specific for this species after removal of antibodies to proteoglycan monomer (38). These antisera showed a strong cross-reaction with these species isolated from epiphyseal and growth-plate cartilages. We prepared proteoglycan monomers and link protein as previously described (44, 45, 48).

Preparation of Growth Plate and Metaphysis for Histochemical and Immunohistochemical Studies

The localizations of proteoglycan monomer core protein and link protein were examined in the growth plates and metaphysis of proximal tibiae which were demineralized with EDTA for all studies. Fetal tibiae were removed by dissection. The perichondrium surrounding the growth plate of the proximal tibia was incised circumferentially. One or more vertical mediolateral slices were made from the articular surface of the proximal tibial epiphysis to the metaphysis. With a twist of the scalpel blade, a fracture was created at the junction of the transverse septa of the last hypertrophic cells and the metaphysis. This fracture yielded two fragments, a metaphyseal fragment containing calcified cartilage, and a fragment containing the cartilaginous epiphysis and the growth plate (Fig. 1 *a* and *b*). The fragment containing the epiphyseal cartilage and growth plate was divided into vertical slices (coronal sections). A part of the epiphyseal cartilage was removed to provide specimens of growth plate which were 4 mm high, 4 mm wide, and 2 mm thick. Coronal sections of the metaphyseal fragment were prepared using a Dremel electric saw with no. 8029 fine blades.

Other specimens consisted of the entire growth plate, including the metaphysis (Fig. 6 *a*). Coronal sections of the proximal tibial growth late and sagittal sections of the distal femoral growth plate were prepared with a Dremel saw (Dremel Manufacturing Co., Racine, Wis.). Where indicated, the specimens were decalcified by slowly oscillating the specimens in test tubes on a Buchler tube shaker (Buchler Instruments Inc., Fort Lee, N. J.), in 0.5 M EDTA, pH 7.5, containing 5-mM concentrations of benzamidine hydrochloride, iodoacetamide, and phenylmethylsulfonyl fluoride, at 5°C for 48 h. For immunohistochemical studies, the specimens were washed for 4 h in 1 M NaCl, 0.01 M MES, pH 7., then embedded in gelatin as described previously (38).

Extraction of Proteoglycans

Specimens were extracted with 4 M GdmCl, 0.15 M sodium acetate, pH 6.3, containing protease inhibitors, at 5°C for 24 or 48 h, to remove proteoglycans and link protein. In other experiments, specimens were extracted with 4 M GdmCl, 0.5 M EDTA, 0.15 M sodium acetate, pH 7.5, containing protease inhibitors, at 5°C for 24 or 48 h.

Safranin 0 Staining

Specimens of undecalcified fresh growth plate, or decalcified or extracted growth plate, were fixed in 10% formalin for 48 to 72 h. Microscopic sections (5-μm thick) were prepared and stained with safranin 0 and fast green, as previously described (41).

Frozen Sectioning, Fixation, and Treatment of Sections with Chondroitinase ABC

4-μm thick frozen sections of demineralized tissues were fixed for 5 min in formaldehyde and treated with chondroitinase ABC to remove chondroitin sulphate, to enhance permeability of immunoreagents, as previously described (38).

Treatment of Fixed Sections with Immunoreagents

5 mM cysteine was used at all times and sections were stained for proteoglycan and link protein with Fab' reagents as described earlier (38). Briefly, the sheep antibody to bovine articular proteoglycan and the rabbit antibody to bovine nasal cartilage link protein were used as Fab' reagents. Fluorescein-labeled pig Fab' anti-sheep Fab' and pig Fab' anti-rabbit Fab' were used to detect the reactions of the first step reagents. Antisera Fab' absorbed with epiphyseal PG or link protein, and nonimmune serum Fab' of the same species were used as controls. These controls gave identical results and only nonimmune serum Fab' results are shown in Results.

RESULTS

The immunofluorescent localization of proteoglycan monomer and link protein was first studied in specimens which included the zones of proliferating and hypertrophic chondrocytes, down to the transverse septa of the last hypertrophic chondrocytes. Fig. 1 *a* shows the appearance of a section from this kind of specimen, stained with safranin 0 and fast green. Safranin 0 is a red cationic dye which binds to the anionic groups of glycosaminoglycans (41). This section shown in Fig. 1 *a* was prepared after fixation in 10% formalin, without prior decalcification. Similar results were obtained after decalcification (Fig. 6 *a* and *b*). Staining can be seen thoughout the growth plate.

