On the Role of Type IX Collagen in the Extracellular Matrix of Cartilage: Type IX Collagen Is Localized to Intersections of Collagen Fibrils

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Abstract. The tissue distribution of type II and type IX collagen in 17-d-old chicken embryo was studied by immunofluorescence using polyclonal antibodies against type II collagen and a peptic fragment of type IX collagen (HMW), respectively. Both proteins were found only in cartilage where they were co-distributed. They occurred uniformly throughout the extracellular matrix, i.e., without distinction between pericellular, territorial, and interterritorial matrices. Tissues that undergo endochondral bone formation contained type IX collagen, whereas periosteal and membranous bones were negative.

The thin collagenous fibrils in cartilage consisted of type II collagen as determined by immunoelectron microscopy. Type IX collagen was associated with the fibrils but essentially was restricted to intersections of the fibrils.

These observations suggested that type IX collagen contributes to the stabilization of the network of thin fibers of the extracellular matrix of cartilage by interactions of its triple helical domains with several fibrils at or close to their intersections.

THE biochemical composition of cartilage is rather unique and comprises a large variety of collagens, proteoglycans, and glycoproteins. The major components are type II collagen and the large aggregating proteoglycans. Type II collagen is thought to form the bulk of the fibrillar network and, hence, to confer mechanical stability to the tissue. The proteoglycans can bind large amounts of water due to their high negative charge density. Upon compression, some of this water is thought to be lost from the tissue and to be taken up again upon decompression. Thus, the proteoglycans are responsible for the characteristic resilience of cartilage. This is important, for example, in the lubrication of articular surfaces in joints.

A number of minor components of cartilage have recently been discovered. These include the collagenous 1α, 2α, and 3α chains, type IX and type X collagen (reviewed by Mayne and von der Mark, 1983), and several minor proteoglycans (Heinegård et al., 1985). The tissue distribution of 1α, 2α, and 3α collagen is not known and there is controversy over that of type IX collagen (Duance et al., 1982; Ricard-Blum et al., 1982; Hartmann et al., 1983; Irwin et al., 1985). Type X collagen seems to be a specialized minor component of bone-forming cartilages (Capasso et al., 1984; Schmid and Linsenmayer, 1985; Gibson and Flint, 1985). The functions of these minor components are unknown.

Both major cartilage components, type II collagen and the aggregating proteoglycans, have a number of properties that make their ultrastructural appearance in the tissues difficult to understand. Under appropriate conditions, purified type II collagen in solution can be reconstituted into large fibril-like structures (Lee and Piez, 1983). However, in the tissue, the protein forms very thin fibrils. Likewise, the self assembly of cartilage proteoglycans to large aggregates is well documented (for review, see Hascall and Lowther, 1982). However, no large areas occur in cartilage matrix where proteoglycans or collagens are found exclusively. These features would be readily explained if type II collagen and the major proteoglycans, in addition to their self-assembly, could also interact with each other. However, efforts to demonstrate specific interactions directly by physicochemical techniques have failed (Smith et al., 1985) or produced controversial results (reviewed by Comper and Laurent, 1978). Therefore, it seems conceivable that the minor cartilage components mediate the interaction and/or the organization of the major components in the tissue.

In this context, type IX collagen is especially interesting since it has both several collagenous domains and covalently bound glycosaminoglycans (Bruckner et al., 1985; Vaughan et al., 1985). These structural properties make it an excellent candidate as a possible mediator of contacts between collagens and proteoglycans. Therefore, we investigated the distribution of type IX collagen in several tissues by immunofluorescence and by immunoelectron microscopy.

This paper is dedicated to Dr. C. Martius on the occasion of his 80th birthday.

Materials and Methods

Materials

17-d-old chicken embryos were purchased from a local hatchery. Protein A and ovine testicular hyaluronidase were from Serva (Heidelberg, FRG). Polylysine ($M_r > 150,000$) and polyvinyl alcohol was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were purchased from Fluka AG (Buchs, Switzerland) or from E. Merck (Darmstadt, FRG). Protein A–coated gold particles were prepared according to the procedure described by Horisberger and Rosset (1977). Antisera specific for a peptic fragment (HMW) of type IX collagen (Reese and Mayne, 1981) were raised in rabbits. Their purification and specificity has been described previously (Bruckner et al., 1983). Affinity purified antibodies against chicken type II collagen were a gift from Dr. K. von der Mark.

Preparation of the Tissues for Light Microscopy

Tissues were cut with razor blades into 1–2-mm slices. Specimens from the eye and the liver were fixed at 4°C for 1 h in 1% (wt/vol) of freshly depolymerized paraformaldehyde in 0.15 M sodium chloride buffered with 20 mM sodium phosphate to pH 7.4 (PBS). The tissue fragments were then treated with 0.1 M lysine hydrochloride in PBS and dehydrated through an ethanol series at room temperature. Infiltration and embedding with hydroxypropyl methacrylate was done as described by Franklin (1984). The hard mixture formulation was used for all tissues except for liver. Adequate tissue penetration was obtained by extending the time for infiltration, i.e., overnight at 4°C and then for 4 h at room temperature. The specimens were then immersed in cold embedding mixture and polymerized at 4°C for 12 h.

