Corneal epithelial cell cultures on stromal carriers

Judith Friend, Shigeru Kinoshita, Richard A. Thoft, and Joseph A. Eliason

Exposure of denuded rabbit corneal stromal carriers for 24 hr at 37°C to suspensions of rabbit corneal epithelial cells grown in tissue culture resulted in the establishment of a cell layer on the carriers. The cell layers persisted for at least 1 week of incubation and were one to three cells thick. They consisted of healthy-appearing cells with normal intracellular organelles and intercellular desmosomal connections. After 2 to 7 days of incubation the cells were still capable of DNA replication and produced hemidesmosomes and basement membrane. This system is useful for in vitro studies of substrate requirements for hemidesmosome and basement-membrane formation by corneal epithelial cells. (INVEST OPHTHALMOL VIS SCI 23:41-49, 1982.)

Key words: corneal epithelium, tissue culture, hemidesmosomes, basement membrane

Tissue culture of corneal epithelial cells is a well-established technique used in many laboratories for the study of a variety of factors, including collagen and collagenase production,\textsuperscript{1-4} proteoglycan production,\textsuperscript{5, 6} effects of viral infections,\textsuperscript{7} and cellular differentiation,\textsuperscript{8, 9} as well as for basic studies of cells in culture and the effects of various agents (e.g., growth factors) on epithelial behavior in culture.\textsuperscript{10, 11} Feeder layers, such as layers of collagen\textsuperscript{11} or of irradiated fibroblasts\textsuperscript{8, 9} are frequently used to enhance growth of epithelial cell cultures and result in the development of multilayered corneal cultures that respond to growth factors in a manner similar to that of normal in vivo corneal epithelium.\textsuperscript{10-13} However, although those methods are useful for studies of epithelial cells in vitro, they do not permit in vivo analysis of the interactions between epithelium and subjacent tissue.

The work to be presented here demonstrates the feasibility of using suspensions of cultured rabbit corneal epithelial cells to repopulate denuded corneal stroma in vitro. Such a system may then be used for in vitro studies of the role of subjacent tissue composition, of basement membrane, and of hemidesmosomes in epithelial attachment.

Materials and methods

Epithelial cell cultures from explants and epithelial cell suspensions. Rabbits weighing between 1.5 and 3.0 kg were killed by an overdose of sodium pentobarbital. The eyes were enucleated and removed to a tissue culture hood. With sterile techniques, a 180-degree incision was made along the limbus. A cotton-tipped applicator was inserted into the anterior chamber and the corneal endothelium was wiped away leaving Descemet's membrane intact. The remaining 180-degree section of limbus was then incised, and the cornea was laid, epithelial side up, on a Teflon block. The edges of
Table I. Epithelial cells on denuded corneal stroma after in vitro incubation

<table>
<thead>
<tr>
<th>Stroma sample</th>
<th>Time (incubated with cells)</th>
<th>Time (total)</th>
<th>n</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>4 hr</td>
<td>4 hr</td>
<td>4</td>
<td>No cells on surface, all cases</td>
</tr>
<tr>
<td>K</td>
<td>6 hr</td>
<td>6 hr</td>
<td>4</td>
<td>No cells on surface, all cases</td>
</tr>
<tr>
<td>S</td>
<td>6 hr</td>
<td>6 hr</td>
<td>1</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>K</td>
<td>8 hr</td>
<td>8 hr</td>
<td>4</td>
<td>No cells on surface, all cases</td>
</tr>
<tr>
<td>K</td>
<td>24 hr</td>
<td>24 hr</td>
<td>4</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>S</td>
<td>24 hr</td>
<td>24 hr</td>
<td>3</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>K</td>
<td>48 hr</td>
<td>1 week</td>
<td>5</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>S</td>
<td>48 hr</td>
<td>1 week*</td>
<td>3</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>K</td>
<td>4 days</td>
<td>4 days</td>
<td>9</td>
<td>Cell layer, all cases</td>
</tr>
<tr>
<td>S</td>
<td>4 days</td>
<td>4 days</td>
<td>3</td>
<td>No cells on surface, all cases</td>
</tr>
<tr>
<td>K</td>
<td>0</td>
<td>48 hr</td>
<td>2</td>
<td>No cells on surface, all cases</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>48 hr</td>
<td>2</td>
<td>No cells on surface, all cases</td>
</tr>
</tbody>
</table>

S = scraped; K = keratectomized.

