Organization of Collagen Types I and V in the Embryonic Chicken Cornea

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The distribution and organization of type I and type V collagens were studied in the embryonic chicken cornea using anti-collagen, type specific, monoclonal antibodies and immunoelectron microscopy. These studies were performed on lathyritic 17-day corneas treated at 4°C or 37°C. At the lower temperature, collagen fibril structure is disrupted; at the higher temperature, normal fibril structure is maintained. Corneas from non-lathyritic 17-day chick embryos, reacted at the two different temperatures, were studied for comparison. In Bowman's membrane, the thin (20 nm) fibrils were labelled by antibodies against both type I and type V collagen under all conditions studied. In the corneal stroma, the striated collagen fibrils (25 nm) were labelled with the antibodies against type I collagen in all cases, and by antibodies against type V collagen under conditions where fibril structure was disrupted. These results are consistent with the concept of heteropolymeric fibrils consisting of both type I and type V collagen molecules assembled such that the epitopes on the type V molecule are unavailable to antibody unless the fibrillar structure is disrupted. We suggest that the interaction of type V collagen with type I collagen may be responsible for the small diameter fibrils and the rigid control of fibril structure found in the cornea. Invest Ophthalmol Vis Sci 27:1470-1477, 1986

The size and organization of collagen fibrils within the extracellular matrix are important determinants of tissue structure and function. The embryonic chick corneal stroma is a well-organized collagenous matrix composed of uniform, small diameter collagen fibrils (25 nm) with a relatively constant interfibrillar spacing. The collagen fibrils are arranged as lamella with adjacent layers oriented at approximately 90° to one another.1-3 The rigid control of both collagen fibril micro-architecture and matrix macro-architecture is necessary for corneal transparency.

Type I collagen is the predominant collagen in the cornea,4 with type V collagen being a quantitatively minor component.5-9 However, the content of type V collagen in the cornea (5-20% of the total collagen) is high when compared to other tissues containing predominantly type I collagen such as sclera, tendon, bone, and dermis.8,10,11

Immunocytochemical studies of the chick cornea, using anti-collagen monoclonal antibodies, have demonstrated that type I collagen is distributed throughout Bowman's membrane and the corneal stroma.12-14 In contrast, in the normal chick cornea, type V collagen appeared to be confined to Bowman's membrane, a subepithelial, acellular structure containing small (20 nm) collagen fibrils.12-14 However, further analysis showed that the pretreatment of corneas with agents which disrupt collagen fibril structure (i.e. dilute acid), or which remove type I collagen (i.e. vertebrate collagenase) permitted the visualization of type V collagen throughout the corneal stroma.13-15

In addition, it was observed that the immersion of lathyritic corneas in cold buffer also made stromal type V collagen accessible for antibody binding.15 The in ovo administration of the lathyrogen β-aminopropionitrile (βAPN), which prevents the formation of crosslinks through the inhibition of lysyl oxidase16 results in the deposition of collagen fibrils whose structure is sensitive to temperature manipulations.17 In such tissues, normal fibril structure is maintained at 37°C while at 4°C fibrils dissociate.18,19 This alteration of fibril structure in the cold can be reversed when the tissue is returned to 37°C.17

When taken together, these studies indicate that type V collagen is masked within the corneal stroma due to some structural arrangement within fibrils. The unmasking of type V collagen by digestion with vertebrate...
collagenase, an enzyme which degrades type I but not type V collagen, further indicates that a fibrillar organization involving type I collagen is responsible for the masking of type V collagen. This suggests that, within extracellular matrices in situ, type I and type V collagens may be co-assembled within a single heteropolymeric fibril.

In this paper, we have employed collagen type-specific monoclonal antibodies and immunoelectron microscopy to directly visualize collagen type I and type V in the normal chick embryo cornea and lathyritic corneas in which fibril structure has been altered. The results support the presence of heteropolymeric fibrils composed of collagen types I and V.