Immunofluorescence

The procedures of Franklin (1984) were modified slightly. 2-μm sections were cut dry with glass knives and then spread on coverslips coated with polylysine. They were etched with tetrahydrofurane and rehydrated by subsequent treatment with 100% (vol/vol) and 70% (vol/vol) ethanol in water. Some sections were decalcified in 0.1 M EDTA, pH 7.4. Reaction with von Kossa's stain showed that decalcification was complete within 2 h. The sections were rinsed with PBS, pH 5.3, and digested with 1% (wt/vol) hyaluronidase in PBS, pH 5.3. After washing with 0.15 M sodium chloride in 20 mM Tris HCl buffer, pH 7.4 (TBS) and blocking of unspecific binding sites for antibody molecules for 30 min with 10% fetal calf serum, 0.2% Tween 20, 40 mM $MgCl_2$ in TBS (blocking buffer, Zeller et al., 1983), sections were reacted with 80 ng of anti–type IX collagen antibodies per milliliter of blocking buffer. Normal rabbit serum diluted 1:20 in blocking buffer was used in negative controls. After washing with blocking buffer the sections were exposed for 1 h to rhodamine-conjugated goat antibodies against rabbit IgG diluted 1:100 in blocking buffer. They were rinsed consecutively with blocking buffer and PBS and mounted in a medium that contained polyvinyl alcohol (Lennette, 1978). The tissue sections were examined on a Zeiss Universal microscope equipped with phase contrast, epifluorescence (filterpack: 510–560/580/590 nm), and with 16/0.40, 40/0.65, and 100/1.32 (oil immersion) objectives. Kodak Tri X Pan film (400 ASA) was exposed for 60 s.

Histology

Unetched, dry tissue sections were stained with toluidine blue (1% in 1% borax), van Gieson's stain, or von Kossa's stain (counterstained with 0.5% nuclear fast red in 5% $Al_2[SO_4]_3$).

Immunoelectron Microscopy

Excised tissue was placed into PBS and cut with razor blades into 1-mm slices. Treatment of the tissue fragments was at room temperature unless stated otherwise, and all solutions contained 0.2 mg/ml of sodium azide. Some tissues were decalcified overnight at 4°C in 0.1 M EDTA, pH 7.4. After washing for 30 min in PBS (pH 5.3), slices were digested with hyaluronidase. They were washed for 90 min in TBS and 30 min in blocking buffer (see above). 80 ng or 400 ng of antibodies against type IX collagen, 20 or 100 μl of solutions of antibodies against type II collagen, or 100 μl of normal rabbit serum per milliliter of blocking buffer were applied to the tissue fragments overnight at 4°C. The slices were washed for 4 h in blocking buffer and then reacted overnight at 4°C with solutions that contained adequate amounts of protein A–gold suspensions in blocking buffer and washed with blocking buffer and PBS for 4 h. They were dehydrated through an ethanol series and embedded in Epon. Thin sections were cut from the surfaces of the tissue slices, mounted on slotted grids coated with formvar film, and stained with uranyl acetate and lead citrate. Electron micrographs were taken at 80 kV in a Zeiss EM 10 CR electron microscope.

Results

The distribution of type IX collagen in various tissues of 17-d-old chicken embryo was studied by indirect immunofluorescence on sections of tissues embedded in a polymethacrylate resin (Franklin, 1984). Polyclonal antibodies specific for the HMW domain of type IX collagen (Bruckner et al., 1983) were used. Only cartilage exhibited a positive reaction, whereas all other connective tissues tested, i.e., skin, cornea, tendons, ligaments, long bones, and calvaria, were negative. Tissues such as skeletal muscle, liver, large blood vessels, and brain were also negative. In every type of cartilage studied, the protein was found in all histologically distinct zones and appeared to be homogeneously distributed. In chick embryo cartilage, no distinct pericellular, territorial, and interterritorial regions of the matrix were detected by the various histological stains used. As reported previously for immunofluorescence staining of type II collagen (von der Mark, 1981), removal of the proteoglycans by treatment of the tissue with hyaluronidase was necessary before reaction with antibodies against type IX collagen. No appreciable further increase in the intensity of the immunofluorescence could be obtained by swelling of collagen fibers through treatment of the sections with acetic acid (Linsenmayer et al., 1983) or after decalcification with EDTA (von der Mark, 1981).

A uniform distribution of type IX collagen was observed in scleral cartilage in the eye (Fig. 1) with a sharp distinction of cartilage and adjacent fibrous tissue evident with respect to type IX collagen. In femur the following regions from the epiphysis towards the mid-diaphysis could be distinguished histologically (Figs. 2 and 3). A fibrous layer continuous with the perichondrial layer of the diaphysis was followed by hyaline cartilage. This bordered on a growth zone with disc-

