Embryonic Corneal Epithelial Actin Alters Distribution in Response to Laminin

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In the current study, laser scanning confocal image analysis was used to investigate the actin distribution in whole-mount preparations of freshly isolated and cultured corneal epithelia. Actin staining defined the cell borders and microvilli of the periderm cells. The actin was prominent as an organized network at the interface between the basal and periderm cells and in the basal compartment of the basal cells (actin cortical mat) when isolated with the basal lamina (BL). In epithelia isolated without BL, the actin in the periderm cells and the network at the periderm-basal cell interface was the same as in epithelia isolated with BL. However, the actin in the basal compartment of the basal cells was localized in the cellular blebs that projected from the basal cell surface. Epithelia isolated without BL and cultured in the presence of laminin reorganized the actin cortical mat within 2 hours. However, epithelia isolated without BL and cultured without BL proteins continued to have basal cell projections, sometimes into the pores of the filter. Treatment of epithelia with 2 μM cytochalasin D prevented the reorganization of the actin by laminin. In conclusion, the use of confocal analysis increased our understanding of actin distribution in the epithelial sheets. These results confirmed and extended previous studies using electron microscopy to determine that corneal epithelial cells respond to extracellular matrix molecules by an actin-dependent mechanism. Invest Ophthalmol Vis Sci 33:324–333, 1992

Recently, we developed a technique for viewing structural proteins in freshly isolated or cultured epithelial sheets with confocal microscopy. The chick embryonic corneal epithelium was used for these studies. This epithelium has been well characterized1 as a two-cell-layer structure with a thin flat outer layer, the periderm, and a cuboidal-to-columnar inner layer, the basal cells. The basal epithelial cells have a flat basal surface with a well-defined basal lamina (BL). Both cell layers are characterized by abundant secretory organelles that are necessary for the synthesis of the secreted glycoproteins that form the primary stroma.1,2 The chick corneal epithelial basal cell surface interacts with the underlying stroma through extracellular matrix (ECM) binding proteins in the lamina lucida of the BL, such as the integrin molecules (α6β4), previously described.3 These molecules are composed of two subunits with three domains: a cytoplasmic region, a membrane spanning region, and the extracellular domain that resides in the BL lamina lucida.4 The basal area of the cell, the BL, and the ECM immediately below the lamina densa will be referred to as the basement membrane zone (BMZ) in this study. The basal epithelial compartment is the area between the nucleus and the basal plasmalemma. This area is rich in many cell organelles, such as rough endoplasmic reticulum (RER), Golgi apparatus, secretory granules, mitochondria, and cytoskeletal structures that change as the epithelium develops. We examined the actin cytoskeleton throughout both the periderm and basal cell layers, but the filamentous actin (F-actin) network resident in the BMZ was emphasized. It has been established that an extensive actin cortical mat is present in the basal epithelial compartment, and furthermore, it is a dynamic structure that responds to ECM molecules.5–9 Other actin-associated proteins also may be present in the basal epithelial area, including α-actinin, vinculin,10 talin,11 fodrin, and ankyrin.12

Previous knowledge of the actin cortical mat was based on cross-sectional data from electron micrographs.5–9 Although the actin network is visible in many micrographs of the corneal epithelium, it is not present in all micrographs. It is known that the embedding medium, plastic, hides some cytoskeletal elements because the structures are the same optical density as the plastic and would not be seen in routine transmission electron microscopy. A new technique is
needed to view these structures in whole corneas and isolated corneal epithelia.

The cultured corneal epithelium has not been studied extensively with fluorescent markers because several problems had to be overcome. First, the support material had to be changed to a fluorescently negative substance. The epithelia have been cultured on Milli-pore filters (Millipore Corp., Bedford, MA) composed of nitrocellulose for many years,8,9,13 but this filter autofluoresces. Furthermore, the freshly isolated epithelial sheets have to be kept flat through many incubations and washing steps. When the epithelia are placed on glass cover slips, they curl up and float away. Black membranes (Poretics Corp., Livermore, CA) solve both problems. The black filters (polycarbonate membranes) are fluorescently negative. They were developed originally for microbial water assays. After the filter substrate problem was solved, routine epifluorescence and video-enhanced microscopy were attempted,14 but the tissue out of the plane of focus caused a background blur. Although the video tapes were promising, still photographs were disappointing. The confocal microscope solves this problem by only recording the fluorescent data at the plane of focus.15

