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Expression and Role of VEGF in the Adult Retinal Pigment Epithelium

Knatokie M. Ford,1,2 Magali Saint-Geniez,1,3 Tony Walshe,1 Alisar Zahr,1 and Patricia A. D’Amore1,2,3,4

Purpose. Despite a lack of active angiogenesis, VEGF is expressed in nearly every adult tissue, and recent evidence suggests that VEGF may serve as a survival factor for both vascular and nonvascular tissues. VEGF blockade is a widely used treatment for neovascular diseases such as wet age-related macular degeneration (AMD). Therefore, it was sought in this study to evaluate the expression and role of endogenous VEGF in RPE.

Methods. VEGF and VEGFR2 expression in the murine retina were assessed during development. Bevacizumab was used to neutralize VEGF in ARPE-19 cells, and the effects on cell survival and apical microvill were assessed by TUNEL and SEM, respectively. VEGF was systemically neutralized in vivo by adenoviral-mediated overexpression of soluble VEGFRI (sFlt1). RPE and choriocapillaris were analyzed by transmission electron microscopy (TEM). Changes in gene expression were evaluated by quantitative real-time PCR.

Results. VEGF expression was detected in the developing RPE as early as embryonic day (E) 9.5, whereas VEGFR2 expression by RPE began nonuniformly between postnatal (P) day 6.5 and P8.5. VEGF neutralization in vitro led to increased apoptosis and reduced microvilli density and length. Systemic VEGF neutralization led to transient degenerative changes; RPE were vacuolated and separated from photoreceptor outer segments, and choriocapillaris fenestrations were decreased. VEGF levels were elevated in RPE of AdsFlt1 mice at day 4 postinfection, and there was increased expression of the neurotrophic factor CD59a at day 14.

Conclusions. These results indicate that VEGF plays a critical role in survival and maintenance of RPE integrity. Potential undesired off-target effects should be considered with chronic use of anti-VEGF agents. (Invest Ophthalmol Vis Sci. 2011;52: 9478–9487) DOI:10.1167/iovs.11-8353

The retinal pigment epithelium (RPE) is a polarized epithelial monolayer located between the photoreceptor outer segments and the choroid, a highly fenestrated vascular bed. Separated by Bruch’s membrane (BrM), the RPE and choroid each play a vital role in normal eye physiology. At its apical surface, RPE extend long microvilli that facilitate the interaction between RPE and photoreceptor outer segments. The RPE is responsible for the phagocytosis of shed outer segments, ensuring photoreceptor renewal and maintenance of their excitability.

The RPE also maintains the choriocapillaris, a specialized capillary bed that lies beneath the RPE on the opposite side of BrM. The fenestrations of the choriocapillaris are preferentially localized toward the RPE. These fenestrations, which are characteristic of tissues that are involved in secretion and/or filtration, are vital to facilitating the passage of nutrients and oxygen from the choroidal blood supply. In light of the high metabolic activity of the photoreceptors, the integrity of the choriocapillaris is essential to meeting the metabolic demands of the photoreceptors. It has been previously shown that selective destruction of the RPE leads to a secondary atrophy of the choriocapillaris.1 Our laboratory has shown that this effect is due, at least in part, to the dependence of the choriocapillaris on RPE-derived vascular endothelial growth factor (VEGF).2 In addition, a study using mice bearing an RPE-specific deletion of VEGF revealed that the absence of RPE-derived VEGF during development leads to impaired choriocapillaris development and a discontinuous RPE monolayer.3

VEGF has also been implicated in pathologic subretinal vessel growth of wet age-related macular degeneration (AMD), termed choroidal neovascularization (CNV). Accordingly, VEGF neutralizing agents such as bevacizumab (Avastin; Genentech, San Francisco, CA) have proven to be successful in controlling the vascular leakage associated with CNV. Despite the fact that many patients experience an improvement in vision with anti-VEGF therapy, recent observations raise questions regarding the efficacy of these treatments beyond 2 years.4 In addition, there have been reports of RPE tears after administration of VEGF modulating therapies, with an incidence as high as 17%, suggesting that neutralization of VEGF may have adverse effects.5,6 RPE tears may occur as a part of the natural progression of AMD; however, in most cases, the tears arise within 6 weeks of the first injection, lending to the suspicion that the anti-VEGF therapy may be causative.7,8 Furthermore, a recent report described significant vision loss after 2 years of anti-VEGF treatment that appeared to be unassociated with the primary pathology, raising the possibility of damage to the RPE and photoreceptors from “off-target” effects.

