Hemidesmosome Formation In Vitro

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ABSTRACT Intact, viable sheets of adult rabbit corneal epithelium, 9 mm in diameter, were
Vis. Sci. 23:269-273). The sheets, freed of the basal lamina, retained their desmosomes and
stratified epithelial characteristics, but lacked hemidesmosomes (HD). Epithelial sheets were
placed on fresh segments of corneal stroma with denuded basal laminae and incubated in
serum-free media for 1, 3, 6, 18, or 24 h. Tissue was processed for electron microscopy, and
the number of HD/#m membrane, the number of LiDs with anchoring fibrils directly across
the lamina densa from them, and the number of anchoring fibrils not associated with HDs
were counted.

After 6 h in culture, the number of newly formed HD was 82% of controls (normal rabbit
corneas), and by 24 h the number had reached 95% of controls. At all time periods studied,
80-86% of HDs had anchoring fibrils directly across the lamina densa from them. Anchoring
fibrils not associated with HDs decreased with culture time. These data indicate that the sites
where anchoring fibrils insert into the lamina densa may be nucleation sites for new HD
formation.

Corneal epithelial sheets placed on two other ocular basal laminae, Descemet's membrane
and lens capsule, had not formed HDs after 24 h in culture. These two laminae do not have
anchoring fibrils associated with them.

Rabbit epithelial sheets placed on the denuded epithelial basal lamina of rat and human
corneas formed new HDs. Thus, at least in these mammalian species, HD formation may
involve some of the same molecular components.

Cell-substrate adhesion is of fundamental importance in a
variety of developmental, homeostatic, and pathologic proc-
esses. Such adhesion has been studied extensively, particularly
in regard to adhesion of cultured cells to artificial substrates
(see review by Kleinman et al. [1]). By comparison, little
information is available on the adhesion of cells to "native"
substrates. The hemidesmosome (HD), a specialized adhesion
junction, is an important means by which stratified squamous
epithelial cells (i.e., epidermal and corneal epithelium) adhere
to their native substrate, the basal lamina (basement mem-
brane) (2-5).

HDs, or half-desmosomes, are structurally similar to cell-
cell adhesion junctions, desmosomes. However, in the HD
complex, the plasmalemma and electron-dense plaque of the
apposing cell of the desmosome complex is replaced by the
lamina densa of the basal lamina (6-8). Anchoring fibrils
insert into the lamina densa on the side opposite the basal
cell plasmalemma, and splay out among the collagen fibrils
(5, 9-13). The anchoring fibrils function to hold the basal
lamina and the epithelium to the subjacent connective tissue
strata (14-16).

Krawczyk and Wilgram (17) studied HD formation in a
mouse epidermal wound-healing model and proposed four
ultrastructural stages. Their first stage was the extension of
fine filaments between the external leaflet of the basal plasma
membrane and the lamina densa. These extracellular fila-
ments became increasingly dense, and a narrow electron-
dense line, parallel to the basal cell plasma membrane and
the lamina densa, appeared within the extracellular filaments
(stage 2). Thereafter, an intracellular electron-dense zone (at-
tachment plate) appeared in exact apposition to the extracel-
lar fibrils (stage 3). The final stage was the insertion of
tonofilaments into the attachment plate (stage 4). Beyond
these morphologic studies, little is known of HD formation.

We have developed an in vitro system for study of HD
formation, using intact, viable sheets of adult rabbit corneal
epithelium (18). Epithelial sheets can be removed from their basement membrane by incubation of excised corneas for 1 h in the culture medium containing Dispase II. These fully differentiated, stratified epithelial sheets maintain their epithelial characteristics and have viable basal cells, but lack HDs. When placed on corneas with freshly denuded epithelial basal laminae and cultured, new HDs form along the basal cell membrane. Using this system, we examined (a) the time requirements for HD formation, (b) the relationship between HD formation and anchoring fibrils, (c) HD formation on basal laminae of different ocular tissues, and (d) species specificity of HD formation. A preliminary account of this work has been presented (19).

