Different Corneal Epithelial Healing Mechanisms in Rat and Rabbit: Role of Actin and Calmodulin

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The authors investigated the effects of calmodulin inhibitors, trifluoperazine (10–20 µM) and W-7 (25–50 µM), and of cytochalasin B (5 µg/ml) on the F-actin distribution, surface morphology, and migration of rat and rabbit corneal epithelial cells in tissue culture. In the rat, actively migrating cells have abundant F-actin-containing stress fibers and numerous cytoplasmic extensions of the plasmalemma. These features, and ultimately cell migration, are inhibited by calmodulin inhibitors and cytochalasin B. In the rabbit, migrating cells are devoid of stress fibers and cytoplasmic extensions. Cell migration is not inhibited by calmodulin inhibitors but is arrested by cytochalasin B. The cell-to-substrate adhesion is reduced by calmodulin inhibitors in both rat and rabbit. These findings corroborate our earlier observations in organ culture studies and support the view that corneal epithelial cell migration is calmodulin-dependent in the rat, while it is not in the rabbit. The complete blockage of migration in both species by cytochalasin B suggests that actin polymerization is critical for corneal epithelial locomotion in both species. Invest Ophthalmol Vis Sci 26:838–848, 1985

Actin and its role in nonmuscle cell motility is well documented.1,2 Its presence, distribution, and role in epithelial movement during wound healing have been studied with myosin subfragment-1 labeling in the rat corneal epithelium by Gipson and Anderson.3 They observed that actin filaments in normal rat corneal epithelium form an apical network under microvilli of superficial cell layers. In contrast, actin filaments in migrating epithelium were concentrated in basal regions of cells. Here they were arranged in parallel bundles of filaments extending back into the cell from the leading edge and as dense networks at leading edges and within cellular processes. Gipson et al also reported that corneal epithelial movement in rat is inhibited by cytochalasins B and D, both antagonists of actin polymerization.4

Calmodulin (CaM) found in all eukaryotic cells so far examined, is a multifunctional, calcium-binding regulatory protein that mediates many Ca2+-related cellular events.5 The involvement of CaM in the control of cell motility in nonmuscle cells is well documented.6 Also, CaM is intimately associated with actin-binding proteins7-9 and has been identified as a regulator of myosin light chain kinase and Ca2+-activated myosin ATPase activities.6,10,11 The effects of CaM inhibitors, trifluoperazine (TFP) and N-6-aminohexyl-5-chloro-1-naphthalene sulfonamide (W-7), on corneal epithelial migration in rat and rabbit have been studied in wounded organ-cultured corneas.12 In that study, TFP and W-7 was found to inhibit migration in rat in a dose-dependent manner but had no effect in rabbit. Also, cytochalasin B (CB) was found to completely inhibit corneal epithelial cell migration in both species.4,13

We report here striking differences between the healing corneal epithelium in rat vs rabbit, vis-a-vis the filamentary actin (F-actin) distribution and the alterations of this distribution in response to CaM inhibitors in cell culture. These findings corroborate our previous wound-healing studies in organ culture and demonstrate that the corneal epithelial movement in rat is CaM-dependent, while it is not in rabbit.

Materials and Methods

Cells

Adult male Sprague-Dawley rats and albino rabbits were treated in accordance with the ARVO Resolution on the Use of Animals in Research. Full-thickness
sheets of pure rat and rabbit corneal epithelium were removed using Dispase II. The sheets were allowed to adhere to the substratum on tissue culture slides (Miles Laboratories; Naperville, IL) and the cells were allowed to migrate for 2 to 3 days. Confluency was avoided. Cells were cultured at 37°C and 5% CO₂ in Eagle's minimum essential medium (M. A. Bioproducts; Walkersville, MD) with nonessential amino acids, L-glutamine, penicillin, streptomycin, amphotericin B, and 10% fetal calf serum. Cells were grown on both glass and collagen (Vitrogen 100, Flow Laboratories; McLean, VA) in order to assure that our results were not influenced by peculiarities in one type of substratum. The cells were initially cultured in drug-free medium for 2 to 3 days until there was sufficient spreading and migration onto the substratum. For the drug studies, this medium was then replaced with medium containing TFP, W-7, or CB, and the cells were cultured for an additional 24 hr before examination.

