Integrins in the Wounded and Unwounded Stratified Squamous Epithelium of the Cornea

Mary Ann Stepp,* Sandra Spurr-Michaud,* and Ilene K. Gipson*

Purpose. The authors determined the synthesis, cell surface expression, and localization of integrins in the rat corneal epithelium to detect whether any changes in integrins occur during epithelial migration in response to simple debridement wounding.

Methods. Immunoprecipitation analysis of extracts from either metabolically or surface-labeled rat epithelia was done to assess the synthesis and cell surface expression of integrins in the normal cornea. The localization of integrins was determined by indirect immunofluorescence of frozen sections obtained from control corneas and from those after debridement wounding. Immunoblotting of extracts from time course experiments was done on organ cultures of rat corneas after debridement to determine if any changes in the amounts of integrins occurred. The cell adhesion function of integrins on control and migrating epithelial cells was evaluated by cell adhesion assays.

Results. The data indicated that the corneal epithelium has a variety of distinct integrin subunits including $\beta_1$, $\beta_4$, $\alpha_2$, $\alpha_5$, $\alpha_6$, and $\alpha_v$. Although $\beta_1$, $\beta_4$, $\alpha_5$, and $\alpha_v$ were localized to sites of apparent cell–cell contact, $\alpha_2$, $\alpha_6$, and $\beta_4$ were localized specifically to the basal membrane of the basal cells. Little change occurred in the localization of integrins in the migrating epithelial sheets. At 3, 6, 9, 12, 18, and 24 hr after wounding, the amount of the $\beta_1$, $\beta_4$, $\alpha_5$, and $\alpha_v$ integrin subunits (as measured by immunoblots) was not altered relative to that of the control corneas. Adhesion assays also showed no differences in adhesion of stationary versus migrating corneal epithelial cells to fibronectin and laminin.

Conclusions. Integrin localization, production, and cell adhesion function in the stratified squamous epithelium of the cornea are not dramatically altered during epithelial cell migration over simple debridement wounds. Integrins in the cell membrane at sites of cell–cell interaction and as components of the hemidesmosomes in stationary epithelia may be available for rapid recruitment as epithelial cell migration proceeds. Invest Ophthalmol Vis Sci. 1993;34:1829-1844.

The stratified squamous epithelia (SSE)\(^1\) of the body, including the epidermal, corneal, vaginal, esophageal, and oral mucosal epithelia, border the outside world and are continually subjected to abrasive forces and other environmental assaults. To maintain their integrity, these tissues have developed a variety of cell–cell and cell–substrate adhesion mechanisms, including desmosomes at the sites of cell–cell interaction and hemidesmosomes at the sites of cell–substrate interaction. Recently, several groups have reported the presence of members of the integrin family of receptors at the sites of cell–cell and cell–substrate interactions in the epidermis of the skin\(^1-7\) and the epithelium of the cornea.\(^8,9\) The integrins are a family of integral mem-

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brane glycoproteins that function as \(\alpha\beta\) heterodimers.\(^{10-13}\) Since their original characterization,\(^{14}\) the known integrin family has been expanded to include at least 8 \(\beta\) and 14 \(\alpha\) subunits. Integrins are bifunctional molecules; they simultaneously interact extracellularly with extracellular matrix (ECM) molecules or counterreceptors on adjacent cells and intracellularly with components of the actin-based cytoskeleton. Increased expression of certain integrins can cause a decrease in motility in some cell types;\(^{15}\) other investigators have suggested that integrins are elevated during cell migration.\(^{16,17}\)

The integrins reported in the SSE include \(\alpha_6\beta_1\), \(\alpha_3\beta_1\), \(\alpha_6\beta_3\), \(\alpha_6\beta_4\), and low but detectable amounts of \(\alpha_6\beta_1\). Although experiments done on cultured keratinocytes suggest a functional role for the \(\beta_1\)-containing integrin heterodimers in maintaining epithelial cell–cell interactions,\(^6\) the exact nature of the integrin-containing cell–cell adhesion structures and their relative contribution to the overall integrity of the SSE of the body remain to be determined. Somewhat more progress has been made in understanding integrin involvement in the adhesion of the epithelium to its basement membrane. Several groups have reported that the integrin heterodimer \(\alpha_6\beta_4\) is an integral membrane protein component of the hemidesmosome;\(^{18-20}\) however, the extracellular ligand in the basement membrane and the intracellular ligand in the cytoplasm with which the hemidesmosomal \(\alpha_6\beta_4\) integrin interacts remain to be identified.

One of the intrinsic properties of SSE is the ability to respond rapidly to injury.\(^{21-23}\) Shortly after injury, the metabolic activity of the individual SSE cells increases dramatically. The basal cells at the wound margin begin to lose their hemidesmosome attachment sites and start to change their shape from columnar to a more elongated morphology as they begin to send out lamellipodia. The strength of cell–cell adhesion interactions in the migrating epithelial sheet must be sufficient to withstand the forces generated during this process. As the basal cells change shape and begin to move out over the wound bed, the neighboring cells appear to be pulled along behind them. The result is that the entire sheet of cells moves, and the epithelium becomes progressively thinner as cells flatten out in an attempt to cover the exposed wound bed. After the wound edges meet and epithelial integrity is again established, the metabolic activity of the cells decreases, and a wave of mitosis begins as the tissue begins to reestablish.

Corneal epithelial cell migration has been extensively characterized using both in vivo and in vitro wound-healing models in rabbits and rodents.\(^{24-26}\) Shortly after epidermal and corneal wounds occur in vivo, a fibronectin- and fibrin-rich matrix is laid down on the exposed basement membrane or stroma.\(^{27,28}\) It is this matrix that the epithelial cells first encounter as they begin to migrate. In vivo and in vitro experiments using an in vitro organ culture model in rat corneas showed that, shortly after cells began to migrate, their production of vinculin relative to total protein increased dramatically.\(^{29}\) Fibronectin and vinculin indirectly interact by binding to integrin receptors in focal contacts.\(^{30}\) We evaluated integrin production and localization in control and migrating corneal epithelium and, in a series of time course experiments using rat corneal epithelium, quantified the integrins in sodium dodecyl sulfate (SDS) extracts of epithelial sheets before, during, and shortly after wound closure. In addition, we evaluated the ability of integrins on the surface of control and migrating epithelial cells to function in cell binding to fibronectin and laminin using cell adhesion assays.

