Enhancement of Vinculin Synthesis by Migrating Stratified Squamous Epithelium

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Abstract. A 110-115-kD protein is present at levels 27-fold higher in migratory epithelium in the rat cornea than in stationary epithelium. This protein represents 2.7% of the total protein in migratory epithelium 6-h postabrasion wound and 0.1% of the total protein in stationary epithelium. Our findings demonstrate that this 110-115-kD protein is vinculin. In Western blots comparing proteins from migratory and control epithelium, antibody against vinculin cross-reacted with the 110-115-kD protein. Using immunoslot blots, vinculin was determined to be present at maximal levels 6 h postabrasion wound, at levels 22- and 8-fold higher than control at 18 and 48 h, respectively, returning to control levels 72 h postwounding. Vinculin was also localized by indirect immunohistochemistry in migrating corneal epithelium.

Vinculin is a structural protein with an apparent molecular mass of 130 kD in nonreducing conditions and a slightly lower apparent molecular mass in reducing conditions (8). It was first purified from smooth muscle isolated from chicken gizzard and has been found to be localized in focal contacts of cultured fibroblasts (3, 8, 36), dense plaques of smooth muscle (10, 11), intercalated disks of cardiac muscle (11, 26, 31), and zonula adherens of epithelium (9, 10). The localization of vinculin adjacent to the plasma membrane and actin bundles suggests that it is a "linker" protein between the two structures. In this link, vinculin binds to talin (4, 30), which in turn binds to integrin (20), anchoring the cell to its substrate. The mechanism of the linkage between vinculin and actin bundles remains unclear. Initial reports suggested that vinculin could bind directly to actin (23, 41); however, this binding was later shown to result from impurities in the vinculin preparation (6, 34, 42). The finding that vinculin can be phosphorylated has led to speculation that phosphorylation might be a mechanism by which adherens-type junctions could be altered during transformation as well as in cell migration (35). This correlation of phosphorylation of vinculin and alteration of cell contacts resulting in altered cell activity has not been documented conclusively (1, 21, 24, 28, 33).

Although vinculin has been detected in epithelium in cell culture, its localization and synthesis by an intact sheet of migrating epithelium have not been elucidated. To determine whether vinculin levels change as cells migrate, we used a wound healing model in which the epithelium migrates as a unified sheet to cover the wound. Migration is dependent on protein synthesis and does not require cell mitosis (13, 18). We have demonstrated in this system that during corneal epithelial migration there is a large (≈15-fold) increase in protein synthesis relative to stationary control epithelium (16, 44). We detected a protein that is synthesized at enhanced levels by migrating epithelium, has an apparent molecular mass of 110-115 kD in reduced SDS-PAGE and a slightly higher molecular mass in unreduced conditions, and which we postulated to be vinculin (44). The present study was undertaken to test this identification.

Materials and Methods

Model Wounds and Organ Culture

The organ culture system used in this investigation has been described (16). Briefly, Sprague-Dawley rats with clear, healthy corneas were killed with an overdose of sodium pentobarbital. An epithelial wound 3 mm in diameter was created by demarcating an area on the cornea with a 3-mm trephine and removing the epithelium within the circle with a small scalpel, leaving an intact basement membrane (38). Following wounding, the corneas were dissected and placed into organ culture in a completely defined medium (15). Corneas were pinned to rounded paraffin posts that maintain the corneal...
curvature and provide a solid support for harvest of the epithelium. To harvest migrating epithelium, the corneas were stained with Richardson's stain (32), which reveals the remaining defect, the 3-mm trephine was placed on the cornea concentric to the defect, and the migrating epithelium was removed with a small scalpel. For controls, epithelium was removed from a central area of unwounded corneas. In organ culture 3-mm wounds heal in 22 h; in vivo wound closure occurs at 24 h.

