The Spatial Organization of Descemet's Membrane–associated Type IV Collagen in the Avian Cornea

John M. Fitch,* David E. Birk,† Cathy Linsenmayer,* and Thomas F. Linsenmayer*

*Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111; and †Department of Pathology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Abstract. The organization of type IV collagen in the unconventional basement membrane of the corneal endothelium (Descemet's membrane) was investigated in developing chicken embryos using anti–collagen mAbs. Both immunofluorescence histochecmetry and immunoelectron microscopy were performed. In mature embryos (>15 d of development), the type IV collagen of Descemet's membrane was present as an array of discrete aggregates of amorphous material at the interface between Descemet's membrane and the posterior corneal stroma. Immunoreactivity for type IV collagen was also observed in the posterior corneal stroma as irregular plaques of material with a morphology similar to that of the Descemet's membrane-associated aggregates. This arrangement of Descemet's membrane-associated type IV collagen developed from a subendothelial mat of type IV collagen-containing material. This mat, in which type IV collagen–specific immunoreactivity was always discontinuous, first appeared at the time a confluent endothelium was established, well before the onset of Descemet's membrane formation.

Immunoelectron microscopy of mature corneas revealed that the characteristic nodal matrix of Descemet's membrane itself was unreactive for type IV collagen, but was penetrated at intervals by projections of type IV collagen-containing material. These projections frequently appeared to contact cell processes from the underlying corneal endothelium. This spatial arrangement of type IV collagen suggests that it serves to suture the corneal endothelium/Descemet's membrane to the dense interfacial matrix of the posterior stroma.

Epithelia are separated from stromal connective tissues by basement membranes, which are sheets of extracellular matrix whose components are derived largely from the epithelial cells themselves. The molecular components of these matrices typically include a variety of glycoproteins (e.g., laminin, entactin), heparan sulfate proteoglycan, and type IV ("basement membrane") collagen (see references 18, 29, 55 for reviews). Whereas most of these components are found in basement membranes throughout an organism, components with a more restricted tissue distribution (e.g., references 33, 53) have been described. Moreover, regional diversity in the content or assembly of a variety of undefined antigens (e.g., 8, 20) and even of the "ubiquitous" molecular components of basement membranes (10, 21, 30, 50) have been observed. In the electron microscope, basement membranes typically appear as thin (∼50–100 nm) amorphous sheets of electron-dense lamina densa sandwiched between two thin electron lucent zones (lamina lucida interna and externa; see references 18, 29, 60 for reviews). But variations on this theme also exist. For example, in some basement membranes, the external electron-lucent layer is essentially absent; in others (e.g., Reichert's membrane and the anterior lens capsule) the electron-dense layer is thickened and has a filamentous morphology.

Among the more unique basement membranes is Descemet's membrane of the vertebrate cornea. This is a thick, dense, acellular matrix that separates the corneal endothelium from the overlying stroma. In sections viewed by EM, Descemet's membrane appears as a hexagonal array of electron-dense "nodes" embedded in an amorphous ground substance (16, 17, 22, 43, 47). The internodal separation is ∼100 nm; the nodes appear to be connected by a network of thin filaments. Unlike most basement membranes, Descemet's membrane progressively increases in thickness with age. A matrix with a similar structure can be deposited in vitro by isolated corneal endothelial cells (38, 48, 49).

The molecular composition of Descemet's membrane is controversial, and may be unique among the basement membranes. Biochemical studies on isolated Descemet's membrane and biosynthetic studies on cultured corneal endothelium have identified the usual basement membrane components, such as type IV collagen and laminin (1, 14, 28, 32), but many of these investigations and others (e.g., 46, 57) have also identified a variety of interstitial collagens and other macromolecules normally associated with stromal matrices. More recent studies have indicated that the major collagen of Descemet's membrane may be neither "basement membrane" nor interstitial, but endothelial collagen (EC), now termed type VIII (1, 2, 23, 24, 32). Many of the apparent

1. Abbreviation used in this paper: EC, endothelial collagen.
differences may be due to phenotypic modulation of protein expression by endothelial cells in vitro (25, 27), which may in turn mirror similar modulations observed in a variety of corneal endothelial pathologies (26, 61).

Immunohistochemical observations of corneal sections with antibodies against extracellular matrix components have also demonstrated an association of Descemet's membrane with interstitial collagens, type IV collagen, and several other basement membrane–specific macromolecules (15, 19, 31, 38, 48, 49, 51, 54, 59). Using a series of mAbs against chicken type IV collagen in immunofluorescence histochemistry, we have observed discontinuous immunoreactivity in the region of Descemet's membrane of corneas from mature chicken embryos (9) that could be resolved as an array of fine dots (36). This report describes the spatial organization of the Descemet's membrane–associated type IV collagen in mature corneas, its relationship to the nodal matrix of Descemet's membrane, and its appearance during development.

