Distributional Change of Fodrin in the Wound Healing Process of the Corneal Epithelium

Masayo Takahashi,* Toyoshi Fujimotof, Yoshihito Honda,* and Kazuo Ogawat

Distribution of fodrin, F-actin, and E-cadherin in the wound healing process of the mouse corneal epithelium was examined by immunolabeling techniques. In the normal epithelium, fodrin, F-actin, and E-cadherin were observed only in the cell periphery. After wounding, however, fodrin was observed diffusely in the basal cell cytoplasm. Distribution of the other two proteins was not changed. Fodrin in the upper cell layers did not show cytoplasmic labeling. The distributional change of fodrin in the basal cell was seen not less than 1 mm apart from the wound edge and as early as 5 min after the wounding, lasted for about 12 hr, and then gradually disappeared. The change in fodrin localization is one of the earliest phenomena which occur in the corneal epithelium after wounding. Because fodrin is an actin-binding protein and is believed to interact with E-cadherin, this change may be important in modulating the cytoskeleton and the intercellular junction before cell migration occurs. Invest Ophthalmol Vis Sci 33:280-285, 1992

The corneal epithelium after artificial wounding provides a valuable model to study the migration of stratified epithelial cells in vivo. The process of wound closure has been divided in two phases, that is, an initial latent phase and a subsequent cell migration phase.1 Cell mitosis begins after the cell migration phase.2 During the latent phase (which lasts for about 6 hr), movement of epithelial cells is not observed, but the structure of the epithelial cell is reorganized extensively. The columnar contour of the basal cells is lost, and the hemidesmosomes on the basal plasma membrane decrease in number.1,3,4

It is well known that the cytoskeleton plays a major role in cell migration.5,6 In the stratified epithelium, however, how each cytoskeletal component works has not been examined in detail. Because of tight intercellular adhesion provided by desmosomes, the stratified cells may move differently from others, especially such isolated cells as leukocytes and amebas. In the corneal epithelium, a difference in distribution of actin filaments was observed between normal and migrating cells. They were present as an apical network in the normal cell but were located in the basal region of migrating cells as parallel bundles.7 Moreover, cytochalasins affected the ultrastructure of migrating cells and inhibited their movement; colchicine had no effect on either morphology or motility.8-10 These findings indicate that actin filaments, but not microtubules, are critical for corneal epithelial migration.

We explored the mechanism of corneal epithelial movement by focusing on one membrane skeletal protein, fodrin, which was found to change its localization in migrating neutrophils.11 We examined its distribution during wound healing by immunolabeling techniques. Because fodrin is an actin-binding protein, distribution of F-actin was studied simultaneously. In addition, localization of E-cadherin was studied because of a recent report that fodrin forms a complex with this Ca2+-dependent cell–cell adhesion protein. We found that fodrin undergoes a drastic distributional change in the wounded corneal epithelium, and thus we suggest that this protein may play an important role in the healing process.

Materials and Methods

Animal care and treatment in this investigation were in compliance with the ARVO Resolution on the Use of Animals in Research. We used BALB/c mice (average weight, 50 g) tranquilized by intraperitoneal pentobarbital injection. A central corneal ablation of approximately 2 mm in diameter was created with a razor blade.

Antibodies

Anti-rat brain fodrin antibody was raised in rabbits and purified by affinity chromatography.12 Rat monoclonal antibody to mouse E-cadherin was provided by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). Fluorescein isothiocyanate (FITC)-conjugated goat antibodies to rabbit immunoglobulin G and rat immunoglobulin G antibodies (Cappel, Pennsylvania)
were used as secondary antibodies for immunofluorescence microscopy. A combination of biotinylated goat anti-rabbit immunoglobulin G (Immunotech, Marseilles, France) and colloidal gold (5 nm)-conjugated streptavidin (BioCell, Cardiff, UK) was used to visualize antigenic sites by immunoelectron microscopy.

Indirect Immunofluorescence Microscopy

After the central ablation, the corneas were fixed at various times in 4% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4, for 60 min at 4°C. After infusion of 2.3 M sucrose, the tissue was frozen quickly in liquid nitrogen, and frozen sections of 0.5–1 μm in thickness were prepared in a Reichert (Vienna, Austria) Ultracut ultramicrotome equipped with a cryochamber FC4.1 Sections pretreated with 1% bovine serum albumin for 10 min were incubated with antifodrin or anti-E-cadherin antibody for 30 min at 37°C. Then they were treated with a mixture of a FITC-conjugated goat antibody and rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 30 min at 37°C. Sections were observed with a VANOX photomicroscope (Olympus, Tokyo, Japan) equipped with epifluorescent illumination and a phase-contrast device. Photographs were taken on Kodak Tri-X film (Rochester, NY).

