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# Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement

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## SUMMARY

**At the leading edge of healing embryonic epithelium, cables of actin filaments appear to extend from cell to cell, forming a ring around the wound circumference. It has been hypothesized that this actin filament cable functions as a contractile 'purse string' to facilitate wound closure. We have observed this cable in large, circular healing epithelial wounds in corneas of adult mice. To elucidate the role of the actin filament cable, we characterized the molecular components associated with the cell-cell junction where the actin filament cable inserts and with the actin filament cable itself, and we studied the effect of disruption of the cable using an E-cadherin function-blocking antibody, ECCD-1. Localization of E-cadherin and the direct association of catenins with actin filament cable at the cell-cell interface of the actin cable confirmed that the cell-cell junction associated with the actin filament cable is an adherens junction. The E-cadherin function-blocking antibody caused disruption of the actin filament cable and induction of prominent lamellipodial extensions on cells at the leading edge, leading to a ragged uneven epithelial wound margin. These data demonstrate that cell-to-cell associated E-cadherin molecules link the actin filament cable, forming a functional adherens junction, and that the actin filament cable plays a role in coordinating cell movement.**

Key words: Actin cytoskeleton, Cell adhesion molecule, Cell movement, Corneal epithelium, Wound healing

## INTRODUCTION

The crawling or movement of cells is a fundamental activity in living tissue. It is now well accepted that motile cells utilize lamellipodial extension as a standard mode of cell movement (Condeelis, 1993; Small, 1994; Stossel, 1993). This is the method of movement of separate motile cells with which a number of studies of cytoskeletal organization have been conducted. However, little is known about movement of cells which keep contact with one another, for example, the epithelial cells which move as a sheet during wound healing. These cells have contact with neighboring cells as well as underlying extracellular matrix, requiring coordinated cell movement for correct wound closure.

The forefront of the epithelial wound border, termed the leading edge, formed by a single layer of flattened cells, has been thought to play a pivotal role in determining the orientation and rate of healing and in coordinating cell movement. This hypothesis is indirectly supported by several lines of evidence: (1) an actin filament network was observed in cells at the leading edge (Gipson and Anderson, 1977), (2) cytochalasin caused drastic alteration in the structure of cells at the leading edge (Gipson and Keezer, 1982) and inhibited their movement (Gipson et al., 1982), and (3) an actin filament cable is present at the leading edge of the migrating epithelial sheet in embryonic skin wounds, where it appears to run from cell to cell (Martin and Lewis, 1992). A similar actin filament cable was demonstrated along the border of wounds created on monolayer sheets of the intestinal epithelial cell line, Caco-$2_{BBe}$ (Bement et al., 1993). Presence of the actin filament cable in both of these wound-healing models (Martin and Lewis, 1992; Bement et al., 1993) led to the hypothesis that the actin filament cable acts as a contractile 'purse string' to facilitate wound closure. The localization of myosin II at the wound margins provided further evidence for the presence of contractile machinery associated with the actin filament cable (Bement et al., 1993). Reports of studies of embryonic epidermal wounds state that the 'purse string' mechanism functions only in embryonic epidermis and not in adult keratinized epidermis, where lamellipodia function to close the wound (Martin and Lewis, 1992; Brock et al., 1996).

More recently, cadherins were localized by immunohistochemistry to regions where the actin filament cables insert into adjoining cell membranes of cells of the leading edge, suggesting that the junction anchoring the neighboring cells may be an adherens junction (Brock et al., 1996). What distinguishes the adherens junctions from the other cell-cell junctions is the presence at the membrane site

of E-cadherin, α- and β-catenins, and γ-catenin (plakoglobin) (Geiger and Ginsberg, 1991). At sites of adherens junctions, E-cadherin molecules bind homotypically at their extracellular domain in a $Ca^{2+}$-dependent manner, and their cytoplasmic domain is linked indirectly to actin filaments via catenins (Takeichi, 1991; Geiger and Ayalon, 1992; Kemler, 1993). Both the homotypic interactions of their extracellular domains and their association with actin filaments at their cytoplasmic domains through catenins are required for cadherins to function as adhesion molecules (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). Brock et al. (1996) showed punctate localization of a pan cadherin antibody to the wound margin on the whole mounts of healing embryonic epidermis. They suggest that the punctate binding represents clustering of cadherin at sites of cell-cell association of the actin filament cable. The presence of cadherin at such sites would imply the presence of an adherens junction. However, in sections of stratified epidermal epithelium and corneal epithelium, E-cadherin localizes along the entire cell membrane of all cell layers (Shimoyama et al., 1989; Gipson and Sugrue, 1994). It is surprising, therefore, that cadherin localization was restricted to punctate spots along whole mounts of wounded embryonic epidermis. Furthermore, it is reported that ZO-1, a component of tight junctions, is localized at the wound margins of cells of an epithelial cell line (Bement et al., 1993; Yonemura et al., 1995). The localization of ZO-1 was not tested by Brock et al. (1996). Thus, while the data that have been published by Brock et al. (1996) suggest the presence of adherens junctions at the sites where the actin filament cable inserts, definitive proof that the cell-cell junction associated with the actin filament cable is an adherens junction and whether this junction has a functional activity is lacking.

In this study, using adult mouse corneas with central epithelial debridement wounds, we established that: (1) fully developed adult stratified epithelia exhibit actin filament cables at their leading edge during healing; (2) the cell-cell junction associated with actin filament cable at the leading edge of migrating corneal epithelium is an adherens junction, by showing a direct association of catenin molecules with the junctions; and that (3) this adherens junction has an adhesive activity, by showing that function-blocking antibodies to E-cadherin disrupt the actin filament cable, leading to formation of lamellipodia and uncoordinated cell movement.

## MATERIALS AND METHODS

### Wounding and tissue processing

All investigations in this report were approved by an institute Animal Care and Use Committee. Central corneal wounds were created as described previously (Gipson and Kiorpes, 1982). Briefly, after centrally marking corneas of anesthetized Balb/c mice (6-8 weeks of age, male) with 2 mm trephine (Storz Instrument Co. St Louis, MO), corneal epithelium was debrided with a blunted blade within the marked area, leaving the basement membrane intact (Spurr-Michaud et al., 1988). Animals were anesthetized by intraperitoneal injection of a 50 μl per 10 g weight cocktail of a ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, IA), xylazine (Phoenix Pharmaceutical, Inc., St Joseph, MO), and phosphate-buffered saline (PBS) (1:1:6). Since circadian rhythm affects corneal epithelial proliferation (Lavker et al., 1991), wounding was performed between 4 pm and 5 pm for most experiments. Immediately after euthanasia by $CO_2$ asphyxiation, eyes were fixed in situ, as described below, and enucleated.