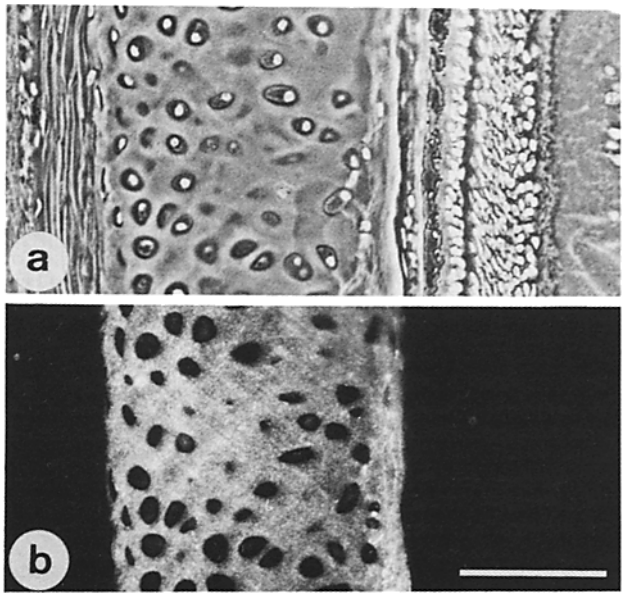

Figure 1. Posterior sclera of eye of 17-d-old chicken embryo. (*a*) Phase contrast micrograph of section shown in *b* by indirect immunofluorescence with anti–type IX antibodies. Note exclusive staining of cartilage. Bar, 50 μm.

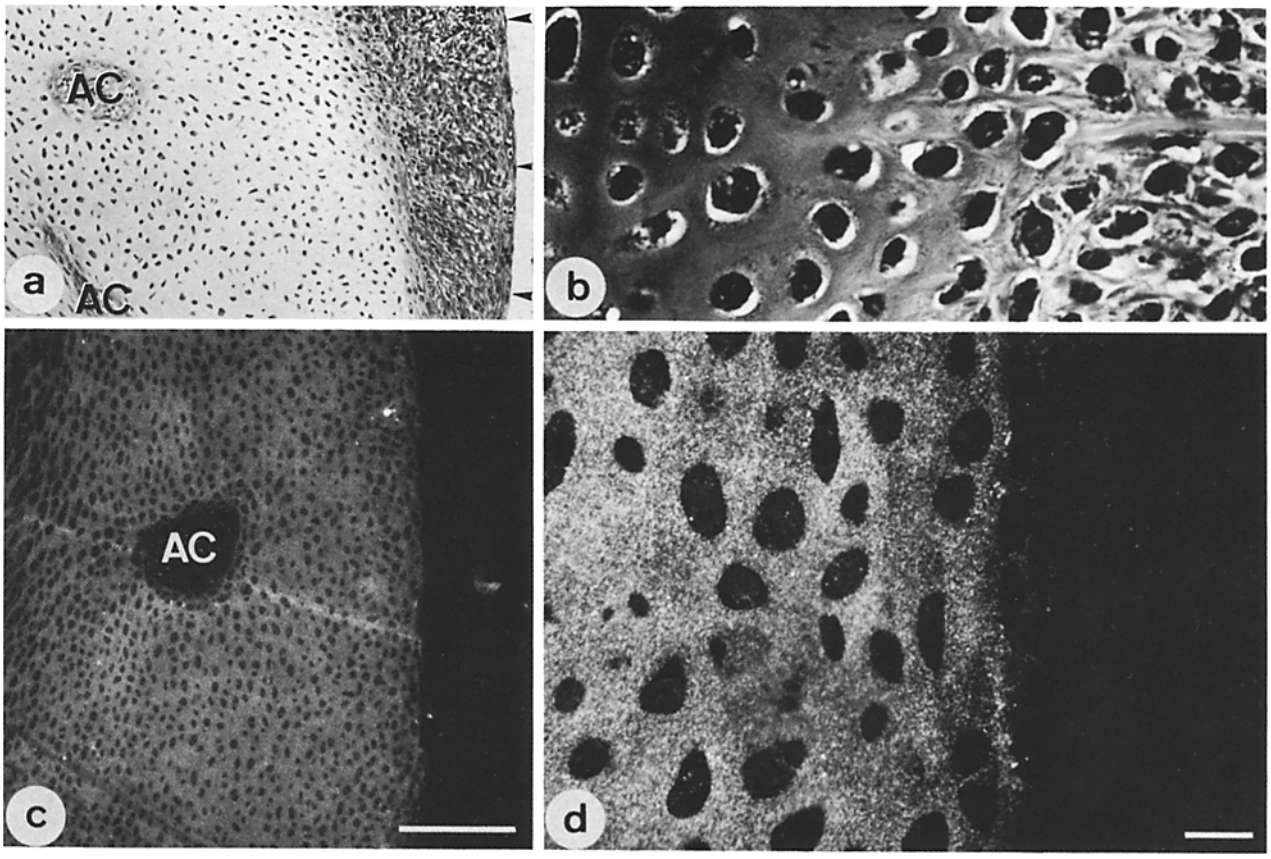

Figure 2. Epiphyseal cartilage of knee condyle of femur of 17-d-old chicken embryo (*a* and *c*) and details of the border between fibrous surface layer and hyaline cartilage (*b* and *d*). Stains: (*a*) van Gieson; (*b*) toluidine blue; (*c* and *d*) immunofluorescence (type IX collagen). Note fluorescence restricted to hyaline cartilage. *AC*, Angiogenic center; arrowheads, surface of condyle towards joint cavity. Bars: (*a* and *c*) 100 μm; (*b* and *d*) 10 μm.

shaped chondrocytes occasionally arranged in small stacks. This in turn was followed by a zone with large hypertrophic chondrocytes. Cartilage breakdown and/or endochondral ossification took place in a part of this region. Type IX collagen was found throughout these histologically distinct regions (Figs. 2 and 3). At high magnification, the matrix presented a uniform, finely grained fluorescence (Figs. 2*d* and 3*d*) without a differentiation into pericellular, territorial, and interterritorial regions. However, occurrence of type IX collagen abruptly ceased at the border to the fibrous surface layer in the joint (Fig. 2) as well as at the perichondrium of the diaphysis (Fig. 3). Confirming previous reports (Irwin et al., 1985), similar observations were made with sternal cartilage (not shown).

