

Cartilage Contains Mixed Fibrils of Collagen Types II, IX, and XI

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Abstract. The distribution of collagen XI in fibril fragments from 17-d chick embryo sternal cartilage was determined by immunoelectron microscopy using specific polyclonal antibodies. The protein was distributed throughout the fibril fragments but was antigenically masked due to the tight packing of collagen molecules and could be identified only at sites where the fibril structure was partially disrupted. Collagens

II and IX were also distributed uniformly along fibrils but, in contrast to collagen XI, were accessible to the antibodies in intact fibrils. Therefore, cartilage fibrils are heterotypically assembled from collagens II, IX, and XI. This implies that collagen XI is an integral component of the cartilage fibrillar network and homogeneously distributed throughout the tissue. This was confirmed by immunofluorescence.

To suit their functions, connective tissues must have distinct biomechanical properties that critically depend on the molecular structure of the components of the extracellular matrix as well as their supramolecular assembly. As the principal tensile elements, fibrils play a key role in structural stabilization. The distribution of the diameters and the organization of the fibrils into meshworks, fiber bundles, or highly ordered layers are characteristics of different tissues. For the stabilization of the tissue structure, fibrils require unique surface properties enabling them to specifically interact with themselves as well as other matrix components, such as proteoglycans and matrix glycoproteins. The morphological diversity of connective tissues is paralleled by a variety of the major molecular fibril components, the collagens (25). Recent reports have established the existence of heterotypic fibrils assembled from more than one collagen type (2, 12, 17, 18, 20, 23, 37, 38). The interaction of different collagens during fibrillogenesis may well be crucial in the regulation of the fibril architecture and the modulation of the fibril surface properties.

Cartilage is unique in that it contains a tissue-specific set of collagens (25); i.e., collagens II, IX, and XI. Collagen X is predominantly found in cartilage but also occurs at low levels in intramembranous bone (31). In cartilage, however, collagen X is restricted to hypertrophic zones and, therefore, may play a role in the transition of cartilage to bone (32). Collagen II is the major fibril component and is similar to collagens I and III of other tissues in that the molecule essentially consists of a single uninterrupted helical domain 300 nm in length. Collagen II comprises three identical $\alpha 1(\text{II})$ -chains. Collagen XI probably is the structural analogue in cartilage to collagen V because, in the tissue form, both proteins contain a large amino-terminal noncollagenous domain

in addition to a 300-nm triple helix (4, 26). Collagen XI is a heterotrimer (26). The $\alpha 1(\text{XI})$ - and $\alpha 2(\text{XI})$ -chains are structurally similar to the $\alpha 1(\text{V})$ - and $\alpha 2(\text{V})$ -chains (6, 11). Curiously, the $\alpha 3(\text{XI})$ -chain is similar if not identical with an overglycosylated form of the $\alpha 1(\text{II})$ -chain (14). Because of their structural similarities and their ability to form fibrils with a D-periodic banding pattern, collagens I, II, III, V, and XI have been designated as the fibril-forming types (25). By contrast, collagen IX is only 190 nm in length and comprises three triple-helical domains linked together by flexible regions, and an amino-terminal globular domain (36). The protein probably does not form fibrils by itself (Mendler, M., and P. Bruckner, unpublished observations) but, nevertheless, is a fibril component (16, 27, 38). Collagen IX is assembled from three disulfide-bonded polypeptides, which have been designated $\alpha 1(\text{IX})$ -, $\alpha 2(\text{IX})$ -, and $\alpha 3(\text{IX})$ -chains, respectively. Intriguingly, this protein can be considered as a proteoglycan since the $\alpha 2(\text{IX})$ -chain contains a single covalently bound glycosaminoglycan chain (19, 24, 36).

The molecular organization of cartilage fibrils is complex. They contain at least two types of collagens. Recently, we have shown that collagen IX is arranged D-periodically along fibril fragments isolated from chick embryo sternal cartilage (38). The close association of collagens II and IX has been demonstrated by the finding of aldehyde-derived cross-links between the two collagen types (12, 37). Our preparations of cartilage fibril fragments also contained collagen XI (38) but with unknown localization at suprastructural level. That cartilage fibrils are composed of even three collagen types, therefore, is an interesting possibility. Alternatively, collagen XI could be part of fibrils lacking collagen II and/or IX. Here, we describe the distribution of collagen XI in fibril fragments by immunochemical localization.

