Human Retinal Pigment Epithelium Cells as Functional Models for the RPE In Vivo

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PURPOSE. The two most commonly used in vitro models of the retinal pigment epithelium (RPE) are fetal human RPE (fhRPE) and ARPE-19 cells; however, studies of their barrier properties have produced contradictory results. To compare their utility as RPE models, their morphologic and functional characteristics were analyzed.

METHODS. Monolayers of both cell types were grown on permeable membrane filters. Barrier function and cellular morphology were assessed by transepithelial resistance (TER) measurements and immunohistochemistry. Protein expression was evaluated by immunoblotting and ELISA assays, and retinoid metabolism characterized by HPLC.

RESULTS. Both cultures developed tight junctions. However, only the fhRPE cells were pigmented, uniform in size and shape, expressed high levels of RPE markers, metabolized all-*trans* retinal, and developed high TER ($>400 \Omega\text{cm}^2$). The net secretion of pigment-epithelium-derived factor (PEDF) was directed apically in both cultures, but fhRPE cells exhibited secretion rates a thousand-fold greater than in ARPE-19 cells. The net secretion of vascular endothelial growth factor (VEGF) was significantly higher in fhRPE cultures and the direction of this secretion was basolateral; while net secretion was apical in ARPE-19 cells. In fresh media, VEGF-E reduced TER in both cultures; however, in conditioned media fhRPE cells did not respond to VEGF-E administration, but retreatment of the conditioned media with anti-PEDF antibodies allowed fhRPE cells to fully respond to VEGF-E.

CONCLUSIONS. Properties of fhRPE cells align with a functionally normal RPE in vivo, while ARPE-19 cells resemble a pathologic or aged RPE. These results suggest a utility for both cell types in understanding distinct, particular aspects of RPE function.

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The RPE plays a key role in maintaining normal vision due to its anatomic location between the photoreceptors and the choriocapillaries and the specific biochemical processes that support phototransduction. An essential component in facilitating this function is the tight junction complexes between the cells,¹ which constitute the outer blood-retina barrier. The tight junctions dynamically interact with numerous other proteins to regulate the paracellular permeability, the polar orientation of the membrane proteins, and protein expression.^{2,3} Disrupting this barrier not only disturbs normal fluid flow between the neural retina and the choriocapillaries, resulting in edemas,⁴ but also alters the metabolic circuits and function of the RPE.

VEGF is required for choroidal vasculogenesis,⁵ and it is the principle cytokine responsible for neovascularization in the mature eye.⁶ However, VEGF is also a potent modulator of barrier function in both the retinal endothelia and the RPE.^{7–9} Several clinical studies have shown that anti-VEGF therapies can treat ocular neovascularization and retinal edema.⁶ Although the RPE is a primary source of VEGF in the eye,¹⁰ and the receptors for VEGF are expressed by the RPE,^{11,12} there is a lack of adequate in vivo models that could distinguish between VEGF effects on the retinal vasculature and the RPE. Indeed, it is generally thought that retinal degeneration associated with diabetic retinopathy or wet forms of AMD are caused mostly by vascular tissue and RPE failure has received limited attention. Yet, it is well recognized that RPE forms a tight barrier between the retina and the highly vascular choroid. Part of the problem is that RPE function is mostly investigated in vitro by studying the human ARPE-19 cell line. ARPE-19 cells are simple to maintain, but they are limited by exhibiting only a low ($< 50 \Omega\text{cm}^2$) transepithelial resistance (TER).¹³ Primary human, porcine, and bovine cells have also been investigated,^{14–17} but only fetal human RPE (fhRPE) cells developed tight monolayers with TER above $200 \Omega\text{cm}^2$.^{18,19} The literature on VEGF modulating the permeability of different RPE cultures shows a confusing array of responses: VEGF can increase, decrease, or not affect paracellular permeability.^{14,20–22} Therefore, a specific role for VEGF in the regulation of RPE function has not been established, leaving a critical void in our understanding of how the RPE prevents edema and subsequent neuroretinal damage.

Pigment-epithelium-derived factor (PEDF) has been identified as an important antagonist that limits the mitogenic activity of VEGF and is secreted in large quantities by the native RPE. This secretion takes place predominantly from the apical side^{19,23}; however, a basal accumulation of PEDF in aged eyes has also been observed.²⁴ Similarly to VEGF, the role of PEDF in the RPE is also not well understood. We have recently demonstrated that apically administered VEGF induces a failure

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of RPE barrier function, which is blocked by apically provided PEDF.¹¹ In light of this discovery, we hypothesized that the large amount of PEDF naturally secreted apically from the RPE, maintains barrier integrity even in the presence of a significant quantity of VEGF. Therefore, the observed VEGF responses need to be interpreted in the context of endogenous PEDF secretion.