### Migration rate and appearance of actin cable

To assess the migration rate, wounds were allowed to heal 0, 3, 6, 12, 18, or 24 hours in vivo, eyes were immediately fixed in situ with 4% paraformaldehyde in PBS for 20-30 minutes. They were able to be fixed in situ without difficulty since surface tension kept the fixative drops on the surface of the eye. Surface tension also facilitated the application and retention of solutions to the in vivo mouse eyes, as described below in this study. Shortly thereafter, left eyes were stained with Richardson's stain (Richardson et al., 1960) to visualize and quantify the remaining epithelial defect by photography. Developed negatives of healing eyes were scanned, and areas of epithelial defect were calculated by using NIH Image (National Institutes of Health, Bethesda, MD). Right eyes were placed in 4% paraformaldehyde and processed for labeling of F-actin.

### Labeling of F-actin and immunolabeling

Fixed eyes were washed three times for 10 minutes each in PBS and incubated with rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) diluted at 1:50 in PBS, for 2 hours at room temperature. They were then washed three times for 10 minutes each in PBS.

Wounded eyes processed for immunolabeling were allowed to heal for 18 hours in vivo. Immediately after euthanasia, eyes were fixed in situ, as described above, with 4% paraformaldehyde for at least 20-30 minutes, followed by enucleation of whole eyes and further fixation in 4% paraformaldehyde for an additional 30-45 minutes. Tissues were usually processed immediately for immunolabeling or they were stored in 4% paraformaldehyde overnight. Whole eyes were washed three times for 10 minutes each in PBS, pre-blocked with 1% bovine serum albumin (BSA) for 30 minutes, and incubated with primary antibodies for 2 hours, followed by incubation with secondary antibodies for 2 hours at room temperature. The following primary antibodies were used: rat monoclonal anti-E-cadherin antibody, ECCD-2 (Zymed Laboratories, Inc., South San Francisco, CA) diluted at 1:200 in PBS; rat monoclonal anti-P-cadherin antibody, PCD-1 (Zymed Laboratories, Inc.), diluted at 1:200 in PBS; goat polyclonal anti-αE-, β-, γ-catenin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:100 in PBS; rat monoclonal anti-ZO-1 antibody (Chemicon International, Inc., Temecula, CA) diluted at 1:100 in PBS; rabbit polyclonal anti-occludin antibody (Zymed Laboratories, Inc.) diluted at 1:100 in PBS; rabbit polyclonal anti-human platelet myosin-II antibody (Biomedical Technologies, Inc., Stoughton, MA) diluted at 1:25 in PBS. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat, rabbit, or goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted at 1:50 in PBS. For double labeling, rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) was used at the dilution of 1:50 together with the above FITC-conjugated secondary antibody. Before incubation with individual primary antibodies, 0.3% Triton X-100 in PBS was applied for permeabilization except for anti-E- and P-cadherin antibodies, which recognize extracellular regions of respective cadherin molecules. Three washes with PBS were performed between each step. For controls, primary antibodies were omitted.

For studies of catenin and myosin II localization and for studies of actin filaments in cell layers behind the leading edge, an extraction buffer designated cytoskeleton (CSK) buffer (Fey et al., 1984; McNeill et al., 1993; Näthke et al., 1994; Adams et al., 1996; Angres et al., 1996), which extracts proteins not associated with actin filaments while retaining actin filament-bound molecules, was applied topically in situ on the mouse eye prior to fixation. The CSK buffer used in this study was modified and included 50 mM NaCl, 10 mM Pipes (Boehringer Mannheim Corp., Indianapolis, IN), pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, and 30 mM sucrose. Immediately after euthanasia, ice-cold CSK buffer was placed on the eyes in situ so that

the droplets were kept on the surface of eyes with the help of surface tension. After 5 minutes of extraction, eyes were washed with PBS for 5 minutes and subsequently fixed in situ with 4% paraformaldehyde for 20-30 minutes. Whole eyes were enucleated and processed for the above immunolabeling with no further permeabilization.

### E-cadherin function-blocking experiments

Eighteen hours after wounding, mice were anesthetized and one of the following solutions was placed on the surface of the eyes for 30 or 60 minutes, with replacement at 10 minute intervals: rat monoclonal anti-E-cadherin antibody (ECCD-1, generously provided by Dr Masatoshi Takeichi, Kyoto University), 200 μg/ml diluted in Hanks’ balanced salt solution (HBSS) containing calcium and magnesium ion, but no Phenol Red (designated as HBSS with $Ca^{2+}$) (Gibco BRL, Grand Island, NY); rat pure IgG (Zymed Laboratories, Inc.), 200 μg/ml diluted in HBSS with $Ca^{2+}$; HBSS with $Ca^{2+}$. ECCD-1 disrupts cell-cell adhesion by binding to E-cadherin in the presence of $Ca^{2+}$ (Yoshida-Noro et al., 1984). Additional anesthesia was required for mice who received 60 minute applications. After euthanasia, eyes were fixed in situ, enucleated, and processed for labeling of F-actin as described above. To test reversibility of test reagents, eyes were washed with HBSS with $Ca^{2+}$ after 30 or 60 minute applications of the above individual solutions and allowed to heal for an additional 30 or 60 minutes, followed by euthanasia and in situ fixation.

### Confocal laser scanning microscope

Following either F-actin labeling or immunolabeling procedure, whole eyes were fixed for an additional 30 minutes in 4% paraformaldehyde in PBS and washed three times for 10 minutes each in PBS. Corneas were dissected off from whole eyes under a dissecting microscope. Care was taken not to allow the corneas to dry. The peripheral corneas were trimmed away as closely to the leading edge as possible, with a razor blade, on a wax sheet, under a dissecting microscope. The trimmed corneas were mounted in VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) with the epithelium side up in 18 × 18 mm wells made of nail polish on glass slides (3-4 applications), covered with coverslips (18 × 18 mm, No. 1 1/2 thickness; VWR Scientific, Inc., Media, PA), and sealed with nail polish. The height of the well was crucial to obtaining good en face confocal images of the leading edge because the sample thickness originated from a convexly contoured cornea. Corneas that had healed 12 hours or less were often cut into quarters prior to mounting, to facilitate imaging of the leading edge, which was more peripheral to the center of the curved corneas than those that had healed 18 and 24 hours.