Materials and Methods

Tissue

White Leghorn chicken eggs were obtained from Spafas, Inc. (Norwich, CT) and incubated for 5–19 days at 38°C. Unfixed anterior eyes used for immunofluorescence histochemistry were removed in HBSS, soaked in 7-8% sucrose in PBS for 5–10 min, embedded in OCT (Miles Laboratories, Elkhart, IN), and frozen in liquid nitrogen. The blocks were stored at −20°C until use. Some tissue was fixed lightly (2–4% paraformaldehyde in PBS, 2–5 min) before being immersed in sucrose. No differences were seen between unfixed and lightly fixed material. 8-μm-thick frozen sections were mounted on 12-spot slides (Shandon Scientific Instruments, Sewickley, PA) coated with albumin or polylysine (mol wt ~250,000; Sigma Chemical Co., St. Louis, MO), dried for 2–4 h, and stored at −20°C.

Other tissue was prepared for sectioning in an ultracryostome (Reichert FC4; Reichert, Vienna, Austria). These corneas were fixed for 1 h with 2% paraformaldehyde containing periodate and lysine in phosphate buffer (PLP; see reference 42), or with 3.5% paraformaldehyde in PBS. They were then rinsed in PBS and infused in a graded series of buffered sucrose solutions, to 2.3 M sucrose, in PBS. Wedges of corneal tissue were then frozen on stubs in liquid freon cooled to liquid nitrogen temperatures, and stored in liquid nitrogen until used. Thick (0.5-1 μm) sections from these specimens, for use in immunofluorescence studies, were mounted on 12-spot slides coated with Cell-Tak adhesive (Biopolymers, Inc., Farmington, CT); 100-nm thin sections for use in immunoelectron microscopy (see below) were transferred to formvar-coated grids via a drop of 2.3 M sucrose, and stored at 4°C on 2% gelatin in PBS until used.

Antibodies

The production and characterization of type-specific mAbs against collagen types I (BA1, see 35), IV (IBA8, see 9; IBI12 and ID2, see 41), and X (AC9, see 52) have been described previously. In addition, another of our mAbs against chicken type I collagen (DD4) was frequently used. This antibody arose from the same fusion as did I-BA1, but recognizes an epitope at the other end of the triple helical molecule (unpublished observations). All of the antibodies used in this study have been tested for cross-reactivity to the other collagen types and are collagen type specific; different antibodies within each type-specific group recognize different epitopes on the triple helical molecule (see references cited above, and unpublished observations).

All antibodies were stored at 4°C. In the immunofluorescence experiments described here, the antibodies were used in the form of either undiluted supernatant from spent hybridoma cultures or ascites fluid diluted 1/300–1/500 with PBS. For coating gold colloidal particles for use in immunoelectron microscopy (see below), IgG was purified on a preparative protein A column (Bio-Rad Laboratories, Richmond, CA) using the Affi-Prep reagents and protocol, and was dialyzed into PBS and stored at 4°C until used.

Immunofluorescence Histochemistry

The pattern of anticolagen immunoreactivity in 8-μm frozen sections was revealed using an indirect immunofluorescence procedure described previously (9). Sections of unfixed material were blocked with 0.1% BSA in PBS for 10–30 min and then covered with a drop of the primary antibody for 4–6 h at room temperature (RT) or overnight at 4°C. Sections were lightly fixed (2–4% paraformaldehyde in PBS, 5–10 min) tissue were quenched for 30–60 min with 0.14 M Tris buffer containing 0.8% BSA and 0.12 M glycine, pH 7, 90, before their exposure to the primary antibodies. The slides were then washed thoroughly with PBS and incubated with a rhodamine-conjugated goat anti-mouse IgG second antibody (1 h, RT), washed in PBS, and mounted in glycerol/PBS (65-3). Essentially the same procedure was used to label 0.5–1-μm frozen sections for observation by immunofluorescence, except that the sections were quenched with PBS containing glycine (50 mM) and lysine (50 mM) and then 10% FCS in PBS.

In most experiments, several different mAbs against each collagen type were used, along with a mixture of all of the antibodies in each collagen type-specific group. The type IV collagen–specific mixture in particular gave an enhanced immunofluorescent signal, but without any change in the pattern of immunoreactivity in the posterior corneal region. Background immunofluorescence was assessed by the use, as a negative control, of the antibody against type X collagen (AC9), which is specific for hypertrophic cartilage.

To enhance further the intensity of the fluorescent signal, many of the sections received two rounds of reaction with the primary and secondary antibodies (36). In this procedure, 45-min incubations with primary and secondary antibodies were used in the second round.

In some experiments, the tissue sections were first pretreated with dilute acetic acid (0.2M HAc, 20 min, RT), or testicular hyaluronidase (type IV-S, Sigma Chemical Co.; 1 mg/ml in PBS, 30 min, 37°C) before the application of the primary antibody. The first procedure has been shown to expose epitopes masked by their fibrillar organization (34); the second, by matrix glycosoaminoglycans (58). We also used mixed glycosidases (Miles Laboratories, 5–10 mg/ml, 0.1 M PO4, pH 5.5, 15–30 min, 37°C) or elastase (Sigma Chemical Co., type II, 15–75 U/ml, 0.1 M PO4, pH 8.2, 15 min, 37°C) as unmasking reagents. In other experiments, potential masking by fibrillar organization was tested in another way by using corneal sections from embryos made lathyritic by the administration of β-aminopropionitrile during development, using a procedure described previously (11). Finally, sections digested with vertebrate interstitial collagenase (kindly provided by Dr. Karen Hasty and Dr. Carlo Mainardi, University of Tennessee School of Medicine, Memphis, TN) for 12–48 h as described previously (12) were examined for unmasking of type IV collagen by other collagen types susceptible to this protease.