In the current investigation, confocal laser scanning microscopic instruments were used to reexamine the spatial distribution of actin in epithelia from whole corneas and in freshly isolated corneal epithelia with and without the BL. These experiments showed that the distribution of actin in freshly isolated corneal epithelium was extensive throughout both cell layers, with a remarkable actin cortical mat both in whole-cornea preparations and in epithelium isolated with the BL. These studies also demonstrated that the actin reorganizes rapidly in the presence of laminin in the cultured epithelia. In addition, aggregation of actin by cytochalasin D (CD) inhibited the laminin-stimulated reorganization of the actin cortical mat, and the actin aggregates appeared as intensely staining spots throughout all cell layers of the epithelium.

Materials and Methods

White leghorn chicken eggs were obtained from Spafas (Norwich, CT) and incubated for 6 days at 38°C. The embryos were removed and rinsed in Hank’s balanced saline solution (BSS). The whole corneas were removed from the embryos with fine forceps. At this stage of development (Hamburger-Hamilton stage, 27–29), the embryonic cornea is characterized by three regions.1 The anterior or apical surface is covered by the two-cell-layer epithelium, the center is composed of the primary stroma, and the posterior surface is covered by a single endothelial cell layer. These whole corneas were either fixed and stained immediately for actin, or the epithelial layer was removed either with the basal lamina (+BL) or without the basal lamina (−BL) with appropriate enzyme treatments. To remove the epithelium from the stroma, the whole corneas were incubated in enzyme in calcium-, magnesium-free BSS. After the appropriate enzyme treatment for 2–3 min at 37°C, the whole corneas were rinsed in calcium-, magnesium-free BSS, then the epithelia were removed from the stroma with fine forceps. Epithelia were isolated from the stroma +BL using 0.6 mg/ml Dispase II (Boehringer Mannheim, Indianapolis, IN) or −BL using 0.1 mg/ml trypsin and collagenase (Sigma, St. Louis, MO). The animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Actin Labeling

To prepare the embryonic corneal epithelia as whole mounts, each isolated epithelial sheet was placed on a black filter 3 mm in diameter (4-μm pore size; Poretics). Actin was visualized with fluorescently tagged phalloidin (Molecular Probe Inc., Eugene, OR) in phosphate-buffered saline (PBS) with 3.7% paraformaldehyde and 0.1% lysopalmitate. The epithelia were incubated in the rhodamine phalloidin for 30 min at room temperature followed by three 10-min rinses in PBS. The filters were placed epithelial-side up in antifade mounting media on slides that had four nail polish spacers, cover slipped, and viewed on an Axioplan microscope. The antifade mounting media was 90% glycerol/10% PBS/1 mg/ml p-phenylenediamine, pH 9.0,16 stored at −20°C. The nail polish spacers prevented compression of the epithelia, which was important for the determination of the height of cells with the motorized focus control. Whole corneas were incubated with the phalloidin fix mixture for 1 hr, washed, and viewed with the laser scanning microscope.

Extraction Buffers and Permeabilization

Several techniques were used to determine the best method for permeabilization of the cells with minimal disruption of the cytoarchitecture. The “cytoskeletal” extraction method was modified from Dr. Sheldon Penman’s laboratory.17,18 Briefly, freshly isolated epithelia were rinsed in PBS, then extracted with cytoskeletal buffer (100 mM NaCl/300 mM sucrose/10 mM PIPES (pH 6.8)/3 mM MgCl2/0.5% Triton X-100/1.2 mM phenylmethylsulfonyl fluoride, 100 mM NEM, and 4 mM vanadyl ribonucleoside complex) for 5 min on ice.17,18 Another extraction method was used in some experiments. The tissue was rinsed in PBS, dipped in PMET buffer (20 mM PIPES (pH
6.8)/5 mM EGTA/2 mM MgCl₂/0.5% Triton X-100) at 37°C five times for 2 sec each, and rinsed in PBS. In addition, some epithelia were fixed for 5 min in ice-cold methanol, rinsed in PBS, and then stained as described.

