Effects of cytochalasins and colchicine on the ultrastructure of migrating corneal epithelium

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To determine the effects of cytochalasins and colchicine on the ultrastructure of migrating corneal epithelium of the rat, abraded corneas healing in organ culture were cultured for 30 min in the presence of these cytoskeletal-perturbing drugs. Transmission and scanning electron microscopy of these corneas indicate the following. (1) Cytochalasins B (1 µg/ml of medium) and D (0.1 µg/ml of medium) caused drastic alteration in structure of cells of the leading edge only. Stratified layers of cells behind the leading edge were unaffected. This observation may indicate that it is the cells of the leading edge which have a rapid turnover of actin filament formation during epithelial sheet movement. (2) Both cytochalasins caused surface blebs or zeiotic processes to form on cells of the leading edge. The shape of the processes caused by the cytochalasins differed, however. Actin filaments accumulated in the cytoplasm under the zeiotic processes. (3) Colchicine had no effect on the ultrastructure of migrating epithelium. (INVEST OPHTHALMOL VIS SCI 22:643-650, 1982.)

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In the preceding paper we reported that cytochalasins B and D reversibly inhibited both initiation and continuation of corneal epithelial wound healing and that colchicine did not. Cytochalasin B (cyto B) inhibits actin filament formation by preventing addition of monomeric actin molecules at the forming end of the filament.1 2 Cytochalasin D (cyto D) has similar effects,3 but its action is less clear.2 Colchicine, on the other hand, binds to tubulin monomers, the protein subunits of microtubules, and brings about microtubule depolymerization. Thus morphological changes caused by each of these drugs may indicate where the greatest activity in actin filament or microtubule function, formation, and turnover occurs in normal and migrating corneal epithelial cells.

This report describes the short-term effects of these drugs on the surface ultrastructure of migrating and normal epithelial cells and on the filament distribution within their cytoplasm. The cytochalasins caused blebbing of the cell surfaces and disruption of cytoplasmic filament arrangement of only the leading edge of migrating epithelium. Cells behind the leading edge were unaffected. Colchicine had no visible effect on the ultrastructure of migrating or normal cells.

Materials and methods

Male Sprague-Dawley rats with clear healthy corneas, as determined by biomicroscopy, were
Fig. 1. Electron micrographs (EMs) of healing control corneas organ-cultured 12 hr after receiving a 3 mm, central epithelial abrasion. A, Low-magnification scanning EM of entire wound area shows remaining central denuded basal lamellae (BL and arrows) and leading edge (LE) of migrating epithelium. The basal lamellae appear wrinkled and folded due to critical-point drying. Superficial cells (SC), which appear to be loosened or desquamating, are inside the abraded 3 mm area. (×260.) B, Scanning EM of flattened cells of leading edge (LE) and denuded basal lamellae (BL). Cells behind LE are oblong. (×320.) C, Transmission EMs of flattened lamellipodia of cell adjacent to denuded basal lamellae. (×12,500.) Inset, Another lamellipodium with actin filament (AF) network in cytoplasm. (×45,000.) D, Higher-magnification scanning EM of flattened cells at leading edge. Broad lamellipodia (LP) are present on these smooth-surfaced cells. Cells directly behind leading cells have sparsely arranged microvillae (MP). (×1450.)

Rats were killed by intraperitoneal injection of phenobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.). For studies on migrating epithelium, 3 mm diameter central abrasions were made on the corneal epithelium with eyes still in place. The area was outlined with a trephine, and epithelium within the circle was scraped away with a rounded Bard-Parker blade. Abraded and unabraded eyes for studies on normal epithelium were enucleated, corneas with scleral rims excised with a razor blade, and irides removed with a forceps.
Fig. 2. EMs of abraded corneas cultured 12 hr and placed in medium containing 1 μg/ml cyto B for 30 min. A, Low-magnification scanning EM of entire wound area. BL and arrows, Remaining denuded basal lamellae. As in Fig. 1, a ring of loose, superficial cells surrounds the leading edge. (×26). B, Scanning EM of leading-edge cells (LE) and denuded basal lamellae (BL). Cyto B caused rounding and blebbing of leading edge cells, but cells just behind the leading edge are smooth-surfaced (between arrows). (×375). C, Transmission EMs of cell at leading edge. Note surface blebs, or zeiotic processes (ZP). Just below ZP surface is an electron-dense accumulation of actin filaments (AF) (arrows). These dense areas are connected under the membrane by a thin, less dense strand of filaments. The tip of the cell, which probably represents the retracted lamellipodium, is electron-lucent with sparse cytoplasm and no filaments. (×4800.) Inset, Higher-magnification of dense actin filament mass beneath a ZP. (×18,500.) D, Higher-magnification scanning EM of grapelike clusters of ZPs of leading edge cells. (×1300.)
Organ culture and drug treatment. Excised abraded corneas were organ-cultured to obtain actively migrating corneal epithelium. Culture medium and antibiotic-antimycotic additives have been described. Corneas were cultured for 12 or 15 hr at 35°C with 5% CO₂ and then placed in medium containing cyto B, cyto D, or colchicine. Normal unabraded corneas were placed in medium for 30 min to 1 hr before exposure to the drugs. Cyto B was used at a concentration of 1 μg/ml of medium and was solubilized in dimethylsulfoxide (DMSO). Cyto D was used at 0.5 μg/ml of medium and was also dissolved in DMSO. The final DMSO levels were 0.01% and 0.04%, respectively. Colchicine was used at a concentration of 40 μg/ml of medium. Corneas were prepared for either transmission or scanning electron microscopy after culture for 30 min with drugs. Corneas cultured without drugs or with 0.04% DMSO served as controls. Each experimental group contained two corneas.