**MATERIALS AND METHODS**

**Materials**

The antisera used in immunohistochemical analysis, immunoblotting, and immunoprecipitation were gathered from a variety of sources. The laboratory of Richard O. Hynes (Center for Cancer Research and the Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA) donated several antibodies, including those directed against the \(\beta_1\) subunit and \(\alpha_5\) integrins (161); these antisera were used in immunohistochemical, immunoprecipitation, and immunoblot analyses as indicated in the text. An antiserum against the ectodomain of the rat \(\beta_1\) integrin was provided by Donald Gullberg (Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA) and used to localize this subunit by immunohistochemical analysis. Polyclonal antisera against the cytoplasmic domains of the human \(\beta_3\), \(\alpha_5\), \(\alpha_6\), and \(\alpha_4\) integrin molecules were provided by F. Giancotti (Department of Pathology, New York University School of Medicine, New York, NY). A polyclonal antibody prepared in rabbits against the denatured human \(\beta_4\) antigen and an antiserum that recognizes the cytoplasmic domain of \(\beta_8\) integrin were donated by Martin Hemler (Dana Farber Cancer Institute and Harvard Medical School, Boston, MA). Telios Pharmaceuticals (San Diego, CA) provided the polyclonal antiserum \(\alpha_5\beta_3\) against human vitronectin receptor. The \(\alpha_6\) subunit was localized and precipitated using \(\text{G}_{\text{6 H}}\), a rat monoclonal antibody that recognizes the \(\alpha_6\) subunit in many species; \(\text{G}_{\text{6 H}}\) was obtained from Arnoud Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands). A monoclonal antibody recognizing the human \(\alpha_5\) integrin molecule, used in immunohistochemical techniques, was obtained from F. Watt (Imperial Cancer...
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Research Foundation, London, UK). A monoclonal antibody against vinculin was obtained from Boehringer Mannheim (Indianapolis, IN). Table 1 provides additional information about the antisera.

Human plasma fibronectin for the cell adhesion assays was obtained from the New York Blood Center (New York, NY); all other ECM proteins were obtained from Telios Pharmaceuticals, including human collagen types I, III, IV, V, and VI; rat laminin; bovine vitronectin; and human tenasin. GRGDSP (gly-arg-gly-asp-ser-pro) and GRGESP (gly-arg-gly-glu-ser-pro) peptides were also obtained from Telios Pharmaceuticals.

The Organ Culture Model

All investigations described in this report conformed to the ARVO Resolution on the Use of Animals in Research. Rats (Sprague-Dawley) and mice (Balb/c) were killed by intraperitoneal lethal injection of sodium pentobarbital. For studies of the migrating corneal epithelium, a 3-mm central corneal area was demarcated with a dulled trephine, and the epithelium within this region was removed by gentle scraping with a dulled scalpel. After wounding, the eyes were removed, and the corneas were excised and placed in organ culture in serum-free medium supplemented as described previously. Controls included both epithelial sheets from unwounded corneas subjected to the same organ culture conditions as were the wounded corneas and also epithelia removed from corneas immediately after the death of unwounded animals. For experiments involving metabolic labeling (immunoprecipitation and cell adhesion studies), methionine-free medium, supplemented with 35S-methionine and 35S-cysteine (Trans 35S-label; ICN, Irvine, CA) at 250 μCi/ml (1 μCi = 37 KBq), was used for organ culture. Rat corneas were used in most experiments; mice were used for experiments involving G0H3.

Immunohistochemical Analysis

The excised corneas were frozen in Tissue Tek II OCT (Lab Tek Products, Naperville, IL) compound, and 6-μm cryostat sections were placed on gelatin-coated slides and dried overnight at 37°C. The sections were rehydrated in phosphate-buffered saline (PBS), pH 7.2, and washed in PBS with 1% (wt/vol) bovine serum albumin (BSA) for 10 min. The primary antibody was then applied for 1 hr at room temperature in a moist chamber. The slides were rinsed with PBS followed by 10 min in PBS with 1% (wt/vol) BSA, and the secondary antibody applied for 1 hr at room temperature in a moist chamber. After a PBS wash, cover slips were

<table>
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<th>Antibody Specificity</th>
<th>Name</th>
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<tr>
<td>β1</td>
<td>363</td>
<td>Cytoplasmic domain peptide from chicken β1</td>
<td>Rabbit</td>
<td>Marcantonio and Hynes. J Cell Biol. 1988;106:1765</td>
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<td>Mouse</td>
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<td>α2</td>
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<td>Mouse</td>
<td>F. Watt, Imperial Cancer Research Foundation, London, U.K.</td>
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<td>α6</td>
<td>GoH3</td>
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<td>Rat</td>
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<td>Purified human vitronectin receptor</td>
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<td>β5</td>
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<td>Cytoplasmic domain peptide from human β5</td>
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<td>Vinculin</td>
<td>V284</td>
<td>Human vinculin</td>
<td>Mouse</td>
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mounted with a medium of PBS, glycerol, and para-
phenylenediamine. Negative control tissue sections
( primary antibody omitted) were run with each anti-
body-binding study.

The secondary antibody was either tetramethyl
rhodamine isothiocyanate-conjugated goat anti-rat im-
munoglobulin (Ig) G, fluorescein isothiocyanate-con-
jugated goat anti-human IgG (both from Boehringer
Mannheim), fluorescein isothiocyanate-conjugated rab-
bit anti-mouse IgG (Calbiochem, La Jolla, CA), or
dichlorotriazinyl amino fluorescein-conjugated don-
key anti-rabbit IgG (Jackson Laboratories, West
Grove, PA). All secondary antibodies had been affinity
purified and had minimal cross reaction to other spe-
cies.

**Immunoprecipitation**

Unwounded corneas were incubated in organ culture
for 18 hr to label cellular proteins. After labeling, the
epithelium was removed from the underlying stroma
by incubation for 1 hr at 35°C in Ca2+- and Mg2+-free
minimum essential medium (MEM) supplemented
with 0.5 mol/l ethylenediaminetetraacetic acid. The
freed sheets were then placed in buffer A (50 mmol/l
Tris, pH 8.0, 0.15 mmol/l NaCl, 0.5 mmol/l CaCl2,
with 0.5 mol/l ethylenediaminetetraacetic acid. The
precipitated proteins were sus-
dained in Laemmli sample buffer, boiled, and run
nonreduced on 20-cm SDS 6% (wt/vol) polyacryl-
amide gels and processed for autoradiography.

Experiments involving immunoprecipitation of
surface-labeled integrins required modifications of
this procedure. Epithelial sheets from organ cultured
corneas were removed from underlying stroma by in-
cubation of the corneas in 2.4 mg/ml Dispase (Boeh-
ringer Mannheim, Grade II) in MEM for 1 hr. Indi-
vidual cells were obtained from the intact sheets by
trypsinization of Dispase-released sheets of epithelium
obtained as described. The resultant cell suspension
was washed extensively in 0.5 mg/ml soybean trypsin
inhibitor (Sigma) in PBS. The cells were labeled
with 125I and lactoperoxidase (Sigma) as previously de-
scribed. After labeling, the cells were extracted as
described previously, the trichloroacetic acid-precipi-
table counts of 125I were determined by gamma count-
ing, and equal counts per minute of surface-labeled
extracts were immunoprecipitated.