MATERIALS AND METHODS

Animals and Tissues: Corneal epithelial sheets (Fig. 1 a) were obtained from adult albino New Zealand rabbits. In the time course studies, rabbit epithelia were placed on small segments of denuded Descemet's membrane or denuded lens capsule, the thick basal laminae of the corneal endothelium and lens epithelium, respectively. These laminae, obtained from rabbits, were prepared by removing cells by scraping with a scalpel.

To study species specificity of HD formation, rabbit corneal epithelia were placed on segments of denuded rat (Sprague-Dawley) and human corneas. Human corneas were obtained from the New England Eye Bank (Massachusetts Eye and Ear Infirmary, Boston, MA 02114).

Epithelial Sheet Removal, Culture, and Tissue Processing: Corneal epithelia were removed from their underlying stroma by a method previously described (18). Briefly, 9-mm-diameter corneal buttons from which the posterior half of the stroma had been removed were incubated for 1 h at 35°C in a completely defined culture medium (20) containing 1.2 U/ml of Dispase II, a bacterial neutral protease (Boehringer Mannheim, Indianapolis, IN). The culture medium contained Eagle’s minimum essential medium with Earle’s balanced salt solution, 2 mM glutamine, trace elements (0.46 µM CoCl2, 0.28 µM MnCl2, 1.7 µM FeSO4, 0.5 µM ZnSO4, 0.097 µM (NH4)6Mo7O24), 0.1 mM nonessential amino acids, and also 100 U penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B per 100 ml of medium. To remove the epithelium, the cut edge of the corneal stroma is grasped with jeweler’s forceps, and a second, closed forceps is used to gently free the epithelium from the stroma. The edges of the epithelial sheet roll inward, scroll fashion, toward the apical side of the epithelium (Fig. 1 a). Thus, in transferring sheets to new substrates, one can discern apical from basal surface.

The 9-mm-diameter sheets (Fig. 1 a and b) were transferred to fresh media, washed three times, and placed on smaller-diameter segments of cornea with denuded epithelial basal lamina (Fig. 1 e and f). Smaller substrates were used so that the basal cells of the epithelium would remain columnar and would not migrate to resurface uncovered basement membrane. Actively migrating cells do not form mature HDs (17, 21). To study the time of HD formation, epithelial-sheet-denuded basal lamina combinations were cultured for 1, 3, 6, 18, or 24 h. All other combinations (corneal epithelium on Descemet’s membrane, lens capsule, and rat or human corneal epithelial basement membranes) were cultured for 24 h. Controls were normal rabbit, rat, and human corneas. All tissues were fixed for 1 h in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 2% OsO4 in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C. After en bloc staining with aqueous 0.5% uranyl acetate, the tissue was dehydrated in a graded acetone series followed by propylene oxide and then embedded in Epon-araldite.

Thick sections (1 µm) were stained with toluidine blue, a suitable area containing columnar basal cells was chosen. The blocks were trimmed accordingly, thin sectioned, stained with 2% uranyl acetate and lead citrate, and photographed using a Philips 200 or 300 electron microscope.

The basal cell membrane and lamina region of a minimum of eight cells per cornea, four corneas per experimental group, or an equal number of controls, were photographed. Final prints were enlarged × 25,000.

