A New Method of Culturing and Transferring Iris Pigment Epithelium


Purpose. To optimize a culture technique and transfer iris pigment epithelial (IPE) cells for cellular studies in vitro.

Methods. Porcine iris tissues were obtained, and IPE cells were isolated and cultured at high densities by plating them in the form of drops. Spherically shaped structures containing a high concentration of cells were formed after 7 to 10 days of culture. Cells were subcultured by transferring spheres to new culture dishes without employing enzymatic dissociation. The purity of IPE cells was determined by pigmentation and cytokeratin labeling. Proliferation was assessed by incorporation of 5-bromo-2'-deoxyuridine. Cellular structure was analyzed under the light and electron microscopes and function was assayed by rod outer segment phagocytosis.

Results. Iris pigment epithelial cells, when cultured at high densities, tended to form elevated spherical structures containing viable cells. The cultured cells were pigmented and showed positive labeling with a monoclonal cytokeratin antibody. The IPE cells proliferated and migrated from the spheres to form monolayers. Cells originating from the transferred spheres also continued to proliferate and to migrate in a similar manner to the originally cultivated cells to form monolayers after 7 to 10 days. These cells were able to phagocytose rod outer segments.

Conclusions. This new method provides a simple method of culturing a large quantity of IPE cells. The high yield of pure IPE cells and the ease of transfer provide an ideal means to study them at the cellular level. Invest Ophthalmol Vis Sci. 1997;38:2255-2260.

The iris pigment epithelium (IPE) covering the posterior layer of the iris tissue possesses the same embryonic origin as the retinal pigment epithelium, both of which are derived from the neuroectoderm.1, 2 The two cell types are classified as stable, maintaining their proliferative origin. Although retinal pigment epithelial cells have been extensively studied, relatively little is known about IPE cells. The isolation and cultivation of IPE cells have been previously documented.3-6 However, it is not known whether IPE cells can form three-dimensional culture arrangements called spheroids. The advantages of the spheroid model include a highly proliferative activity and resistance to dedifferentiation through a long period.7 Furthermore, spheroids can be easily transferred from one culture dish to another and used as a source of cells to migrate and form monolayers.

In this study, we have developed a new technique to cultivate IPE cells in a three-dimensional arrangement. The structure of the cultures was examined, using a scanning and transmission electron microscope. The purity of IPE cells isolated from these cultures was tested with anticytokeratin antibody. Their proliferation was assessed using 5-bromo-2'-deoxyuridine (BrdU) labeling, and their functional capability was examined by their phagocytosis of rod outer segments (ROS). This method of culturing provides a simple and reproducible means by which large quantities of pure IPE cells can be cultivated.

MATERIALS AND METHODS

Twenty-two porcine eyes were obtained from a slaughterhouse. The eyes were enucleated immediately after

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the death of the animals, stored on ice, and processed within 2 hours after death.

Isolation and Culture

Dissection and processing of tissue was performed under aseptic conditions in a laminar-flow hood. The eyes were opened with a scalpel just anterior to the ora serrata, 1.5 mm posterior to the limbus. A 360° excision of the iris from its root, along with the anterior segment, was performed. The lens and its anterior capsule were separated from the posterior surface of the iris. The iris was then removed from the remainder of the anterior segment with forceps and washed with phosphate-buffered saline (PBS, pH 7.4). The isolated iris tissues were placed with their posterior surface facing upward in a Petri dish containing trypsin-ethylendiaminetetraacetic acid (EDTA) solution (2.5% trypsin and 0.2% EDTA; Sigma Chemical, St. Louis, MO) for 20 minutes at 37°C, after which each iris tissue was placed in a Petri dish containing growth medium. The growth medium consisted of Ham's F12 Nutrient Mixture (Gibco; Grand Island, NY), 15% fetal bovine serum (Gibco) and 1% glutamine, supplemented with an 1% antibiotic–antimycotic (gentamicin and amphotericin; Sigma) mixture. The periphery of the iris tissue was held with forceps under a dissecting microscope, and the IPE cells were gently brushed and separated from the stroma, using the fire-polished tip (L shaped) of a Pasteur pipette. The isolated cells were transferred to a conical centrifuge tube containing growth medium. Each tube contained isolated cells from two eyes. The number of cells in each tube was counted in a hemocytometer and the average calculated. The cell suspension was centrifuged for 5 minutes at 1900 rpm. The supernatant was discarded, and 0.75 ml of growth medium was added to each tube. Using a pipette, the cells were plated in the form of drops, with each drop approximately 1 mm in diameter, into laminin-coated Petri dishes. Two to three drops of cell suspension were plated in each dish. They were incubated in a humidified CO₂ incubator with a mixture of 95% air and 5% CO₂ for 24 hours. Growth medium (1.25 ml) was then gently added to the dishes, and the cells were further incubated for 7 to 10 days. From each pair of eyes, 10 to 12 spheres were obtained. The spheres were subcultured by aspirating them from the dishes and transferring them to an eight-chamber slide (Nunc, Naperville, IL) using a Pasteur pipette. Trypsinization was not employed to dissociate the cells from the culture dishes. They were further incubated in the humidified CO₂ incubator. The cultures were examined daily, and micrographs were taken with a Zeiss (Carl Zeiss, Oberkochen, Germany) phase-contrast microscope.