Immunoelectron Microscopy

Both preembedding immunoelectron microscopy with 8-μm cryosections and postembedding immunoelectron microscopy with 100 nm ultracryosections, using monoclonal antibodies indirectly labeled with colloidal gold as well as directly-conjugated antibody colloidal gold probes (see below), were performed. The procedure for preembedding immunoelectron microscopy was as described previously (12). In brief, unfixed or lightly fixed thick (8 μm) cryosections were quenched BSA in Tris/glycine buffer, reacted with antibody-gold complexes, and washed extensively. In some experiments, the sections were incubated with vertebrate collagenase, as described (12), for 12–48 h before application of the antibodies. They were then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde and postfixed with 1.25% osmium tetroxide, dehydrated, and embedded in an Epon-araldite mixture. Blocks were removed from the surface of glass slides, sectioned, and observed with a Phillips CM-10 transmission electron microscope.

Postembedding immunoelectron microscopy on thin ultracryosections was performed using immunogold complexes in both directly as well as indirectly labeled procedures. For direct labeling of epitopes, thin ultracryosections on formvar-coated grids were first quenched in PBS containing glycine (50 mM) and lysine (50 mM) followed by 10% FCS in PBS. They were then immersed in dilution fluid (PBS containing 1% BSA, 1% gelatin, 0.1% Tween, 0.1% Triton X-100) before being incubated in antibody–gold complexes (diluted in dilution fluid) for 1-6 h (usually 5–6 h) at RT. The sections were immersed for 30 min in six changes of washing solution (PBS containing 0.1% Tween, 0.1% Triton), and then four times in H2O. They were then contrasted and protected following the method of Tokuyasu (56) by immersion in 0.15 M oxalic acid containing 2% uranyl acetate, pH 7, washing with water, and infusion with 1% methyl cellulose (25 ps, Sigma Chemical Co.) containing 0.2% uranyl acetate. Dried grids
Figure 1. Thick (8 μm) frozen sections of anterior eyes from 19-20-d chicken embryos reacted with mAbs for type IV (A, B, and D) or type X (C) collagen. Bar, (A) 100 μm; (B-D) 25 μm. In the cornea, immunoreactivity for type IV collagen is associated with the basement membranes of the corneal epithelium (A, small arrows) and endothelium (Descemet's membrane; large arrows in A), and in corneal nerves (A, arrowhead) within the corneal stroma (cs). In the posterior stroma (towards the endothelial surface), scattered plaques of immunoreactivity can be seen (faint specks in A; more prominent flecks in B and D). Elsewhere, type IV collagen-specific immunoreactivity is found in the basement membranes of the striated muscle and blood vessels of the iris (i) and in the thick lens capsule surrounding the lens (l). In the posterior cornea viewed at higher magnification (B), Descemet's membrane-associated immunoreactivity for type IV collagen can be resolved as a compact array (arrows) of fine punctate fluorescence ("dots"). Coarser flecks of immunofluorescence (e.g., arrowheads) in the overlying stroma (s) are also observed. These structures do not react with an irrelevant antibody against type X collagen (C). In corneas sectioned tangentially, approximately parallel to the endothelial surface, and reacted for type IV collagen (D), the array of dots is generally wider and more irregular, but the punctate pattern of immunofluorescence within this array is maintained.

were observed and photographed with a Philips CM-10 transmission electron microscope.

In the indirect labeling procedure, thin ultracyrosections were quenched, labeled with primary mAb, diluted in dilution fluid, for 1-3 h, RT, and then washed for 20 min in four changes of washing solution and once in dilution fluid. Bound antibody was then labeled for 3-4 h with either 10 or 15 nm colloidal gold particles coated with goat anti-mouse IgG (Janssen Pharmaceutica, Piscataway, NJ) diluted 1:20 with dilution fluid. The sections
were then rinsed for 30 min with washing solution (six changes) and processed as described above.

In some experiments, we attempted to expose potentially masked epitopes by treating the sections with enzymes (mixed glycosidases [Miles, 7 mg/ml in 20 mM Tris, 60 mM NaCl, 2 mM CaCl₂, pH 6.8, 15 min, 37°C], or testicular hyaluronidase [Sigma Chemical Co., IV-S, 0.1% in PBS, pH 7.3], and/or heparitinase [ICN Radiochemicals, 50 units/ml, in 50 mM Tris, 150 mM NaCl, 5 mM CaCl₂, pH 6.8]) at 37°C for 20-30 min/enzyme, or with 4 M guanidine (in 50 mM Tris, pH 7) for 5 min at RT. The unmasking procedure was performed after the quenching steps described above, and was followed by two to three rinses in PBS or Tris buffer and then dilution fluid before labeling with the antibody-gold complexes.

