Immunoelectron Microscopic Studies of Type X Collagen in Endochondral Ossification

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Abstract. Immunofluorescence and immunoelectron microscopy were used in conjunction with a monoclonal antibody to investigate the localization of type X collagen in the proximal tibial growth plate of 7-d-old chicks. This molecule was detected throughout the hypertrophic zone first appearing when chondrocytes exhibited hypertrophy: it was absent from the proliferative zone. Type X collagen was primarily associated with type II collagen fibrils as demonstrated by immunogold staining. Type X collagen was not concentrated in the focal calcification sites nor was it associated with matrix vesicles. These observations suggest that type X collagen may play a role other than that directly related to the nucleation of calcification.

TYPE X collagen is synthesized by hypertrophic chondrocytes of calcifying cartilages and represents a major gene product (Schmid and Conrad, 1982*a*,*b*; Gibson et al., 1982; Reginato et al., 1986). It has been detected by light microscopy in the extracellular matrix using monoclonal (Schmid and Linsenmayer, 1985) and polyclonal (Gibson et al., 1986) antibodies. The molecule isolated from chick cartilages is composed of a triple helix of three apparently identical α chains, with a molecular mass of 59 kD, at the carboxy terminus of which there is a globular nonhelical domain of molecular mass 15–20 kD (Schmid and Conrad, 1982*a*,*b*; Schmid et al., 1984). A relatively short nontriple helical segment is located at the amino terminus (Ninomiya et al., 1986; LuValle et al., 1988). In bovine cartilage the α chains are of similar size and the triple helical domain contains interchain disulfide bonds (Remington et al., 1984; Grant et al., 1985).

The function of this molecule is unknown although it has been reported to be associated with isolated matrix vesicles (Habuchi et al., 1985) which are considered by some workers to be the primary nucleation sites involved in the calcification of cartilage (Bonucci, 1967; Anderson, 1969). More recent studies have demonstrated that cartilage calcifies in focal sites which are not directly associated with matrix vesicles and which contain focal concentrations of the C-propeptide of type II collagen (Poole et al., 1984; Poole and Rosenberg, 1987) and cartilage proteoglycans (Shepard and Mitchell, 1985; see also Poole et al., 1989).

The purpose of this study was to use a monoclonal antibody to type X collagen to determine with immunofluorescence and immunoelectron microscopy the relationship of extracellular type X collagen to cartilage calcification in the calcifying proximal growth plate of the newborn chick tibia.

Materials and Methods

Chicks and Growth Plates

These were 5 and 7 d old and were obtained from the Simetin Hatchery (St. Canut, Quebec). The proximal tibial growth plate was removed by dissection.

Immunolocalization of Type X Collagen

Immunofluorescence Microscopy. Whole growth plates were dissected free of bone and fixed for 1 h at room temperature in freshly prepared 4% formaldehyde in PBS (Poole et al., 1980), rinsed in PBS, and then placed in 0.5 M ammonium chloride for 1 h at 4°C. After rinsing in PBS, tissue was frozen in OCT embedding compound (Miles Laboratories, Inc., Elkhart, IN) in liquid nitrogen and frozen sectioned at 6 μm. Sections were attached to microscope slides by fixation for 5 min in fresh 4% formaldehyde at room temperature. After washing in PBS, sections were routinely decalcified in 0.25 M EDTA in 50 mM Tris, pH 7.4, for 1 h at room temperature. Otherwise nonspecific binding of IgG to mineral is observed. Sections were then treated with chondroitinase ABC to remove chondroitin sulfate chains of proteoglycans (Poole et al., 1980).

Sections were then treated either with monoclonal antibody X-AC9 to type X collagen (Schmid and Linsenmayer, 1985) (which was generously donated by these authors) at an IgG concentration of 2 μg/ml or with nonimmune IgG (Miles Scientific Div., Naperville, IL) at the same concentration. After washing in PBS for 10 min, sections were treated for 30 min with fluorescein-labeled pig $F(ab')_2$ directed against mouse IgG. This antibody has been previously described (Champion and Poole, 1981). $F(ab')_2$ preparation and fluorescein labeling were performed as earlier reported (Poole et al., 1980). Cells were counterstained with the dye eriochrome black (Difco Laboratories Inc., Detroit, MI) which fluoresces red (Poole et al., 1974). After a final rinse in PBS for 30 min, sections were examined with a fluorescence microscope (Poole et al., 1980). Undecalcified sections were stained with von Kossa's stain which demonstrates mineral deposits in the extracellular matrix.

Immunoelectron and Electron Microscopy. For routine electron microscopy, growth plate was cut into longitudinal slices ~1 mm thick. These were fixed in a mixture of 2% glutaraldehyde and 2% formaldehyde (freshly prepared) in PBS at 4°C for 24 h. Tissue was rinsed in PBS, postfixed in