**Immunoblotting**

For immunoblotting time course experiments,
wounded corneas were maintained in defined medium
for increasing time intervals from 0–24 hr; previous
experiments have shown that a 3-mm rat corneal de-
bridement wound heals in this organ culture model by
22 ± 2 hr. Epithelial sheets, harvested by scraping,
were extracted with 2% SDS sample buffer without
dyes; the protein concentrations were determined us-
ing the BCA protein assay reagent (Pierce, Rockland,
IL). Controls for these experiments were epithelial
sheets obtained by scraping from freshly killed rats.
We ran 60 µg of total protein from control and mi-
grating epithelial extracts on 6% SDS polyacrylamide
gels. The proteins were transferred to nitrocellulose
filters (NC BA83, 0.2 µm; Schleicher and Schuell,
Keene, NH) at 4°C overnight at 100 mA constant volt-
age in an electroblot buffer containing 0.2 mol/l gly-
cine, 25 mmol/l Tris base, and 20% methanol. Nitro-
cellulose filters were blocked for 1 hr with 10% milk in a
0.1% solution containing 25 mmol/l Tris, pH 7.5,
150 mmol/l NaCl, and 0.1% Tween-20 (TBST) and
washed with 0.3% TBST (0.3% Tween-20) for 15 min
followed by two washes for 10 min each with 0.1%
TBST. Then, the blots were incubated for 1 hr with a
1:1000 dilution of horseradish peroxidase-conjugated
donkey anti-rabbit IgG (NA.9340; Amersham, Arling-
ton Heights, IL), the wash steps were repeated, and
the antigens were detected using the ECL western
blotting detection system (Amersham). The film was
scanned by densitometry.

**Cell Adhesion Assays**

Microtiter plates (Costar, 96-well tissue-culture plas-
tic, Fisher Scientific, Pittsburgh, PA) were coated
overnight at room temperature with ECM proteins di-
luted in PBS at 10 mg/ml. The plates were rinsed thor-
oughly in PBS and uncoated plastic blocked with 2
mg/ml of heat-deactivated BSA (Sigma, 96–99% pure)
in PBS. The plates were rinsed again and temporarily
stored at 4°C until needed.

Both normal control and migrating epithelium
were cultured for 18 hr in 35S-containing medium to
radio-label the cells. Individual cells were obtained by
tryptsinization of Dispase-released sheets of epithelium
obtained as described. The resultant cell suspension
was washed extensively in 0.5 mg/ml soybean trypsin
inhibitor (Sigma) in PBS both to deactivate the trypsin
and to remove unincorporated radioactive methionine
and cysteine; the final cell suspensions were prepared

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in serum-free MEM. The cell numbers were counted manually using a hemocytometer, and the counts per minute per cell were determined by liquid scintillation counting. The cells were plated at 10^5 cells/well and allowed to adhere for 60 min at 37°C under 5% CO₂, followed by extensive washing with MEM to remove nonadherent cells. For peptide addition assays, GRGDS or GRGES was added at the time of plating. Cells still adherent after multiple washes were solubilized in 2% SDS (Bio-Rad, Richmond, CA), and the counts per minute extracted were quantified by liquid scintillation counting. The data were expressed as the fold increase in cell adhesion to BSA over the background cell adhesion.

DNA Assay

DNA was quantified in SDS extracts of epithelial sheets using a fluorometric assay based on the binding of the fluorochrome H33258 (catalog no. TK0310, Hoeffer Scientific, San Francisco, CA) to DNA as described previously. Fluorescence was detected using a Perkin Elmer LS-5B luminescence spectrometer (Norwalk, CT) with the excitation at 365 nm and the emission at 455 nm. Calf thymus DNA (TKO102, Hoeffer Scientific) was used as a standard.

RESULTS

Integrin Synthesis and Cell Surface Expression in Stationary Corneal Epithelial Cells

The ability of integrins to function in cell adhesive events requires the synthesis of αβ heterodimers and their expression on the cell surface. To determine which integrin heterodimers are being synthesized by these cells, immunoprecipitation experiments were done on metabolically labeled epithelial extracts under conditions whereby heterodimers were maintained. Figure 1A shows the results of experiments in which corneas from rats (or mice as indicated in the legend) were incubated overnight in a defined medium containing 250 μCi/ml ^35S-methionine-^35S-cysteine, the epithelial sheets harvested, the proteins extracted, and immunoprecipitation done using the antiserum indicated. The precipitated proteins were then run on a 6% SDS polyacrylamide gel under nonreducing conditions. The β1 antiserum precipitates three bands corresponding to pre-β1, mature β1, and a single band assumed to represent several comigrating α chains. The α5 antiserum precipitates two bands, α5 and the mature β1 subunit. Using a polyclonal antisera against the vitronectin receptor, a broad band migrating in the α chain region of the gel and a minor band migrating more slowly than β1 were observed. We saw no bands migrating in the expected position of β1, indicating that the amount of αβ1 integrin in these tissues is minor. This pattern was consistent with the presence of α6 integrin in heterodimers with β5. Immunoprecipitation of epithelial extracts with β4 antiserum detected both the large β4 subunit and an α subunit known to be α6. Using a mouse corneal epithelial extract, α6 antiserum precipitates both α6 and β4 integrin. No bands migrating in the expected position of β1 were observed, confirming that minor amounts of α6β1 heterodimers are present in these epithelial tissues. As reported previously, immunoprecipitation with antibodies against α6 does not detect quantitative amounts of the associated β4 chain. This could be the result of the instability of the heterodimer induced by antibody binding and/or the degradation of the β4 subunit in some immunoprecipitation reactions but not others. It explains the differences in the ratios of the α6 and β4 subunits observed in lanes 4 and 5 of Figure 1A. We were unable to precipitate α1 and α4 integrins in these tissues (Fig. 1A). Not shown are the results of experiments in which extracts were unsuccessfully immunoprecipitated with α4 and β3 antisera.