*Medium changed on day 4.

the cornea were trimmed and the tissue was cut into four approximately equal-sized, pie-shaped wedges. These corneal wedges were placed, epithelial side up, in a 60 mm culture dish (Falcon 3002) and 4.0 ml of medium were added. The medium used was medium 199, supplemented with fetal calf serum (to 20%) and gentamycin (33 μg/ml). The tissues were then incubated in a 37°C, 5% CO2-95% air, water-jacketed incubator for 1 week, with one change of medium on day 4. Epithelium cells then grew from the corneal explants onto the plate. After 1 week the epithelial cultures were examined under an inverted microscope. A few cultures (less than 5% of the plates) had a small endothelial cell or fibroblast contamination and were discarded. No cultures had extensive contamination.

Epithelial cell suspensions were prepared from the remaining epithelial cultures as follows. The explants and medium were discarded. Trypsin, 5 mg/ml in 1 to 1.5 ml of versene (1:5000), was added to each culture and the excess was immediately removed. The cells were then stirred up gently with a disposable glass pipette, transferred to a sterile conical centrifuge tube, and centrifuged for 15 min at 1000 rpm (ca. 200 x g). The supernatant was discarded, the cells were resuspended in medium, and that suspension was placed into the wells, which contained denuded corneal stromal carriers adherent to the bottom of the dish.

In general, the cell cultures from two eyes were used for each carrier. The cell suspension applied to the stromal carriers had an average of 7.2 ± 1.5 x 10⁵ cells/ml, with a range of 4 to 12.5 x 10⁵ cells/ml. In most cases, cells in the suspensions were individual but there were some clumps of two to five cells present.

Preparation of stromal carriers. Rabbits were killed as described above. Under a surgical microscope the corneal epithelium was scraped away. The cornea was then split with a corneal splitter, and an 8 mm diameter lamellar keratectomy, marked by a trephine, was done. Carriers so prepared were designated scraped samples and were left with intact basement membranes. An incision was then made at the limbus and a cotton-tipped applicator was inserted into the anterior chamber and used to remove the endothelium, after which the remainder of the 8 mm diameter button was incised and the carrier was removed. This second type of carrier was the keratectomized sample, differing from scraped samples in that it had no basement membrane remaining. The 8 mm de-epithelialized stromal carriers were then placed, Descemet's-membrane side down, in individual wells in a Multiwell tissue culture plate (Falcon 3008) and were covered with 100 μl of supplemented medium until the cell suspensions were prepared and added (30 min to 1 hr).

Incubation of stromal carriers with epithelial suspensions. One milliliter of epithelial cell suspension was placed into each well containing a stromal carrier after removal of the cell-free medium. The carriers were then incubated at 37°C in
Fig. 1. Light micrograph of epithelial cells on stromal carrier after 2 days of incubation. There are two to three cell layers, and no keratocytes are visible in this anterior portion of the stromal carrier. (Toluidine blue; ×330.)

Fig. 2. Scanning electron microscopic appearance of cell layers. A, Scanning electron microscopy shows an intact cell layer (EP) on the stromal carrier after 48 hr of incubation. The stromal carrier in this case had been de-epithelialized by scraping prior to incubation. The remaining basement membrane (BM) is visible at the cut edge of the sample. (Bar = 1 mm.) B, Scanning electron microscopy shows microplicae on the surface of the cells after 48 hr of incubation. (Bar = 1 μm.)

the 5% CO₂-95% air, water-jacketed incubator for 4 hr to 1 week. If the tissues were incubated 24 hr or longer, the cell suspensions were replaced by cell-free medium after 24 hr in most cases but after 4 days in others. After incubation, the carriers were evaluated morphologically.

**Histology and ultrastructure.** Routine hematoxylin and eosin staining on 7 μm paraffin sections was performed on 71 carriers after fixation in 10% buffered formalin: 2.5% glutaraldehyde (1:1).

Scanning electron microscopy was performed on six samples. The tissues were prepared by fixation in glutaraldehyde (2.5%), followed by critical-point drying and gold coating. They were examined under an AMA 1000 scanning microscope.

Transmission electron microscopy was per-
Fig. 3. For legend see facing page.
formed on 14 samples. Tissues were fixed in 2.5% glutaraldehyde in 0.15M phosphate buffer, pH 7.4, postfixed for 60 min in 1.0% osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under a Philips 200 electron microscope.

Uptake of tritiated thymidine by epithelial cells

In vitro. Tritiated thymidine (2 μCi; New England Nuclear, 20 Ci/mMol) in 1.0 ml medium 199 plus serum and antibiotic was placed over the stromal carriers in the wells of the Falcon 3008 dishes 3 hr before harvesting. One hour before harvesting, that medium was replaced by 1 ml of isotope-free supplemented medium. The carriers were then harvested, fixed in formalin-glutaraldehyde, and sectioned. After deparaffinization, slides were dipped in Kodak NTB-2 emulsion, stored for 14 days at −20°C, developed in Kodak D-19 developer, and stained with hematoxylin. The number of labeled cells per three to four fields of 100 epithelial cells in the center of the carrier was counted. Two keratectomized and two scraped samples after 48 hr of incubation (24 hr with cells, 24 hr without) were counted.