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of VEGF neutralization. VEGF antagonists are widely used as a treatment for other ocular pathologies with new vessel growth and excess permeability, such as branch vein occlusions, diabetic macular edema, and retinopathy of prematurity. Motivated by increasing use of anti-VEGF therapies and the many nonvascular functions of VEGF, this study explores the effects of VEGF neutralization on RPE structure, survival, and gene expression both in vitro and in vivo.

**Materials and Methods**

**Animals**

Pups obtained from timed-pregnant Swiss-Webster VEGF-lacZ mice (provided by Andras Nagy, Samuel Lunenfeld Research Institute, Toronto, Canada) were used to determine the time course of VEGF expression by RPE during development. Pregnant mothers were euthanized by CO2 inhalation and embryos collected at embryonic day (E) 9.0, E9.5, and E10.5 were fixed for 2 hours at room temperature (RT) in 4% paraformaldehyde in PBS. Tissues were washed extensively in PBS to remove residual paraformaldehyde.

The time course of VEGF expression by RPE during postnatal development was examined by immunohistochemistry in retinas from wild type Swiss-Webster mice. Postnatal pups were euthanized by decapitation, and eyes were immediately enucleated and fixed in 4% paraformaldehyde in PBS overnight at 4°C. After fixation and extensive washing in PBS, the tissues were subjected to a sucrose gradient of 5%–20% sucrose for 4 hours, followed by embedding in compound (OCT; Sakura Finetecchnical, Torrance, CA). Samples were cryosectioned (10 μm), and stored at −20°C until further use.

**Cell Culture**

ARPE-19 cells, purchased from ATCC (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 1:1 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (GluMax; Invitrogen, Carlsbad, CA) (GSP), and used through a maximum of 30 passages. Cells were incubated at 37°C in a 5% CO2 and cultured with 1 x noncytotoxic cell dissociation solution or trypsin. ARPE-19 cells were allowed to polarize by plating at high density (1.7 × 105 cells) on laminin-coated 0.4 μm-pore 12-well plates (Costar Transwells, Thermo Fisher Scientific, Cambridge, MA), and maintained in DMEM/F12 supplemented with 1% FBS and 1% GSP. Media were changed twice a week for 4 weeks before use in experiments.

**Adenoviral-Mediated sFlt1 Overexpression**

For in vivo neutralization of VEGF, 6- to 8-week-old adult CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA) were injected via the tail vein with adenovirus expressing either soluble VEGFR1 (Ad-sFlt1) or empty vector (Ad-null) as follows: 2.5 × 109 viral particles (VP) for Ad-null and 2.5 × 109 VP for Ad-sFlt1. sFlt1 plasma levels were determined by ELISA (R&D Systems, Minneapolis, MN). Plasma levels of sFlt1 of at least 200 ng/mL were measured at days three, six, and 12, just before animal euthanization. Ad-null-infected mice showed no detectable sFlt1. All animal experiments were conducted according to the ARVO statement for the use of animals in Ophthalmic and Vision research under protocols that were approved by the Schepens Eye Research Institute Institutional Animal Care and Use Committees (IA-CUC).

**TUNEL Assay**

ARPE-19 cells cultured on membranes (Transwell; Corning, Inc., Lowell, MA) for 4 weeks were treated with 10 μg/mL of bevacizumab or control human IgG (R&D Systems) for 1, 2, or 3 weeks. Cells were fixed in 4% paraformaldehyde for 10 minutes at RT and then washed in PBS. The membranes (Transwell; Corning, Inc.) were then cut in half using a razor blade, removed from the well and transferred to a 24-well plate with tweezers. Half of the membrane was used for the TUNEL assay and the other half was immunostained for ZO-1. Apoptotic cells were detected using a kit (In Situ Cell Death Detection TMR red; Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s instructions. DAPI labeling was used to identify cell nuclei. Processed membranes (Transwell; Corning, Inc.) were mounted on slides, and images were taken of 10 randomly selected fields with a microscope (Axio- scope Mot 2; Carl Zeiss Meditec, Inc., Dublin, CA). The number of apoptotic cells detected was represented as the sum of apoptotic cells detected in 10 fields for each membrane, and then averaged for three membranes per condition and time point.