To detect proteoglycan immunologically, control sections were reacted first with nonimmune sheep serum Fab', then reacted with fluorescein-labeled pig Fab' reactive against sheep

FIGURE 3 Decalcified sections of the growth plate to show mainly the hypertrophic zone up to its junction with the metaphysis. In *b* the matrix stained brightly with sheep antibody Fab' to proteoglycan in longitudinal and transverse septa. Stronger staining was seen in the hypertrophic zone closest to the metaphysis. In *c*, the growth plate was extracted with both EDTA and guanidine HCl before staining with antibody Fab' to proteoglycan. There is reduced staining but some unextrable proteoglycan is demonstrable compared with *a* where decalcified sections were initially treated with nonimmune sheep Fab'. Here, no matrix staining was observed but cells showed some weak staining. Sections *e* and *f* were stained for link protein with (*f*) and without guanidine HCl extraction (*e*). Strong staining was seen in *e* which was again strongest in interterritorial (arrowhead *IT*) and pericellular (arrowhead *P*) sites. Transverse septa were weakly stained. Guanidine and EDTA extraction partly reduced staining, particularly in pericellular sites, but enhanced transverse septa staining and territorial staining. No separate territorial and interterritorial zones were observed after extraction. In *d*, the decalcified section was first treated with nonimmune rabbit Fab'. No staining was seen. Bars, 50 μm. *a–d*, × 250. *e*, × 425. *f*, × 270.