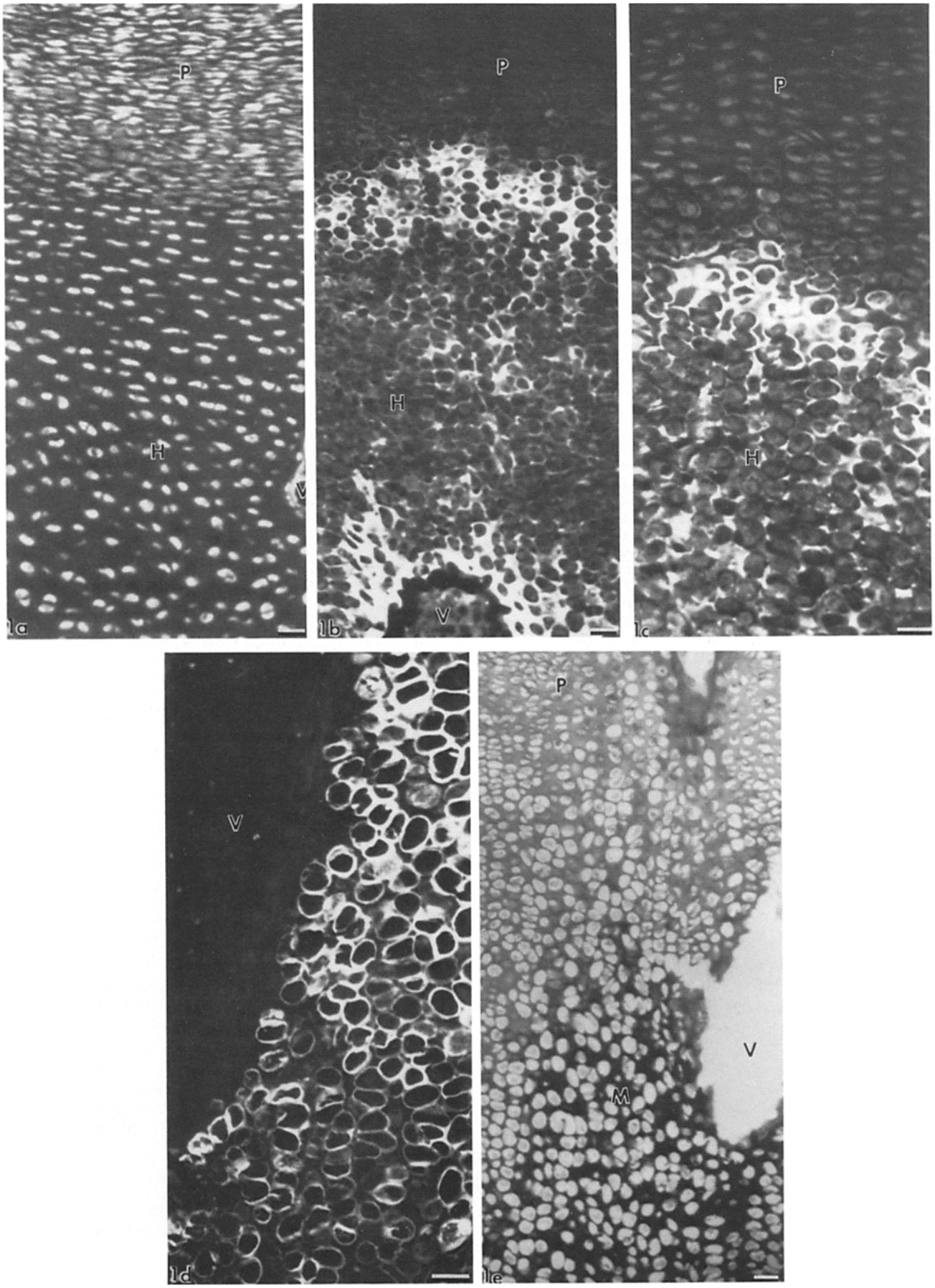
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Table I. Electron Microscopic Analysis of Distribution of Antibody to Type X Collagen

Site	Percentage distribution of colloidal gold particles		
	Over collagen fibrils	Within one fibril width	More than one fibril width
Upper hypertrophic zone (uncalcified pericellular territorial matrix) n = 1,000	75.2	19.6	5.2
Lower hypertrophic zone (calcified pericellular territorial matrix) n = 1,000	86.7	11.7	1.6

1% osmium tetroxide in PBS for 1 h, rinsed in deionized water, dehydrated, and embedded in Spurr resin (Poole et al., 1984).

For immunoelectron microscopy similar tissue slices were fixed in 4% formaldehyde for 30 min at 4°C. After rinsing in PBS they were washed in 0.5 M ammonium chloride for 1 h at 4°C. After a wash in PBS some specimens were decalcified overnight at 4°C (where indicated) in 0.5 M EDTA containing protease inhibitors used as before (Poole et al., 1984). Tissue was then sectioned in 50 μm with a tissue chopper (Sorvall Instruments Div., Newton, CT). Sections were digested for 3 h at 37°C in testicular hyaluronidase (type IS; Sigma Chemical Co., St. Louis, MO) in the presence of the same protease inhibitors as described above. After rinsing in PBS containing 0.1% BSA either mouse monoclonal antibody IgG_1 to type X collagen or nonimmune IgG_1 in PBS were added at 2 μg/ml. After an overnight incubation at 4°C on a rotary shaker, sections were washed on a shaker for 5 h first with PBS containing 0.1% BSA and then with 20 mM Tris and 0.15 M sodium chloride at pH 8.2 also containing 0.1% BSA. Sections were then incubated overnight at 4°C with goat anti-mouse IgG/IgM antibody labeled with 5-nm colloidal gold (Janssen Life Sciences Products, Beerse, NJ) diluted 1/10 in 0.1% BSA in PBS. After a final 3-h wash in Tris saline containing 0.1% BSA, tissue was dehydrated in graded ethanols and embedded in Spurr resin. Thin sections were cut, stained lightly with lead citrate and uranyl acetate, and examined in a Phillips 400 electron microscope.

Quantitation of Distribution of Antibody Binding in Electron Micrographs. This was determined by measuring the distribution of colloidal gold particles over collagen fibrils, within one fibril width of a fibril, and between fibrils at distances which were greater than one fibril width. A graticule (1 × 1 cm) was used to examine electron micrographs printed at a magnification of 78,400.

Results

Immunofluorescence Microscopy and Staining for Calcium Phosphate

Histological examination of the chick growth plate with von Kossa stain revealed that calcification of cartilage occurs in the lower hypertrophic zone after the cells of the proliferative zone have enlarged in size (Fig. 1 *e*). This hypertrophy of cells was accompanied by the appearance of staining for type X collagen in cells and in the surrounding extracellular matrix (Fig. 1, *b*, *c*, and *d*). Immunostaining for type X collagen was intense in precalcified (Fig. 1 *c*) and in calcified cartilage (Fig. 1, *b*, *c*, and *d*). Staining was strongest in pericellular sites. Sections pretreated with nonimmune mouse immunoglobulin exhibited no green extracellular staining: only red cellular counterstain was visible (Fig. 1 *a*).

