Collagen Type I and Type V Are Present in the Same Fibril in the Avian Corneal Stroma

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Abstract. The distribution, supramolecular form, and arrangement of collagen types I and V in the chicken embryo corneal stroma were studied using electron microscopy, collagen type–specific monoclonal antibodies, and a preembedding immunogold method. Double-label immunoelectron microscopy with colloidal gold–tagged monoclonal antibodies was used to simultaneously localize collagen type I and type V within the chick corneal stroma. The results definitively demonstrate, for the first time, that both collagens are codistributed within the same fibril. Type I collagen was localized to striated fibrils throughout the corneal stroma homogeneously. Type V collagen could be localized only after pretreatment of the tissue to partially disrupt collagen fibril structure. After such pretreatments the type V collagen was found in regions where fibrils were partially dissociated and not in regions where fibril structure was intact. When pretreated tissues were double labeled with antibodies against types I and V collagen coupled to different size gold particles, the two collagens colocalized in areas where fibril structure was partially disrupted. Antibodies against type IV collagen were used as a control and were nonreactive with fibrils. These results indicate that collagen types I and V are assembled together within single fibrils in the corneal stroma such that the interaction of these collagen types within heterotypic fibrils masks the epitopes on the type V collagen molecule. One consequence of the formation of such heterotypic fibrils may be the regulation of corneal fibril diameter, a condition essential for corneal transparency.

THE structure of collagen fibrils, the grouping of fibrils into bundles, and the organization of fibril bundles within the stroma are all important in the determination of extracellular matrix structure and function. The corneal stroma is a highly organized connective tissue in which the rigid control of fibril diameter and the precise arrangement of the collagen fibrils is essential for optical transparency (9). Corneal collagen fibrils have a monotony of small diameter fibrils (~25 nm) with a constant center to center spacing. The fibrils are further organized into orthogonal lamellae (3, 18–20, 45, 46). This uniformity and order at all levels of matrix architecture makes the corneal stroma an ideal tissue for studies on the in situ regulation of collagen fibrillogenesis.

Collagen fibril formation is a complex process that is regulated by a number of different factors, including: the collagen type or types present (2, 37), the sequence and extent of propeptide processing (14, 15, 38), interactions with other matrix components (7, 41), as well as a direct involvement of the cells (3, 4, 45). The collagen composition, distribution of collagen types, and fibril organization are characteristic of different connective tissues. The earliest corneal anlagen in the embryonic chicken is the primary stroma, an acellular orthogonal fibrillar meshwork composed of collagen types I and II, produced by the corneal epithelium (20, 21, 30). This is invaded by mesenchymal cells which deposit a matrix, termed the secondary stroma. With the migration of fibroblasts into the primary corneal stroma types I, V, and VI collagen are found throughout the secondary or mature corneal stroma (5, 13, 20, 29–32). In the secondary stroma, type I collagen is the predominant collagen, with types V and VI contributing quantitatively less. However, the content of type V collagen in the corneal stroma is relatively high when compared with other tissues containing predominantly type I collagen such as sclera, tendon, dermis, and bone (6, 8, 10, 11, 16, 23, 26, 47, 50).

Immunocytochemical studies of the chick cornea using anti–collagen monoclonal antibodies have demonstrated that type I collagen is distributed throughout the corneal stroma (5, 13, 30–32). In contrast, type V collagen could not be detected unless normal collagen fibrillar structure was disrupted with agents such as dilute acid or guanidine-HCl, or when the type I collagen was removed by digestion with vertebrate collagenase. After such treatments type V collagen, like type I collagen, could be detected uniformly throughout the stroma (13, 30–32, 49).

In addition, fibril disruption by temperature manipulation of lathyritic corneas in which the collagen molecules have re-

duced covalent cross-linking, also was shown to expose the epitopes on the type V collagen within the stroma (5, 13, 30). In these studies, the in ovo administration of the lathyrogen, β-aminopropionitrile (βAPN),[1] was used to prevent the formation of covalent cross-links through the inhibition of the enzyme lysyl oxidase (44). In such embryos, the newly formed collagen fibrils have a structure that is sensitive to temperature manipulation. In such tissues, normal fibril structure is maintained at 37°C while at 4°C the fibrils dissociate. This dissociation of fibril structure in the cold can be partially reversed by returning the tissue to 37°C (27, 28, 48). We observed that when immunohistochemical staining was done on such tissues at 37°C, there was no reactivity for type V collagen while at 4°C reactivity was observed.

These results indicated that the helical epitopes on the type V molecule recognized by the two monoclonal antibodies used are masked within the corneal stroma. A structural arrangement for fibrils in which type I collagen molecules are responsible for the antigenic masking of the type V collagen is suggested by the observation that digestion of tissues with vertebrate collagenase which degrades type I collagen but not type V, also will unmask the type V epitopes (13, 15, 30). Fitch et al. (Fitch, J. M., D. E. Birk, A. Mentzer, R. A. Hasty, C. Mainardi, and T. F. Linsenmayer, manuscript submitted for publication) also have demonstrated that type V collagen is not hydrolyzed by a type V-specific collagenase unless fibrillar structure is disrupted.

In the present study, double-label immunoelectron microscopy with colloidal gold–tagged collagen type–specific monoclonal antibodies was used to simultaneously localize collagen type I and type V within the fibrils of the chick corneal stroma. The results definitively demonstrate for the first time that both collagens are codistributed within the same collagen fibril and that these fibrils are, therefore, heterotypic structures.

Materials and Methods

White leghorn chicken embryos were incubated at 37.5°C in a humidified atmosphere and staged according to Hamburger and Hamilton (17). Embryos were made lathyritic by the injection of 0.1 ml β-aminopropionitrile fumarate (βAPN; Sigma Chemical Co., St. Louis, MO) in PBS on days 8, 9, and 10. Concentrations of 1.0 mg/ml on day 8, 1.5 mg/ml on day 9, and 3.0 mg/ml on day 10 were used. The embryos were sacrificed on day 11 with a viable yield of 40–60%.

Antibodies

Two different monoclonal antibodies against type V collagen, V-AB12 and V-DH2 (32); two different monoclonal antibodies against type I collagen, I-BA1 and I-IB6 (33, 34); as well as two different antibodies against type IV collagen, IV-IIB12/C2 and IV-IA8/H8G8 (12, 36) were used in these experiments. These collagen type–specific antibodies have been characterized previously and show no cross-reactivity with any other known collagen. They also are species specific and conformation dependent. In most experiments described, the two different type V-specific monoclonal antibodies, V-AB12 and V-DH2, were mixed to increase the observed labeling. These antibodies react with different epitopes; however, the epitopes occur at the same axial location along the helical region of the type V molecule, ∼60 nm from one end (30).