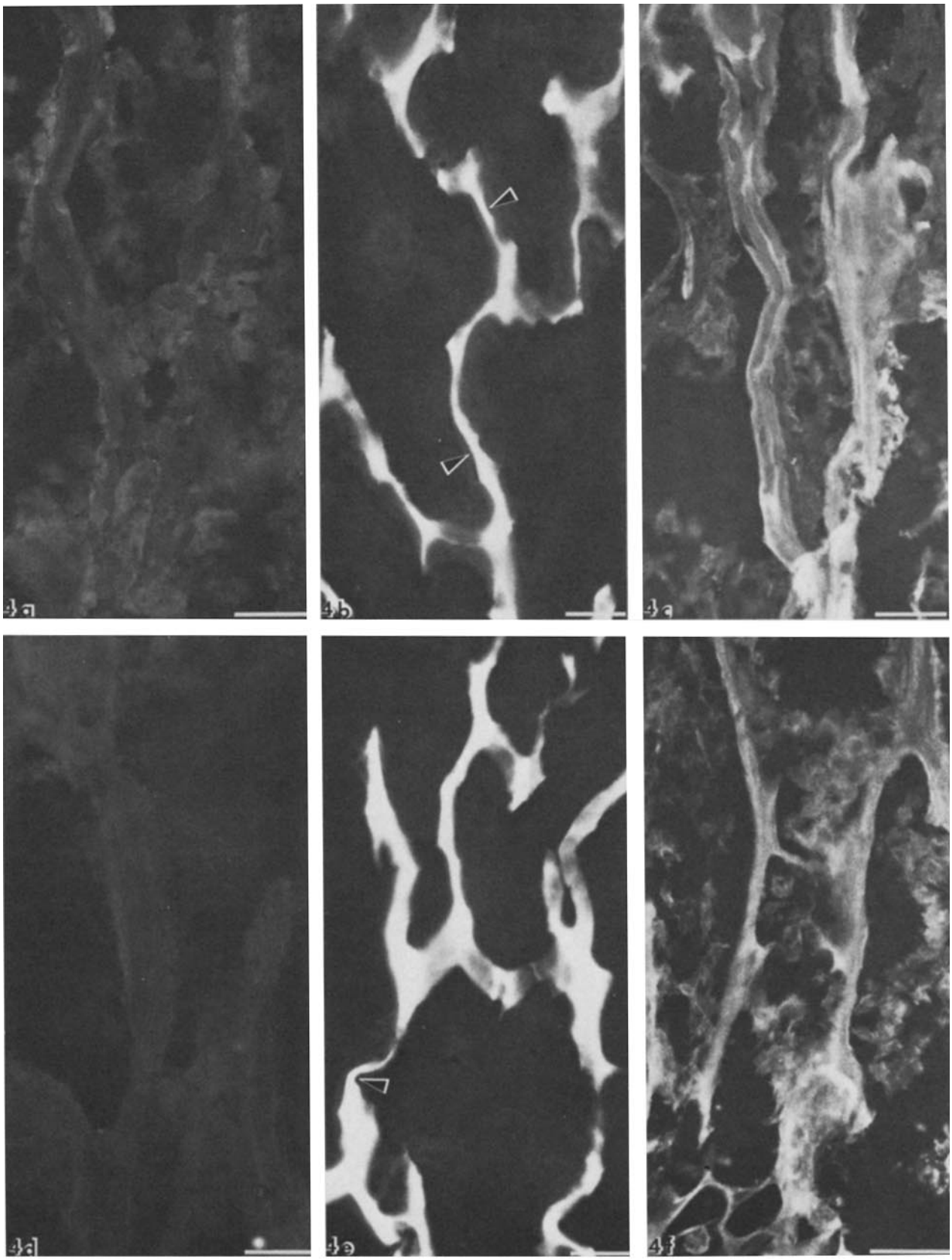


FIGURE 4 Decalcified sections of the metaphysis just below its junction with the hypertrophic zone. In *b* can be seen staining for proteoglycan in the trabeculae (arrowheads) on which osteoid and bone are being deposited. In *c*, tissue was first extracted in EDTA plus guanidine HCl. This left some residual staining. *a* is a decalcified section initially treated with a nonimmune Fab' and shows