Tissue culture of human retinal pigment epithelium

Jean Mannagh, Dharmendra V. Arya, and A. Ray Irvine, Jr.

Adult human retinal pigment epithelium has been enzymatically isolated and established in tissue culture. Primary cultures survived three to six months. Occasionally, cell lines spontaneously evolved from primary cultures. Melanin granules decreased with the age of the culture, became less pigmented but retained some characteristics of melanin-producing cells. Lipofuscin granules are present, but differ from lipofuscin of testicular origin. Mitotic activity is present throughout the life of the cultures.

Key words: retinal pigment epithelium, tissue culture, primary culture, cell line, mitosis, melanin granules, lipofuscin granules.

Because the epithelial and endothelial tissues of the eyes are adjacent to glassy, acellular membranes, they are particularly suitable for isolation and establishment in tissue culture. The method for enzymatic removal and primary culture of rabbit corneal endothelium has been reported, and similarly, a modification of that technique successful in the culture of human corneal endothelium has been published.

This paper reports the results of isolation and culture of the retinal pigment epithelium from 182 human eyeballs, 119 of which were established in primary culture. Of these, seven spontaneously evolved into cell lines.

Observations and comparisons of the cells of primary culture with those of the cell lines are made. Cell morphology, motility, growth, mitotic indices, and generation times were evaluated by phase microscopy and time-lapse cinemicrography. Cell morphology with particular emphasis on cytoplasmic granules was also studied by light microscopy of fixed and stained specimens utilizing both tinctorial and histochemical techniques. In addition, the granules were evaluated electron microscopically and melanogenesis measured autoradiographically by the uptake of labeled tyrosine.

Materials and methods

Tissue culture techniques. Donor eyes not suitable for corneal transplant and eyes from which the corneas were removed for preservation were used. Sterile technique was maintained.

Each eyeball was incised about 6 mm. behind the limbus and the anterior segment removed. The posterior globe was inverted and gelled vitreous
with retina was pulled from the vitreous cavity. Retinal remnants were removed from the optic disc with a rounded Bard-Parker blade. The specimen was then filled with about 5 ml of 0.03 per cent Pronase,* dissolved in a calcium and magnesium-free balanced salt solution, placed in a Petri dish, covered and incubated at 37° C. for 20 to 30 minutes.

The cells were freed from Bruch's membrane and harvested by repeated aspirations of the Pronase solution through an 18-gauge needle attached to a 5 ml syringe. The suspension was placed in a 5 ml conical tube and centrifuged at 1,000 r.p.m. for 3 minutes. The supernatant solution was aspirated with a Pasteur pipette, and the button of pigmented cells resuspended in 1 ml of tissue culture medium (15 per cent fetal bovine serum in Eagle’s minimum essential medium). The cell suspension was then placed in a Rose chamber or in a T-15 flask, and the culture vessel was filled with the medium.

Cultures forming a confluent monolayer were divided by exposure to 0.25 per cent trypsin in calcium and magnesium-free balanced salt solution and were subcultured.

Cell morphology, motility, growth, mitotic index, and generation time were documented by time-lapse cinemicrography and phase photomicrography.

Histologic techniques. In order to document the purity of the primary culture, the posterior eyeball was fixed in formalin, the remaining choroidal tissue removed from the sclera, flattened by radial incision, dehydrated with alcohol, cleared with xylene, and mounted flat on a glass slide, lamina vitrea uppermost. This preparation was examined by light and phase microscopy to determine that the remaining denuded Bruch's membrane was microscopically intact.

Most cultures were eventually fixed and stained. The following stains were used selectively: hematoxylin and eosin for cytology of the fixed specimen, May-Grünwald-Giemsa for cytology and chromosome studies, Polak stain for mitochondria, Sudan Black B for lipids, Nile blue sulfate and/or the Armed Forces Institute of Pathology (A.F.I.P.) lipofuscin stain for granules, and Becker’s stain, and/or Masson-Fontana stain for melanin. The dopa reaction was done to detect the presence of melanin precursor. Specimens were also bleached to help determine the nature of cytoplasmic granules.

Autoradiographic technique. Cover slip cultures were used for autoradiography to study the active tyrosinase system. The cells were incubated in phosphate-buffered saline containing 0.1 μCi per milliliters of L-tyrosine-2-C-14 (Calbiochem, Los Angeles, Calif.) for 24 hours. For controls, a tyrosinase inhibitor, sodium diethyldithiocarbamate, which blocks copper, was added at a concentration of 0.01 M to one-half of the above cultures.

