High-Voltage Electron Microscopy of Normal Human Cornea

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Conventional transmission electron microscopy (CTEM) was compared with high-voltage electron microscopy (HVEM) on 11 normal human corneas (age range, 30 weeks of gestation to 92 yr). Epithelial anchoring fibrils were noted between the basal epithelial cells and Bowman's layer (BL) as previously reported. Parallel pairs of fibers, 27.5 nm in diameter, were observed crossing into the anterior stromal lamellae from BL; their termination sites, however, were not identified. The lateral termination of BL was marked by the presence of a keratocyte lying directly below the end of the multilaminar epithelial basal lamina. In this region, BL tapered and became interwoven with the scleral collagen fibrils in the substantia propria. The HVEM accentuated the orthogonal relationship of collagen bundles apparently emerging from the stromal keratocytes. The posterior corneal stroma appeared to be attached to the anterior surface of Descemet's membrane (DM) by fibers 22.3 nm in diameter that were associated frequently with a dense amorphous material. In the periphery, DM tapered to a thin strand, 0.5 μm in thickness, containing cable-like strands of banded collagen. The posterior nonbanded portion continued laterally and anteriorly in a series of folds between the fibrous collagen sheets of the anterior trabecular meshwork. In addition, HVEM enhanced the visibility of extracellular matrix interactions in the lateral terminations of BL and DM, attachment fibers from BL to the stroma and from the stroma to DM, and keratocyte and collagen fiber orientations not seen easily by CTEM. Invest Ophthalmol Vis Sci 32:2234-2243, 1991

Conventional transmission electron microscopy (CTEM) uses tissue sections that vary in thickness from 70–120 nm and accelerating voltages of 60–80 kV to produce two-dimensional ultrastructural views. In high-voltage electron microscopy (HVEM), accelerating voltages of 1 million or more eV permit thicker tissue specimens, up to 5 μm, to be evaluated. Use of the thicker sections produces a three-dimensional ultrastructural view of cellular and extracellular matrix interactions and also allows greater stain uptake, making small structures more visible.1-2 These characteristics of HVEM previously were used to illuminate the relationship of collagen fibers and bundles to adjacent keratocytes and to tendon fibroblasts of the chick embryo.1,2

The ultrastructure of the attachment of corneal epithelium to its basal lamina and to Bowman's layer (BL) has been well characterized.3 Recent evidence suggests there also is a mechanism for the attachment of BL to the anterior stroma.4 Little is known of the nature of the attachment sites of Descemet's membrane (DM) to the posterior corneal stroma or of the anterior attachment of the endothelium to DM.5 Furthermore, previous EM studies of the normal human cornea have not defined the ultrastructural features of the termination of BL or DM in the limbal area clearly.5-8 Using HVEM, we previously described some of the ultrastructure of these portions of the normal human cornea,9 and we now present our definitive findings.

Materials and Methods

Eleven human eyebank eyes or corneas (age range, 30 weeks of gestation to 92 yr) were received in a moist chamber or in Dexsol (Chiron, Irvine, CA) and were fixed within 96 hr post mortem. Corneas with scleral rims were divided into halves with steel razor blades over Teflon blocks (Weck, Research Triangle Park, NC) and placed in half-strength Karnovsky's fixative10 (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, 300 mOsm) for 24 hr at 4°C. The specimens were rinsed after fixation with 0.1 M cacodylate buffer three times for 5 min each, postfixed in 2% osmium tetroxide for 1 hr at 4°C, and dehydrated in a graded series of ethanol followed by immersion and two changes in propylene oxide for 20 min each. The samples then were infiltrated and embedded in Polybed 812 (Polysciences, Warrington, PA).
One block from the central cornea and one block from the peripheral cornea of each specimen were evaluated by light microscopy and HVEM. One-micron sections were cut from each specimen and stained with toluidine blue-o11 or Mallory's azure II-methylene blue12 for light microscopic analysis. The specimens also were sectioned in thicknesses ranging from 0.25-1.0 \( \mu \text{m} \) on a AO/Reichert ultramicrotome (Buffalo, NY) and placed onto 1 \( \times \) 2-mm slot grids coated with 0.7% formvar substrate (polyvinyl formal). These sections were stained in 4% aqueous uranyl acetate for 30 min for each 0.25 \( \mu \text{m} \) of thickness and then in Reynolds's lead citrate stain for 10 min for each 0.25 \( \mu \text{m} \) of thickness.13 A stabilizing layer of carbon was evaporated onto both sides of the grid using a Denton carbon evaporator (Cherry Hill, NJ) to enable the section to withstand the intense electron beam of the microscope.14 Micrographs were taken on a JEM 1000 electron microscope (JEOL, Tokyo, Japan), located on the University of Colorado campus (Boulder, CO), at an accelerating voltage of 1 million V.