Morphometric Analysis of Hemidesmosomes and Anchoring Fibrils: For all experiments two independent counts were made of HDs, HDs associated with anchoring fibrils, and anchoring fibrils not associated with HDs. An 8X magnifying lens was used to facilitate counting. Specifically:

(a) The total number of HDs per micrometer of cell membrane was counted, and each HD was classified into one of three ultrastructural types. We attempted to categorize them into the four “stages of formation” proposed by Krawczyk and Wilgram (17). We used their stage I as our type 1, but we could not identify any HDs that were similar to their stage 2. In no instance did we find the extracellular dense line that is parallel to the cell membrane without the presence of the intracellular attachment plate. However, we did find that in the cornea most HDs fell into a structural type not described by Krawczyk and Wilgram. In this type the extracellular anchoring filaments traversed the space between plasmalemma and lamina densa and there was no electron-dense line transecting them, but an intracellular attachment plate was present. We categorized such HDs as type 2. In addition, we found observation of tonofilament insertion (Krawczyk and Wilgram stage 4) into HD attachment plates to be quite subjective. We therefore lumped their stage 3 and 4 into type 3. Our three ultrastructural types are shown in Fig. 2.

(b) As HDs per micrometer of membrane were counted and typed, HDs with anchoring fibrils directly across the lamina densa were also tabulated.

c) Anchoring fibrils not associated with or across from HDs/µm membrane were counted. Since anchoring fibrils can branch and splay out, we counted anchoring fibrils only at the point of their insertion into the lamina densa.

Figure 1 Light and electron micrographs of tissues used for in vitro model to study hemidesmosome formation. (a) 9-mm-diameter corneal epithelial sheet after removal from underlying stroma using Dispase II. Edges of sheet roll inward, scroll fashion, toward apical surface of epithelium. × 6. (b) Cross section of freed epithelial sheet. Sheets removed with Dispase II retain their epithelial architecture; basal cells are columnar and blebs are present on their basal membranes. × 760. (c) Electron micrograph of free surface of basal cell. Basal blebs and a few remnant intracellular hemidesmosome plaques are present (arrows). Bar, 1.0 µm. × 25,000. (d) Denuded basal lamina of cornea; upon which freed epithelial sheets were placed. The lamina densa is intact and anchoring fibrils (arrows) are present. Bar, 1.0 µm. × 25,000. Inset: Higher magnification showing cross-banding of anchoring fibril (arrows). Bar, 0.1 µm. × 81,000. (e) Section through the corneal segment upon which the epithelial sheet had been placed. To minimize migration, corneal segments or buttons were made smaller than the sheet. Micrographs for hemidesmosome counts were taken along columnar basal cells only. × 38.
RESULTS

Rabbit corneal epithelium is a nonkeratinizing, stratified, squamous epithelium, five to seven cells thick. Cells of the basal layer are columnar. Desmosomes are present along all membranes that abut other cell membranes, and hemidesmosomes are present along the basal cell plasma membrane adjacent to the basement membrane. Beneath the epithelial basement membrane is a thick, collagenous stroma. Anchoring fibrils similar in appearance to those found along the basement membrane of rat skin (12) and oral mucosa (13) extend from the lamina densa into the underlying stroma (Fig. 1 d, inset). The rabbit corneal anchoring fibrils have a banding pattern similar to that reported for epidermis (12), and although their total length cannot be determined because they branch and curve out of the plane of section, we have measured anchoring fibrils that extended 1.2 \( \mu \text{m} \) into the stroma.

**Characteristics of Freed Epithelial Sheets**

Sheets of epithelium removed from corneas by the Dispase II method retain their stratified epithelial characteristics (Fig. 1 b) (18), and desmosomes along cell membrane are intact. Basal cells remain columnar in shape; however, the lateral membrane that interdigitates with adjacent membranes in normal epithelium becomes quite straight upon removal of the sheet (18). Upon culture in the presence of Dispase II, basal cells send out cytoplasmic blebs at their basal surface (Fig. 1 b and c). These blebs are similar in appearance to those present on basal cells of embryonic chick corneal epithelia removed from their basement membranes by brief incubation in the presence of trypsin and collagenase or EDTA (22).

In our adult epithelial sheets removed with Dispase II, a few remnant intracellular HD plaques are present on the basal cell plasmalemmata (0.6 plaques/\( \mu \text{m} \) membrane as compared with 2.5 on normal epithelia) (Fig. 1 c). These remnant plaques were counted along membrane regions outside the cytoplasmic blebs, and this number was considered the zero time point for studies of time required for HD formation.