Immunoelectron Microscopy

Colloidal gold staining for type X collagen was clearly visible throughout the extracellular matrix of hypertrophic cartilage and not just in pericellular sites. It was primarily associated with the thin type II collagen fibrils (Table I) characteristic of growth plate cartilages (Fig. 2 *a*). These fibrils had a mean fibril width of 17.1 nm (n = 36, range 12.8–25.6 nm) which did not vary significantly from zone to zone (data not shown). There was no recognizable pattern to this staining to suggest a periodic distribution on collagen fibrils. There was also no evidence that type X collagen was associated with cartilage proteoglycans. Since tissues have to be pretreated with hyaluronidase to permit penetration of antibody it is not possible to detect these molecules in these tissues by their characteristic morphology. Further experiments using dual localization with monospecific antibodies to proteoglycans and other matrix proteins would be required to establish whether there is any evidence for an association of type X collagen with other such matrix molecules. Cartilage matrix of the proliferative zone also contained the same thin type II collagen fibrils but there was no staining for type X collagen (Fig. 2 *b*). Also there was no immunogold staining seen in hypertrophic cartilage when first stained with nonimmune mouse immunoglobulin (data not shown).

Early calcification of cartilage could be clearly identified in the lower hypertrophic zone where focal nucleation sites were characterized by the presence of the electron dense needle-like crystals of mineral (Fig. 3, *a* and *b*). Type X collagen could be detected in this matrix again mainly in association with type II collagen fibrils (Table I). There was no evidence of any specific association of type X collagen with these focal nucleation sites. In general they were unstained whether or not the tissue had been decalcified before immunostaining. Calcification sites could be recognized in decalcified sections by the presence of electron dense heavy metal staining in these sites (Poole et al., 1984). Using conventional fixation with glutaraldehyde and osmium tetroxide, matrix vesicles could be clearly identified in the calcifying cartilage in close association with calcification sites but not ordinarily as a central component of the nucleation site (Fig. 4 *a*). The majority of the mineral was separate from the vesi-

Figure 1. 6-μm-thick sections of decalcified 7-d-old chick proximal tibial growth plate were stained with monoclonal antibody to type X collagen (*b*, *c*, and *d*) or nonimmune IgG (*a*) followed by fluorescein-labeled pig anti-mouse ($F[ab']_2$). Sections were then counterstained with eriochrome black which stains cells and they fluoresce red. Counterstaining was stronger in *a* to demonstrate cellular detail. Other nondecalcified sections (*e*) were stained with von Kossa's stain for mineral (*M*) and counterstained with Safranin O. The proliferative zone (*P*), hypertrophic zone (*H*), and vascular cavities (*V*) are indicated. Bars, 25 μm.

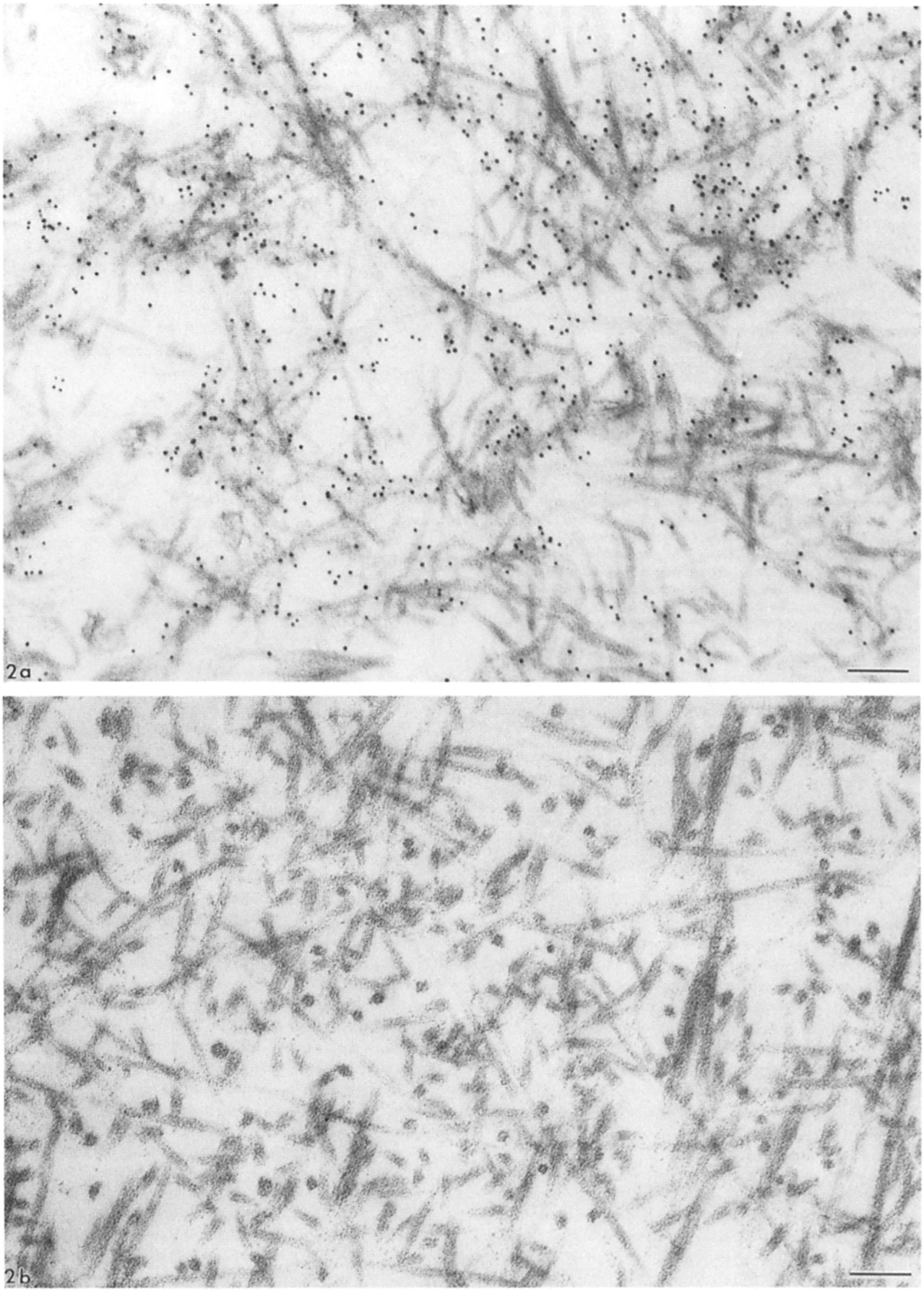
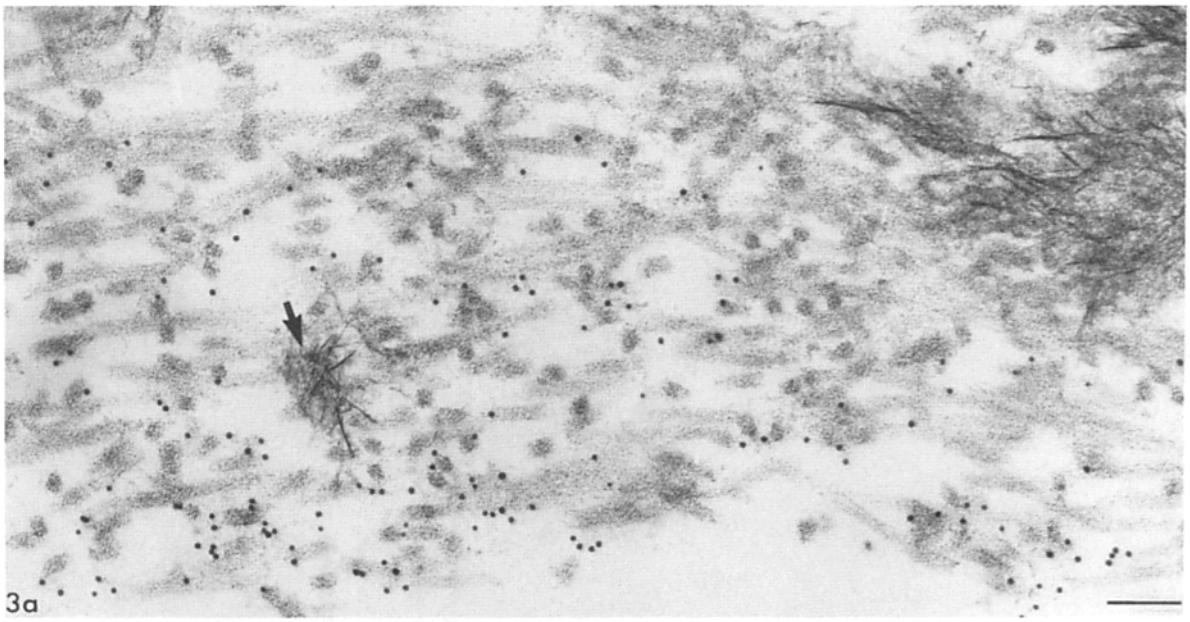