Preembedding Immunoelectron Microscopy

Lathyritic or normal corneas were dissected from staged embryos, rinsed in PBS, incubated with 10% DMSO for 30 min, embedded in OCT Compound (Tissue Tek, Miles Laboratories, Inc., Naperville, IL), and frozen

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1. *Abbreviation used in this paper*: βAPN, β-aminopropionitrile.

in liquid nitrogen. Sections were cut on a cryostat at 10 μm and picked up onto albumin-coated slides (5).

To unmask the type V epitopes, the sections were incubated in PBS at 4°C for 9–24 h depending on the experiment. Controls were incubated at 37°C for the same times. Fixation was with 4% paraformaldehyde in PBS, pH 7.4 for 15 min at 4°C. The sections were washed three times in PBS and incubated in PBS with sodium borohydride (50 mg/100 ml) for 60 min at 4°C to reduce any free aldehydes (35). In some cases, the tissues were digested with 500 U/ml ovine testicular hyaluronidase (Sigma Chemical Co.) in PBS at 37°C for 30 min to facilitate penetration of the antibody-gold complexes.

The sections were washed several times in PBS and blocked in 2% normal goat serum for 2 h at 4°C followed by PBS with 1% BSA, 0.05% Tween 20, 0.05% sodium azide, pH 7.3. The sections were then incubated overnight at 4°C with gold-conjugated antibody. The antibodies were diluted in PBS with 1% BSA and 0.05% Tween 20 and centrifuged for 5 min in a microfuge (Brinkmann Instruments Co., Westbury, NY). Antibodies conjugated to 5 nm gold were used at 0.20–0.30 OD_{600} units while the antibodies conjugated to 10 nm gold particles were used at 0.30–0.40 OD_{600} units. After incubation, the sections were washed five times over 1 h with PBS/0.05% Tween, three times with PBS, and rinsed with 0.1 M cacodylate buffer.

The sections were fixed in 2.5% glutaraldehyde, 0.1 M cacodylate pH 7.4 for 1 h followed by postfixation in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h. The tissues were dehydrated in a graded ethanol series. In some cases the corneal stroma was stained en bloc with ethanolic uranyl acetate which significantly enhances the staining of the partially dissociated collagen fibrils. After postfixation in osmium, the tissues were washed with cacodylate buffer, and dehydrated through 30 and 50% ethanol. At this stage the tissues were transferred to 2% uranyl acetate in 50% ethanol and en bloc stained with filtered ethanolic uranyl acetate for 1 h at 4°C. Dehydration was continued through 70, 85, 95, and 100% ethanol, propylene oxide, and the sections were embedded in Epon-Araldite. The embedded sections were mounted on epoxy cylinders and polymerized. The sections were "popped off" by gently rocking the epoxy cylinder after placing the polymerized slide on a 100°C hot plate for 15 s (5).

Electron Microscopy

For transmission electron microscopy, sections were cut with a pale gold interference color, picked up onto mesh grids, stained with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid pH 3.2. Sections were examined and photographed using a Philips 420 transmission electron microscope.

Preparation of Colloidal Gold-conjugated Antibodies

Colloidal gold suspensions containing particle sizes of 5 and 10 nm were purchased from Janssen Life Sciences Products (Piscataway, NJ). The gold colloid preparations had no overlap of particle distributions. The gold particles were coated with affinity-purified antibodies using a procedure modified from Roth (40) and Slot and Geuze (43). The antibodies were dialyzed overnight against 10 mM phosphate buffer with 0.05% sodium azide pH 8.3, a pH slightly greater than the isoelectric point of the antibody. The antibodies, centrifuged at 100,000 g for 60 min to remove aggregates, were added to the colloidal gold suspension.

