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, 1982a,b; Gibson et al., 1982; Regnato 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, 1982a,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.
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 presence of the same protease inhibitors as described above. After rinsing and embedded in Spurr resin (Poole et al., 1984).

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

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 IgG1 to type X collagen or nonimmune IgG 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 ethanol 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.

**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 vesicles.

**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 celluar counterstain was visible (Fig. 1 a).

![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.](image-url)
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.
icles 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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