The goal of this article is to understand the function of the RPE cell layers, to determine the utility of ARPE-19 and fhrPE cells for the study of RPE function, and to address the highly contradictory results with respect to the action of VEGF in these cells. Our premise is that the reported differences in VEGF responsiveness are due to the intrinsic properties of the culture systems. Therefore, we have begun to systematically compare ARPE-19 and fhrPE monolayers in terms of structural parameters, secretory profiles, and VEGF-induced responses. Our conclusion is that while both models have their place in studying RPE function, fhrPE cells model the function of the normal RPE cell layer more closely.

MATERIALS AND METHODS

Cell Culture

ARPE-19 cells (American Type Culture Collections, Manassas, VA) were cultured as described previously.¹⁴ Briefly, ARPE-19 cells were maintained and passaged in standard culture dishes (Corning; Thermo Fisher Scientific, Fair Lawn, NJ) using DMEM/F12/HAM media (Sigma Chemical Co., St. Louis, MO) with 1% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 1.2 g/L sodium bicarbonate (Thermo Fisher Scientific), and 10 mL/L L-glutamine-penicillin-G-streptomycin (2 mM, 100 U/mL, 0.1 mg/mL, respectively; Sigma Chemical Co.) in a humidified incubator, kept at 37°C in 5% CO₂. The media was changed every 2 to 3 days.

To establish fhrPE cultures, fetal human eyes were acquired from Advanced Bioscience Resources (Alameda, CA). The human fetal tissue was used in accordance with the guidelines of the Medical University of South Carolina Institutional Review Board and the tenets of the Declaration of Helsinki. After arrival, the eyes were rinsed with low calcium culture medium developed by Hu and Bok,¹⁸ the periorcular tissue was trimmed, and the anterior segment, vitreous, and neural retina were removed. The eye cup containing the RPE was then incubated with the low-calcium culture medium for 30 minutes at 37°C, and sheets of RPE cells were removed and cultured for up to 4 weeks in the low-calcium medium in a humidified incubator at 37°C, in 5% CO₂ and 95% air in standard culture dishes. In low-calcium media, the RPE sheets dispersed and produced suspended cells, floaters, which were collected every 2 days and frozen for use in establishing monolayers for future studies.

RPE Monolayers

To establish cell monolayers for study, ARPE-19 cells were plated on permeable membrane inserts (3 × 10⁵ cells per insert; Costar Clear Transwell, 0.4 μm pore, 24 mm; Thermo Fisher Scientific) and fhrPE cells were plated either on millipore (7.5 × 10⁴ cells/insert; Millicell HF, 12 mm; Thermo Fisher Scientific) or corning transwell inserts (3 × 10⁵ cells/insert) interchangeably. The type of the used filter for fhrPE cells did not significantly affect experimental outcomes. Fetal human RPE cells were cultured in the normal (calcium containing) culture media developed by Hu and Bok.¹⁸ After the cells had attached, the media were replaced every 2 to 3 days. Barrier function was assessed by monitoring TER by means of an epithelial volt/ohm meter using an electrode (STX2; World Precision Instruments, Sarasota, FL). The resistance values for individual monolayers at specific times (Ωcm²) was determined from the average of four independent measurements, and corrected for background resistance produced by the blank filter and culture medium. A minimum of six independent monolayers were tested in each experimental condition. The results were expressed as

the mean ± standard error (SE) and compared with the unpaired Student *t* test. Significance was set at *P* ≤ 0.05.

Only confluent monolayer cultures with stable TER values greater than 40 Ωcm² for ARPE-19 cells and 400 Ωcm² for fhrPE cells were used. Confluent monolayers with stable TER were treated with 5 ng/mL VEGF-E (VEGF-R2 specific agonist viral VEGF analog; Fitzgerald, Concord, MA). In selected cultures, the cells were pretreated with 1 μg/mL PEDF antibody (Bioproducts MD, Middletown, MD) 60 minutes before VEGF-E. TER was measured 1 hour before VEGF-E treatment, and at 0.5, 1, 2, 3, and 5 hours post administration.

Enzyme-Linked Immunoassays

Aliquots of the medium from the upper and lower chamber wells were collected at regular intervals (1 μL at 0, 0.5, 1, 1.5, and 2 hours for PEDF in fhrPE cells; and 100 μL at 0, 6, 24, 30, and 48 hours for all other measurements). The VEGF and PEDF concentrations were assayed by means of the human VEGF ELISA kit (R&D Systems, Minneapolis, MN) and PEDF ELISA kit (Bioproducts MD), respectively. The volume changes during the experiment were corrected in the calculations for VEGF and PEDF concentrations. The rate of secretion was determined from the slope of the curve (linear) fitted to the experimental points.