The lowest concentration of antibody which fully protects the colloidal gold particles from precipitating in a high ionic strength solution plus 10% was chosen for coating the gold. The stock colloidal gold solution was adjusted to pH 8.3 with 0.2 M K_2CO_3 and the antibody in 10 mM phosphate buffer of the same pH was added dropwise to the colloidal gold solution, over a 30-s period, with gentle stirring. After 5 min of stirring it was determined if an aliquot was fully protected against precipitation by 1 M NaCl. If so, 0.3 ml of 5% polyethylene glycol (15,000–20,000 mol wt), 2.0 ml of PBS solution containing 1.0 M NaCl, and 3.0 ml of PBS containing 1% BSA were added. Large aggregates were removed by centrifugation (20 min, 5,000 g). The supernatant was centrifuged for 45 min at 34,000 rpm for 5 nm gold or 25,000 rpm for 10 nm gold in a rotor (type 50; Beckman Instruments, Inc., Palo Alto, CA). The pellet was resuspended in PBS, 0.1% BSA, 0.05% Na azide pH 8.3, applied to a 10%–35% glycerol gradient in PBS with 0.1% BSA, and centrifuged (45 min at 125,000 g for 5 nm gold or 50,000 g for 10 nm gold; SW-41 Ti rotor; Beckman Instruments, Inc.). The upper one-third to one-half of the gradient contained antibodies coupled to the single gold particles as determined by transmission electron microscopy. The fractions from the gradient containing antibody coupled to single gold particles were washed by centrifugation in PBS, 0.1% BSA, 0.05% Na azide
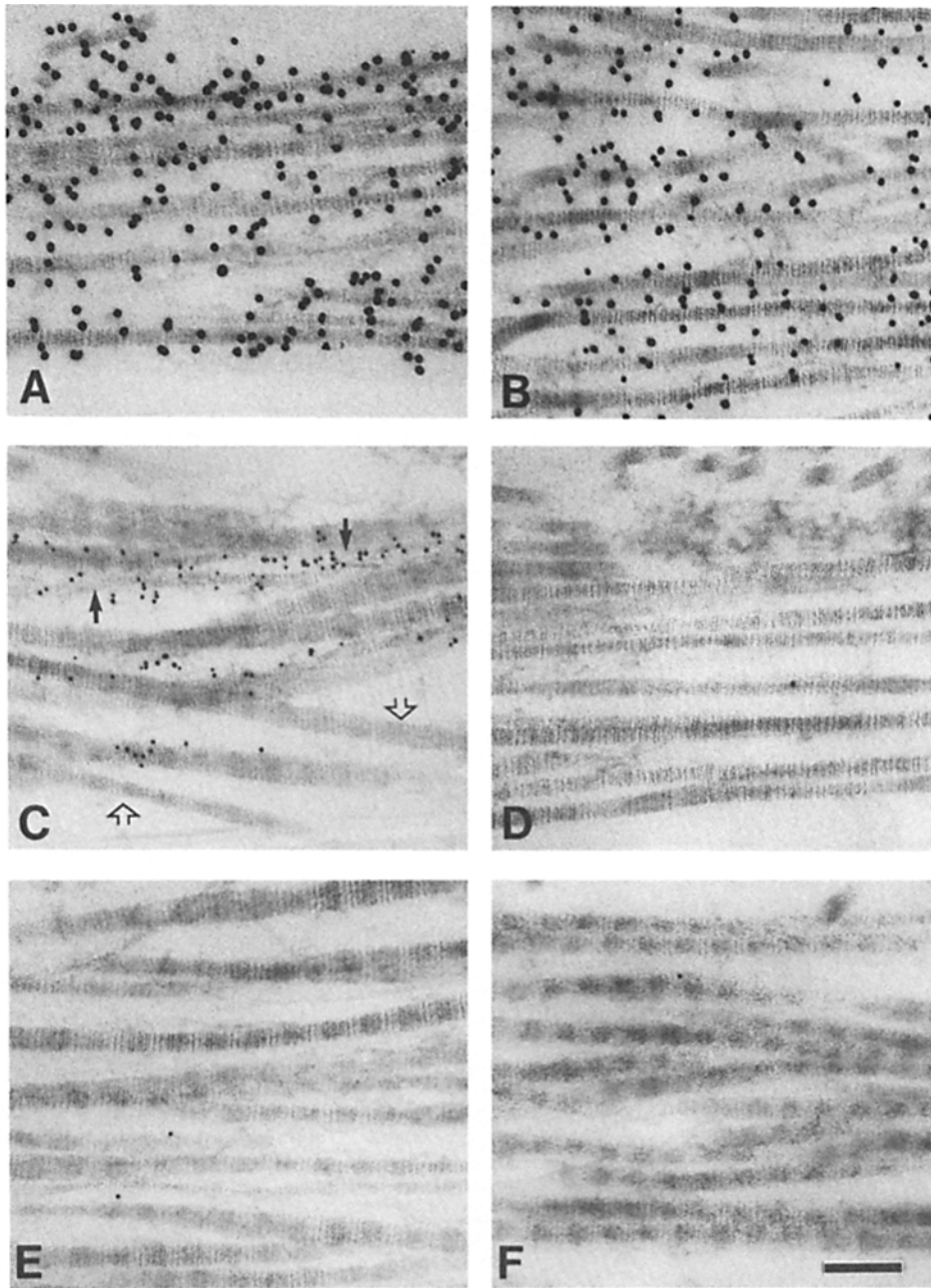
Figure 1. Localization of collagen types I and V in the lathyritic cornea pretreated at 4°C or 37°C. Chicken embryos were made lathyritic beginning on day 8 of development by the in ovo administration of βAPN. The corneas were dissected on day 11 and cryostat sections were cut. Sections from the same cornea were incubated for 15 h at either 4°C, a condition where fibril structure is partially disrupted (*A*, *C*, and *E*) or 37°C, a condition where fibril structure is maintained (*B*, *D*, and *F*). The sections were fixed in paraformaldehyde and incubated with monoclonal antibodies coupled directly to either 5 or 10 nm colloidal gold particles. *A* and *B* were incubated with monoclonal antibodies against type I collagen coupled to 10 nm gold; *C* and *D* were incubated with monoclonal antibodies against type V collagen coupled to 5 nm gold; and *E* and *F* were incubated with monoclonal antibodies against type IV collagen coupled to 5 nm gold. (*A* and *B*) The anti–type I monoclonal antibodies label all of the fibrils present. (*C*) The anti–type V monoclonal antibodies label only those lathyritic fibrils whose structure has been partially disrupted by the pretreatment with cold PBS (*solid arrows*). Nondisrupted fibrils, presumably formed before the inhibition of cross-linking (*open arrows*) are not labeled by the antibodies against type V collagen. (*D*) All of the fibrils maintain their structure at 37°C and there is no labeling of the fibrils with antibodies against type V collagen. (*E* and *F*) The antibodies against type IV collagen, which is normally confined to basement membranes, show no reactivity. Bar, 100 nm.

pH 7.4 (as described above). The washed fractions were diluted to a concentration corresponding to 0.6 OD$_{600}$ unit and stored.

Results

Monoclonal antibodies against collagen types I and V were used individually or simultaneously in a series of single- and double-label immunoelectron microscopic experiments.