Pigment formation. ACTH at a concentration of five-tenths to one unit per milliliter was used to stimulate pigment formation in the cultured cells.

Electron microscopy.* Preliminary electron microscopic studies were done to determine the nature of the granules in cultured pigment epithelial cells.

Results

Tissue culture. Successful primary cultures of retinal pigment epithelium were obtained from 65 per cent of donor eyes (119 of 182 cultures) (Table I). Viable cultures were obtained in 75 per cent of donors under the age of 60 years (57 of 76 cultures). By contrast, only 58 per cent of cultures from eyes over age 60 grew (62 of 106 cultures). Table II correlates the success of cultures with age of the donor.

In eight pairs of eyes it was possible to grow the pigment epithelium in only one of each pair.

Out of 24 pairs of eyes from which the retinal pigment epithelium could not be grown, 10 yielded viable cultures of corneal endothelium and in five of these when culture of lens epithelium was attempted, three were successful. This leaves 14 pairs of eyes from which it was not possible to obtain a viable culture of corneal endothelium, lens epithelium, or retinal pigment epithelium.

Verification of purity of initial cultures. Examination of flat preparation of posterior segments after trypsinization in all cases indicated that only pigment epithelium had been removed for culture and that the remaining denuded Bruch’s membrane was microscopically intact (Fig. 1).

Morphology. The first significant findings of cultured retinal pigment epithelial cells occurred after 48 hours when small clusters of rounded, heavily pigmented cells ad-

*We are indebted to Dr. Russell P. Sherwin, Department of Pathology, University of Southern California Medical School for electron photomicrographs of the pigment epithelial cultures.
Table I. Data concerning successful cultures of retinal pigment epithelium

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<td>Cell death</td>
<td>Yeast</td>
</tr>
<tr>
<td>1050 A</td>
<td>79 years</td>
<td>60 days</td>
<td>0</td>
<td>Fixed for electron microscopy</td>
<td>Yeast</td>
</tr>
<tr>
<td>B</td>
<td>60 days</td>
<td>0</td>
<td>Fixed for electron microscopy</td>
<td>Yeast</td>
<td></td>
</tr>
<tr>
<td>872 B</td>
<td>82 years</td>
<td>60 days</td>
<td>0</td>
<td>Cell death</td>
<td>Yeast</td>
</tr>
<tr>
<td>1102 A</td>
<td>82 years</td>
<td>60 days</td>
<td>A and B pooled</td>
<td>Cell death</td>
<td>Yeast</td>
</tr>
<tr>
<td>B</td>
<td>60 days</td>
<td>A and B pooled</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>583 A</td>
<td>88 years</td>
<td>30 days</td>
<td>0</td>
<td>Cell death</td>
<td>Yeast</td>
</tr>
<tr>
<td>B</td>
<td>60 days</td>
<td>0</td>
<td>Moj-Cheung et al. 1968</td>
<td>Yeast</td>
<td></td>
</tr>
<tr>
<td>451 B</td>
<td>90 years</td>
<td>72 days</td>
<td>3</td>
<td>Fixed and stained</td>
<td>Yeast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>May-Grünwald-Giemsa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

herent to the chamber floor and numerous free-floating pigment granules were seen. The medium was changed at this time to clear the chamber of the floating pigment.

Mitotic activity usually became apparent after 48 hours (Fig. 2). In primary culture the cells remained diploid. The generation time (the time necessary for cells to double) was approximately 32 hours. The mitotic time (prophase through division) was 75 minutes.

During mitosis, the pigment granules surrounded the chromosomes in metaphase and moved in opposite directions with the
cytoplasm at telophase. As a result undivided cells in the center of the cluster showed numerous heavily pigmented granules, whereas more recently divided cells at the periphery of the colony had fewer pigment granules and these were less heavily pigmented (Fig. 3).

After 72 hours, the cells were seen singly and in small clusters growing in an epithelial-like pattern from a pigmented nidus.
Round and rod-shaped pigmented granules were massed around the nucleus, leaving the peripheral cytoplasm relatively free of granules (Fig. 4). The cell nuclei were rounded with a well-defined nuclear membrane and had one to three nucleoli. Bineucleated cells were not uncommon.

It took about 30 days for a confluent monolayer to fill the floor of a Rose chamber. When this occurred, the cells were trypsinized at $37^\circ$ C. and subcultured. (In some cases, cultures from the two eyes of an individual were pooled and transferred to a T-flask). Some cultures were stained for morphology and for histochemical study of cytoplasmic granules. The successful primary cultures were maintained by this method for two to six months, after which the cells became vacuolated and died. Thirty-eight primary cultures were terminally stained with May-Grünwald-Giemsa for assessment of their morphology.

