Regulation of Keratinocyte Intercellular Junction Organization and Epidermal Morphogenesis by E-Cadherin

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Abstract. Elevation of the calcium concentration in human keratinocyte culture rapidly induces the redistribution of E-cadherin, P-cadherin, vinculin, β1 integrin, and desmoplakin to the cell–cell borders. Antibody to E-cadherin that blocks its functional activity delays the redistribution of each marker by several hours. Furthermore, antibody to E-cadherin interferes with normal, calcium-induced stratification of keratinocytes. Although several uneven vertical layers of cells can be detected in the presence of anti-E-cadherin antibody, the superficial cells appear defective in their adhesion. They do not flatten upon the basal layer nor do they enlarge, as do the controls; but rather they remain in groups of small cells connected by a line of single cells or by very long processes. In spite of the deformed appearance of the superficial cells in the presence of anti-E-cadherin IgG, these cells express the differentiation marker filaggrin, do not express P-cadherin, and concentrate desmoplakin at their cell–cell borders, consistent with the pattern in normally stratified cultures and in epidermis. These studies suggest a central role for E-cadherin in the regulation of keratinocyte intercellular junction organization as well as in epidermal morphogenesis.

Cell–cell adhesion of keratinocytes involves numerous molecular species that are organized into distinct structures in a process regulated by calcium ion concentration. In low calcium (i.e., 30 μM), keratinocytes grow as a monolayer and do not exhibit adherens junctions or desmosomes; however, elevation of the calcium concentration induces the rapid formation of both of these intercellular junctions and subsequently initiates stratification (O'Keefe et al., 1987; Hennings et al., 1980; Hennings and Holbrook, 1983; Watt et al., 1984; Tsao et al., 1982). The integrin receptors α2β1 and α3β1 have also been implicated in cell–cell adhesion in keratinocytes by several recent studies (Larjava et al., 1990; Carter et al., 1990; Marchisio et al., 1991). Little is known about the mechanisms by which intercellular junctions are assembled or about the functional relationships between the various structures and components. Previous studies have suggested that E-cadherin, a calcium-dependent homophilic adhesion protein (Takeichi, 1988), may play an important role in the assembly of the intercellular junctional complex in the canine kidney line MDCK (Gumbiner et al., 1988) and in the establishment of intercellular communication via gap junctions in mouse epidermal cells (Jongen et al., 1991). In addition to their role in junction formation, cadherin-mediated cell–cell interactions may be important in limiting the invasiveness of epithelial cells during tumor formation (Chen and Obrink, 1991; Vleminckx et al., 1991; Frixen et al., 1991).

In the present study we have used early passage normal human keratinocyte cultures to explore the relationships among E-cadherin, P-cadherin, vinculin, β1 integrin, and desmoplakin during the formation of intercellular junctions and the initiation of stratification. The ability to control with calcium concentration the state of organization of the intercellular junctions in the normal keratinocyte makes it an ideal system for exploration of the specific components and steps involved in junction formation and stratification. Both E-cadherin and P-cadherin as well as vinculin, β1 integrin, and desmoplakin accumulate at the cell–cell borders upon elevation of the calcium concentration. E-cadherin can regulate the redistribution of all the other markers. Furthermore, keratinocytes maintained in the presence of blocking antibody to E-cadherin for several days exhibit grossly abnormal stratification, suggesting that E-cadherin function is required for normal epidermal morphogenesis.