Type IX collagen could be detected in calcified cartilage, but was absent in the portion of bone that was most likely derived from periosteal tissue. The two layers formed a well defined border (Fig. 4, *c–e*). Similar observations were made in zones of endochondral ossification in neck vertebrae (Fig. 4, *a* and *b*).

A bright fluorescence is apparent in regions of endochondral ossification (Figs. 3 and 4), which suggests a possibly elevated level of occurrence of type IX collagen. To test this hypothesis we also studied the distribution of type II collagen in the same tissue. This protein has been shown to occur homogeneously in similar tissues (Schmid and Linsenmayer, 1985). However, we did not observe a homogeneous distribution of type II collagen but found that immunofluorescence patterns of type II collagen closely resembled those of type IX collagen (not shown). Proteoglycans are lost in such areas due to tissue remodeling as revealed by a sudden absence of stain of the tissue by toluidine blue (compare Fig. 3*f* with 3 *g* and 4*c* with 4*e*). We concluded that the apparent increase in immunofluorescence of both type II and type IX collagen probably was due to a drastically enhanced exposure of the antigen towards the antibodies rather than an increase of the amount of these proteins in calcifying regions of femoral cartilage.

Taken together these observations clearly demonstrated that type IX collagen was restricted to cartilage where it occurred homogeneously in all histologically distinct regions. At the resolution of light microscopy, the distribution of type IX collagen coincides with that of type II collagen as observed here and as reported (von der Mark, 1981; Duance et al., 1982; Irwin et al., 1985).

To define more precisely the localization of type IX collagen in cartilage we studied its occurrence in cartilage of 17-d-old chicken embryos by immunoelectron microscopy using the protein A–gold technique (Horisberger and Rosset, 1977). The tissue consisted of faintly banded, fine collagenous fibrils in essentially random orientation except for the outermost regions of the tissue where they tended to lie more parallel to the surface of the organ. No network of thicker fibrils typical for the interterritorial matrix of mammalian cartilage could