Figure 1A shows that a variety of integrin heterodimers are present in epithelial tissue and that αβ1 heterodimers constitute a major portion of them. In skin epidermis, α6β1 heterodimers are abundant. Because we lacked an α6 antisera that cross reacted with rodent tissues, we took an indirect approach to identifying the relative amounts of non-α6-containing β1 heterodimers. We used metabolically labeled epithelial extracts in a series of pre-clearing experiments. The extracts were subjected to three rounds of preclearing with the indicated antiserum, and then the proteins remaining in the extract were precipitated with a different integrin antiserum. Figure 1B shows the results of these experiments. After a pre-clearing with α6 antisera to remove all the α6 heterodimers from the extract and then precipitation of the remaining integrins in the extract with an antisera against the β1 chain, approximately one half of the total β1 was precipitated with the α6 antiserum. The α chains present in the β1 heterodimers that remained after removal of the α6 migrated slightly differently; they moved in a much broader, more diffuse band, which on occasion, resolved into a doublet. This result was consistent with the findings of other integrin α chains being present in the β1 immunoprecipitates. In Figure 1A, we show that there was little if any α1 and α6 integrin in these extracts and that α6 and α4 formed complexes with other β subunits besides β1. Therefore, the data presented in Figure 1B are consistent with the conclusion that approximately one half of the β1-containing heterodimers was α6β1 and most of the rest was α4β1. Also shown in Figure 1B is the inverse pre-clearing experiment, in which metabolically labeled extracts were first precipitated by three rounds of β1 antiserum to re-
FIGURE 1. Immunoprecipitation analyses of integrins in the corneal epithelium. In all cases, immunoprecipitates underwent electrophoresis on 6% SDS separating gels under nonreducing conditions, and the dried gels were visualized using autoradiography. (A) Epithelial extracts, metabolically labeled with $^{35}$S-methionine and $^{35}$S-cysteine in organ culture, were immunoprecipitated with antisera against the integrin subunits as indicated. Lane 1, $\beta_1$; Lane 2, $\alpha_5$; Lane 3, $\alpha_5\beta_3$; Lane 4, $\beta_4$; and Lane 5, $\alpha_6$. Immunoprecipitation experiments using antisera against $\alpha_7$, $\alpha_4$, and $\alpha_3$ were unsuccessful (not shown). Rat epithelial extracts were used for Lanes 1–4; the extract used in Lane 5 was from mouse epithelia. (B) Lanes 1–4 contain metabolically labeled rat epithelial extracts that were subjected to three rounds of preclearing with an antiserum against $\alpha_3$. Lanes 3, 2, and 1 show the $\alpha_3$-containing heterodimers immunoprecipitated in the first, second, and third rounds of preclearing respectively; Lane 4 shows immunoprecipitation of the $\alpha_3$-depleted extract with an antiserum against $\beta_1$. Note that after the third round of preclearing, no $\alpha_3$ remained in the extract (Lane 1). As indicated in the text, the likely identity of the $\alpha$ chain in Lane 4 is $\alpha_2$. Lanes 5–8 contain metabolically labeled rat epithelial extracts that were also subjected to three rounds of preclearing with an antiserum against $\beta_1$. Lanes 7, 6, and 5 show the $\beta_1$-containing heterodimers immunoprecipitated in the first, second, and third rounds of preclearing. Lane 8 shows immunoprecipitation of the $\beta_1$-depleted extract with an antiserum against $\alpha_3$. Note that all the $\alpha$ present appears to be associated with $\beta_1$. Lanes 7–8 contain metabolically labeled rat epithelial extracts that were subjected to three rounds of preclearing with an antiserum against $\beta_1$. Lanes 7, 6, and 5 show the $\beta_1$-containing heterodimers immunoprecipitated in the first, second, and third rounds of preclearing. (C) Individual epithelial cells were surface labeled with $^{125}$I, the cells were extracted, and the extracts were immunoprecipitated with $^{125}$I using a lactoperoxidase procedure. To obtain individual corneal epithelial cells from the intact tissue, corneas were incubated with Dispase to allow removal of intact sheets of epithelium. The sheets were then trypsinized to obtain single cells for surface-labeling studies. It has been reported that integrins are trypsin resistant; however, their sensitivity to Dispase is not known.

Lane 1 of Figure 1C shows that, even after Dispase...
and trypsin, at least a portion of the \( \beta_1 \) heterodimers could be found intact on the cell surfaces. Although a band migrating just under the 77,000 Dalton marker was observed in Lane 1, which could represent a cleavage product of \( \beta_1 \), most of this integrin did appear to migrate as though it were intact. The inability to resolve the integrin \( \alpha \) and \( \beta \) chains completely in Lane 1 of Figure 1C was the result of a diffuse fuzzy appearance caused by the 123I surface label on the integrins. Lane 2 of Figure 1C shows that intact \( \beta_4 \) integrin could also be immunoprecipitated after Dispase and trypsin treatment. Because \( \beta_4 \) is localized to the basal surface of the basal cells where Dispase acts to cause the release of the epithelial sheet, these data imply that the \( \beta_4 \) integrin is not cleaved during the Dispase-induced release of epithelial sheets from the stroma. The relative lack of coprecipitating \( \alpha_6 \) in Lane 2 of Figure 1C could be caused by degradation of \( \alpha_6 \) integrin. A more likely explanation, however, is the \( \alpha_6\beta_4 \) heterodimer instability during the immunoprecipitation reaction. The results in Figure 1C confirm the conclusion that integrin heterodimers are indeed on the surface of corneal epithelial cells and that Dispase, used as described in these experiments, appears to leave most of the \( \beta_1 \) and \( \beta_4 \) integrins on the surface of cells intact.

In summary, these biochemical analyses indicate that a variety of integrins, including \( \beta_1, \beta_4, \alpha_2, \alpha_5, \alpha_6 \), and \( \alpha_\varepsilon \), are synthesized and expressed on the cell membranes of epithelial cells. We found no evidence for \( \beta_8, \alpha_1, \alpha_4 \), or \( \alpha_8 \) integrins in these tissues by immunoprecipitation. The possible significance of our inability to detect the \( \alpha_6 \) and \( \beta_4 \) integrins by immunoprecipitation when they are detectable by other methods will be addressed subsequently.

### Localization of Integrins in the Stationary SSE

The corneal epithelium is a SSE of five to seven cell layers. The basal cells are columnar, and two to three layers of suprabasal cells have wing-like lateral extensions. There are three to four layers of flattened outer squames. The turnover of the rat corneal epithelium occurs in 5–7 days. Figure 2A shows a light microscopy of the corneal epithelium. Integrin distribution was assessed by indirect immunofluorescence microscopy within frozen sections of intact corneal tissues using a variety of antisera specific to distinct integrin subunits. Rat corneas were used in all instances except when cross reactive subunit-specific antisera were not available for a given integrin. For analyses of \( \alpha_6 \) integrin localization, mouse corneas were used, and for \( \alpha_6 \), human corneal tissues were used. We have stained rat, mouse, hamster, and human corneas with various integrin antisera (data not shown) and have not observed any differences among species. Using \( \beta_1, \alpha_2, \alpha_5, \) and \( \alpha_\varepsilon \) vitronectin receptor antibodies (Figs. 2B–E), the patterns of localization were similar. The binding of the antibody to these integrins was most intense on the membranes of the basal cells and on two to three layers of suprabasal cells. There was an apparent diminution of binding as the cells moved away from the basal layer. In addition, \( \beta_1, \alpha_2, \alpha_5, \) and \( \alpha_6 \) were prominently localized in regions of cell–cell interaction with little binding observed along the basal aspect of the basal cell membrane where the cells were in contact with the basement membrane. We found \( \beta_5 \) (Fig. 2F) showed no evidence of polarized expression or localization within the corneal epithelium; it was found around all membranes of the cells of the epithelium and did not localize to the basal aspect of the basal cells. The distributions of \( \alpha_4, \alpha_5, \) and \( \alpha_\varepsilon \) (Figs. 2G, H, I, respectively) were distinctly different from those of \( \beta_1, \alpha_2, \alpha_5, \) and \( \alpha_\varepsilon \). In addition, \( \beta_4, \alpha_5, \) and \( \alpha_\varepsilon \) integrins were found almost exclusively at the basal aspect of the basal cells; some \( \alpha_6 \) and \( \alpha_\varepsilon \) were found in the basal-most lateral margins of some cells. As reported previously, \( \alpha_6 \beta_4 \) integrin is a component of the hemidesmosomes found at the basement membrane zone beneath the basal cells of all stratified squamous epithelia.