**Real-Time PCR Analysis**

Total mRNA was purified using RNeasy kit solution (Isotext Diagnostic, Inc., Friendswood, TX) under RNase-free conditions, according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed (Superscript III; Invitrogen, Carlsbad, CA), and cDNA that was diluted 1:20 (50 ng of equivalent RNA) was used in each amplification reaction. Reactions were performed using the SYBR Green Master mix (ABI Prism 9700 Sequence Detection System; Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. PCR cycles consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each sample was subjected to melting curve analysis to confirm amplification specificity. Samples were run in duplicate, and each experiment included two nontemplate control wells. Samples were normalized to HRPT, and expressed as the relative expression using the 2−ΔΔCt method. The highest and lowest values from each Ad-null and Ad-sFlt1 group at each time point were omitted to eliminate potential outliers. Primer sequences included: ciliary neurotrophic factor (CNTF) (forward, 5′-TCTTGTAGCCGGTCTACCTG-3′; reverse, 5′-GGTAGACCATCATCACGATCAA-3′), HPRT (forward, 5′-TCAGTCACACGGGGCATAAA-3′; reverse, 5′-GGGGCTGTACTGCTTAACCACT-3′), HIF-1α (forward, 5′-ATTGTTGATACCCATCTCCTACCC-3′; reverse, 5′-CATA-TGAGGCTGTGACTGAG-3′), CD59a (forward, 5′-GTTAGCCTCACTGTCACACCC-3′; reverse, 5′-AGAGAGAAATGTCGCTGTTTACA-3′), and VEGF A (forward, 5′-GACATAAGAGAATGAGCTTCC-3′; reverse, 5′-TCGGCTTCAGAAAGGCT-3′).

**Figure 1.** Expression of VEGF during eye development. Cryosections (10 μm) of E9.0, E9.5, and E10.5 VEGF-LacZ/+ mouse embryos were stained for β-galactosidase (blue) and counterstained with eosin (red). VEGF was nearly undetectable at E9.0, but was evident at E9.5, primarily in the proximal optic vesicle. By E10.5, VEGF was expressed predominantly in the dorsal portion of the optic cup in the primitive RPE. Scale bar, 100 μm. NR, neural retina; OV, optic vesicle.
Semi-Quantitative PCR Analysis

Total mRNA and cDNA were prepared as described above for real-time PCR analysis. One microcroliter of cDNA was used as a template in a 25 μL amplification mixture containing 200 nM dNTPs, 1 U Taq DNA polymerase (Roche Diagnostics), and 0.2 μM of the primer pair capable of amplifying all three VEGF isoforms. Samples were amplified for 35 cycles, and amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV light. Primer sequences include: GAPDH (forward, 5'- CAAATCTCATGGCAGCG-3'; reverse, 5'-GGAGTGTTGTTGCTGTGTA-3') and VEGF (forward, 5'-AGCCATGCGACCTGGCAAT-3'; reverse, 5'-ACTGCTGCTTCCTCAGCG-3').

β-Galactosidase Histochemistry

LacZ, as a reporter of VEGF expression, was visualized in cryosections of whole embryos (E9.0, E9.5, E10.5) by staining for LacZ using a staining kit (In Situ β-galactosidase Staining Kit; Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Embryos were stained for LacZ immediately after fixation, followed by postfixation for 5 minutes at RT, and rinsing in PBS. After embedding (OCT; Sakura Finetechanical), embryos were cryosectioned (10 μm), and counterstained with cosin.

Electron Microscopy

Four, seven, and 14 days after adenovirus infection, mice were deeply anesthetized by injection with ketamine (75 mg/kg) and xylazine (1.8 mg/kg). Live animals were perfused over 2 minutes with 10 mL of sodium cacodylate buffer (0.2 M, pH 7.4), followed by 10 mL of half-strength Karnovsky’s fixative (Electron Microscopy Sciences, Hatfield, PA) via a 21-gauge cannula inserted into the aorta via the left ventricle. Fluid drained through the right atrium, and animal death was immediate on perfusion. Eyes were enucleated, fixed in half-strength Karnovsky’s fixative, and dissected to remove a quadrant containing the posterior portion of the eye. A secondary fixation in 2% osmium tetroxide was performed, followed by dehydration and embedding. Ultrathin sections were treated with uranyl acetate and visualized by transmission electron microscopy (TEM) using a transmission electron microscope (Philips 410; Philips/FEI Corporation, Eindhoven, Holland).