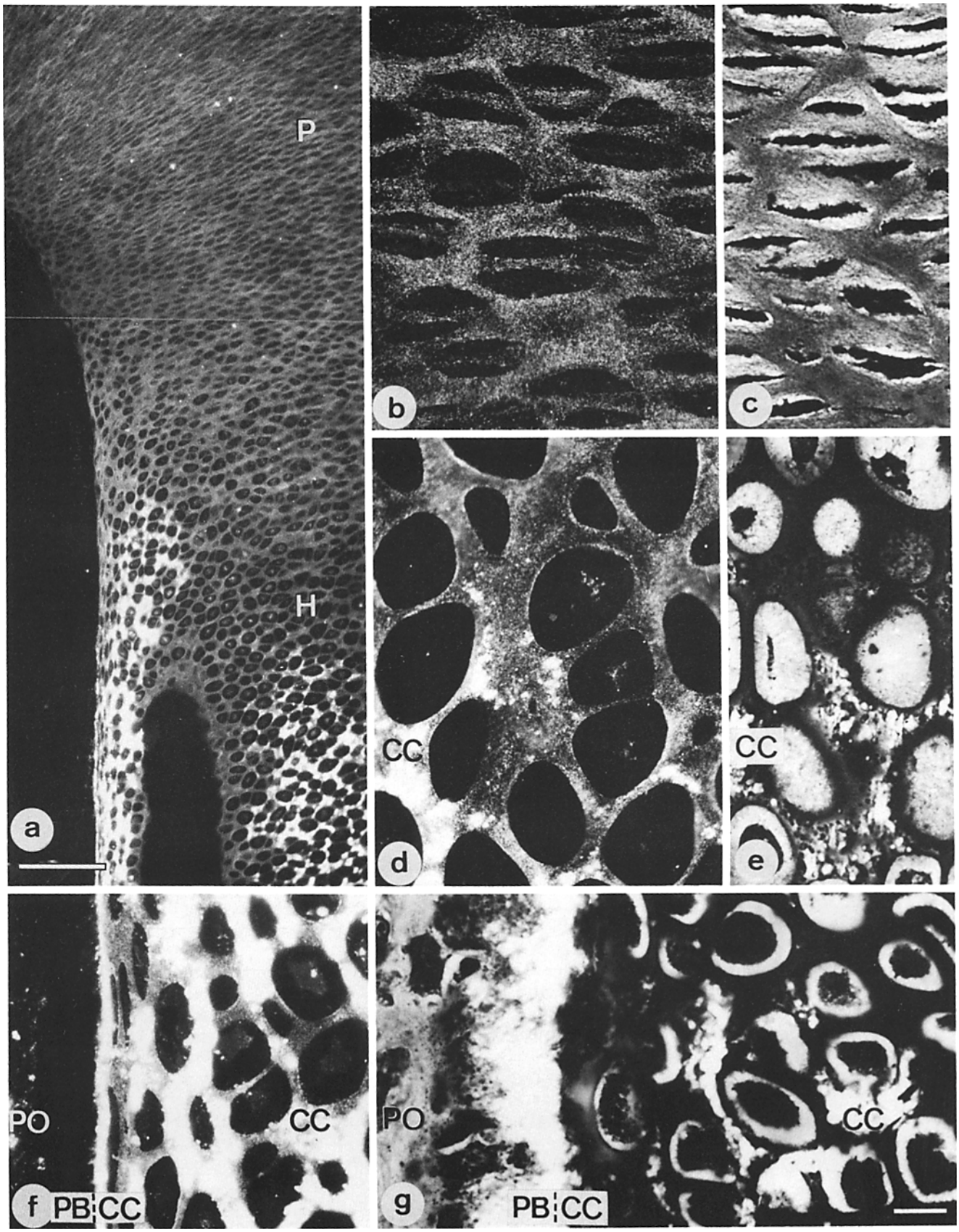

Figure 3. Longitudinal sections through the distal part of femur of 17-d-old chicken embryo. Stain: Immunofluorescence for type IX collagen (*a*, *b*, *d*, and *f*); toluidine blue (*c*, *e*, and *g*). The fluorescent stain occurs in both proliferative (*P*) and hypertrophic (*H*) zones (*a*). Details of proliferative zone (*b* and *c*) and of central (*d* and *e*) and peripheral (*f* and *g*) part of hypertrophic cartilage. *CC*, calcifying cartilage stained brightly in immunofluorescence (*d* and *f*) but only slightly with toluidine blue (*e* and *g*). *PB*:*CC*, marks the border between periosteal bone (*PB*) and calcifying cartilage (*CC*) in *f* and *g*. *PO*, periosteum. Bars: (*a*) 100 μm; (*b*–*g*) 10 μm.