Electrophoresis, Fluorography, and Immunoblotting

Epithelial protein samples were analyzed by SDS-PAGE using the buffer system described by Miles Laboratories (27). The gels were fixed in 50% methanol, and protein was detected with ammoniacal silver reagent as described by Wray et al. (43). Fluorography of [35S]methionine-labeled proteins was done using ENHANCE (New England Nuclear, Boston, MA) as previously described (45). Proteins of known molecular mass (43-200 kD) (Bio-Rad Laboratories, Richmond, CA) were used to determine molecular masses of unknown components.

Protein samples used for immunoblotting were transferred to nitrocellulose paper as described by Towbin et al. (39). The paper was then placed in a blocking solution containing 3% gelatin in TBS. The paper was washed and then incubated for 2 h in a preparation of monoclonal antivinculin (ICN Immuno Biologicals, Lisle, IL) at a 1:30 dilution. Antibody binding was detected by reaction (1 h) with peroxidase-conjugated goat anti-mouse IgG (Organon Teknika-Cappel, Malvern, PA) at a 1:3,000 dilution. Color was developed using 0.05% diaminobenzidine, 0.015% H2O2. Specificity of the commercial antivinculin was determined by preabsorption of the antibody with purified chicken vinculin (100 mg/ml) overnight at 4°C.

Purification of Chicken Gizzard Vinculin

Vinculin was purified from 50 g of frozen chicken gizzards as described by Feramisco and Burridge (7), except that we used a DEAE 5 PW (Waters Associates, Milford, MA) column for the last purification step. Vinculin appeared as a single band in SDS-PAGE migrating slightly ahead of purified β-galactosidase (116 kD). This molecular mass is in agreement with the report of Burridge et al. (5).

Quantification of Vinculin in Epithelial Protein Samples

Rats were anesthetized with an intraperitoneal injection of chloralhydrate (35 mg/100 g of body weight) (Squibb, Princeton, NJ) followed by topical application of proparacaine HCl (Akorn, Metairie, LA). 3-mm scrape wounds were made in the central cornea and allowed to heal in vivo for 1, 3, 6, 18, 48, and 72 h and 7 d. The rats were killed with an intraperitoneal injection of sodium pentobarbital, and the eyes were stained and epithelium harvested as described above. Epithelium from unwounded corneas served as control. The epithelium was immediately frozen in liquid nitrogen and proteins solubilized in 2% SDS, 2 M urea, 0.1 mM phenyl methyl sulfonyl fluoride in 0.064 M Tris-HCl, pH 8.9. Protein amounts were quantitated by proteins solubilized in 2% SDS, 2 M urea, 0.1 mM phenyl methyl sulfonyl fluoride in 0.064 M Tris-HCl, pH 8.9. Protein amounts were quantitated using a bidimensional gel apparatus and En3HANCE (New England Nuclear, Boston, MA) as substrate. Proteins of known molecular mass (43-200 kD) (Bio-Rad Laboratories, Richmond, CA) were used to determine molecular masses of unknown components.

For localization in cross sections of migrating epithelium, rat corneas were frozen in Tissue Tek II OCT compound (Lab Tek Products, Naperville, IL). 6-μm cryostat sections were placed on gelatin-coated slides and air-dried overnight at 37°C followed by rehybridization in PBS and washing in 1% BSA for 10 min. Alternatively, sections were fixed in 3% paraformaldehyde in PBS and used immediately. Monoclonal antivinculin was applied and incubated 1 h in a moist chamber. The sections were washed with PBS, incubated for 1 h in FITC-conjugated goat anti-mouse IgG (Organon Teknika-Cappel), washed, and mounted with a medium consisting of PBS, glycerol, and paraphenylenediamine. Then they were viewed and photographed using a photomicroscope III equipped for epi-illumination (Carl Zeiss, Inc.). To test for nonspecific binding, sections were viewed when either the primary or secondary antibodies were omitted or when the antivinculin was preabsorbed with purified chicken vinculin.