Scanning electron microscopy (SEM) was used to visualize the apical microvilli of RPE cells in vitro. ARPE-19 cells that had been cultured for 4 weeks on membranes (Transwells; Corning, Inc.) and treated with 10 μg/mL of bevacizumab or control IgG for 4 weeks were washed three times in PBS and fixed in half-strength Carnovsky’s fixative overnight at 4°C. After rinsing three times in PBS, samples were dehydrated through graded ethanol and critical point dried (Au-purified CO2; Quorum Technologies, Manchester, UK) and mounted on specimen holders, and sputter coated with carbon in an ion beam coater (model 681; Gatan, Pleasanton, CA). Images were mounted on specimen holders, and sputter coated with carbon in an ion beam coater (model 681; Gatan, Pleasanton, CA). Images were viewed with a scanning electron microscope (Philips 410; Philips/FEI Corporation, Eindhoven, Holland).

Quantification of Apical Microvilli

The center of each membrane (Transwell; Corning, Inc.) was marked by a single dot, and 10 SEM images were taken around the center of each membrane (Transwell; Corning, Inc.). An image program (Photoshop CS5; Adobe Systems Inc., San Jose, CA) was used to distinguish apical microvilli from the cell surface by setting the luminance threshold. Microvilli pixels were selected using the ‘Color Range’ function, and the ratio of microvilli pixels to total pixels per image was calculated. The sum of the 10 images was averaged among replicates.

Immunohistochemistry

VEGFR2 expression was visualized using the avidin-biotin-peroxidase method. Eyes were collected from postnatal (P) pups (P2.5, P6.5, P7.5, and P8.5) as well as adult mice and fixed in 4% paraformaldehyde in PBS overnight at 4°C, and then washed extensively in PBS to remove residual paraformaldehyde. Pup and adult eyes were subjected to the 5%-20% sucrose gradient before cryosectioning. Cryosections (10 μm) were air-dried, washed in PBS, then PBS containing 0.1% Tween (PBST) and were then pretreated with 1% H2O2 in methanol for 10 minutes to neutralize endogenous peroxidase activity. Samples were then incubated with avidin and biotin for 15 minutes each, followed by blocking for 1 hour at RT in 1.5% goat serum PBST. Samples were incubated overnight at 4°C in a humid chamber with rabbit anti-mouse VEGFR2 (Abcam, Cambridge, MA) and biotinylated anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA) followed by the avidin-biotinylated enzyme complex (Vector ABC kit; Vector Laboratories). VEGFR2 labeling was localized with the 3,3′-diaminobenzidine (DAB) substrate chromogen system (Dako, Dako North America, Inc., Carpinteria, CA). As a negative control, sections were incubated in blocking buffer in the absence of the primary antibody overnight, and then were treated in the same manner as the experimental slides. After mounting, images were taken with a microscope (Axioskop Mot 2; Carl Zeiss Meditec, Inc.).

ZO-1 was localized in polarized ARPE-19 cells. Cells were washed with PBS and fixed in 4°C-4% PFA for 5 minutes. After three washes in PBS, samples were blocked in 0.1% Trition/5% goat serum in PBS for 2 hours at RT, and incubated with the ZO-1 antibody (1:100, Zymed Laboratories, San Francisco, CA) overnight at 4°C. After washing with PBS, samples were incubated with a mixture of FITC-conjugated goat anti-rabbit fluorescent secondary antibody (1:300, Jackson Immunoresearch Laboratories; West Grove, PA) and DAPI (1:100) for 2 hours at RT. Membranes (Transwell; Corning, Inc.) were cut from wells, mounted, and images were taken with a fluorescence microscope (Axioskop Mot 2; Carl Zeiss Meditec, Inc.).

Statistical Analysis

Values are expressed as the mean ± SD, unless otherwise indicated. Differences between means of the experimental group relative to its corresponding control at the indicated time points were evaluated for statistical significance using an unpaired Student’s t test (*P < 0.05; not significant: P > 0.05).

RESULTS

VEGF Expression in RPE during Ocular Development

As a first step in addressing the role of VEGF in the RPE, the temporal expression of both VEGF and its primary receptor VEGFR2 were characterized during eye development. Ocular development, which begins around E8.5 in mouse, proceeds from two principal tissue components: the neural ectoderm and surface ectoderm. The neural ectoderm buds from the wall of the optic vesicle to form the optic vesicle, whereas the surface ectoderm eventually gives rise to the lens.13 When the optic vesicle comes in contact with the overlying surface ectoderm, it invaginates, forming the optic cup at E10.5. The optic cup consists of an inner layer, which gives rise to the neural retina and an outer layer, which forms the RPE.