no stain. *e* shows staining for link protein in decalcified tissue (arrowhead). The tissue in *f* was decalcified with guanidine and reveals residual staining for link protein. *d* shows a section of decalcified tissue treated first with nonimmune Fab': there is no staining. Bars, 50 μm. *a* and *c*, × 260. *b* and *e*, × 220. *d*, × 240. *f*, × 290.

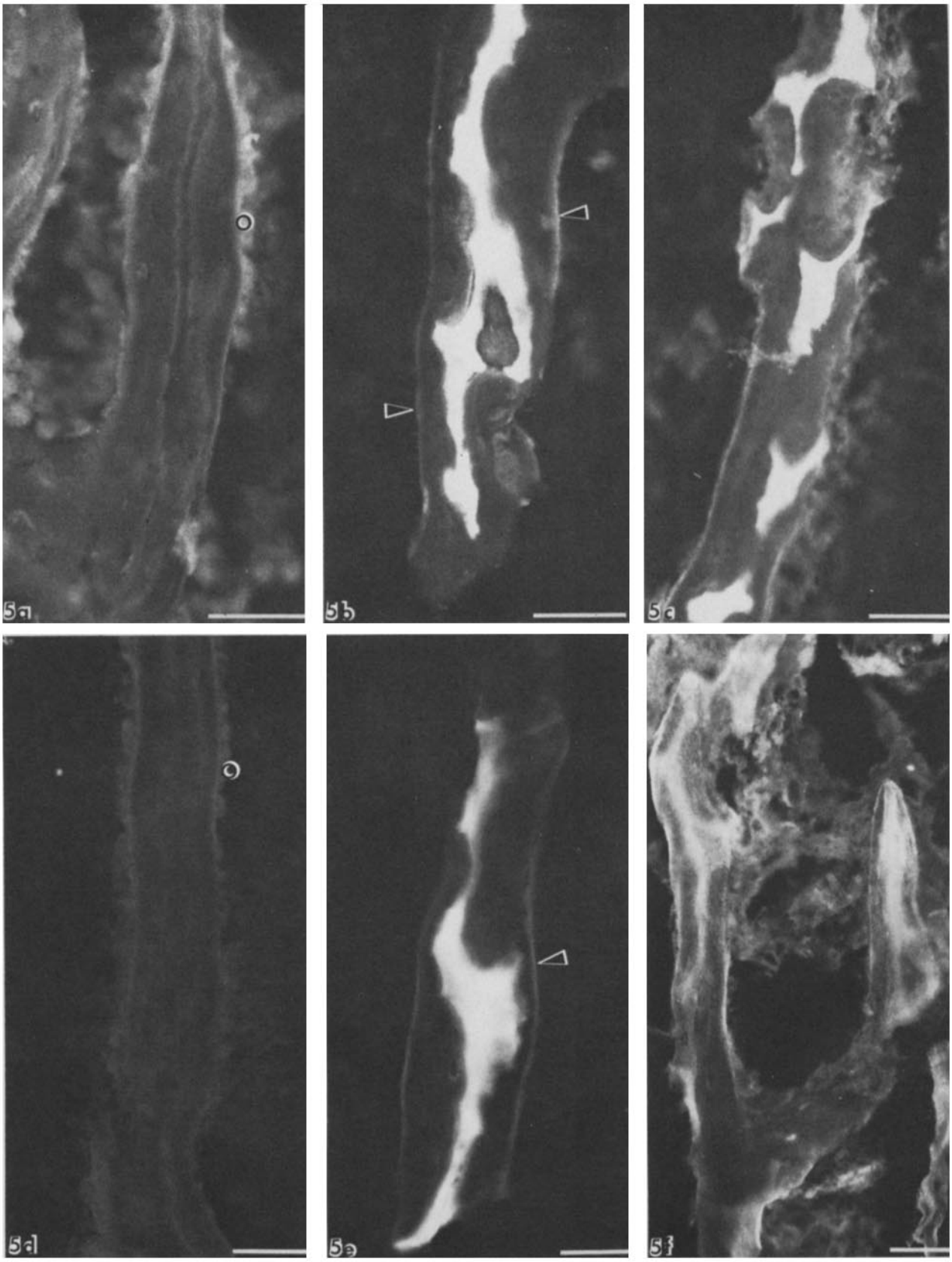
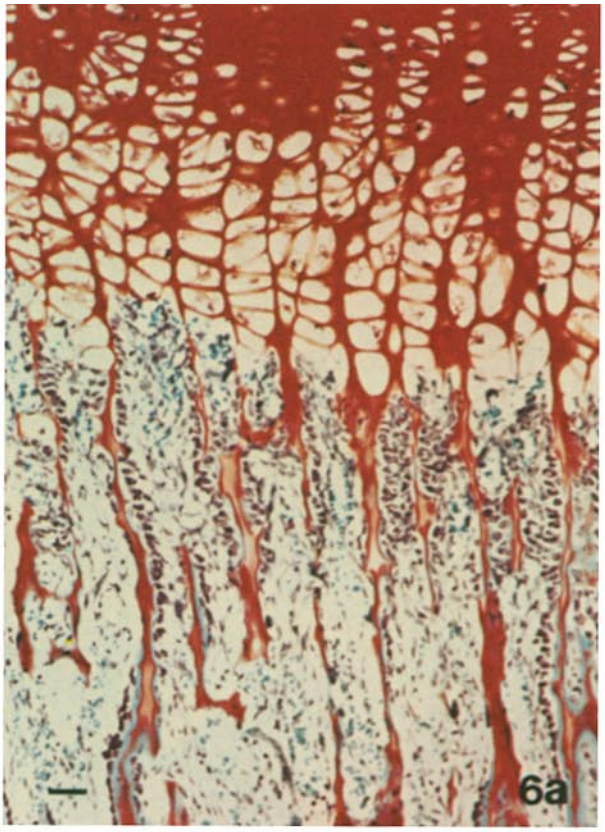