Materials and Methods

Cell Culture

Human keratinocytes from neonatal foreskin were propagated in MCDB 153 medium with 30 μM calcium and the following additives: bovine pituitary extract, insulin, EGF, hydrocortisone, and high amino acids, as previously described (Shipley and Pittelkow, 1987; McNeill and Jensen, 1990); this medium is referred to as "complete medium." Cultures were used between the first and third passage. MCDB 153 medium was purchased from Irvine Scientific (Santa Ana, CA), Sigma Chemical Co. (St. Louis, MO), and Clonetics (San Diego, CA; KGM with 30 μM calcium). EGF was from Collaborative Research (Bedford, MA); insulin and hydrocortisone were from Sigma Chemical Co. Bovine pituitaries were from Pelfreeze Biological (Rogers, AK).
**Immunofluorescence**

Keratinocytes were plated in complete medium on glass coverslips and grown until almost confluent. Calcium was elevated in the indicated coverslips to 1.0 mM for the final 12 h of incubation, unless stated otherwise. The cells were fixed in 1% paraformaldehyde buffered with HBSS (Gibco Laboratories, Grand Island, NY) and 10 mM Hepes (Sigma Chemical Co.) and then permeabilized and blocked by incubating in PBS containing 10% goat serum, 0.1 M glycine, and 0.05% saponin. They were stained for 1 h with the primary antibody, washed with PBS, and then stained for 1 h with FITC-labeled secondary antibody (Organon Technica-Cappel, West Chester, PA) diluted 1:25 in PBS containing 10% goat serum. The coverslips were washed, mounted, and observed with a Zeiss Axioshot microscope equipped with epifluorescence.

**Electron Microscopy**

Keratinocytes were grown on Falcon cyclopore membrane filters. After preincubation with normal rabbit IgG or anti-E-cadherin IgG, calcium was elevated to 1.0 mM for 4 d. Cultures were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and processed for routine EM.

**Antibodies**

Rat monoclonal (E-9) and rabbit polyclonal antibodies against human E-cadherin (Cell-CAM 120/80) have been previously described (Wheelock et al., 1987). Mouse monoclonal anti-vinculin and anti-desmoplakin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Mouse monoclonal anti-human transferrin receptor was purchased from Chemicon (Temecula, CA). Mouse monoclonal anti-human filaggrin was purchased from Biomedical Technologies Inc. (Stoughton, MA). Rat monoclonal anti-β1 integrin (A11B2) was a gift of Dr. Caroline Damsky (University of California, San Francisco, CA). Rat monoclonal anti-P-cadherin (NCC-CAD-299) was a gift of Dr. Setsuo Hiroshashi (National Cancer Center Research Institute, Tokyo, Japan); anti-P-cadherin did not recognize E-cadherin (Shimoyma et al., 1989).

**Detergent Extractions**

Semiconfluent monolayers of keratinocytes were maintained in complete medium containing either 30 μM or 1.0 mM calcium for 12 h. After washing three times with PBS saturated with PMSF at 0°C, they were extracted at 0°C with 1 ml per 25 cm² flask of 10 mM Tris acetate, pH 8.0, 0.5% NP-40, saturated with PMSF and containing either 30 μM CaCl₂ or 1 mM CaCl₂. The cells detached from the tissue culture flask after a few minutes in this buffer and they were then agitated by vigorous pipetting for 10 min on ice. NP-40 insoluble material was removed by centrifugation at 15,000 g for 45 min.

**Immunoprecipitation**

Semiconfluent monolayers of keratinocytes were maintained in complete medium containing either 30 μM or 1.0 mM calcium for 12 h. The monolayers were then washed with PBS and incubated for 6 h in medium without methionine, followed by a 1-h incubation in 3 ml of the same medium supplemented with 0.5 mM 35S-methionine + cysteine (Translabel, ICN Biomedicals, Irvine, CA). Detergent extracts of labeled cells were adjusted to 0.25 M NaCl and 0.3% BSA (Sigma Chemical Co.); 100 μl of extract was mixed with 50 μl Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C for 30 min to remove nonspecifically bound material. The Sepharose was removed by microfuging for 1 min at 4°C, and the supernatant was mixed with 25 μl monoclonal rat anti-E- or P-cadherin at 4°C. After 1 h, anti-rat IgG attached to Sepharose (Organon Technica-Cappel) was added and mixing was continued for another 30 min. The Sepharose-bound immune complexes were washed five times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS. The remaining pellets were boiled in sample buffer, resolved on SDS-PAGE as described below, enhanced with En3 Hance™ (New England Nuclear, Boston, MA) and autoradiographed at −70°C overnight.