Morphologic Analysis

For examination by transmission electron microscope, eight cultures were fixed for 60 minutes in a mi of 2% glutaraldehyde and 2% paraformaldehyde (Electron Microscopy Sciences [EMS], Fort Washington, PA) buffered with PBS (pH 7.4). The slides were washed with PBS and postfixed in 1% osmium tetroxide (aqueous) for 60 minutes. Samples were subsequently washed with deionized water and stained en bloc with 3% uranyl acetate (EMS). Cultures were dehydrated in a series of increasing graded ethanol. The dehydrated cultures were treated and embedded in Epon to be sectioned, stained, and examined under a transmission electron microscope (100 CX-II; JEOL, Tokyo, Japan).

For study by scanning electron microscope, cultures were fixed for 60 minutes in 2% glutaraldehyde and 2% paraformaldehyde (pH 7.4). They were washed with PBS and postfixed with 1% osmium tetroxide (EMS) for 90 minutes. Cells were washed with deionized water and left in 0.5% uranyl acetate (EMS) overnight. The cultures were dehydrated in a series of increasingly graded ethanol. Dehydrated cultures were incubated with hexamethyldisilazane (Si three times, for 10 minutes each, at room temperature. Next, most of the hexamethyldisilazane was moved, and the remaining volume was allowed to evaporate in a fume hood. Finally, specimens were coated with gold and visualized with a scanning electron microscope (JEOL 840 A).

Purity Assay: Cytokeratin Labeling

For cytokeratin labeling, a sample of isolated from each pair of eyes was plated into chamber slides. Before labeling, the medium was removed, and cells were washed with PBS. They were fixed in molar for 10 minutes at —20°C and transferred to a 1°C environment for 1 minute at —20°C. Fresh cultures were fixed three times with PBS for 5 minutes each. Fixed cells were treated with 0.5% Triton X (Sigma) for 10 minutes at room temperature. Three washes with PBS, they were incubated for minutes at room temperature with a monoclonal mouse anticytokeratin antibody (Sigma) at a 1:10 dilution. After incubation, the cells were rinsed three times with PBS and incubated with rhodamine mouse antibody (Sigma) at a 1:20 dilution for 30 minutes at room temperature, washed with PBS, and examined using a Zeiss fluorescence microscope with rhodamine selective filter set. Photomicrographs taken (Ektachrome 320 T; Eastman Kodak, Rochester NY). Cultured retinal pigment epithelial cells served as the positive control.
Culturing Iris Pigment Epithelial Cells

Proliferation Assay: 5-Bromo-2'-Deoxyuridine (BrdU) Labeling

For BrdU labeling, eight cultures were incubated with BrdU labeling agent (Boehringer-Mannheim, Indianapolis, IN) at 37°C and 5% CO₂–95% O₂. After incubation, the cultures were washed three times with washing buffer (Boehringer-Mannheim) and were fixed in 70% ethanol at -20°C for 30 minutes. Cultures were washed again three times with washing buffer and were incubated with mouse anti-BrdU antibody at 37°C for 30 minutes (Boehringer-Mannheim). They were incubated with antimouse polyclonal–fluorescein isothiocyanate solution (Boehringer-Mannheim) for 30 minutes at 37°C. After incubation with the secondary antibody, the cells were washed with washing buffer several times and were observed with a Zeiss microscope, using a fluorescein isothiocyanate selective filter set.

