Comparison of Central and Peripheral Human Corneal Epithelium in Tissue Culture

Bunshu Ebato, Judith Friend, and Richard A. Thoft

Past attempts to grow human corneal epithelium in culture had limited success, with confluence rarely attained. This work is to determine whether different areas of human corneal epithelium grow better in tissue culture. We compared the extent, the mitotic rates, and morphology of outgrowths and histology of explants from central and peripheral human corneas in culture. Explants, 2 mm in diameter, removed from eye bank eyes, were placed epithelial side up on a culture dish with modified SHEM tissue culture medium (Jumblatt et al, 1983). After 7 days, the tissues were fixed, stained and the area of outgrowths from explants measured using an image processor. For eight eyes from donors averaging 66 yr old, the average area of central outgrowths was 7.8 ± 1.1 mm², while that of peripheral outgrowths was 52.8 ± 5.2 mm² (P < 0.001). The mitotic rate of outgrowths of central epithelium was significantly less than that of peripheral epithelium (1.1 ± 0.5% vs 18.8 ± 0.8%) (P < 0.001). After 14 days, central outgrowths had not attained confluence and consisted of large cells. Peripheral outgrowths had attained confluence and consisted of small polygonal cells. Histology of explants showed that only one layer of epithelium remained on the stroma in central explants, but several layers were present on the peripheral explants. Thus, peripheral human corneal epithelium grows better in culture than does central human corneal epithelium. Invest Ophthalmol Vis Sci 28:1450-1456, 1987

It has been proposed that peripheral human corneal epithelium is important in the normal maintenance of the cell mass covering the cornea.9,10 The implication of this hypothesis is that peripheral epithelium, as a more actively growing area, may grow better in tissue culture than central epithelium. This suggests that the culture of peripheral corneal epithelium in SHEM may optimize the growth of human cells and permit sufficient production of the epithelium to allow clinical ocular surface replacement using cultured cells.

The purpose of this work is to (1) establish cultures of pure human corneal epithelium using enriched medium, and (2) test the hypothesis that different areas of human corneal epithelium grow better in tissue culture than others. To do so, primary cultures of central or peripheral epithelium were established, and (1) the area of outgrowth from the explants, (2) the mitotic rates of outgrowth, (3) the histology of explants, and (4) the morphology of the outgrowths from the two areas were compared.

The work clearly demonstrates that peripheral human corneal epithelial cells grow better in vitro than central ones when grown under the same conditions.

Materials and Methods

Human eyes deemed unsuitable for grafting were provided for this study by the Medical Eye Bank of Western Pennsylvania (Pittsburgh, PA).
Primary Cultures of Human Corneal Epithelium

Primary cultures of human corneal epithelium were started using endothelium-free explants. Under a tissue culture hood and using a dissecting microscope and sterile techniques, 2 mm diameter explants were prepared from the central or peripheral areas of corneas using a trephine and spring scissors. Six to eight 2 mm diameter explants were removed from the central area of each donor eye, and 10 to 12 2 mm diameter explants were removed from the peripheral cornea. Four central or peripheral explants were placed epithelial side up on a 35 mm tissue culture dish (Corning [New York, NY] 25000 or on a 22 x 22 mm sterile coverslip [Gold Seal, Becton Dickinson and Co., Rutherford, NJ] placed in a 35 mm tissue culture dish) and left covered for approximately 15 min in the tissue culture hood. Then, 1 ml enriched supplemented tissue culture medium was added to the culture dishes. The next day, another 1 ml medium was added to the culture dishes. Explants that did not remain attached or that floated off the dish were removed from the dish. Thus, approximately two dishes using central and three dishes using peripheral explants were prepared from each donor eye.

The medium was modified SHEM which contained Hams F12 and Dulbecco's Modified Eagles Medium (DMEM) (1:1), mouse-epidermal growth factor (m-EGF, 10 ng/ml), insulin (5 μg/ml), cholera toxin (0.1 μg/ml), 1-glutamine (1 μg/ml), fetal calf serum (to 15%), donor horse serum (to 5%), dimethylsulfoxide (0.5%) and gentamycin (40 μg/ml). The cultures were incubated in a 37°C, 5% CO₂-95% air, water-jacketed incubator for 7 to 14 days with the medium changed twice a week. Explants were removed and discarded. The coverslips with epithelial cell outgrowths were mounted cell side up on glass histology slides using Permoun® (Fisher Scientific, Fair Lawn, NJ). The slides were dipped in Kodak NTB-2 emulsion (Kodak, Rochester, NY), stored for 14 days at —20°C, developed in Kodak D-19 developer, fixed, and stained with hematoxylin (Gill #3, 5 min). After washing and dehydration, the slides were soaked in xylene, and the coverslips were removed from the slides and remounted cell side down on new glass slides.