**Cell lines.** Seven primary cultures of retinal pigment epithelium spontaneously transformed into cell lines. The first indication of transformation was a change of cell morphology (Fig. 5). They became smaller, more uniform, more closely approximated, and remained in a monolayer. They grew more rapidly and gradually replaced cells in the primary culture. Squash preparations indicated they had changed from a diploid to a heteroploid chromosome number, the prime indica-

**Table II. Results of retinal pigment epithelial cell growth by age group**

<table>
<thead>
<tr>
<th>Donor's age</th>
<th>Number of eyes by age groups</th>
<th>Successfully grown cultures</th>
<th>Percentage success</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days-19 years</td>
<td>15</td>
<td>11</td>
<td>73</td>
</tr>
<tr>
<td>20-29 years</td>
<td>4</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>30-39 years</td>
<td>4</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>40-49 years</td>
<td>18</td>
<td>13</td>
<td>72</td>
</tr>
<tr>
<td>50-59 years</td>
<td>35</td>
<td>27</td>
<td>77</td>
</tr>
<tr>
<td>60-69 years</td>
<td>34</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>70-79 years</td>
<td>57</td>
<td>36</td>
<td>63</td>
</tr>
<tr>
<td>80-89 years</td>
<td>15</td>
<td>9</td>
<td>60</td>
</tr>
</tbody>
</table>
Fig. 3. Thirteen-day-old living culture showing nidus of heavily pigmented cells adjacent to peripheral cells more recently divided and having fewer granules. Note that many of the pigment granules appear clear because they are slightly out of focus. Mitotic activity is apparent. (Phase optics. Magnification x200.)

Fig. 4. Perinuclear arrangement of pigmented granules with clearer peripheral cytoplasm. Eighteen-day-old living culture. (Phase optics. Magnification x500.)
tion of cell transformation. The mitotic index roughly doubled and the generation time approximately halved. Data concerning the cell lines are presented in Table III. Two of the cell lines have survived and details of their growth and culture are as follows: Culture No. 505 was started in 1965 from a 70-year-old eyebank donor and is presently in its one hundred seventieth passage. The primary cultures from the two eyes were pooled after 15 days and the cells placed in a T-60 flask. Cell transformation occurred in the second passage. The culture was transferred at six to eight day intervals, a monolayer having formed. The new vessel was seeded at 10^6 cells per milliliter. The media was replaced twice a week. During the first year, a Rose chamber was prepared at each passage and these were fixed and stained with May-Grünwald-Giemsa for cell morphology. Pigmented granules were almost absent in the fourth passage, and in subsequent transfers only an occasional cell containing pigment granules was seen in all passages by phase microscopy. Chromosome preparations were done on the twenty-second passage. Counts of spreads varied from 42 to 132 in the 20 cells counted. The mitotic index of this cell line was determined on a 24-hour-old culture at the sixth, seventh, and eighth passages and averaged 66 cells per 1,000 (6.6 per cent). The generation time was 22 hours which represented the average of four cells studied while simultaneously

Fig. 5. Culture No. 505 showing early transformation. Note the more epithelial pattern of transformed cells compared to cells of primary culture which are less cohesive and less epithelial in appearance. Thirteen-day-old subculture in third passage. (Phase optics. Magnification ×100.)
Table III. Data concerning cell lines

<table>
<thead>
<tr>
<th>Culture</th>
<th>Donor's age</th>
<th>Culture both eyes combined</th>
<th>Passage at which culture transformed</th>
<th>Age of culture at transformation</th>
<th>Number of passages</th>
<th>Mitotic index (%)</th>
<th>Generation time</th>
<th>Chromosome count</th>
<th>Fate of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>504</td>
<td>89</td>
<td>Yes</td>
<td>Second</td>
<td>65 days</td>
<td>12</td>
<td>66</td>
<td>Not done</td>
<td>70-121</td>
<td>Contaminated</td>
</tr>
<tr>
<td>505</td>
<td>70</td>
<td>Yes</td>
<td>Third</td>
<td>75 days</td>
<td>176</td>
<td>66</td>
<td>22 hours</td>
<td>42-132</td>
<td>Still carried</td>
</tr>
<tr>
<td>506</td>
<td>61</td>
<td>No</td>
<td>First</td>
<td>21 days</td>
<td>19</td>
<td>64</td>
<td>Not done</td>
<td>54-74</td>
<td>Contaminated</td>
</tr>
<tr>
<td>698</td>
<td>13</td>
<td>Yes</td>
<td>Third</td>
<td>90 days</td>
<td>24</td>
<td>Not done</td>
<td>66-146</td>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td>698</td>
<td>32</td>
<td>No</td>
<td>First</td>
<td>55 days</td>
<td>36</td>
<td>Not done</td>
<td>60-87</td>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td>718</td>
<td>20</td>
<td>Yes</td>
<td>Seventh</td>
<td>5 months</td>
<td>12</td>
<td>Not done</td>
<td>46-86</td>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td>923</td>
<td>21</td>
<td>Yes</td>
<td>Third</td>
<td>73 days</td>
<td>156</td>
<td>79</td>
<td>20 hours</td>
<td>46-76</td>
<td>Still carried</td>
</tr>
</tbody>
</table>