Figure 2. Topography of the endothelial surface of thick (8 µm) corneal sections at loci with a punctate pattern of immunofluorescence versus a linear pattern of immunofluorescence for type IV collagen. Bar (A), 10 µm. A shows the posterior region of a transverse section of a cornea containing adjacent regions with linear and punctate patterns of immunofluorescence. If this section was embedded in plastic, removed from the glass slide, and then sectioned perpendicular to the endothelial surface (as was the case for the sections in Fig. 4), thin sections through the linear (e.g., b) versus punctate (e.g., c) regions would appear as diagrammed in B and C. The region showing a linear pattern of fluorescence (B) would show an erect orientation of the endothelium (arrowhead) and Descemet's membrane. Sections through the region containing an array of dots (C) would show an endothelium (arrowhead) and Descemet's membrane that has "toppled" to varying extents. Thus, for thick (8 µm) frozen sections of corneas reacted for type IV collagen, in regions where the posterior surface of the cornea has topped, one is viewing the pattern of type IV collagen-specific immunofluorescence essentially en face (horizontal to the endothelial surface), showing a discontinuous array of punctate immunofluorescence; in untopped (erect) regions, the array is viewed edge-on, with the superimposed fluorescent dots giving an apparently continuous linear pattern of immunofluorescence. s, stroma.

Preparation of Colloidal Gold–Antibody Complexes
Colloidal gold particles with diameters of 5 or 15 nm were obtained from Janssen Pharmaceuticals. They were coated with affinity-purified monoclonal IgG preparations in 2 mM borate buffer at pH 8.5–9, following a procedure essentially as described previously (3), with minor modifications from the Janssen protocol.

Antibody–gold complexes were centrifuged through a 10–35% glycerol gradient in 20 mM Tris, 150 mM NaCl, pH 8.2, with 1% BSA, as described (3); material from the middle of the gradient was removed and diluted with 1% BSA in Tris/NaCl buffer to give an absorbance at 510 nm of 2.5 (5-nm particles) or 3.5 (15-nm particles). These stocks were stored at 4°C until used. For use in single- or double-label experiments on thin frozen sections, the antibody–gold particle stocks were diluted with dilution fluid (see above) to 1:12–1:15 (A_10 = 0.2–0.3) for each antibody.

Results
Our observations on the arrangement of endothelial type IV collagen in "mature" corneas (>15 d of development), in which a substantial nodal Descemet's membrane matrix is present, are presented first. These are then followed by our immunofluorescence histochemical findings on the development of this complex at stages before and including the onset of Descemet's membrane deposition.

Immunofluorescence Histochemistry
In 8-µm frozen sections of 17–20-d anterior eyes reacted with antibodies against type IV collagen, linear type IV collagen-specific immunofluorescence was seen associated with basement membranes of blood vessels, muscle, the lens (Fig. 1A), and the corneal epithelium (Fig. 1A, small arrows), and nerves (arrowhead, Fig. 1A). In the posterior region of the cornea, however, we observed immunoreactivity for type IV collagen as an array of fine punctate fluorescence (which we term "dots") in the region of Descemet's membrane (large arrows, Fig. 1A and B); measurements in photographic enlargements gave an interdot separation of ~1 µm. This punctate pattern of type IV collagen–specific immunofluorescence was observed in no other basement membranes (e.g., those of blood vessels, muscle, nerve, lens), whether these structures were sectioned transversely or tangentially (not shown). In the overlying stroma, we also saw occasional irregular flecks of coarse immunoreactivity (e.g., arrowheads, Fig. 1B). None of these structures reacted with an antibody against type X collagen (Fig. 1C).

Attempts to expose potentially masked epitopes by reacting sections of corneas from lathyritic chicken embryos with antibodies at low temperatures, or by treating sections with dilute acetic acid, testicular hyaluronidase, mixed glycosidases, vertebrate collagenase, elastase, or 4 M guanidine, before application of the primary antibody did not change the punctate pattern of immunoreactivity for type IV collagen (not shown).

A variety of observations indicate that, in such 8-µm-thick cryosections, the array of fine Descemet's membrane–associated dots reflects the distribution of type IV collagen in the
Figure 3. Semithin (0.5–1 μm) transverse frozen section of a cornea from an 18-d chicken embryo, reacted for two rounds with a mixture of three mAbs against type IV collagen. Bar, 10 μm. In the posterior cornea, the pattern of subendothelial immunoreactivity (e.g., arrows) is discontinuous. The length of the arrows approximates the width of the underlying corneal endothelium. s, stroma. (Inset) Epithelial basement membrane (arrowheads) from the same section, showing a pattern of continuous linear immunofluorescence. epi, epithelium.