Both normal 11-d chick embryo corneas or those made lathyritic were used. In single-label experiments, all the striated collagen fibrils of tissue sections labeled with gold-conjugated antibodies against type I collagen were decorated. The labeling of the fibrils was identical regardless of whether normal corneas (data not shown) or lathyritic corneas after pretreatment in PBS at either 37°C, a condition in which fibril structure is maintained or 4°C, a condition in
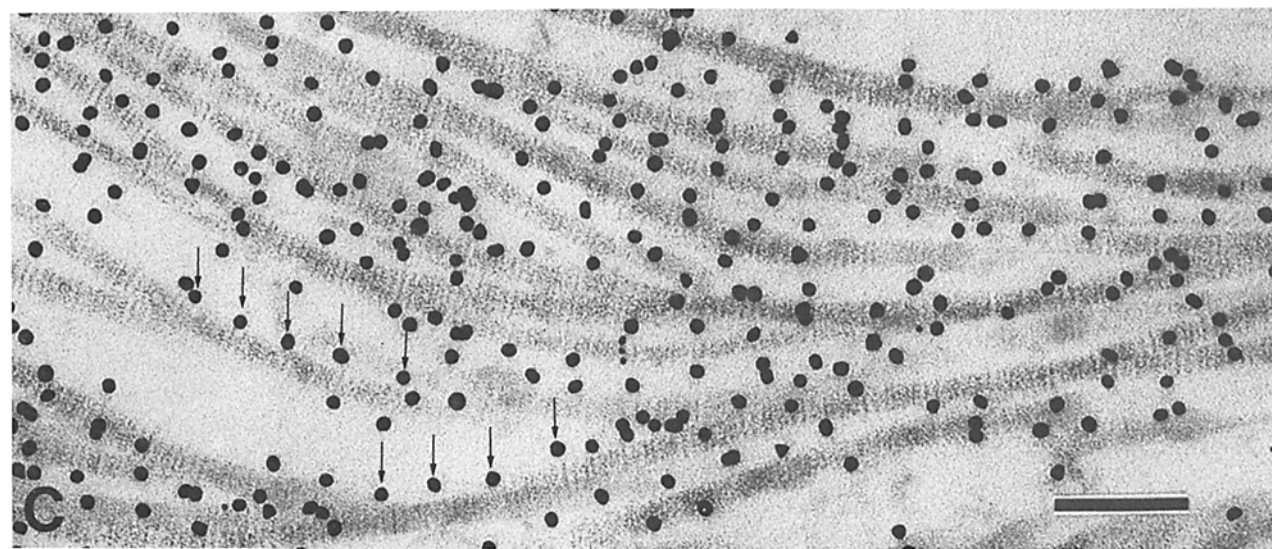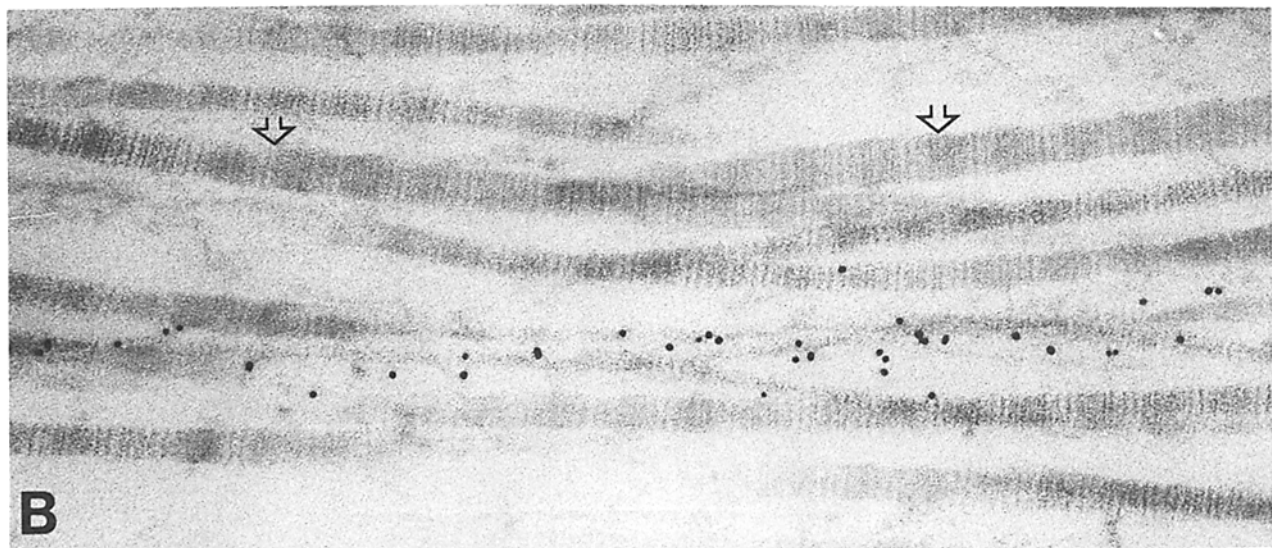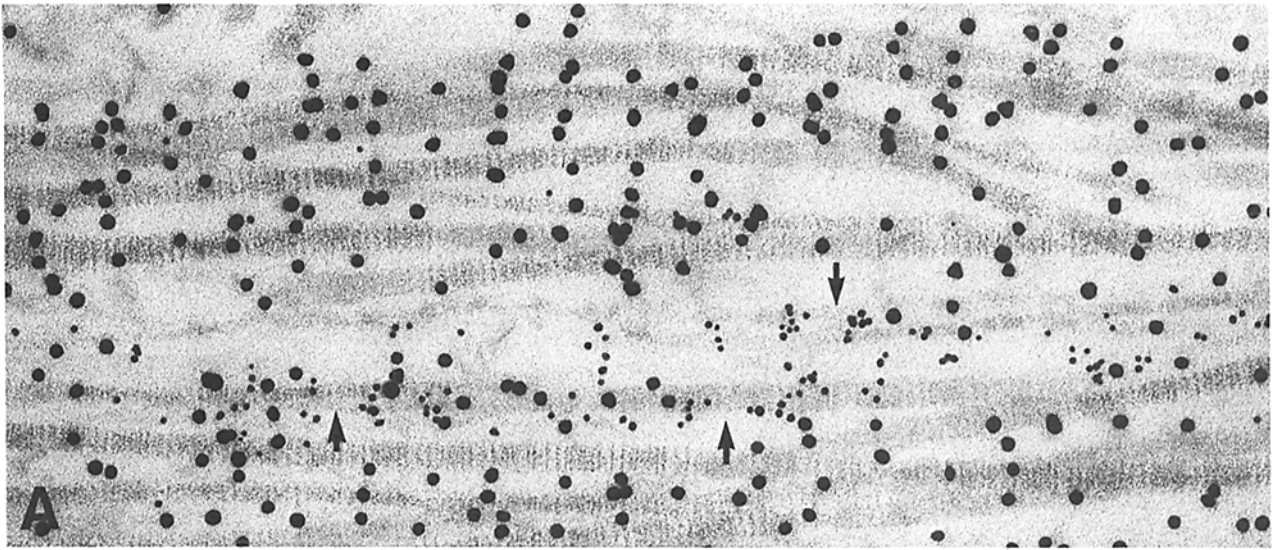
Figure 2. Colocalization of collagen types I and V in the lathyritic cornea pretreated at 4°C. Sections from lathyritic corneas were incubated in PBS for 15 h at 4°C, a condition where fibril structure is partially disrupted, fixed in paraformaldehyde, and incubated simultaneously with two different monoclonal antibodies coupled directly to either 5 or 10 nm colloidal gold particles. *A* was incubated with monoclonal antibodies against type I collagen coupled to 10 nm gold and against type V collagen coupled to 5 nm gold particles; *B* was incubated

which noncross-linked fibril structure is disrupted, were studied (Fig. 1, A and B). When antibodies specific for type V collagen were used to label normal (data not shown) or lathyritic cornea sections pretreated in PBS at 37°C, no reactivity was observed (Fig. 1 D). However, when lathyritic corneal sections were pretreated for 12 h at 4°C in PBS, the fibrils that had become partially dissociated were now heavily labeled with the anti–type V collagen antibodies (Fig. 1 C). The fibrils that maintained their structural integrity remained unlabeled presumably because they had been formed before administration of the lathyrogen. In control preparations reacted with antibodies directed against type IV collagen, a type thought to be restricted to basement membranes, the striated stromal fibrils were unreactive under all conditions (Fig. 1, E and F).

To localize simultaneously collagen type I and type V, a series of double-label experiments were performed. In these experiments, lathyritic corneal sections were subjected to pretreatment in PBS at 4°C for 12 h. The sections were then double labeled with anti–type I monoclonal antibodies conjugated to 10 nm gold particles and anti–type V monoclonal antibodies coupled to 5 nm gold particles (Fig. 2 A). As can be seen in Fig. 2 A, all fibrils were labeled with the anti–type I collagen antibody coupled to 10 nm gold particles. However, only those fibrils whose structure had been partially disrupted by the pretreatment at 4°C also demonstrated labeling with the 5 nm particles coupled to the type V collagen antibody. The codistribution of both labels on such fibrils indicates that they are composed of both type I and type V collagen molecules.

As a control for possible nonspecific interactions between the different size colloidal gold particles, double-label experiments were done with one size gold particle coupled to antibodies against either type I or type V collagen and the other size gold particle coupled to an antibody against type IV collagen. In such combinations only the antibodies against the type V (Fig. 2 B) or type I (Fig. 2 C) collagen labeled the stromal fibrils, so the labeling appeared identical to that seen in the single label experiments with antibodies against types I or V collagen. As a further control for these double-label experiments, sections were examined in which both 5 and 10 nm gold particles were coupled to antibodies against type IV collagen. In these cases, the collagen fibrils had essentially no label (data not shown).