After preliminary analysis of sections by HVEM, it was determined that 0.5- \( \mu \text{m} \) sections (nearly ten times thicker than CTEM sections) produced optimal visualization of the specimens. Measurements were made

![Fig. 1. Junction of epithelium (EP) and Bowman's layer (BL) (HVEM). Fibris (arrows) are seen traversing the cell membrane of the basal cells into the anterior aspect of Bowman's layer. Note the epithelial origin of the fibrils (solid arrow), the fanning out of the fibrils as they cross the lamina densa (arrowheads), and the anchoring point (plaque) in BL (open arrow). Bar = 1 \( \mu \text{m} \), specimen age is 75 years-old, thickness 0.5 \( \mu \text{m} \).](image-url)
from micrographs using a Bausch & Lomb magnifying loop (Rochester, NY) and a Pelco Ultrasize Calculator (Ted Pella, Tustin, CA). Fibers oriented in cross-section to the plane of section were selected for diameter measurements. Statistical analysis was done using SYSTAT programs (Evanston, IL).

Results

Epithelial Anchoring Fibrils

Intermediate filaments extending from the basal epithelium to BL (Fig. 1) were sometimes visible with HVEM even in the absence of special processing techniques. The fibers appeared in a fan-like arrangement spreading out from a point in a basal epithelial cell approximately 0.25 µm to a densely staining region on or in the basal lamina. A similar set of fibers fanned out from a point in BL and anchored 0.25 µm away in the same region of densely staining material for a total length of 0.5 µm.

Attachment of BL to the Anterior Stroma

Attachment fibers that could be followed for up to 1.5 µm (average diameter, 27.5 nm) were seen leaving BL in parallel pairs perpendicular to the ocular surface and crossing anterior stromal lamellae (Fig. 2, 3).
woven with the anterior scleral collagen in the sub-peripheral third.

Termination of BL and DM

Corneas, they were observed more frequently in the older specimens we studied; it may be an age-related change. In all the specimens we examined, the most distinct feature was a keratocyte or fibrocyte lying in the same area, adjacent to and directly below the termination of the multilaminar basa lamina (Fig. 7).

### Keratocyte and Extracellular Matrix Interactions

The relationship between keratocytes and the extracellular matrix can be seen when the corneal stroma is examined with CTEM (Fig. 3A). With the greater specimen thickness allowed by HVEM, the close association of collagen fibers with the cells became apparent (Fig. 3B). Corneal stromal collagen fibers appeared to be produced and organized by keratocytes into bundles that were perpendicular to each other (Fig. 3B). The stereo pair (Fig. 4) emphasized the orthogonality of the collagen bundle relationships. This three-dimensional image helped to clarify the organization of the cell-fiber interaction and provided a broader view of the functional dynamics of this system.

### Attachment of DM to the Posterior Corneal Stroma

The attachment of DM to the posterior stroma appeared to be accomplished in part by fibers 22.3 nm in diameter (Table 2) that ran perpendicular to DM (Fig. 5). The depth of penetration of the fibers into DM was 0.16–0.21 µm (Table 3). They were associated frequently with a dense, amorphous mass at the interface between DM and the posterior stroma (Fig. 6). Although these fibers were found across the entire cornea, they were observed more frequently in the peripheral third.

### Termination of BL and DM

In the limbal zone, BL tapered and became interwoven with the anterior scleral collagen in the substantia propria (Fig. 7). From a normal thickness of 10–15 µm, BL tapered over a variable distance of 20–45 µm until the characteristic collagen fibers became indistinguishable at the light and electron microscopic level from those of the sclera. In this zone, the overlying epithelial basal lamina of the peripheral cornea changed in appearance from a thickened multilaminar variety to a smooth surface, typical of the conjunctival epithelium’s basement membrane (Figs. 7, 8). Transition from an interdigitating corneal basal epithelial cell membrane to a more linear membrane of the limbal epithelium was observed more frequently in the older specimens we studied; it may be an age-related change. In all the specimens we examined, the most distinct feature was a keratocyte or fibrocyte lying in the same area, adjacent to and directly below the termination of the multilaminar basal lamina (Fig. 7).