Materials and Methods

Antigen Preparation

The triple-helical domains of collagens II, IX, and XI were obtained by limited pepsin digestion of adult chicken sternal cartilage and were purified by differential salt fractionation as described (28). During these procedures, terminal nonhelical segments of the collagens are lost and, in addition, collagen IX is cleaved into the HMW and LMW fragments. Collagens II and XI were further purified by ion-exchange chromatography on DEAE-cellulose and carboxymethyl-cellulose under non-denaturing conditions (14). A mixture of $\alpha 1(XI)$ -, $\alpha 2(XI)$ -, and $\alpha 3(XI)$ -chains was obtained by molecular-sieve chromatography of thermally denatured collagen XI. Purified $\alpha 1(XI)$ - and $\alpha 2(XI)$ -chains were prepared by chain separation on carboxymethyl-cellulose under denaturing conditions (14).

Antigen conjugates were prepared by a similar procedure as described in reference 29. All steps were performed at 4°C. Antigens were dissolved in 0.5 M acetic acid containing 0.2 M NaCl at a concentration of 1 mg/ml and dialyzed against PBS. Keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at a concentration of 10 mg/ml and dialyzed against PBS. 0.2 ml of the antigen and 0.1 ml of the hemocyanin solution were mixed and diluted with 0.5 ml PBS. The coupling reaction was initiated by addition of 0.4 ml 0.25% (vol/vol) glutaraldehyde. The reaction mixture was stirred overnight and then dialyzed against PBS. For immunization, the final solution was mixed 1:1 (vol/vol) with complete or incomplete Freund's adjuvant.

SDS-PAGE and Immunoblots

Gradient gels of 4–10/15% (wt/vol) polyacrylamide and immunoblots were run according to standard methods (21, 35).

Antisera to Native Collagens and Antigen Conjugates

Animals were immunized following the protocol of Timpl (34). Rabbits were immunized with 1 mg native collagens II or XI. When antigen conjugates were used, rabbits were immunized with 0.1 mg of either native collagen XI, or of $\alpha 1(XI)$ - or $\alpha 2(XI)$ -chains conjugated to hemocyanin. Antisera were affinity purified sequentially on pepsin fragments of collagen II, IX, and XI immobilized on Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). Antisera were assayed by ELISA (8) and by immunoblotting. Antibodies directed against the HMW fragment of collagen IX are described elsewhere (5).

Preparation of Fibril Fragments

The isolation of fibril fragments has been described previously (38). In brief, after homogenization and extraction of the tissue in a neutral 1 M NaCl, the fibril fragments were obtained by differential centrifugation. The final pellet containing the fibril fragments was resuspended in neutral 1 M NaCl or was reextracted with either 8 M urea or 4 M guanidinium hydrochloride and recentrifuged. This procedure was repeated to remove contaminants.

Enzymatic Digestions

Trypsinized fibril fragments were prepared as previously described (38). To partially unwind their tight molecular packing, fibril fragments were digested with 0.5 mg/ml of pepsin in 0.5 M acetic acid containing 0.2 M NaCl. After 2 h, the digestion was stopped by neutralization with solid Tris and the mixture was centrifuged at 115,000 g for 2 h. The pellet was resuspended in the acetic acid–NaCl mixture and recentrifuged. This procedure was repeated to remove single collagen molecules solubilized during the digestion. The final pellet was resuspended in a 1 M NaCl solution buffered to pH 7.4 with 0.05 M Tris-HCl. All steps were performed at 4°C.

Immunoelectron Microscopy

Suspensions of fibril fragments were absorbed onto carbon-coated copper grids, washed with TBS, treated for 30 min with 2% (wt/vol) dried skimmed milk in TBS (blocking solution), and for 2 h with collagen type-specific antibodies at various dilutions in blocking solution. After washing three times for 5 min with TBS, the grids were floated for 2 h on drops of blocking solution containing colloidal gold particles (10 nm) coated with goat antibodies to rabbit immunoglobulins at concentrations recommended by the

supplier (Janssen Life Science Products, Beerse, Belgium). After washing three times with TBS, the grids were negatively stained with uranyl acetate and examined in an electron microscope (type 300; Philips Electronic Instruments, Inc., Mahwah, NJ). Control experiments were done without the first antibody or with preimmune serum. Rotary-shadowed specimens were prepared as described (38).