Immunohistochemistry

Monolayers were washed in PBS, and fixed in 2% paraformaldehyde (Mallinckrodt Baker, Phillipsburg, NJ) in PBS at 4°C for 1 hour. Then the samples were washed three times in PBS at room temperature (RT) and permeabilized by exposure to 0.2% Triton X-100 (VWR, West Chester, PA) in PBS for 30 minutes at 4°C. Samples were washed three times in PBS (at RT), and incubated with primary antibody (mouse anti-ZO-1 or mouse anti-occludin, diluted 1:100; Chemicon, Temecula, CA) for 1 hour at 4°C (negative controls were incubated with PBS only). The slides were again washed three times in PBS, and incubated with secondary antibody (fluorescein-conjugated goat anti-mouse, diluted 1:50; Chemicon) for 30 minutes at RT. The slides were treated with nuclear stain and examined using a fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and software (Axiovision 4.7; Zeiss).

Immunoblots

Monolayers of ARPE-19 and fhrPE cultures were washed with ice cold PBS and lysed (4 × 10⁶ cells; 100 μL; pH 7.5; 2.42 g/L Tris Base, 1 mM EGTA, 2.5 mM EDTA, 5 mM dithiothreitol, 0.3 M sucrose, 1 mM Na₃VO₄, and 20 mM NaF—all from Sigma Chemical Co.; 1 complete mini protease inhibitor tablet from Roche Applied Science, Indianapolis, IN), scraped, and collected in a centrifuge tube. Then the samples were sonicated twice for 10 seconds each, centrifuged for 5 minutes at 10,000g, and the supernatant collected. Thirty micrograms of the samples (determined by protein assay; Bio-Rad, Hercules, CA) were separated on 4–12% Bis-Tris Gel, transferred to a blotting membrane (Hybond-ECL; GE Healthcare, Piscataway, NJ), blocked with 5% nonfat dry milk, and incubated at 1:1000 with anti-CRALBP (Abcam, Cambridge, MA), anti-RPE65 (PETLET,²⁵ a generous gift from Rosalie Crouch), or anti-β-actin (Sigma Chemical Co.) overnight at 4°C. After washing three times in TBST, the membranes were incubated at 1:10,000 with HRP-conjugated goat secondary antibody (anti-rabbit for RPE-65, anti-mouse for the rest; Sigma Chemical Co.) for 1 hour, washed again four times in TBST, and the lanes were visualized (VersaDoc 5000 imager; Bio-Rad) after treatment with chemiluminescent reagent (Thermo Fisher Scientific).

Retinoid Profiling

Monolayer cultures of ARPE-19 and fhrPE cells were treated with all-*trans* retinal (Sigma Chemical Co.) in phosphatidylcholine (PC; Avanti Polar Lipids Inc., Alabaster, AL) vesicles at a concentration of 5

FIGURE 1. RPE cell monolayer development and the establishment of barrier function. RPE paracellular permeability was assessed by trans-epithelial resistance measurements (TER) in ARPE-19 and fhRPE cells. In both types of cells, the TER began to increase after reaching confluence. ARPE-19 cells became confluent within 5 days after plating and reached a maximum resistance of $43 \pm 5 \Omega\text{cm}^2$ within 2 weeks ($n = 12$). fhRPE cells became confluent within 3 to 5 weeks after plating and reached a maximum resistance of $1046 \pm 43 \Omega\text{cm}^2$ within 6 to 8 weeks ($n = 10$). Values are mean $\pm$ SE of individual wells.

$\mu\text{mol/L}$ at one time only. The vesicles were prepared using a mini-extruder kit (Avanti Polar Lipids Inc.) and all-*trans* retinal from a 6 mM solution of ethanol was incorporated according to the instructions of the kit manufacturer. The total incorporation and final concentration of all-*trans* retinal was determined from absorption at 382 nm with a spectrophotometer (Cary 300, Agilent, Santa Clara, CA). PC vesicles without all-*trans* retinal administered to cells and all-*trans* retinal-loaded PC vesicles in cell-free tissue culture media were used as controls. At least three independent wells were used for each experimental condition. All-*trans* retinal and all-*trans* retinal loaded vesicles and samples were handled under dim red light.

