The Growth of Endothelium from Human Corneal Rims in Tissue Culture

Shankar K. Nayak and Perry S. Binder

The authors report the successful in vitro growth of human corneal endothelium derived from 12 of 31 corneal-scleral rims obtained following corneal transplantation. The average age of donors whose endothelium grew was 17.6 ± 3.2 years. The average interval between death and culturing was 51.5 ± 10.1 hr. The cells migrated from the explants within 3–7 days and were characterized by a flattened, polygonal shape with a centrally located nucleus. Cell growth exhibited a doubling time of 72–96 hr in the second and third tissue culture passages. A reduced growth rate was observed by cell lines maintained in vitro for over 6 months. Fibroblast growth factor, epidermal growth factor, and endothelial cell growth supplement all exerted a positive influence on cell proliferation. Corneal-scleral rims can be a valuable source of endothelial cells for corneal research. Invest Ophthalmol Vis Sci 25:1213–1216, 1984

Because of a chronic shortage of donor human corneas, there has been increasing interest in the possibility of transplanting healthy corneal endothelial cells grown in tissue culture, but only a limited number of attempts have been made to grow this cell type in tissue culture. The tasks of growing pure corneal endothelium in tissue culture is difficult; with few exceptions, endothelium from donors older than 20 years of age has been exceedingly difficult to grow. One of the reasons for the slow progress in growing endothelium has been the limited availability of good quality tissue from donors younger than 20 years of age. We report for the first time, to our knowledge, the successful growth of human endothelium obtained from corneas with a scleral rim obtained following corneal transplantation.

Materials and Methods. Establishment of endothelial cell cultures: Thirty-one, human, corneal-scleral rims were obtained following corneal transplantation. The donor age ranged from 14 months to 57 years. The interval between death (TOD) and culture (TOC) is presented in Table 1.

The corneal-scleral rim was placed endothelial side up in a 60 × 15 mm petri dish containing serum-free medium 199. The petri dish was placed under a dissecting microscope and the endothelium was separated from the stroma. Two fine jeweler’s forceps were employed, one to anchor the rim from the scleral edge and the second to lift gently Descemet’s membrane. Care was taken not to touch the exposed stromal edge. Descemet’s membrane with intact endothelial cells was established as a primary explant culture in a 25-cm² tissue-culture flask coated with 0.2% gelatin. The explants were allowed to adhere to the surface for 30 min to 1 hr at 37°C in a 5% CO₂ + 95% air and humid atmosphere. Growth medium next was added to cover the explants without dislodging them and incubated undisturbed for 2–4 days. Medium NCTC-199 (Irvine Scientific; Irvine, CA) containing 1% nonessential amino acids; 1 mM/ml sodium pyruvate; 3 mg/ml glutamine: 10 mM/ml HEPES buffer; and 1% antibiotic solution consisting of 100 unit/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone solution supplemented with 20% fetal calf serum (FCS) was our standard growth medium. The medium was changed twice a week and the cell growth was subcultured upon reaching confluency by the Trypsin-EDTA treatment according to the method described by Baum et al.5

Measurement of cell growth: Following trypsinization, the number of cells in suspension was determined using a hemacytometer and adjusted to a concentration of 5 × 10⁴ cells/ml. One ml of the suspension was distributed into a Co-star, six-well cluster dish (M.A. Bioproducts; Walkersville, MD), each containing 4 ml of growth medium. Cell growth was monitored by counting Trypsin-EDTA detached cells from triplicate dishes at regular intervals.