Immunofluorescence

Cryostat sections of 17-d chick embryo sterna were spread on coverslips coated with polylysine and were treated at room temperature for 60 min with 1 mg/ml of pepsin dissolved in 0.2 M NaCl, 0.5 M acetic acid, pH 2.0. After washing with PBS, the sections were incubated for 30 min with 10 mg/ml of ovalbumin in 0.1 M MgCl₂, 0.01 M Tris-HCl, supplemented with 5% goat serum (blocking buffer), and subsequently incubated for 12 h with antibodies to collagen XI in blocking buffer. Sections were then rinsed with blocking buffer and exposed for 60 min to fluorescein-labeled goat antibodies to rabbit immunoglobulins (Tago, Inc., Burlingame, CA). After washing with PBS, the sections were mounted in a medium containing polyvinyl alcohol. The tissue sections were examined on a Leitz Ortholux III epifluorescence microscope.

Results

Antibodies to Collagens II and XI

To compare the distribution of collagen XI in cartilage fibril fragments with that of collagens II and IX, it was necessary to prepare specific antibodies to collagens II and XI. The major difficulty here is that pepsin-treated collagens are notoriously poor immunogens. This is particularly the case for unfolded collagenous polypeptides. To further compound these difficulties, the $\alpha 1(II)$ -chain of collagen II and the $\alpha 3(XI)$ -chain of collagen XI are highly homologous. For this reason, antibodies to collagen XI commonly cross-react with collagen II and vice versa.

As a consequence, several approaches were chosen to obtain specific antisera directed against collagen XI. Firstly, rabbits were immunized with salt-fractionated collagen XI following standard procedures. Secondly, purified collagen XI was coupled to key hole limpet hemocyanin, a carrier known to enhance the immune response against the bound protein (9). This conjugate was used as an immunogen in rabbits. Finally, separated $\alpha 1(XI)$ - and $\alpha 2(XI)$ -chains, respectively, were also coupled to hemocyanin. We expected to raise chain-specific antisera reacting with native collagen XI as well. If that approach was successful, possible cross-reactions between collagen XI and II could be circumvented.

The crude antisera to collagen XI were tested by ELISA and reacted similarly with collagens XI, II, and IX. This nonspecificity was confirmed by immunoblotting. Therefore, the antisera were preadsorbed on columns containing immobilized collagen II or HMW/LMW fragments before adsorption on and elution from a column containing collagen XI. The affinity-purified antibodies were strongly positive for collagen XI in ELISA, did not respond to collagen II, and reacted 64 times weaker with HMW/LMW fragments than with collagen XI (Fig. 1 A). This was expected since our preparations of HMW/LMW fragments contained traces of collagen XI visible on overloaded gels (not shown). The specificity of the antibodies was further tested by immunoblotting (Fig. 2, lanes 4–6). Reactivity was observed against the $\alpha 2(XI)$ -chain and, to a lesser extent, against the $\alpha 1(XI)$ -chain. However, no reactivity against HMW/LMW fragments and the homologous $\alpha 1(II)$ - and $\alpha 3(XI)$ -chains was de-

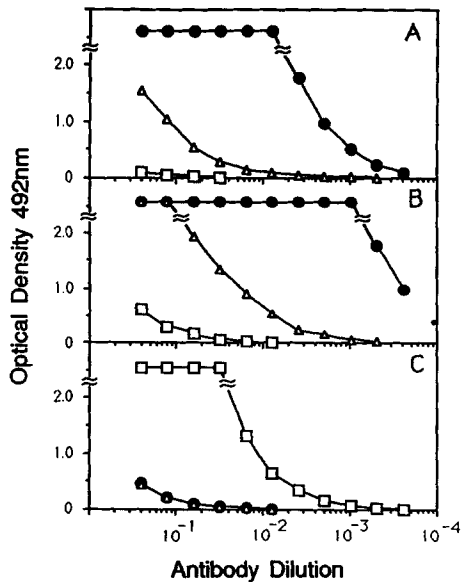


Figure 1. Characterization by ELISA of (A) affinity-purified antibodies to collagen XI obtained from an antiserum raised against native collagen XI; (B) crude antisera raised against native collagen XI conjugated to hemocyanin; (C) affinity-purified antibodies to collagen II. The antigens tested were (●) collagen XI, (□) collagen II, and (Δ) a mixture of the HMW and LMW fragments of collagen IX.

tected. We concluded that the affinity-purified antibodies were specific for collagen XI.

Antisera against purified native collagen XI coupled to hemocyanin had the same specificity in ELISA as the affinity-purified antibodies described above (Fig. 1, compare B with A). The antibody titer was about eight times higher than that of the affinity-purified antibodies and this immune response was elicited with 10 times less collagen XI. Therefore, cross-linking of collagen XI to hemocyanin led both to an increase in the immunogenicity and to an improvement in the specificity of the crude antisera. Purified $\alpha 1(XI)$ - and $\alpha 2(XI)$ -chains, respectively, coupled to hemocyanin, elicited specific antisera in rabbits which, unfortunately, did not react with the native protein and, hence, were unsuitable for the immunochemical identification of collagen XI in fibrils. However, coupling of intact collagens or of purified collagenous polypeptides to hemocyanin proved to be a powerful approach for obtaining specific antisera.