Confocal Scanning Microscopy

The specimens were analyzed with either the Bio-Rad MRC 600 Lasersharp confocal system (BioRad Microscience Division, Cambridge, MA) mounted on a Zeiss Axiophot microscope or the Leitz (Leica, Deerfield, IL) confocal laser scanning microscope. The images collected with the Bio-Rad instrument were accumulated without filtering (n = 50), averaged, and scaled by the computer. The aperture (pinhole) was set at the minimum size for optimal signal. The z-series optical sections were taken at either 1- or 2-μm intervals. These images then were analyzed, enhanced, and stored on an optical disc. Black-and-white photographs and pseudocolor images were generated and photographed. A description of the design and operating principles of the confocal microscope developed in England was published previously. The images collected on the Leica instrument also were accumulated without filtering (n = 8-10) and were not enhanced further by computer processing. The figure legends for each figure state which system was used to obtain the images.

Organ Culture

Corneal epithelia were removed as a sheet of cells from 6-day-old chick embryos by treating with 0.1 mg/ml trypsin and collagenase (Sigma) at 37°C for 2–3 min (–BL). The epithelia were dissected from the underlying stroma and placed basal-side down on a black polycarbonate filter (Poretics). The epithelial cultures were maintained at the air-media interface of organ culture dishes in Hank’s F12 media containing antibiotic and antymycotic agents. Cultures were incubated at 37°C in a humidified gas mixture (5% CO₂ and 95% air) for 2 hr in control media, in laminin (5 μg/ml), or in laminin with 2 μM CD. The laminin was extracted from the Engelbreth-Holm-Swarm mouse tumor (Collaborative Research, Bedford, MA). The CD (Sigma) was kept in a stock solution of 2 mM in dimethyl sulfoxide (DMSO, Sigma). Appropriate DMSO controls were cultured in parallel to the untreated control and CD treatment groups.

Results

General Characteristics of the Embryonic Corneal Epithelium

Unlike the adult corneal epithelium, the chick embryonic epithelium is two cell layers thick and contains a thin flat outer layer, the periderm, and a cuboidal basal cell layer (Fig. 1). The basal epithelial cells have a flat basal surface with a well-defined BL. Both cell layers are characterized by abundant secretory organelles that are necessary for the synthesis of the secreted glycoproteins that form the primary stroma. The chick corneal epithelium begins to differentiate after the lens vesicle completely buds off on day 3. Endothelial cells migrate between the primary stroma and lens on day 5 of development. Then, neural crest-derived fibroblast cells invade the primary stroma 1 day later and begin producing the secondary stroma.

Before the complicated attachment system of hemidesmosomes and anchoring fibrils develops on day 10, the chick epithelial basal cell surface interacts with the underlying stroma by connections to the BL through ECM receptor molecules such as integrin. The basal area of the cell is rich in many cell organelles such as RER, Golgi apparatus, secretory granules, mitochondria, and organized cytoskeletal structures. The cytoskeletal structures, such as tonofilaments, increase in number and complexity as the epithelium develops.

The periderm cells are large polygonal-shaped cells that can be more than 25 μm in diameter and 6–8 μm thick. These cells have an F-actin in the microvilli and another actin network at the basal cell junction area. The basal cells of the embryonic cornea range from 15–20 μm in height and are 5–10 μm in diameter, depending on the level of the optical section. There are approximately four basal cells beneath each periderm cell. The ER in the periderm cells appears to form a reticular network. These characteristics have been established with transmission electron microscopy and confocal analysis and are illustrated in the diagram in Figure 1.