Figure 4. Preembedding immunoelectron microscopy of 15-d corneal thick (8 μm) frozen sections labeled with colloidal gold particles coated with type IV collagen-specific antibodies, and then embedded and sectioned for EM. Bars, 250 nm. Bound 5-nm gold particles were observed mostly in small discrete clusters (arrows) at the interface between Descemet’s membrane (asterisk) and the corneal stroma (s), and in irregular plaques (arrowheads and inset) of amorphous material within the stroma itself. The matrix of Descemet’s membrane is unlabeled.
immunoelectron microscopy did not allow us to determine whether the Descemet's membrane and its endothelium has largely toppled over to varying membrane-stroma interface were distributed largely in clusters through application of α-aminopropionitrile during development. The Descemet's membrane itself. and in sections digested with vertebrate collagenase retic by application of α-aminopropionitrile during development was identical. Together these observations indicate that, in transverse 8-μm frozen sections, Descemet's membrane and its endothelium has largely toppled over to varying extents, thus allowing the posterior region of the cornea to be viewed en face (diagrammed in Fig. 2). This interpretation was bolstered by observations of semithin (0.5–1 μm) frozen sections of 18 day corneas reacted for type IV collagen, which gave patterns of immunofluorescence resembling a broken line or a string of beads (Fig. 3); by comparison immunofluorescence in the epithelial basement membrane appeared as a thin continuous line (inset, Fig. 3). While useful in elucidating the spatial organization of the array of Descemet related dots, the semithin cryostat sections still did not allow us to determine whether the Descemet's membrane-associated type IV collagen present within Descemet's membrane itself.

**Immunoelectron Microscopy**

To localize precisely type IV collagen in the posterior cornea, we performed preembedding and postembedding immunoelectron microscopy using colloidal gold particles as electron-dense markers. The preembedding procedure revealed gold-labeled type IV collagen distributed along the interface between Descemet's membrane and the corneal stroma (Fig. 4) and in irregular plaques in the stroma itself (Fig. 4, inset). The gold particles in the Descemet's membrane–stroma interface were distributed largely in clusters (arrows, Fig. 4), which likely correspond to the Descemet's membrane-associated "dots" of type IV collagen-specific immunofluorescence described above. Between the clusters of immunoreactivity, label for type IV collagen was either sparse or absent. This pattern of type IV collagen-specific immunoreactivity was observed also in corneas made lathyritic by application of β-aminopropionitrile during development, and in sections digested with vertebrate collagenase (not shown), which will degrade most interstitial collagens but not type IV. In all cases, Descemet's membrane itself was not labeled, but it is possible that in these preparations this dense matrix was not penetrated by the applied antibodies. To obviate the penetration problem, immunolabeling of ultrathin frozen sections was used. Corneas from 18-d-old chicken embryos were embedded in 2.3 M sucrose, frozen, and thin sections (>100 nm) were labeled with the antibodies, using both indirect and direct procedures that used colloidal gold particles as electron-dense markers. Both procedures yielded the same results. In some experiments, a double-label antibody mixture was used that included anti-type IV collagen antibody coupled to one size gold along with another antibody on a different-sized gold that served either as a negative control (anti-type X collagen) or a positive control (anti-type I or VI collagen) for stromal collagens. Again, type IV collagen–specific gold particles were distributed in the interfacial matrix, mostly in small (0.5 μm or less) clusters (Fig. 5, A, C, and D), and in stromal plaques (Fig. 7). In these preparations, however, we observed in addition that the type IV collagen–containing aggregates at the interface in many cases penetrated the underlying Descemet's membrane, and in some cases extended all the way to the endothelial surface (Fig. 5, A and C; Fig. 6). We also frequently found such aggregates to be associated with discrete endothelial cell processes projecting upward into Descemet's membrane (Fig. 5, A and C; Fig. 6). Occasionally a cluster of type IV collagen–specific immunogold was observed that appeared to be entirely embedded within the nodal matrix of Descemet's membrane (e.g., arrowhead, Fig. 5 F). We interpret such a profile as probably representing an obliquely sectioned finger of penetrating material whose stromal and endothelial continuities were not included in the section. Likewise, clusters of immunoreactivity confined to the stroma–Descemet's membrane interface (e.g., Fig. 5 D) may represent grazing sections of units of penetrating type IV collagen-containing material. The characteristic nodal matrix of Descemet's membrane itself was not labeled by the type IV collagen–specific antibodies, nor by antibodies against collagen types I or VI (not shown), or X (Fig. 5, B and G; Fig. 6).

Attempts to expose potentially masked epitopes by pre-

**Figure 5.** Postembedding immunoelectron microscopy of thin (>100 nm) frozen sections of 18-d embryonic chicken corneas labeled for collagen type IV (A, C–F) or type X (B and G) after incubation with elastase (E–G) or buffer (A–D). Descemet's membrane-associated immunoreactivity. Bars, 0.5 μm. (Bar A is also applicable to B and E–G; bar C is also applicable to D.) In sections incubated with buffer alone (A–D), type IV collagen–specific antibodies, identified by 15-nm gold particles coated with a second antibody, are found largely at the interface between Descemet's membrane (asterisk in A) and the stroma (s), in a pattern of discrete clusters (arrows) of immunoreactivity. Some of these type IV collagen–containing structures appear to penetrate the nodal matrix of Descemet's membrane, but the Descemet's membrane matrix itself is negative. After digestion with elastase (E–G), the endothelium becomes generally separated from Descemet's membrane, leaving hollow depressions in the matrix where cell processes have pulled away (curved arrows). But the characteristic morphology of the Descemet's membrane matrix appears similar to that of controls. In such preparations, a noticeably higher level of labeling for type IV collagen is achieved, but the general pattern of discontinuous immunoreactivity (arrows) is still clearly evident, and the nodal Descemet's membrane matrix remains negative for type IV collagen. The binding of antibody against type X collagen is negligible in both control (B) and digested (G) sections. s, stroma; e, endothelium.