These immunohistochemical data demonstrate that, as occurs in skin epidermis, most integrin subunits localized in a polarized pattern in the corneal epithelium. Most of these subunits (\( \beta_1, \beta_4, \alpha_5, \alpha_6, \) and \( \alpha_\varepsilon \)) polarized to the specific cell layers within the tissue, but \( \beta_5 \) did not. Many integrins (\( \beta_1, \beta_4, \alpha_5, \alpha_\varepsilon \)) localized to regions of cell–cell interaction. The only integrin subunits that appeared to be abundant at the site of cell–substrate interaction were \( \alpha_6 \) and \( \beta_4 \). Although \( \alpha_6 \) integrin could be detected by immunofluorescence at a low level in the membrane at the basal aspect of basal cells, we were unable to demonstrate it by immunoprecipitation (Fig. 1A), possibly because of low levels of synthesis, rapid proteolysis, or insolubility in the immunoprecipitation buffer. Attempts to show that the \( \alpha_6 \) or \( \alpha_\varepsilon \) integrin subunits were expressed in these epithelial tissues were unsuccessful.

### Localization of Integrins During Active Epithelial Migration

After wounding, cells at the wound margin of the SSE flattened and elongated to form the leading edge of migration. Approximately 25–30 cells behind the leading edge, normal stratification and morphology remained evident. To determine whether localization of the epithelial integrins was altered during migration, an in vitro organ culture wound-healing model was employed. Debridement wounds (3 mm) were made in rat corneas after the animals were killed, and the corneas were allowed to heal in organ culture in a defined medium. These debridement wounds involved removal of the epithelial cell layers only; the underlying basement membrane was left intact. Figures 3A
FIGURE 2. Immunofluorescent localization of integrin subunits in the corneal epithelium. (A) Light micrograph of a section of rat cornea demonstrates the five to seven cell layers of SSE. (B-I) Immunofluorescence micrographs of sections of rat (B, D-G, I), human (C), and mouse (H) corneas showing binding of antibodies to integrin subunits indicated on micrographs. In B-E, \( \beta_1 \), \( \alpha_\text{V} \), \( \alpha_5 \), and \( \alpha_6 \) have similar patterns of localization; the binding is primarily in basal and suprabasal cells. The basal cell membrane of the basal cell has less bound antibody (arrows). In F, \( \beta_4 \) is localized to all cell layers. Micrographs in G-I demonstrate that the localization of \( \alpha_5 \), \( \alpha_6 \), and \( \beta_5 \) integrin is primarily restricted to the basal cell membrane associated with the basement membrane. Some binding of \( \alpha_5 \) and \( \alpha_6 \) extends up the basolateral membrane of the basal cell. Bar = 10 \( \mu \text{m} \).

and B show a section of the leading edge migrating to cover such a wound. The phase micrograph (Fig. 3A) is the same section in which \( \beta_1 \) subunit was localized by indirect immunofluorescence microscopy (Fig. 3B). In Figure 3C, a micrograph of a similar leading edge is shown after staining with an antiserum against \( \alpha_5 \) integrin. Compared with the staining for \( \beta_1 \) and \( \alpha_5 \) observed on the control nonmigrating epithelium (Figs. 2B, 2D), no difference was observed on the cells behind the leading edge. At the tip of the leading edge, the cells bind antibodies to integrins primarily on their substrate-associated membranes. Staining of sections of leading-edge wounds was also performed with several other integrin antisera, including \( \beta_5 \), \( \alpha_5 \), \( \alpha_6 \), and \( \alpha_\text{V} \). Neither the pattern of localization nor the relative intensity of the fluorescence signal changed during migration for any of the integrins analyzed by immunohistochemical tests. Because we lacked cross reactive antisera for \( \alpha_2 \) chain directly in the rodent corneal wound-healing model.
**Integrins in Corneal Epithelium**

**Time Course Experiments and Quantitative Immunoblot Analyses**

To quantify the integrins present in the epithelial cells during migration in vitro, a series of immunoblot analyses were done at various times after wounding. After 3-mm debridement wounding, the corneas were placed in organ culture in serum-free media for 3, 6, 9, 12, 18, or 24 hr. The controls were unwounded corneas obtained immediately after death. At the indicated times, the entire corneal epithelium was removed by incubation of the corneas with ethylenediaminetetraacetic acid followed by extraction with SDS-sample buffer. The samples were normalized by protein content and run nonreduced on 7% SDS polyacrylamide gels. Because previous work showed that vinculin levels increased during corneal epithelial migration, the vinculin levels in the extracts were also determined to compare them with any changes in the integrins. The immunoblot results to these experiments are shown in Figure 4. For each integrin, multiple exposures of the films were obtained, each with varying densities, and all were scanned to ensure that this observation was consistent for the range of densities tested. These data are presented in Table 2. For all of the integrins analyzed and for all of the time points tested, no significant difference in the amount of integrins was observed relative to the control unwounded epithelium findings. As expected, vinculin levels increased during migration.

We know that the rate of protein synthesis in these cells increases as they migrate and that migration proceeds in the absence of mitosis. To relate the data presented in Figure 4, which have been normalized for protein, to a value related to the cell number, the DNA content of extracts was determined using a fluorometric assay, and the ratio of protein to DNA was calculated for each time point and for the control unwounded epithelium. These results are presented in Table 3. With the possible exception of the 12-hr time point, the ratio of protein to DNA did not appear to be altered during migration. Therefore, the total amount of the integrin subunits $\beta_1$, $\alpha_5$, $\alpha_6$, $\alpha_7$, and $\alpha_8$ as a percentage of total protein and per cell did not change during active cell migration.