Figure 2. Immunoelectron microscopic localization of cartilage stained for type X collagen showing its association with collagen fibrils in the matrix of (*a*) precalcifying hypertrophic zone and (*b*) its absence from the proliferative zone. Bars, 100 nm.



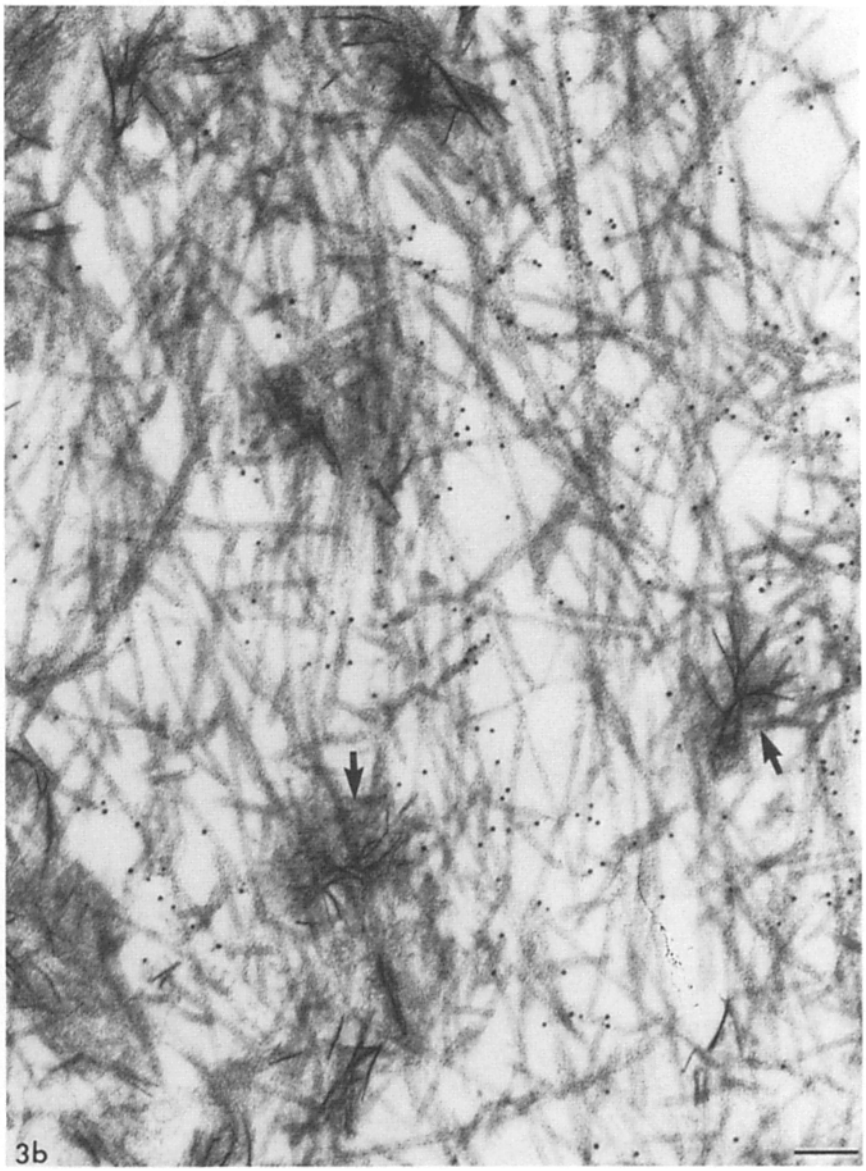
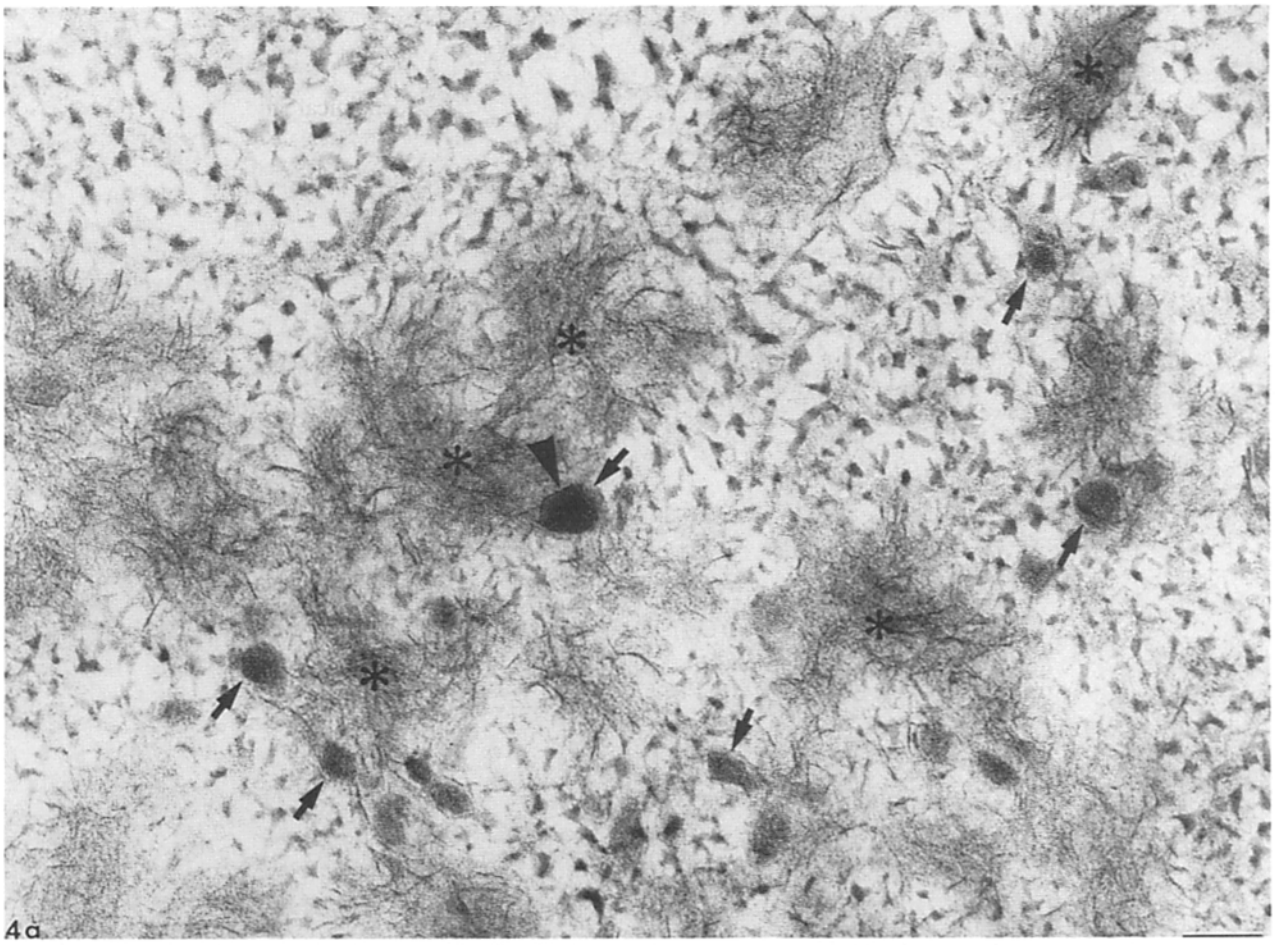


Figure 3. (*a* and *b*) Immunoelectron microscopic localization of type X collagen in the early calcifying matrix of the lower hypertrophic zone. Focal mineral deposits are indicated (*arrows*) which do not exhibit any specific staining for type X collagen. Bars, 100 nm.