Immunogold Electron Microscopy

Ultrathin frozen sections of 50–100 nm in thickness were prepared similarly.12 After pretreatment, they were incubated consecutively with antifodrin antibody, biotinylated anti-rabbit immunoglobulin G, and colloidal gold-conjugated streptavidin (each step for 30 min at 37°C). The sections were postfixed with 2% glutaraldehyde, absorptively stained with 2% uranyl acetate, and embedded in a mixture of 0.2% methylcellulose, 2% carbowax, and 0.01% uranyl acetate as described.13

Results

Normal (Uninjured) Corneal Epithelium

In the normal corneal epithelial cell, labeling for fodrin at the light microscopic level was localized in
the cell periphery of all epithelial cells (Fig. 1A). All cells from the basal cells to the surface cells were labeled with approximately the same intensity. Labeling for F-actin and E-cadherin in the normal corneal epithelial cells appeared almost identical with that for fodrin (Figs. 1B–C). They also were localized in the cell periphery. A notable exception was the basal cell surface facing the basement membrane, which was labeled for F-actin but not for fodrin and E-cadherin (arrows, Fig. 1). Keratocytes were labeled for fodrin along the plasma membrane.

**Injured Corneal Epithelium**

Five minutes after epithelial ablation, fodrin was observed diffusely in the basal cell cytoplasm, although there was some concentration along the cell surface (Fig. 2A). The intensity of labeling in the basal cells was much higher than that in the uninjured epithelium. The basal cells retained their columnar appearance at this time. By contrast, in most cells of the upper layers, fodrin was confined to the cell periphery, as in the control tissue (Fig. 2A). This pattern of labeling was seen not less than 1 mm apart from the wound edge (wound edge not shown). However, F-actin and E-cadherin were localized in the cell periphery in all layers of the corneal epithelium even after injury (Figs. 2B–C).

The distributional change in fodrin was confirmed by immunoelectron microscopy. Whereas the immunogold particles in the normal basal cells were observed primarily along the plasma membrane (arrows, Fig. 3A), those in the basal cells of the wounded epithelium were seen diffusely in the cytoplasm (Fig. 3B). The labeling in the latter specimen was not localized to any particular structure in the cytoplasm. At this time, hemidesmosomes had not disappeared, and many could be observed in the basal cell surface in the same cryosection (photograph not shown).

Twelve hours after ablation, fodrin in the basal cell still was observed in the cytoplasm, but the intensity of the cytoplasmic labeling was decreased compared with earlier stages (Fig. 4A). The basal cells were flattened in shape and separated from each other. The distribution of fodrin returned to its normal peripheral pattern within 48 hr, although the time required seemed to vary according to the size of the initial wound. The distribution of F-actin did not change at this site (Fig. 4B).

**Discussion**

Fodrin is an analogue of erythrocyte spectrin and thought to constitute the membrane skeleton in various nonerythroid cells. In vitro experiments
showed that the protein binds to actin filaments, microtubules, and intermediate filaments. Moreover, in MDCK cells, fodrin was found to form a tertiary complex with another membrane skeletal protein, ankyrin, and a Ca^{2+}-dependent cell-cell adhesion protein, E-cadherin (uvomorulin). Although it is not known whether the latter complex formation occurs ubiquitously, fodrin is localized at the interface of the

Fig. 3. Immunelectron microscopy of fodrin in (A) the normal corneal epithelium and (B) the epithelium 5 min after wounding. Ultrathin frozen sections. Immunogold particles are observed mostly along the plasma membrane in the normal epithelial cells, but after wounding, they are distributed in the cytoplasm without marked concentration. The plasma membrane is marked with arrowheads. Bars = 0.5 μm.
extracellular environment and the intracellular structure, possibly to mediate interaction of the cell adhesion protein and the cytoskeleton.