Double-label immunoelectron microscopic localization of collagen types I and V also was performed on lathyritic corneas pretreated at 37°C (Fig. 3, A, C, and E) and on normal corneas (Fig. 3, B, D, and F). The results confirm the single-label studies. The fibrils labeled with the antibodies against type I collagen, but demonstrated, little, if any reactivity for

type V[2] (Fig. 3, A and B). Controls also were performed in which one of the antibodies was replaced with anti-type IV collagen antibody (Fig. 3, C–F). When these different combinations were used on either normal or lathyritic corneas pretreated at 37°C, the only fibrillar labeling was for type I collagen.

To characterize further the effect of low temperature on the structure of lathyritic collagen fibrils, we determined the time course of unmasking of the epitopes of type V collagen. Adjacent sections from the same lathyritic cornea were pretreated at 4°C in PBS for 9, 12, 15, and 18 h followed by double-label immunolocalization of collagen types I and V. At all time points, all fibrils labeled with the antibody against type I collagen. However, reactivity for type V collagen could not be conclusively detected until after 12 h of pretreatment (Fig. 4 A). In sections pretreated for longer periods of time there was a progressive increase in the amount of label against type V collagen (Fig. 4, B and C). The increase in the labeling was correlated with an increase in the number and extent of the dissociated collagen fibrils.

To exclude the possibility that the secondary corneal stroma might have two populations of collagen fibrils, one containing type I collagen and heterotypic fibrils containing collagen types I and V, very lathyritic corneas were dissociated at 4°C and labeled with antibodies against either type I or type V collagen (Fig. 5). In these experiments all of the fibrils were labeled with antibody against type I collagen (Fig. 5 A) and most of the fibrils were labeled with antibodies specific for type V collagen (Fig. 5 B). The pretreatment protocol used with these very lathyritic fibrils dissociated all susceptible fibrils. A small proportion of the fibrils did not label with antibody against type V collagen presumably because they were formed before the inhibition of covalent cross-linking.

To determine whether, within these fibrils the type V collagen was arranged in the "quarter-staggered" array, the distribution of the gold particles was determined from micrographs similar to those presented in Figs. 1 C and 2 B. As can be seen in Fig. 6 B, the label for type V occurred with a clear periodicity of 65 nm. The relative frequency distribution of these measurements were nearly identical to those determined for type I collagen (Fig. 6 A). This periodic arrangement of type V collagen provides evidence that the type V molecule can fit in a fibrillar packing arrangement along with type I collagen.

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2. The labeling for type V collagen in normal and lathyritic corneas pretreated at 37°C was often slightly above the type IV control level. This suggests that a small portion of the type V collagen molecules may be exposed on a fibril surface, however, there was never enough label to conclude this with certainty.

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with monoclonal antibodies against type V collagen coupled to 5 nm gold and against type IV coupled to 10 nm colloidal gold; and C was incubated with monoclonal antibodies against type I coupled to 10 nm particles and antibodies against type IV collagen coupled to 5 nm gold. (A) Both collagen types I and V are found within a single fibril as demonstrated by the decoration of fibrils with both 5 and 10 nm gold particles. Most of the fibrils are labeled with antibodies against type I collagen coupled to 10 nm gold, while only those fibrils whose structure has been partially disrupted by the pretreatment with cold PBS are labeled with the antibodies against type V collagen coupled to 5 nm gold (arrows). In some cases the fibril is so dissociated that the labeled structure is difficult to identify. (B) The compact fibrils (open arrowheads) are not decorated with 5 nm gold particles coupled to anti–type V collagen. Those fibrils formed in the presence of βAPN, and therefore not fully cross-linked, have been partially dissociated by the cold treatment, and are labeled with antibodies specific for type V collagen. Note the absence of 10 nm gold particles conjugated to anti–type IV collagen. (C) All fibrils are labeled by the antibodies against type I collagen. Note the absence of 5 nm gold particles which were conjugated to anti–type IV collagen. The periodicity of collagen type I (small arrows) is readily apparent in this panel. Bar, 100 nm.
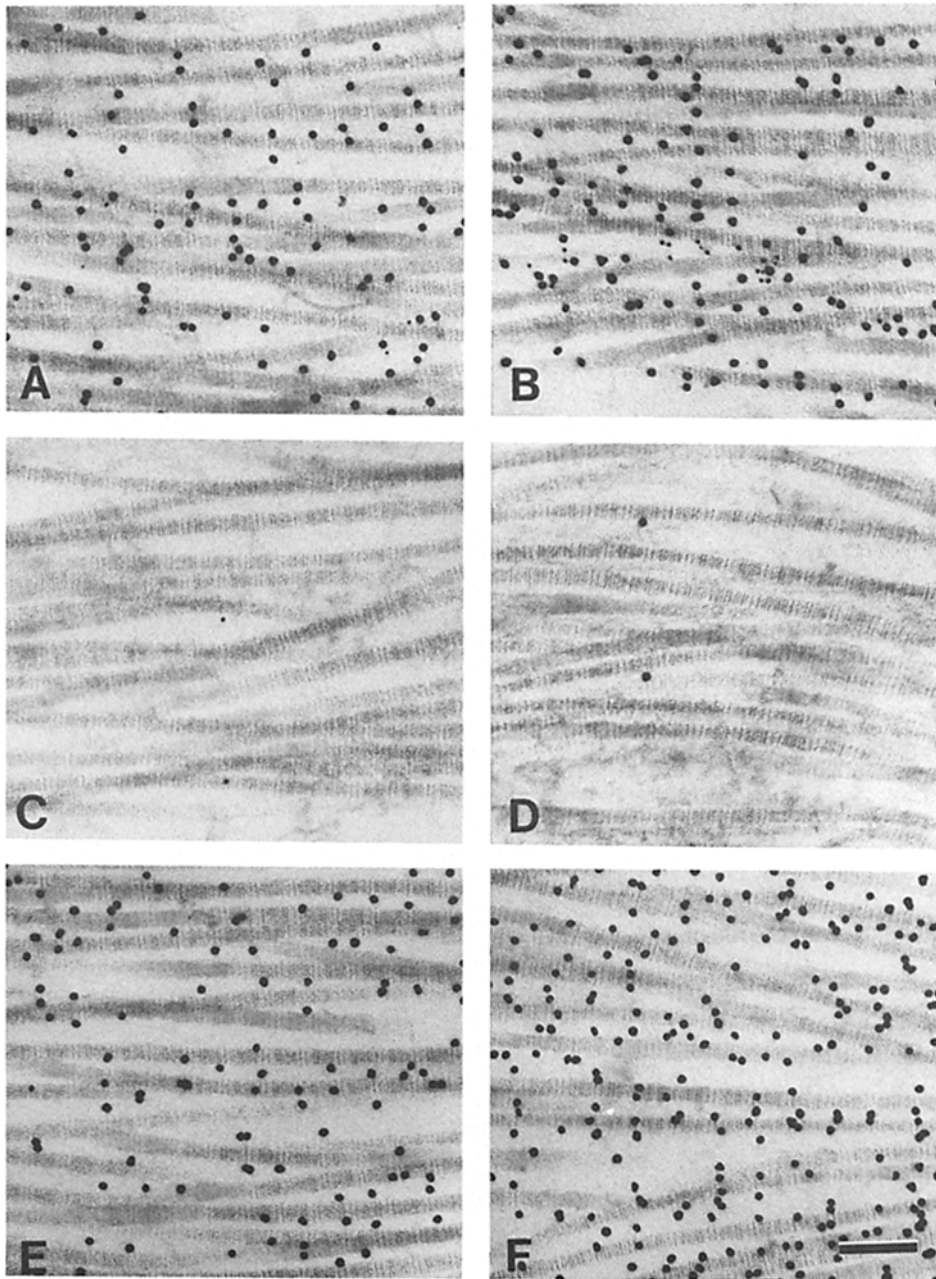
Figure 3. Colocalization of collagen types I and V in the lathyritic cornea pretreated at 37°C and nonlathyritic corneas. Sections from lathyritic corneas (*A, C,* and *E*) or nonlathyritic corneas (*B, D,* and *F*) were incubated in PBS for 15 h at 37°C. The sections were fixed and incubated simultaneously with two different monoclonal antibodies coupled directly to either 5 or 10 nm colloidal gold particles. *A* and *B* were incubated with monoclonal antibodies against type I collagen coupled to 10 nm gold and against type V collagen coupled to 5 nm gold particles; *C* and *D* were incubated with monoclonal antibodies against type V collagen coupled to 5 nm gold and against type IV coupled to 10 nm colloidal gold; and *E* and *F* were incubated with monoclonal antibodies against type I coupled to 10 nm particles and antibodies against type IV collagen coupled to 5 nm gold. In nonlathyritic corneas and in lathyritic tissues pretreated at 37°C, collagen fibril structure is maintained and therefore type V collagen is not labeled. (*A* and *B*) Most of the fibrils are labeled with antibodies against type I collagen coupled to 10 nm gold. Note the relative absence of 5 nm gold particles coupled to type V antibodies. It is a common observation that the type V label is slightly above that seen for the type IV control. (*C* and *D*) The compact fibrils are not decorated with 5 nm gold particles coupled to anti–type V collagen or 10 nm gold particles coupled to anti–type IV antibodies. (*E* and *F*) All fibrils are labeled by the antibodies against type I collagen. Note the absence of 5 nm gold particles which were conjugated to anti–type IV collagen. Bar, 100 nm.