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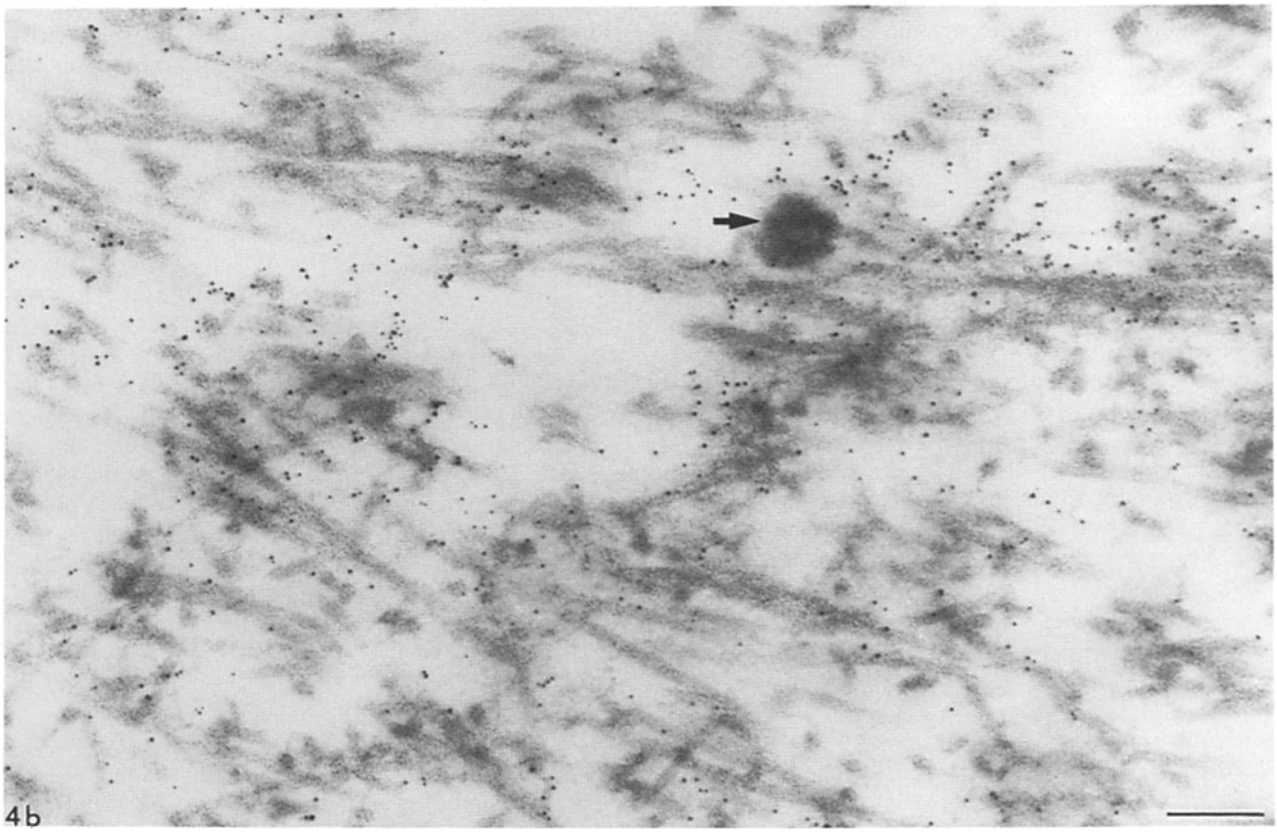

4b

cles in that it did not surround the vesicles. Vesicles were often found, however, immediately adjacent to focal mineral deposits. Sometimes very fine crystals could be seen outlining part of the edge of a matrix vesicle (*arrowhead* in Fig. 4 *a*). When fixation for immunoelectron microscopy was used, the membranes of matrix vesicles were poorly preserved but they could still be identified by their electron density. There was no evidence of any specific association of type X collagen with matrix vesicles. A single representative vesicle is shown in Fig. 4 *b*. At least 50 vesicles were examined in electron micrographs. None exhibited any staining for type X collagen even when prints were made at reduced contrast to facilitate the detection of immunogold against the ordinarily electron dense vesicle.

Discussion

Our observations demonstrate that the use of immunoelectron microscopy permits an analysis of the distribution of type X collagen in hypertrophic cartilage that has not previously been reported. The antibody X-AC9 binds to a site close to the globular COOH-terminal domain of type X collagen. It also recognizes the major fragment produced by collagenase-mediated cleavage of type X collagen (Schmid et al., 1986; Gadher et al., 1988). Therefore even if there is cleavage in situ of type X collagen produced by collagenase, a major degradation product will also be recognized by the antibody. Collagenase has been demonstrated within the hypertrophic zone (Dean et al., 1985). Type X collagen was detected both in the precalcifying upper hypertrophic zone as well as in the calcifying lower hypertrophic zone.

The association of type X collagen with type II collagen fibrils has been observed by independent immunoelectron microscopic studies of 14-d embryonic chick tibio-tarsus (Schmid, T., and T. Linsenmayer, personal communication). In addition, these authors also observed an association of antibody with weakly stained "fine filamentous mat-like material" which was not observed in our studies of young chicks. We were unable to identify any association of type X collagen with any other matrix structures. The majority of antibody binding was over or immediately adjacent to type II fibrils. It is possible that type X collagen may also interact with type IX collagen (Eyre et al., 1987; van der Rest and Mayne, 1988) or with type XI collagen (Mendler et al., 1989) which have both been reported to be closely associated with type II fibrils: in the case of type IX a covalent linkage has been observed. Both type IX (Müller-Glauser et al., 1986) and type XI (Burgeson and Hollister, 1979) collagens are present within the growth plate.

The binding of type X collagen does apparently involve the formation of covalent cross-links since addition of the lathyrogen β-aminopropionitrile (which inhibits lysyl oxidase and cross-link formation in types I, II and III collagens) (Tanzer, 1976) enhances extractability of newly synthesized type X collagen (Reginato et al., 1986). These observations also favor a direct or indirect association of type X with type II collagen fibrils which may involve type IX and/or XI collagens.

Earlier immunohistochemical studies using light microscopy drew attention to the correspondence between type X collagen, the hypertrophy of chondrocytes and the subsequent calcification of cartilage (Schmid and Linsenmayer, 1985; Gibson et al., 1986). This raised the possibility that type X collagen may be involved in the nucleation of calcification. The reported selective association of type X collagen with isolated matrix vesicles (Habuchi et al., 1985) led to the suggestion by these workers that the roles of matrix vesicles and type X collagen in calcification were coupled. Our present studies indicate that there is no evidence for a specific association of type X collagen with matrix vesicles nor with nucleation sites.