Materials and Methods

White leghorn chick embryos were incubated at 37.5°C in a humidified atmosphere and staged according to Hamburger and Hamilton.20

Tissue Preparation

Chick embryos were made lathyritic beginning on day 14 by injection of β-aminopropionitrile fumerate (βAPN; Sigma Chemical Co., St. Louis, MO). Eggs were injected with 1 mg βAPN in 0.1 ml of phosphate buffered saline (PBS) on day 14, 1-2 mg on day 15, and 2 mg on day 16. On day 17 of development, normal or lathyritic corneas were carefully dissected with a ring containing the scleral ossicles. The corneas were washed in PBS, incubated in 1% dimethyl sulfoxide (DMSO) in PBS, and embedded in OCT compound (Tissue-Tek; Miles Scientific, Naperville, IL), all at room temperature. The embedded tissue was frozen in liquid nitrogen and 8-10 μm cryostat sections were cut perpendicular to the corneal surface. Sections were picked up onto albumin-coated microscope slides, air dried, washed with PBS, and incubated with PBS for approximately 16 hr at either 4°C or 37°C. Following this pre-incubation, the sections were routinely fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min at 4°C. This was followed by washing in PBS, incubation in sodium borohydride (50 mg/100 ml PBS) for 1 hr,21 and three washes in PBS, all done at 4°C.

Antibody Labelling

The sections were incubated with 2% normal goat serum for 2 hr at room temperature. The serum was decanted and the sections were incubated with the affinity purified monoclonal antibodies (35 μg/ml) at 4°C for 8-12 hr. The antibodies were isolated by protein A chromatography of the hybridoma culture supernatants. Monoclonal antibodies against type I and type V collagens were prepared and characterized as described previously.13,14,22 Two antibodies against type V collagen (V-DH2 and V-AB12) and two antibodies against type I collagen (IIB6 and I-BA1) were used. Affinity purified non-immune mouse IgG (Cappel Laboratories, Malvern, PA) was used in control preparations. The sections were washed five times over 1 hr with PBS, and processed for either immunofluorescence or immunoelectron microscopy.

In addition, a variety of controls and variations on this experimental protocol were performed. These included substitution of the primary antibody with non-immune mouse IgG, anti-type IV collagen antibodies, or normal mouse serum; two different well-characterized monoclonal antibodies were used against each collagen type. We have unmasked type V collagen using temperature manipulation of lathyritic tissue as well as pretreatment with dilute acetic acid, examined penetration into the cornea by looking at unwashed preparations and by serially sectioning heavily labelled blocks, used unfixed tissues and those fixed under a variety of conditions, and used a ferritin bridge method as well as indirect labeling with a secondary ferritin or gold conjugated antibody. These experiments and other combinations have been done over 40 different times.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy, the sections were incubated with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA), diluted at 1:150 to 1:250 with PBS. Incubation was for 4 hr at room temperature followed by five washes in PBS over 1 hr. The sections were examined and photographed using a Zeiss Photomicroscope III with epifluorescence optics. The film was developed and micrographs were printed under identical conditions to permit comparison.

Immunoelectron Microscopy

The corneal sections were prepared for immunoelectron microscopy using a modified ferritin bridge technique.23 After incubation with the primary antibody, the sections were incubated for 8-12 hr at 4°C, with goat anti-mouse IgG (anti H & L chain) gamma globulin fraction from whole antiserum (Jackson Laboratories, Avondale, PA), diluted 1:100 with PBS, followed by five washes in PBS over 1 hr. This was followed by incubation with affinity purified mouse anti-horse spleen ferritin (20 μg/ml; Jackson Laboratories, Avondale, PA) for 8-12 hr at 4°C. The tissues were washed and incubated in horse spleen ferritin (10 μg/ml PBS) for 8-12 hr at 4°C. The tissues were washed five times over 1 hr in PBS, one time with 0.1 M sodium cacodylate pH 7.4, and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate pH 7.4.
for 1 hr, washed with 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide in 0.1 M cacodylate pH 7.4, dehydrated in ethanol, cleared in propylene oxide, and embedded in epon-araldite. An epon-araldite filled Beem capsule was inverted on the section and polymerized at 68°C for 18-36 hr. The slides and Beem capsules were removed from the oven and allowed to cool. The slide was then placed on a 100°C hot plate for 10-15 sec and the Beem capsule “popped off” the slide with the tissue section.

Sections with a pale gold interference color were cut for electron microscopy. The sections were picked up onto mesh grids, stained with 2% aqueous uranyl acetate, examined, and photographed using either a Philips 300 or Philips 420 transmission electron microscope.