Whole-mounted corneas were viewed with a Leica TCS 4D confocal laser scanning microscope (Leica, Inc., Heidelberg, Switzerland) equipped with an argon-krypton laser. For single-channel measurements using the rhodamine detector channel, excitation wavelength was 568 nm and the emission barrier filter was at 590 nm. For dual-channel measurements using both FITC and rhodamine detector channels, excitation wavelengths were 488/568 nm and emission barrier filters were 525/590 nm, respectively. Z-series, consisting of eight optical sections, were collected from the apical side to the basal side of the cells at the leading edge.

## RESULTS

Fig. 1 shows a histological structure of the leading edge of migrating corneal epithelium in an in vivo healing eye of an adult mouse. The tip of the leading edge is a single cell layer. Just behind the leading edge cell, two to three cell layers are present, and the cell immediately behind the leading edge cell overlies the posterior apical cell membrane of the leading edge cell. The time required for in vivo healing of the 2 mm wound is shown in Fig. 2A. After a 6 hour lag, the denuded area decreased linearly until complete wound closure was achieved at around 24 hours. The experiments of the present study were conducted on in vivo healing corneas due to the absence of actin filament cable formation in organ culture (data not shown).

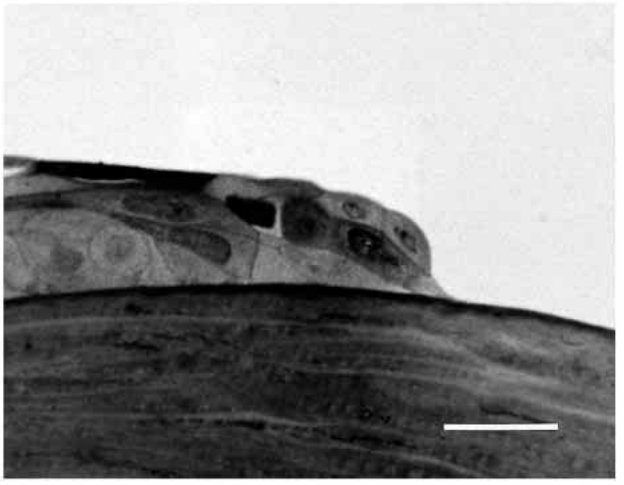
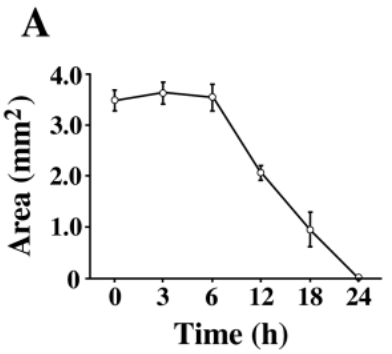


**Fig. 1.** Cross-sectional view of a representative section of the leading edge of migrating corneal epithelium in an in vivo healing eye of an adult mouse 18 hours after wounding. The leading edge cell is migrating over the denuded basement membrane and its apical membrane is exposed at the anterior part of the cell. The epithelium just behind the leading edge cell consists of two to three cell layers and the cell directly behind the leading edge cell overlaps the posterior part of that. 1% Toluidine Blue stain. Bar, 20 μm.

### Formation of an actin filament cable at the leading edge of migrating corneal epithelium

In healing eyes of adult mice, the actin filament cable formed within 3 to 6 hours after wounding and appeared to run continuously from cell to cell at the leading edge of migrating corneal epithelium (Figs 2B and 3). The cable was present until wound closure. Small lamellipodial-like extensions were also present along cytoplasm of leading edge cells, ahead of the actin filament cable. In contrast to the actin filament cable, stress fibers, which insert into focal adhesions at the basal cell membrane (Burridge et al., 1988; Jockusch et al., 1995), could not be observed at the leading edge.

To determine whether this actin filament cable is specific to the cells at the leading edge only, we examined the cells behind the leading edge where two to three cell layers were present in contrast to the single cell layer of the leading edge. No structure similar to the actin filament cable could be observed in cells in any cell layer behind the leading edge (Fig. 3), even in eyes taken 18 hours after wounding and permeabilized in situ with ice-cold CSK buffer prior to fixation (see Materials and Methods) so that phalloidin could more easily penetrate to the basal cells behind the leading edge (not shown). Although actin filaments occasionally ran parallel to the wound margin along the cell membranes of basal cells behind the leading edge, they were not continuous from cell to cell. These data



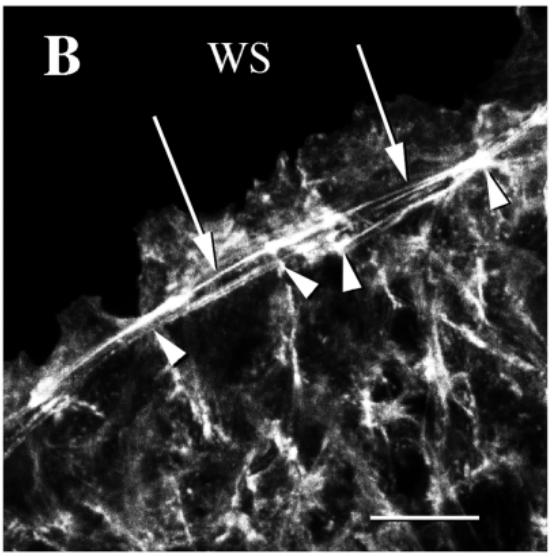




**Fig. 2.** Wound healing profile of mouse corneal epithelial wounds healing in vivo. The central 2 mm diameter of corneal epithelium was debrided, leaving the basement membrane intact. (A) At 0, 3, 6, 12, 18, or 24 hours after wounding, the area of the remaining epithelial defect was assessed and plotted. Note that there was a lag of 6 hours before the wounded epithelial sheets initiated forward movement. It was at this 3 to 6 hour time point that the actin filament cable appeared, as shown in B. The results are expressed as mean ± s.d. ($n$=6) of duplicated separate experiments. (B) The leading edge cells 6 hours after wounding labeled with rhodamine phalloidin. Note presence of actin filament cables (arrows), which appear to extend from cell to cell at cell boundaries (arrowhead). Note also the presence of broad, but indistinct lamellipodia-like extensions of the leading edge membrane. A stacked image consisting of 8 sequential optical sections is shown. Wound surface (WS). Bar, 10 μm.

indicate that the actin filament cable is specific to cells at the leading edge.