We observed that the distribution of fodrin in the corneal epithelial cell changed rapidly when an artificial wound was made. Fodrin, primarily localized just beneath the plasma membrane in the normal cell, was distributed in the cytoplasm in the wounded epithelium. Although fodrin generally is localized in the cell periphery, there have been reports that show it occurs in the cell interior.\(^1\) For example, in the mouse epidermis, although fodrin was distributed only in the cell periphery in the upper spinous cell, it occurred diffusely in the cytoplasm of the basal cell.\(^2\) It is therefore likely that the role of fodrin may not be limited to the plasma membrane, and the protein may change distribution depending on the physiologic condition of the cell.

The distributional change of fodrin occurred within a few minutes after wound formation. Among several reports of fodrin's redistribution, we found that this change occurred quickest. The underlying molecular mechanism is not clear, but the rapidity of the reaction makes de novo synthesis of the protein an unlikely cause because the maximal rate of protein synthesis in the migrating epithelium occurred 16 hr after wounding.\(^3,4\) Other possible causes of the change are degradation of the protein by proteases, such as calpain I (Ca\(^{2+}\)-dependent neutral proteinase), and chemical modification of the protein, such as phosphorylation, or Ca\(^{2+}\)-dependent calmodulin binding.\(^1\)

Similar dual distribution of fodrin, that is, plasmalemmal versus cytoplasmic, has been shown to occur in vitro in MDCK cells\(^5\) and in transformed keratinocytes.\(^6\)

In these cells, fodrin was seen in the cytoplasm in a low Ca\(^{2+}\) medium, but it was localized to the plasma membrane in a standard Ca\(^{2+}\) medium. It is known that the intracellular Ca\(^{2+}\) concentration is also higher in standard Ca\(^{2+}\) medium than in the low Ca\(^{2+}\) medium.\(^7\) It may be that a change of intracellular Ca\(^{2+}\) concentration is related to the distributional change of fodrin in the wounded epithelium.

In the wounded corneal epithelium, a decrease in hemidesmosome number was reported to be the earliest phenomenon; 2 hr after wounding, hemidesmosomes virtually disappeared within 180 \(\mu\)m from the wound edge.\(^8\) At the same time, a decrease of desmosomes, widening of intercellular spaces, and reduction of the surface interdigitations were observed in the vicinity of the wound. The change in fodrin distribution we observed occurred much earlier after wounding than these phenomena. Because redistribution of fodrin may affect desmosomes and hemidesmosomes through its linkage to plectin and tonofilaments,\(^9\) it is possible that the observed change of fodrin might have a causal relationship with the decrease of the junctional apparatus.

Another remarkable feature of the change in fodrin distribution was that it happened exclusively in the basal cell layer and not in the upper layers of the epithelium. If the change is related to subsequent cell motility, this result is consistent with an earlier hypothesis that only the basal cells migrate actively to close the denuded area and cells in the upper layer are carried along passively.\(^10\) Although highly speculative, the following mechanism is possible. The distribution of F-actin and E-cadherin did not change notably even after wounding. Because fodrin most likely binds to them, however, it is probable that the redistribution caused modulation of the actin-based structure for migration at the same time. It may have affected E-cadherin and thus changed the property of cell-cell adhesion. Conversely, it may be that wounding induced some change in cell-cell adhesion first,
which in turn made fodrin change its distribution. In either case, the changes may be prerequisites for basal cells to start migration.

The last thing we wish to mention about our observations is that the change was seen in a wide area of the epithelium, which extended not less than 1 mm from the wound edge. This also is consistent with the earlier hypothesis that the power of cell migration is not derived only from a “pulling force” of the cells adjacent to the wound but also from a diffuse “pushing force” generated throughout the epithelium. The widespread change also showed that there was a means to transmit information rapidly to distant cells when only a small portion of the epithelium was injured. The most likely mechanism is communication through gap junctions. The proportion of the cell surface occupied by the junction was reported to increase during healing of the epidermis. As reported in other epithelia, Ca²⁺ or inositol 1,4,5-triphosphate might be messenger molecules carrying information through gap junctions.

In summary, we showed a distributional change of fodrin, which occurred rapidly and specifically in the basal cell layer in a wide area of the wounded corneal epithelium. Although the molecular detail awaits further study, the phenomenon is most likely related to the mechanism of cell migration in the stratified epithelium.

Key words: corneal wound healing, fodrin, actin, cadherin, cell migration

Acknowledgments

The authors thank Dr. Masatoshi Takeichi (Faculty of Science, Kyoto University) for providing rat monoclonal antibody to E-cadherin.

References