Anchoring Fibrils Form a Complex Network in Human and Rabbit Cornea

Ilene K. Gipson, Sandra J. Spurr-Michaud, and Ann S. Tisdale

Anchoring fibril distribution, depth of penetration into the stroma, and pattern of histochemical localization of type VII collagen (the anchoring fibril collagen) were studied in normal human and rabbit corneas. Electron micrographs of cross sections and sections taken parallel to the basement membrane demonstrate that anchoring fibrils insert into the basal lamina and then splay out laterally. They are more readily seen in sections taken parallel to the basal lamina, where they are observed to form a complex branching and anastomosing network below the basal lamina. Distal to the basal lamina, anchoring fibrils appear to insert into patches of dense extracellular matrix termed “anchoring plaques.” Average depth of penetration of anchoring fibrils into stroma is 0.60 and 0.54 μm for human and rabbit, respectively. Monoclonal antibodies to type VII collagen localize only to the basement membrane-anchoring fibril zone of both human and rabbit corneas. No obvious differences in anchoring fibril structure or distribution were observed between human corneas, which have a Bowman’s layer, and rabbit corneas, which do not.


Anchoring fibrils are uniquely cross-banded fibrils present on the connective tissue side of the basal lamina of all stratified squamous epithelia. They are a component of the adhesion complex required for tight adherence of these epithelia, which are subject to abrasion. The adhesion complex consists of the hemidesmosome, which is the adhesion junction joining the basal cell membrane to the basal lamina. The hemidesmosome junction is connected in an as yet undefined manner through the basal lamina to another portion of the complex, the anchoring fibril network. This network splays out into the subjacent connective tissue strata.

The structure and distribution of anchoring fibrils (formerly termed Special Fibrils) have been most thoroughly studied at the epidermis-dermis junction.1-3 Studies of amphibian skin demonstrate that the fibrils have a symmetrical transverse band pattern, are ~750 nm long and attach at both free ends to the undulating basement membrane of the epidermis.3 Evidence that these fibrils function in holding the overlying epithelium and basement membrane to the dermis comes from studies of forms of epidermolysis bullosa dystrophica in which epidermal blisters form due to the absence of the fibrils (for reviews, see references 4 and 5).

Other stratified squamous epithelia in which anchoring fibrils have been studied include oral mucosa,6 ectocervix,7 and cornea.8-10 Jakus in 196211 described “fine-filaments” that extend from protuberances of the corneal epithelial basement membrane. Later reports12,13 described accumulations of short fibrous material in pockets of duplicated basement membrane in Meesmann’s corneal dystrophy. Kenyon and Maumenee14 ascribed the term “anchoring fibrils” to crossbanded fibers in the central cornea of a case of congenital corneal dystrophy. Subsequently, Kenyon15 and Alvarado et al16 described accumulations of anchoring fibrils in duplicated epithelial basement lamina of diabetic and aged humans, respectively.

A study using an in-vitro corneal system indicated that new hemidesmosomes form over specific sites on basal laminae where anchoring fibrils insert from the stromal side.10 This site may be a “nucleation” site for hemidesmosome reformation as basal cells of stratified epithelium divide and constantly change positions in relation to the basal lamina. It is not known whether the anchoring fibril itself passes through the basal lamina to form the nucleation site or whether there are linking elements between anchoring fibrils and hemidesmosomes.

Bentz et al16 reported isolation of a new type of collagen, designated type VII, purified from human amnion. Immunohistochemical and immunoelectron microscopy studies demonstrated that antibodies to type VII collagen localize to epidermal anchoring fi-
brils. Type VII collagen seems to have a tripartite globular nonhelical head region at the carboxyl terminus of the filamentous triple helix portion of the molecule. Triple helices of individual molecules associate to form the cross-banded fibril, and globular domains associate in the basal lamina and in patches of electron-dense extracellular matrix (anchoring plaques) located in the connective tissue region subjacent to the basal lamina. Despite the reports regarding the accumulation of anchoring fibrils in corneal pathologies and in aged humans, little morphological and morphometric data are available on the distribution of anchoring fibrils in the normal human cornea. We report studies of the distribution and depth of penetration of anchoring fibrils in normal human corneas as well as in rabbit corneas, which do not have a Bowman's layer. In addition, we report the pattern of immunohistochemical localization of monoclonal antibodies to type VII collagen in the epithelial basement membrane zone of both species.