**Electrophoresis and Immunoblotting**

Polyacrylamide slab gel electrophoresis in the presence of SDS was done according to the procedure of Laemmli (Laemmli, 1970) with a 7% resolving gel and a 3.5% stacking gel. Immunoblotting was done as described (Wheelock et al., 1987). Bound antibodies were detected by incubation with alkaline phosphatase-labeled anti-rabbit IgG (Promega, Madison, WI), followed by washing, and then development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma Chemical Co.).

**Antibody Blocking**

Purified IgG was prepared by protein A affinity chromatography of a previously described blocking rabbit antiserum against the external fragment of human E-cadherin (Wheelock et al., 1987). Normal rabbit IgG was purchased from Organon Technica-Cappel. Anti-E-cadherin IgG and normal rabbit IgG were diluted to 20 μg/ml in complete MCDB 153 with 30 μM calcium. After an overnight incubation with antibodies, the calcium was raised to 1 mM and incubation was continued for the indicated length of time. For long-term experiments, the IgG was replenished every 24 h with fresh complete medium containing 1 mM calcium. To harvest for histology, cultures in 24-well plates were incubated with dispase (2.4 mg/ml; #165-859; Boeringer Mannheim Biochemicals) at 37°C. Upon detachment, the epithelial sheet was fixed in 10% formalin in phosphate buffer; paraffin sections were stained with hematoxylin and eosin.

**Results**

**Distribution of E-Cadherin, and Other Adhesion Molecules**

The presence of E-cadherin and P-cadherin in cultured human keratinocytes at low and high calcium was quantified in two ways. Immunoblots of cell extracts demonstrated that similar amounts of E-cadherin were present in keratinocyte cultures maintained at 30 μM calcium or transferred to 1.0 mM calcium for the final 12 h of incubation; both sparse and confluent cultures were examined, with comparable results (Fig. 1a). Immunoprecipitations with anti-E-cadherin antibody or anti-P-cadherin antibody of keratinocytes labeled with 35S-methionine in the presence of low or high calcium further demonstrated the consistent level of these molecules under both calcium conditions (Wheelock and Knudsen, 1991; Fig. 1b).

In contrast to the consistency of the total levels of E-cadherin and P-cadherin, their cellular distributions were dra-

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matically altered upon calcium elevation, as previously observed for E-cadherin in other epithelial cells (Gumbiner et al., 1988). Under low calcium conditions, both cadherins were diffusely distributed throughout the keratinocyte (Fig. 2, a and d). By 15–20 min after elevation of the calcium concentration, redistribution of both the cadherins to the cell–cell borders was apparent (Fig. 2, b and e). After 1 h in high calcium, all fields showed cell–cell border staining for both cadherins (data not shown), the intensity of which was enhanced after overnight incubation (Fig. 2, c and f).

Redistribution of E-cadherin from a diffuse localization to a concentration at the cell–cell borders was likewise observed when nonpermeabilized cells were stained (data not shown); furthermore, the intensity of staining was similar in permeabilized and nonpermeabilized cells. These results suggest that most E-cadherin was present on the cell surface even when the cells were in 30 μM calcium.

In agreement with previous reports (O’Keefe et al., 1987; Larjava et al., 1990; Carter et al., 1990; Marchisio et al., 1991; Watt et al., 1984), we noted that several other markers involved in cell–cell adhesion also redistributed upon calcium elevation. Vinculin (a component of adherens junc-

### Figure 2

Time course of calcium-induced translocation of E-cadherin, P-cadherin, vinculin β1 integrin, and desmoplakin. Nearly confluent keratinocyte cultures were left in 30 μM calcium (a, d, g, j, and m); switched to 1.0 mM calcium for 20 min (b, e, h, k, and n); or switched to 1.0 mM calcium overnight (c, f, i, l, and o) before fixation, permeabilization, and immunofluorescent staining. The following antibodies were used: (a–c) anti-E-cadherin; (d–f) anti-P-cadherin; (g–i) anti-vinculin; (j–l) anti-β1 integrin; (m–o) anti-desmoplakin. Bar, 50 μm.