3. Each experiment included reciprocal combinations of double-label mixtures (e.g., IV5 + X15 and IV15 + X5). The results obtained for each combination did not differ, but for each antibody the level of labeling achieved by the 15-nm complex was about three- to fivefold lower than that observed for the corresponding 5-nm complex.
Figure 6. Structure of penetrating aggregates of type IV collagen-containing material in thin (~100 nm) frozen sections of 18-d corneas double-labeled with colloidal gold particles coated with antibodies against collagen type IV (5 nm) or type X (15 nm). Bars, 250 nm. A and B show two aggregates (arrows) of material containing type IV collagen that appear to extend all the way to the surface of the endothelium. The gold particles are associated with moderately electron-dense amorphous material with very little linear (filamentous) structure. However, in some loci (B) where the penetrating matrix material appears to meet the apical tip of an endothelial cell process, thin (<5 nm) filaments (arrowheads) are observed.

Development

The avian cornea develops in a series of well-defined stages involving the deposition of two stromal matrices and the differentiation of its endothelial and stromal cell populations from precursors in the surrounding mesenchyme (see references 16 and 17 for reviews). The corneal endothelium arises from periocular mesenchymal cells that migrate centripetally between the acellular primary stroma and the underlying lens capsule beginning at ~4 d of development (stage 22). These cells form a continuous endothelium by 5 d (stage 26). Soon afterwards (stage 27), the primary stroma swells and is invaded by another wave of mesenchymal cells which then secrete a secondary corneal stroma. Deposition of the characteristic nodal matrix of Descemet's membrane by the endothelium begins at ~9 d of development, and continues into adulthood.

In frozen sections of eyes from stage 22–24 embryos, the migrating presumptive endothelial cells had little, if any, immunofluorescence for type IV collagen associated with them (data not shown). At stage 25/26, however, when the formation of an intact endothelium is under way, these cells were decorated with scattered, discontinuous immunofluorescence for type IV collagen (e.g., small arrows, Fig. 8 A). Most of this appeared to be localized on their basal surfaces, adjacent to the acellular primary stroma. Sections reacted for type X collagen (negative control, Fig. 8 B) showed only nonspecific binding to the corneal epithelium. This immunoreactivity rapidly increased in density, forming a loose network of fine type IV collagen-containing strands (arrows, Fig. 8, C and D). This network, which we term the "subendothelial mat," remained intact as the corneal stroma expanded greatly (Fig. 8 D), suggesting its anchorage to the endothelial surface. With time, immunoreactivity for type IV collagen continued to increase in density at this site, so that by the time that the nodal matrix of Descemet's membrane first becomes detectable (9 d of development), it has assumed the appearance of a finely woven fabric, but with discontinuous filaments (arrowheads) are observed.4

4. At about this time, the primary stroma swells and becomes invaded by periocular mesenchymal cells. These events also correlate with the presence throughout the corneal stroma of numerous profiles of type IV collagen-specific immunoreactivity (Fig. 8, C–F). Some of these structures, which are transitory, appear identical to the "stromal flecks" described earlier. Others, however, differ in their morphology, immunoreactivity with other antibodies, and, probably, origin (work in progress).
Figure 7. Structure of stromal plaques of type IV collagen-containing material in thin (~100 nm) frozen sections of 18-d corneas directly labeled with antibodies against collagen types IV and X or VI. Bars, 250 nm. A shows a section double-labeled with antibodies against collagen types IV (5 nm) and X (15 nm). A stromal plaque (arrow) containing type IV collagen is located just above Descemet’s membrane (asterisk), where diffuse interfacial labeling for type IV collagen is found. Close examination of the plaque reveals a filamentous component (e.g., arrowhead) within the amorphous matrix. B shows another stromal plaque in a section double-labeled with antibodies against collagen types IV (15 nm) and VI (5 nm). The absence of 5-nm gold particles within the plaque suggests that its filamentous component is unlikely to contain type VI collagen.

ties, or “holes” (Fig. 8 E). With continued development (and ongoing deposition of the nodal matrix of Descemet’s membrane) the associated type IV collagen-specific immunoreactivity gradually lost its meshwork pattern, and assumed by 11–12 d (Fig. 8 F) a more punctate pattern of immunofluorescence. This became more sharply defined in mature corneas (e.g., Fig. 1, B and D).