Cells from the twenty-second passage were frozen at -21°C in 15 per cent glycerol media. At this temperature, the cells could be stored for 45 days and remain viable. After that time, however, they could not be cultured. For the past three years, the cells have been stored at -70 to -100°C in glycerol media and have remained viable.

Culture No. 923 was started in 1968 from a 21-year-old eyebank donor and now is in its one hundred sixtieth passage. Cells from the two primary cultures were pooled after three weeks and placed in a T-60 flask. Cells grew slowly and a monolayer was formed after 60 days. Cell transformation occurred in the third passage. After the fourth passage, the cells divided rapidly and formed a monolayer in the T-flask in seven to ten days. The cells were transferred weekly, and new vessels seeded at 10⁶ per milliliter. Rose chambers were prepared for cell morphology at each passage. Chromosome analysis was done on the eleventh passage, and count of the spreads varied from 46 to 76 in the 20 cells studied. The mitotic index was 7.9 per cent on a 24 hour culture in the one hundred fifty-eighth passage, and the generation time was 20 hours, determined by four days of consecutive time-lapse cinemicrography. Cells have been stored at -70 to -100°C in 15 per cent glycerol media and can be recultured.

**Histology.** Thirty-eight primary cultures were terminally stained with May-Grünewald-Giemsa for assessment of their morphology. One of a paired specimen in the cell lines was fixed and stained at each passage for morphologic study. In all such preparations, the cells were epithelial in appearance and grew in a monolayer. Mitochondria could be detected by phase time-lapse cinemicrography and their presence verified in the fixed specimen with Polak's mitochondrial stain.

Cytoplasmic granules appeared to be of two types. The majority were uniform in size, rod-shaped, and brown in color and were characteristic of melanin granules of retinal pigment epithelium. A second type of granule was noted. They were larger than the melanin granules, varied in size, tended to be round, and were less densely brownly pigmented. These were thought to represent lipofuscin granules. All granules decreased in number as the cultures aged, and the granules remaining were less pigmented. In the oldest primary cultures and in the cell lines, the granules became colorless but were still visible by phase microscopy.

In an effort to further elucidate the nature of the granules, special stains and histochemical tests were done. Lipofuscin granules of testis were used as a control. These did not bleach after 24 hours exposure to 0.25 per cent potassium permanganate; they stained red with the A.F.I.P. lipofuscin stain, stained blue with Nile blue sulfate, and gave a positive reaction to lipid with Sudan black B. In
Fig. 6 A and B. A. Nonpigmented granules in A which seem to be dopa positive in B. Five-day-old culture. (Phase optics. Magnification ×200.)

Contrast, all granules from five primary cultures bleached and there were no red-staining granules with the A.F.I.P. lipo-fuscin stain. However, the lipofuscin granules stained blue with Nile blue sulfate and gave a positive reaction for lipid with Sudan black B. Therefore, lipofuscin of retinal pigmented epithelium is similar to lipofuscin of testis in that it gives a similar reaction with Nile blue sulfate and Sudan black B, but differs from the control pigment in that it bleaches and gives a negative reaction to the A.F.I.P. lipofuscin stain.

In five primary cultures in which the melanin granules had become less pigmented or colorless, the dopa reaction was positive, suggesting that melanin precursor was still present (Fig. 6). The dopa reaction was negative on one cell line tested. Two primary cultures and two cell lines with lightly colored or colorless granules stained positive for melanin with the Masson-Fontana silver stain.

Autoradiography indicated that in the presence of a tyrosinase inhibitor the incorporation of labeled tyrosine-2-C-14 was inhibited extensively in the cells of a primary culture, to a lesser degree in a cell line, and least in a cell line of human lens epithelium, thus indicating a more active tyrosinase system in the pigment epithelium than in the lens epithelium.

Preliminary electron microscopy studies revealed melanosomes in a primary culture and in a cell line (Fig. 7).