(continued)

tions), β1 integrin, and desmoplakin (a desmosomal plaque protein) all redistributed to the cell–cell borders when calcium concentration was raised to 1.0 mM (Fig. 2, g–o). The redistribution occurred rapidly; within 15–20 min, a change in localization of each of the molecules was detectable. By 60 min after calcium elevation, the redistribution was apparent in nearly every field (data not shown).

As a control, cells at low and high calcium were also stained with antibody to transferrin receptor. The distribution of the transferrin receptor was similar under both low and high calcium conditions and did not localize at the cell–cell borders (data not shown).

Effect of Anti-E-Cadherin IgG on Marker Redistribution

To explore the functional relationship between E-cadherin translocation and that of the other adhesion molecules, we employed a rabbit anti-E-cadherin IgG that blocks functional activity (Wheelock et al., 1987). Cultures were preincubated with immune or nonimmune IgG overnight; calcium concentration was then adjusted to 1.0 mM and incubation continued for various periods of time before immunofluorescent staining.

Preincubation of cells with nonimmune IgG did not alter the time course of translocation of any of the molecules upon calcium elevation; i.e., the time course was identical to that shown in Fig. 2, where obvious redistribution of P-cadherin, vinculin, β1 integrin, and desmoplakin had begun by 15–20 min after calcium elevation and was present in nearly every field by 60 min.

Preincubation with anti-E-cadherin IgG delayed the calcium-induced translation of all the molecules. In the case of P-cadherin, no cell–cell border stain was observed 30–60 min after elevation of calcium in the presence of anti-E-cadherin IgG. By 2 h after elevating calcium in the presence of anti-E-cadherin IgG, P-cadherin redistribution (Fig. 3 a) had begun and was similar to that observed in controls incubated for 15–20 min in high calcium (compare Figs. 3 a and 2 e). After 4 h in high calcium with anti-E-cadherin IgG, many but not all fields showed P-cadherin localization at the

Figure 3. Antibodies against E-cadherin delay the calcium-induced translocation of other molecules. Polyclonal rabbit anti-E-cadherin IgG was added to nearly confluent keratinocyte cultures in complete medium with 30 µM calcium for 12 h. Without changing the medium, the calcium concentration was then increased to 1.0 mM for varying lengths of time before processing for immunofluorescent staining as in Fig. 2. a, d, g, and j depict cells 2 h after raising the calcium to 1 mM; b, e, h, and k depict cells 4 h after raising the calcium to 1 mM; c, f, i, and l depict cells after an overnight incubation in 1 mM calcium. The following antibodies were used: (a–c) anti-P-cadherin; (d–f) antivinculin; (g–i) anti-β1 integrin; (j–l) antidesmoplakin. Bar, 50 µm.
permeabilized, and stained with FITC-labeled anti-rabbit IgG and then stained with FITC-labeled anti-rabbit IgG. Bar, 50 μm.

Effect of Anti-E-Cadherin IgG on Stratification

When the calcium concentration is elevated, keratinocytes growing as a monolayer are induced to stratify, forming a structure resembling a multilayered epithelial sheet (Tsao et al., 1982; Boyce and Ham, 1983). To determine if E-cadherin plays a role in the initiation of stratification, keratinocytes were incubated for several days with 1.0 mM calcium in the presence of blocking anti-E-cadherin IgG or control IgG (Figs. 5 and 6). Nonimmune rabbit IgG did not alter the normal stratification process. However, inhibitory anti-E-cadherin IgG led to gross disruption of stratification.