Actin Distribution in Epithelia From Whole Corneas

To visualize the organization of the cytoskeleton in freshly isolated whole-mount corneal epithelia, fluorescently tagged phalloidin was used. The spatial distribution of the phalloidin was analyzed by the Leica confocal laser scanning microscope, and the scanned images were used to generate two- and three-dimensional views of the corneal epithelial actin cytoskeleton. The confocal microscope allowed viewing of a sequential series of focal planes along the vertical axis of the whole corneal epithelium, with each plane containing exclusively the fluorescence in focus. Figure 1 shows eight horizontal planes between the apical (Fig. 1A) and basal (Figs. 1G–H) regions of a typical field of cells, documenting the distribution of actin in epithelial cells from a whole cornea. The plane of each optical section is indicated in the diagram in Figure 1. Each cornea was isolated with some surrounding ectoderm. Therefore, an area in the center of the epiclulm...
Fig. 1. Diagram: schematic drawing of cells in an embryonic corneal epithelium showing the general characteristics of both periderm and basal cells. The periderm cells are large and flat (P), and the basal cells (B) are columnar. Both cell types have abundant RER and Golgi apparatus, and a well defined cytoskeleton. The fine lines represent actin, and thicker lines represent cytokeratin filaments. Each lettered (A–H) horizontal line indicates the level of each optical section in the z series. Micrographs: micrographs of the epithelial cell layers from a whole cornea viewed with the Leica laser scanning microscope. This z series is obtained after staining the freshly isolated whole cornea with phalloidin for 1 hr, then washing out excess stain. The actin in the periderm cells (A, B) is present in the microvilli and as a network surrounding the nucleus. At the periderm–basal cell interface another actin network is present (C), and in the center of the basal cells the phalloidin stain defined the cell borders (D, E). In the basal compartment the actin organized into bundles (arrow, G) that appear to be the actin cortical mat described previously with TEM methods (F, G). Scale bar = 10 μm.
Fig. 2. Micrographs of corneal epithelial cells that were stained with rhodamine phalloloidin, and optically sectioned with a Bio-Rad MRC 600 confocal microscope. In this figure all optical sections from one z series are illustrated. These micrographs correspond to the optical section levels indicated in the accompanying diagram. The corneal epithelium was isolated with the BL intact, stained with phalloloidin, optically sectioned (~2 μm), and photographed. The sections illustrate an area through periderm cells (A–C), the junction between the periderm cells and basal cells (D, E), the center of the basal cell (F–J), and the base of the basal cell (K–N). The arrow points to the same area in each picture at the different levels. The actin cortical mat (arrow, K, L) projects through several cells. The optical sections at the level of the filter (N) are negative for actin staining (N). Scale bar = 10 μm.
D–E). Below the basal cell nuclei, the actin formed another extensive network (Figs. 1F–H). This actin network corresponded to the previously described basal actin cortical mat.6,8,9 In the cross-sectional transmission electron micrograph, this actin network was present in many cells and appeared to align from cell to cell. The confocal images also indicate that the actin was aligned from cell to cell (Fig. 1G, arrow).

### Actin Distribution in Epithelia +BL

Embryonic corneal epithelia were removed from the underlying stroma +BL, placed on a black polycarbonate membrane, and immediately stained with fluorescently tagged phalloidin. The spatial distribution of phalloidin was analyzed with the Bio-Rad MRC 600 confocal scanning laser microscope. Figure 2 shows 14 horizontal planes between the apical (Fig. 2A) and basal (Figs. 2J–N) regions of a typical field of cells. The plane of each optical section is illustrated in the diagram. Each epithelium was isolated with some surrounding ectoderm. Therefore, a flat plane in the center of the epithelia was selected for confocal scanning. Because the epithelial sheets were not perfectly flat, the cells in the upper left quadrant appeared to be in a different plane than the cells in the lower right quadrant. Also, not all periderm cells absorbed the phalloidin marker equally. The F-actin distribution in this epithelium (+BL) was similar to the whole-cornea sample. The F-actin was prominent in the microvilli of the periderm cells (Fig. 2A) and in the organized network beneath the microvilli. As demonstrated in the whole-cornea confocal scans, another extensive actin network was found at the junction of the periderm and the basal cell layers (Figs. 2D–E). In the central region of the basal cells, the actin was limited to the cell membrane area (Figs. 2F–J). Below the basal cell nuclei, the actin formed another extensive network in the +BL isolated epithelia similar to the whole corneas. The confocal images also indicate that the actin was aligned from cell to cell. In addition, the area of greatest linear actin staining included four basal cells (Figs. 2K–M, arrows).

In the rest of this report, selected micrographs are chosen to illustrate the objectives of each experiment. A diagram with the focal planes indicated accompanies each series of micrographs.