Discussion

Descemet’s membrane is a specialized extracellular matrix that is structurally unique among the basement membranes, appearing as a hexagonal array of nodes and internodes (22). Our observations suggest that this nodal matrix may be unique in its molecular composition as well: unlike most basement membranes, it appears not to contain type IV (basement membrane) collagen. We did, however, find type IV collagen intimately associated with Descemet’s membrane, as a component of material that penetrates at regular intervals the characteristic hexagonal (nodal-internodal) matrix of this structure. But the Descemet’s membrane matrix itself was consistently unreactive for type IV collagen.

Our immuno-electron microscopic observations revealed binding of anti-type IV collagen antibodies to large irregular plaques of amorphous/filamentous material in the posterior corneal stroma. These likely correspond to the coarse flecks of type IV collagen-specific immunoreactivity observed by immunofluorescence histochemistry. By morphological and developmental criteria, they appear quite similar to the transitory “interstitial bodies” described in early embryos by Low (37) and others (7, 39, 41) and suggested to function in cell migration (4, 40). The stromal plaques described here appear to differ from the “basement membrane-like plaques” (51) or the microfibril-associated type IV collagen (44) observed throughout adult mouse corneal stromas, in that they lack a linear morphology, are transitory, and become confined to the posterior third of the cornea during development.

The absence of immunoreactivity for type IV collagen in Descemet’s membrane does not necessarily imply absence of the protein, so we cannot completely exclude the possibility that it might be present in a masked form. It seems likely, however, that these results do in fact reflect the true distribution of type IV collagen, at least in avian species. Our three mAbs against type IV collagen recognize epitopes located on separate domains of the molecule (9, 37). We consider it unlikely that in Descemet's membrane all of these epitopes are masked, and remain masked after treatment with acetic acid, β-aminopropionitrile, 4 M guanidine, vertebrate collagenase, elastase, glycosidases, and glycosaminoglycan-degrading enzymes. It is of interest that elastase digestion did appear to increase binding of the antibodies to the interfacial matrix and its penetrating fingers. This suggests that the type IV collagen at this interface might closely associated with microfibrils, which are probably enriched in Descemet's membrane-associated matrix (45) and are a substrate of elastase (5).

Our findings appear to be compatible with recent biochemical analyses of isolated bovine and rabbit Descemet's membrane showing type IV collagen to be a minor component of the matrix, the major collagen being type VIII (2, 23). Such preparations would undoubtedly include the penetrating domains of type IV collagen-containing material along with the predominant nodal Descemet's membrane matrix. We also found that avian Descemet’s membrane did
Figure 8. Thick (8 μm) frozen sections of anterior eyes from chicken embryos at various stages of development reacted with antibodies against collagen type IV (A, C-F) or type X (B). Bar (A), 10 μm. A and B show eyes at stage 26 (5 d of development) labeled for type IV (A) or type X (B) collagen. The endothelial cells, which have completely covered the posterior surface of the primary stroma (s), show discontinuous immunoreactivity for type IV collagen (e.g., small arrows), but not for type X (B). (Large arrows) Anterior lens capsule. By stage 28 (6 d, C), the endothelial-associated immunoreactivity becomes organized into a loosely-woven mat (arrows) of type IV collagen-containing material. The overlying stroma also contains abundant immunoreactivity for type IV collagen. At stage 30 (7 d, D), rapid stromal expansion has dispersed somewhat the stromal immunoreactivity for type IV collagen, but not that of the subendothelial mat (arrows). The pattern of subendothelial immunoreactivity becomes more dense with time, so that by 9 d (E), individual strands are difficult to resolve. With further maturation, a punctate pattern of immunofluorescence (F, 11 1/2 d) begins to emerge.
not react with any of our antibodies against the interstitial collagen types I and VI (this report), nor with antibodies against collagen types II, III, and V (unpublished observations). Some of these antibodies react strongly, however, with the stroma and the dense interface between Descemet's membrane and the stroma. From other work on Descemet's membrane (2, 23), we surmise that the collagenous component of Descemet's membrane proper is largely, and perhaps exclusively, type VIII. It is possible that most of the interstitial collagens that have been identified in extracts of isolated Descemet's membrane may be derived from this tightly adherent interfacial matrix (see below).

The results of this study appear to contradict some published immunohistochemical studies (15, 48) that have localized type IV collagen throughout the nodal matrix of Descemet's membrane. We have no explanation for this, other than to cite differences in species, developmental age, and antibodies used. On the other hand, we consider the studies of others that have found by immunohistochemistry only faint or restricted immunoreactivity for type IV collagen in the region of Descemet's membrane (13, 23, 49) to be compatible with our observations.

Most of the Descemet's membrane-associated type IV collagen is present in the interfacial region in aggregates that frequently appear to penetrate the Descemet's membrane matrix and, at least in some cases, extend to the endothelial surface; lesser amounts are distributed diffusely along this interface. The fingers of penetrating type IV collagen-containing material may correspond to the domains of fine filamentous ("cloud-like") material observed by Jakus (22) in thin sections of Descemet's membrane. The association of these aggregates of type IV collagen-containing material with endothelial cell processes suggests an attachment to those processes. It is noteworthy that the projection of these processes into Descemet's membrane creates dimples in the posterior surface of Descemet's membrane that are similar to those described recently in the developing rabbit cornea (6).