Results

Rat corneal epithelium consists of 5–7 layers of cells: a basal cell layer, 2–3 layers of wing cells, and 2–3 layers of flattened superficial cells. After an abrasion wound in which basement membrane is left intact, epithelium migrates into the wound area as a unified sheet with the leading edge of the sheet tapering down to a single cell layer. Migration does not require mitosis; cell elongation and flattening provide coverage of the wound. After epithelial wound closure, the rate of mitosis increases, and the epithelium regains its normal stratification (18).

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A 110–115-kD protein is present in greater amounts in epithelium migrating over denuded basement membrane than in stationary epithelium (Fig. 1). Fig. 1 shows the incorporation of [35S]methionine into proteins by the epithelium during organ culture; the 110–115-kD protein is radiolabeled in migrating epithelium to a far greater extent than in stationary, unwounded corneal epithelium. This protein could be extracted in low-ionic-strength buffers such as 0.1 M ammonium acetate (Fig. 2). It could also be extracted in 0.1 M phosphate buffer, pH 7.0, or 10 mM Tris-HCl, pH 7.4.

To determine whether the protein was vinculin, protein samples from migratory epithelium inside the 3-mm wound area (W), migratory epithelium from outside the original 3-mm wound periphery (WP), and unwounded control epithelium (C) were reacted with a monoclonal antibody against chicken vinculin. After staining of the blot for total protein, the 110–115-kD protein could be detected in greater amounts in migratory epithelium (W + WP) than in C epithelium (Fig. 3 a). In an identical blot, antibody against vinculin reacted with bands corresponding to the 110–115-kD protein in W and WP with no detectable reaction in control epithelium (Fig. 3 b). To ensure specificity of the antibody, it was preabsorbed with purified chicken gizzard vinculin and then reacted with proteins from migratory epithelium in Western blots. The reaction of antivinculin and the 110–115-kD protein was completely blocked (Fig. 3 c). No reaction was seen when the primary or secondary antibody was omitted (data not shown).

To quantify alterations of vinculin levels during epithelial migration, rat epithelium was assayed with immunoslot blots (Fig. 4). In both W and WP epithelium migrating over a scrape wound, vinculin was present at maximal levels 6 h after wounding representing 2.72 ± 0.14 µg and 1.60 ± 0.001 µg/100 µg of total epithelial protein, an increase of 27- and 16-fold over control levels, respectively. The increase in vinculin levels in WP was seen as early as 1 h after wounding; the level was 11-fold higher than control. Vinculin levels in W could not be measured at 1 and 3 h since only very small amounts of epithelium have migrated into the original 3-mm wound area at these time points. By 48 h after wounding, 24 h after wound closure, levels in W and WP dropped to 0.84 ± 0.11 and 0.90 ± 0.11 µg of vinculin/100 µg of total epithelial protein, and, by 72 h, the level of vinculin returned to control levels. The amount of vinculin remained at control levels 7 d after wounding.

In whole amounts of rat corneas, 20 h postscrape wound in vivo, vinculin was detected as punctate spots near the leading edge of migrating epithelium around the entire wound margin (Fig. 5). Behind the leading edge where the epithelium is more stratified, fewer punctate spots could be detected. Punctate spots were not detected when the primary antibody was omitted or when the antibody was preabsorbed with vinculin.

Vinculin was also immunolocalized in cross sections of cryostat sections (Fig. 6). Intensity of binding was far greater in migrating than in stationary epithelium, corroborating the immunoslot blot data (Fig. 4). In corneas 6 and 18 h postabrasion wound, vinculin was localized, at the leading edge of migrating epithelium, along the basal cell membranes adjacent to the basement membrane and in patches

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**Figure 2.** 5–15% SDS-PAGE of corneal epithelium harvested 18 h postabrasion wound. Epithelium was extracted in 0.1 M ammonium acetate in a glass–glass homogenizer. Homogenate was centrifuged at 12,000 g for 10 min. Supernatant (S), 20 µg/lane, and pellet (P), 40 µg/lane, were applied to SDS-PAGE. Arrow indicates 110–115-kD protein. Silver stain. Molecular masses (in kilodaltons) determined from standard proteins are noted.