Transmission electron microscopy. Cultured corneas were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, washed three times, and cut into halves. They were then postfixed in osmium tetroxide in the same buffer and stained en bloc with 2% aqueous uranyl acetate. After a final wash, the corneas were dehydrated through an acetone series and embedded in Epon-Araldite.

Scanning electron microscopy. Whole corneas were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, for 1 hr. They were then washed three times in the same buffer and dehydrated through an ethanol series. Ethanol was exchanged for anhydrous CO₂ in a critical-point drying apparatus during a 10 min purge. Mounted samples were coated uniformly with gold-palladium for 3 min at 22 mA. Photographs were taken on a JEOL-35 or AMR 1000A scanning electron microscope.

Results

Controls. Corneal epithelium that migrated across the central, round, 3 mm abraded area in organ culture resembled that in vivo, except that loose desquamating cells were present behind the leading edge, probably owing to the absence of lid abrasion. Cells at the leading edge of migration were flattened and had smooth-surfaced, broad lamellipodia at the edge adjacent to the denuded basal lamellae (Fig. 1). The surface of the leading cell appeared entirely smooth, whereas cells behind it had sparsely distributed microvilli (Fig. 1, D). Thin fingerlike protrusions known as filopodia were rarely seen on the migrating cells of rat corneal epithelium. As has been described previously, within the cytoplasm of cells of the leading edge of rat corneal epithelium, actin filaments were found in networks in the lamellipodia and in bundles either at the basal surface of cells or both basally and apically. Occasionally microtubules are also seen in the cytoplasm of cells of the leading edge but not in leading lamellipodia.

Cultured healing corneas had a ring of loose superficial cells around the leading edge of migrating epithelium. This ring (as determined by planimetry measurements) did not coincide with the trephine line made at the time of abrasion. These loose cells were within the 3 mm region and may represent the original wound edge, carried into the wound by mass epithelial migration. If so, it appears that superficial cells do not move down to participate in epithelial sheet movement. DMSO had no effect on the ultrastructure of migrating epithelium.

Cytochalasins. Cultured healing corneas exposed 30 min to cyto B and cyto D showed drastic ultrastructural changes in the cells of the leading edge (Figs. 2 and 3). The effects of the two cytochalasins were quite different, however. In corneas exposed to 1 μg/ml cyto B, cells of the leading edge developed grape-like clusters of round protrusions, or blebs, on their apical surfaces (Fig. 2). This phenomenon, called zeiosis, has been observed in other cell types. Cells at the leading edge were rounded or retracted from their normal, flattened state, and a marked alteration and redistribution of membrane-associated actin filaments accompanied the rearrangement at the apical cell surface. Fig. 2, C, shows masses of actin filaments at the base of the surface blebs, or zeiotic processes. These filaments were identified as actin on the basis of size and similarity to structures previously reported and histochemically labeled after cyto B treatment.

Cyto B affected only cells of the lead-
Fig. 3. EMs of healing corneas incubated for 30 min with cyto D (0.5 μg/ml) before fixation. A, Low-magnification scanning EM of whole abraded area. Remaining denuded basal lamellae (BL) are indicated by arrows. Ultrastructural alteration of leading edge (LE) can be seen even at this low magnification. Ring of superficial cells (SC) can be seen also. (×26.) B, Scanning EM of leading edge (LE) shows drastic effects of cyto D on these cells. Zonotic processes (ZP) are structurally different from those caused by cyto B. These processes are cytoplasmic blebs with long cytoplasmic strands connecting them to the cell body. As with cyto B, cyto D did not affect cells behind the leading edge; they have a flat, normal structure (between arrows). (×440.) C, Transmission EM of leading edge cell shows zonotic process (ZP) connected to cell body by a thin cytoplasmic strand (CS). As with the cyto B–induced ZPs, a mass of actin filaments (AF) is present at the ZP base. ZP cytoplasm is sparse and no filaments are discernible. (×8500.) D, Higher-magnification scanning EM of leading edge shows layers of ZPs. (×800.)
Fig. 4. EMs of abraded rat corneas cultured 15 hr and then placed in 40 μg/ml colchicine for 30 min. A, Scanning EM of entire wound area. Remaining defect is indicated by BL (basal lamellae) and arrows. As in all other corneas shown at this magnification, loose, superficial cells (SC) are present behind the leading edge (x26.) B, Cells of leading edge are flat and have broad lamellipodia (LP) as in controls. BL, Basal lamellae. (x480.) C, Transmission EM of lamellipodia shows flattened state and filament network in the presence of colchicine. (x15,000.) D, Higher-magnification scanning EM demonstrates flattened lamellipodia (LP). (x1400.)