Results

As a control and a confirmation for the specificity of the immunoelectron microscopy studies, in each experiment parallel tissue sections were processed for immunofluorescence microscopy. Representative results are presented in Figure 1. Types I and V collagen were localized by indirect immunofluorescence microscopy in non-lathyritic corneas and in lathyritic corneas after pretreatment in PBS at 37°C and 4°C. In all cases, type I collagen was localized throughout Bowman’s membrane and the corneal stroma (Fig. 1). In non-lathyritic corneas and lathyritic corneas incubated at 37°C, type V collagen was localized to Bowman’s membrane with very little, if any, staining of the corneal stroma (Fig. 1). However, in lathyritic corneas pretreated at 4°C, type V collagen was now detected throughout the corneal stroma as well as Bowman’s membrane, giving a pattern identical to that of type I collagen (Fig. 1). In all cases, the control preparations, non-immune mouse IgG substituted for the primary antibody, showed no reactivity (Fig. 1).

The structure and relationship of collagen types I and V within Bowman’s membrane and the corneal stroma was then visualized by immunoelectron microscopy using a ferritin bridge technique. Bowman’s membrane of both non-lathyritic and lathyritic 17-day corneas treated at 4°C and 37°C was uniformly labelled with antibodies against type I and type V collagen (Fig. 2-4). Both collagen types were localized to the small diameter (20 nm) collagen fibrils comprising Bowman’s membrane. In no case did any of the antibodies against type V collagen label the corneal epithelial basement membrane. The control preparations were not reactive (Figs. 2-4).

In the corneal stroma, type I collagen was localized to the striated collagen fibrils in all preparations (Figs. 2-4). Labelled fibrils were found throughout the entire stroma. In the non-lathyritic corneas and lathyritic corneas pretreated at 37°C, the stromal fibrils were typically cross-striated; however, when lathyritic corneas were cooled in PBS to 4°C, numerous areas within the stroma were observed in which collagen fibrillar striations were not apparent or in which the fibrils had partially dissociated into thin strands. These fine fibrillar structures were derived from striated fibrils as evidenced by the continuity between striated and dissociated portions of fibrils in some regions (Fig. 4).

When reacted with anti-type V collagen antibodies, the stroma of non-lathyritic corneas or lathyritic corneas maintained at 37°C (Figs. 2, 3) showed labelling of the typical striated collagen fibrils which was only slightly greater than the level seen in the control preparations. The pretreatment of the non-lathyritic cornea at 4°C (data not shown) or 37°C gave identical results. When the lathyritic corneas were pretreated at 4°C prior to localization of type V, the corneal stroma was now heavily labelled by the anti-type V collagen antibodies (Fig. 4). The label was localized chiefly to collagen fibrils that appeared to be at least partially dissociated. The regions containing typical, compact, striated collagen fibrils were not as heavily labelled.

Discussion

Monoclonal antibodies against collagen types I and V, an immunoferritin technique, and electron microscopy were used to localize these collagens and to study their morphology within the embryonic chicken cornea.

Both collagen types are found within the small diameter fibrils of Bowman’s membrane, as well as the larger diameter, striated fibrils of the stroma. These results are the first direct evidence that collagen types I and V are deposited together within the same heteropolymeric fibril. This conclusion is based on the assumption that morphologically identical fibrils are also chemically homogeneous. Electron microscopic analysis of corneas doubly labelled with antibodies against type I and V collagen, now underway, should demonstrate unequivocally the spatial relationship between these collagen types.

Type V collagen could be detected within the small diameter fibrils (20 nm) of Bowman’s membrane without any pretreatment of the tissue. However, to detect immunohistochemically the type V collagen within the stroma, it was necessary to disrupt collagen fibril structure to “unmask” type V collagen. This was true for both of the anti-type V collagen monoclonal antibodies we used. As previously described, “unmasking” can be effected by pretreatment of tissue sections with dilute acid or vertebrate collagenase, or by the pre-incubation of lathyritic tissues in cold saline.12-15 These treatments are all known to disrupt collagen fibril structure, either by swelling/dissociating the fibrils, or by selectively re-
Fig. 1. Immunofluorescent localization of collagen types I and V. Normal chick embryos and those made lathyritic beginning on day 14 of incubation were dissected on day 17 and the corneas were prepared for immunocytochemical localization of collagen types I and V using type-specific monoclonal antibodies and a Rhodamine-conjugated goat anti-mouse IgG. Pre-treatment of lathyritic tissues in cold PBS (C, F, and I) disrupts type I fibril structure, while at 37°C (B, E, and H) fibril structure is maintained. The pre-incubation has no effect on the normal cornea (A, D, and G). In A, B, and C, the corneas were incubated with a monoclonal antibody against type I collagen; in D, E, and F, the corneas were incubated with an antibody against type V collagen; and in G, H, and I, the primary antibody was replaced with affinity purified non-immune mouse IgG. The corneal stroma is labelled (S) while Bowman’s membrane is indicated by the arrows.