### Actin Distribution in Epithelia Isolated −BL

Embryonic corneal epithelia were removed from the underlying stroma −BL, placed on a black polycarbonate membrane, and immediately stained with fluorescently tagged phalloidin. As described before, a central flat area was selected for optical sectioning. The level of the optical sections is illustrated in the diagram. The actin distribution in the periderm cells and at the periderm–basal cell interface appeared nearly the same in epithelia isolated +BL or −BL (compare Figs. 2A–C with Figs. 3A–D). By contrast, the actin in the basal compartment of the basal cells had a completely different distribution in cells isolated by the two methods. In epithelia isolated −BL, the basal actin cortical mat was disorganized, and the actin appeared as dense foci, indicating that the basal surface was convoluted into cellular projections or blebs (Figs. 3F–H). Heavy meromyosin staining of corneal epithelia isolated in a similar manner also revealed that the cell blebs contained abundant actin filaments.8,9 The confocal images showed that there were many blebs on the basal surface of each basal cell (Figs. 3G–H, arrows). In addition, the pseudocolor image at this plane of focus indicated that the intensity of staining was still as high or higher than cells isolated with the BL intact. These epithelial images were recorded at a lower magnification and smaller intervals than those in Figure 2. However, both sets of data (Figs. 2, 3) were obtained with the Bio-Rad MRC 600 confocal microscope.

### Actin Distribution in Epithelia −BL, Cultured in the Presence of Laminin

It previously was shown that the corneal epithelial cells reorganized the basal actin cortical mat in the presence of laminin within 2 hr.6,9 We asked, “Can this dynamic change in the actin organization be demonstrated with confocal microscopy?” The epithelia were isolated −BL and cultured on black membranes (0.4-μm pore size) for 2 hr, either with or without laminin (5 μg/ml). They were fixed and stained with phalloidin, then scanned with both the Bio-Rad and Leica confocal laser scanning microscopes, with similar results. Our data shown here were obtained with the Leica confocal microscope. The levels of the optical sections for Figure 4 are illustrated in the accompanying diagram. The letters on the diagram correspond to the micrograph of the same letter. There did not appear to be any difference in the actin distribution in the periderm cell and periderm–basal cell junction after epithelia cultured in control media or with laminin (Figs. 4A–B, 4E–F). However, the reorganization of the actin cortical mat was visible in cells incubated with laminin for 2 hr (Fig. 4H) compared with epithelia cultured in control media (Fig. 4D). The epithelia in control media continued to have a blebbing basal surface, and some of the blebs appeared to be in the pores of the filter (0.4 μm in diameter; Fig. 4D, arrows). The quantity of actin in the basal compartment appeared to be greater in epithelia cultured in the presence of laminin and more organized into a filamentous network (Fig. 4H).
Actin Distribution in Epithelia Isolated - BL, Cultured in the Presence of CD and Laminin

Previous experiments found that the laminin-stimulated actin reorganization of the basal compartment can be blocked by treating the cells with CD. The confocal image from a similar experiment showed that 2 μM CD aggregated the actin into brightly staining clumps in both cell layers (Figs. 4I-L). The periderm cell actin was found along cell borders (Fig. 4I); at the periderm-basal cell junction, however, the actin aggregated appear throughout the cytoplasm (Fig. 4J). In the center of the basal cells, the cell membrane-associated actin disappeared almost completely, with only a few isolated clumps delineating the cell borders (Fig. 4K). In the BMZ region of the epithelium, the abundant actin was seen as small intense dots (Fig. 4L). These brightly staining areas were actually cellular projections into the pores of the filter (Fig. 4L), indicating that the basal cell surface extended processes or blebs into the filter and was not responding to the presence of laminin in the media.