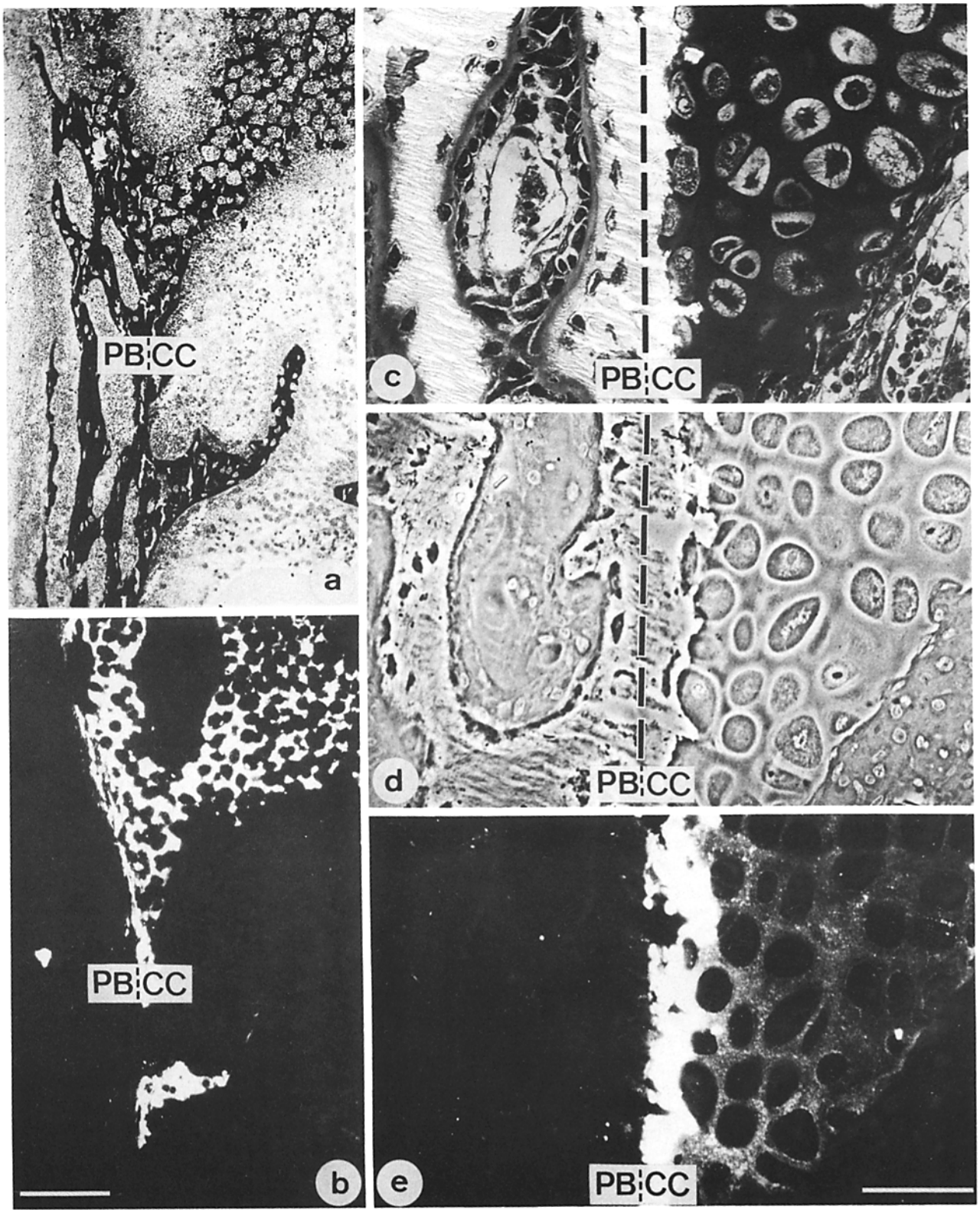

Figure 4. Periosteal and endochondral ossification in neck vertebrae (overview in *a* and *b*) and in distal part of femoral diaphysis (details in *c–e*) of 17-d-old chicken embryo. Stain: von Kossa (*a*), toluidine blue (*c*), immunofluorescence for type IX collagen (*b* and *e*). (*d*) Phase contrast micrograph of area shown in *e*. Note the lack of fluorescence in bone derived from periosteum (*PB*) and sharp border (dashed line) towards calcified cartilage (*CC*). Bars: (*a* and *b*) 100 μm; (*c–e*) 10 μm.

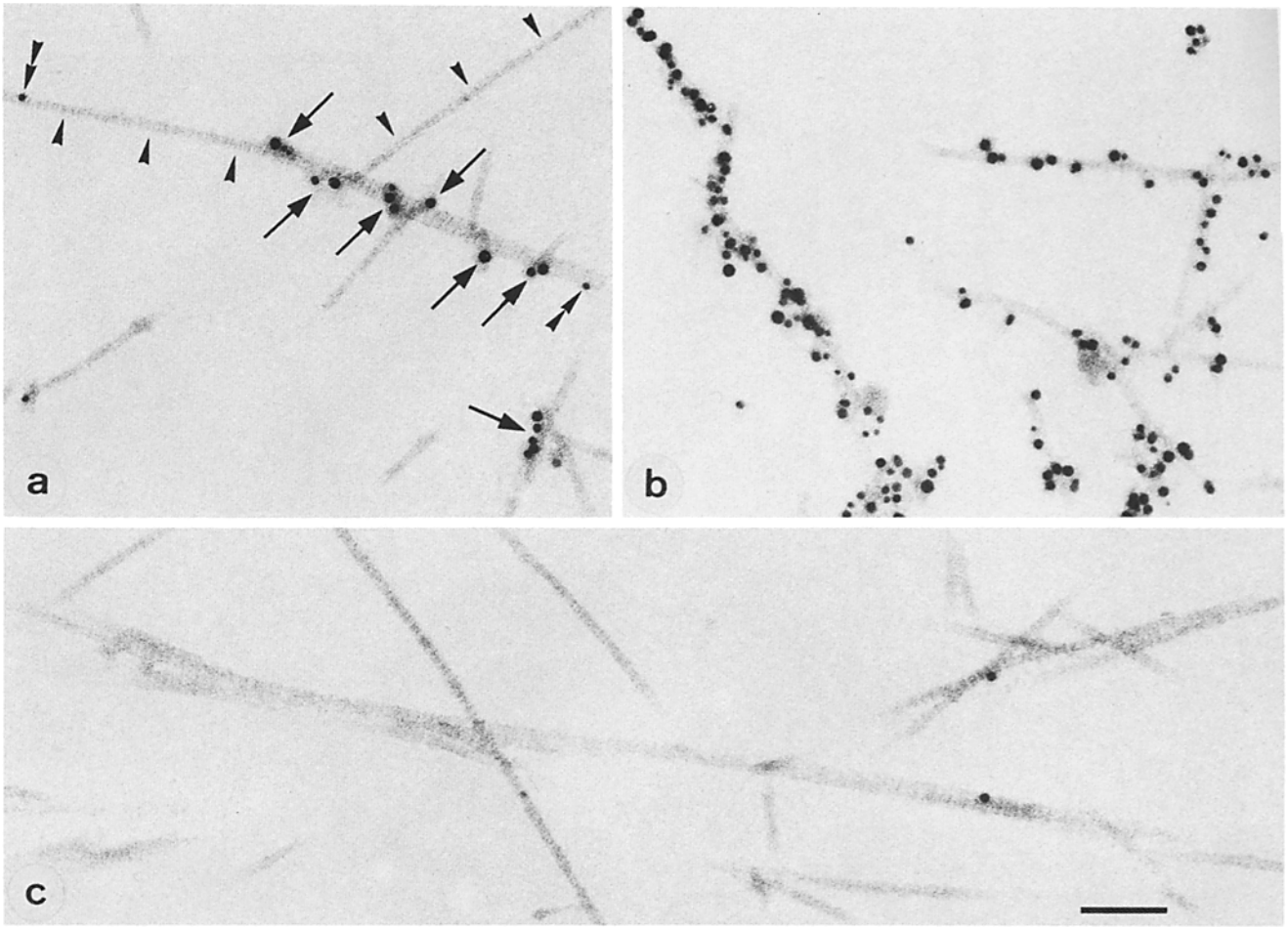

Figure 5. Matrix of sternal cartilage of 17-d-old chicken embryo labeled by the immunogold technique using antibodies against type IX collagen (*a*), type II collagen (*b*), and nonimmune serum (*c*). Note in *b* uniform decoration of fibrils, in contrast to *a* where gold particles are concentrated at intersections of fibrils (arrows). Arrowheads in *a*, long parts of fibrils that lack label (exceptions: double arrowheads). Bar, 100 nm.

be observed. This finding is consistent with the absence of distinct pericellular, territorial, and interterritorial regions in chick embryo cartilage as described above.