**Figure 3.** (a) Blot of 5–15% SDS-PAGE: W, 20 µg of protein from epithelium harvested 18 h postabrasion wound that has migrated into original 3-mm abrasion wound area as described (16); WP, 20 µg of protein from epithelium harvested 18 h postabrasion wound outside original 3-mm abrasion wound periphery; C, 20 µg of protein from control, unwounded epithelium. Blot was stained with 0.1% naphthol blue-black in 45% methanol and 10% acetic acid to reveal total proteins present. Arrow indicates 110–115-kD protein. Molecular masses (in kilodaltons) determined from standard proteins are noted. (b) Immunoblot of 5–15% SDS-PAGE identical to blot on left. Blot was reacted with antivinculin as described in Materials and Methods. Arrow indicates immunoreaction with protein comigrating with 110–115-kD protein. (c) Immunoblot of 5–15% SDS-PAGE identical to W in a and b reacted with antivinculin preabsorbed overnight with purified chicken vinculin (100 µg/ml). Note lack of reaction to 110–115-kD protein.

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between cells as well as diffusely throughout the cell (Fig. 6b). Behind the leading edge at 18 h, vinculin was localized as punctate spots along the basal cell membranes adjacent to the basement membranes (Fig. 6c). In unwounded corneas, vinculin was localized in far lesser amounts with only diffuse binding and along cell membranes of superficial cells. Vinculin was not localized in corneas reacted with antivinculin preabsorbed with purified chicken vinculin. Identical localizations of vinculin were seen when the tissue was cryostat sectioned, fixed in 3% paraformaldehyde, and used immediately for immunolocalization.

**Discussion**

Corneal epithelial migration in response to a wound involves major changes in epithelial architecture and protein synthesis. The hemidesmosomes, the normal cell-substrate adherens junctions, disappear (25), the number of cell layers decreases, the cells flatten and elongate, and mitosis diminishes (18). In addition, the rates of protein and glycoprotein synthesis increase dramatically during epithelial migration (16, 44). The enhanced synthesis of vinculin suggests that a new method of cell-substrate adhesion is activated during epithelial migration.

Although our data do not demonstrate that vinculin is localized in focal contacts in our system, the localization of vinculin along the basement membrane zone is consistent with reported localization of focal contacts in cultured cells.

**Figure 4.** Vinculin concentrations in W (black bars) and WP (cross-hatched bars) epithelium quantitated as described in Materials and Methods. Wound, epithelium that has migrated into original 3-mm wound area; WP, epithelium harvested from outside original WP; control, epithelium removed from unwounded corneas. Error bars are ± SEM. n = 8. Note: SEM for 6 h WP is ± 0.001 and is contained in border of bar. Vinculin concentrations in 1- and 3-h W could not be determined since only very small amounts of epithelium have migrated into the original 3-mm wound area at these time points.