Measurement of the Extent of Outgrowths

After 7 days of incubation, the epithelial outgrowths and explants were fixed in absolute ethanol for 15 min and stained with hematoxylin (Gill #3, Sigma Chemical Co., St. Louis, MO) for 5 min followed by rinsing in tap water and air drying. Photographs of the outgrowths were taken and the area of each outgrowth measured using computerized image processing on an IS 2000 Image Processor (New Hartford, NY). The outgrowths from eight eyes (two single, three paired) were measured (Table 1).

Mitotic Rate

Uptake of ³H-thymidine determined by analysis of autoradiographs was used as a measure of the mitotic rate of the tissues. After 7 days of incubation, cultures were prepared for autoradiography as follows: tritiated thymidine (1.0 μCi, New England Nuclear, Boston, MA, 20 Ci/nMol) in 1.0 ml modified SHEM was placed over the cultures growing on coverslips in 35 mm culture dishes, and the tissues were incubated at 37°C for 30 min. After incubation, the cultures were rinsed in phosphate buffered saline (PBS), fixed in absolute ethanol for 15 min, washed three times in ice cold trichloroacetic acid (TCA), washed two times with distilled water and then air dried. The dried explants were removed and discarded. The coverslips with epithelial cell outgrowths were mounted cell side up on glass histology slides using Permoun® (Fisher Scientific, Fair Lawn, NJ). The slides were dipped in Kodak NTB-2 emulsion (Kodak, Rochester, NY), stored for 14 days at —20°C, developed in Kodak D-19 developer, fixed, and stained with hematoxylin (Gill #3, 5 min). After washing and dehydration, the slides were soaked in xylene, and the coverslips were removed from the slides and remounted cell side down on new glass slides.

The number of labeled cells (those which incorporated tritiated thymidine) and the total number of cells in various fields were counted. The fields were located in the center (adjacent to the site of the explant), middle or leading edge of the outgrowths. Approximately 20 fields at X400 magnification were counted for each sample. Since there were no statistically significant differences in mitotic rate between different areas of the outgrowths on the culture dishes, the results are averages of all the areas counted for any one eye.

The mitotic rate is expressed as the labeling index:

\[
\text{Labeling index} = \frac{\text{Labeled cells}}{\text{Total cells}} \times 100
\]

Mitotic rates were measured in outgrowths from four (two single, one pair) eyes (Table 2).

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Table 1. Donor eyes used for measurement of area of outgrowths

<table>
<thead>
<tr>
<th>Donor eye #</th>
<th>Donor</th>
<th>Age (years)</th>
<th>TOD-TOC* (hours)</th>
<th>Number of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>17</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>65</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>65</td>
<td>32.5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>70</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>70</td>
<td>20.5</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>D</td>
<td>73</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>D</td>
<td>73</td>
<td>20.5</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>E</td>
<td>96</td>
<td>34.5</td>
<td>8</td>
</tr>
</tbody>
</table>

Average: 66 ± 8 34.5 ± 9.1

* Time of death to time of culture.
Table 2. Donor eyes used for autoradiography of outgrowths

<table>
<thead>
<tr>
<th>Donor eye #</th>
<th>Donor</th>
<th>Age (years)</th>
<th>TOD-TOC* (hours)</th>
<th>Labeling index†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>69</td>
<td>26</td>
<td>1.2 ± 0.1 (3)</td>
</tr>
<tr>
<td>2</td>
<td>G</td>
<td>79</td>
<td>18</td>
<td>1.6 ± 1.0 (6)</td>
</tr>
<tr>
<td>3</td>
<td>G</td>
<td>79</td>
<td>19</td>
<td>1.2 ± 0.4 (5)</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>81</td>
<td>22</td>
<td>0.5 ± 0.4 (4)</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>77 ± 5</td>
<td>21 ± 4</td>
<td>1.1 ± 0.5 (20)</td>
</tr>
</tbody>
</table>

* Time of death to time of culture.  † Mean ± SD (number of samples).

Histology of Explants

Explants removed from cultures after 7 days of incubation were fixed in 10% buffered formalin and sectioned. The sections were stained with hematoxylin and eosin using standard procedures. Explants from four cultures were studied.

Morphology of Outgrowths

Cultures were examined every day and photographed after 14 days incubation using a phase contrast microscope (Nikon AFX-II, Tokyo, Japan).

Results

Area of Outgrowths

The areas of outgrowths of epithelium from central and peripheral explants of eight donor eyes were examined after 7 days in culture. The average age of the donors was 66 ± 8 yr and the average time of death to time of culture was 34.5 ± 9.1 hr (Table 1). In all eight donor eyes from patients, the epithelial outgrowths from peripheral explants were significantly larger than those from central explants. The average area of outgrowths for all eight eyes was 52.8 ± 5.2 mm² for growth from peripheral explants and 7.8 ± 1.1 mm² for growth from central explants (P < 0.001) (Figs. 1 and 2).