### E-cadherin localizes along the lateral cell membranes of the leading edge cells

E- and P-cadherins are expressed in the epidermis of embryonic mice (Hirai et al., 1989b) as well as in human adult stratified epithelia of skin and esophagus (Shimoyama et al., 1989). Their distributions are distinct from each other; E-cadherin is present in all cell layers, whereas, P-cadherin is restricted to basal cells. In corneal epithelium, E-cadherin is present along all cell membranes that abut other cell membranes in rabbits (Gipson and Sugrue, 1994) and in mice (data not shown); in our study, P-cadherin was not localized in either wounded or unwounded epithelia (data not shown). In whole mounts of corneas, E-cadherin was localized along the cell membranes where the neighboring leading edge cells contacted each other (Fig. 4). It appears that the localization of E-cadherin is not specifically intense at the sites where the actin filament cable inserts, since the exposed lateral cell membranes, those not covered with the overlying cells from behind the leading edge, were stained with anti-E-cadherin antibody, ECCD-2.

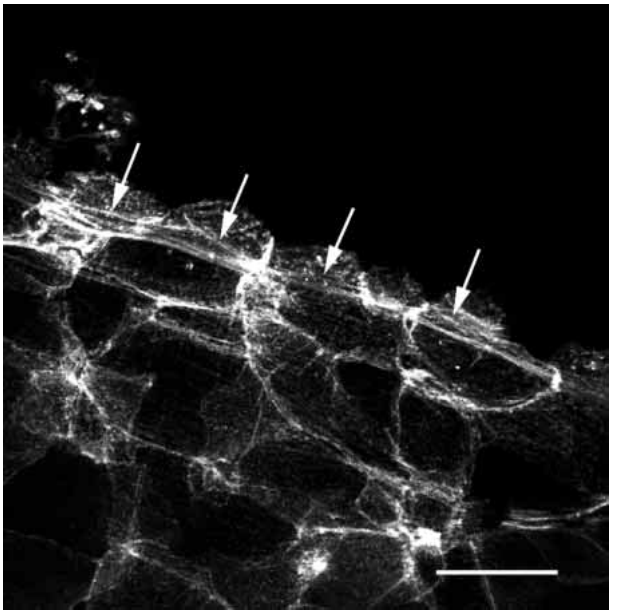


**Fig. 3.** Actin filament cable (arrows) in leading edge cells of migrating corneal epithelium healing for 18 hours and labeled with rhodamine phalloidin. An actin filament cable appears to run continuously from cell to cell at the leading edge (arrows). Note that the cable is present in leading edge cells only. A stacked image consisting of 8 sequential optical sections is shown. Bar, 20 μm.

Although cytoplasmic fluorescence was observed, neither ZO-1 (Fig. 5) nor occludin (data not shown), which are components of tight junctions, was localized at the site where the neighboring leading edge cells contacted each other, indicating that the cell-cell junction associated with actin filament cable is not a tight junction. By contrast, the apical cells behind the leading edge had tight junctions, as evidenced by localization of ZO-1 (Fig. 5) and occludin (not shown). The presence of these tight junctions may explain why E-cadherin antibody binding was not detected along cell membranes behind the leading edge on the whole-mount corneal tissues. In MDCK cell cultures, tight junctions that form between cells block penetrance of antibodies to lateral membrane proteins (McNeill et al., 1990). As described above, E-cadherin was present on all cell membranes associated with adjacent cell membranes in the entire corneal epithelium. Since no localization of cadherin was detected behind the leading edge on the whole-mount tissues in this study, we interpret this to indicate that the antibodies did not penetrate past the tight junctions. The observation of Brock et al. (1996) of intense localization of cadherin only at the leading edge of their embryonic wound model might also have been confounded by the difficulty in penetration of cadherin antibody into deeper cell layers. Thus, from the cadherin localization data alone, we could not conclude that the cell-cell junction associated with actin filament cable is an adherens junction.

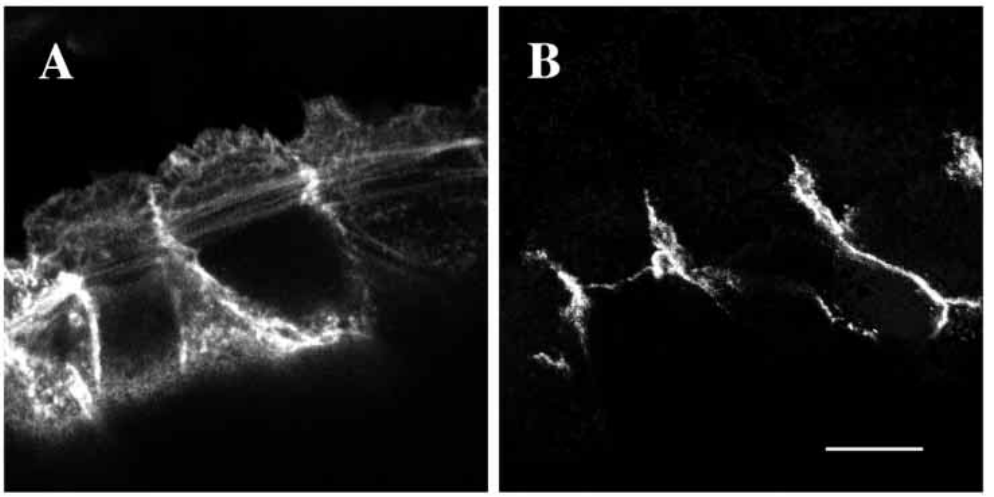



**Fig. 4.** Immunofluorescence localization of E-cadherin at a leading edge of migrating corneal epithelium. The leading edge cells, 18 hours after wounding, were double labeled with phalloidin (A) and anti-E-cadherin antibody (B). Note that E-cadherin was localized along the cell membranes where the neighboring leading edge cells make contact, but is not limited to the sites where the actin filament cable inserted. The apical cells behind the leading edge cells overlie the leading edge cells and conceal the adjoining lateral cell membranes, preventing penetrance of the anti-E-cadherin antibody to the underlying binding sites. Stacked images consisting of 2 and 8 sequential optical sections are shown in (A) and (B), respectively. The two optical sections stacked in (A) are at the level of the actin filament cable. Bar, 10 μm.