Rabbit antisera against pepsin-solubilized collagen II were similarly adsorbed on columns containing immobilized collagen XI or HMW/LMW fragments followed by adsorption and elution from a collagen II column. The antibodies recognized collagen II in ELISA and reacted ~ 64 times less in intensity with both collagen XI and HMW/LMW fragments (Fig. 1 C). As expected, the homologous $\alpha 1(II)$ - and $\alpha 3(XI)$ -chains were entirely cross-reactive in immunoblots (Fig. 2, lanes 7-9), but no reaction with HMW/LMW fragments, the $\alpha 1(XI)$ - and $\alpha 2(XI)$ -chains was apparent. We concluded, therefore, that our antibodies to type II collagen specifically recognized that protein in native state but, as expected, did not distinguish between denatured $\alpha 1(II)$ - and $\alpha 3(XI)$ -chains.

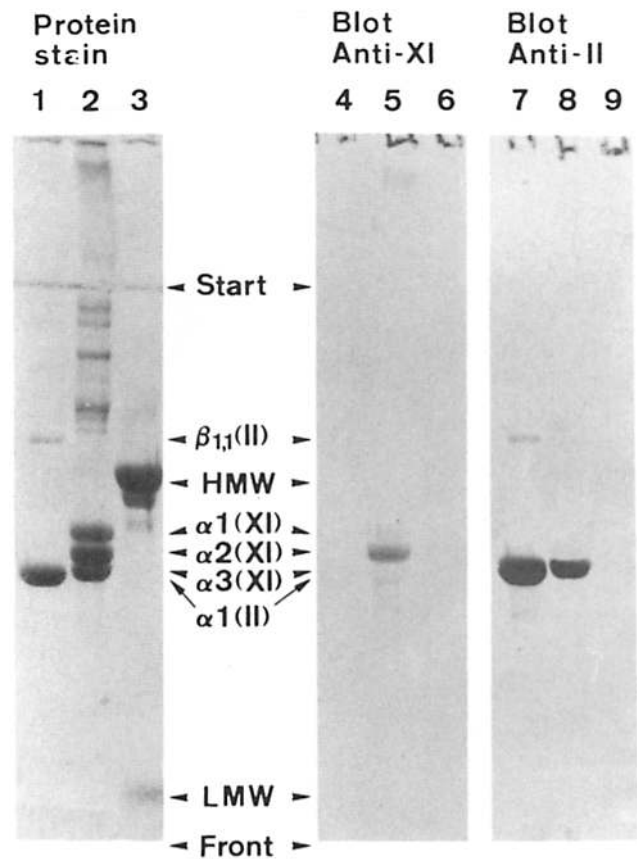


Figure 2. Characterization of affinity-purified antibodies to collagen II and XI by immunoblotting. Pepsin-extracted collagen II (lanes 1, 4, and 7), collagen XI (lanes 2, 5, and 8), and the fragments of collagen IX (lanes 3, 6, and 9) were subjected to electrophoresis in 4-10% polyacrylamide gels. Gels were stained with Coomassie Blue (Protein stain) or subjected to immunoblotting using affinity-purified antibodies to collagen XI (Blot Anti-XI) or collagen II (Blot Anti-II).

Immunoelectron Microscopy

Fibril fragments were mechanically generated by homogenization of sternal cartilage and purified by differential centrifugation and treatment with chaotropic agents. In spite of these rather drastic conditions, the fragments strikingly resemble the native fibrils. The fragments also represent well the fibril populations found in tissues and are attractive objects for the study of the architecture of fibrils. For example, immunochemical masking due to other tissue components is expected to be less prominent than in tissue sections. They also offer advantages for the biochemical assessment of fibril components.

Fibril fragments were stained by the indirect immunogold technique using antibodies to collagens II, IX, and XI directly after adsorption to EM grids. Immunolabeling was associated with patches of fuzzy material which were absent in negative controls and which, therefore, represented bound antibody molecules.

