Basement Membrane Synthesis by Human Corneal Epithelial Cells In Vitro

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Purpose. Collagen gels may prove to be potential carriers for transplantation of cultured corneal epithelial cells. The purpose of this study was to evaluate the suitability of collagen gels in comparison with corneal stromal blocks as the substrate to support the growth of human corneal epithelial cells in culture and the synthesis and deposition of the basement membrane components by these cells.

Methods. Conical epithelial sheets, freed from the culture dishes using Dispase II (Boehringer Mannheim, Indianapolis, IN), were cultured on corneal stromal blocks. Deposition of laminin, type IV collagen, type VII collagen, and perlecan (heparan sulfate proteoglycan) were evaluated immunohistochemically after 4 days, 7 days, 2 weeks, and 3 weeks. Human limbal explant cultures were established on collagen gels prepared from bovine type I collagen with or without addition of cultured human corneal fibroblasts. After 1, 2, 3, and 4 weeks, the deposition of the basement membrane components was evaluated immunohistochemically.

Results. Corneal epithelial cells, cultured on corneal stromal blocks as well as on collagen gels with or without fibroblasts, deposited laminin, type IV collagen, perlecan, and type VII collagen at the interface of the cells and the substrates. However, different substrates differentially influenced the temporal pattern of the deposition of various basement membrane components. On the stromal blocks, deposition of laminin, type IV collagen, and perlecan by the epithelial cells was evident at 1 week. Type VII collagen was detected at 2 weeks. On the collagen gels with fibroblasts, deposition of laminin, type IV collagen and perlecan was detectable at 1 week. In the epithelial cultures on the collagen gels without fibroblasts, only perlecan was detectable at 1 week. At 2 weeks, all of the basement membrane components, including type VII collagen were detectable on the collagen gels, either with or without fibroblasts.

Conclusion. Human corneal epithelium cultured on collagen gels or on corneal stromal blocks can synthesize and deposit basement membrane components, including laminin, type IV collagen, type VII collagen, and perlecan within 2 weeks in culture. Therefore, collagen gels may serve as potential carriers for human corneal epithelial transplantation.


Keratoepithelioplasty and conjunctival transplantation are sometimes successful in the treatment of ocular surface disorders, such as chemical burns and atopic keratoconjunctivitis. However, allograft rejection and reopacification of the ocular surface after keratoepithelioplasty and failure of conjunctival epithelium to attain corneal epithelial-like properties after conjunctival transplantation are problems that are often encountered. Use of cultured epithelial cells to replace ocular surface cells may serve as a new therapeutic method.

In rabbits, transplantation of sheets of corneal epithelium has been reported to be successful when the basement membrane was intact in the recipient cornea. However, transplanted corneal epithelial sheets disappeared in a few days in most cases without intact basement membranes. Because most patients who need ocular surface replacement do not have an intact basement membrane, poor attachment of corneal epi...
The Eye Bank of Western Pennsylvania. Four 2X2 carriers for cultured corneal epithelium was evaluated. Ana, CA), 10 ng/ml human recombinant epidermal growth factor (Earth Chemical, Hyogo, Japan), 5 µg/ml insulin (Sigma, St. Louis, MO), 0.1 µg/ml cholera toxin (Sigma), 5 mM L-glutamine (Gibco), 0.5% dimethylsulfoxide (Fisher, Pittsburgh, PA) and 20 µg/ml gentamicin (Elkins-Sinn, Cherry Hill, NJ). Limbal explants were removed at 10 days. Corneal epithelial cells reached confluency by 12 to 15 days. Corneal epithelial sheets were freed from culture dishes using Dispase II (1.5 U/ml, Boehringer Mannheim, Indianapolis, IN) and washed twice with Supplemental Hormone Epithelial Medium. Corneal stromal blocks, which had been prepared by removing epithelium, superficial stroma, Descemet's membrane, and endothelium from the human donor eyes (aged 65 to 75 years), were placed under freed corneal epithelial sheets. One milliliter of Supplemental Hormone Epithelial Medium was added to the dish and next day two milliliters of the medium was added. Medium was changed every 2 to 3 days during the experiments. Corneal epithelial sheets along with the corneal stromal blocks were rinsed three times with phosphate-buffered saline (PBS) and embedded in Tissue Tek OCT compound (Miles, Elkhart, IN). At 4 days, 7 days, 2 weeks, and 3 weeks, the cultures were analyzed immunohistochemically.