To examine further the pattern of stratification, cross-sections were prepared from cultures incubated with and without anti-E-cadherin IgG. Cultures with no antibody or with nonimmune rabbit IgG stratified evenly, with three to four vertical layers of cells readily observed after several days in high calcium (Fig. 6, a–c). A similar pattern, resembling in vivo stratification, has been observed previously (Banks-Schlegel and Green, 1981; Pillai et al., 1988). Cultures in the presence of anti-E-cadherin IgG also revealed several vertical layers, but the pattern of stratification was highly irregular and abnormal (Fig. 6, d–f); the superficial cells were not usually flattened against the lower layer, but rather appeared bulbous and deformed (Fig. 6, e and f), in marked contrast to the flat upper cells in the controls (Fig. 6, b and c). In some cases the superficial cells in the cultures treated with anti-E-cadherin IgG were connected to the underlying cells by only a thin process (Fig. 6, f, arrow).

In spite of their abnormal appearance, these suprabasal cells showed intense staining for desmoplakin at the cell–cell borders (Fig. 7), strongly suggesting that at least some desmosomal organization occurred in the presence of anti-E-cadherin IgG. Further examination at the electron microscopic level revealed the presence of desmosomes in cultures incubated with blocking antibodies to E-cadherin (Fig. 7 e).
that ultrastructurally resembled those present in the control cultures (Fig. 7 d). These initial studies thus suggest that desmosomal organization alone may not be sufficient for normal stratification and emphasize the crucial role for E-cadherin. Extensive electron microscopic analysis will be necessary to quantify and characterize intercellular junctions in these cultures before we can definitively evaluate the roles of adherens junctions and desmosomes in stratification.

Figure 5. Morphology of keratinocytes stratified in the presence or absence of anti-E-cadherin IgG. Nearly confluent keratinocytes in 24-well plates were incubated overnight with normal rabbit IgG (a and c) or with rabbit anti-E-cadherin IgG (b and d) in complete medium with 30 μM calcium. The calcium was then elevated to 1.0 mM and incubation continued for 3 d with replenishment of antibodies every 24 h. Cultures were examined and photographed under phase-contrast microscopy. Bar, 50 μm.

Figure 6. Histological cross-sections of keratinocyte cultures stratified in the presence or absence of anti-E-cadherin IgG. Cultures were treated as in Fig. 5; incubation with 1.0 mM calcium was continued for 4 d in the presence of normal rabbit IgG (a and c), no IgG (b), or rabbit anti-E-cadherin IgG (d-f). Cultures were then treated with dispase, an enzyme that has been shown to detach the epithelium without disrupting the junctions between cells (Green et al., 1979; Banks-Schlegel and Green, 1981). Dispase treatment results in contraction and thickening of the epithelium. The detached epithelium was fixed in formalin, processed for histology, and stained with hematoxylin and eosin. In b, c, e, and f the top of the epithelium is oriented toward the top of the figure. In a and d the epithelium has twisted around, permitting observation of many fields. In f, a suprabasal cell attached by a very thin process to the underlying layer is pointed out by an arrow. Bar, 50 μm.
Incubation with anti-E-cadherin IgG did not cause any change in the total number of cells adherent to the plates even after 4 d in 1.0 mM calcium (Table I). At all times examined, the number of cells released into the medium was only a very small fraction of the number of adherent cells. However, more than twice as many cells were released into the medium in the presence of anti-E-cadherin IgG as in the presence of normal rabbit IgG.