Collagen XI was detected predominantly at the mechanically generated ends of the fragments. Occasional labeling was seen where the fibril body was damaged, presumably by shear forces during tissue homogenization, but the fragments remained undecorated where the fibril structure was intact

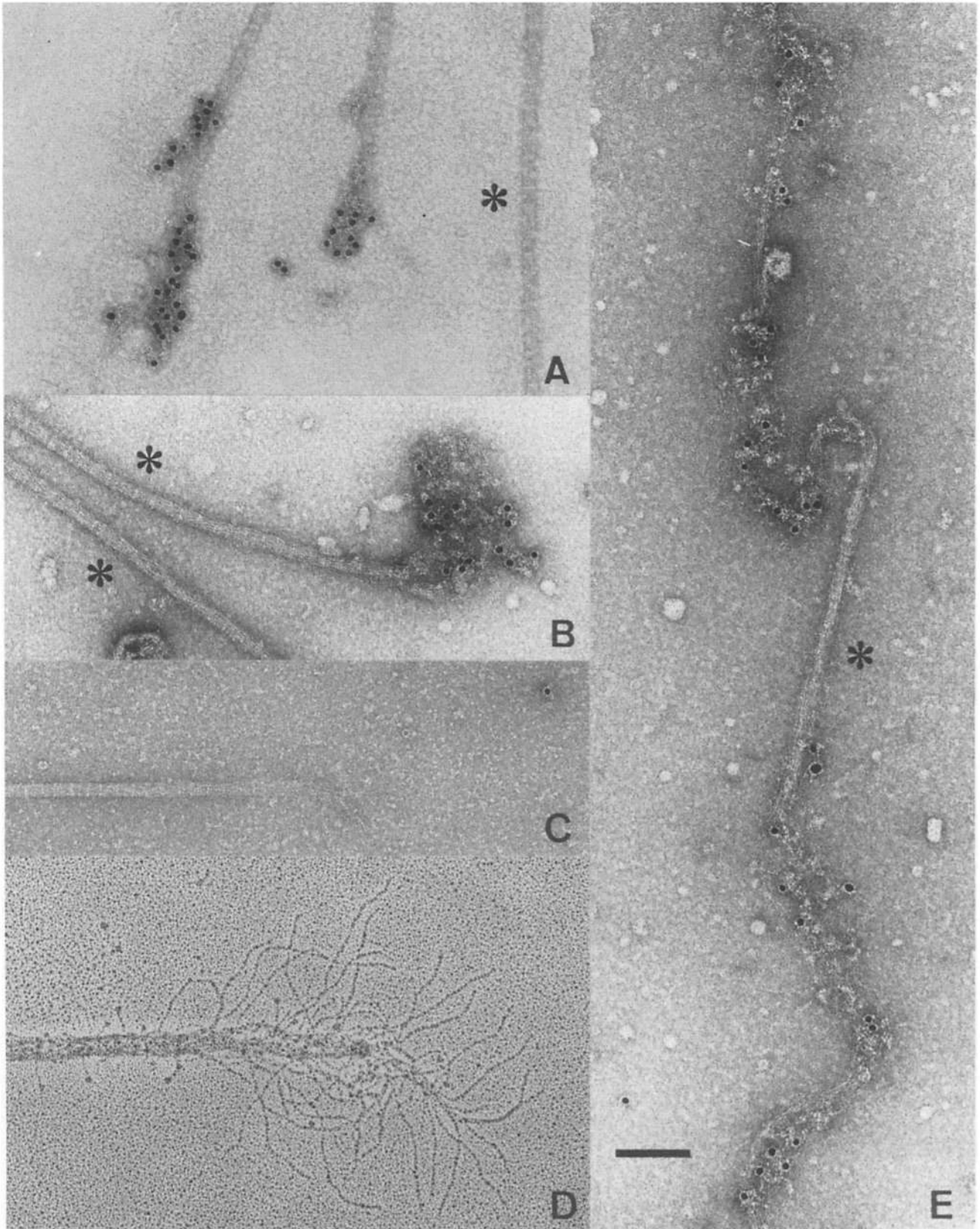


Figure 3. Ultrastructural immunolocalization of collagen XI in fibril fragments from 17-d chick embryo sternal cartilage. (*A* and *B*) Sheared fibril ends. (*C*) Negative control with preimmune sera. (*D*) Sheared fibril end after rotary shadowing showing individual collagen molecules fanning out from the intact fibril body. (*E*) Partially digested fibril. Intact fibril segments (*) are continuous with partially disrupted fibril stretches. Note the absence of gold particles on intact fibril segments in *A*, *B*, and *E* (*). Bar, 100 nm.