These observations indicate that one role of the type IV collagen in this region may be to suture the endothelium and its basement membrane to the overlying stroma, via the extremely dense network of fibrils and filaments that comprises the interfacial matrix. The intimate intermingling of stromal and basement membrane elements at this interface seems to enhance the stability and mechanical properties of the matrix. For example, we have found that the interfacial type I collagen in situ is more stable to thermal denaturation (35) and more resistant to degradation by vertebrate collagenase (11) than is type I elsewhere in the stroma. Likewise, we have observed that in regions of thin frozen sections where Descemet's membrane has torn away from the stroma, the fracture plane is usually not at the interface between Descemet's membrane and the stroma, but just above it (i.e., within the stroma itself). The dense interfacial matrix remains tightly associated with Descemet's membrane/endothelium as a cohesive unit (unpublished observations).

During development, the unique array of type IV-containing aggregates appears to be derived from a subendothelial mat of type IV collagen-containing matrix that arises just after a confluent endothelium is established. Although it is likely that this structure is a product of the endothelium, we cannot eliminate the possibility of some contribution from adjacent stromal cells. These appear to secrete type IV collagen for a limited period during development (unpublished results). The pattern of this type IV collagen–specific immunofluorescence differs from that of conventional basement membranes in that it is never continuous, appearing instead as a network of fine strands that may be associated with the endothelial surface. As development proceeds, this pattern gradually assumes the punctate pattern seen in mature corneas. This transition correlates temporally with the continued production of the nodal matrix of Descemet's membrane. It seems likely that Descemet's membrane proper is deposited between this mat and the endothelium such that focal contacts between the mat and the endothelial cells are retained. These then become more sharply defined as Descemet's membrane increases in thickness. We do not know whether type IV collagen continues to be added to the aggregates in situ as Descemet's membrane accretes, but it is clear that corneal endothelial cells in culture can synthesize type IV collagen, along with a variety of other matrix macromolecules (I, 2, 20, 24, 26, 35, 41). If this reflects the situation in situ, the corneal endothelium might prove to be a useful model for investigating certain of the mechanisms by which matrix macromolecules become deposited in a spatially segregated manner.

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References

1. Benya, P. D. 1980. EC collagen: biosynthesis by corneal endothelial cells and separation from type IV without pepsin treatment or denaturation. Rev. Neurol. (Basel). 3:30-35.

2. Benya, P. D. and S. R. Padilla. 1986. Isolation and characterization of type VIII collagen synthesized by cultured rabbit endothelial cells. J. Biol. Chem. 261:4160-4169.

3. Birk, D. E., J. M. Fitch, J. P. Babiarz, and T. F. Linsenmayer. 1988. Collagen type I and type V are present in the same fibril in the avian corneal stroma. J. Cell Biol. 106:999-1008.

4. Brauer, P. R. and R. R. Markwald. 1988. Specific configurations of fibronectin-containing particles correlate with pathways taken by neural crest cells at two axial levels. Anat. Rec. 222:69-82.

5. Carlson, E. C. and G. O. Waring. 1988. Ultrastructural analyses of enzyme-treated microfibrils in rabbit corneal stroma. Invest. Ophthalmol. & Vis. Sci. 29:578-585.

6. Cintron, C., H. I. Covington, and C. L. Kublin. 1988. Morphogenesis of rabbit corneal endothelium. Curr. Eye Res. 7:913-929.

7. Cohen, A. M. and E. D. Hay. 1971. Secretion of collagen by embryonic neuroepithelium at the time of spinal cord-somite interaction. Dev. Biol. 26:578-605.

8. Fitch, J. M. and T. F. Linsenmayer. 1983. Monoclonal antibody analysis of ocular basement membranes during development. Dev. Biol. 95:137-153.

9. Fitch, J. M., E. Gibney, R. D. Sanderson, R. Mayne, and T. F. Linsenmayer. 1982. Domain and basement membrane specificity of a monoclonal antibody against chicken type IV collagen. J. Cell Biol. 95:641-647.

10. Fitch, J. M., R. Mayne, and T. F. Linsenmayer. 1983. Developmental acquisition of basement membrane heterogeneity: type IV collagen in the avian lens capsule. J. Cell Biol. 97:940-943.

11. Fitch, J. M., J. Gross, R. Mayne, B. Johnson-Wint, and T. F. Linsenmayer. 1984. Organization of collagen types I and V in the embryonic chicken cornea: monoclonal antibody studies. Proc. Natl. Acad. Sci. USA. 81:2791-2795.

12. Fitch, J. M., D. E. Birk, A. Mentzer, K. A. Hasty, C. Mainardi, and T. F. Linsenmayer. 1988. Corneal collagen fibrils: section with specific collagens and monoclonal antibodies. Invest. Ophthalmol. & Vis. Sci. 29:1125-1136.

13. Fujikawa, L. S., C. S. Foster, I. K. Gipson, and R. B. Colvin. 1984. Base-