### The actin filament cable forms a complex with catenins where it inserts

To determine if catenins, components of adherens junctions, are present at cell-cell junction sites associated with the actin filament cable, we performed whole-mount confocal immunofluorescence microscopy using antibodies to α-, β- and γ-catenins. All catenin molecules tested were localized in the cytoplasm as well as along the cell membranes of the leading edge cells (not shown). Although the degree of fluorescence seems intense at the sites where the actin filament cables insert, these findings of catenin localization do not provide proof that the cell-cell junction associated with actin filament cable is an adherens junction, because of the lack of exclusive colocalization of catenins with the actin filament cable at the insertion site. Since catenins link the cytoplasmic domains of cadherin to actin filaments, thereby forming a stable complex, they become resistant to extraction (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990). In order to extract the non-complexed form of catenins, CSK buffer was applied to corneas prior to fixation. Imaging of the whole-mounted tissues which were extracted with CSK buffer prior to fixation revealed that all three catenins were localized at the sites where the actin filament cables inserted. Both α- and β-catenins appeared more prominent at the junction since γ-catenin was localized equally all along the cell membranes of the neighboring leading edge cells including the insertion sites of the actin filament cables (Fig. 6). γ-catenin is known to be a component of desmosomes and thus this distribution is not surprising (Cowin et al., 1986). These data showing presence of catenins at the junction site provide direct evidence that the cell-cell junction associated with actin filament cable is a type of adherens junction.

### E-cadherin function-blocking antibody disrupts the actin filament cable

Although the colocalization of catenins at the junction site indicates that the actin filament cable is associated through a type of adherens junction, it is unclear whether E-cadherin molecules mediate the junction linkage through homotypic interactions at their extracellular domains (Takeichi, 1991; Geiger and Ayalon, 1992). We performed a function-blocking study with an E-cadherin function-blocking antibody, ECCD-

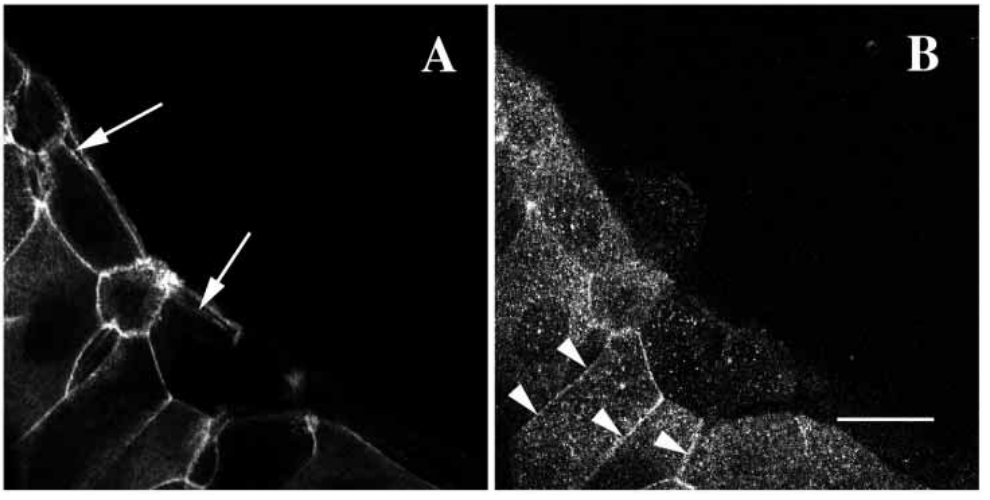



**Fig. 5.** Immunofluorescence localization of ZO-1 at a leading edge of migrating corneal epithelium. The leading edge cells, 18 hours after wounding, were double labeled with phalloidin (A) and anti-ZO-1 antibody (B). Note that the apical cells directly behind the leading edge have tight junctions, as shown by localization of ZO-1 (arrowheads). A part of the actin filament cable is visible in A (arrows). A single optical section, which corresponds with the plane of the apical cells just behind the leading edge, is selected so that the localization of ZO-1 can be visualized. Bar, 20 μm.

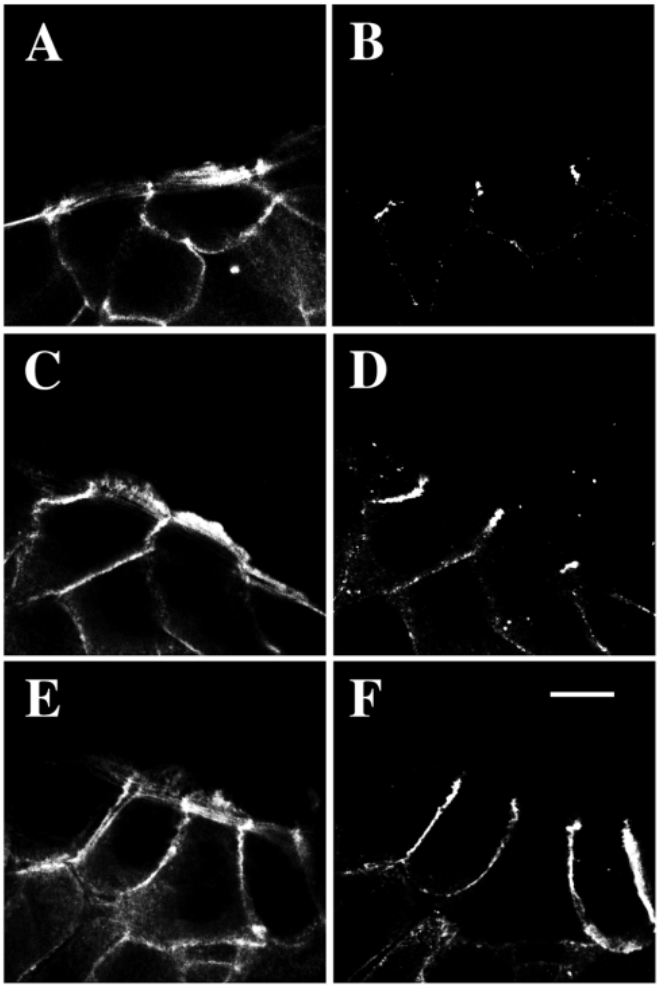



**Fig. 6.** Immunofluorescence localization of α-, β-, and γ-catenins at an 18 hour leading edge of migrating corneal epithelium, after CSK buffer extraction prior to fixation. Tissues were double labeled with phalloidin (A,C,E) and respective anti-catenin antibodies (B,D,F). (A,C,E) Corresponding optical sections with (B) anti-α-catenin antibody, (D) anti-β-catenin antibody, and (F) anti-γ-catenin antibody, respectively. The three sets of single optical sections are at the level of actin filament cable insertion sites. α- and β-catenin are more prominently localized at actin filament cable insertion sites, and γ-catenin is localized equally along the cell membranes of leading edge cells, including actin filament cable insertion sites. Bar, 10 μm.