Corneal Epithelial Cells Cultured on Collagen Gels

Bovine dermal collagen I (0.65 ml Vitrogen 100 at a concentration of 3.0 mg/ml, Celtrix, Palo Alto, CA), 0.5 ml 1.7X concentrated Dulbecco's modified Eagle's medium and 0.2 ml fetal bovine serum were mixed and the pH was adjusted to 7.4 with 0.1 N sodium hydroxide. A suspension of 5 X 10^5 cultured human corneal fibroblasts (prepared as described later) in 0.2 ml Dulbecco's modified Eagle's medium (for the collagen gels with fibroblasts) or 0.2 ml Dulbecco's modified Eagle's medium (for the collagen gels without fibroblasts) was added to collagen mixture. The mixture was poured into a 35 mm culture dish (Falcon 3001, Becton Dickinson, Lincoln Park, NJ) and polymerized at 32°C for 1 hour. Explant cultures of human corneal fibroblasts were derived from the central corneas from donor human eyes (aged 65 to 75 years) obtained from The Eye Bank of Western Pennsylvania as described by Stopak et al, and corneal fibroblasts, in passages 4 to 7, were used in the collagen gels, as described earlier. Four 2 x 2 mm human corneal limbal explants were placed with the epithelial side up on the surface of collagen gels with/without human corneal fibroblasts to grow the explant cultures using modified Supplemental Hormone Epithelial Medium. After 1, 2, 3, and 4 weeks, the epithelial cultures along with the gels were embedded in OCT compound and the deposition of laminin, collagen IV, collagen VII, and perlecan was evaluated immunohistochemically.

Preparation of Antibodies

The monoclonal antibody to human laminin was developed by the Hybridoma Core Facility in our department. Monoclonal antibody to collagen IV was developed as described previously. Monoclonal antibody to collagen type VII was donated by Dr. Robert Burgeson (Cutaneous Biology Research Center, Massa-
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Polyclonal antibodies to perlecan were prepared in rabbits as described previously.19

**Immunohistochemistry**

The deposition of laminin, collagen IV, collagen VII, and perlecan by sheets of corneal epithelial cells cultured on corneal stromal blocks and on the collagen gels was evaluated immunohistochemically. Six-micrometer-thick cryostat sections of the samples were placed on gelatin-coated slides and fixed with cold \((-20^\circ C)\) acetone for 10 minutes. For blocking nonspecific binding of the second antibody, the sections were treated with 10% goat serum in PBS for 30 minutes. The sections were then treated for 1 hour with primary antibodies including monoclonal antibodies to laminin (undiluted hybridoma culture supernatant), collagen IV (1:50 diluted ascites fluid), and collagen VII (1:2 hybridoma culture supernatant), or rabbit polyclonal antibodies to perlecan (1:5), diluted in 10% goat serum in PBS. After washing the sections three times with PBS, secondary antibodies, consisting of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappel, Durham, NC) or fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Cappel) diluted in 10% goat serum in PBS, were applied for 45 minutes. After rinsing three times with PBS, the sections were mounted with Aquamount (Lerner, Pittsburgh, PA). As a negative control, tissue sections were treated with mouse immunoglobulin G or preimmune rabbit serum instead of specific primary antibodies. The sections were viewed and photographed using a photomicroscope with attachment for fluorescence microscopy (Olympus, Tokyo, Japan). Intensities of fluorescence were evaluated using photomicrographs taken with a fixed exposure time and were graded as follows:

- = no staining
+ = weak staining
++ = moderate to marked staining

**RESULTS**

**Deposition of Basement Membrane Components by Corneal Epithelial Sheets Cultured on Corneal Stromal Blocks**

None of the basement membrane components were detectable in the epithelial cultures on the stromal blocks at 4 days. Depositions of laminin, type IV collagen, and perlecan were evident under the epithelial cells by day 7 in culture, however, deposition of type VII collagen was not evident at day 7 (Fig. 1). At 2 weeks, deposition of all the basement membrane components including type VII collagen was detectable (Fig. 1). The intensities of the staining of the basement membrane components in these cultures at 3 weeks were significantly stronger than those at 2 weeks, however, they were significantly weaker than those in the basement membranes in the normal cornea (not shown).

**Corneal Epithelial Cells Cultured on Collagen Gels**

Collagen gels mixed with human corneal fibroblasts started to contract in 2 days. The gel diameters gradually decreased by 30 to 40% of the initial diameters at 1 week and did not change thereafter (Fig. 2A). The same changes in the collagen gels with fibroblasts were seen when corneal explants were not cultured on
A

FIGURE 2. Corneal epithelial cultures (each derived from 4 pieces of limbal explants) at 3 weeks on collagen with corneal fibroblasts (A) and on collagen gel without corneal fibroblasts (B).

B

Histologic analysis indicated that cultured corneal epithelial cells on collagen gels had grown as single layers of flat cells at 1 week (Fig. 3) and stratified to 2 to 3 layers after 2 weeks in culture (Fig. 4). Fibroblasts were seen in collagen gels throughout the experiments. There were no marked differences between the growth patterns of epithelial cells on collagen gels with and without corneal fibroblasts.

Immunohistochemical Analysis

One-Week Cultures. Weak immunostaining for laminin and type IV collagen was detectable under the epithelium on the collagen gels with fibroblasts, but
not on the collagen gels without fibroblasts (Fig. 5). Type VII collagen was not detectable. Staining for perlecan was evident under the epithelium on the collagen gels with and without fibroblasts.

**Two-Week Cultures.** The intensities of staining of laminin and type IV collagen were greater than at 1 week on collagen gels with fibroblasts (Fig. 6). These components were now detectable also on the gels without fibroblasts. The intensities of staining of perlecan were greater than at 1 week on gels with and without fibroblasts. Deposition of type VII collagen in the basement membrane zone was evident on gels with and without fibroblasts.

**FIGURE 5.** Immunofluorescence staining of cross-sections of human corneal epithelium cultured for 1 week on collagen gels with fibroblasts (A), (C), (E), (G), and (I) and collagen gels without fibroblasts (B), (D), (F), and (H), stained for laminin (A) and (B), type IV collagen (C) and (D), type VII collagen (E) and (F), perlecan (G) and (H), and negative control (I). Bar = 20 \( \mu \)m.

**FIGURE 6.** Immunofluorescence staining of cross-sections of corneal epithelium cultured for 3 weeks on collagen gels with fibroblasts (A), (C), (E), (G), and (I) and collagen gels without fibroblasts (B), (D), (F), and (H) stained for laminin (A) and (B), type IV collagen (C) and (D), type VII collagen (E) and (F), perlecan (G) and (H) and negative control (I). All of the four basement membrane components were detected on collagen gels with or without fibroblasts. Bar = 20 \( \mu \)m.

**Three- and Four-Week Cultures.** The pattern of immunofluorescence staining of these four basement membrane components in the cultures at 3 and 4 weeks was not different than at 2 weeks.

The data are summarized in Table 1.