Measurement of growth stimulation: Growth stimulation of the endothelial cells by fibroblast growth factor (FGF), epidermal growth factor (EGF) and endothelial cell growth supplement (ECGS) (Collaborative Research, Inc.; Waltham, MA) was studied according to the method of Gospodarowicz et al.7 Microcultures were established by adding 5 × 10³ cells in 0.2 ml growth medium to wells of a 96-well

<table>
<thead>
<tr>
<th>Donor age (years)</th>
<th>Number of specimens</th>
<th>Average donor age (years)</th>
<th>TOD-TOC*</th>
<th>Average Time (hours)</th>
<th>Number: successful cultures†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–10</td>
<td>5</td>
<td>3.8</td>
<td>44</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>11–20</td>
<td>9</td>
<td>15.9</td>
<td>44</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>21–30</td>
<td>5</td>
<td>23.4</td>
<td>41</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>31–50</td>
<td>6</td>
<td>38.8</td>
<td>80</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>51–57</td>
<td>6</td>
<td>54.3</td>
<td>68</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td></td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>30.6 ± 21.1</td>
<td>54.7 ± 26.3</td>
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</tr>
</tbody>
</table>

* Time of death to time of culture.
† Number of pure endothelial cell cultures. Other attempts resulted in either no growth or growth contaminated with fibroblasts.
Fig. 1. A, Migration of endothelial cells HCE-25 from the explants of endothelium from a 17-year-old donor. Note Descemet's membrane (DM) with viable sheet of endothelium (E). Phase contrast photomicrograph day 6 (original magnification, ×145). B, Formation of confluent monolayer of HCE-25 cells migrating out of explant shown in A, at day 23. Cells are polygonal in shape forming a compact monolayer. Phase contrast photomicrograph (original magnification, ×150). C, Confluent growth of endothelial cells HCE-28 derived from a 3-year-old donor in second passage. Note the compact monolayer formed by polygonal shaped endothelial cells. Phase contrast photomicrograph day 7 (original magnification, ×120). D, Growth of HCE-5 in sixth passage established from the corneal endothelium of a 37-year-old donor. Note the enlarged, mosaic pattern and multinucleated cells (arrow). Photomicrograph of 6-week-old monolayer. Phase contrast (original magnification, ×150).

Microplate. Twenty-four hours later, medium was replaced containing growth factors. The individual growth factor concentrations used were 50 ng/well FGF, 100 ng/well EGF and 200 ng/well ECGS. After 48 hr, cultures were pulse-labeled with 0.5 µCi of [3H]-thymidine (sp. Act. 47 Ci/mM; Amersham; Chicago, IL) for 18 hr. For each parameter, triplicate wells were used.

Results. Pure endothelial cell growth was obtained in 12 of 31 samples (Table 1) and was contaminated with fibroblasts in 17 samples. No endothelial cell growth occurred in two samples. The average age of the donors whose endothelium grew in tissue culture was 17.6 ± 3.1 years and the average time of death to time of culture was 51.5 ± 10.1 hr. In the 1–10 year age group, two of the five donors' endothelium grew. The age of these two donors was 3 years and the TOD to TOC was 72 hr. One donor's cells have grown in tissue culture for over 18 weeks (six passages). The other culture was discontinued at the fourth passage after 12 weeks due to bacterial contamination. In the 11–20-year age group, endothelium from seven of nine donors grew. The average donor age was 15.4 ± 2.4 years and the average TOD-TOC was 37.7 ± 9.9 hr. Five of the cultures did not grow beyond 7 months or six passages. One culture is presently growing after 12 months and 14 tissue culture passages, and one is growing in the fourth passage after 5 months. Three of 17 samples grew in tissue culture from the other donor age group. One culture from a 37-year-old donor (TOD-TOC = 96 hr) grew for 9 passages over 12 months. The other two cultures, one established from the endothelium of a 21-year-old donor (TOD-TOC = 36 hr) and one from a 35-year-old donor (TOD-TOC = 76 hr) failed to grow beyond its primary stage.

Cells began to migrate from the explant within 3–7 days (Fig. 1A). These cells were characterized by a flattened, polygonal shape with a centrally located nucleus. Cellular growth continued and reached confluence by days 14–21 (Fig. 1B). A sample growth pattern was recorded for cell line HCE-28 growing in its second passage (Fig. 1C). In most instances, cell growth drastically decreased after the sixth passage and 7–10 months in culture (Fig. 1D). In these samples, there was no indication of mitotic activity, the cells were large and flat with more than one nuclei and formed a mosaic pattern.