Two days post all-*trans* retinal or control administration, the tissue culture media was collected and the cells were lysed by scraping in 200 μL of PBS and sonicated two times for 10 seconds each. Methanol (300 μL ; Sigma Chemical Co.) and hydroxylamine (60 μL , 1 mol/L in PBS; Sigma Chemical Co.) were added and samples were vortexed (30 seconds). After 5 minutes at 22°C, methylene chloride (300 μL ; Thermo Fisher Scientific) was added, and the samples were vortexed (30 seconds) and centrifuged (14,000g, 1 minute). The media samples (2 mL each) went through the same treatment, receiving proportionately larger volumes of reagents (3 mL methanol, 600 μL hydroxylamine, 3 mL methylene chloride). Thereafter, the lower phase was separated, dried under argon, and dissolved in HPLC mobile phase (11.2% ethyl acetate; Thermo Fisher Scientific; 2.0% dioxane; Sigma Chemical Co.; 1.4% octanol; Sigma Chemical Co.) in hexane (Sigma Chemical Co.; 90 mL). Separation of retinoids was performed (Lichrosphere SI-60, 5 mm column; Alltech, Lexington, KY) and quantified by comparison of retention times and absorption properties with pure retinoid isomeric standards as before.²⁶

RESULTS

Transepithelial Resistance and Morphology

Monolayer cultures of fhRPE and ARPE-19 cells were established on permeable membrane filters. Both cell types show increases in TER after confluence 5 to 7 days after plating ARPE-19 cells, and 3 to 5 weeks after plating fhRPE cells (Fig. 1). For the ARPE-19 cells, maximum resistance developed within 2 weeks and ranged from 35 to 55 Ωcm^2 with a mean of $43 \pm 5 \Omega\text{cm}^2$ ($n = 12$). In contrast, the resistance

plateaued between 6 and 8 weeks reaching a maximum of 600 and 1200 Ωcm^2 with a mean of $1046 \pm 43 \Omega\text{cm}^2$ ($n = 10$) in fhRPE cultures.

The morphologic features of the two cultures were also considerably different as presented in Figure 2. Cultured monolayers of ARPE-19 cells were roughly hexagonal in shape, developed limited pigmentation, and contained several multinuclear cells (Fig. 2A). Immunofluorescent staining for the tight junction protein, ZO-1, showed continuous junctions between cells (Fig. 2B); however, staining for occludin was diffuse through the cytosol (Fig. 2C).

Like ARPE-19 cells, subconfluent fhRPE cells were only lightly pigmented (data not shown); but on reaching confluence, cellular pigmentation began to increase, paralleling the rise in TER. By the time the cells reached maximal TER, all cells were highly pigmented (Fig. 2D). Monolayers of fhRPE cells formed a uniform hexagonal lattice that was similar to native RPE tissue in vivo. The presence of well-developed tight junctions was evidenced by intense membrane staining against both ZO-1 (Fig. 2E) and occludin (Fig. 2F).

Retinoid Metabolism

A principle biochemical function of the RPE in vivo is its participation in the retinoid visual cycle. To determine whether components of the visual cycle were expressed by

FIGURE 2. Epithelial morphology in established RPE cell monolayers. Epithelial morphology was assessed by phase contrast and immunofluorescence microscopy in ARPE-19 and fhRPE cells. Phase-contrast micrographs of (A) ARPE-19 and (D) fhRPE cells provide evidence of differences in morphology and pigmentation. Immunofluorescent micrographs were prepared with fluorescein-labeled secondary antibody (mouse): (B) ARPE-19 and (E) fhRPE cells with anti-ZO-1 primary antibody indicate that ARPE-19 cells have inferior structure and organization. Micrographs of (C) ARPE-19 cells and (F) fhRPE cells with anti-occludin primary antibody show that ARPE-19 cells have inferior occludin staining at the cell-cell contacts, indicating limited tight junctions (as at the same time ZO-1 staining is clearly present). The images were prepared using a 20 $\times$ objective lens; the scale bar represents 50 μm .

these culture systems, immunoblots of cellular retinal aldehyde binding protein (CRALBP) and RPE-65 as well as enzymatic conversion of all-*trans* retinal to 11-*cis* retinal were evaluated. Western blot analysis show that ARPE-19 cells expressed considerably lower levels of CRALBP than fhrPE cells (Fig. 3A, bottom left panel) and unlike the latter, express almost no RPE65 (Fig. 3A, bottom right panel).

As seen in Figure 3B, the HPLC profile of ARPE-19 cells treated with all-*trans* retinal is dominated by unconverted all-*trans* retinal. In addition, a detectable level of all-*trans* retinol was also recovered from both the cells and the media

(data not shown for media). Like ARPE-19, fhrPE cultures converted most of the added all-*trans* retinal into all-*trans* retinol, but then they further esterified it yielding a large retinyl-ester peak and isomerized it resulting in detectable amounts of 11-*cis* retinal (Fig. 3C). In cell free systems no conversion of all-*trans* retinal was measured (data not shown).

VEGF and PEDF Secretion

Barrier function is thought to be maintained through a balance of VEGF and anti-VEGF agents. Therefore, when comparing the barrier function of ARPE-19 and fhrPE monolayers, it is essential to understand the endogenous apical and basal secretion patterns of VEGF and the principal anti-VEGF cytokine, PEDF. These results are summarized in Figure 4 and Table 1.