In sternal cartilage (Fig. 5*a*) and in femoral cartilage (not shown), labeling for type IX collagen was restricted to the thin collagenous fibrils. However, the fibrils were labeled not uniformly but mainly at their intersections (arrows in Fig. 5*a*). Long stretches of fibrils without intersections remained without label (arrowheads in Fig. 5*a*). On the other hand, not all intersections were labeled (* in Fig. 6). Of the 188 particles visible in Fig. 6, 177 were localized to or within 30 nm of fibril intersections. (The distance of 30 nm about corresponds to the size of an antibody molecule plus the average diameter of a gold particle coated with protein A.) Three particles were found in the ground substance (arrowheads in Fig. 6), and five particles were on regions without intersections (outside 30-nm limit; arrows in Fig. 6). For three particles, the localization was ambiguous, which compares well with the number of particles found in a typical negative control (Fig. 5*c*). These results were reproduced in three independently stained specimens.

When the tissue was reacted with antibodies directed against type II collagen, a different pattern was observed. As shown in Fig. 5*b*, uniform decoration of the entire fibrils was apparent.

Discussion

Type IX collagen is a newly discovered minor component of cartilage tissue. Its structure has been elucidated recently in part by chemical characterization of the native molecule (Gibson et al., 1983; Bruckner et al., 1983; Noro et al., 1983; von der Mark et al., 1984; Bruckner et al., 1985; Vaughan et al., 1985). However, most of the structural information of type IX collagen comes from its pepsin fragments due to the poor extractability from cartilage of the intact protein (Shimokomaki et al., 1980; Ayad et al., 1981; Reese and Mayne, 1981; von der Mark et al., 1982; Reese et al., 1982; Ricard-Blum et al., 1982; Mayne et al., 1985; van der Rest et al., 1985). A further substantial part of the information on the structure comes from studies on cDNA and genomic DNA clones for type IX collagen (Ninomiya and Olsen, 1984; Ninomiya et al., 1985; Lozano et al., 1985). The protein is composed of three disulfide bonded polypeptide chains that form together three triple helical domains interrupted by nonhelical sequences which confer flexibility to the molecule visible on electron micrographs after rotary shadowing (Irwin et al., 1985). Chondroitin sulfate and sometimes also dermatan sulfate are linked to one of the three composite polypeptide chains (Noro et al., 1983; Huber et al., 1986).