1, to determine whether this adherens junction has an activity mediated by E-cadherin. As stated earlier, a distinct actin filament cable does not form in organ culture, thus the function-blocking study was done on corneas healing in vivo.

After a 30 minute application of ECCD-1 to the corneas of anesthetized healing mice, the actin filament cable disappeared from the leading edge and instead prominent lamellipodial extension appeared (Fig. 7A). The leading edge morphology changed from a comparatively smooth shape to a jagged one in which some cells were in advance of others (Fig. 7A). This morphologic conversion from a smooth to a jagged wound border demonstrates that the actin filament cable anchored by E-cadherin-mediated adherens junctions plays an important role in coordinating cell movement at the leading edge. There was no indication that the cells at the leading edge dissociated from each other or were detached from the underlying substrate, and no morphologic change was observed in the apical cells behind the leading edge. These effects of ECCD-1 on the leading edge cells were similar between 30 minute and 60 minute applications (Fig. 7B). Furthermore, the effects were completely reversible. After a 60 minute but not a 30 minute recovery period following a 30 or 60 minute application of ECCD-1, the actin filament cable reappeared and lamellipodial extensions receded (Fig. 7C).

For control experiments, rat normal IgG in HBSS with $Ca^{2+}$ and HBSS with $Ca^{2+}$ were tested. After a 30 minute application, these solutions did not cause disruption of the actin filament cable, but moderate lamellipodial extension appeared (Fig. 7D and G). After a 60 minute application of these solutions, the actin filament cable became less distinct and lamellipodial extension began to appear (Fig. 7E and H), but these effects were completely reversed after a 30 minute recovery period (Fig. 7F). Although actin filament cable disruption and lamellipodial extension sometimes occurred even with the use of a control solution, the effects of ECCD-1 were distinguishable from those of controls. With ECCD-1, the disruption of actin filament cable occurred after a 30 minute application, and reformation of actin filament cable required longer than 30 minutes. This E-cadherin function-blocking experiment demonstrated clearly that E-cadherin molecules associated with actin filament cable at the leading edge serve as a functional component of the adhesion molecule complex at the adherens junction of this cell-cell contact site.

### Myosin II colocalize along the actin filament cable

Since it has been proposed that actin-myosin II interaction generates forces to drive cell movement (Condeelis, 1993; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996), it is conceivable that for the actin filament cable to contribute to coordinated cell movement during wound healing, myosin II molecules must be integrated into the structure of actin filament cable anchored by the adherens junction. Although myosin II localization at the leading edge was demonstrated in earlier studies (Bement et al., 1993; Brock et al., 1996), it is unclear from previous work whether these myosin II molecules are associated with the actin filament cable. Since myosin II is an actin-binding protein, the CSK buffer extraction method used in this study for catenin localization is also useful for demonstration of a direct association of myosin II with actin filaments (Conrad et al., 1989; Kolega 1997; Svitkina et al., 1997). Fig. 8 shows that the myosin II retained after CSK buffer extraction is localized along the actin filament cable in a beaded intermittent pattern, indicating direct binding with actin filament cable. In sharp contrast to catenin localization, myosin II is excluded from the sites where the actin filament cables associate with the cell membrane.

## DISCUSSION

The major conclusion that can be drawn from these studies is that an actin filament cable specific to the cells at the tip of the leading edge of migration is associated by adherens junctions,