**Figure 5.** Micrographs showing immunolocalization of vinculin in flat mounts of corneas allowed to heal 20 h in vivo after 3-mm-diam abrasion. Arrows indicate leading edge of migrating epithelial sheet. (a) Phase-contrast of area shown in b. Bar, 50 μm. (b) Same area as a showing punctate binding of vinculin antibody at leading edge. Bar, 50 μm. (c) Lower magnification showing punctate localization around wound edge. Bar, 125 μm. (d) Preabsorption control showing lack of punctate staining. Bar, 50 μm. Defect area = area of wound remaining to be covered by migrating epithelium.
Figure 6. Micrographs showing immunolocalization of vinculin in cryostat sections. (a) Control, unwounded cornea. Large arrows indicate punctate binding along membranes of superficial cells. Bar, 50 µm. Note: a is printed at 6x the intensity of b, c, and d to allow detail to be seen. (b) Leading edge of migrating sheet of epithelium 6 h postabrasion wound. Large arrows indicate binding along basement membrane; small arrows indicate punctate binding between cells. Note overall increase in binding compared to controls. Similar localization was seen 18 h postabrasion wound. Bar, 50 µm. (c) Migrating epithelium behind leading edge, 18 h postabrasion wound. Arrows indicate punctate binding along basement membrane. Bar, 50 µm. (d) Preabsorption control showing reaction of antivinculin preabsorbed with chicken vinculin in leading edge of migrating epithelium. Bar, 50 µm. In b and d, note that tip of leading edge of migrating epithelium has been artefactually separated from basement membrane during sectioning.

Since the first report by Geiger (8) that vinculin is localized in focal contacts in cultured chick fibroblast cells, vinculin has been used as a marker of focal contacts in epithelial and nonepithelial tissues. The increased presence of vinculin and its localization along the basement membrane zone in migratory corneal epithelium suggest that focal contacts might be involved in the linkage between the epithelial sheets and their substrate during migration. The present report of increased synthesis of vinculin and its localization at the leading edge is, to the best of our knowledge, the first in an in vivo system showing migration of an intact epithelial sheet. Focal contacts and vinculin have been demonstrated in various in vitro systems involving cell spreading and growth. Soong (37) localized vinculin in rat corneal epithelial cells grown on collagen-coated glass slides. He found that vinculin was localized at the termini of actin bundles in the most motile cells at the edge of the cell colony. Vinculin and focal contacts have also been demonstrated in cell culture in the motile cells of neurites (17), human erythroleukemia cells (22), platelets (2), retinal pigmented epithelial cells (29), and chick fibroblasts (11). In all of these systems, vinculin has been hypothesized to be involved in linking the cell cytoskeleton to its substrate during cell movement.

During migration, vinculin is localized along the basement membrane between cells and also diffusely throughout the cells (Figs. 5 and 6). The diffuse localization may represent the cellular pool of vinculin required for the formation of cell–cell and cell–substrate junctions. In flat mounts, the localization of vinculin is maximal at the rear of the first cell, perhaps providing an anchor for the cell to push against. This localization differs from that of Soong (37) who found in cultured cells that the localization was at the front of the cell.

The localization of vinculin in cell–cell junctions in the suprabasal cells of the migrating sheet correlates with the report of Gipson and Anderson (14) who found actin bundles in the same area in migrating epithelium. These data suggest that the upper layers of cells are actively involved in the migration process forming junctions and that they are not merely passively carried along by actively migrating basal cells.

Perhaps our most important finding is the increase up to 27-fold in the amount of vinculin present in migratory compared with stationary epithelium. This type of increase was also observed in cultured fibroblasts (40) where fibroblasts grown on a substrate promoting focal contacts expressed up to 20-fold more vinculin than fibroblasts grown on a poorly adherent substrate. Geiger et al. (12) postulate that vinculin may control its own synthesis and that increased synthesis follows sequestering of the vinculin into junctions as would occur during the onset of migration. Another possible expla-
nation for the increase in vinculin synthesis is suggested by the report of Herman et al. (19) who found that, following disruption of focal contacts, vinculin was not recycled, and that the return of vinculin to adhesion plaques required de novo mRNA transcription and protein synthesis. If vinculin could not be reused during a period of rapid turnover of focal contacts such as would be expected in migration, large amounts of vinculin would have to be synthesized. Finally, it is of interest that using SDS-PAGE we detected a large increase in the amount of a protein corresponding to vinculin. No increase in any other focal contact component (e.g., integrin, talin, α-actinin, or actin) was detected (44). Although this may reflect only the limitation of our assay, it is intriguing to speculate that vinculin may be the central point of focal adhesion formation.

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