Human Ciliary Body Epithelium in Culture

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Human ciliary body epithelial cells have been maintained in vitro and have been partially characterized by the determination of growth rate, morphology, and ultrastructural parameters. The dissection technique employed allows the separation of pure ciliary body epithelium with a predominance of cells being from the nonpigmented layer. Growth curves indicate this cell population follows a prolonged rate of growth compared to other primary cell cultures. Loss of pigment granules noted by light microscopy were documented by morphometric analysis of electron micrographs. Thirty-two percent of the cultures attempted were successful. Maintenance of these cells in vitro may provide a means for studying their enzyme systems, growth factors, reactions to various stimuli, and the effects of this cell population on other intraocular tissues. Invest Ophthalmol Vis Sci 24:687-696, 1983

The isolation and maintenance of pure human ciliary body epithelium (CBE) in vitro has not been reported previously. Literature describing the function and physiology of this essential intraocular cell population has dealt primarily with data gained from animal experimentation, microanatomical evaluation, and biochemical analysis of the metabolic products present in aqueous humor.1-3 The opportunity to investigate these cells in tissue culture now exists. Possible approaches to the study of this cell population are numerous, and the information to be gained has potential significance from both a basic science standpoint and clinically.

Materials and Methods

Human CBE was obtained from fresh donor eyes available through the Central Texas Lions Eye Bank. The tissue was processed within 4 to 30 hrs after enucleation. All eyes were stored at 4 C in a moist chamber containing Neosporin solution and normal saline until processing.

Eyes were obtained from donors ranging in age from 10 months to 72 years. They were approximately equally distributed according to sex, and the cause of death did not rule out an attempt to culture the CBE.

All tissue dissection and processing was carried out under sterile conditions in a laminar flow hood. Pure human CBE, primarily the nonpigmented layer, was separated from adjacent tissue by superficially incising the CBE epithelium just posterior to the pars plicata (the anterior most portion of the pars plana), and dissecting anteriorly to include the epithelium overlying the ciliary body processes. The dissection was terminated posterior to the root of the iris. Meridional sections of selected globes were prepared before and after removal of the CBE for histologic examination.

Tissue Culture

Following removal, the CBE was minced immediately in L-15 tissue culture medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum, gentamycin 40 μg/ml, and amphotericin B 2.5 μg/ml. The minced CBE and mono-dispersed cells were explanted to 25 cm² Falcon flasks with 2 ml of medium and incubated undisturbed for 48 hrs at 37 C in a nonhumidified atmosphere without the addition of CO₂. The flasks containing the fine minces were examined after three days and fed with 5 ml of growth medium. From then on, all flasks were fed once per week by complete removal of the spent medium and refed with 5 ml of fresh medium. Cell lines were passed routinely by removing supernatant media and overlaying the monolayer with 0.25% trypsin-0.1% versene solution in phosphate buffered saline pH 7.8. Cell counts were recorded at each passage, and the viability was determined by the trypan blue exclusion technique.

Growth Curves

A growth curve was carried out for 56 days without refeeding. Three 25 cm² plastic tissue culture flasks were inoculated with 1 X 10⁶ cells in 5 ml of L-15

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medium for each time period and incubated at 37 C. At weekly intervals, the supernatant from each flask was removed and the cells in each flask were harvested with 0.25% trypsin-0.1% versene, and the final population of viable cells was determined by the trypan blue exclusion technique.

Microscopy
Photomicrographs taken with an inverted phase contrast Nikon microscope, were obtained on initial culture and on subcultures to document cell changes that occurred in vitro. For electron microscopy, cells at various passage levels were fixed and embedded in situ. Thin sections were stained with uranyl acetate and lead citrate before examination with an Hitachi H-600 electron microscope at 50 kV. Evaluation of changes in CBE morphology with adaptation to in vitro growth was made by morphometric analysis of electron micrographs. Seven to 13 electron micrographs of random cells from one subculture were made at a magnification of 2,000× at each passage level. Because of the destructive nature of the test, it was necessary to use a different subculture for each passage level. The point counting method of Weibel was used to determine the average percentage of cell volume occupied by the nucleus, pigment granules (melanin), mitochondria, and glycogen granules. Morphometric values were analyzed by one-way analysis of variance (ANOVA) and nonparametric analyses, including Kruskal-Wallis Test on ranks.