To determine the onset of VEGF expression in the RPE, mice expressing a LacZ reporter gene containing a nuclear localization signal in the 3′ untranslated region (UTR) of the VEGF locus were used.13 VEGF was previously reported to be expressed in the primitive RPE at E10.5, the earliest time point initially assessed.15 Histochemical localization of β-galactosidase activity revealed that VEGF was nearly undetectable in the optic vesicle at E9.0. However, robust VEGF expression was observed throughout the optic vesicle at E9.5, primarily in the proximal region (Fig. 1), indicating...
that VEGF expression in the eye commences between E9.0 and E9.5.

VEGFR2 Expression during Eye Development

The time course of VEGFR2 expression during RPE development was next determined. Previous studies have reported that VEGFR2 is absent from the developing RPE through P7,16 and our laboratory has shown that VEGFR2 is expressed by adult RPE.15 Consistent with the previous reports, VEGFR2 was absent from the RPE at P2.5 (data not shown), but present in the adult (Fig. 2). However, in contrast to the previous report that indicated that VEGFR2 was absent through P7,16 VEGFR2 expression was detected at P6.5 in discrete patches of the RPE with some regions of RPE negative for VEGFR2 adjacent to areas showing strong expression (Fig. 2). A similarly discontinuous pattern of VEGFR2 expression was observed through P7.5, and by P8.5, VEGFR2 was expressed more robustly and consistently throughout the RPE (Fig. 2). Therefore, it appears that VEGFR2 expression by the RPE commences gradually in the RPE during postnatal development, beginning at least at P6.5 until it is fully expressed by virtually all RPE by P8.5.

VEGF Neutralization In Vitro Leads to RPE Apoptosis and Reduced Apical Microvilli

ARPE-19 cells, an immortalized human RPE cell line, recapitulate many of the characteristics of RPE in vivo and are thus a useful tool for studying the RPE in vitro.17 ARPE-19 cells cultured on membranes (Transwell; Corning, Inc.) in low serum for 4 weeks formed tight junctions reminiscent of those formed by RPE in vivo (Fig. 3). Furthermore, ARPE-19 cells reproduced the pattern of VEGF isoform expression that is seen by RPE in vivo, with VEGF165 being the predominant isoform, and VEGF189 nearly undetectable (Fig. 3). Because adult RPE express both VEGF and its receptor, we evaluated the effect of VEGF neutralization on RPE in vitro. ARPE-19 cells grown for 4 weeks on membranes (Transwell; Corning, Inc.) were treated for 1 to 3 weeks with 10 μg/mL of bevacizumab or a human IgG control, a concentration that reflects the vitreal concentration of bevacizumab that has been reportedly maintained for 30 days after a single intravitreal injection.18

The effect of VEGF neutralization on cell survival was assessed using a TUNEL (TMR Red; Roche Diagnostics, Indianapolis, IN) assay to visualize apoptotic cells. A twofold

Figure 2. Expression of VEGFR2 during RPE development. Cryosections (10 μm) of eyes from (A) P6.5, (B) P7.5, (C) P8.5, and (D) adult Swiss-Webster albino animals were immunostained using a polyclonal VEGFR2 antibody (brown) or incubated in the absence of primary antibody as a negative control. At (A) P6.5 and (B) P7.5, VEGFR2 is observed primarily in the choroid/choriocapillaris as well as in patches in the RPE. (C) By P8.5, robust VEGFR2 expression is observed throughout the RPE. (D) In adult RPE, VEGFR2 is detected throughout the RPE. The negative control is shown only for adult. (A–C) Different areas of the same mouse eye. Black asterisks: VEGFR2-negative RPE; white asterisks: VEGFR2-positive RPE. Scale bar, 20 μm.

Figure 3. ARPE-19 cells display characteristics of RPE in vivo. (A) ZO-1 (green) and DAPI (blue) immunolocalization in ARPE-19 cells cultured for 4 weeks on membranes (Transwell; Corning, Inc.) to induce polarization, illustrating the presence of extensive tight junctions. (B) Semiquantitative PCR of VEGF isoform expression by undifferentiated ARPE-19 cells demonstrated that VEGF165 and VEGF121 were the predominant isoforms expressed, with VEGF189 virtually undetectable. Scale bar, 50 μm.
increase in the number of apoptotic cells was observed after 2 weeks of bevacizumab treatment (Fig. 4). The effect of VEGF neutralization on RPE proliferation, ZO-1 localization, expression of a number of genes, including VEGF, VEGF C, VEGFR2, placenta growth factor (PlGF), and pigment epithelium-derived factor (PEDF), as well as transepithelial resistance (TER) was also evaluated; however, none of these variables were affected by VEGF neutralization in vitro (data not shown).