Mitotic Rate of Outgrowths

The mitotic rate of outgrowths from four donor eyes was measured after 7 days in culture, expressed as the labeling index. The average age of donors was 77 ± 5 yr and the average time of death to time of culture was 21 ± 4 hr (Table 2). In all cases, the labeling index of peripheral outgrowths was much higher than that of central outgrowths. No cultures had reached confluence, nor did outgrowth from any
Fig. 3. Tritiated thymidine uptake by 1 week old central (A) and peripheral (B) epithelial outgrowths. Only two labeled cells are present in the central outgrowth while approximately 20 labeled cells are present in the peripheral outgrowth (Hematoxylin stain, original magnification ×800).

Histology of Explants

After 7 days in culture, several layers of epithelium were present on peripheral explants, but only one thin layer of epithelium remained on the stroma of central explants (Fig. 4).

Morphology of Outgrowths

 Cultures from four donor eyes (four dishes each from peripheral and central explants) were cultured for a total of 14 days (Table 1, #5–8). By that time, all the outgrowths from peripheral epithelium were confluent in the 35 mm culture dishes and consisted of
small polygonal cells. Central outgrowths had not attained confluence, consisted of large cells, had many holes, and floating dead cells were present in the medium (Fig. 5).

Discussion

These results show that it is possible to create and maintain viable cultures of human corneal epithelium if certain criteria are met. The enriched SHEM medium used in these studies contains growth factors and components which have been shown to be useful in culture of rabbit corneal epithelium or of uveal melanoma and other tissues. In these studies, the medium was arrived at empirically, and it is not clear whether all of the ingredients are essential for growth.

Most importantly, it appears that the location of donor cornea from which explants are taken is critical. In these studies, at 3 to 4 days the cultures did not appear to be very different, but the differences became quite apparent soon thereafter. Cultures derived from peripheral epithelium had larger outgrowths and higher mitotic rates after 1 week than...
Fig. 5. Phase contrast micrographs of 2 week old central (A) and peripheral (B) epithelial outgrowths. Central outgrowths never attained confluence and consisted of large cells. Peripheral outgrowths attained confluence and consisted of small polygonal cells (original magnification X400).

comparable cultures derived from central epithelium. Moreover, cultures of peripheral epithelium became confluent within 2 weeks while cultures from central epithelium died within 2 weeks. Studies of the plating efficiency and doubling time of passaged cells from the periphery are underway.

It appears that the human central epithelial cells are not capable of replication in culture, at least under the conditions studied in these experiments, and that the cells spread out as much as they can from the explant to cover the surface of the culture dish but the cultures do not thrive. This poor replication of central corneal epithelium supports the hypothesis that there may be a centripetal migration of corneal epithelial cells from the periphery to the center of the cornea to maintain the cell mass. It has recently been reported that there are cells located in the limbus that are stem cells for the corneal epithelium, further demonstrating that there are differences between ocular surface epithelial cells from different areas. These studies suggest that the central epithelial cells may be more highly differentiated than peripheral epithelial cells. If that is the case, it is perhaps not unexpected that peripheral cells should
be capable of more division, and, therefore, be more likely to survive in culture than central epithelial cells.

This discovery of the different growth characteristics of central and peripheral corneal epithelial cells may explain some of the previous inconsistencies in the reports of cultures of human corneal epithelium. It is possible that some investigators used explants of peripheral tissue while others used explants of central epithelium.

There are important differences between rabbit and human corneal epithelium.17 Therefore, having a readily available, reproducible system with which to grow cultures of human corneal epithelium is important for studies of epithelial biochemistry and physiology. Since only peripheral explants are needed, it appears that the corneal rim left after penetrating keratoplasty can be used as a source of tissue for culture (as is the case for human corneal endothelium,18 which will greatly expand the availability of tissue and therefore of cultures. For example, use of these cultures will permit us to determine whether the adhesion characteristics of human tissue are similar to those found in rabbit.19,20

There are several important clinical implications of this work. First, it indicates that the lenticules which are used as an epithelial source in keratoepithelioplasty21 should be oriented so that the peripheral corneal epithelium is directed toward the center of the cornea. This will permit the most active peripheral cells to grow onto and populate the corneal surface, rather than the sluggish central epithelial cells. Second, disturbance of peripheral corneal epithelium, such as inhibition of mitosis, may lead to a reduction of the central epithelial mass, leading to central epithelial defects. Third, central penetrating keratoplasty with epithelial retention is not an efficient way to treat diseases of epithelial origin, since the host epithelium will eventually repopulate the surface of the donor tissue, as has already been shown in rabbits.22

Finding that peripheral human corneal epithelium grows well in culture encourages us to explore the use of such cultured sheets for ocular surface replacement in patients. Studies to characterize the adhesive properties of these sheets to human corneal stroma are underway to explore the possibility of cultured epithelial cell transplantation.

Key words: human, corneal epithelium, tissue culture

Acknowledgments

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References