In summary, these data indicate that collagen types I and V are present within the same fibril. A staggered relationship of collagen type I with type V within these heterotypic fibrils results in masking of the helical determinants recognized by the two different monoclonal antibodies against type V collagen.

Discussion

The data presented here represent the first direct demonstration that collagen fibrils in situ are composed of more than one collagen type. We have established by double-label immunoelectron microscopy using collagen type specific monoclonal antibodies directly coupled to different size colloidal gold particles that in the avian cornea, both collagen types I and V are present within the same fibril. Determination of this colocalization of type V collagen with type I collagen required pretreatment of the tissue to partially disrupt fibril structure, indicating that the epitopes recognized by the type
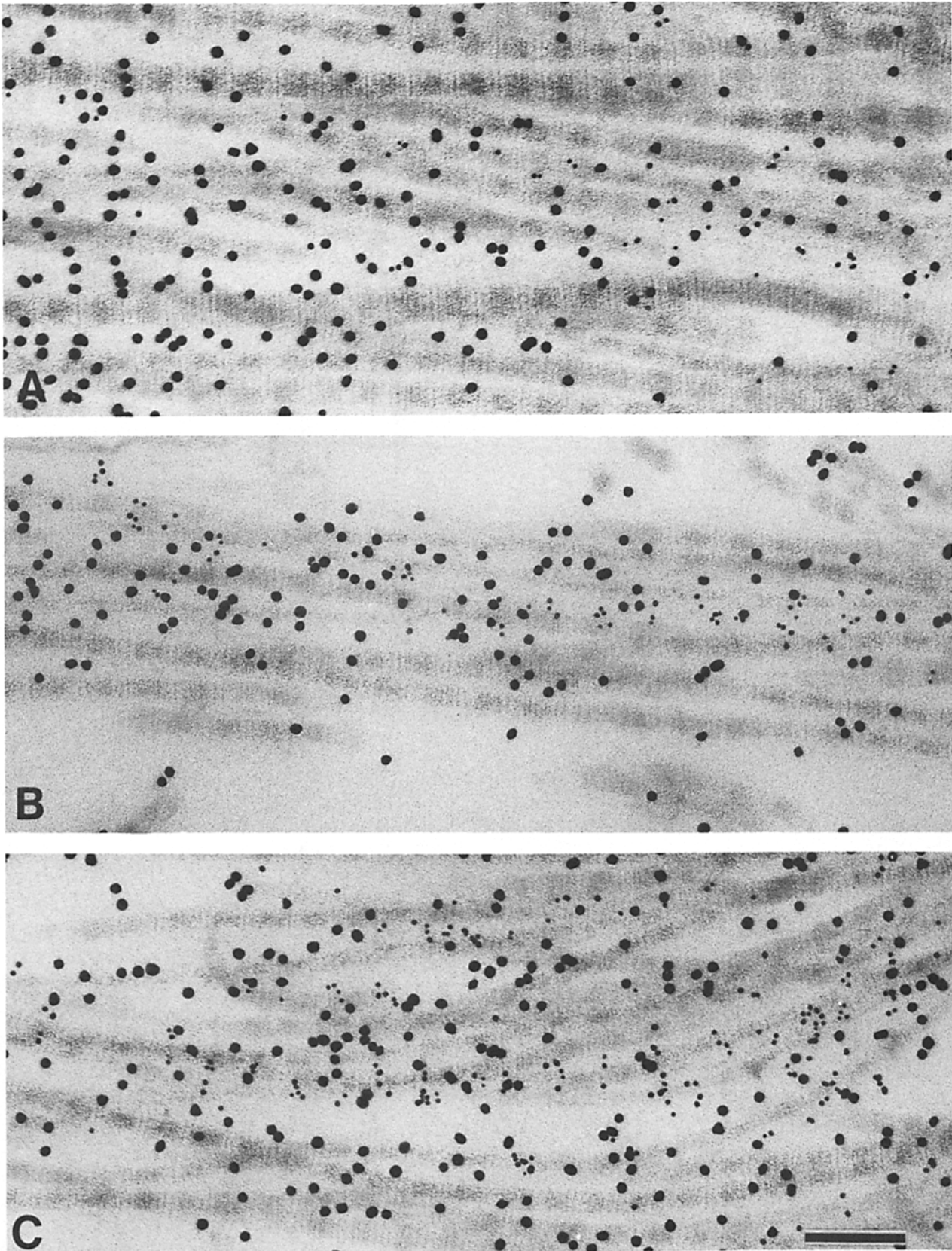
Figure 4. The time course of colocalization of collagen types I and V in the lathyritic cornea pretreated at 4°C. Chicken embryos were made lathyritic beginning on day 8 by the in ovo administration of βAPN and dissected on day 11 of development. Sections from the same cornea were pretreated in PBS at 4°C for 12 (*A*), 15 (*B*), or 18 (*C*) h, a procedure that progressively disrupts fibril structure. Double immunochemical–labeling experiments were done to localize collagens type I and V simultaneously. Sections were incubated simultaneously with monoclonal antibodies against type I collagen coupled to 10 nm gold and against type V collagen coupled to 5 nm gold particles. Both collagen types I and V are found within a single fibril as demonstrated by the decoration of fibrils with both 5 and 10 nm gold particles. Most of the fibrils are labeled with antibodies against type I collagen coupled to 10 nm gold while only those fibrils whose structure has been partially disrupted by the pretreatment with cold PBS are labeled with the antibodies against type V collagen coupled to 5 nm gold. The amount of type V label increases with time in the cold and is associated with an increase in number and extent of dissociated fibrils. Bar, 100 nm.
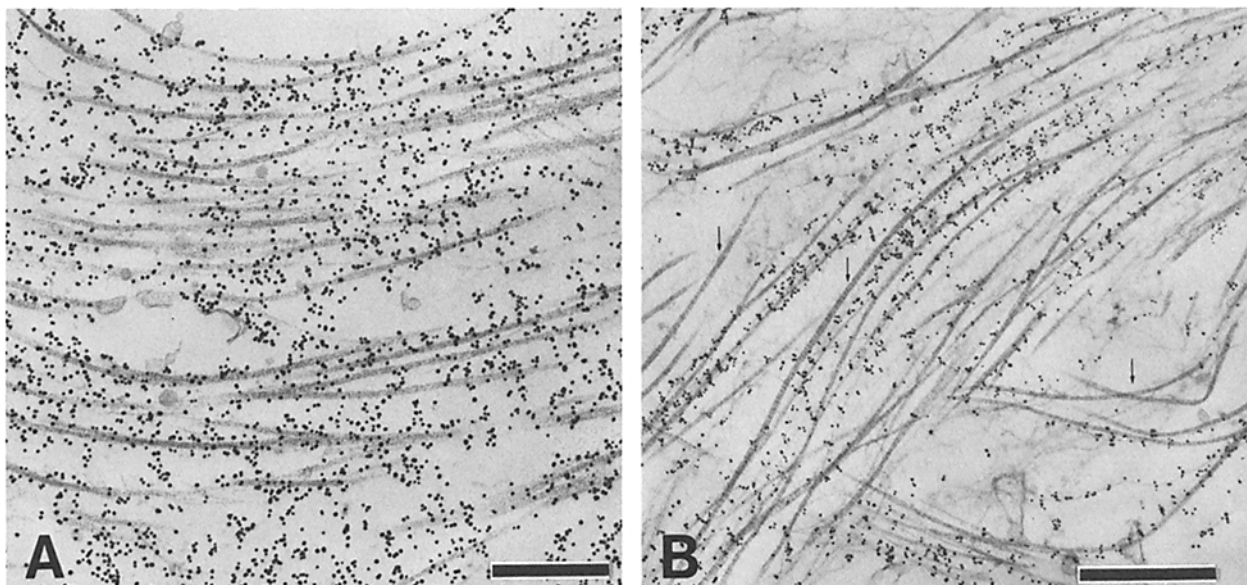
Figure 5. Heterotypic fibrils predominate in the secondary stroma. Lathyritic 11-d corneas were dissected, cryostat sections were cut, and the sections were incubated for 18 h at 4°C to disrupt all susceptible fibrils. The pretreated sections were fixed and incubated with monoclonal antibodies coupled to 10 nm colloidal gold particles. (*A*) The anti–type I monoclonal antibodies label all of the fibrils present. (*B*) The anti–type V monoclonal antibodies label most of the fibrils present. A few nondisrupted fibrils, presumably formed before the inhibition of cross-linking (*arrows*), are not labeled by the antibodies against type V collagen. The labeling of most of the fibrils in both fields indicates that most, if not all, corneal fibrils are heterotypic. Bar, 500 nm.