Growth Factors
Immunocytochemical localization of growth factors in relation to CBE cells was performed on cells grown on the carbon-coated lids of BEEM capsules. These plastic capsules used routinely in electron microscopy were presterilized by a 30-min soak in 95% ETOH, followed by three rinses in sterile distilled water. Cell suspensions of CBE were prepared by trypsinizing stock CBE cultures and resuspending the cells in L-15 culture medium. Each BEEM capsule was filled with 0.8 ml of cell suspension (20,000–50,000 cells), covered with the lid, inverted, and immobilized in double-sided masking tape in a petri dish. After incubation for 3 days to allow attachment and growth of the cells, the medium was decanted from the capsule and the pointed tip cut off. The wall of the capsule was replaced in the lid to form wells around the cells. Cell fixations and reactions were performed in the wells.

The cells were prefixed in situ for 30 min with 2% paraformaldehyde in PBS, rinsed three times in PBS, and reacted with the primary antibody to growth factor. One hour at room temperature at a dilution of 1:20 in PBS was used for the Nerve Growth Factor (NGF) and Epidermal Growth Factor (EGF) made in rabbit (Collaborative Research, Lexington, MA). After three rinses in PBS, biotinylated antibody to rabbit serum (Vector Laboratories, Burlingame, CA) was added for 30 min. Three more rinses preceded incubation in horseradish peroxidase-avidin complex (Vector Laboratories) for 30 min. The peroxidase was visualized using the diaminobenzidine reagent of Graham and Karnovsky for 10 min. After three PBS rinses, the cell sheet was fixed 30 min in 1% OsO4 in Millonig's PBS. Dehydration was accomplished with graded alcohols (70–100% ETOH) and Hydroxypropyl Methacrylate (HPMA) 10 min, each in 90%, 95%,
Fig. 2. Meridional section of the ciliary body epithelium showing the separation of the nonpigmented cell layer (NPE) from the pigmented cell layer (PE), as it occurs during dissection. No stromal (S) or other extraneous tissue is involved (x320).

and 100% HPMA. Infiltration was with graded mixtures of Epoxy 812 and HPMA. Finally, each well was filled with Epoxy 812, incubated at 37 C overnight, and polymerized at 56 C at least 8 hrs. The blocks were removed from the capsules, and an area of cells was selected for sectioning from the monolayer embedded on the flat surface. Thin sections were poststained with uranyl acetate and lead citrate before they were examined and photographed on a Hitachi HS-600.

Results

Forty-four cultures were attempted during the study period. Fourteen of these were maintained in culture for an extended period of time, but only seven reached a sufficient cell population to study growth curves. No definite correlation has been found with respect to the effects of the age of the donor, the sex of the donor, death to enucleation time, or specimen storage time, and the success of a CBE culture.

Tissue Culture

The zone of CBE taken for culture is shown in Figure 1. Histologic sections of globes before and after removal of CBE showed the dissection technique to have removed primarily the nonpigmented epithelium with some adherent cells from the pigmented layer included in the specimens (Fig. 2).

In the initial explants (P/O) cells began to migrate from the explants within 2-5 days. Early migratory cells were usually nonpigmented, although heavily pigmented cells could be distinguished (Fig. 3). As growth continued, the cells varied in size, shape, and the number of pigmented granules. When passaged the cells would initially have a spindle-cell configuration, however, after attachment the cells tended to elongate and enlarge.

The length of time from initiating the cultures to monolayer confluency was 8-12 weeks. Passage of the cells by trypsinization thereafter could be accomplished at 2- to 3-week intervals. Some density dependence was noted as successful passage was rarely accomplished at a greater than 1:2 dilution.