A major function of RPE is phagocytosis of shed outer segments, which is facilitated by the microvilli at the apical RPE surface. Therefore, we determined the effect of VEGF neutralization on the appearance and abundance of RPE apical microvilli. ARPE-19 cells grown for 4 weeks on membranes (Transwell; Corning, Inc.) were treated for 4 weeks with 10 μg/mL bevacizumab or a human IgG control. The apical microvilli were analyzed by SEM. RPE microvilli appeared blunted, and quantification of the microvilli revealed a strong trend toward a reduction (20%) in microvilli density in bevacizumab-treated ARPE-19; however, the difference was not statistically significant (Fig. 5).

VEGF Neutralization In Vivo Leads to Transient RPE Damage

In light of these in vitro observations, we hypothesized that VEGF may have an autocrine function in RPE in vivo. To test this possibility, mice were injected intravenously with Ad-sFlt1, a VEGF inhibitor, or Ad-null, an empty vector control. The effect of VEGF neutralization on the RPE was evaluated at days 4, 7, and 14 postinjection. Ultrastructural analysis of RPE at these time points revealed numerous vacuoles within the RPE as well as evidence of a separation of the RPE from photoreceptor outer segments at their apical surface at days four and seven (Fig. 6). RPE retained their association with BrM. There were spaces between the RPE cells; however, the tight junctions appeared to remain intact.

The underlying choriocapillaris also displayed alterations; the endothelium was noticeably thicker in some regions, correlating with a loss of fenestrations at days four and seven (Fig. 7). These results are consistent with the proposed role of RPE-derived VEGF in maintenance of choriocapillaris fenestrations. However, the fenestrations in the Ad-sFlt1-infected animals appeared to recover by day 14 (Fig. 7),...
which corroborated previous findings by our laboratory where there was no significant difference in the number of choriocapillaris fenestrations between the Ad-null and Ad-sFlt1-expressing animals at day 28.7 We speculated that RPE in vivo may have the ability to recover from the early deleterious effects of VEGF neutralization and therefore tested the idea that RPE recovery and the associated restoration of choriocapillaris fenestrations might be due to the upregulation of VEGF in response to VEGF blockade.

RNA isolated from the RPE-choroid complex of mice injected with Ad-null or Ad-sFlt1 was analyzed at days four, seven, and 14 postinjection. We have previously shown that the RPE is the only source of VEGF in the RPE-choroid complex.15 At day four, there was a 30% increase in VEGF mRNA in Ad-sFlt1 animals compared with Ad-null-injected animals (Fig. 8). At day seven and day 14, the VEGF levels were comparable in both Ad-sFlt1 and Ad-null animals (Fig. 8). We speculate that the transient increase in VEGF may be the result of reduced nutrient access due to the loss of fenestrations. In support of this concept, low glucose has been shown to lead to the induction of VEGF.19 Interestingly, there was also a similar increase in hypoxia-inducible factor 1α (HIF-1α) in Ad-sFlt1 animals at day four; however, the difference did not reach statistical significance (Fig. 8). HIFs have previously been shown to be sensitive to nonhypoxic stimuli, such as hypoglycemia.20 Therefore, it is possible that impaired nutritional support due to choriocapillaris fenestration loss and subsequent endothelial wall thickening may account for the increase in HIF-1α observed.

The expression of a series of RPE survival factors including brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), CNTF, CD59a, and PEDF, as well as other VEGF family members, VEGF C and PlGF, was also analyzed. Of the genes assessed, there was a trend toward increased CNTF at each time point assessed, and there was a 32% increase in CD59a at day 14 in Ad-sFlt1-infected animals (Fig. 8). No change was detected between Ad-null and Ad-sFlt1-infected animals at any time point for VEGF C, bFGF, BDNF, PEDF, or PlGF (data not shown). Thus, it appears that VEGF expression by RPE is upregulated in response to VEGF blockade, potentially mediated by reduced nutrient access after the transient loss of choriocapillaris fenestrations and associated endothelial wall thickening. The increase in CNTF and CD59a may also play a role in RPE recovery in the face of persistent VEGF neutralization.