In vivo. Tritiated thymidine (20 μCi; New England Nuclear, 20 Ci/mMol) was injected into the anterior chambers of two anesthetized rabbits 2 hr before they were killed. Corneas were prepared for autoradiography as described above.

Results

Attachment of epithelial cells to stromal carriers. After 24 hr of exposure to cell suspensions in vitro, 43 of 47 denuded carriers, both after keratectomy and after scraping, had a one to three cell-thick layer of epithelial cells on the surface (Table I and Fig. 1). That layer persisted at least 4 days (14 cases). Further incubation (1 week) resulted in the absence of cells if the medium was not changed (four of four cases) but a retention of the cell layer if the medium was changed (four of four cases). There was no tendency for the cells to form more than one to three overlapping layers on the stromal carriers in vitro. Longer exposure of the stromal carriers to the epithelial cell suspensions (4 days) resulted in no epithelial cell layer on the carriers (seven of seven cases) after 4 days. Therefore all the data on cell layers reported below are on samples incubated 24 hr with cells, followed by 1 to 6 days further incubation in cell-free medium.

Samples that were incubated with no added cell suspension showed no cells on the epithelial or endothelial surfaces (four cases).

Cells were absent in the anterior stromal portion of all scraped and keratectomized carriers (71 cases).

Electron microscopy of cell layers. Scanning electron microscopy confirmed that there was an intact cell layer present on the carriers that had been exposed to the cell suspension for 24 hr and incubated a total of 1 day to 1 week, both after scraping and after keratectomy (Fig. 2, A). Microplicae were visible on the cell surfaces, with a density only slightly lower than that present on normal epithelial cells (Fig. 2, B).

Transmission electron microscopy showed that the cells on the carriers had characteristics of normal epithelium. Desmosomes could be seen between the cells, and the intracellular organelles appeared normal for up to

Fig. 3. Epithelial cells on scraped stromal carrier. A, Two days after removal of the epithelium from the stromal carrier by scraping and placement of the cell suspension in the well with the carrier for incubation, cells are present on the stromal carrier (St). Note normal-appearing desmosomes (d), mitochondria (m), Golgi apparatus (G), rough endoplasmic reticulum (rer), filaments (f), nuclei (N), and the presence of basement membrane (BM). There is some intercellular edema and no hemidesmosomes are present. (Bar = 1 μm.) B, After 1 week of incubation, mitochondria (m), filaments (f), nuclei (N), and rough endoplasmic reticulum (rer) are present. In addition, occasional hemidesmosomes (hd, marked by arrows) are present along the basement membrane (BM) and undersurface of the basal cells, and a pinocytic vesicle (p) is also present. (Bar = 1 μm.) C, Enlargement of one of the hemidesmosomes (hd, arrow) shown in B. (Bar = 0.2 μm.) D, Enlargement of pinocytic vesicle seen in B. (Bar = 0.2 μm.)
Fig. 4. For legend see facing page.
1 week (Figs. 3 and 4). Specifically, Golgi apparatus, mitochondria, tonofilaments, endoplasmic reticulum, nuclei, and surface microvilli were present and appeared essentially normal. There was, however, some intercellular edema, and only one to three cell layers were present.

The basement membrane was present in scraped samples at all times, but no hemidesmosomes could be seen after 24 to 48 hr of incubation (Fig. 3, A). After 1 week of incubation, however, the cells were still viable and hemidesmosomes were present along the basement membrane (Fig. 3, B and C).

Similarly, on the keratectomized stromal carriers, the cells had normal-appearing intracellular organelles and desmosomes between the cells. These samples showed processes (blebs) extending from the cells into the stroma. There was no basement membrane after 48 hr of incubation (Fig. 4, A). By 4 days, basement-membrane formation had begun, but there were no hemidesmosomes. The cells at 4 days appeared to be normal, with normal microorganelles and intercellular connections (Fig. 4, B and C).

**Uptake of tritiated thymidine.** Table II shows that 48 hr after incubation, 1% of the epithelial cells on the stromal carriers had taken up tritiated thymidine, but it was not possible to determine which cell layers were labeled. The uptake was the same for cells on scraped and keratectomized stromal carriers.