Materials and Methods

All investigations involving animals reported in this study conform to the ARVO Resolution on the Use of Animals in Research. Normal human donor corneas and corneas from adult (2.5 kg) New Zealand White rabbits were used. The rabbits were killed with an intravenous overdose of sodium pentobarbital.

Transmission Electron Microscopy

Donor human corneas (N = 9) and excised rabbit corneas (N = 7) were fixed in either half-strength Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) or 3.75% osmium tetroxide (OsO4) in 0.2 M s-collidine buffer, pH 7.4. In addition, 3 human stromas and 43 rabbit corneas were incubated 1–24 hr in a completely defined organ culture medium consisting of PBS, glycerol, and paraphenylene-diamine. Negative control tissue sections (primary antibody omitted) were routinely run with every antibody-binding study. The sections were viewed and photographed using a Zeiss photomicroscope III equipped for epi-illumination. The primary antibodies were the kind gift of Dr. Robert E. Burgeson (Shriners Crippled Children's Hospital, Portland, OR), who has verified their specificity. The antibodies recognize the nonhelical globular domain of the type VII collagen molecule.

Results

Anchoring fibril structure and distribution is difficult to discern in corneas fixed immediately after dissection in routinely used glutaraldehyde-paraformaldehyde fixatives (i.e., half-strength Karnovsky's fixative). We noted in previous studies that anchoring fibrils were more discernible in corneal tissue that had been organ-cultured for 24 hr prior to fixation in half-strength Karnovsky's fixative. Also, trials of various fixatives demonstrated that anchoring fibrils were more discernible after fixation with osmium tetroxide in collidine buffer. All electron micrographs in Figures 1 to 4 are sections of tissues processed after 24 hr culture or after osmium fixation.

Immunofluorescence

Normal rabbit and human corneas were frozen in Tissue Tek II O.C.T. Compound (Lab Tek Products; Naperville, IL). Six-micrometer cryostat sections were placed on gelatin-coated slides and air-dried overnight at 37°C. The sections were rehydrated in phosphate-buffered saline (PBS), pH 7.2, and washed in PBS with 1% bovine serum albumin (BSA) for 10 min. The primary antibody (mouse anti-human type VII collagen, clone NP76 or 185) was then applied for 1 hr in a moist chamber. The slides were rinsed with PBS followed by 10 min in PBS with 1% BSA. The secondary antibody (fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse IgG; Cooper Biomedical Inc.; Malvern, PA) was applied for 1 hr in a moist chamber. After a PBS wash, coverslips were mounted with a medium consisting of PBS, glycerol, and paraphenylene-diamine. Negative control tissue sections (primary antibody omitted) were routinely run with every antibody-binding study. The sections were viewed and photographed using a Zeiss photomicroscope III equipped for epi-illumination. The primary antibodies were the kind gift of Dr. Robert E. Burgeson (Shriners Crippled Children's Hospital, Portland, OR), who has verified their specificity. The antibodies recognize the nonhelical globular domain of the type VII collagen molecule.

Morphometric Analysis

A Zeiss Videoplan Image Analysis System (Rainin Instruments; Woburn, MA) was used to measure the depth of penetration into the stroma of the five longest anchoring fibrils on each of 8–10 electron micrographs per cornea (N = 9 for humans, N = 50 for rabbits). The anchoring fibrils were measured perpendicular to the basal lamina from their deepest point in the stroma to their insertion into the lamina densa. The mean and standard error of the mean were then computed for each group of corneas.
of the fibrils insert distally from the basal lamina in patches of amorphous extracellular matrix (anchoring plaques), which have the appearance of isolated bits of basal lamina (Figs. 1, 2). These patches, as demonstrated by antibody localization, represent the accumulated globular head regions of the type VII collagen molecule plus type IV collagen (Keene et al, unpublished data).

We could not identify anchoring fibrils in cross section. The width of anchoring fibrils is variable in longitudinal section; anchoring fibrils with widths up to 0.15 \( \mu \)m have been observed. If the anchoring fibrils of this width were round, they should be visible in cross section. We could not, however, identify such shapes in cross section.