**Adhesion to Fibronectin and Laminin**

Our results indicate that, although one component of focal contacts, vinculin, increased severalfold during migration, the amount of the epithelial integrins per cell did not appear to increase. To begin to evaluate integrin function in control and migrating corneal epithelial cells, a series of cell adhesion experiments were done. First, the optimal concentrations of ECM proteins required for support of cell adhesion and the optimal concentrations of RGD peptides required for inhibition of cell adhesion for stationary epithelial cells were established. Control RGE peptides had no effect on cell adhesion to any of the ECM molecules tested (data not shown). In the surface-labeling experiments (Fig. 1C), we showed that Dispase and trypsin can be used to obtain single-cell suspensions of epithelial cells without apparent proteolytic cleavage of integrins. Therefore, we did in vitro cell adhesion experi...
FIGURE 4. Immunoblotting analyses of epithelial integrins at various times after wounding. We used 60 μg total protein from SDS extracts of corneal epithelium for each of the time points indicated. The proteins were transferred to nitrocellulose, and the integrins were visualized using subunit-specific antisera followed by horseradish peroxidase-conjugated secondary antiserum and development against vinculin. The more rapid mobility of vinculin in the control samples was not reproducible.

ments using single cells obtained using a similar protocol.

Figure 5A shows the results in control cells. The cells most preferred to adhere to fibronectin followed by adhesion to the various fibrillar collagens (I, III, V, and VI), laminin, and vitronectin. Collagens IV and VII, components of the basement membrane, were among the least preferred ligands. Tenascin was no better at supporting cell adhesion than was BSA. The adhesion of cells to fibronectin and vitronectin was partially inhibited by RGD peptides but not by control peptides, indicating that integrins are involved in mediating these adhesion events. The substrates most preferred by the cells were those that the epithelial cells might encounter during in vivo migration over a wound where fibronectin is deposited at the wound site. In the cornea, in vivo wounds, fibronectin from the tear film is deposited after wounded basement membrane.

TABLE 2. Analysis of Epithelial Integrins and Comparison With Vinculin As a Function of Time After Wounding

<table>
<thead>
<tr>
<th>Hours</th>
<th>$\beta_1$ (μg)</th>
<th>$\beta_4$ (μg)</th>
<th>$\alpha_5$ (μg)</th>
<th>$\alpha_6$ (μg)</th>
<th>Vinculin (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6360 (1314)</td>
<td>5626 (759)</td>
<td>6035 (293)</td>
<td>1535 (280)</td>
<td>6161 (226)</td>
</tr>
<tr>
<td>3</td>
<td>6089 (2902)</td>
<td>5829 (619)</td>
<td>6408 (121)</td>
<td>1667 (101)</td>
<td>5859 (208)</td>
</tr>
<tr>
<td>6</td>
<td>5580 (390)</td>
<td>6521 (534)</td>
<td>6380 (85)</td>
<td>1500 (270)</td>
<td>6056 (160)</td>
</tr>
<tr>
<td>9</td>
<td>7048 (1447)</td>
<td>6428 (115)</td>
<td>6052 (389)</td>
<td>1922 (144)</td>
<td>6441 (205)</td>
</tr>
<tr>
<td>12</td>
<td>4996 (1027)</td>
<td>4319 (27)</td>
<td>6944 (227)</td>
<td>1555 (502)</td>
<td>6707 (580)</td>
</tr>
<tr>
<td>18</td>
<td>4255 (790)</td>
<td>5100 (192)</td>
<td>6842 (361)</td>
<td>1743 (39)</td>
<td>7704 (318)</td>
</tr>
<tr>
<td>24</td>
<td>5544 (2031)</td>
<td>5385 (851)</td>
<td>6095 (474)</td>
<td>1758 (28)</td>
<td>7789 (445)</td>
</tr>
</tbody>
</table>

These values were obtained by densitometric analysis of films obtained from Western blots similar to those presented in Figure 4. Data are expressed in arbitrary units (μg [SD]) and represent the average of at least three separate determinations.
TABLE 3. Protein: DNA in Stationary and Migrating Epithelial Sheets

<table>
<thead>
<tr>
<th>Variable</th>
<th>Protein (µg/µl)</th>
<th>DNA (µg/µl)</th>
<th>Protein: DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7</td>
<td>0.08</td>
<td>46.3</td>
</tr>
<tr>
<td>3 hr</td>
<td>3.7</td>
<td>0.08</td>
<td>46.3</td>
</tr>
<tr>
<td>6 hr</td>
<td>2.5</td>
<td>0.06</td>
<td>41.7</td>
</tr>
<tr>
<td>9 hr</td>
<td>4.0</td>
<td>0.08</td>
<td>50.0</td>
</tr>
<tr>
<td>12 hr</td>
<td>2.2</td>
<td>0.08</td>
<td>27.5</td>
</tr>
<tr>
<td>18 hr</td>
<td>2.9</td>
<td>0.06</td>
<td>48.3</td>
</tr>
<tr>
<td>24 hr</td>
<td>2.5</td>
<td>0.06</td>
<td>41.7</td>
</tr>
</tbody>
</table>

Values are the means of three experiments in which 5–6 corneal epithelial sheets were extracted for each time point and the protein and DNA concentrations were determined as described in "Methods."

membranes and denuded stroma. Collagens I, III, V, and VI are found in the dermis of the skin and the stroma of the cornea. The percentage of cells that adhered in this assay was low; the cells containing the most integrin subunits were the basal and suprabasal cells, which constituted approximately 30–40% of the total cell population.

For experiments on migrating cells, 3-mm corneal scrape wounds were made, and the corneas were placed in organ culture in media containing 35S-methionine and 35S-cysteine for 18 hr to label the cells metabolically. Control corneas were also placed in organ culture overnight; as shown in Figure 4 and Table 3, epithelial sheets from the organ cultured control corneas possessed the same amount of integrins per cell as did the epithelium from the corneas obtained immediately after death. The adhesion to fibronectin and laminin with and without RGD peptides was assessed. Epithelial cells migrating over an intact basement membrane adhered to fibronectin and laminin at the same percentage as did control nonmigrating cells (Fig. 5B); furthermore, there was no difference in the RGD sensitivity of the ECM adhesion. These data indicate that the integrins in the cells of the control epithelium appear to be as capable of mediating attachment to fibronectin and laminin as are the integrins found in the membranes of migrating cells. The α5β1, α5β3,
and \( \alpha_\beta_4 \) integrins in the corneal epithelium localized to the sites of apparent cell–cell interaction (Fig. 2); none of the ligands involved in mediating this cell–cell interaction have been discovered. Our data indicate that attachment of cells to fibronectin and laminin is unaltered during migration, but the adhesion functions of \( \alpha_\beta_1, \alpha_\beta_5, \) and \( \alpha_\beta_3 \) integrins toward other ligands or counterreceptors are not addressed by these experiments.

**DISCUSSION**

We found integrins in the corneal epithelium at sites of cell–cell interaction and at sites of cell–substrate interaction, and this distribution changed little during active migration of the epithelial sheet to cover a wound in vitro in organ culture experiments on rat cornea. In addition, the total amount of the integrin subunits \( \beta_1, \beta_6, \alpha_5, \alpha_6, \) and \( \alpha_4 \), as a percentage of the total protein and per cell did not change during active cell migration. We have also shown that the integrin-mediated epithelial cell adhesion to the ECM molecules fibronectin and laminin were not upregulated during epithelial migration. What, if any, role then are integrins playing in the corneal epithelium and in its ability to respond rapidly to migrate and cover a wound?