At the corneal periphery, DM tapered from the normal 7–10 µm to a thin strand measuring 0.5 µm thick (Fig. 9). This taper occurred over a distance of approximately 50 µm, varying among corneal samples from 26–60 µm. In the tapered configuration, DM contained cable-like strands of banded collagen (Fig. 9). At the first trabecular endothelial cell, the posterior nonbanded portion became interlaced laterally and anteriorly in a series of folds that could be detected between the sheets of the anterior trabecular meshwork. The endothelium adjacent to this tapered form of DM was highly attenuated. The corneal endothelium was characteristically 5 µm in height and 15–20 µm in width, with an oval, centrally located nucleus. By comparison, the endothelium of the trabeculum

### Table 1. Diameter of fibers between Bowman’s layer and anterior corneal stroma

<table>
<thead>
<tr>
<th>Donor age</th>
<th># Fibers measured</th>
<th>Mean diameter (nm)</th>
<th>Standard deviation</th>
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<tbody>
<tr>
<td>4 days</td>
<td>7</td>
<td>30.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14 years</td>
<td>10</td>
<td>28.75</td>
<td>2.1</td>
</tr>
<tr>
<td>44 years</td>
<td>21</td>
<td>22.3</td>
<td>2.5</td>
</tr>
<tr>
<td>47 years</td>
<td>11</td>
<td>25.6</td>
<td>2.0</td>
</tr>
<tr>
<td>62 years</td>
<td>17</td>
<td>25.3</td>
<td>4.2</td>
</tr>
<tr>
<td>92 years</td>
<td>13</td>
<td>33.3</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Average fiber diameter = 27.5 nm; SD = 3.9 nm; n = 6. Magnification ranged from 10,000 to 40,000×.

### Table 2. Diameter of fibers between Descemet’s membrane and posterior corneal stroma

<table>
<thead>
<tr>
<th>Donor age</th>
<th># Fibers measured</th>
<th>Mean diameter (nm)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 week gestation</td>
<td>3</td>
<td>24.4</td>
<td>4.2</td>
</tr>
<tr>
<td>31 years</td>
<td>21</td>
<td>20.7</td>
<td>2.0</td>
</tr>
<tr>
<td>44 years</td>
<td>21</td>
<td>22.4</td>
<td>5.4</td>
</tr>
<tr>
<td>92 years</td>
<td>6</td>
<td>21.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Average fiber diameter = 22.3 nm, SD = 1.6; n = 4. Magnification ranged from 20,000 to 60,000×.

### Table 3. Depth of penetration of posterior corneal stromal fibers into Descemet’s membrane

<table>
<thead>
<tr>
<th>Donor age</th>
<th># Fibers measured</th>
<th>Mean depth into Descemet’s membrane</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>31 yr</td>
<td>5</td>
<td>0.16 µm</td>
<td>0.016 µm</td>
</tr>
<tr>
<td>44 yr</td>
<td>6</td>
<td>0.21 µm</td>
<td>0.05 µm</td>
</tr>
</tbody>
</table>

Magnification ranged from 40,000 to 60,000×.
had a large central nucleus, an abundance of rough endoplasmic reticulum, a more developed Golgi apparatus, and cytoplasmic extensions both anterior and posterior to the trabecular sheet. Immediately anterior to the endothelium, wide spacing collagen appeared as free-floating bundles in the extracellular matrix, combined with an increase in fibrillar collagen in masses and bundles (Fig. 9).

Discussion

Epithelial cell anchoring structures were reported using CTEM in rabbit and human corneas. These structures, identified with monoclonal antibodies to type VII collagen, form a complex of fibers branching into an anastomosing network below the basal lamina which then inserts into patches of dense extracellular matrix, termed anchoring plaques. We sometimes were able to observe these fibrils using HVEM without special staining or processing techniques, although they are reported to be seen more easily in corneas fixed with collidine-buffered osmium tetroxide or cultured before fixation. We determined a total length of the attachment structure from the origin in the epithelial cell to the observed ending in BL to be 0.5 μm, somewhat less than the measurement previously reported. The reduced fibril length that we observed could be due to fixation differences or differences in the cutting angle of the section. The changes observed in the corneal-limbal basal epithelial membranes of the older specimens may be related to slower epithelial turnover rates and/or to the increasing thickness of basal lamina over time.

The parallel fibrils extending from BL into the anterior stroma were described earlier using HVEM, in a preliminary report from our laboratory, and previously by others using a frozen resin cracking preparation for scanning electron microscopy. These investigators observed strata in BL in the central cornea that were absent in the periphery. Their proposed structure of BL is consistent with our observation of a decided tapering of the layer and its inter-

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Fig. 3. Comparative CTEM and HVEM of corneal stroma. In this CTEM of a 30-week fetal cornea, the keratocytes (K) and the collagen fibers (CF) have a flat two-dimensional appearance (A). Bundles of collagen fibers can be seen emerging from pits in the keratocyte surface (arrow). Bar = 1 μm. In contrast, the HVEM view of a 30-week fetal cornea shows more depth of field, with collagen fibers (CF) appearing to emerge from the keratocyte (K) cell membrane (arrows) at right angles to each other (B). Bar = 1 μm.
weaving into the adjacent scleral fibers, appearing to give structural continuity between the sclera and BL. Visualization of this relationship is enhanced by the increased depth of field offered by HVEM.