The relationship of matrix vesicles to focal sites of mineral deposition in the calcifying growth plate has been the subject of many investigations since Bonucci (1967) and Anderson (1969) first described the association of early mineral deposits within or close to matrix vesicles. Since then further studies using freeze substitution, cryoultramicrotomy and ethylene glycol treatment, coupled with energy dispersive x-ray analysis have demonstrated mineral deposits within or in close association with matrix vesicles (see for example Morris et al., 1983). Other studies using cryoultramicrotomy, unfixed tissues, and electron probe x-ray microanalysis failed to find evidence for a solid phase of calcium phosphate within or associated with matrix vesicles (Landis and Glimcher, 1982). Both Shepard and Mitchell (1985) and Thyberg (1974) found that early crystals were deposited in the matrix and not in vesicles. Others have reported a reduced matrix vesicle concentration when matrix mineralization occurs (Engfeldt et al., 1982; Buckwalter et al., 1987). Clearly there are widely divergent viewpoints concerning the initial sites of mineral deposition in the lower hypertrophic zone. There can, however, be little doubt that alkaline phosphatase is closely associated with mineralization (Robison, 1923; Fell and Robison, 1929, 1930; see also Poole et al., 1989). It can produce the inorganic phosphate required for the formation of mineral. It is concentrated in calcifying matrix in matrix vesicles, as well as being present in the plasma membranes of chondrocytes in these sites (de Bernard et al., 1986). The presence and activity of this enzyme within calcifying sites is thus favored by its localization within matrix vesicles.

In cartilage calcification, early nucleation takes place focally in interfibrillar sites where proteoglycans are concentrated (Shepard and Mitchell, 1985; Arsenault and Ottensmeyer, 1983). In this and earlier studies (Poole et al., 1984), we found no evidence of a specific association of early nucleation with matrix vesicles. Type X collagen, in contrast, was mainly associated with collagen fibrils in sites where there is no evidence for early calcification (in contrast to osteoid in bone where collagen fibrils calcify as an early event). Hence the question arises concerning the role of type X col-

Figure 4. (*a*) Electron microscopy of the calcifying lower hypertrophic zone to show focal calcifying sites (*) and matrix vesicles (*arrows*) when the tissue was fixed in glutaraldehyde and osmium tetroxide. Very fine crystals were sometimes seen at the edges of matrix vesicles (*arrowhead*). (*b*) Immunoelectron microscopic localization of type X collagen in the decalcified lower hypertrophic zone to show a less well-preserved matrix vesicle (*arrow*) and its lack of association with type X collagen which is mainly associated with type II collagen fibrils. Bars, 100 nm.

lagen. Does it prevent the calcification of collagen fibrils thereby directing initial calcification to focal interfibrillar sites? There is yet no practical evidence for this. But clearly this hypothesis requires examination by further experimentation. It has also been suggested that type X collagen may "target" skeletal tissues for eventual removal (Linsenmayer et al., 1986). This is an important process in endochondral ossification which is mediated by osteoclasts after woven bone has been deposited on the calcified cartilagenous trabecula. It has been proposed by others that the appearance of type X collagen immediately before calcification may somehow prepare the cartilage for this event. Our suggestion concerning a possible inhibitory effect of type X on the calcification of type II collagen would fall into this category. Direct roles for type X collagen in nucleation and matrix vesicle function are not supported by our observations.

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References

Anderson, H. C. 1969. Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J. Cell Biol.* 41:59–72.

Arsenault, A. L., and F. P. Ottensmeyer. 1983. Quantitative spatial distributions of calcium, phosphorus and sulfur in calcifying epiphysis by high resolution electron spectroscopic imaging. *Proc. Natl. Acad. Sci. USA.* 80: 1322–1326.

Bonucci, E. 1967. Fine structure of early cartilage calcification. *J. Ultrastruct. Res.* 20:33–50.

Buckwalter, J. A., D. Mower, and J. Schaeffer. 1987. Differences in matrix vesicle concentration among growth plate zones. *J. Orthop. Res.* 5:157–163.

Burgeson, R. E., and D. W. Hollister. 1979. Collagen heterogeneity in human cartilage: identification of several new collagen chains. *Biochem. Biophys. Res. Comm.* 87:1124–1131.

Champion, B. R., and A. R. Poole. 1981. Immunity to homologous collagens and cartilage proteoglycans in rabbits with chronic inflammatory arthritis. *Collagen Relat. Res.* 1:453–473.

Dean, D. D., O. E. Muniz, I. Berman, J. C. Pita, M. R. Carreno, J. F. Woessner, Jr., and D. S. Howell. 1985. Localization of collagenase in the growth plate of rachitic rats. *J. Clin. Invest.* 76:716–722.

de Bernard, B., P. Bianco, E. Bonucci, M. Constantini, G. C. Lunazzi, P. Martinuzzi, C. Modricky, L. Moro, E. Panfili, P. Polleslo, N. Stagni, and F. Vittur. 1986. Biochemical and immunohistochemical evidence that in cartilage an alkaline phosphatase is a Ca^{2+}-binding glycoprotein. *J. Cell Biol.* 103:1615–1623.

Engfeldt, B., A. Hjerpe, F. P. Reinholt, and B. Wikström. 1985. Distribution of matrix vesicles in the lower part of the epihyseal growth plate. *In* The Chemistry and Biology of Mineralized Tissues. WT. Butler, editor. Ebsco Media Inc., Birmingham, AL. 356–359.

Eyre, D. R., S. Apone, J. -J. Wu, L. H. Ericsson, and K. A. Walsh. 1987. Collagen type IX: evidence for covalent linkages to type II collagen in cartilage. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 220:337–341.

Fell, H. B., and R. Robison. 1929. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated *in vitro*. *Biochem J.* 23:767–784.

Fell, H. B., and R. Robison. 1930. The development and phosphatase activity *in vivo* and *in vitro* of the mandibular skeletal tissue of the fowl. *Biochem. J.* 24:1905–1921.

Gadher, S. J., D. R. Eyre, V. C. Duance, S. F. Wotton, L. W. Heck, T. M. Schmid, and D. E. Woolley. 1988. Susceptibility of cartilage collagens types II, IX, X, and XI to human synovial collagenase and neutrophil elastase. *Eur. J. Biochem.* 175:1–7.

Gibson, G. J., C. H. Bearman, and M. H. Flint. 1986. The immunoperoxidase localization of type X collagen in chick cartilage and lung. *Collagen Relat. Res.* 6:163–184.

Gibson, G. J., S. L. Schor, and M. E. Grant. 1982. Effects of matrix molecules on chondrocyte gene expression: synthesis of a low molecular weight collagen species by cells cultured within collagen gels. *J. Cell Biol.* 93:767–774.