The Effect of Detergents and Other Membrane Disruptors on Phalloidin Staining

The cell membranes were permeabilized to allow the phalloidin molecule to reach the F-actin. Many procedures have been developed for this purpose, from fixing cells in cold alcohols to using various types of detergents in different stabilizing buffers. The phalloidin manufacturer (Molecular Probes) recommended the use of phosphatidyl choline or palmitate as the permeabilizing detergent. In addition, the cells were fixed with formaldehyde at the same time that they were stained. As part of this study, several other permeabilizing methods were used. Cold methanol fixation inhibited phalloidin staining nearly com-
Fig. 4. Corneal epithelia were isolated without a basal lamina (–BL) and cultured for 2 hr on black filters (Poretics). All micrographs were taken at 1 μm optical sections starting with the periderm (P) cells, the periderm–basal cell junction (P–B), in the center of the basal cells (B), and at the level of the actin cortical mat (Mat) in the basal compartment of the basal cells. The schematic drawing of the corneal epithelial cell shows the level of each optical section that corresponds to the micrographs. The actin distribution in the periderm cells and at the periderm–basal cell junction of the epithelium cultured –BL NT and –BL + Lam were similar, but the intensity of staining was higher in the –BL NT group than the –BL + Lam. This difference in intensity could be the result of not getting the same level of optical section in the two samples. The microvilli are not shown in (E) because this section is below the level of the microvilli; this does not indicate that the microvilli were absent. The periderm cells in the culture treated with CD (–BL + Lam + CD), however, are represented by large clumps or aggregates (1). The central region of the epithelia represented in this plate (B, C, F, G) are from similar optical planes and do illustrate that the actin distribution is similar between epithelia cultured in the presence or absence of laminin. But the epithelium cultured in the presence of CD and laminin (J, K) does have a different actin distribution. The periderm basal cell junction network was replaced with aggregates of actin (J), and the basal cell membrane-associated actin is almost absent (K). The basal compartment of the basal cells shows the most remarkable changes in response to culturing the cells in the presence of laminin. The epithelia cultured without ECM molecules (–BL NT) had blebs on the basal cell surface, seen as intense “dots” with the Leica confocal laser scanning microscope (arrow, D), whereas epithelia cultured in the presence of 5 μg/ml laminin (–BL + Lam) had an organized actin cortical mat in the basal cytoplasm (arrow, H). Epithelia that were treated with cytochalasin D and laminin (–BL + Lam + CD) did not have an organized actin cortical mat (arrow, L), and the actin is aggregated throughout the cytoplasm (I–L). Scale bar = 10 μm.

Discussion

Two confocal laser scanning systems were used to visualize the spatial distribution of actin in corneal...
epithelia from whole corneas, freshly isolated epithelia (+ or -BL), and cultured epithelia. In addition, the effect of permeabilizing agents and CD on actin distribution was determined. These experiments gave a fresh view of actin distribution in embryonic corneal epithelia. Optical sectioning through an epithelial sheet that is 20–30 μm thick was not possible a few years ago because of the thickness of the tissue. The current investigation showed that corneal epithelial actin was the same in the periderm cells and upper basal cells in whole cornea, freshly isolated epithelia, and cultured epithelia. By contrast, the basal compartment of the basal cells had a different actin distribution, depending on the presence or absence of BL. These experiments also found that the actin distribution changes in response to laminin were extensive in the basal compartment of the cultured epithelia. Furthermore, CD caused clumping of the actin throughout the tissue and prevented the laminin-stimulated reorganization of the actin cortical mat.

The actin distribution in the BMZ or basal area of the epithelial cells was different when the BL (Figs. 1, 2) was present versus when it was absent (Fig. 3H). These images are the first (to our knowledge) to indicate the extensive nature of the actin cortical mat. It appeared to connect cells and extend through several optical sections. In three-dimensional reconstructions, the actin cables projected basally from the nuclear region to form these bundles. This technique also illustrated the extensive actin network at the periderm–basal cell junction area (Figs. 1B, 2C–D) that was not described previously and was present regardless of the isolation method. Experiments also were done in which the cells were permeated with various detergents or other solvents. These permeation protocols caused a change in actin distribution, and cold methanol permeation completely degraded the actin staining, indicating that the actin may be indirectly or directly linked to the plasma membrane.