Morphometric analyses demonstrate that average maximum depth of penetration of anchoring fibrils into the corneal stroma of human and rabbit were 0.54 ± 0.01 and 0.60 ± 0.01 \( \mu \)m, respectively. Maximum penetrations measured were 2.05 \( \mu \)m for human corneas and 2.69 \( \mu \)m for rabbit. Anchoring fibrils were most discernible in sections of both human and rabbit corneas taken tangential to the basal lamina (Figs. 3, 4). These sections demonstrate an elaborate interconnecting network of anchoring fibrils in this plane. The filamentous regions of anchoring fibrils appear to branch and anastomose, forming the complex interwoven network.

The networks appear equally complex in human and rabbit corneas. By electron microscopy we could discern no significant difference of anchoring fibril penetration and distribution in the human corneas, which have a Bowman's layer, and in rabbit corneas, which do not. Basement membranes of human corneas do,
Fig. 2. Cross sections of human (A) and rabbit (B) corneas through zone of anchoring fibrils. Most anchoring fibrils appear directly adjacent to the basal lamina; an occasional fibril penetrates deeply into stroma (B, large arrow). Anchoring plaques at distal insertion of anchoring fibril are indicated by small arrows. Tissue incubated 24 hr prior to fixation (A, ×31,200; B, ×25,000).

however, appear to undulate as compared with the flat basal lamina of rabbits (see Fig. 2).

Type VII Collagen Localization

Monoclonal antibodies, which localize to the tripartite globular nonhelical head region of the type VII collagen molecule, localized to the region of the anchoring fibril network at the basement membrane zone of the corneal epithelium (Fig. 5). The localization pattern in human and rabbit appeared similar, but we did see variation in intensity of localization in human eyes (Fig. 5A, B). The pattern of localization on a 9-month-old human eye is shown in Figure 5B. The region of and below the basal lamina fluoresces intensely with a diminution of intensity toward the stromal side. At the
Fig. 3. Sections of 9-month-old human cornea taken tangential (parallel) to the basal lamina. The basal lamina in this cornea was undulating, not flat. Thus, sections through tips of basal cells are present. A: Branching and anastomosing networks of anchoring fibrils are visible in this plane of section (arrows) (×42,000). B: Higher magnification of region of anchoring fibril network demonstrating insertion of fibrils into basal lamina (arrows) (×93,000). Tissue fixed in osmium collidine fixative.

interface of the stroma with the anchoring fibril network, discrete small patches of localization are obvious. These patches may correlate to the patches of amorphous extracellular matrix (anchoring plaques) in which the anchoring fibrils appear to insert (Figs. 1, 3).
Fig. 4. Electron micrographs of sections of rabbit cornea taken tangential (parallel) to basal lamina (BL). A: Low magnification demonstrating the density of anchoring fibril network in this plane (×31,200). B: Higher magnification micrograph of anchoring fibril network. Branching insertion of anchoring fibril into basal lamina is evident at arrow. Branching and anastomosing of fibrils is very obvious in this plane of section (×164,400). Tissue was cultured 24 hr prior to fixation.
Fig. 5. Light micrographs demonstrating immunofluorescent localization of monoclonal antibodies to nonhelical region of the type VII collagen molecule on cryostat sections of human and rabbit corneas. A: Human (age 54) cornea. Binding of antibody appears as a thin band along basement membrane zone only. Although not visible, the epithelium is above the fluorescent band (×600). B: Nine-month-old human cornea. Localization in this cornea was particularly intense and the zone of localization was wide. Localization pattern appears as parallel strands of beads extending into stroma. Electron micrographs in Figure 3 are from this cornea (×600). C: Adult rabbit cornea. Binding of antibody is intense at region near basal lamina. Beaded strands reach into stroma (×600).