In contrast to the rather well known molecular structure,

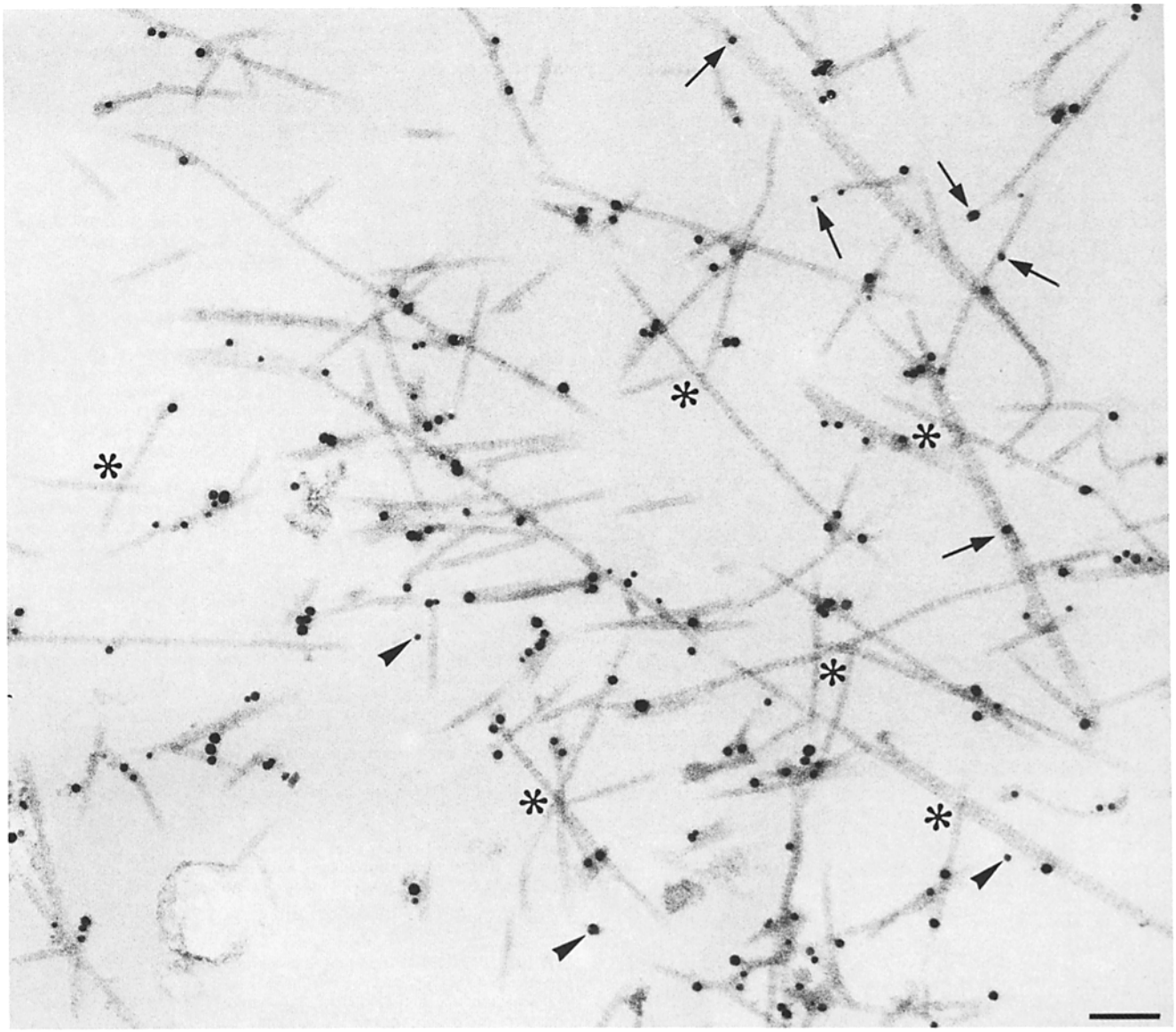

Figure 6. Matrix of sternal cartilage of 17-d-old chicken embryo labeled with antibodies against type IX collagen. Note the scarcity of particles not localized at intersections (arrows). Arrowheads, particles not localized in fibrils; *, intersections without label. Bar, 100 nm.

very little is known about the function of type IX collagen. As a first step in the elucidation of the function of type IX collagen, we studied its distribution in the tissue. Our results demonstrated that in hyaline cartilage of 17-d-old chicken embryo, type IX collagen is found homogeneously in cartilage matrix and was co-distributed with type II collagen at the level of resolution of light microscopy. These results agree with those reported by Noro et al. (1983) and by Irwin et al. (1985) on sternal cartilage of chicken embryo and adult quail. However others (Duance et al., 1982; Ricard-Blum et al., 1982; Evans et al., 1983; Hartmann et al., 1983) found type IX collagen located preferentially in close vicinity of the chondrocytes in various mammalian hyaline cartilage. This difference in the observations could be explained by the histological differences between mammalian and avian hyaline cartilage. The former tissue consists of pericellular, territorial, and interterritorial zones, which differ from each other in the density and the arrangement of the collagen fibrils as suggested by Szirmai (1969) and recently confirmed by Eggli et al. (1985). Such distinctions are less apparent in cartilages of chick embryos. Therefore, the differences of the intensity of the stain for type IX collagen in mammalian cartilage may simply reflect the differences of the amounts of collagenous fibrils deposited in the different regions of the tissues. We consider these differences in the tissue distribution of type IX collagen important because the protein could not serve a generalized function in the architecture of the extracellular matrix such as postulated below if the protein was restricted to specialized regions.

Type IX collagen did not co-distribute with type X collagen. This is best illustrated by the absence of type IX collagen from calvaria, a bony tissue formed by intramembranous ossification. The same holds for periosteal tissue. Conversely, type X collagen is absent from epiphyseal cartilage (Schmid and Linsenmayer, 1985) where type IX collagen was present.

At the ultrastructural level, type II collagen was detected

uniformly along cartilage fibrils. Type IX collagen was also localized to the fibrils but was concentrated near intersections in the fibrillar network. This observation was in part unexpected since the dimensions of the rigid, triple helical domains of the molecule are significantly larger than the intersections of the fibrils themselves. It is tempting to speculate that the important antigenic sites of type IX collagen are restricted to the nonhelical kink regions of the molecule. In this context, it is interesting to note that in major interstitial collagens the large triple helical domains represent relatively poor antigens in rabbits, whereas most of the antibody response is directed towards the nonhelical, so called telopeptides (Timpl, 1976).

From the unique molecular structure, the ultrastructural organization, and the tissue distribution of type IX collagen, we propose the following model for the function of the protein. The three independent triple helical domains, which hinge about flexible nonhelical regions, allow the protein to interact with two or more fibrils in the collagenous network of cartilage. The central triple helical domain, which is 100-nm long, may cross-bridge two fibrils separated in space by that distance or less. Through such interactions, type IX collagen could contribute to the stability of the three-dimensional organization of the individual fibrils. Furthermore, it is conceivable that lateral growth of the fibrils through the addition of further type II collagen molecules is sterically hindered by the association of individual triple helical domains of type IX collagen to the fibril surfaces. In the absence of type IX collagen, type II collagen assumes supramolecular structures different from the network of cartilage fibrils. Purified type II collagen has been shown to form large fibrillar aggregates when solutions of the protein were incubated in vitro under appropriate conditions (Lee and Piez, 1983). Parallel fibrils of type II collagen form the inner sheath of lamprey notochord (Eikenberry et al., 1984). Curiously, no type IX collagen has been found in this tissue by biochemical techniques (Eikenberry, E. F., personal communication).

The presence of glycosaminoglycans in type IX collagen is a particularly interesting property of this material. Unfortunately, from the studies described here it is not possible to draw conclusions about the significance of the proteoglycan character of type IX collagen. It is important to find answers to this question.

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