The zeiotic processes produced by cyto D were structurally different from those produced by cyto B (Fig. 3). The former were present at the extreme tip of leading edge cells, as if formed from the lamellipodium. They consisted of cytoplasmic blebs connected to the cell of origin by a long, thin, membrane-bound cytoplasmic strand. As with cyto B zeiotic processes, actin filament masses

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were present at the base (Fig. 3, C). Cyto D had no effect on microplicae of superficial cells behind the leading edge (not shown) or on overall structure of cells behind the leading edge (Fig. 3, B).

**Colchicine.** Culture of migrating corneal epithelium in 40 μg/ml colchicine had no effect on the ultrastructure of cells at the leading edge (Fig. 4). Cells maintained their flattened lamellipodia and smooth apical surface and showed no change in filament distribution. As with the cytochalasins, colchicine had no effect on stratified cells behind the leading edge.

**Discussion**

Movement of an epithelial sheet is clearly of considerable importance in corneal wound healing. It remains unresolved, however, where within the sheet of cells the force for such movement is generated. At least four possibilities exist. (1) All the cells of the sheet contribute to its movement. (2) The majority of the sheet is towed by the locomotory activity of cells of its leading edge. (3) The motile force is provided by a push from cells behind the leading edge, with cells of the leading edge functioning to explore the substrate and gather information. (4) Cells behind the leading edge push, and cells of the leading edge pull.

Data from this and the preceding paper, plus data on actin filament distribution in cells of normal and migrating epithelium previously reported, support the hypothesis that cells of the leading edge tow the sheet (possibility No. 2) or provide a portion, but a required one, of the force needed for movement (possibility No. 4). We define the leading edge as those cells adjacent to the wound area which are flattened with their long axis oriented parallel to the basal lamellae. Although the very tip of the leading edge is only one cell extending out from under other cells, the major portion of the leading edge is two, three, or four cell layers thick, grading into the normal stratified epithelial tissue. Cells of this leading edge, including basal cells and cells lying above them, are differentiated from nonmigrating, stratified epithelial cells in that they have within their cytoplasm an arrangement of actin filaments which suggests that these cells are capable of movement. This arrangement includes actin filament networks and actin filament bundles, and they are present within the cytoplasms of all cells of the leading edge. Cells behind the leading edge do not have such an arrangement of actin filaments. In normal epithelium, the only area with dense actin filaments is the region beneath the microplicae of superficial cells. The cytochalasin experiments reported here demonstrate that an intact actin filament system is required for sheet movement, and since that network is present only in the leading edge, it follows that the leading edge provides motile force for movement of the sheet. Buck states "In the absence of firm attachment of the leading cell (i.e., absence of hemidesmosomes) it is difficult to conceive of any significant traction exerted by them". If one examines closely electron micrographs of the leading edge cells adjacent to the basal lamellae, one can see very close and tight association of the membrane to the basal lamellae. Perhaps such areas of tight adhesion are of a different biochemical and structural nature than hemidesmosomes, but yet serve the same function.

Cytochalasins prevent addition of G (globular) actin monomers to the forming end of actin filaments; thus regions of tissue that show ultrastructural disruption by these drugs probably are regions in which there is active turnover and formation of actin filaments. The fact that only cells of the leading edge are structurally affected by cyto B and cyto D indicates that there is rapid formation of actin filaments in these cells. In contrast, microplicae of nonmigrating, superficial cells are unaffected by cyto B and cyto D at 30 min exposures, which indicates that actin filament networks under microplicae are stable or have a slower turnover rate and hence are not affected by the 30 min exposures we used. Perhaps such a stable microplicae structure is required to maintain a stable tear film.

Microtubules are essential for development and maintenance of cell shape, polarity...
and orientation of organelles, directionality of movement, intracellular translocation of cellular organelles, and restriction or regulation of cell contractility. Most of these functions are thought to be performed in combination or association with actin filaments, and Godman and Miranda state "The proposal that microtubules constitute an opposing skeletal system, upon or against which the contractile elements may act, appears to be generally valid." Data presented here and in the previous paper showing that colchicine had no effect on motility and corneal epithelial cell structure indicate that microtubules do not provide such an "opposing skeletal system" for corneal epithelial cells. Epidermal epithelial cells do not require microtubules for motility either.

In summary, an actively forming actin filament system appears necessary for motility and shape of cells at the leading edge of migrating corneal epithelium. Our data indicate that cells of the leading edge provide a motile force required for pulling the epithelial sheet.

REFERENCES