FIGURE 5. Effect of VEGF neutralization on RPE apical microvilli. SEM micrograph of ARPE-19 cells cultured for 4 weeks on membranes (Transwell; Corning, Inc.) and treated with (A) IgG or (C) bevacizumab for 4 weeks revealed less dense microvilli in bevacizumab-treated cells. Higher magnification of (B) IgG and (D) bevacizumab-treated ARPE-19 cells demonstrated that microvilli appear blunted in bevacizumab-treated cells. (E) Quantification of microvilli (n = 3) illustrated a trend of decreased microvilli abundance in bevacizumab-treated cells. Data are represented as the mean ± SD. Scale bar, 5 μm. ns, P = 0.0713.
DISCUSSION

VEGF expression begins very early during eye development, between E9.0 and E9.5. At this stage, the optic vesicle is multipotent, with each cell expressing the same transcription factors, and accordingly, with the capacity to become any of the various ocular cell types. Hence, VEGF expression in the eye commences before the formation of specialized eye structures. At least one of the functions of the early expression of VEGF during eye development is to support choroidal development, which begins around the time of optic cup formation.3 In further support of the role of early VEGF expression in mediating choroidal vascularization, mice with an RPE-specific deletion of VEGF displayed failed choriocapillaris development, and the RPE itself became a discontinuous monolayer.3

RPE begin to synthesize VEGFR2 during the first week of postnatal development with nonuniform expression among the RPE. Such heterogeneous expression within the RPE monolayer has been observed for other proteins, including E-cadherin and vimentin.21–22 P6.5 to P8.5 is considered a “priming” period during which the visual system prepares for the onset of vision after eye opening between P10 and P13. Photoreceptors begin to form outer segments and synapses at approximately P7, and the deep retinal vascular plexus commences development at approximately P8.23 Nevertheless, the lag between VEGF and VEGFR2 expression during RPE development suggests that autocrine VEGF signaling in the RPE is not required during early RPE development.

Neutralization of VEGF in cultured RPE led to increased cell death whereas VEGF neutralization in vivo resulted in transient defects in the RPE-choroid complex, including vacuolization, loss of association with photoreceptor outer segments, and decreased choriocapillaris fenestrations. Taken together, these results provide evidence for an autocrine role of VEGF in RPE and a paracrine function for the choriocapillaris. A recent report demonstrated that autocrine VEGF signaling enhances the survival of RPE cells under oxidative stress via the VEGFR2/PI3K/Akt pathways.24 VEGF not only affects RPE cell survival, but it can also influence RPE function, as treatment of cultured RPE cells with bevacizumab reduced RPE phagocytosis.25 In addition, we found that VEGF blockade in cultured RPE cells led to blunted and less dense microvilli. RPE microvilli are composed of an internal core bundle of densely packed actin filaments,26 along with EBP50 and ezrin,27–30 a member of the ezrin/moesin/radixin (ERM) family that functions as a bridge between actin filaments and plasma membrane proteins. In support of a role for VEGF in the maintenance of microvilli, patients with pre-eclampsia, which is mediated in part by overexpression of soluble VEGFR1, displayed reduced levels of ezrin in the podocytes.31 Similarly, mice with targeted deletion of ezrin exhibit substantially reduced apical microvilli and basal

**FIGURE 6.** Effect of VEGF neutralization on RPE in vivo. Ultrastructural analysis of eyes from mice 4 days and 14 days after infection with Ad-null revealed normal RPE ultrastructure. At days four and seven after infection with Ad-sFlt1, RPE displayed numerous vacuoles (asterisks), and appeared to separate within the RPE monolayer (arrowhead) and from photoreceptor outer segments (arrows). At day 14 and day 28 post Ad-sFlt1 infection, the RPE appeared normal. Scale bar, 4 μm. CC, choriocapillaris; OS, outer segments.
Interestingly, we did not detect an effect of VEGF neutralization on nonspecific phagocytosis by ARPE-19 cells; however, similar to a previous report, we did note a reduction in the capacity of human fetal RPE to phagocytize outer-segment-opsonized particles after 2 weeks of bev-acizumab treatment (data not shown). These results suggest that VEGF inhibition may influence the specific phagocytosis of outer segments but not nonspecific phagocytosis, which is particularly relevant in light of the wide usage of anti-VEGF agents in the treatments of AMD because phagocytosis of shed photoreceptor outer segments is critical to photoreceptor function.

The recovery of the RPE and choriocapillary fenestrations in the face of sustained VEGF neutralization in vivo was

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933457/)

**Figure 7.** Effect of VEGF neutralization on choriocapillary endothelial cell fenestrations. High magnification of the choriocapillaris from mice four days postinfection with Ad-null revealed numerous fenestrations (arrows) on the capillary endothelial membrane adjacent to the RPE. At four and seven days post Ad-sFlt1 infection, the RPE retained their association with BrM, but the chorioidal capillary endothelium was thickened. At 14 days postinfection, the fenestrations appear to have recovered in the Ad-sFlt1 animals. Scale bar, 0.5 μm.