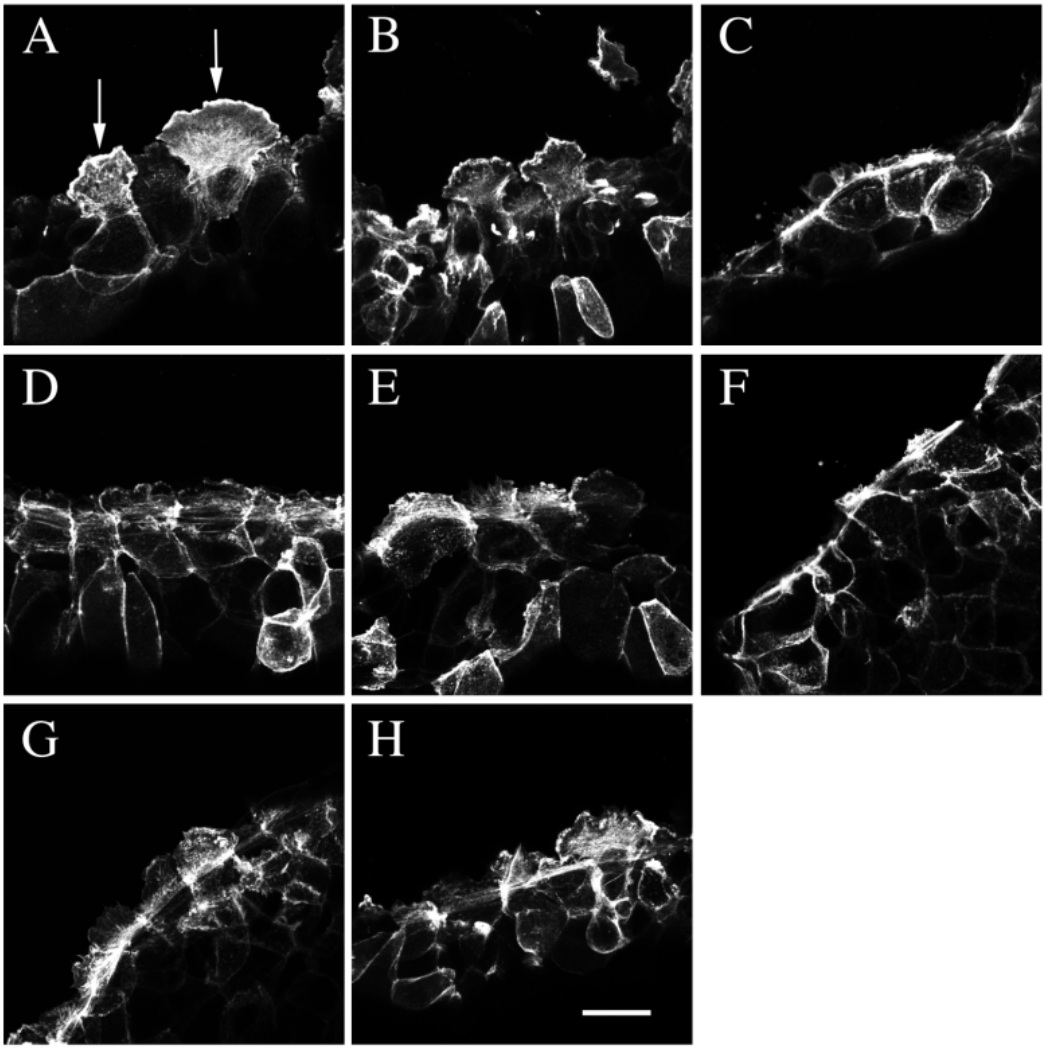



**Fig. 7.** E-cadherin function-blocking experiment on a leading edge of an 18 hour migrating corneal epithelium. Wounds were treated with an E-cadherin function-blocking antibody, ECCD-1 (A-C), rat normal IgG (D-F), and HBSS with $Ca^{2+}$ (G,H) for either 30 (A,D,G) or 60 minutes (B,E,H). In contrast with rat normal IgG and HBSS with $Ca^{2+}$, ECCD-1 caused dramatic changes in the leading edge as early as 30 minutes after application (A). Note disruption and lack of presence of actin filament cable and the prominent lamellipodial extension (arrows). These effects of ECCD-1 were reversed with actin filament cable assembly after a 60 minute recovery (C). The actin filament cable became less distinct and lamellipodial extension began to appear after a 60 minute application of rat normal IgG (E) or HBSS with $Ca^{2+}$ (H). These changes were completely reversed after a 30 minute recovery (F), earlier than those of ECCD-1. Stacked images consisting of 8 sequential optical sections are shown. Bar, 20 μm.

which are functionally linked by E-cadherin. We confirmed that the cell-cell junction associated with actin filament cable is an adherens junction by showing direct association of catenins with the actin filament cable. As demonstrated by the function-blocking experiments, an E-cadherin function-blocking antibody caused disruption of the actin filament cable and induction of prominent lamellipodial extensions leading to an irregular arrangement of cells at the leading edge. This indicates that E-cadherin molecules link the actin filament cables of adjoining cells to coordinate cell movement. This

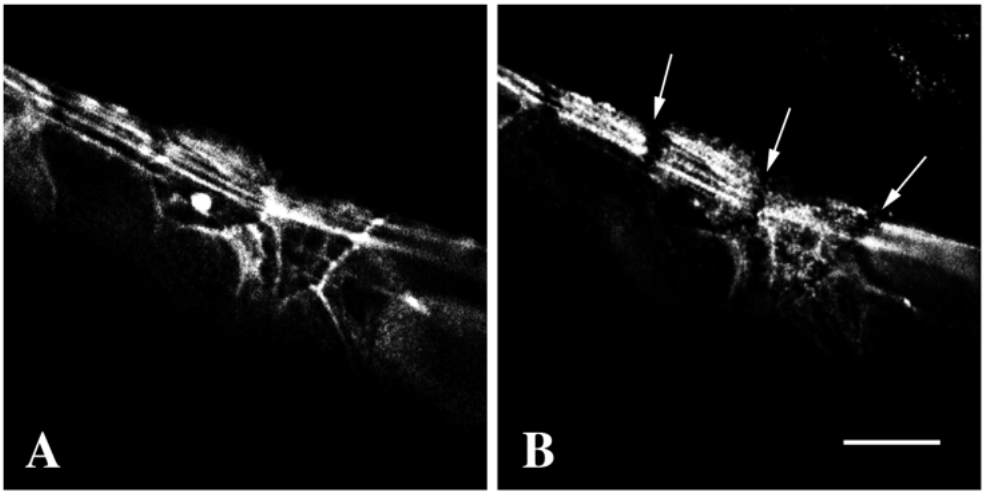



**Fig. 8.** Immunolocalization of myosin II in leading edge cells of 18 hour migrating corneal epithelium. The leading edge cells, 18 hours after wounding, were double labeled after extraction with CSK buffer and fixation with phalloidin (A) and anti-myosin II antibody (B). Myosin II colocalized exactly along the actin filament cable in a beaded pattern. Myosin II is excluded from the membrane insertion sites (arrows). This single optical section is at the level of the actin filament cable. Bar, 10 μm.

function-blocking study also demonstrates a new role played by the actin filament cable, coordination of cell movement, which may be a part of the contractile 'purse string' mechanism. Direct colocalization of myosin II along the actin filament cable further indicates that these structures provide the force to contract the wound in a 'purse string' fashion. Thus, we propose that cells of the tip of the leading edge are unique in their function in that the actin filament cable, the adherens junctions, and the actin-myosin II interactions are the fundamental elements belonging to contractile 'purse string' machinery, and that the cell movement at the leading edge is coordinated by this 'purse string' machinery.

It has been proposed that large skin wounds in adult animals heal by lamellipodial locomotion of cells at the leading edge, whereas small circular wounds of embryonic epithelia heal by the contractile 'purse string' mechanism (Martin and Lewis, 1992; Bement et al., 1993; Cramer et al., 1994). However, the present study indicates that large wounds, such as the 2 mm in diameter wounds on adult mouse corneas, also use the actin filament cable mechanism. Thus, actin filament cable and lamellipodial extension formation appear irrespective of wound size. Perhaps the epithelium can use either method, with the 'purse string' method being sensitive to extracellular signals, e.g. organ culture can prevent actin filament cable formation. The differences between environmental components of in vivo and organ culture significantly influence actin filament cable formation and the appearance of the leading edge cells, since a distinct actin filament cable does not form in organ culture. In fact, we observed in organ culture a prominent lamellipodial extension similar to that caused by E-cadherin function-blocking antibody, ECCD-1 (unpublished results). In this context, ECCD-1 can be considered an extracellular signal, since it disrupted the actin filament cable and transformed the smooth leading edge into an irregular shape with prominent lamellipodial extensions. However, the actin filament cable and lamellipodia are not mutually exclusive. In the present study, lamellipodia, although not prominent, were sometimes observed in some cells of the leading edge of the in vivo healing cornea. In addition, it is reported that lamellipodia are sometimes seen during the first few hours, usually less than 6 hours, in the in vivo corneal epithelial wound (Buck, 1979). It seems conceivable that the leading edge cells of the cornea move forward by lamellipodia for an initial 6 hours after wounding, and afterwards by both the mechanisms of lamellipodia and actin filament cables. Interestingly, the healing rate of the corneas, as shown in Fig. 2A, accelerates remarkably after 6 hours of wounding when the actin filament cables form in the leading edge cells. Thus, the actin filament cable and lamellipodia can co-exist, but the dominant method of cell extension may be regulated by extracellular signals which affect the adherens junction directly, as do ECCD-1, or indirectly via unidentified receptor-ligand mediated pathways.