The apical and basal VEGF secretion rate from ARPE-19 cultures was 15 ± 1 and 6.7 ± 0.5 pg/mL $\times$ hours, respectively. Roughly 2- and 7-fold (22 ± 1 apical and 41 ± 2 pg/mL $\times$ hours basolateral) higher rates of VEGF secretion was observed in the fhrPE monolayers (Figs. 4A, 4B). Thus, ARPE-19 monolayers secreted VEGF at significantly lower rates ($P < 0.0001$) than fhrPE monolayers. In addition, most VEGF was directed apically in ARPE-19 monolayers, but was directed toward the basal surface in fhrPE cells.

Similar evaluation revealed that unlike VEGF, the polarity of PEDF secretion was biased toward the apical side in both ARPE-19 and fhrPE monolayers (Figs. 4C, 4D). ARPE-19 cells secreted PEDF at rates of 6.8 ± 0.5 pg/mL $\times$ hours apically and 2.8 ± 0.3 pg/mL $\times$ hours basally. Strikingly, however, fhrPE monolayers secreted PEDF at a 4000- to 7000-higher rate than ARPE-19 cells (30 ± 2 ng/mL $\times$ hours apical and 20 ± 1 ng/mL $\times$ hours basal).

VEGF-Induced Breakdown of Barrier Function

In ARPE-19 monolayers, the administration of VEGF-E (a viral VEGF homolog, relatively specific for the VEGF-R2 receptor) produced a significant drop in TER within 1 hour. The maximum reduction (approximately 45%) was observed at 5 hours post VEGF administration. The time courses and magnitudes of the VEGF responses in ARPE-19 cultures incubated in unconditioned or conditioned media were not significantly different (Fig. 5A).

Although the TER in fhrPE cultures was 10- to 20-fold higher, the addition of VEGF-E induced a percent decrease in TER that paralleled ARPE-19 cells with a maximum decrease in resistance of 30% measured at 5 hours (Fig. 5B). However, in fhrPE cultures the response to VEGF-E was only observed when monolayers were incubated in fresh unconditioned media. To determine whether the apical secretion of endogenous PEDF was responsible for suppressing the VEGF-E in fhrPE cultures in the conditioned media, the anti-PEDF antibody (1 μ g/mL) was administered apically to fhrPE cells in conditioned media 1 hour before VEGF-E administration. As shown in Figure 5B, pretreatment with anti-PEDF antibody exposed a VEGF-E response similar to that measured in unconditioned media.

DISCUSSION

To assess the utility of commonly used in vitro models for the study of RPE barrier function and to clarify the role of VEGF in the RPE, we compared baseline parameters and VEGF responses in two culture systems: the ARPE-19 cell line and primary fhrPE cells. The human ARPE-19 cell line is commercially available and has been widely used to evaluate RPE function. Fetal human RPE cells require establishing primary cultures from fetal eyes and using precise culture conditions to promote differentiation and monolayer formation. It is a gen-

FIGURE 3. Retinoid metabolism in monolayer RPE cultures. Expression of RPE visual cycle proteins (A) showing immunoblots against RPE65 and CRALBP in both fhrPE and ARPE-19 cells. While fhrPE cells abundantly express both proteins, ARPE-19 cells express only low quantities of CRALBP. β -Actin indicated approximately equal loading of the lanes. Functional retinoid metabolism was missing from ARPE-19 cells (B); but was present in fhrPE cells (C) as shown by the corresponding retinoid profiles taken by monitoring the HPLC at 360 nm 2 days post administration of 5 μ mol/L all-*trans* retinal (atRal) in phosphatidylcholine (PC) vesicles. The profile for ARPE-19 cells (B), was dominated by unconverted all-*trans* retinal, while the profile for fhrPE cells (C), was dominated by retinyl-esters and 11-*cis* retinal (11cRal), indicating functional retinoid metabolism. The data are representative of three independent experiments. atRol, all-*trans* retinol.

FIGURE 4. Secretion of VEGF and PEDF from established RPE cell monolayers. Time-dependent changes in the concentration of VEGF (A, B) and PEDF (C, D) were measured by ELISA from media collected from the apical (●) and basolateral (○) sides of ARPE-19 (A, C) and fhRPE (B, D) cultures at the indicated time points of 0, 6, 24, 30, and 48 hours in all experiments except fhRPE PEDF, where it was 0, 0.5, 1, 1.5, and 2 hours. The secretion rates were determined from the slopes of curves (linear) fitted to the experimental data. *Solid lines* indicate apical and *dashed lines* basolateral secretions. Values are the mean $\pm$ SE of individual wells ($n = 4$).

eral observation that the ARPE-19 media does not support the growth and development of the fhRPE monolayers (it lacks essential hormones, enzymes, and retina extract). Although the fhRPE media allowed the growth of ARPE-19 monolayers and the development of TER similar to the native ARPE-19 media, it limited the useful lifetime of the cultures with a stable TER from 21 days to 7 days (data not shown). Therefore, to achieve optimal culture performance we have used the media for the two cell types that are preferentially used in the literature. However, it is not expected that the composition of the media alone could explain the observed differences between the two cell types, as neither the use of fhRPE media nor exogenous PEDF induced differentiation in ARPE-19 cells similar to the fhRPE cells (data not shown). As fhRPE cells are not subcultured (they tend to lose their phenotype in the process), it appears that during the numerous generations of subculturing

the ARPE-19 cells lost their ability to properly differentiate (ARPE-19 cells are 30th generation when acquired from the vendor.)