Drugs

TFP (a gift of Smith, Kline, & French; Philadelphia, PA) or W-7 (CAABCO, Inc.; Houston, TX) was dissolved in the cell culture medium and protected from light. Drug-free medium served as the control for TFP and W-7, an inactive analogue of W-7, was used as a control for W-7. CB (Sigma Chemical; St. Louis, MO) was solubilized in 0.1% dimethylsulfoxide (DMSO) before being added to the medium. DMSO at this concentration has been found to have no effect on corneal epithelial migration and ultrastructure. DMSO (0.1%) alone, dissolved in drug-free medium, served as the control for CB.

Fluorescence and Scanning Electron Microscopy

Cultured cells on slides were washed in phosphate-buffered saline (PBS), pH 7.0, fixed in 3.7% formaldehyde for 10 min at 20°C, washed again in PBS, and extracted with acetone at −20°C for 4 min. The cells were then immersed in the dark for 40 min in NBD-phallacidin (Molecular Probes; Junction City, OR), the F-actin binding fluorescent derivative of the mushroom phallotoxin, used at a dilution of 165 ng/ml. As a control for the specificity of the staining, the cells were preincubated with excess nonfluorescent phalloidin (Sigma Chemical; St. Louis, MO) (3.3 mg/ml) to saturate the phallotoxin-binding sites. The cells were then post-treated with fluorescent phalloidin. A faint autofluorescence was seen in the cells of the control specimens (Fig. 1). Fluorescence and phase-contrast microscopy were carried out in a Zeiss photomicroscope III.

Specimens for scanning electron microscopy were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, rinsed in 0.2 M sodium cacodylate buffer following fixation, dehydrated with a graded series of alcohols, and critical-point dried in CO₂. The samples were sputter coated with gold and viewed with an AMR-1000 scanning electron microscope.

Measurement of Epithelial Migration in Cell Culture

The length of the path of cell migration in 48 hr was measured in cell cultures of corneal epithelium of rat and rabbit, on both glass and collagen substrata. For these experiments, cells were cultured on special tissue culture dishes with grid markings of 2-mm squares on the bottom (Nunc C; Roskilde, Denmark). Ten rat and 10 rabbit cell cultures were used for this purpose. Half of the cultures from each species were grown on glass and half were grown on collagen substrata. At 24 hr after explantation, upon sufficient attachment of the cells to the substratum, the position of the leading edge relative to the grid markings was
determined at four points (spaced 500 μm apart). These readings were further facilitated with a micrometer reticle on the ocular eyepiece of the microscope (magnifications of ×100 and ×350). Forty-eight hours after starting the measurements, the linear displacements of the four predetermined points were measured. In performing these measurements, several assumptions were made: (1) the direction of cell movement is perpendicular to the leading edge, and (2) all parts of the leading edge move at the same rate. Measurements of rate were averaged separately by species and substratum type. Statistical analysis was done using the nonparametric Mann–Whitney U-test.

Results

Cell Morphology, F-Actin Distribution, and Migration in the Controls

The drug-free controls of rat and rabbit corneal epithelium in cell culture migrated well on either glass or collagen substrata but displayed prominent differences. In rat, the leading edge of the migrating epithelial cells was lined with cells possessing abundant lamellopodia, filopodia, and ruffled membranes, as characteristic of motile cells. Cells in this region were richly endowed with networks of parallel and crisscrossing arrays of actin-containing stress fibers existing
in multiple focal planes (Fig. 2). Stress fibers often terminated in fluorescence-dense ends on the plasmalemma (Figs. 3, 4) corresponding to membrane-associated adhesion plaques (see Discussion).13-18 Such terminations were especially numerous within lamellopodia and ruffled membranes where stress fibers were abundant. Filopodia stained intensely for F-actin. Within the cytoplasm, stress fibers converged to fluorescence-dense focal points (Fig. 3) corresponding to cytoplasmic dense bodies.15-18 The intracytoplasmic localization of these focal points was confirmed by focusing the microscope up and down. The central region, well behind the leading edge, was at least 4 to 5 cell layers thick, in contrast to the leading edge, which was usually tapered to a single cell layer thickness. The cells in the central region possessed fewer stress fibers (Fig. 5) than their counterparts at the leading edge and there was an increased concentration of F-actin in the cell periphery. The F-actin distribution in cultured rat corneal epithelial cells, especially with respect to stress fiber formation and distribution, was similar to that seen in other cell types.16,19-23