Functional Analysis: Phagocytosis

The phagocytosis of ROS was used as an assay for the functional viability of cultivated IPE cells. The retinas of pig eyes were used as the source of ROS. After removal of the anterior segment (similar to that described earlier), the vireous and the retina were dissected. The rod outer segments were isolated from the excised retina, as described previously. One of the ROS pellets was fixed and processed for identification of isolated ROS with electron microscope. The cultivated IPE cells from six slide chambers were incubated with ROS (10⁷/ml in growth medium) for 4 hours. The supernatant was removed, and the cultures were processed for electron microscopy, as described earlier.

RESULTS

On the basis of results of our initial studies, we have observed that IPE cells could be easily separated from the iris tissues as a monolayer without pretreatment with trypsin. However, the removed layers attached poorly to the culture plates, and the yield of IPE cells was very low. Similar observations have also been reported by Hu et al. Therefore, we trypsinized the iris...
FIGURE 2. The light microscopic structure of isolated iris pigment epithelial cells. (A) All the isolated cells were pigmented as viewed with the phase-contrast filter (magnification, \( \times 20 \)). (B) The pigmented cells were also stained with cytokeratin antibody, indicating their epithelial origin (magnification, \( \times 40 \)). The proliferation was shown (C) by incorporation of 5-bromo-2′-deoxyuridine into their nucleus, using fluorescence filter (magnification, \( \times 20 \)). Note the fluorescence of the nuclei.

Morphologic Studies
Detailed morphologic studies of the cultures were performed with the light microscope and with transmission and scanning electron microscopes. In Figure 1A, the three-dimensional structure of an IPE sphere, in the initial phase, is shown in a micrograph from a scanning electron microscope. After the formation of the sphere, the IPE cells proliferated and migrated from the sphere to form a monolayer, covering the plate (Fig. 1B). The cells located at the basal part attached the sphere to the plate and prevented it from dislodging into the medium. In Figure 1C, the structure of IPE cells inside the sphere is shown. The cells were heavily pigmented, round shaped, and tightly clustered. The IPE cells located inside the sphere built a large number of cell contacts, especially desmosomes, with their neighboring cells (Fig. 1D). The more peripherally migrating cells (Fig. 1E) were more elongated and less pigmented than the primary cells inside the sphere. Distant from the sphere, the IPE cells formed an intact monolayer possessing apical microvilli, intact nuclei, and dense endoplasmic reticulum (Figs. 1F, 1G).

The nonenzymatically transferred spheres attached to the new culture plate for 48 to 96 hours. The cells migrated in a centripetal configuration around the original sphere and covered the culture plate (similar to those in Fig. 1B). These migrating cells exhibited a similar intracellular structure that of the primary IPE cells.

Purity and Proliferation
One important characteristic of IPE cells is their pigmentation. Isolated and subsequently cultured cells were all pigmented as viewed with the phase contrast microscope (Fig. 2A). To differentiate IPE cells from melanocytes, the cultures were stained with anticytokeratin antibodies. Melanocytes do not possess the cytokeratin antigen and therefore would not be expected to label with the antibody. The pigmented cultured cells were also stained with the anticytokeratin antibody when viewed with a rhodamine selective filter (Fig. 2B). The pigmentation of the cultured cells and their cytokeratin expression suggest that the isolated cells were pure IPE cells, lacking any contamination with fibroblasts or melanocytes. No fluorescence was detected in the negative controls.

Before plating, the cells were suspended in a minimal amount of medium and then were plated in drop form. These cells formed an elevated sphere that was darkly colored because of the high concentration of pigmented IPE cells. They proliferated and migrated from the sphere in a tight formation to form a monolayer. The proliferation of the IPE cells was demonstrated by the incorporation of BrdU into their nuclei (Fig. 2C).