**Time Required for Hemidesmosome Formation**

New HDs formed after 1, 3, 6, 18, and 24 h in culture are shown in Fig. 3 and counts of HD/\( \mu \text{m} \) membrane in Fig. 4. After 1 h in culture, cytoplasmic blebs had withdrawn from the free surface of basal cells, and new HDs had formed (Fig. 3 a). The number of HDs/\( \mu \text{m} \) membrane was 1.4, an increase of 0.8 HDs over the remnant HD plaques on the sheet. Only a small increase in HD number was evident between hours 1 and 3 (to 1.42/\( \mu \text{m} \) membrane), but after 6 h in culture the number was 2.2/\( \mu \text{m} \) membrane, which is 82% that of control corneas. Between 6 and 24 h there was a small, steady increase so that by 24 h the number of HDs/\( \mu \text{m} \) membrane was 94.4% of the number of HDs along basal cell membranes of control corneas.

With increasing culture time, the percentage of types 1 and 2 (immature HDs) decreased and the percentage of mature HDs (Type 3) increased (Table I). A major shift from type 1 to types 2 and 3 occurred during the first 6 h of culture, the same time period during which most HD formation was observed. Even in control corneas a large percentage of HDs were types 1 and 2.

**Hemidesmosome Formation and Anchoring Fibrils**

The number of HDs found directly across the basal lamina from anchoring fibrils was constant (Table II); at all time periods studied, such HDs constituted 80–86% of the total number of HDs. The number of anchoring fibrils not opposite HDs decreased with increasing time in culture (Fig. 5). The greatest decreases occurred at the time of most rapid HD formation (compare Figs. 4 and 5). In addition to the morphometric data, one can visually see the association between HDs and anchoring fibrils (Fig. 3 d).

**Table 1**

<table>
<thead>
<tr>
<th>Culture time</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
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<tbody>
<tr>
<td>h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.2 ± 2.6</td>
<td>54.9 ± 2.0</td>
<td>16.6 ± 2.7</td>
</tr>
<tr>
<td>3</td>
<td>22.1 ± 3.4</td>
<td>58.9 ± 2.8</td>
<td>19.0 ± 3.7</td>
</tr>
<tr>
<td>6</td>
<td>16.2 ± 2.4</td>
<td>56.2 ± 1.9</td>
<td>27.5 ± 2.9</td>
</tr>
<tr>
<td>18</td>
<td>20.5 ± 3.2</td>
<td>47.6 ± 1.7</td>
<td>31.8 ± 4.0</td>
</tr>
<tr>
<td>24</td>
<td>17.5 ± 2.1</td>
<td>41.6 ± 1.9</td>
<td>40.9 ± 2.3</td>
</tr>
<tr>
<td>Control</td>
<td>14.4 ± 3.7</td>
<td>47.9 ± 2.6</td>
<td>37.6 ± 5.6</td>
</tr>
</tbody>
</table>

Average ± SEM, \( n \approx 4; 8 \) basal cells/experiment.

Figure 3. Micrographs demonstrating hemidesmosome formation with increasing culture time. In d, anchoring fibrils are particularly obvious under hemidesmosomes (arrows). (f) Control rabbit cornea hemidesmosomes. For morphometry, micrographs of eight columnar basal cells were taken. Two independent counts were made of the total number of hemidesmosomes per micrometer membrane, each ultrastructural type (1–3), hemidesmosomes across the lamina densa from anchoring fibrils, and anchoring fibrils not across from hemidesmosomes. Bar, 1.0 \( \mu \text{m} \). \( \times 25,000 \).
Formation of Hemidesmosomes on Other Ocular Basement Membranes

Basal cells of epithelial sheets placed on Descemet's membrane or lens capsule had not formed HDs after 24 h of culture (Fig. 6). The corneal epithelium adhered to both of these basal laminae and the basal cell cytoplasmic blebs withdrew, but the basal cells did not maintain their columnar shape (Fig. 6, insets). Neither of these thick ocular basement membranes have anchoring fibrils associated with them.