In the drug-free controls of rabbit, the migrating corneal epithelium in cell culture appeared markedly different from rat. In rabbit, the cells at the leading

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**Fig. 3.** Actin staining within a lamellopodium in an epithelial leading edge cell of rat. Numerous stress fibers terminate in fluorescence-dense ends on the cell membrane (arrowheads) and in intracytoplasmic focal junctions (asterisks). Ruffled membranes (large arrows) are visible (bar = 2 µm).

**Fig. 4.** Actin distribution at leading epithelial edge in rat. Note abundance of stress fibers, some with membrane-associated terminals (large arrow). Filopodia containing actin stress fibers (small arrows) and ruffled membranes (curved arrows) are present (bar = 10 µm).
Fig. 5. Actin distribution in corneal epithelial cells (rat) in the central region away from the leading edge. Stress fibers here are fewer than at the leading edge. F-actin is predominantly concentrated in the cortical cytoplasm, thereby delineating the cell borders (bar = 10 μm).

Effects of CaM Inhibitors and CB on Cell Migration and F-actin

CaM inhibitors had a profound effect on cultured rat corneal epithelium but had no effect in rabbit. In rat, incubation of the migrating cells with TFP (10–20 μM) or W-7 (25–50 μM) was associated with changes in F-actin distribution, cell shape, and migration. The morphologic alterations were more pronounced at the leading edge than in the region behind it; but, nevertheless, stress fibers were markedly reduced everywhere, leaving only a diffuse residual cytoplasmic fluorescence (Fig. 8). In the majority of cells, F-actin was now concentrated in the cortical cytoplasm, thus somewhat resembling the appearance of the drug-free controls of rabbit. Lamellopodia, filopodia, and ruffled membranes were markedly reduced in numbers. Scanning electron micrographs of CaM inhibitor-treated epithelium showed a total loss of microvilli in the leading edge cells, with the cell surfaces abnormally smooth in comparison to the drug-free controls (Figs. 9 A, B). Lamellipodia, ruffled membranes, and other cell membrane extensions were conspicuously absent. Advancing edges of cells in this area were often curled off the substratum because of reduced cell-to-substratum adhesion. In contrast, the cell surface morphology in the regions away from the leading edge appeared relatively unaffected by CaM inhibitors, as illustrated by intact microvilli and microvilli (Fig. 9C). Furthermore, epithelial migration was completely inhibited by TFP and W-7 in rat.

When these cells were washed free of the drug, they restored their original shapes; reformed arrays of stress fibers; reextended lamellopodia, filopodia, and ruffled membranes; and resumed migration (micrographs not shown). Concentrations of TFP > 40 μM and W-7 > 50 μM irreversibly inhibited stress fibers and migration.

In contrast to rat, TFP (10–20 μM) or W-7 (25–50 μM) in rabbit produced no discernible changes in actin distribution, cell shape, and migration (micrographs not shown). At higher doses of CaM inhibitors, however, cell migration was irreversibly arrested. Moreover, the cells were often detached as a sheet from the substratum. These irreversible changes are similar to those seen at higher drug concentrations in rat and corroborate the toxic reactions seen in organ cultured corneas at the higher doses of CaM inhibitors used.13

CB (5.0 μg/ml) completely, but reversibly, arrested migration of both rat and rabbit cells in culture. In rat, CB caused loss of stress fibers. In both rat and rabbit, CB caused cell rounding (Fig. 10). In rat, multiple fluorescent spots were diffusely scattered throughout the cytoplasm, while in rabbit, similar fluorescent spots delineated the cell membranes. These spots correspond to focal areas of greatest cell-to-substratum adhesion.24