Both pigmented and unpigmented cells could be identified in early passages. General observations were that the pigmented cells (Fig. 4) attached but did not divide. These cells assumed various shapes but could be distinguished easily by their large number of melanin granules. Cells that appeared nonpigmented grew out in monolayer fashion and were the predominant cell type by the third passage (Fig. 5).
Growth Curve Analysis

Growth curves were initiated by inoculating flasks in triplicate with quantitated suspension. It was found that cells could be cultured for 56 days without feeding if inoculated with $1 \times 10^5$ cells per flask. The cells had doubled by day 14 and continued logarithmic growth until day 28. A plateau stage was observed.

Fig. 3. The initial cellular proliferation from ciliary body epithelial explant (E) consists of elongated spindle-shaped cells and cuboidal cells which contain densely packed to sparse concentrations of melanin granules (arrows) ($\times 240$).

Fig. 4. Pigmented cells of the initial outgrowth (P/O). Attached pigmented cells assume various shapes while adherent to plastic and remain static or degenerate. Note prominent melanin granules around nucleus (arrow) ($\times 240$).
Fig. 5. Nonpigmented cells at the leading edge of the initial outgrowth are uniformly spindle shaped and become the predominant cell type with time (×240).

from day 28 to 42 at which time the cells went into the death phase and were completely dead by day 56 (Fig. 6).

Electron Microscopy

Electron microscopy of the ciliary body and epithelium prior to separation and the epithelium revealed intact tissue similar to that described by Hogan et al. Two types of cells could be distinguished: heavily pigmented cells that contained many melanin granules, and cells of the nonpigmented layer that contained relatively few melanin granules. The cells of the nonpigmented layer were more electronlucent. The predominant form of cellular adhesion was by desmosome, although occasional gap junctions could be identified between cells of the nonpigmented and pigmented epithelium. Lipid droplets were observed in both cell types, although a greater number were found in the nonpigmented epithelium (Fig. 7).

In early passages (P/0 to P/3) the cells were similar morphologically to those observed in intact tissue, although the difference in lucidity between the cell types was lost. Melanin granules decreased in prominence in cells from the pigmented layer and were only rarely observed in cells from the nonpigmented layer. No premelanosomes or their precursors were observed. Cells of the nonpigmented layer contained lipid droplets, and the mitochondria retained their characteristic inclusions (Fig. 8). There was an abundance of smooth and rough endoplasmic reticulum and Golgi apparatus in the cytoplasm.

Desmosomes were the predominant form of cellular adhesion.

Morphometric analysis of one cell line (Table 1) revealed a statistically significant difference between subpassages for all parameters measured with the most notable being a decrease with successive passages in the amount of melanin present ($P < 0.0001$). The number of melanin granules observed per cell decreased markedly with each passage and virtually disappeared by passage four. Since only one subculture was measured at each passage time, additional
Fig. 7. Micrograph of the ciliary body prior to separation of the epithelium showing the nonpigmented cell layer (NPE) and the pigmented cell layer (PE).
Fig. 8. CBE cells in early culture (P/O) resemble cells in vivo. Cells from the pigmented epithelium contain melanin granules (MG). Cells from the nonpigmented epithelium contains lipid droplets (L), and the mitochondria retain their characteristic inclusions (arrow).
Table 1. Morphometric analysis of electron micrographs. Mean count, standard error of mean, and number of observations.

<table>
<thead>
<tr>
<th>Passage</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>9</th>
<th>SD*</th>
<th>P*</th>
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<tbody>
<tr>
<td>Membrane</td>
<td>68.7</td>
<td>116.3</td>
<td>127.8</td>
<td>66.8</td>
<td>128.9</td>
<td>97.2</td>
<td>36.4</td>
<td>0.0003</td>
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<td>18.6</td>
<td>20.6</td>
<td>6.7</td>
<td>8.8</td>
<td>10.2</td>
<td>12.0</td>
<td>9.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Melanin</td>
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<td>2.0</td>
<td>1.8</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2.7</td>
<td>1.9</td>
<td>2.0</td>
<td>0.7</td>
<td>2.8</td>
<td>1.1</td>
<td>1.5</td>
<td>0.02</td>
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<tr>
<td>Glycogen</td>
<td>0.1</td>
<td>2.2</td>
<td>1.2</td>
<td>4.1</td>
<td>4.0</td>
<td>5.4</td>
<td>3.1</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Number of cells examined from specified subculture: 14 7 6 11 9 9

* Standard deviation within passage from ANOVA.
† P value for test against null hypothesis of equal means in all six passages from ANOVA. Nonparametric tests all yielded results similar to those obtained with ANOVA.