The growth pattern of three endothelial cell lines, HCE-25, HCE-28 and HCE-29 in the second and third passages was studied. All cell lines showed an initial drop in cell number, which gradually increased twofold by day 4 (Fig. 2); thereafter an eightfold increase in cell number was observed for HCE-25 on day 6. About a fourfold increase was noticed for HCE-28 and HCE-29 during the same period of time.

The results of proliferation of these cells in response to stimulation by growth factors was studied by
Table 2. Effect of growth factors on incorporation of $^3$H-thymidine by human corneal endothelial cells in vitro

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>Control</th>
<th>FGF 50 ng/well</th>
<th>EGF 25 ng/well</th>
<th>ECGS 100 ng/well</th>
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<tbody>
<tr>
<td>HCE-25 P-3</td>
<td>47,031 ± 3020</td>
<td>86,088 ± 2977</td>
<td>61,605 ± 128</td>
<td>57,938 ± 1557</td>
</tr>
<tr>
<td>HCE-28 P-2</td>
<td>6,259 ± 1411</td>
<td>17,127 ± 1957</td>
<td>11,361 ± 1130</td>
<td>13,608 ± 1267</td>
</tr>
<tr>
<td>HCE-29 P-2</td>
<td>7,983 ± 1976</td>
<td>18,968 ± 1464</td>
<td>15,356 ± 2837</td>
<td>15,771 ± 2239</td>
</tr>
</tbody>
</table>

* Significance of the increased incorporation of $^3$H-thymidine in the presence of growth factors was analyzed by Student’s t-test.

measuring DNA synthesis (Table 2). All three cell lines studied showed a significantly higher $^3$H-thymidine incorporation in the presence of 50 ng/well FGF, 25 ng/well EGF, and 100 ng/well ECGS.

**Discussion.** Culturing of pure human corneal endothelium in vitro is a difficult task. Only a few attempts have been made to grow this cell in culture. The source of the endothelium for these previous investigations was from human donors whose age ranged from prematurity to 80 years, whose cell cultures were established within 24-48 hr of enucleation. In previous studies, investigators used whole corneas that were either not suitable for or not required for transplantation. In spite of utilizing the entire endothelium to establish cultures, investigators only could achieve a limited success in growing the cells in vitro. To the best of our knowledge, this is the first report of the use of endothelium from corneal-scleral rims remaining following transplantation, for establishing cell cultures.

In 1 year, we attempted to culture 31 such specimens (Table 1) and were able to grow pure endothelium in culture from specimens from donors who were 14 months to 37 years of age. Of the 12 successful attempts, 9 were established from donors who were younger than 20 years of age. Similar observations were made by Baum and co-workers. In our study, endothelial cell growth was contaminated by fibroblasts in 55% of the experiments. We believe the source of such contamination is the exposed stromal edge in the corneal specimen.

Growth kinetics of cells studied in early passages showed a stationary phase, which lasted for about 4 days (Fig. 2). Thereafter, the growth increase was moderate and reached a four to eightfold increase by day 6. In an initial attempt to improve the growth properties of these cells, we added various growth factors to microcultures and measured the initiation of DNA synthesis (Table 2). Our preliminary results indicated that these growth factors exhibited a positive influence on the proliferation of human corneal endothelial cells.

We successfully have established cell cultures from the endothelium from the remaining corneal scleral rims obtained immediately following transplantation. One easily can obtain this tissue in good condition at a short death to culture time averaging about 55 hr. This is a valuable source of tissue for culturing corneal endothelium for biochemical, biologic, and morphologic studies.

**Key words:** cornea scleral rim, endothelium, tissue culture, growth factors

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**References**