Both culture models develop into monolayers with tight junctions, as they form continuous cell-cell contacts evidenced by ZO-1 immunostaining. However, the fhRPE monolayers exhibited extensive pigmentation, uniform hexagonal shape, and a mononuclear appearance reminiscent of the RPE in vivo; while the ARPE-19 cells expressed little or no pigmentation, variable cell shape, and some multinuclear appearances. Trans-epithelial resistance measurements demonstrated that fhRPE cells formed a tight monolayer with resistances above 600 Ωcm^2 , which was associated with an intense, circumferential staining of occludin. In contrast, the average TER of ARPE-19 monolayers was only $43 \pm 5 \Omega\text{cm}^2$ with a limited development of tight-junctional complexes (Figs. 1, 2).

TABLE 1. Levels of VEGF and PEDF in Media from Established RPE Cell Monolayer Cultures

Cell Monolayers	Apical VEGF	Basal VEGF	Apical PEDF	Basal PEDF
ARPE-19	15 $\pm$ 1 pg/mL $\times$ h	6.7 $\pm$ 0.5 pg/mL $\times$ h	6.8 $\pm$ 0.6 pg/mL $\times$ h	2.8 $\pm$ 0.3 pg/mL $\times$ h
fhRPE	22 $\pm$ 1 pg/mL $\times$ h	41 $\pm$ 2 pg/mL $\times$ h	30 $\pm$ 2 ng/mL $\times$ h	20 $\pm$ 1 ng/mL $\times$ h

Bold text indicates three orders of magnitude higher value than in the rest of the table.

FIGURE 5. VEGF-induced barrier breakdown in RPE cell monolayers. VEGF-E (a selective VEGF-R2 agonist) was administered apically to ARPE-19 (A) and fhRPE (B) cells at a concentration of 5 ng/mL in cultures that had been maintained in either fresh, unconditioned media (●), or in conditioned media (○) for 24 hours. In the fhRPE cells, a third experiment was also performed in conditioned media, where the cells were pretreated with anti-PEDF 1 hour before the treatment with VEGF-E (□). TER was measured starting 1 hour before VEGF-E administration, to 5 hours post VEGF-E administration. To show the trend of the change, the *closed circles* were connected with *solid lines*, the *open circles* were connected with *dashed lines*, and the *open squares* were connected with a *dotted line*. Values are the mean $\pm$ SE of individual wells ($n = 6$) normalized to the TER at $t = -60$ min.

Both fhRPE and ARPE-19 cells expressed the RPE marker visual cycle protein, CRALBP, but the expression in ARPE-19 cells was greatly reduced. The other important RPE marker protein (RPE65) was, however, only expressed in fhRPE cells, indicating that these cells could retain native RPE function as well. This conclusion is consistent with other studies, which have shown that fhRPE cultures retain expression of normal RPE markers *in vivo*, such as Best1, RPE65, and CRALBP expression,²⁷ which is limited or absent in ARPE-19 cells.¹³ The functional response of these proteins was tested with retinoid profiling of the two cell types after short-term all-*trans* retinal administration. Both cell types were able to internalize all-*trans* retinal and also to convert it into all-*trans* retinol (testifying to functional retinal dehydrogenase activity), but only fhRPE cells had acyl-transferase, -hydrolase, and 11-*cis* isomerase activities. The retinoid profile of the fhRPE cells was somewhat reminiscent of those of RPE65-deficient animals, as a lot of retinyl-ester accumulated. However, the accumulation of the ester in our case is attributed not to the lack of RPE65, but to the lack of carriers and consumption of 11-*cis* retinal. Thus, in contrast to ARPE-19 cells, fhRPE cells have RPE-specific metabolic functions as well (Fig. 3).