The in vitro wound model presented here has been well characterized. The migration of the rat corneal epithelium in organ culture resulted in complete closure of a 5-mm debridement wound within 24 hr. We know that corneal epithelial sheet migration (1) requires an increase in overall metabolism with increased protein synthetic activity,21 (2) occurs only if serine proteases are functional,38 (3) is accompanied by an increased production relative to total protein of the cytoskeletal protein vinculin,29 and (4) occurs without addition of serum or exogenous growth factors.21 To these data, we add that migration of the epithelial sheet does not result in a change in the amount of the integrins \( \beta_1, \beta_4, \alpha_5, \alpha_6, \) and \( \alpha_4 \), as a percentage of total protein or per cell. As originally proposed39 and based on their experimental results,6 the integrins could add cohesive strength to the stationary epithelial sheet and, in the event of injury, are immediately available to function in cell–substrate adhesion and migration. During wound healing, epithelial sheet movement requires that the entire sheet withstand the forces generated by the dramatic cell shape changes occurring in the basal cell layer at the leading edge. The shape of the basal cells change rapidly from columnar to a more flattened morphology, forcing lateral membranes formerly in contact with other cells to begin to come in contact with the underlying basement membrane. Because of their unique ability to mediate interaction both between adjacent cells and between cells and their underlying ECM substrate and with components of the actin-based cytoskeleton, integrins are likely to be important components of several aspects of epithelial cell migration, including the cell shape changes that must occur, the migration and transient adhesion of the sheet to the underlying ECM, the ability of the sheet to remain intact and cohesive as it stretches and thins, and the disassembly and subsequent reassembly of the \( \alpha_\beta_4 \)-containing hemidesmosomes after migration is complete. Direct data in support of these assumptions in a SSE are not yet available, but there is some suggestive indirect evidence.

The technique we used to quantify integrins involved the complete solubilization of the SSE in a SDS buffer followed by electrophoresis and immunoblotting of equal micrograms of total cellular protein for each time point. A limitation of this technique was that it might miss a small but significant change in the synthesis or turnover of integrins in response to wounding, which could only be detected by pulse-chase experiments followed by quantitative immunoprecipitation and/or messenger RNA quantitation. We did quantitative immunoprecipitation experiments on metabolically labeled epithelial extracts from mouse corneas 12 hr after debridement and compared the synthesis of integrins by these cells to that of control unwounded corneas, which had also been incubated in \( ^{35} \)S-methionine and \( ^{35} \)S-cysteine-labeled culture media for 12 hr. We also found no difference in \( \beta_1 \) and \( \alpha_5 \) integrin synthesis by this technique (Gipson IK et al. Submitted for publication, 1993). Quantitative immunoprecipitation of integrins from the SSE of the cornea is difficult because of the low level of incorporation of labeled amino acids compared with that in cultured cell lines and the relative insolubility of the tissue, presumably as a result of the keratin-rich intermediate filament network. Not all integrins in the SSE of the cornea are soluble in the nonionic detergent buffers that work best for immunoprecipitation; the relative insolubility of the \( \alpha_5 \) integrin in these buffers appears to explain our inability to detect this integrin by immunoprecipitation.40 In addition, metabolic labeling experiments require that the control unwounded epithelium be incubated for long periods in the labeling medium, which could permit induction of migration of the epithelium off its basement membrane. Because of these limitations in the analysis of integrin synthesis at the protein level, future efforts should use molecular techniques to address whether the messenger RNA levels for the integrins change during cell migration.

**Integrins and Cell Shape**

Integrins are components of the focal contacts in cultured cells.41 By mediating the interaction of \( \alpha \)-actinin and talin to the actin filaments of the cytoskeleton,
Integrins participate in the establishment of cell shape. In culture, well-spread cells show extensive colocalization of integrins with cytoskeletal elements such as vinculin within focal contacts, whereas cells with evenly dispersed integrins appear more rounded, less adherent, and more likely to be migratory. Cell shape also appears to affect integrin function and expression. Keratinocytes forced to maintain a round morphology by culturing in low Ca²⁺ rapidly shut off some of their integrins. First, a deactivation step occurs that results in a loss of integrin function, and second, integrins disappear from the cell surface.

Thus, experimental findings suggest that integrins participate in maintaining the cell shape and that changes in the shape of cultured epidermal cells alter integrin function and expression. Dramatic changes in the shape of the cells at the tip of the leading edge occur in the corneal epithelium during migration (Fig. 3). The basal cells convert from a columnar to a more flattened morphology, and the cells at the tip of the leading edge redistribute β₁ integrins to their basal cell surface. Although the amount of integrins present within the migrating cells is unchanged relative to control cells (Fig. 4 and Table 2), the activation state of these integrins could, in fact, be altered along with their shape. Although we saw no differences in the adhesion of control and migrating cells to the ECM molecules fibronectin and laminin (Fig. 5), integrin interaction with as yet uncharacterized ligands could be upregulated. In addition, the ability of the integrins within the migrating cells to interact with intracellular ligands such as cytoskeletal proteins may be altered relative to stationary cells.

Integrins and Epithelial Sheet Integrity

It was shown that antibody against the ectodomain of the β₁ subunit could cause clusters of epithelial cells to dissociate from one another but remain attached to the substrate. These data imply that, for cultured keratinocytes, the mechanism of cell–cell interaction is β₁ subunit dependent; cell–substrate interaction is not. These data are consistent with those of others, who also blocked keratinocyte cell–cell interaction with an antibody against the β₁ subunit and, in addition, showed that cultured cells can adhere by large patches of the αβ₄ integrin in what were termed “stable anchoring contacts.” It was also demonstrated that keratinocyte cultures grown under low calcium conditions that result in disassembly of desmosomes and adherens junctions still localize β₁ integrins to sites of cell–cell contact. Thus, in cultured keratinocytes, conditions that interfere with at least two different mechanisms of epithelial cell–cell interaction do not disrupt β₁ integrin-mediated cell–cell adhesion. Currently, the exact ligand functioning in these novel cell–cell adhesion events has not been characterized.

Possible ligands include those molecules, such as fibronectins, laminins, and collagens, known to interact with αβ₁ and αβ₃ integrins. Although the presence of any of these ECM molecules between epithelial cells has not been reported, the possibility of novel forms of fibronectins, laminins, and/or collagens between cells cannot be excluded. Alternatively, like the integrin αβ₃, there may be a counterreceptor in the membranes of adjacent cells that functions to mediate cell–cell interaction.