FIGURE 5 Decalcified sections of the metaphysis remote from the hypertrophic zone showing thicker trabeculae on which bone has been deposited. *b* was stained with antibody Fab′ to proteoglycan to reveal a strongly stained core. The staining of the peripheral osteoid (arrowheads) is not considered significant. After extraction with EDTA plus guanidine, strong residual staining persisted in the trabeculae (*c*). Decalcified tissue treated initially with nonimmune sheep Fab′ did not stain (*a*). Some osteoblasts (*O*) can be seen. In *e*, tissue was stained with rabbit antibody Fab′ to link protein which also reacted with the cartilaginous core of the trabeculae. Again, the peripheral staining in osteoid (arrowhead) is not considered significant. Decalcified and guanidine-extracted tissue showed some weak residual staining for link protein in (*f*). Sometimes, more residual staining was observed. There was no staining of decalcified tissue treated with nonimmune rabbit Fab′ (*d*), although osteoblasts could be seen. Bars, 50 μm. *a*, × 350. *b*, × 340. *c*, × 300. *d*, × 280. *e*, × 260. *f*, × 220.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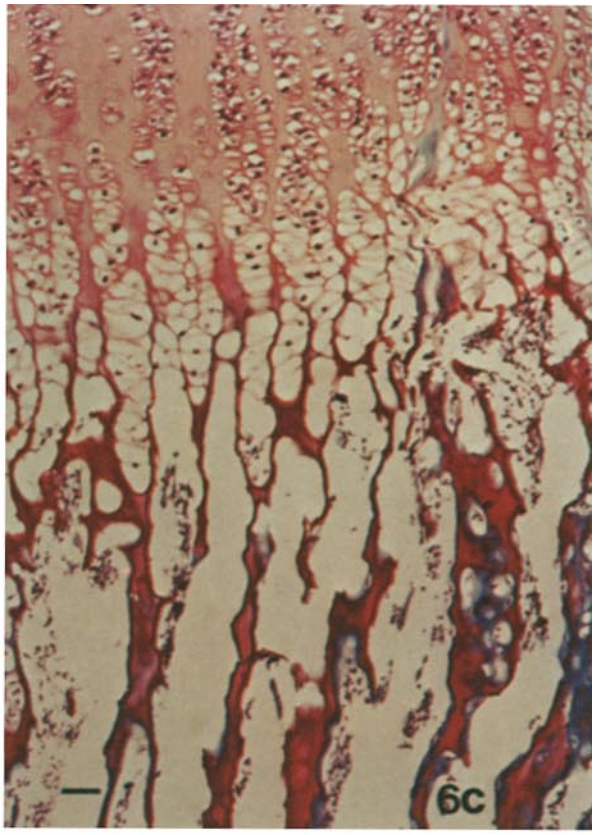
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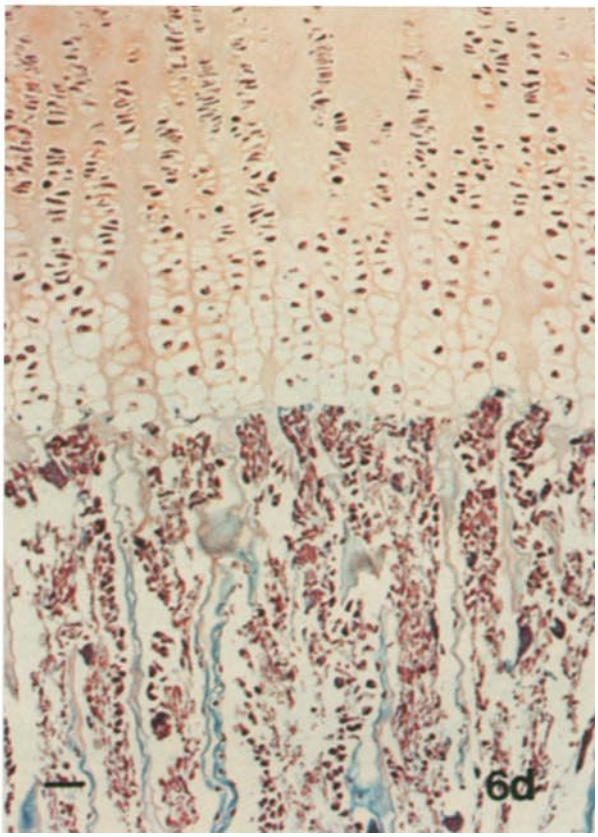
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Fab'. The cells showed some weak fluorescence, but the intercellular matrix did not stain (Figs. 2*a* and 3*d*). Fig. 2*b* and *c* show decalcified sections from the same regions initially treated with sheep antibody Fab' to proteoglycan monomer at the same protein concentration as used previously for sheep nonimmune Fab'. Strong staining was observed throughout the longitudinal and transverse septa of the zone of proliferating chondrocytes (Fig. 2*b* and *c*). Fig. 2*c* shows an area from the zone of proliferating chondrocytes at a higher magnification. The intensity of the immunofluorescence is greater in the pericellular regions than elsewhere. Similar results were obtained with Fab' antibody to link protein (Figs. 2*d*, *e*, and *f*), except that at higher magnification it was apparent that more intensely stained pericellular and interterritorial zones could be identified in the zone of proliferating cells in sections treated with the antibody to link protein (Fig. 2*f*). These observations of different territorial staining for link protein and proteoglycan monomer were very similar to those reported by us previously for articular cartilage (38). Immunoelectron microscopy studies have since revealed that this local reduction of territorial staining for link protein in articular cartilage is directly related to differences in the amount and organisation of link protein (A. R. Poole and L. Rosenberg, manuscript in preparation). In this study the stronger-stained interterritorial zone was restricted to the middle of the longitudinal septa. Although transverse septa of very "flattened" proliferating chondrocytes showed significant link staining (Fig. 2*e*), those nearer and in the hypertrophic zone revealed a reduction in link staining (Figs. 2*f* and 7). Fig. 3 shows decalcified control (Fig. 3*a*) and test sections (Fig. 3*b*) using antibodies to proteoglycan monomer core protein and link protein to stain the zone of hypertrophic chondrocytes, down to the transverse septum of the last hypertrophic chondrocyte. In the test section using antibody to proteoglycan monomer core proteins, fairly uniform intense staining was observed throughout the longitudinal and transverse septa of the zone of hypertrophic chondrocytes with an increase in general intensity towards the metaphysis (Fig. 3*b*).