V collagen–specific monoclonal antibodies are unavailable to the antibodies within an intact collagen fibril.

The temperature manipulation of lathyritic fibrils was used to partially disrupt and therefore "unmask" the type V epitopes. Types I and V collagen molecules are assembled as heterotypic fibrils with the type I molecules blocking the type V epitopes, helical determinants on the type V molecule. Collagen fibrils with a normal complement of covalent intermolecular cross-links have a stable structure over a wide range of physiological temperatures. Lathyritic fibrils, deficient in intermolecular cross-links, are stable at 37°C but at 0–4°C the fibril dissociates exposing molecules within the fibril (27, 28, 48). As this occurs the epitopes on the type V collagen molecule become unmasked.

The labeling of collagen fibrils with antibodies against type V collagen requires the disruption of fibril structure. The degree of fibril disruption observed is dependent on the method used to disrupt fibril structure (lathyrism, dilute acid, collagenase) and the time course and/or concentration used for disruption. Morphologically, fibrils labeled with antibodies against type V collagen vary from no discernable change in fibril structure, to fibrils with a loss of characteristic striations, to thin filaments with no periodicity. This is not dependent on the stage of development but rather on the conditions and time used to disrupt fibril structure.

As a method to dissect collagen fibrils, lathyrism offers several other advantages. Within a tissue, it produces at least two distinct populations of fibrils: those that are highly cross-linked, presumably due to their early deposition, and those that do not have a full complement of cross-links. Thus, the highly cross-linked collagen can serve as internal controls for the ones whose structure can be thermally manipulated. Secondly, the unmasking by pretreatment at 4°C is almost totally reversible when the sections are returned to 37°C (13, 30; unpublished observations). Finally, adjacent sections treated at 4°C versus those treated at 37°C serve as controls for one another.

We used 11-d corneas in this study rather than 17-d corneas which we had used previously (5). The younger corneas were chosen for two reasons: there is a larger proportion of lathyritic fibrils sensitive to temperature manipulation in the younger corneas; and the younger corneas have not compacted and therefore there is more space around fibril bundles so the penetration of antibody–gold conjugates does not present a problem. We found no differences in the secondary corneal stroma in these two developmental stages.