**DISCUSSION**

As evidenced by the results, human corneal epithelial cells cultured on corneal stromal blocks in vitro take more than 4 days to deposit their basement membrane components. The deposition of laminin, type IV collagen, and perlecan is evident by 1 week; however, type
**TABLE 1. Summary of Immunostaining of Corneal Epithelial Cells Cultured on Collagen Gels With or Without Fibroblasts**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Laminin</th>
<th>Collagen Type 4</th>
<th>Collagen Type 7</th>
<th>Perlecan</th>
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</thead>
<tbody>
<tr>
<td>1-Week Culture</td>
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<tr>
<td>Fibroblasts(+)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fibroblasts(-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Week Culture</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fibroblasts(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Fibroblasts(-)</td>
<td>+</td>
<td>+</td>
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<td>3- 4-Week Culture</td>
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<tr>
<td>Fibroblasts(+)</td>
<td>++</td>
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<td>Fibroblasts(-)</td>
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+ = Weak staining; ++ = moderate to marked staining; - = no staining.

VII collagen synthesis and deposition takes longer. These findings may explain the results animal experiments in vivo, in which epithelial sheets did not stay attached to the stromal surface for more than a few days after transplantation (unpublished data). This failure of the transplantation may be due to the absence of basement membrane components, which are likely to be critical for the attachment of the epithelial cells to the underlying stroma.13-16

For the successful transplantation of cultured corneal epithelium, a carrier substratum may be essential because sheets of corneal epithelium, grown on tissue culture dishes, were found not to adhere to corneal stroma (unpublished data). We therefore biofabricated a material consisting of collagen and cultured corneal fibroblasts as a carrier substratum for growing explant cultures of corneal epithelial cells. The analyses of these explant cultures indicated that fibroblasts play a major role in gel contraction. However, corneal epithelial cells are also capable of causing gel contraction to a lesser extent as evident from the gels without fibroblasts also contracted after the epithelial cells were cultured on them for 2 to 3 weeks.

Our study indicated that human corneal epithelial cells, cultured on collagen gels either with or without corneal fibroblasts, synthesized the major basement membrane components, laminin, collagen IV, collagen VII, and perlecan. It was particularly encouraging to note that these epithelial cultures on the collagen gels could synthesize type VII collagen because type VII collagen is the major component of the anchoring fibrils, which are believed to be important in tight adhesion of the epithelial cells to the underlying tissue. The cell types contributing to the synthesis of basement membrane components, especially of type VII collagen, has been debatable.20-28 Our studies clearly show that human corneal epithelial cells can synthesize type VII collagen in culture without the influence of the fibroblasts. This agrees with a recent report that demonstrated that epidermal keratinocytes can secrete type VII collagen on their own.27

Fibroblasts may influence the deposition of basement membrane components by epithelial cells, because laminin and type IV collagen were evident earlier and the concentrations of deposited laminin and type VII collagen were higher in epithelial cultures on collagen gels with fibroblasts than those without. Fibroblasts may contribute either by synthesizing these components or influencing epithelial cells to synthesize these components.29

Evaluating the time course of the deposition of basement membrane components is important in determining the appropriate time at which epithelial cells cultured on the gels are competent for transplantation. Depositions of the components of the basement membrane and adhesion complexes by corneal epithelial sheets have been reported by some investigators using animal models. Laminin and type VII collagen reappeared synchronously at 48 hours in an in vivo experiment using rabbits,30 whereas in bovine cornea, laminin appeared earlier than type VII collagen in an in vivo experiment.31 In fetal tissues, the synthesis of laminin and type IV collagen precedes that of type VII collagen.32 In the current study, the cultured epithelial cells were found to synthesize laminin and type IV collagen by 1 week and type VII collagen by 2 weeks. Based on the minimum time required to get the optimum deposition of basement membrane components, two weeks old culture on collagen gels with fibroblasts would be the most suitable for transplantation.

Rejection of the transplants due to fibroblasts in the gel may be a concern; however, a living skin equivalent, epidermal keratinocytes cultured on collagen gel with fibroblasts, was not rejected.33

In summary, we have shown that human corneal epithelial cells cultured on collagen gels (with or without fibroblasts) are able to synthesize and deposit basement membrane components, including laminin, type IV collagen, type VII collagen, and perlecan. Therefore, collagen gels may prove to be suitable carriers for corneal epithelial transplantation.
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Key Words
corneal epithelium, collagen gel, carrier substratum, tissue culture, basement membrane

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References