Fig. 3*d* and *e* show control and test sections, respectively, stained with antibody to link protein, of the zone of hypertrophic chondrocytes. Again, the immunofluorescent staining is intense and pericellular, and territorial and interterritorial staining zones can often still be seen (Fig. 3*e*). However, staining in transverse septa is strikingly weaker. Using antibodies to either proteoglycan monomer core protein or link protein, there was a general indication of an increase in the intensity of staining of both proteoglycan monomer and link protein in the longitudinal septa of the growth plate as one approached the lowermost hypertrophic chondrocytes. Here, calcification of the matrix begins and staining for these molecules was at its strongest (Figs. 3*b*, and *e* and Fig. 7). These observations cast doubt upon the validity of the hypothesis that degradation and removal of proteoglycan occurs before the onset of mineralization in the longitudinal septa. These longitudinal septa which stained strongly for link protein and proteoglycan extended into the metaphysis (Figs. 6*a* and *b* and 7). Results similar to the above were obtained in studies of undecalcified cartilages of the proliferating and hypertrophic zones. In the interest of brevity these are not shown.

Metaphysis

This is shown in Fig. 1*b*. In the decalcified metaphysis, spicules of calcified cartilage stained intensely with safranin 0. The spicules of calcified cartilage, which are derived from the longitudinal septa of the growth plate, form channels which support the ingrowth of capillaries. On the surfaces of these spicules of calcified cartilage, rows of osteoblasts deposit osteoid and bone, which stains blue with fast green (Fig. 1*b*). The section shown in Fig. 1*b* was prepared after fixation in 10% formalin and decalcification in formic acid. Identical results were obtained when the metaphyseal fragments were decalcified in 0.5 M EDTA, pH 7.5, in the presence of protease inhibitors at 5°C for 48 h, then fixed in 10% formalin. For the immunofluorescent localization of proteoglycan monomer and link protein in specimens of the metaphyseal fragment, the specimens were decalcified in 0.5 M EDTA, pH 7.5, in the presence of protease inhibitors, then embedded in gelatin and sectioned. Fig. 4*a* and *b* show control and test sections from the metaphyseal fragment, containing spicules of calcified cartilage which have been covered by osteoid and osteoblasts stained for proteoglycan. In the control section, the calcified cartilage does not stain. In the test sections, intense staining for proteoglycan is observed in the calcified cartilage. Essentially identical results were obtained using antibodies to link protein (Figs. 4*d* and *e*, and 5*d* and *e*). Farther into the metaphysis, osteoblasts had deposited bone on the cartilagenous trabeculae. Here, the cartilage now formed a central core which stained brightly for proteoglycan (Fig. 5*b*) and link protein (Fig. 5*e*). Control sections were unstained (Fig. 5*a* and *d*). No convincing staining was observed in the osteoid in sections stained with antibody.

Effects of Dissociative Extraction on the Immunofluorescent Localization of Proteoglycan Monomer and Link Protein in Growth Plate and Metaphysis

We conducted studies to assess the effect of dissociative extraction of proteoglycans with 4 M GdmCl on the immunofluorescent localization of proteoglycan monomer and link protein in growth plate. Approximately 85% of the proteoglycan is removed from bovine nasal cartilage or bovine articular cartilage (43–45) or from bovine fetal epiphyseal cartilage (L. Rosenberg, unpublished observations) by dissociative extrac-

FIGURE 6 Sections of the entire growth plate, including the metaphysis, stained with Safranin 0 and fast green. *a* was fixed in 10% formalin, then decalcified in formic acid. *b* was decalcified without prior fixation, in 0.5 M EDTA, pH 7.5, containing protease inhibitors, at 5°C for 48 h. For the immunofluorescent localization of proteoglycan, specimens were decalcified by the latter procedure, then embedded in gelatin and sectioned. There is no discernible decrease in glycosaminoglycan content in the longitudinal septa of the lower hypertrophic zone, based on the intensity of Safranin 0 staining. *c* was extracted with 4 M GdmCl, 0.15 M sodium acetate, pH 6.3, containing protease inhibitors, at 5°C for 24 h, fixed in formalin, then decalcified in formic acid. The proteoglycan entombed in calcified cartilage is resistant to extraction. *d* was extracted with 4 M GdmCl, 0.5 M EDTA, 0.15 M sodium acetate, pH 7.5, containing protease inhibitors at 5°C for 24 h, then fixed in formalin. Most of the proteoglycan has been extracted from the calcified cartilage of the metaphysis. Bars, 50 μm. × 100.