![Figure 8](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933457/)

**Figure 8.** Effect of VEGF neutralization on gene expression in vivo. Real-time PCR of cDNA isolated from RPE-choroid of Ad-null and Ad-sFlt1-injected mice indicating (A) a 30% increase in VEGF mRNA at day four post-Ad-sFlt1 injection, and there was a comparable increase in HIF-1α at the same time point, but it did not reach significance. (B) There was a trend toward increased CNTF expression at each time point, and NPD1 was upregulated 32% at day 14. The experiment was performed in quadruplicate, and data are represented as the mean ± SD. *P < 0.05.
surprising. Restoration of fenestrations during VEGF blockade with VEGF receptor tyrosine kinase inhibitors as well as soluble VEGF receptors has not been noted in a variety of other fenestrated vascular beds, including choroid plexus and kidney, as regrowth of capillaries that had regressed occurred only when VEGF neutralization was terminated. In contrast, our findings indicate that RPE recover from their damage and choriocapillaris reforms its fenestrations in the face of continuous VEGF neutralization. The upregulation of VEGF expression by RPE in vivo during systemic VEGF neutralization suggested that compensatory VEGF upregulation likely plays a role in this recovery. Furthermore, the increased expression of neurotrophins such as CNTF, which has been previously shown to enhance RPE survival under oxidative stress, may also contribute to RPE recovery.

VEGF upregulation in response to neutralization has also been suggested in a rat model of retinopathy of prematurity (ROP); after intravitreal injection of a VEGF-neutralizing antibody there was a significant increase in free intraretinal VEGF protein 1 day postinjection. However, whether the VEGF protein measured in that study represents upregulation in response to anti-VEGF or displacement of cell surface VEGF by the antisera has not been investigated. Compensatory VEGF upregulation was more convincingly observed in a study analyzing the efficacy of combination versus individual angiostatic therapy. Treatment with a VEGF aptamer led to an increase in VEGF, whereas combination therapy significantly reduced that upregulation. That said, VEGF upregulation leading to recovery of anti-VEGF-mediated damage does not appear to be universal. VEGF neutralization has been demonstrated to lead to numerous defects in various other cell types, including decreased vascular perfusion in the choroid plexus of the brain, increased apoptosis in the neuronal retina of the eye, and endotheliosis in the glomerular endothelium. It is noteworthy that the studies in which there appears to be compensatory VEGF expression, including our own work, were performed in young and/or healthy animals so it is unclear whether aged and/or diseased tissues retain this capacity.

VEGF neutralization in vitro did not lead to an increase in VEGF expression (data not shown). We suspect that this is due to the fact that the cultured system does not replicate the complexity of the in vivo system. VEGF blockade resulted in a transient loss of choriocapillaris fenestrations and concomitant endothelial wall thickening, which may well have led to reduced nutritional support for the RPE and photoreceptors. Low glucose has been shown to induce both VEGF as well as HIF-1α expression, and thus may account for the increases in VEGF and HIF-1α observed at day 4 and the lack of VEGF induction in vitro.

An autocrine role for VEGF has been observed in other cell types, including kidney podocytes. In an in vitro differentiation system using immortalized murine podocytes, both VEGF and VEGFR2 expression were shown to be increased with podocyte differentiation. Furthermore, addition of VEGF reduced podocyte apoptosis by 40% whereas neutralization of VEGF led to a twofold increase in apoptosis. One of the first detectable side effects of systemic neutralization in vivo is proteinuria, which has been clearly shown to be the result of damage to glomerular capillaries. In support of this, adenosine-viral infection of rats with sFlt1 has been demonstrated to lead to endothelialitis and proteinuria. Thus, in both kidney podocytes and RPE, systemic VEGF neutralization leads to tissue damage and capillary instability.

The role of VEGF in the RPE is complex. During development, VEGF functions to support choroidal development whereas in the adult it appears to function in an autocrine manner in the promotion of RPE survival as well as in a paracrine fashion in the maintenance of the underlying choriocapillary endothelial cells. Thus, appropriate levels of VEGF are vital for ocular homeostasis and integrity. Given the widespread use of VEGF-neutralizing agents, chronic application of VEGF modulators should be used judiciously to avoid undesired off-target effects.

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