Discussion

Actin is a major constituent of eukaryotic cells, accounting for about 10% of the total proteins in nonmuscle cells.25 Fluorescent antibody techniques have revealed actin, myosin, alpha-actinin, and other accessory contractile proteins within stress fibers.26 Stress fibers have been observed in vitro and in situ,26 and have been strongly implicated in cell migration.25,27 Cytoskeletal shape changes,28 and adhesion to substrata.25,29 Although actin is universally involved.
in eukaryotic cell motility, there is evidence that stress fibers, per se, are not.\textsuperscript{30} Our study compares the migration of cultured corneal epithelial cells from two mammalian species, specifically with respect to F-actin localization, stress fiber formation, and regulation by CaM. In rat, migrating cells were characterized by numerous plasmalemma extensions and rich arrays of stress fibers, especially in the leading edge cells. According to DiPasquale,\textsuperscript{31} the epithelium in a culture is attached to substratum only at the leading edge, where the outward movement of the leading cells generates tensile forces for epithelial spreading. Whether tensile forces are generated by the stress fibers themselves or whether they are formed passively in response to exogenous forces remains unclear. Studies have shown these structures to generate contractile forces in vitro.\textsuperscript{32} In our study, focally fluorescent stress fiber endings corresponding to adhesion plaques (dense plaques) and cytoplasmic dense bodies were abundant in rat. Adhesion plaques were most numerous within cytoplasmic extensions and are associated with focal zones of stress fiber-cell membrane interactions. These interactions may govern movement of the cell membrane and adhesion of the cell to the substratum.\textsuperscript{15} Stress fibers also insert into cytoplasmic dense bodies.\textsuperscript{15,33} These focal junctions may possibly serve as central anchoring points whose function is to organize and distribute tensile forces.

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extensions, stress fibers, adhesion plaques, and dense bodies. Despite these differences, cells from both species migrated equally well on a given substratum. As a rule, migration was significantly faster on collagen than on glass.

Since stress fibers disappear upon treatment with CaM inhibitors in the rat, the formation of stress fibers appears to be either directly or indirectly controlled by CaM. Moreover, the disappearance of these structures is accompanied by the loss of cytoplasmic extensions and ultimately, the complete cessation of migration. In contrast, in the migrating rabbit epithelium, normally devoid of stress fibers and cytoplasmic extensions, CaM inhibitors did not alter the existing F-actin distribution or the cell surface morphology; most remarkably, however, CaM inhibitors did not affect cell migration. These observations corroborate organ culture studies in which TFP and W-7 inhibited corneal epithelial movement in the rat but not in the rabbit. CaM inhibitors reduced cell-to-substratum adhesion in both species and this may be related to the inhibition of stress fibers. In organ cultures of corneas, cell-to-substratum adhesion is further reduced by the inhibition of hemidesmosomes by CaM inhibitors.

Conneal epithelial wound healing consists of a premitotic movement of the epithelium to cover a defect as quickly as possible, followed by a mitotic phase to restore the cell population. A combination of individual cell migration, cell enlargement, and alterations in cell shape may contribute to the initial premitotic movement. Perhaps in the rabbit, the premitotic phase of epithelial wound healing is comprised primarily of cell enlargement and alterations in cell shape to move the tissue in a sliding fashion. In sheet-sliding movements, originally described in rabbit corneal epithelium, no specific cytoplasmic constituents supporting cell movement, such as alterations in the arrangement or density of cytoplasmic filaments, were observed. It is therefore plausible that stress fibers may not play a significant role in these alternative mechanisms. Aside from rabbit corneal epithelium, there are other motile cells, such as neural crest cells, granulocytes, monocytes, and amebas, that do not form stress fibers during migration. Herman et al postulated that stress fibers per se may not be essential for motility, but that these structures