**Effect of Anti-E-Cadherin on Expression of Differentiation Markers**

A number of specific markers, including the histidine-rich matrix protein filaggrin are localized in the superficial, more differentiated keratinocytes in vivo and in vitro (Fleckman et al., 1985). When keratinocytes stratified in response to calcium elevation, filaggrin was readily detectable in the superficial layers; large patches of cells were stained in a punctate pattern that made delineation of the individual cell borders difficult (Fig. 8, a and b, an upper layer cell–cell border is pointed out by an arrow). In the presence of anti-E-cadherin IgG, filaggrin was still detected in the superficial cells, but the cells were rounded and formed uneven arrays that were distinct from control cultures (compare Fig. 8, a and d). Examination with both immunofluorescence and phase-contrast microscopy revealed relatively small, superficial filaggrin-positive cells with clearly delineated borders that did not flatten upon the basal layer (Fig. 8, e and f), in contrast to the controls.

In epidermis in vivo (Shimoyama et al., 1989) as well as in stratified, serum-containing cultures of keratinocytes (Nicholson et al., 1991), P-cadherin was detected only in the basal layer of cells; by contrast E-cadherin was detected in all living layers. Cultures that had been induced to stratify by incubation in high calcium for 4 d revealed distributions of the cadherins in agreement with these findings; i.e., E-cad-

**Figure 7. Localization of desmoplakin in cells stratified in the presence of anti-E-cadherin.** (a–c) Nearly confluent keratinocyte cultures were incubated with normal rabbit IgG (a) or with anti-E-cadherin IgG (b and c) in complete medium with 30 μM calcium for 12 h. Without changing the medium, the calcium concentration was increased to 1.0 mM and incubation continued for 4 d; the antibodies were replenished every 24 h. Cells were then fixed, permeabilized, and processed for immunofluorescence with monoclonal anti-desmoplakin. Arrows point out desmoplakin-positive staining in the suprabasal layer of cells; arrowheads point out desmoplakin-positive staining in the underlying basal layer. (d and e) Cultures were grown on cyclospore membrane filters and then treated as above with normal rabbit IgG (d) or anti-E-cadherin IgG (e). After 4 d in 1.0 mM calcium, cultures were processed for EM; desmosomes are clearly present in both cultures. Bars: (c) 50 μm; (e) 1 μm.
Table I. Anti-E-cadherin IgG Does Not Affect the Growth Rate of Keratinocytes

| Time (h) | Attached | Floating | Attached | Floating |
|----------|----------|----------|----------|----------|
| 0        | 740      | 2.8 (0.4%) | 745      | 4.3 (0.6%) |
| 24       | 750      | 3.9 (0.5%) | 860      | 7.8 (0.9%) |
| 48       | 785      | 5.3 (0.7%) | 720      | 11.8 (1.6%) |
| 72       | 640      | 3.0 (0.5%) | 670      | 12.4 (1.9%) |
| 96       | 662      | 6.8 (1.0%) | 667      | 14.5 (2.2%) |

Just confluent keratinocyte cultures in six-well plates were incubated with normal rabbit IgG or with rabbit anti-E-cadherin IgG in complete medium with 30 µM calcium for 12 h. Without changing the medium, the calcium concentration was increased to 1.0 mM and incubation continued for up to 4 d; the antibody was replenished every 24 h. Before increasing the calcium concentration (0 time) and every 24 h after increasing the calcium concentration, the cells were removed with trypsin from two wells that had been incubated in normal rabbit IgG and two wells that had been incubated in anti-E-cadherin IgG. The attached cells were counted. In addition, the cells floating in the culture medium were counted at each timepoint. The data are presented as the average number of cells per well x 10³, with the percentage of the total number of cells that are found in the supernatant in parenthesis.

Thus, at least with regard to changes in the expression of filaggrin and P-cadherin, the program of keratinocyte terminal differentiation was not blocked by inhibition of E-cadherin function.