(Fig. 3, *A* and *B*). Electron micrographs of sheared fibril ends after rotary shadowing revealed collagen molecules fanning out individually from the fibril body, thus presumably exposing the epitopes (Fig. 3 *D*). Taken together, these observations suggested that the epitopes of collagen XI were unavailable in intact fibrils. This antigenic masking could be due to the presence of noncollagenous fibril components adhering to the surface or, more likely, due to the tight lateral packing of collagen molecules in the fibrils. It is not immediately clear whether collagen XI is hidden in the interior of the fibrils or whether the close proximity of the collagen molecules at the surface effectively obstructs the antigenic sites. In each of these situations, however, collagen XI could be present uniformly along the fibrils. To confirm this hypothesis, we sought to render accessible the epitopes by partial disruption of the fibril architecture by treatment with chaotropic reagents, such as 8 M urea or 4 M guanidium hydrochloride. The fibrils were also pretreated with 0.2 M acetic acid, a solvent successfully used to unmask the epitopes

of collagen V in corneal fibrils (23). Further, we digested the fragments with trypsin which, as shown previously, effectively removes adhering proteins or proteoglycans without disruption of the collagenous fibril backbone. These treatments changed neither the electron microscopic appearance nor the labeling intensity along the intact portions of the fibril fragments. This again illustrates the tight packing of the molecules in the fibrils. By contrast, treatment with pepsin, an enzyme known to disassemble the fibril structure by solubilizing individual collagen molecules, led to partially digested fibril fragments. Intact stretches, identifying the fibrillar origin of the objects, were continuous with segments in which fibrils still could be discerned but in which the well-defined structure was disrupted. The antibody binding to collagen XI was only evident in the disrupted portions (Fig. 3 *E*). No immunogold particles were observed at the sheared ends (Fig. 3 *C*), or in disrupted regions of the fibrils (not shown) in negative controls with preimmune sera, or when the treatment with antibodies to collagen XI was omitted.