### Table 1. Mean length of epithelial migration (μm) at 48 hr on glass and collagen substrata

<table>
<thead>
<tr>
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<th>Rat</th>
<th>Rabbit</th>
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<tr>
<td>Glass</td>
<td>832 (82)*</td>
<td>805 (63)</td>
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<tr>
<td>Collagen</td>
<td>988 (146)</td>
<td>1051 (232)</td>
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* (Standard deviation)
Fig. 9. Scanning electron micrographs of the leading edge in rat. A, Drug-free control shows multiple cytoplasmic extensions of the cell membrane (filopodia, lamellodia, and microspikes). B, After treatment with TFP 20 μM, note the smooth surface morphology, the detachment of the cell edges from the substratum, and the loss of all cytoplasmic extensions. Tiny cracks on the cell surface are artifacts (bar = 1 μm).
which are intimately associated with adhesion plaques, have a primary role in anchoring the cell to the substratum and not in generating the actual loco-
motive force itself.\textsuperscript{39} It would follow that the extensive stress fiber pattern at the leading edge of rat corneal epithelium may signify that this region has the greatest cell–substratum adhesion as DiPasquale\textsuperscript{31} suggested. The region behind the leading edge, having fewer stress fibers and consequently less cell–substratum adhesion, would then be the zone of maximum sliding movement with the leading edge functioning as an anchor. The leading edge itself continuously seeks new sites of adhesion by extending pseudopodia. Stress fibers have been observed around “wounds” made in confluent rat aortic cells in culture\textsuperscript{39} and in corneal endothelium and lens epithelium of frogs and rats in situ adjacent to a wound.\textsuperscript{19} A similar statement does not apply to rabbit corneal epithelium which exhibits no obvious stress fibers or focal contacts.

Our results suggest that corneal epithelial migration in rats is CaM-dependent, while it is not in rabbit. CaM inhibitors appear to block migration best in cells that elaborate stress fibers during spreading. For instance, in cultured HeLa, mouse 3T3, kangaroo PtK2, and rat mammary carcinoma cells, which all have abundant stress fibers during locomotion, TFP (40–80 \(\mu\)M) arrested cell motility and produced changes in actin distribution and cell shape\textsuperscript{40} that were identical to our cultured rat corneal epithelium. Furthermore, CaM inhibitors appeared to affect leading edge cells more than the trailing cells. CaM inhibitors could retard epithelial cell migration either by direct antagonism of actin polymerization, by reducing stress fiber formation, by acting on CaM, by preventing myosin activation, by altering the cell membrane, by blocking cell metabolism, by reducing cell-to-substratum adhesion at the leading edge, or by a combination of these mechanisms. The identical effects of two structurally distinct CaM inhibitors, TFP and W-7, and the reversibility of these effects strongly indicate that the observed results were specific to CaM inhibition and not caused by toxicity or nonspecific side effects. In the rabbit, the specific step(s) blocked by CaM inhibitors appears to be not critical or rate-limiting in the maintenance of cell motility.

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Clinically, several classes of commonly used topical eye medications, including the local anesthetics and the beta-adrenergic blockers, possess strong CaM-inhibitory effects.\textsuperscript{41} Moreover, these medications have been implicated in causing persistent, nonhealing corneal epithelial defects.\textsuperscript{42-45} It is suggested that
epithelial sloughing and delayed wound closure may be due in part to the disruption of epithelial cell microfilaments, whose maintenance and assembly is thought to be CaM-dependent.

Previous organ culture and present cell culture studies have shown striking differences between corneal epithelial healing dynamics in rat and rabbit. We do not yet have sufficient information to elucidate the precise mechanisms responsible for the difference. Time-lapse photography, antibody localization of CaM, and microinjection of CaM, CaM inhibitors, and labelled actin may provide further clues as to the mechanisms. The results of our present tissue culture study may not necessarily represent corneal epithelial healing mechanisms in vivo and caution is urged in attempts to closely correlate these two systems. Moreover, tissue culture requirements may be critically different between rat and rabbit; this possibility may be difficult to rule out.

Key words: actin, calmodulin, cell migration, corneal epithelium, phallacidin

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References