Regardless of the mechanism of cell–cell interaction, in cultured keratinocytes, functional β₁ integrins appear to be required for keeping epithelial cell clusters intact and can at least partially compensate for the loss of desmosomes and adherens junctions. In performing this function, the integrins could provide an additional level of redundancy in cell–cell adhesion in the SSE, which allows the tissue to remain intact under a more diverse array of conditions. The movement of the SSE over a basement membrane during healing occurs through a dramatic reorganization of the basal and suprabasal cells within the leading edge of the migrating epithelium. The cells change shape as the overall thickness of the sheet decreases. We have shown that the amount of integrins in the cells is unaltered by migration, and that, apart from the cells at the tip of the leading edge, the distribution of integrins does not change. Along with desmosomes, integrins appear to function as cell–cell adhesion molecules in the SSE. Desmosome formation in cultured keratinocytes is modulated primarily at the level of assembly of constituent proteins; shifting cells grown in low- to high-calcium media results in a dramatic reassembly of desmosomes that is independent of new protein synthesis. Our data are consistent with the hypothesis that integrin-mediated cell–cell interaction in SSE may also be modulated by regulation of assembly into functional adhesion structures within the membrane. Rather than turn on integrin production during migration, the epithelial sheet maintains the same high level of integrin expression observed in the cells of the stationary epithelium. SSE are subjected to a variety of assaults including chemical burns, bacterial and viral infections, and physical trauma. By including integrins in their repertoire of cell–cell and cell–substrate adhesion mechanisms, the SSE further increase the diversity of their adhesive mechanisms and thus their ability to modulate their response to a wide array of environmental and physical assaults.

Integrins and Migration

The relationship between cellular adhesion to and migration along a given substratum is complex. To migrate, cells must adhere; however, if the adhesion is too great, the cells appear not to be able to move. The integrins mediate the adhesion of many different cell
types to various substrata. Mutations in the \( \beta_4 \) gene results in a disease called Glanzmann's thrombasthenia. Affected patients have platelets that do not aggre-
gate at wound sites. The human genetic deficiency disease called leukocyte adhesion deficiency results from mutations in the \( \beta_2 \) integrin gene. The \( \beta_2 \) integrins are required for the migration of lymphocytes to peripheral tissues after injury; patients with leukocyte adhesion deficiency have severe, eventually fatal, infections at relatively minor injury sites.

Antisera against specific integrin \( \alpha \) or \( \beta \) chain ec-
todomains and peptides that interfere with integrin binding sites on their ligands can be shown to block cell adhesion to various substrata. One group transfected Chinese hamster ovary cells, a transformed cell line with a poorly adhesive migratory phenotype, with the \( \alpha_6\beta_1 \) integrin subunit and showed that overexpression of \( \alpha_6\beta_1 \) by the transformed cells restored a more normal phenotype. Cells expressing the transfected \( \alpha_5 \) gene were significantly less motile than mock-transfected control cells. Others used clonal dilution to select a population of Chinese hamster ovary cells that were significantly reduced in their expression of \( \alpha_6\beta_1 \) integrin. Compared with wild-type cells, the cells expressing 20% of the wild-type level of \( \alpha_5 \) also had reduced migration. Therefore, in Chinese hamster ovary cells, too much or too little \( \alpha_6\beta_1 \) integrin results in a decrease in migration. These data suggested that highly motile cells must carefully regulate their production of integrins. The epithelium appears not to alter significantly its regulation of integrin expression during migration. Perhaps it is easier for the tissue to regulate integrin function by altering the expression of proteins that interact with the integrin cytoplasmic domains (ie, vinculin).

The best-studied adhesion structure known to contain integrins is the focal contact. These structures contain integrins in the cell membrane and talin and \( \alpha \)-actin at their cytoplasmic face. Vinculin associates with \( \alpha \)-actin and talin and is thought to stabilize the structure. \( \alpha \)-Actin binds directly to the actin filament network. Because integrins bind to ligands in the ECM, this adhesion structure links the outside of the cell with the inside. It is this integrating function of these \( \alpha \beta \) heterodimers that is responsible for their ability to participate in signal transduction events. A dramatic increase was reported in the amount of vinculin in the migrating epithelium, and punctate staining was observed both along the basal membrane of the basal cells where they abutted the wound substrate and also between cells in the leading edge. This staining pattern suggested vinculin involvement in focal contact-like adhesion events in the migrating epithelium. Our studies confirm the observation of an increase in the vinculin level. Because vinculin can function to stabilize focal contacts, perhaps the increased vinculin pro-

duced during epithelial migration interacts with the integrins already present in the epithelial cell membranes to modulate their function.

Two immunohistochemical studies have suggested that integrins are elevated in keratinocytes during their migration. One study used antisera against \( \beta_1 \) integrin, and the other used antisera against \( \beta_i \) and \( \alpha_5 \). They detected apparent increases in integrins in actively migrating leading edges. The wound-healing model we used in this study was different from that employed by either group; the differences between our results and theirs could be related to the differences in the systems or to the inherent qualitative nature of immunohistochemical analyses.

### Integrins and Their Role in Hemidesmosomes

The pattern of integrin localization shared by \( \alpha_6 \) and \( \beta_4 \) is distinct from the other integrins discussed. The integrin \( \alpha_6\beta_4 \) is found at the basal surface of the basal cells with some localization to the lateral membranes and is a component of hemidesmosomes in stratified epithelia. Hemidesmosomes are the electron-dense sites of close attachment between the basal cell surface and the underlying basement membrane. The hemidesmosomal ligand(s) for \( \alpha_6\beta_4 \) have yet to be reported, but likely candidates include variant forms of laminin. The fate of \( \alpha_6\beta_4 \) integrin after hemidesmosome disassembly during cell migration and the reas-

semblly of hemidesmosomes after migration is an important topic of interest. Elevated levels of expression of \( \alpha_6\beta_4 \) and \( \beta_4 \) phosphorylation correlate with the metastatic potential of many epithelial-derived tumors. We found little evidence for the existence of proteolytic fragments of \( \beta_4 \) within the corneal basal cells. In fact, Figure 1C shows that, even after treatment of the epithelial sheet with the crude enzyme preparation Dispase followed by trypsin, we were able to surface label intact \( \beta_4 \) molecules. Perhaps the sus-
ceptibility of \( \beta_4 \) integrin to degradation is regulated in a tissue-specific manner. The biochemical data presented here suggest that, like the integrins involved in cell–cell adhesion events, the production of the hemidesmosomal \( \alpha_6\beta_4 \) integrin remains unaltered during migration.

In summary, during the migration of corneal epithelial sheets, the epithelial cells appear to maintain tight control over integrin synthesis. Even though the rate of total protein synthesis is increased, the amounts of \( \beta_1 \), \( \beta_4 \), \( \alpha_5 \), \( \alpha_6 \), and \( \alpha \) integrins per cell remain unchanged compared with that in stationary epithelial sheets. The increased expression of vinculin in the migrating epithelium may act to modulate integrin function by changing the stability of the various integrin-mediated cell–substrate and cell–cell adhesion events.
Integrins in Corneal Epithelium

Key Words

integrins, cornea, epithelium, cell migration, cell–cell interaction

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