Interspecies Hemidesmosome Formation

Rabbit corneal epithelial sheets placed on corneal epithelial basement membranes from rat and human corneas formed HDs that were structurally similar to those in rabbit-rabbit combinations (Fig. 7 a and b). The numbers formed were only 56% and 50%, respectively, of those present in rabbit controls. Rat and human control corneas had fewer HDs/μm membrane than did rabbit corneas (Table III). Thus, the numbers formed in the interspecies combinations were 79% of the normal complement of HDs present in rat corneas and 74% of the normal complement of HDs on human corneas.

DISCUSSION

Our in vitro technique for study of the formation of the cell-substrate adhesion junctions, hemidesmosomes, is reproducible, as demonstrated by the small standard errors of the mean found at each time point studied (Fig. 4). Three critical factors necessary for reproducing the technique are (a) obtaining intact, viable sheets of differentiated adult epithelium, (b) placing the sheet on a completely and cleanly denuded basal lamina of equal or smaller surface area, and (c) counting hemidesmosomes only along areas where basal cells have maintained their columnar shape. The cornea is an excellent tissue for such studies because the epithelial basal lamina is, compared with epidermal basal lamina, quite flat, and rabbits are better suited than other laboratory animals because their corneas are not as steeply curved.

Hay and Dodson (23) and Sugrue and Hay (22) obtained intact sheets of corneal epithelium from 5- to 11-d old chick embryos by short-term incubation in culture media containing 0.04% EDTA (disodium salt), or 0.1% trypsin and 0.1% collagenase. Basal cells of these embryonic epithelia have not yet developed HDs. We were able to obtain sheets of adult rabbit epithelium by incubating corneas for a minimum of 2 h in media containing 0.025 M or 0.0025 M EDTA (disodium salt). In our hands such sheets were less viable, with many disrupted cells, and HD formation was not reproducible (I. K. Gipson, S. M. Grill, S. J. Spurr, and S. J. Brennan, unpublished observations). For these reasons we chose to use Dispase II, even though the specific action of the enzyme is not known. Green et al. (24) reported that the less pure Dispase II was more efficient than Dispase I in removing cultured epidermal epithelia from plastic culture dishes. We found this to be also true for removal of corneal epithelium from its underlying basement membrane.

Although it is not clear how Dispase II acts to remove epithelia, our data (25) indicate that (a) the lamina densa becomes discontinuous during 1 h of incubation with the enzyme; (b) the basal cells send cytoplasmic blebs into the underlying stroma through the disrupted places in the densa; and (c) freed sheets, even though there is no obvious densa along their basal cell edge, bind antibodies to laminin. Thus this “less pure” protease brings about disruption of the lamina densa. On the other hand, basal cells of sheets removed with EDTA do not bind antilaminin. Perhaps epithelial sheets removed with Dispase II provide a better preparation for reproducible study of HD formation because some of the basal lamina components are retained on the sheet, or because the enzyme is less toxic to basal epithelial cells than is EDTA.

We have found that the majority (83%) of new HDs form within the first 6 h of culture, and that there are two distinct periods of rapid formation (Fig. 4), within the first hour and between 3 and 6 h. We hypothesize that during the first period, HDs are formed by reassembly of the pre-existing HD components still present in the basal cell cytoplasm or on the basal cell plasmalemma, and that the second formation period may arise from synthesis of new HD components. We are currently testing this hypothesis by use of protein and glycoprotein synthesis inhibitors.