moving the type I collagen. In lathyritic tissues, only those fibrils formed in the presence of βAPN, in this case during day 14–17 of development, would be susceptible to temperature manipulation. In the present series of immunoelectron microscopic studies, we employed both the dilute acetic acid pretreatments and the cold saline pretreatment of lathyritic corneas. The latter was chosen for routine use, since the preservation
Normal Cornea

Fig. 2. Localization of collagen types I and V in normal chick embryo corneas. Normal 17-day chick embryo corneas were dissected, and frozen sections were prepared. Collagen types were localized by immunoelectron microscopy with type specific monoclonal antibodies and a ferritin bridge method. A, C, and E are of Bowman’s membrane, while B, D, and F are from the corneal stroma. In A and B, the corneas were incubated with a monoclonal antibody against type I collagen; in C and D, the corneas were incubated with an antibody against type V collagen; and in E and F, the primary antibody was replaced with affinity purified non-immune mouse IgG. The epithelial basement membrane is indicated (*). Bar = 100 nm.

of ultrastructural detail was considerably better. We observed that good antibody labelling of fibrils for type V collagen can be achieved in fixed tissues if the fixation is performed after these “unmasking” procedures. If the order is reversed, and the tissues are fixed before the “unmasking” is performed, no type V collagen labelling is observed in the stroma. This is consistent with our interpretation that unmasking requires alterations in fibril structure. Prefixed fibrils apparently are unable to undergo these requisite changes.

The co-localization of type V with type I collagen within the corneal stroma suggests that one role for type V collagen may be in the regulation of collagen fibril structure. Matrices with less type V collagen, such as the sclera, contain fibrils with larger and more variable diameters.8 Bowman’s membrane, on the other hand, contains smaller diameter fibrils in which type V collagen is normally exposed (“unmasked”). It is possible that the smaller fibril diameters are due to the incorporation of a greater proportion of type V collagen into these heteropolymeric fibrils. The interaction of type V collagen with type I collagen within heterotypic fibrils may therefore, be responsible for the small, rigidly controlled diameters of the corneal fibrils.
Evidence exists that other collagens may interact in a similar manner. It has been shown in vitro that types I and III collagen can interact with one another to influence the diameter of fibril bundles. Also, cyanogen bromide peptides of collagen from certain tissues have been isolated which are composed of one peptide from the type I collagen and one peptide from the type III molecule. These data suggest the existence of heterotypic fibrils composed of type I collagen and type III collagen molecules. It seems likely that interstitial collagen is commonly deposited in heteropolymeric form.

Type V collagen also has been described in association with basement membranes and in the pericellular environment. Although we did not observe a similar pattern of distribution, it is possible that different investigators are identifying different molecules. Type V collagen is known to exist in at least three distinct molecular forms; \( \alpha 1(V)_2, \alpha 2(V) \), \( \alpha 1(V)_3 \), and \( \alpha 1(V)_2, \alpha 2(V), \alpha 3(V) \). Our antibodies were produced against the \( \alpha 1(V)_2, \alpha 2(V) \) form of the molecule, and are likely to be specific for it. Possibly, it is molecular diversity which has prompted
some of the considerable controversy surrounding the localization and function of type V collagen.

In one study, type V collagen has been localized to 12 nm fibrils in the subepithelial region of the amnion using immunoelectron microscopy. This, coupled with the observations that type V collagen is sometimes seen to be associated with basement membranes, suggests that another possible function of type V collagen, or at least one species of type V collagen, may be to act as a transitional molecule, anchoring basement membranes to the interstitium. In this case, the molecule would exist in situ as thin fibrils. Type V collagen can exist in this form, since purified type V collagen molecules can form thin fibrils (approximately 22 nm) in vitro in the absence of other collagens. The subepithelial localization of type V collagen to thin (20 nm) fibrils in Bowman’s membrane suggests that type V may serve this function in the cornea. But, even in this location, our data suggest that these thin fibrils also contain type I collagen. The diameter of the fibrils in this region may not be sufficient to mask the epitopes on type V collagen.
The presence of heteropolymeric collagen fibrils in which fibril structure is responsible for the masking of the type V epitopes and the variable chemistry of the collagen molecules themselves is consistent with a view of the extracellular matrix as being chemically and structurally heterogeneous. This heterogeneity may partially explain the diversity of extracellular matrices composed of a limited number of macromolecules.

Key words: collagen type I, collagen type V, cornea, monoclonal antibodies, lathyrism, immunoelectron microscopy

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