Grant, W. T., M. D. Sussman, and G. Balian. 1985. A disulfide-bonded short chain collagen synthesized by degenerative and calcifying zones of bovine growth plate cartilage. *J. Biol. Chem.* 260:3798–3803.

Habuchi, H., H. E. Conrad, and J. H. Glaser. 1985. Coordinate regulation of collagen and alkaline phosphatase levels in chick embryo chondrocytes. *J. Biol. Chem.* 260:13029–13034.

Landis, W. J., and M. J. Glimcher. 1982. Electron optical and analytical observations of rat growth plate cartilage prepared by ultramicrotomy: the failure to detect a mineral phase in matrix vesicles and the identification of heterodispersed particles as the initial solid phase of calcium phosphate deposited in the extracellular matrix. *J. Ultrastruct. Res.* 78:227–268.

Linsenmayer, T. F., E. Gidney, and T. M. Schmid. 1986. Segmental appearance of type X collagen in the developing avian notochord. *Dev. Biol.* 113:467–473.

LuValle, P., Y. Ninomiya, N. D. Rosenblum, and B. R. Olsen. 1988. The type X collagen gene. Intron sequences split the 5′ untranslated region and separate the coding regions for the non-collagenous amino terminal and triple-helical domains. *J. Biol. Chem.* 263:18378–18385.

Mendler, M., S. G. Eich-Bender, L. Vaughan, K. H. Winterhalter, and P. Bruckner. 1989. Cartilage contains mixed fibrils of collagen types II, IX and XI. *J. Cell Biol.* 108:191–197.

Morris, D. C., H. K. Väänänen, and H. C. Andersen. 1983. Matrix vesicle calcification in rat epiphyseal growth plate cartilage prepared anhydrously for electron microscopy. *Metab. Bone Dis. & Relat. Res.* 5:131–137.

Müller-Glauser, W., B. Humbel, M. Glatt, P. Strauli, K. M. Winterhalter, and P. Bruckner. 1986. On the role of type IX collagen in the extracellular matrix of cartilage. Type IX collagen is localized to intersections of collagen fibrils. *J. Cell Biol.* 102:1931–1939.

Ninomiya, Y., M. Gordon, M. van der Rest, T. Schmid, T. Linsenmayer, and B. R. Olsen. 1986. The developmentally regulated type X collagen gene contains a long open reading frame without introns. *J. Biol. Chem.* 261:5041–5050.

Poole, A. R., R. M. Hembry, and J. T. Dingle. 1974. Cathepsin D in cartilage: the immunohistochemical demonstration of extracellular enzyme in normal and pathological conditions. *J. Cell Sci.* 14:139–161.

Poole, A. R., Y. Matsui, A. Hinek, and E. R. Lee. 1989. Cartilage macromolecules and the calcification of cartilage matrix. *Anat. Rec.* In press.

Poole, A. R., I. Pidoux, A. Reiner, H. Choi, and L. C. Rosenberg. 1984. Association of an extracellular protein (chondrocalcin) with the calcification of cartilage in endochondral bone formation. *J. Cell Biol.* 98:54–65.

Poole, A. R., I. Pidoux, A. Reiner, L-H. Tang, H. Choi, and L. Rosenberg. 1980. Localization of proteoglycan monomer and link protein in the matrix of bovine articular cartilage: an immunohistochemical study. *J. Histochem. Cytochem.* 28:621–635.

Poole, A. R., and L. C. Rosenberg. 1987. Proteoglycans, chondrocalcin, and the calcification of cartilage matrix in endochondral ossification. *In* Biology of Proteoglycans. T. N. Wight and R. P. Mecham, editors. Academic Press, Inc., New York. 187–210.

Reginato, A. M., J. W. Lash, and S. A. Jiminez. 1986. Biosynthetic expression of type X collagen in embryonic chick sternum cartilage during development. *J. Biol. Chem.* 261:2897–2904.

Remington, M. C., R. I. Bashey, C. T. Brighton, and S. A. Jiminez. 1984. Biosynthesis of a disulphide-bonded short-chain collagen by calf growth plate cartilage. *Biochem J.* 224:227–233.

Robison, R. 1923. The possible significance of hexose phosphoric esters in ossification. *Biochem J.* 17:286–293.

Schmid, T. M., and H. E. Conrad. 1982*a*. A unique low molecular weight collagen secreted by cultured chick embryo chondrocytes. *J. Biol. Chem.* 257:12444–12450.

Schmid, T. M., and H. E. Conrad. 1982*b*. Metabolism of low molecular weight collagen by chondrocytes obtained from histologically distinct zones of the chick tibiotarsus. *J. Biol. Chem.* 257:12451–12457.

Schmid, T. M., and T. F. Linsenmayer. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissue. *J. Cell Biol.* 100:598–605.

Schmid, T. M., R. Mayne, R. Bruns, and T. F. Linsenmayer. 1984. Molecular structure of short-chain (SC) cartilage collagen by electron microscopy. *J. Ultrastruct. Res.* 86:186–191.

Schmid, T. M., R. Mayne, J. J. Jeffrey, and T. F. Linsenmayer. 1986. Type X collagen contains two cleavage sites for vertebrate collagenase. *J. Biol. Chem.* 261:4184–4189.

Shepard, N., and N. Mitchell. 1985. Ultrastructural modifications of proteoglycans coincident with mineralization in local regions of rat growth plate. *J. Bone Jt. Surg. Am. Vol.* 67:455–464.

Tanzer, M. 1976. Cross-linking. *In* Biochemistry of Collagen. G. N. Ramachandran and A. H. Reddi, editors. Plenum Publishing Corp., New York. 137–157.

Thyberg, J. 1974. Electron microscopic studies on the initial phases of calcification in Guinea pig epiphyseal cartilage. *J. Ultrast. Res.* 46:206–218.

Van der Rest, M., and R. Mayne. 1988. Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. *J. Biol. Chem.* 263:1615–1618.