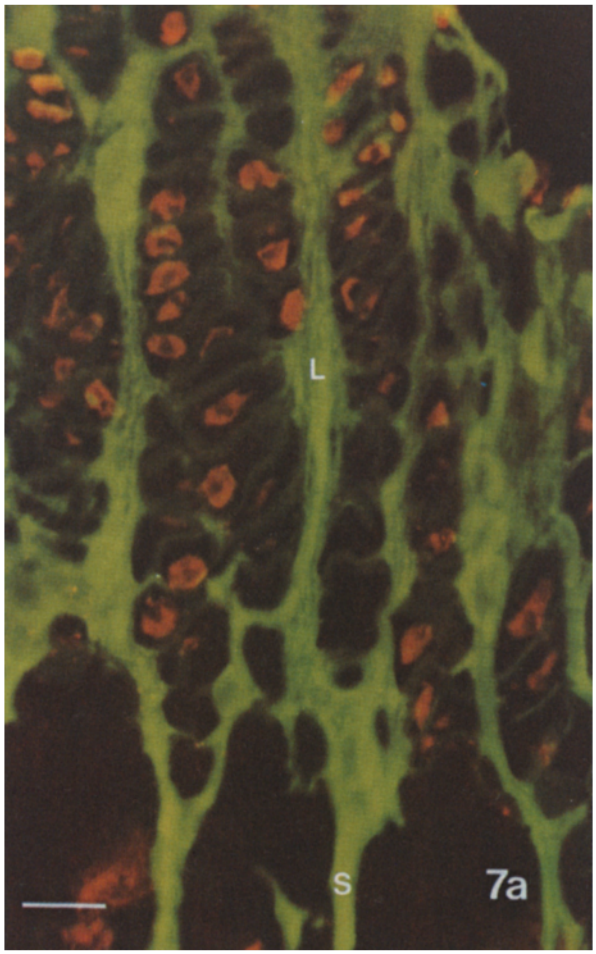
tion with 4 M GdmCl. However, proteoglycans within the growth plate appear to be more resistant to extraction (3, 30). Fig. 6 *c* shows the effect of 4 M GdmCl extraction on growth-plate proteoglycans as revealed by safranin 0 staining. The proteoglycans entombed in the calcified cartilage are resistant to extraction unless the cartilage and metaphysis are extracted with 4 M GdmCl containing 0.5 M EDTA (Fig. 6 *d*). In the noncalcified portion of the growth plate (Fig. 6 *c*, top) containing the zone of proliferating chondrocytes and the zone of hypertrophic chondrocytes, a substantial amount of proteoglycan remains unextracted by 4 M GdmCl, as indicated by the pink staining of the matrix with safranin 0 (Fig. 6 *c*). Even after extraction with 4 M GdmCl containing 0.5 M EDTA, some proteoglycan remains in the noncalcified growth plate (Fig. 6 *d*).

Examination of decalcified and guanidine-extracted sections treated with antibody to proteoglycan also revealed (like safranin 0) significant residual proteoglycan in the hypertrophic region (Fig. 3 *c*). Residual staining for link protein was stronger in territorial zones and transverse septa, with little reduction in overall staining (Fig. 3 *f*).

In the metaphysis, we observed marked residual staining for proteoglycan (Fig. 5 *c*) but often less for link protein (Fig. 5 *f*). Clearly, these molecules are much more tightly bound to growth-plate and metaphyseal cartilages. The reasons for this are not clear, but may be related to stronger structural interactions of proteoglycans and link proteins with collagen, associated with the mineralization of these cartilages.

DISCUSSION

Our results demonstrate for the first time the specific immunofluorescent localizations of both link protein and proteoglycan monomer core protein in the different zones of growth-plate cartilage, using well-characterized monospecific antibodies to these species. The results indicate that, in the longitudinal septa, proteoglycan monomer and link protein persist without significant loss during mineralization of the longitudinal septa of the cartilage matrix. Type II collagen also persists in the cartilage matrix of the longitudinal septa during and after mineralization, without apparent loss (9, 40, 50, 51). Together, these observations indicate that there is no detectable loss of proteoglycans and collagen from mineralizing cartilage before or during mineralization. Instead, they become entombed in calcified cartilage in the longitudinal septa. By capillary ingrowth, up to half of these septa may be eroded (47) together with transverse septa. The remaining longitudinal septa provide a scaffolding on which osteoid and bone are formed in the metaphysis. Finally, the trabeculae are eroded from their surfaces and removed by osteoclasts and chondroclasts, when the primary spongiosa is replaced by the secondary spongiosa.