A trend toward more mature HD stages occurred with increased culture time (Table I). As described in the Materials and Methods section, we found it difficult to stage HDs of the corneal epithelium according to the scheme of Krawczyk and Wilgram (17). We modified their scheme to three ultrastructural types, which probably represent progressive stages of formation. Even with this modification, we found staging of HDs to be more subjective than counting total number of HDs or anchoring fibrils. The standard errors of the mean were greater than in the HD counts, and there was a wider disparity between counts of stages done by two individual

### Table II

<table>
<thead>
<tr>
<th>Culture time (h)</th>
<th>Percentage of Hemidesmosomes Associated with Anchoring Fibrils</th>
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<tbody>
<tr>
<td>1</td>
<td>82.2 ± 4.5</td>
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<tr>
<td>3</td>
<td>80.4 ± 5.5</td>
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<td>6</td>
<td>79.5 ± 4.4</td>
</tr>
<tr>
<td>18</td>
<td>82.3 ± 2.1</td>
</tr>
<tr>
<td>24</td>
<td>85.6 ± 4.6</td>
</tr>
<tr>
<td>Control</td>
<td>82.8 ± 4.6</td>
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</tbody>
</table>

Average ± SEM. n ≥ 4; 8 basal cells/ experiment. C, control rabbit corneas.
FIGURE 6 Micrographs of sections of corneal epithelial sheets cultured for 24 h on two other ocular basal laminae. (a) Epithelium cultured on Descemet's membrane. Light micrograph inset shows sheet on the thick basement membrane and the associated corneal stroma. Electron micrograph demonstrates lack of hemidesmosome formation. (b) Epithelium cultured on lens capsule. Light micrograph inset shows sheet of epithelium on the capsule. Electron micrograph demonstrates lack of hemidesmosome formation. Light micrographs: (a) × 480; (b) × 300. Electron micrographs: bar, 1.0 μm; × 25,000.

FIGURE 7 Rabbit epithelium cultured 24 h on denuded epithelial basement membranes of segments of rat (a) and human (b) corneas. Light micrograph insets demonstrate the tight adherence of the rabbit epithelium to the corneas of the two other species. Electron micrographs demonstrate that hemidesmosomes did form in these interspecies combinations. Light micrographs: a, × 475; b, × 480. Electron micrographs: bar, 1.0 μm; × 25,000.
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In conclusion, we have developed an in vitro system for study of hemidesmosome formation. This system can be used for future studies to determine the factors involved in formation of the cell-substrate adhesion junction.

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TABLE III

<table>
<thead>
<tr>
<th>Hemidesmosome Formation in Interspecies Combinations</th>
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<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Rabbit</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Human</td>
</tr>
<tr>
<td>24 h Co-culture</td>
</tr>
<tr>
<td>Rabbit-Rabbit BL</td>
</tr>
<tr>
<td>Rabbit-Rat BL</td>
</tr>
<tr>
<td>Rabbit-Human BL</td>
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</tbody>
</table>

Average ± SEM. n > 4; 8 basal cells/experiment.
AF, anchoring fibril; BL, basal lamina.

however, that the number of new HDs, expressed as a percentage of controls (normal corneas) after 24 h in culture, was less than the percentage formed in interspecies combinations.

When rabbit epithelia were placed on rabbit basal laminae, the number of new HDs that had formed after 24 h in culture was 95% of controls. When rabbit epithelia were placed on rat or human basement membranes, the numbers formed as a percentage of the rabbit controls were 56% and 50%, respectively. Even taking into account the fact that rat and human controls have fewer HDs and anchoring fibrils/μm membrane, the new HDs expressed as percentages of rat and human controls were 79% and 74%, respectively. Thus, although HDs formed in the interspecies combinations, formation is apparently not as efficient. These lower percentages cannot be fully accounted for by the fact that there are fewer anchoring fibrils per unit membrane on the host basal lamina. We did not do a time study on the interspecies combinations, so we do not know whether the HDs form more slowly. Perhaps with increased culture time the percentage formed would be greater.

REFERENCES