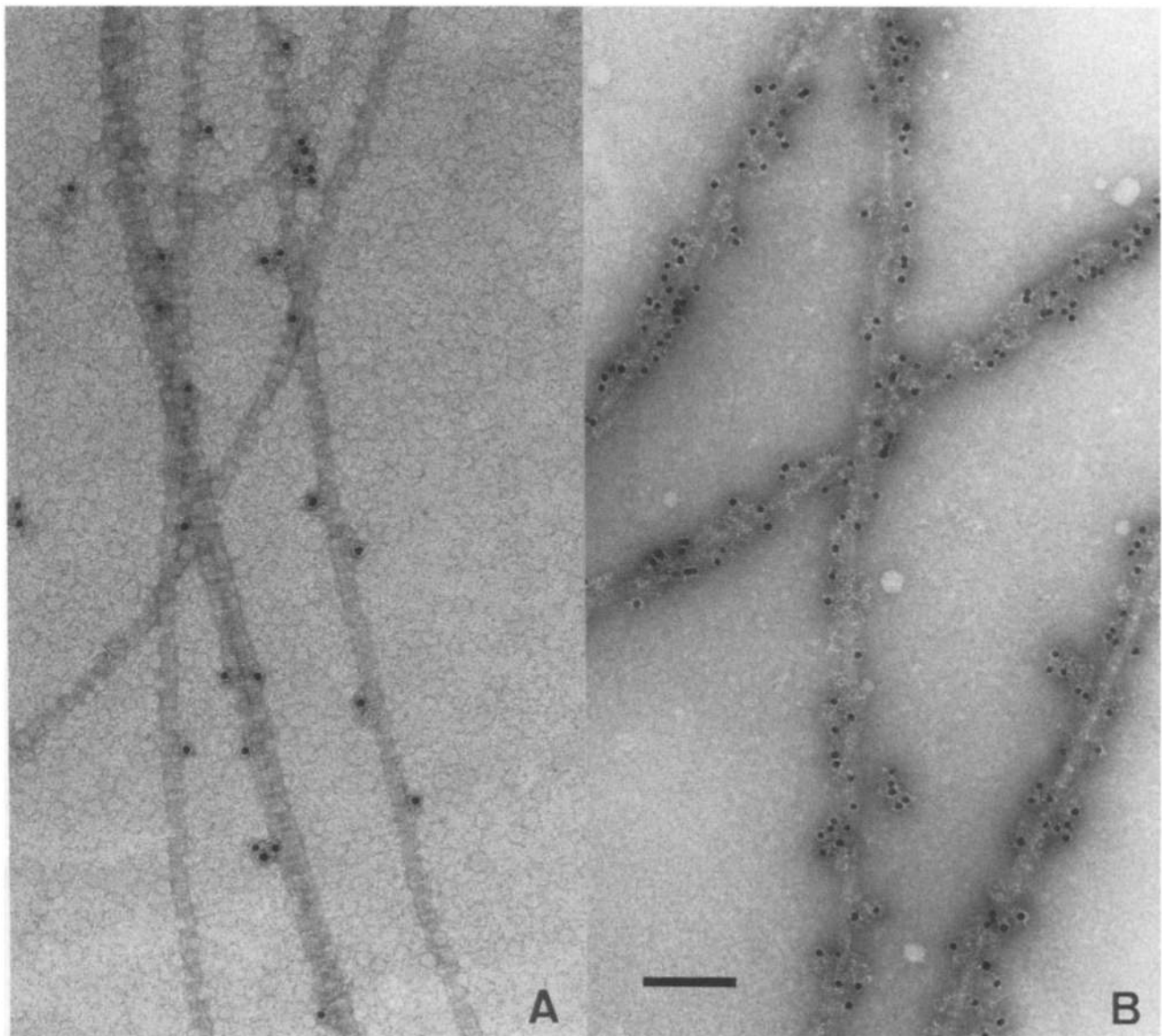


Figure 4. Ultrastructural immunolocalization of collagens II (*A*) and IX (*B*) in fibril fragments from 17-d chick embryo sternal cartilage. Bar, 100 nm.

These observations demonstrated the complete masking of collagen XI in intact fibrils. Since disruption of the fibrils was effected stochastically, either by mechanical shearing or by enzymatic digestion, we concluded that the protein was uniformly present along the cartilage fibrils.

In contrast to collagen XI, immunogold labeling of collagens II and IX did not require pretreatment of the fibril fragments. Uniform labeling of the fibril fragments with antibodies to collagen II was observed, consistent with the immunohistochemical localization reported earlier (Fig. 4 A) (27). The labeling was enhanced if the structure of the fibrils was partially disrupted (not shown), indicating that some but not all antigenic sites of collagen II were masked. Finally, fibril fragments were intensely decorated with antibodies to collagen IX in agreement with our recent finding demonstrating D-periodic distribution of this protein on the surface of fibril fragments (Fig. 4 B) (38).

The presence of collagen XI in all cartilage fibrils is incompatible with an exclusive localization of the protein to pericellular regions of the extracellular matrix as reported earlier (10, 30). Therefore, we reinvestigated the distribution of collagen XI in chick embryo sterna by immunofluorescent labeling of pepsin-treated cryosections. As shown in Fig. 5, the protein is distributed throughout the extracellular matrix. This observation is consistent with the notion that collagen

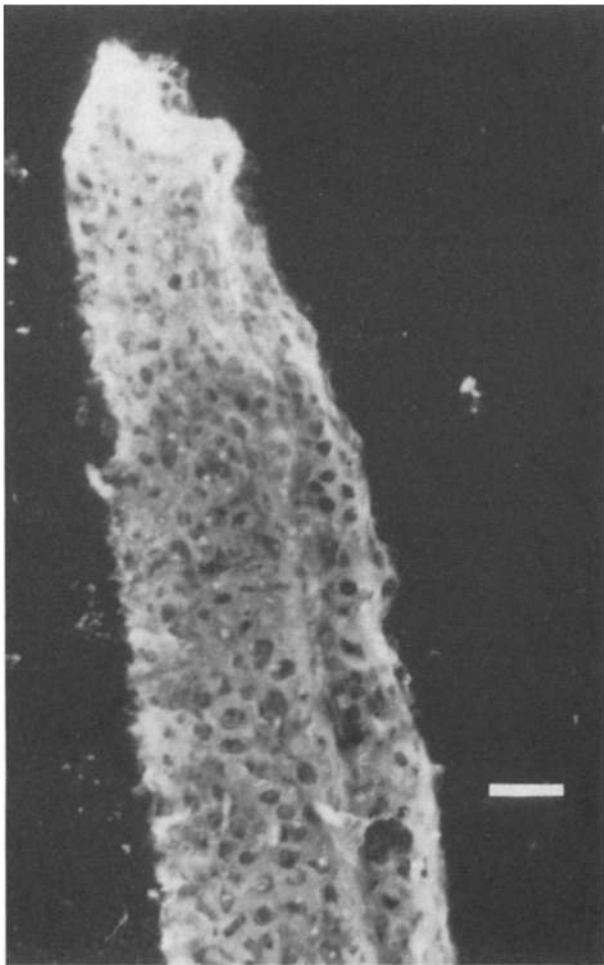


Figure 5. Indirect immunofluorescence of 17-d chick embryo sternum. Portion of a transverse cryosection near to the center of the sternum stained with antibodies to collagen XI. Bar, 10 μ m.