Fig. 6. Schematic diagram of anchoring fibril network in zone below the basal lamina. At top, branching insertions of anchoring fibrils (arrows) into basal lamina below hemidesmosomes (HD) are depicted. Branching and anastomosing cross-banded fibrils splay out among type I collagen fibrils, and some anchoring fibrils insert into dense patches of extracellular matrix which are composed of nonhelical domains of type VII collagen molecules and other basal lamina components. These patches have been termed anchoring plaques (AP).
Discussion

The depth of penetration and the elaborate interconnecting network of the anchoring fibrils in the anterior cornea have not been previously recognized. Conventional fixation and embedding procedures probably obscured the network. Glutaraldehyde and paraformaldehyde fixatives plus osmium postfixation may fix too well. That is, all soluble components surrounding the network may be cross-linked and fixed in place, making the network difficult to differentiate. These soluble components may be eliminated by preincubation in culture medium, and the procedure for osmium fixation in collidine buffer may not fix all components surrounding the network. One could argue that preincubation distorts the anchoring fibril network and that depth of penetration measurements are thus inaccurate. Measurement and distribution patterns do correlate, however, between preincubated tissues and osmium-fixed specimens.

The observation that the anchoring fibril network in cornea is more apparent in tangential section would indicate that a majority of fibrils run in a plane parallel to the basal lamina. However, we did not observe frequent examples of anchoring fibrils with both ends inserting into the basal lamina. These looping fibrils are more prevalent in the epidermis. Indeed such looping anchoring fibrils are more common to squamous epithelium with undulating rather than flat basal lamina. We were unable to recognize anchoring fibrils in cross section. It may be that anchoring fibrils are ribbon-like, with their widest dimension in the center of the fibril. Ribbons in cross section could appear as short segments of filaments or fibers.

Anchoring fibrils insert into dense bodies or patches of extracellular matrix, distal to their insertion into the basal lamina. Other anchoring fibrils deeper in the stroma extend between these patches of matrix. Keene et al. have recently applied the term “anchoring plaque” to these structures. The plaques appear to be the "knots or pivot points" in the three-dimensional network of anchoring fibrils. The plaques are apparently accumulations of the globular domain of type VII molecules in combination with the basal lamina collagen, type IV. Because these plaques have the appearance of bits of basal lamina, other basal lamina components may be present as well.

The role that anchoring fibrils and the network they form have in adhesion of stratified squamous epithelia to underlying connective tissue is becoming increasingly clear. Three examples give insight into the function of the fibrils; in two, epithelial adhesion is faulty.

Epidermolysis bullosa is a group of diseases characterized by epithelial blistering. In some instances, the cornea is involved. Anchoring fibrils are absent or aberrant in these diseases (for review, see references 4 and 5). Whether the anchoring fibril absence is the result of genetic deletion or enzyme degradation is unknown. The epidermal epithelium (and all other squamous epithelium) with its basal lamina easily splits from underlying connective tissue.

A second example indicating the importance of anchoring fibrils in adherence of basal lamina to stroma comes from studies of diabetic corneal epithelium. Kenyon reported that when diabetic corneal epithelium that has become opaque during vitrectomy surgery is scraped off, the duplicated basal lamina remains with the scraped epithelium. In contrast, when normal epithelium is removed by scraping, the basal lamina remains on the stroma. Measurements of depth of penetration of anchoring fibrils from the deepest layer of duplicated basal lamina demonstrate that in diabetics depth of penetration is significantly less than in normal corneas. Basement membrane reduplication, as observed with aging or in diabetes, may interfere with the preservation of the relationship between anchoring fibrils and the stroma, setting up the stage for a loose adhesion as shown by Kenyon.

Finally, in-vitro experiments designed to study conditions for hemidesmosome formation by corneal epithelium provide evidence that the site on the basement membrane where hemidesmosomes form on the basal cell membrane is where anchoring fibrils insert from the stromal surface. These studies indicate that the anchoring fibril network is linked through the basal lamina (directly to the cell or indirectly through linkage molecules) to cell membrane adhesion junctions (hemidesmosomes). This association between hemidesmosomes and anchoring fibrils, previously noted by Susi et al., provides continuity of adhesion from the cell through the basal lamina and into the connective tissue.

In summary, a complex anchoring fibril network is present in the region of the corneal stroma just beneath the epithelial basement membrane. The fibrils insert into the basal lamina and distally into patches of extracellular matrix (anchoring plaques). Monoclonal antibodies to type VII collagen localize to the region occupied by the network. A diagram depicting our concept of the distribution of the anchoring fibril network is shown in Figure 6.

Key words: anchoring fibrils, type VII collagen, hemidesmosomes, corneal epithelial adhesion, cell-substrate adhesion, basement membrane, rabbit cornea, human cornea

References