Discussion

We gave a preliminary report of tissue culture of human retinal pigment epithelium at the Western Section Meeting of the Association for Research in Ophthalmology at Lake Tahoe, Calif., Sept. 16 through 18, 1965. We presented additional work at the Association for Research in Ophthalmology Spring Meeting in Sarasota, Fla., May 1 through 5, 1970. Albert, Tso, and Rabson3 published a report of tissue culture of human retinal pigment epithelium in 1972. Others have cultured these cells with explants containing adjacent choroidal and/or retinal elements.4,5

There is no correlation between failure to establish cells in culture and the cause of the donor's death. Most cultures were prepared within 48 hours postmortem and within that period the successful establishment of cultures was not correlated with the time interval between death and culture preparation. A few cultures were established successfully 72 hours after death.

However, there appeared to be a correlation between the age of the donor and growth of his retinal pigment epithelium,
75 per cent of donors under age 60 yielding viable cultures compared to 58 per cent of eyes over 60 that provided living cultures.

It may be of interest that of the 24 pairs of eyes from which the retinal pigment epithelium could not be grown, other ocular tissues, lens epithelium, or corneal endothelium were successfully cultured in 10 pairs leaving 14 donors from which it was not possible to grow corneal endothelium, lens epithelium, or retinal pigment epithelium. This is in accordance with the experience of Pomerat who stated that cells from some individuals could not be established in tissue culture.

Morphologically, the cells remain epithelial. They actively divide until a monolayer covers the floor of the culture vessel, after which they tend to remain static without mitosis and without becoming multilayered. Mitotic activity and growth resume after subculture. The cells do not undergo metaplasia nor do they form a glassy membrane.

The nature and behavior of cytoplasmic granules was interesting. Pigment granules lessened with age of the culture and the pigment granules remaining were less pigmented. However, in five cultures the colorless granules were dopa positive suggesting that these clear granules contained melanin precursors. This was substantiated by the incorporation of labeled tyrosine in a primary culture and in a cell line.

An autoradiographic study using L-tyrosine-2-C-14 revealed a more active tyro-
sinase system in the pigment epithelium (primary as well as the cell line), as indicated by greater incorporation of labeled tyrosine in this tissue than other ocular tissues. The incorporation of labeled tyrosine was inhibited to a greater degree in pigment epithelium than in lens epithelium by sodium diethylthiocarbamate, a tyrosinase inhibitor.

We were unable to enhance pigment formation by the addition of ACTH (adrenocorticotrophic hormone). This is at variance with the findings of Hu and Chavin who were able to hormonally stimulate melanogenesis in cultured goldfish caudal fin.

There were granules other than those containing melanin. These were thought to be lipofuscin and resembled electron microscopically and tinctorially the lipofuscin granules of retinal pigment epithelium described by Hogan and Feeney and by Streiten. They differed, however, from the lipofuscin of testicular origin used as a control. It is our impression that these lipofuscin granules diminish with the age of the culture and in this way differ from the wear and tear lipofuscin pigment of cardiac muscle and testes which accumulate with age. We considered the possibility that their function might be that of phagosomes in the endocytosis of disintegrating rod outer segments. Their differences from testicular lipofuscin and their decreased concentration with age of the culture suggests that they may have an important role in the cell physiology of retinal pigment epithelium rather than representing a senescent change.

Although the cell characteristics observed in our preparations resemble those reported by Barishak in the epithelial-like sheets of pigmented cells appear within three to four days after explantation, and that peripheral cells in the colonies were less pigmented than cells in the center of the explant, and that isolated cells became rounded, there are some significant differences. Barishak only observed mitosis shortly after explantation, whereas we observed mitotic activity throughout the life of the culture. He was unable to demonstrate the presence of dopa oxidase in the nonpigmented or lightly pigmented cells, whereas we found the dopa reaction to be positive in the five cultures tested. It was of interest to us that the cells retained their granules although the granules were not pigmented.

The spontaneous establishment of seven cell lines was unexpected. The criterion of cell transformation was primarily the development of chromosome heteroploidy. In all cases there was also a morphologic change, the cells of the lines being smaller, more cohesive, and more rapidly growing and dividing than the cells of the primary culture. There seemed to be no relation between the number of subcultures and the development of a cell line, one of them occurring at the first transfer and another at the seventh subculture.

Both primary cultures and cell lines can be stored for two years or longer at -100° C. and still yield viable cultures. Storage at -21° C. will preserve cells up to 45 days. Such cultures may form valuable substrates for microbiologic and pharmaceutical research.

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REFERENCES
6. Pomerat, C. M.: Personal communication.