Evaluation of VEGF and PEDF secretion from ARPE-19 and fhRPE monolayers revealed three major differences: overall VEGF secretion in fhRPE monolayers was significantly higher ($P < 0.0001$) than from ARPE-19 monolayers; the differential secretion of VEGF was basal in fhRPE, but apical in ARPE-19 monolayers; and, the secretion rate of PEDF in fhRPE monolayers was dramatically (4000- to 7000-fold) higher ($P < 0.0001$) than from ARPE-19 monolayers (Fig. 4). *In vivo*, VEGF secretion from the RPE is predominantly basolateral, and likely fosters the development of the choriocapillaris.^{5,17} On the other hand, it is known that RPE cells secrete a large amount of PEDF apically *in vivo*.^{19,23} The high level of apical PEDF secretion from the fhRPE cells is consistent with the amount of PEDF in the vitreous humor *in vivo*.²⁸ Our results suggest that, for PEDF and VEGF, the secretory patterns of the fhRPE monolayers *in vitro* reflects the RPE patterns *in vivo* more accurately than the patterns of ARPE-19 cells. This pattern of PEDF secretion in the RPE could be functionally very important to stimulate vascular health in the choriocapillaries, while maintaining an anti-angiogenic environment in the outer retina (especially in the macular area) and preserving the integrity of the RPE barrier.

Pharmacologically, a primary distinction between monolayers derived from fhRPE cells and other RPE culture systems was that fhRPE cell monolayers were found to be refractory to VEGF-induced changes in permeability.²⁰ As our results, along with those of others, have provided evidence that fhRPE monolayers are a more appropriate model for studying RPE function, one can question if VEGF plays any physiological or pathophysiological role in the RPE *in vivo*. We have hypothesized that VEGF and PEDF play counter-balancing roles in the regulation of RPE function.¹¹ The high expression of PEDF in fhRPE cells and the data in Figure 5 are consistent with this hypothesis. VEGF was unable to disrupt the TER in the presence of conditioned media containing the equivalent of approximately 660 ng/mL PEDF. We further confirmed that PEDF secreted by fhRPE cells inhibits VEGF-induced barrier breakdown by blocking PEDF with an anti-PEDF antibody. VEGF was able to disrupt the TER in the presence of the anti-PEDF antibody, indicating that PEDF was the primary (if not the only) VEGF regulator secreted by the fhRPE cultures. In summary, monolayers derived from both ARPE-19 and fhRPE cells can undergo a rapid breakdown of barrier function in response to VEGF, but this response is kept in check by PEDF. These results seem to be consistent with previous observations in the eye *in vivo*. The predominantly basal secretion of PEDF in aged eyes²⁴ may indicate an impairment of PEDF secretion toward the apical side, thus limiting its protective anti-VEGF abilities with aging. Furthermore, the clinical relevance of this pathway is highlighted by the substantial reduction in PEDF levels in diabetic retinopathy where retinal edema is prevalent.²⁸

The results of this study point to the significant limitations of ARPE-19 cells as a model of RPE function *in vivo*. ARPE-19 cells have a reduced capacity to express critical proteins that regulate and maintain a barrier with strong tight junctions. These results together with the low expression of several RPE markers and the inability to metabolize vitamin A, provide accumulating evidence that ARPE-19 cells are unable to fully differentiate into RPE-like layers. Nevertheless, ARPE-19 cells remain valuable as the above changes appear to be quantitative rather than qualitative in terms of barrier function. Moreover, their hypersensitivity to VEGF action, loss of pigmentation, and weaker tight junctions, are all properties which somewhat resemble the aged eye or pathologic conditions. However, for ultimate confirmation of agents that may alter VEGF signaling, it is necessary to check the results obtained in ARPE-19 cells against similar experiments in fhRPE monolayers or *in vivo* models.

In conclusion, our experiments reinforced previous observations that substantial differences exist between ARPE-19 and fhRPE cells and provided new evidence that monolayers derived from fhRPE cells provide a superior model of native human RPE function. Although fhRPE cells may appear at first to be insensitive to VEGF treatment, our studies indicate that the cells are not inherently deficient in their capacity to respond to VEGF. Instead, the ultimate reason is that PEDF secretion from this cell line is high, resembling the normal eye; whereas in fact, ARPE-19 cells are deficient in this anti-VEGF molecule and more likely resemble an aged or pathologic eye. Thus, fhRPE cells can serve as a particularly excellent model to study agents that disrupt normal RPE function and PEDF action; while ARPE-19 cells can be used to study how certain pathologic changes can be reversed by administering exogenous agents. The study of ARPE-19 cell barrier properties was instrumental in identifying conditions in which the loss of RPE barrier function can be studied in fhRPE cells (or in vivo) by eliminating PEDF. Finally, these studies provide strong support for the hypothesis that the RPE serves as an active protective barrier for the neural retina.