Discussion

The formation of adherens junctions and desmosomes, two distinct multi-component structures that mediate cell–cell adhesion, is rapidly induced in keratinocyte culture by elevation of calcium concentration (O'Keefe et al., 1987; Hennings et al., 1980; Hennings and Holbrook, 1983; Watt et al., 1984). The organization of these junctional structures has been demonstrated by EM and interference reflection microscopy to precede the development of a stratified epithelium in culture. Immunofluorescence experiments have demonstrated that translocation of specific junctional molecules to the cell–cell borders accompanies the formation of these structures. In addition, other investigators (Larjava et al., 1990; Carter et al., 1990) have presented evidence that β₁ integrin plays a role in cell–cell adhesion of keratinocytes.

In this study, we demonstrate that blocking E-cadherin activity with antibodies delays the redistribution of P-cadherin, vinculin, β₁ integrin, and desmoplakin to the cell–cell borders during the calcium-induced organization of cell–cell adhesion. Our results suggest that E-cadherin plays a regula-

Figure 8. Localization of Filaggrin in cells stratified in the presence of anti-E-cadherin. Nearly confluent keratinocyte cultures were incubated with normal rabbit IgG (a–c) or with rabbit anti-E-cadherin IgG (d–f) in complete medium with 30 µM calcium for 12 h. Without changing the medium, the calcium concentration was increased to 1.0 mM and incubation continued for 4 d. The antibodies were replenished every 24 h. Cells were then fixed, permeabilized, and processed for immunofluorescence with monoclonal anti-filaggrin antibody. b and c are corresponding fluorescence and phase micrographs. An arrow points out a cell–cell border in the upper layer of filaggrin-positive cells. e and f are corresponding fluorescence and phase micrographs. Bar, 50 µm.
Figure 9. Localization of cadherins in cells stratified in the presence of anti-E-cadherin IgG. Nearly confluent keratinocyte cultures were incubated with normal rabbit IgG (a-e) or with rabbit anti-E-cadherin IgG (f-h) in complete medium with 30 μM calcium for 12 h. Without changing the medium, the calcium concentration was increased to 1.0 mM and incubation continued for 4 d, with antibody replacement every 24 h. The cultures were then fixed, permeabilized, and processed for immunofluorescence. Cells in a and b were stained with monoclonal anti-E-cadherin. In a, the plane of focus was on the uppermost layer of cells. An arrow points out E-cadherin at a cell border. b is a photomicrograph of the same field but with the plane of focus on the basal layer of cells. Cells in c-h were stained with monoclonal anti-P-cadherin. In c, the plane of focus was on the uppermost layer of cells; no P-cadherin staining is detectable. d is a phase-contrast photomicrograph of the same cells in the same plane of focus. A cell-cell border is pointed out by an arrow. e is a fluorescence photomicrograph of the same field but with the plane of focus on the lower layer. f-h, P-cadherin was localized in cells stratified in the presence of rabbit anti-E-cadherin IgG. In f, the plane of focus was on the uppermost layer of cells; no P-cadherin staining is detected. g is a phase-contrast photomicrograph of the same cells in the same plane of focus. A cell-cell border is pointed out by an arrow. h is a fluorescence photomicrograph of the same field but with the plane of focus on the basal layer of cells, revealing P-cadherin localization. Bar, 50 μm.

Our results are consistent with a previous report by Gumbiner et al. (1988) using the polarized epithelial line MDCK; this study shows that inhibition of E-cadherin function with specific antibodies delays the calcium-induced organization of the intercellular junctional complex, including adherens junctions, desmosomes, and tight junctions. After several hours, MDCK monolayers are able to overcome the effects of anti-E-cadherin antibody, as demonstrated by the eventual redistribution of adhesion molecules. Antibodies to E-cadherin also cause a transient decrease in transepithelial electrical resistance of confluent MDCK monolayers.