Phagocytosis
We have previously reported that IPE cells possess phagocytic activity for ROS, which is 76% of the retinal pigment epithelial cells. Therefore, to assay the functional activity of the IPE cells grown from the spheres, they were incubated with isolated ROS. The ROS were initially attached to the surface of the cells, and after the interaction with ROS, the IPE cells were capable of phagocytizing them. Intracellularly ingested ROS could be observed within 4 hours after their exposure to ROS (Figs. 3A, 3B).

DISCUSSION
This new method of culturing and transferring IPE cells is simple, reproducible, and may be employed for...
obtaining large quantities of these cells in monolayer formation.

In the iris, the stroma and IPE cells are separated by a basement membrane, allowing the isolation of pure IPE cells from the stroma and avoiding any contamination with stromal cells. Although the IPE cells could be directly dissected from the iris, our experience showed that a 20-minute immersion of the iris tissue in trypsin before microdissection would provide us with a superior yield and much easier separation. As suggested by Hu et al, trypsinization decreased the mechanical injury induced by direct dissection. A further advantage of using trypsin in our study was its apparent enhancement of the attachment and induction of proliferation of the IPE cells. Interestingly, these results were similar to those reported by other investigators when culturing adult human retinal pigment epithelium patches.

Most of the previous methods in the culturing of epithelial cells employed the dilution and resuspension of the cells in growth medium after centrifugation of the isolated cells. Cells were plated by dispensing them directly onto the dish and spreading the suspension over the entire floor of the dish. In our study, after the trypsin-assisted microdissection and centrifugation, we simply resuspended the IPE cells at high densities in a minimal amount of growth medium, which induced the formation of spheres. Factors important for the formation of the spheres include the collision of the cells and the biologic properties of the interacting cell surfaces. These parameters determine the probability that the cells will remain attached after their collision, form aggregates. The plating of IPE cells in high densities would increase the probability of their collisions. In addition, trypsinization presumably favored the properties required for interaction of cell surfaces (the reformation of junctional complexes, for example). The IPE cells in the iris are tightly attached to each other by junctional complexes and to the basement membrane to form a cellular layer. After their trypsinization and dissociation, the cells may seek to return to their original spatial configuration. In the suspension form, because of the lack of basement membrane and the high concentration of cells, the junctional complexes of the IPE cells have probably reattached them to each other to form the aggregates.

Different culture techniques have been described to demonstrate the formation of spheroids: stirred suspension cultures or liquid overlay cultures. These models were originally developed for the study of tumor cells. These three-dimensional structures represent aggregates of cells derived from normal tissues or tumors that were capable of proliferation. However, because of the lack of proliferation in many normal cell lines, many cells would not meet the criteria to be called spheroids. One of the most important features for the formation of the spheroids by IPE cells was their subsequent proliferation and migration onto the culture plate. Direct cell-to-cell contact may provide the regulatory signals for cells to induce such proliferation or differentiation. For example, Schwann cells obtain a mitogenic signal from their contact with neurite cells. The proliferation of the cells may also be controlled by the shape of the cell. The shape of the cell, through modulation of the cytoskeleton, plays an important role in the control of the growth. In monolayers, cells are restricted to a two-dimensional space, which largely limits intercellular interaction. However, in aggregate cultures, no particular shape is imposed on the cell. Some cells spread extensively on the external surface of the aggregates, whereas others maintain a spheroidal shape. It has been postulated that the spherical shape of the aggregates is the result of the intrinsic adhesive properties of the interacting cells and the nature of the extracellular matrix produced by the cells.

In our study, unlike in traditional methods, the IPE spheres could be easily transferred to another dish without trypsinization. They were aspirated with a mi-
cropipette and transferred from one plate to another. The transferred spheres attached to the plates, and the IPE cells proliferated and migrated from them to form monolayers covering the plates. The transfer did not appear to affect the functional viability of the cells as they reattached to the culture plate, proliferated, and phagocytosed ROS.

In summary, we have described a simple method of culturing and transferring IPE cells. This method produces IPE cells as spheroids that could be used as a source for generating high concentrations of IPE cells in the form of monolayers. Additionally, the formation of spheroids may have additional applications for cellular-based studies.

**Key Words**

iris pigment epithelial cells, phagocytosis, proliferation, spheroids, transfer

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**References**