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References

- Vinore SA, Derevanjanik NL, Ozaki H, Okamoto N, Campochiaro PA. Cellular mechanisms of blood-retinal barrier dysfunction in macular edema. *Doc Ophthalmol*. 1999;97:217-228.
- Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev*. 2004;84:869-901.
- Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol*. 2004;286:C1213-C1228.
- Marmor MF. Mechanisms of fluid accumulation in retinal edema. *Doc Ophthalmol*. 1999;97:239-249.
- Marneros AG, Fan J, Yokoyama Y, et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. *Am J Pathol*. 2005;167:1451-1459.
- Ciulla TA, Rosenfeld PJ. Anti-vascular endothelial growth factor therapy for neovascular ocular diseases other than age-related macular degeneration. *Curr Opin Ophthalmol*. 2009;20:166-174.
- Murata T, Ishibashi T, Khalil A, Hata Y, Yoshikawa H, Inomata H. Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthalmic Res*. 1995;27:48-52.
- Lara-Castillo N, Zandi S, Nakao S, et al. Atrial natriuretic peptide reduces vascular leakage and choroidal neovascularization. *Am J Pathol*. 2009;175:2343-2350.
- Antcliff RJ, Marshall J. The pathogenesis of edema in diabetic maculopathy. *Semin Ophthalmol*. 1999;14:223-232.
- Kvanta A. Expression and regulation of vascular endothelial growth factor in choroidal fibroblasts. *Curr Eye Res*. 1995;14:1015-1020.
- Ablonczy Z, Prakasam A, Fant J, Fauq A, Crosson C, Sambamurti K. Pigment epithelium-derived factor maintains retinal pigment epithelium function by inhibiting vascular endothelial growth factor-R2 signaling through gamma-secretase. *J Biol Chem*. 2009;284:30177-30186.
- Ohno-Matsui K, Yoshida T, Uetama T, Mochizuki M, Morita I. Vascular endothelial growth factor upregulates pigment epithelium-derived factor expression via VEGFR-1 in human retinal pigment epithelial cells. *Biochem Biophys Res Commun*. 2003;303:962-967.
- Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res*. 1996;62:155-169.
- Ablonczy Z, Crosson CE. VEGF modulation of retinal pigment epithelium resistance. *Exp Eye Res*. 2007;85:762-771.
- Hartnett ME, Lappas A, Darland D, McCollm JR, Lovejoy S, D'Amore PA. Retinal pigment epithelium and endothelial cell interaction causes retinal pigment epithelial barrier dysfunction via a soluble VEGF-dependent mechanism. *Exp Eye Res*. 2003;77:593-599.
- Jin M, Barron E, He S, Ryan SJ, Hinton DR. Regulation of RPE intercellular junction integrity and function by hepatocyte growth factor. *Invest Ophthalmol Vis Sci*. 2002;43:2782-2790.
- Blaauwgeers HG, Holtkamp GM, Rutten H, et al. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris. Evidence for a trophic paracrine relation. *Am J Pathol*. 1999;155:421-428.
- Hu J, Bok D. A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. *Mol Vis*. 2001;7:14-19.
- Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. *Invest Ophthalmol Vis Sci*. 2006;47:3612-3624.
- Peng S, Adelman RA, Rizzolo LJ. Minimal effects of VEGF and anti-VEGF drugs on the permeability or selectivity of RPE tight junctions. *Invest Ophthalmol Vis Sci*. 2010;51:3216-3225.
- Ghassemifar R, Lai CM, Rakoczy PE. VEGF differentially regulates transcription and translation of ZO-1alpha+ and ZO-1alpha- and mediates trans-epithelial resistance in cultured endothelial and epithelial cells. *Cell Tissue Res*. 2006;323:117-125.
- Miyamoto N, de Kozak Y, Jeanny JC, et al. Placental growth factor-1 and epithelial haemato-retinal barrier breakdown: potential implication in the pathogenesis of diabetic retinopathy. *Diabetologia*. 2007;50:461-470.
- Tombran-Tink J, Shivaram SM, Chader GJ, Johnson LV, Bok D. Expression, secretion, and age-related downregulation of pigment epithelium-derived factor, a serpin with neurotrophic activity. *J Neurosci*. 1995;15:4992-5003.
- Bhutto IA, McLeod DS, Hasegawa T, et al. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. *Exp Eye Res*. 2006;82:99-110.
- Ma J, Zhang J, Othersen KL, et al. Expression, purification, and MALDI analysis of RPE65. *Invest Ophthalmol Vis Sci*. 2001;42:1429-1435.
- Groenendijk GW, De-Grip WJ, Daemen FJ. Quantitative determination of retinas with complete retention of their geometric configuration. *Biochim Biophys Acta*. 1980;617:430-438.
- Gamm DM, Melvan JN, Shearer RL, et al. A novel serum-free method for culturing human prenatal retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2008;49:788-799.
- Spranger J, Osterhoff M, Reimann M, et al. Loss of the antiangiogenic pigment epithelium-derived factor in patients with angiogenic eye disease. *Diabetes*. 2001;50:2641-2645.