The E-cadherin function-blocking experiments provide direct proof that E-cadherin molecules link the actin filament cables at the adherens junction of the leading edge and thereby function as cell-cell junctions, joining the cells to form the 'purse string.' Previous studies showed that ECCD-1 caused cell dissociation in vitro (Ogou et al., 1983; Yoshida-Noro et al., 1984; Hatta et al., 1985; Hirai et al., 1989a,b). However, we detected no indication of cell dissociation at 30 or 60 minute applications of ECCD-1, in the present study. In turn, we observed the concurrent disruption of actin filament cable and morphologic conversion of the leading edge into prominent lamellipodial extension. Studies cited above reported that cell dissociation by ECCD-1 takes from several hours to overnight. In this regard, it seems likely that our observation is an early response of the leading edge cells to ECCD-1, although we could not test the long-term effects of ECCD-1, because long-term experiments in our in vivo wound model were not possible.

Control solutions cause some disruption of the actin filament cable albeit slight and quickly reversible, and, as stated previously, organ culture prevents formation of the actin filament cable. It is difficult to explain these data based on current knowledge. Two intriguing possibilities for explanation of these control experiments can be drawn. First, there may be an extracellular signal necessary for maintenance and formation of the actin filament cable. The vehicle solution will dilute the responsible extracellular signal in tear fluids, which cover the corneal epithelium, or possibly the reagents remove the protective and absorbent tear mucoid components. The results of the recovery experiments support this hypothesis. Second, although quite speculative, it is possible that the control IgG, because of its similarities in structure, may compete with the E-cadherin molecule to prevent assembly at the junction site. In fact, it has been reported that the structure of the amino-terminal domain of E-cadherin has structural homology to immunoglobulin tertiary structure without sequence homology (Overduin et al., 1995).

In fully polarized epithelial cells, ZO-1 is localized exclusively at tight junctions, but not adherens junctions (Stevenson et al., 1986). However, at the initial phase of polarization of epithelial cells from mouse mammary tumor, ZO-1 is colocalized with E-cadherin (Yonemura et al., 1995). Furthermore, it is reported that in non-epithelial cells such as cardiac muscle cells and fibroblasts, ZO-1 interacts with the cadherin-catenin complex and the actin filament cytoskeleton through direct interaction with α-catenin and actin filaments at its amino- and carboxyl-terminal halves, respectively (Itoh et al., 1993, 1997). In epithelial cells, ZO-1 associates with α-, β-, γ-catenins only at the initial phase of junctional formation in MDCK cells (Rajasekaran et al., 1996). Itoh et al. (1997) speculate that in epithelial cells ZO-1 functions as a cross-linker between α-catenin and the actin filament cytoskeleton at the initial stage of the cell-cell junctional formation. In the present study, neither ZO-1 nor occludin was localized at the adherens junctions where the actin filament cable inserted, in contrast with the reports by Bement et al. (1993) and Yonemura et al. (1995). Bement et al. (1993) showed colocalization of ZO-1 with actin filament cable, while Yonemura et al. (1995) showed that ZO-1 was highly concentrated at the cell-cell contact sites where actin filament cable inserted. Considering the spatial and temporal association of ZO-1 with E-cadherin at the early stage of cell-cell junctional formation (Itoh et al., 1993, 1997; Yonemura et al., 1995; Rajasekaran et al., 1996), the adherens junctions demonstrated in the present study may be more mature than those in the wounds created on monolayer sheets of the epithelial cell line (Bement et al., 1993; Yonemura et al., 1995), or the junctions of adult healing epithelium may differ.

It is interesting that in healing corneal epithelium, the

leading edge cells have no tight junction despite their apical position at the leading edge. In contrast, the apical cells just behind the leading edge have tight junctions. It seems that there is a sophisticated regulation of junction formation at the leading edge of migrating corneal epithelium, the leading edge cells exhibiting adherens junctions and the apical cells behind them exhibiting tight junctions. This junctional hierarchy in cell-cell junction formation is likely to be involved in the mechanism of stratification of stratified epithelium and seems to be an important subject for future studies. The leading edge cells appear to be specialized for coordinating migration and cells following behind them are specialized to quickly restore the epithelial barrier function.

Together with the elucidation of adherens junctions, immunocolocalization of myosin II along the actin filament cable supports the idea that the actin filament cable generates force and acts as a contractile 'purse string.' The contractile 'purse string' generates force in a different way than that of the lamellipodia. The latter requires force generation involving cell-matrix junctions and stress fibers. It remains to be determined whether there is a relationship or functional interaction between the adherens junction and the cell-matrix junction, and whether the actin filament cable anchored by an adherens junction can generate a significantly stronger mechanical contractile force than the cell-matrix junction.

A wide range of intracellular signaling pathways involving a wide range of extracellular signals have been implicated in regulating adherens junction assembly and function (Tsukita et al., 1992; Yap et al., 1997). Interestingly, Brock et al. (1996) demonstrated that loading the leading edge cells with C3 transferase, which inactivates endogenous Rho, prevents assembly of the actin filament cable and causes a failure of healing. More recently, the small GTPases, Rho and Rac, were shown to regulate the establishment of cadherin-dependent cell-cell contacts in human keratinocytes (Braga et al., 1997), and in MDCK cell lines (Takaishi et al., 1997). These studies may provide new insights into the role played by the actin filament cable in wound healing. However, since the Rho GTPases are also involved in organization of cell-matrix junctions, i.e. stress fibers and focal contacts (Ridley and Hall, 1992), it seems important to distinguish the effects of Rho GTPases on cell-cell and cell-matrix junctions and to determine whether the effects are consequences resulting from a direct association of functional components with the actin filament cable or with the stress fiber.

The fate of the leading edge cells during epithelial sheet movement remains elusive as discussed by Brock et al. (1996). Since wound size decreases, it is unlikely that all the leading edge cells remain at the leading edge. We observed wedge-shaped cells only sporadically at the leading edge, and the size of the individual cells of the leading edge does not appear to change significantly during healing. If cells at the leading edge drop behind, assembly and disassembly of the actin filament cable and the adherens junction should take place. In this dynamic organization of the adherens junction, it seems likely that this adherens junction may not be fully stabilized and may have different characteristics than adherens junctions of non-motile cells. However, to make matters more complicated, in the case of wounds in a stratified epithelium, it remains to be determined whether the cells excluded from the leading edge stay directly behind the leading edge on the basement membrane or whether they depart the basement membrane for the apical layer. Specific labeling of cells of the leading edge may provide information on the fate of the cells and may also provide clues to the mechanisms of epithelial stratification.

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