Fluoresceinated phalloidin binds specifically to F-actin and has been used widely in many cell types since the first description of the fluoresceinated compounds.20,21 The phallloidin molecule we used is a synthetic analogue of the natural poison, phallotoxin. Phalloidin is small bicyclic peptide containing seven amino acids (789 Daltons). Phalloidin and its derivatives bind both large and small F-actin filaments but do not bind monomeric actin.21

One advantage of confocal versus transmission electron microscopy is the time it takes to analyze the experiments. For example, previous experiments show that corneal epithelia responded to ECM molecules in 2 hr. These experiments were analyzed with transmission electron microscopy, a lengthy procedure. We asked, “Can the confocal microscope be used to assess the change in actin distribution in cultured epithelial cells?” Staining the cells after completion of the 2-hr laminin incubation took less than 1 hr. The findings were recorded on the same day as the experiment. These experiments, not only added new information to our knowledge of the widespread changes in actin reorganization, but also confirmed previous transmission electron microscopic observations. They also demonstrated the resolution that can be achieved in a short time. In addition, they established that the images seen with this technique reliably reproduced findings from previously used procedures. In the future, studying the actin distributional changes in living cells as they respond to ECM molecules might be possible.

As stated earlier, the actin cytoskeleton changes its distribution in response to many stimuli.11,22,23 For this discussion, our focus will be on how these proteins change in response to ECM and BL proteins. Most cells grown on plastic or glass produce an abnormal cytoskeleton that includes focal contacts and stress fibers.11,24 This in vitro cytoskeletal structure has been used to study cell-substrate adhesion. When the ECM receptors are bound to the substrate, they change the actin cytoskeleton distribution by an unknown mechanism.11,24 This is well documented in the corneal epithelial organ culture system5,8,9 and in cells grown in traditional tissue culture.11,22–24 The proteins involved may include α-actinin, vinculin, talin, and a calcium-binding protein,11 but the exact link has not been established. Vinculin and α-actinin disassociate from the focal contacts when the cells lose their attachment to the integrin receptor.23 In adult rat corneal epithelium, vinculin synthesis increases 8–27-fold in response to wounding,10 and the actin cytoskeleton reorganizes,23 indicating that these proteins are necessary for the corneal epithelial cells to migrate over the denuded stroma. In future experiments, we hope to determine the identity of actin-associated proteins that are localized to the basal actin cortical mat of the embryonic avian epithelial cells with this method.

In previous studies, evidence was provided for the hypothesis that actin reorganization mediates the metabolic stimulation of corneal epithelia.8,9,13 Corneal epithelial sheets isolated +BL or −BL were cultured in the presence of laminin and CD. These experiments found that laminin-mediated bleb retraction, basal actin cortical mat reformation, and increase in collagen production required intact actin microfilaments.9 Epithelia isolated with +BL maintained a flat basal surface when treated with CD, even though the F-actin cortex was destroyed and collagen production decreased. Morphometric analysis of these epithelia showed that the RER was displaced from the basal
cell compartment, presumably because the attachment to F-actin was lost. It also was determined that disrupting the microtubules with nocodazole did not inhibit bleb extension or retraction, suggesting that the actin cytoskeleton could organize independently of microtubule integrity. We showed that treating the cells with CD disrupts actin distribution throughout the epithelium. The actin aggregates were seen in both the periderm and basal cell layers.

In conclusion, confocal laser scanning microscopy allows us to examine the in situ actin cortical mat in whole corneas and freshly isolated epithelia +BL and -BL. This technique also provides a rapid method for assessing the actin cytoskeleton during and after culture experiments. The results obtained with this method confirm and increase the information obtained with transmission electron microscopy about the distribution of actin in embryonic corneal epithelial cells. Finally, this method allows us to examine the system by optical sectioning en face rather than using the cross-sectional view commonly seen when the cornea is sectioned for transmission electron or light microscopic analysis.

**Key words:** embryonic, cornea, actin, confocal, basal lamina

**Acknowledgments**

The author thanks Miss Laura Rooney for technical assistance; Dr. Douglas Cotanche for help in preparing the video tape and encouragement throughout this project; the Leica representatives, Tim Kearney, and Dr. Martin Hoppee for the use of their confocal laser scanning microscope and their assistance in producing these images; Charles Blanchard, the Bio-Rad representative, and Dr. Karl Matlin from Harvard Medical School for their assistance and use of the Bio-Rad microscope; and Dr. Elizabeth D. Hay for guidance, encouragement, and the use of Harvard Medical School's Bio-Rad confocal microscope.

**References**