Normal keratinocyte cultures offer an additional parameter for study not present in MDCK or most other epithelial lines: the development of stratification. To stratify, the keratinocyte must develop contacts not only with neighboring cells in the same layer but also with cells in the layers immediately above and/or below its own. Our analysis of stratification over several days in the continued presence of anti-E-cadherin IgG indicates that the keratinocyte can not completely compensate for the loss of E-cadherin function during this morphogenetic process.
Our morphological studies of keratinocytes incubated for 3–5 d with 1.0 mM calcium in the presence of blocking antibody to E-cadherin reveal several vertical layers of cells in most fields, but the cells do not appear to have formed the appropriate cell–cell connections. In the presence of anti-E-cadherin IgG, the superficial cells remain in small groups connected by a string of single cells or by very elongated processes (Fig. 6). The superficial cells in immune IgG-treated wells appear much smaller in surface area than those in the control wells, mostly because the former have not flattened to cover a large surface area. This is most clearly demonstrated by comparing the pattern of staining for desmoplakin in the two groups (Fig. 7), although it is also apparent upon close examination with phase contrast microscopy. Some of the superficial cells after 3–5 d in the presence of anti-E-cadherin IgG are grossly elongated and attached very tenuously by a single long process to the underlying cell layer, consistent with a defect in the cell–cell adhesion mechanisms.

It is intriguing that stratification is abnormal even though the basal-like keratinocytes recover the ability to redistribute all their adhesion molecules except E-cadherin to the cell–cell borders after 24 h in high calcium in the presence of antibodies to E-cadherin. One hypothesis consistent with this finding is that the intercellular contacts between cells of adjacent layers may be more dependent upon E-cadherin than the contacts between neighboring cells in the basal layer. Recent data on the distributions of E-cadherin and P-cadherin suggest a potential mechanism for such differential dependence (Shimoyama et al., 1989; Nicholson et al., 1991): E-cadherin is present in all living layers of stratified keratinocytes in vivo and in vitro, but P-cadherin is limited to the basal cells. We suggest that in the basal layer P-cadherin may substitute for E-cadherin to organize cell–cell adhesion. In the suprabasal layers however, where P-cadherin expression is lost, the cells are exclusively dependent upon E-cadherin for organizational function. When E-cadherin activity is blocked with the specific antibody, suprabasal cell–cell organization is disrupted because neither cadherin is available and abnormal stratification results. Previous work with fetal mouse lip organ cultures has suggested that E-cadherin and P-cadherin cooperate in epithelial morphogenesis, although in this study (Hirai et al., 1989) a more significant role was proposed for P-cadherin.

Despite the abnormal stratification, cell–cell borders are stained with antibodies to desmoplakin in the lower layer as well as in the superficial layer of cells incubated in the presence of anti-E-cadherin antibody (Fig. 7); furthermore, ultrastructural analysis demonstrates the presence of desmosomes with apparently normal structure between layers of cells. Further experiments are required to determine whether the desmosomes and other junctional elements formed in the presence of anti-E-cadherin antibodies are completely normal and to establish which adhesive interactions are necessary and/or sufficient for normal epidermal morphogenesis. Nonetheless, our results show that E-cadherin plays a critical regulatory role in the normal organization of cell–cell adhesion and in the development of stratification that accompanies epidermal differentiation. The ability to control the state of organization of the intercellular junctions with calcium concentration and the availability of specific blocking antibodies against components of the adhesion structures makes normal keratinocyte culture an excellent model system for further investigations.

We are grateful to Dr. George F. Murphy and two members of his laboratory, Mr. Brett Telegan and Ms. Diana Whitaker-Menezes, for generously performing the EM. The authors wish to thank Dr. Caroline Damsky for antibodies against β1 integrin and Dr. Setsuo Hirohashi for antibodies against P-cadherin. We acknowledge the helpful comments of the reviewers.

This work was supported by grants CA 44464 and AR-39674-sub-06 from the National Institutes of Health to M. J. Wheelock and P. J. Jensen, respectively, by DIR-R804006 from the National Science Foundation to M. J. Wheelock, and by the Ohio Chapter of the National Heart Association.

Received for publication 15 October 1991 and in revised form 7 January 1992.

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