### Discussion

These results show that it was possible to repopulate a denuded corneal stromal carrier with cells by exposure of the carriers to suspensions of epithelial cells in vitro and that the cells so populating the surface had characteristics of normal epithelial cells. Previous investigators, using other substrates, have demonstrated basement-membrane collagen deposition by corneal epithelial cells in vitro and by other (noncorneal) epithelial cells in vitro (see refs. 15 and 16 for review) and have noted the presence of processes (blebs) extending from cells into substrate when basement membrane is absent (see refs. 15 and 16 for review). Hemidesmosome formation in vitro by noncorneal epithelial cells in culture has also been observed previously. In those cases, the cells were growing either directly on plastic or on collagen substrates. To our knowledge, this is the first demonstration of formation of hemidesmosomes by cultured corneal epithelial cells in vitro or by any epithelial cell plated onto its own natural substrate in vitro. The ability of our cells to form hemidesmosomes and basement membrane, coupled with the mitotic activity present after 2 days of incubation, shows that the cells have formed a synthetically active layer.

In epithelial tissue cultures, overgrowth by fibroblasts is a constant threat. In these experiments, several factors contribute to our

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**Table II.** Uptake of tritiated thymidine by epithelial cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Labeled cells*</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cornea (in vivo)</td>
<td>2.9 ± 0.29</td>
<td>4</td>
</tr>
<tr>
<td>Normal cornea (in vitro)</td>
<td>2.1 ± 0.55</td>
<td>2</td>
</tr>
<tr>
<td>48 hr cell layers (in vitro)</td>
<td>1.0 ± 0.26</td>
<td>4</td>
</tr>
</tbody>
</table>

*Values are given as averages ± S.E.M.
certainty that the resulting new cell layer is epithelial. First, during preparation of the cell suspensions, there was systematic rejection of any culture that showed fibroblast growth when examined under an inverted microscope. The second was the relatively brief culture time—1 week for the original cell cultures plus 1 week or less additional time for the stromal-epithelial combinations. Fibroblast proliferation to an extent necessary to be significant in the cultures would take much longer. Most importantly, the resultant cell layer was ultrastructurally epithelial, showing microvilli on the cell surfaces, intercellular desmosomal attachments, hemidesmosome formation, and basement-membrane production.

In these experiments, the denuded stroma on which the cells are plated probably acts as a feeder layer. Serum and a feeder layer, for example, collagen I or fibroblasts (e.g., irradiated 3T3 cells or conditioned medium from fibroblast cultures) are usually required for successful corneal epithelial cell culture. Some investigators have advocated the use of medium containing D-valine (and no L-valine), but no feeder layer, in order to ensure fibroblast-free epithelial cultures. However, such cultures develop only monolayers of cells. Growth for 2 to 3 weeks with feeder layers, on the other hand, results in multilayered cultures. Because of the ease of handling these tissues for subsequent morphologic or biochemical analysis and of the fact that the corneal stroma is the natural base on which corneal epithelial cells grow, this technique may prove to be more useful than the usual methods of corneal epithelial tissue culture for some specific applications. For example, this system is useful for study of the relationship between corneal epithelial cells and subjacent tissue, especially since the subjacent tissue is their own natural substrate.

The importance of stromal cell viability for epithelial regrowth has not been quantitatively assessed. In these experiments, as shown previously, both scraping and keratectomy resulted in a loss of many of the kerato-cytes in the anterior portion of the stroma. The observation that epithelial regrowth can occur over the nonviable stromal layer in vitro (as it does in vivo) indicates that keratocyte viability in the layer immediately subjacent to the epithelium is, in fact, not essential for regrowth of the epithelium.

Another factor that can be studied effectively is the development of adhesive elements (e.g., hemidesmosomes) and basement membrane by epithelial cells, without obligate prior migration of the epithelial layer. This gentle seeding of the stromal surface by a cell suspension probably eliminates the phase of cell sliding, which occurs when epithelial defects heal. Our work shows no differences in the viability or stability of the epithelial cell layer, whether or not the basement membrane is present. This is in contrast to conventional in vivo corneal wound healing experiments, which have shown that re-epithelialization and the degree of subsequent adhesion are adversely affected by the absence of basement membrane. These divergent results may, in part, be due to the rapid development of basement membrane, beginning within 4 days in this system. Others have reported that the appearance of basement membrane in vivo after keratectomy takes more than 1 week. The appearance of hemidesmosomes within a few days in this system, on the other hand, is in agreement with the results of in vivo studies showing these structures present within a few days of healing after corneal scraping.

Current research is directed at evaluating the role of growth factors, cell surface proteins (e.g., fibronectin), and varied conditions of incubations (e.g., decreased temperature or absence of serum) on cell growth and attachment in this system. These epithelial-stromal combinations may be used clinically as sources of replacement epithelium in ocular surface disease. The ability to shape and manipulate these stromal carriers for optimal surface coverage and to use nonocular epithelium from the patient, thus eliminating immunologic problems, may prove to be clinically valuable.
We thank Ilene Gipson, Ph.D., for helpful discussions, and Patricia Pearson and Yasuo Ishii for histology and electron microscopy.

REFERENCES