Although DM has been studied extensively, the short fibrils extending from the posterior stroma into the anterior aspect of DM that we found have not been reported previously to our knowledge. Lectin histochemical studies indicate the presence of a posterior corneal filamentous network, which may be related to the fibrils we observed. Use of cuprolinic blue dye and scanning electron microscopy also suggested the existence of fibers at the posterior stroma–DM interface. Because we know clinically that DM can detach easily from the posterior cornea during cataract surgery and can be made to “reattach” by unrolling it back on to the posterior cornea with or without the aid of sutures or viscoelastic substances, we can conclude that the attachment sites of these fibrils are relatively weak. We are planning further studies to assess the significance of the newly visualized fibrils seen in this study and their relationship to the dense staining masses at the stromal–DM interface.

The fibers extending perpendicularly to the corneal...
Fig. 4. HVEM stereo micrograph created by tilting the specimen stage 11 degrees, then rotating 180 degrees azimuth between exposures. Some collagen fibers completely cross the keratocyte (K) while others appear to start at the keratocyte. The cytoplasm contains a lightly staining ribbonlike membranous structure. To view in 3-D use stereo viewers or +3 diopter lens pair in a trial frame to merge the images. Donor age 47 years. Bar = 1 μm.

Fig. 5. Posterior corneal stroma and anterior Descemet’s membrane (HVEM). Short, parallel, nonuniform fibers (arrows) larger in diameter than corneal collagen, appear between Descemet’s membrane (DM) and the stroma (S). Specimen age is 44 years, section thickness 0.5 μm. Bar = 1 μm.
Fig. 7. Termination of Bowman's layer (HVEM). This micrograph shows the keratocyte (K), which typically appears just posterior to the basal epithelium (E) at the tapering of BL (BL) into the scleral substantia propria (arrows). The fiber/bundle organization is more obvious by use of 0.5 μm sections and the HVEM. Specimen age 31 years, section thickness 0.5 μm. Bar = 1 μm.
layers and appearing to interconnect BL and the anterior stroma and the posterior stroma and DM are reminiscent of the sutural fibers of the Elasmobranch eye.\textsuperscript{20} In the cartilaginous fishes, the sutural fibers prevent corneal swelling.\textsuperscript{21} Whether these fibers in the human eye act similarly, whether they have been adapted for another function, or if they represent nonfunctional vestigial structures is not clear from these morphologic observations. Analogous fibers made up of type IV collagen were described in the

![Fig. 8. Montage of Bowman's layer (BL) termination (HVEM). The transition in the basal epithelial cell membrane (arrows) from the corneal to the scleral epithelium and the keratocyte (K) associated with the limbal transition are shown. Specimen age 85 years, section thickness 0.5 \( \mu \)m. Bar = 1 \( \mu \)m.](image)

![Fig. 9. Montage of the posterior limbal region of the eye depicting the termination of Descemet's membrane (DM) (HVEM). Associated with the taper of DM there is a noticeable increase in the collagen present in both fibrillar form (FF) and also in the wide spacing collagen (WS) configuration. Note the bands of lightly staining material located anteriorly (arrows) and its similarity to the material making up DM, supporting the theory that DM is continuous with the trabecular meshwork. Specimen age 47 years, section thickness 0.5 \( \mu \)m. Bar = 1 \( \mu \)m.](image)
embryonic chick cornea extending from the endothelium to the posterior stroma.22 Sutural fibers are found throughout the entire stroma of the cartilaginous fish cornea.20 It would be interesting to determine if such fibers occur between the stromal lamellae of the human cornea.

The apparent emergence of collagen fiber bundles from keratocytes, although visible by CTEM, was more obvious by HVEM. The orthogonal relationship of the emerging fiber bundles was emphasized by HVEM. The orientation of the emerging collagen fiber bundles in these human keratocytes supported previous studies of embryonic chick keratocytes.1

The termination of DM showed not only tapering of the layer, but also association of the amorphous extracellular material with banded collagenous material. In this tapered configuration, the posterior nonbanded amorphous portion interweaves throughout the adjacent trabecular meshwork. This type of interaction of the terminus of DM with adjacent tissue would give strong support to the posterior cornea and help to preserve the integrity of the globe in response to trauma.

The use of the HVEM helped us to visualize small attachment fibrils and assemblies between the corneal layers that previously were difficult to detect with CTEM.3 The thicker sections permitted with HVEM produced a three-dimensional view of cellular relationships and allowed greater stain uptake; cellular-extracellular matrix interactions and structural fibers in the human cornea could be seen more easily.

Key words: high-voltage electron microscopy, corneal ultrastructure, Bowman’s layer, Descemet’s membrane, corneal stroma

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References