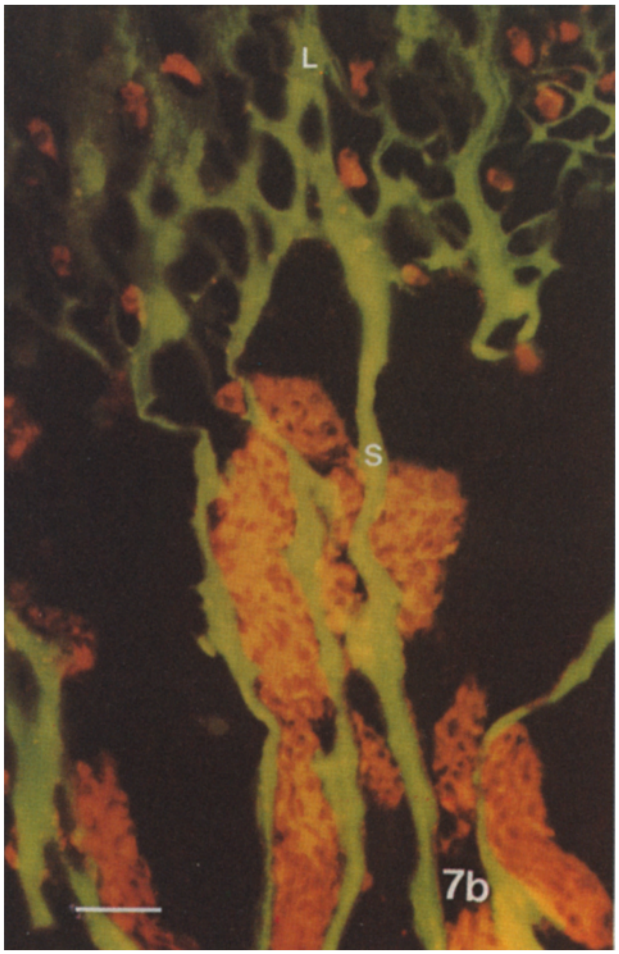


FIGURE 7 Sections of the decalcified hypertrophic zone of growth plate (*a*) and growth-plate hypertrophic zone plus metaphysis (*b*). Intense staining for link protein can be seen in the longitudinal septa (*L*) and spicules (*S*) of calcified cartilage emerging from them, but little staining was present in transverse septa. There is an overall increase in intensity of staining in the lower hypertrophic region of the growth plate. *a*, × 261. *b*, × 235.

This process has no relation to the question of how a noncalcifiable matrix is transformed into a calcifiable matrix just before the onset of mineralization in the lower hypertrophic zone. Some biochemical studies have revealed a net loss of proteoglycan and collagen (54). This would be expected if the metaphyseal cartilage contaminated the hypertrophic cartilage. Studies of growth plate down to its junction with the metaphysis have clearly revealed either no loss of chondroitin sulphate (53) or an increase in galactosaminoglycan in ossifying hypertrophic cartilage (31). Using electron-probe x-ray microanalysis, an increase in sulphated proteoglycan accompanying a larger increase in mineral phosphorus has been observed (19). Although others (17) have reported with immunofluorescence a loss of proteoglycan in the mineralizing region of growth plate, we cannot confirm their observations. Other work by us on endochondral ossification induced in rats by bone matrix protein has also indicated that there is no net loss of proteoglycan and link protein before mineralization of cartilage matrix—only after (39).

Proteoglycan aggregates and high proteoglycan concentrations have been reported to inhibit mineral growth in vitro (2, 22, 35, 36). Experimental removal by papain of proteoglycans from growth plates in vivo can produce extensive mineralization right up to the proliferation zone (8, 18), indicating that in vivo proteoglycan may also have an inhibitory role. Electron microscopy studies (33, 49) have indicated reductions in proteoglycan size and density as the lower hypertrophic zone approaches. These observations have been supported by biochemical studies indicating a reduction in proteoglycan monomer size (32). Others have observed that proteoglycan monomers in rachitic growth-plate cartilage are smaller than normal (46). Together, these observations indicate that changes in the structure and organization of proteoglycans occur in growth plate presumably to permit calcification. These changes may result from both the degradative activities of proteinases (10, 23) and changes in the biosynthesis of proteoglycans as the mineralization front approaches. Thus a dynamic controlled phase of remodeling can be envisioned without net loss of proteoglycan.

Support for this concept has been provided by electron microscopy studies of monolayer preparations of proteoglycan aggregates from epiphyseal cartilage, noncalcified growth plate, and from the calcified cartilage of the metaphysis, which will be published separately (J. Buckwalter and L. Rosenberg, unpublished results). These studies have shown that the monomer contour lengths of core protein in proteoglycan monomers isolated from calcified cartilage are essentially the same as those of proteoglycan monomers from epiphyseal cartilage or from noncalcified growth plate but that the aggregation of these monomers changes in that the monomers are more widely spaced on hyaluronic acid.

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