Experiments will be needed to distinguish the variation between passage times from the variation between subcultures.

By late passages the cells were devoid of melanin granules and lipid. The mitochondria no longer contained the characteristic granules. No type of cellular adhesion could be observed. These very large cells contained many tertiary lysosomes and linear tonofilaments were observed. Prominent Golgi apparatus were apparent suggesting these cells retain their metabolic activity (Fig. 9).

**Growth Factors**

Peroxidase labeled antibody identified varying amounts of NGF and EGF on the surface of CBE cells (Fig. 10). NGF appeared to be present in greater quantities and was more cellbound. EGF often appeared to be associated with material loosely bound to the cell surface, and at high magnification the peroxidase deposits are punctate more than disperse. NGF was visualized in microvesicles near the cell surface as well as on the surface by peroxidase de-
Discussion

The surgical dissection technique used to obtain human CBE from donor eyes assures the establishment of a pure culture of this tissue. The dissected tissue is primarily nonpigmented epithelium as this layer separates from the pigmented layer with only a few adherent pigmented cells included in the specimen. No stromal tissue of the ciliary body is involved at any time. The presence of pigmented epithelium in the culture is not considered inappropriate as these...
two cell layers act in microanatomical and physiologic concert in vivo. The cells that can be maintained in vitro are phenotypically nonpigmented. Whether only nonpigmented cells proliferate or the pigmented cells lose their capacity to produce pigment in vitro is unknown, however, we do know that nonpigmented epithelium is the predominant cell type obtained at dissection and that this cell type has a greater propensity to regenerate in vivo.7

Most of the unsuccessful cultures did not progress to confluency from the initial explants. Two discarded cultures survived through one passage and one culture survived through three passages. Since these cells are derived from normal intraocular tissue, a preprogrammed senescence is expected as with other normal tissues.

The reason, or reasons, why some human CBE dissections adapt to in vitro growth while others, with similar parameters fail, is unknown at this time.

Results of the growth curves confirmed the visually observed protracted turnover time of these cells. This slow growth rate is a critical factor since the number of cells available for study at any one time is limited.

The cell doubling time of 2–3 weeks was confirmed by growth curve analysis. The use of the CBE in tissue culture grown continuously without changing media should prove beneficial in measuring reactions to different stimuli during the different phases of the growth cycle.

Another constraint is that all studies must be completed before senescence. The in vitro duration of viability of CBE is variable, with the expected endpoint as yet undetermined, but probably is between 18 and 24 months.

Ultrastructural studies confirmed the apparent morphologic changes observed in vitro. The loss of pigmentation, the change in cell configuration, and the loss of desmosomes is compatible with the adaptation of these cells to a tissue monolayer in vitro.

The ability of these cells to elaborate growth factors will provide the opportunity to investigate human CBE produced EGF and NGF and their influence on other intraocular structures and cells. Both EGF and NGF are present in early and late cultures, which may indicate that in vitro CBE retains a substantial complement of its metabolic activity even after numerous passages. Several recent publications indicate that growth factors are important in corneal endothelial repair and proliferation.8–10 The EGF and NGF produced by human CBE cultures may play a more specific and critical role than those reported by virtue of their site of production and may ultimately prove to be obligatory factors for corneal endothelial cell function and viability.

The successful in vitro growth and maintenance of human CBE has the potential for providing information not previously available relative to the physiology and biochemical function of this essential intraocular cell population. The effects of these cells and their metabolites on other intraocular structures will be the basis of further investigational work.

Key words: ciliary body epithelium, tissue culture, ultrastructure, human eye

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References