Like most dense stromal connective tissues, the cornea contains predominantly type I collagen (20). However, unlike other tissues containing predominantly type I collagen, the corneal fibrils have uniformly small diameters (~25 nm). This unique morphology may be related to the fact that in several species, it has been shown that the corneal stroma contains significantly more type V collagen than do other tissues whose stromal matrix collagen is predominantly type I (8, 10, 11, 16, 23, 47, 50). Immunohistochemical studies have demonstrated that type V collagen is distributed uniformly throughout the corneal stroma as an interstitial collagen (5, 13, 30–32, 49). These biochemical and immunohistochemical observations suggest that type V collagen may interact with type I collagen to regulate corneal fibril diameter. The current demonstration that the two collagen types physically interact, and that type V collagen is arranged in the quarter-staggered array typical of fibrillar collagens, further strengthens this argument.
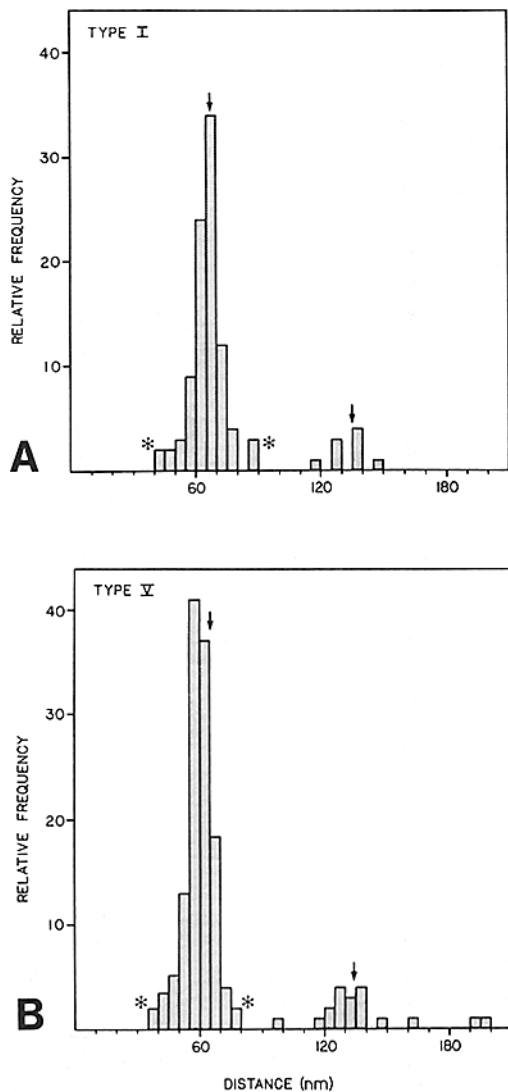
Figure 6. Periodicity of monoclonal antibody–labeled collagen types I and V. The periodicity of collagen type I and type V was determined by measuring the distance between adjacent gold particles on well-defined collagen fibrils. The measurements were done from micrographs similar to Fig. 1, *A–C* and Fig. 2, *B* and *C* and were normalized to the 67-nm macroperiod of the type I collagen fibril. (*A*) The relative frequency distribution of type I collagen measurements is shown. As expected, labeling with the monoclonal antibody against type I collagen demonstrates a clear 67-nm periodicity. (*B*) The relative frequency distribution of type V collagen measurements is presented. The labeling of type V collagen with monoclonal antibodies against type V collagen exhibits a periodicity identical to that of type I collagen. The arrows indicate the macroperiod of type I collagen and two times the macroperiod. The peaks bounded by the asterisk have a range of 47–86 nm and a mean of 66 ± 7.5 nm (±SD) for type I collagen while for type V collagen the range is 39–79 nm and the mean is 65 ± 7.5 nm (±SD).

There are a number of ways in which a quantitatively minor collagen type could function to regulate fibril diameter. Type V collagen may inhibit the lateral growth of the corneal fibrils because of its longer helix (42) and/or because of the persistence of a terminal globular domain (6, 11) even after assembly into a fibril. These features may permit the co-assembly of type V collagen with type I collagen only until a certain "critical" type V concentration is reached, after which the continued addition of type I collagen is inhibited by the 'nonfitting' type V molecule. Another possibility is that type V collagen forms thin core filaments which serve as nucleation sites for type I collagen assembly. The presence of more nucleation sites for a given quantity of type I collagen might result in smaller fibril diameters than in a tissue where there were fewer nucleation sites for the same amount of type I collagen. Further elucidation of the physical arrangement of type I and type V within fibrils is required to distinguish between these and other possible regulatory mechanisms.

In vitro studies have demonstrated that type V collagen interacts (1; Birk, D. E., J. M. Fitch, J. P. Babiarz, and T. F. Linsenmayer, manuscript in preparation) or copolymerizes (Birk, D. E., J. M. Fitch, J. P. Babiarz, and T. F. Linsenmayer, manuscript in preparation) with type I collagen to produce fibrils with smaller diameters. Increasing the amount of type V collagen available progressively decreases the mean fibril diameter. These studies along with other studies involving the copolymerization of collagen types I and III in vitro (25) implicate heterotypic assembly as a general regulatory mechanism in the control of fibril structure. In vivo immunochemical localization studies have shown that collagen types I and II (21, 30), types II and IX (39), and types I and III (24) are possibly present as heterotypic fibrils. A covalent peptide of collagen types I and III was isolated which further supports this conclusion (22). We believe it likely that the various collagen types present within a tissue are responsible, in a major way, for the control of tissue architecture. For example, type I collagen generally forms thicker fibrils than types II and III collagen both in tissues (37) and in reassembly studies in vitro (2, 25). Type V collagen has the ability to form thin filaments with no apparent periodicity (6) or to interact with type I collagen as striated fibrils (1; Birk, D. E., J. M. Fitch, J. P. Babiarz, and T. F. Linsenmayer, manuscript in preparation). With this in mind it is possible that varying the major collagen type present and/or modulating the proportions of the various collagen types being coassembled a large number of fibril and tissue architectures would be possible.

We have shown that collagen types I and V copolymerize during corneal fibrillogenesis. There are a number of possible ways in which these two distinct collagen types could be arranged structurally to form a heterotypic fibril. For example, type V collagen could form a core filament and type I collagen molecules polymerize along these preformed fibrils. Another possibility is that type I and type V collagen could form as alternating layers or some other patterned manner. Also, there could be a dispersed incorporation of type V collagen with type I during polymerization either as individual molecules or as some intermediate subassembly. Finally, type V collagen might even be present on the surface of a type I fibril, but have specific epitopes on the molecule which are unavailable. At present, the data can not definitely rule out any of these or other possibilities.

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