XI is a constituent of all cartilage fibrils. The apparent pericellular binding of antibodies to collagen XI observed earlier most probably was due to antigenic masking of the protein in other regions. In fact, extensive proteolytic pretreatment of the tissue sections was necessary in our immunofluorescence studies to render the protein accessible to the antibodies. Treatment with hyaluronidase as usually used in immunofluorescence studies of cartilage collagens did not, in this case, result in detectable immunofluorescence.

Discussion

In chick embryo hyaline cartilage, fibrils are tightly controlled in diameter. They are thin (17 nm) and form an essentially random network entrapping large aggregates of proteoglycans and glycoproteins (27, 38). The molecular mechanisms involved in the formation of the fibrils and their stabilization through specific interactions remain largely unknown. Therefore, we have isolated fibril fragments from cartilage and studied the composition and the organization of their collagens.

Collagen II is the bulk component of cartilage fibrils. Recently we have described the D-periodic distribution of collagen IX at their surface (38). Here, we have directly demonstrated that the same fibrils comprise even a third collagen; i.e., collagen XI.

We have previously found that preparations of mechanically generated fibril fragments contained collagens II, IX, and XI in a ratio of 8:1:1 (38). Based on these results and those reported here we assume that, in chick embryo hyaline cartilage, there is a single population of fibrils containing 10% of collagens IX and XI, with the remainder being collagen II. From this we conclude that collagen XI must be uniformly distributed throughout hyaline cartilage matrix. This is inconsistent with earlier immunohistochemical observations suggesting that the protein occurred predominantly in pericellular regions (10, 30). Our own histochemical results, however, clearly demonstrate that the entire matrix was labeled with antibodies to collagen XI, supporting the concept of the protein as a universal cartilage fibril component.

The fibril fragments exhibited a remarkable stability towards reagents known to dissociate the fibrillar packing of collagen molecules. Even digestion with trypsin at high enzyme to substrate ratios did not perceptibly alter the fibril morphology as observed in the electron microscopy after negative staining. Treatment with acid or chaotropic agents such as 4 M guanidine hydrochloride or 8 M urea was equally ineffective. This explains, at least in part, the immunochemical masking of cartilage collagens when they reside in fibrils. However, the masking effect was most prominent for collagen XI. Antibodies to this protein exclusively reacted at regions where the fibril structure was partially disrupted, either at the sheared fibril ends or where the fibrillar packing was partially disorganized by pepsin treatment. Labeling of collagens II and IX was also more intense at such locations but, in contrast to collagen XI, both proteins were also detected by our antibodies at sites where the fibril body remained intact. We favor, therefore, the notion that collagen XI is buried in the interior of the fibrils although we still cannot exclude its presence on the fibril surface where antigenic sites are merely obstructed by the close proximity of collagens II and/or IX.

Cartilage fibrils, therefore, seem to share a number of structural similarities with heterotypic fibrils of collagen I and V identified in the chicken corneal stroma (2). These fibrils have a uniform diameter of 25 nm and the localization of collagen V also required disruption of the fibril structure. Lathyrin fibrils in tissue sections could partially be disrupted by temperature treatment only and, therefore, it was concluded that the masking effect was due to the fibril architecture. These results were supported by a study using collagen type-specific collagenases (13). Furthermore, in vitro reconstitution studies led to the suggestion that collagen V limits the lateral growth of collagen I fibrils (1). This lends further support to the notion that collagens II and XI are the cartilage analogues of collagens I and V in other tissues. In this context, it is interesting to note that collagen XII, a molecule structurally resembling collagen IX (15), has been discovered in minute quantities in tissues containing collagens I and/or V (7).

The role of the various cartilage collagens still remains largely unknown. When collagen II alone was subjected to conditions of in vitro reconstitution of fibrils the protein formed large tactoidal aggregates (22) vastly different from the fibrils seen in situ with a uniform diameter of 17 nm. Similar tactoids were observed when mixtures of collagen II and IX were reconstituted (Mendler, M., and P. Bruckner, unpublished observations), indicating that collagen IX alone is not able to limit the lateral growth of collagen II aggregates. By contrast, intact as well as pepsin-treated collagen XI alone has been reported to reconstitute into thin fibrils with a diameter of ~20 nm (3, 33). It is tempting to speculate that collagen XI may be a factor controlling the lateral growth of fibrils and, therefore, may be important in the regulation of fibrillogenesis in vivo.

This paper is dedicated to Professor G. Semenza on the occasion of his 60th birthday.

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