Pigment Epithelium-Derived Factor Decreases Outflow Facility

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PURPOSE. Pigment epithelium-derived factor (PEDF) regulates blood-retinal barrier function. As a constituent of aqueous humor, the role of PEDF in conventional outflow function is unknown. The goals of the study were to examine the effects of PEDF on barrier function of cultured Schlemm's canal (SC) endothelia and outflow facility in mouse eyes in situ.

METHODS. To model the inner wall of SC, transendothelial electrical resistance (TEER) of human SC and porcine angular aqueous plexus (AAP) cells was monitored. To examine an intact conventional outflow pathway, enucleated eyes from culled C57BL/6 mice were perfused with PEDF using a computer-controlled system. Purified PEDF (0.1 and 1 µg/mL) was perfused at four different pressure steps (4, 8, 15, 20 mm Hg), measuring flow to determine outflow facility (slope of flow/pressure relationship).

RESULTS. Pigment epithelium-derived factor increased TEER of porcine AAP cells in a dose-dependent fashion (0.3–3 µg/mL), and 1 µg/mL recombinant PEDF or conditioned media from pigmented retinal pigment epithelial monolayers stabilized TEER of human SC monolayers over time (0–48 hours). In perfusion experiments, we observed a 43.7% decrease in outflow facility (0.016 vs. 0.029 µL/min/mm Hg, $P = 4.5 \times 10^{-5}$) in eyes treated with 1 µg/mL PEDF compared to vehicle-perfused controls, and a 19.9% decrease (0.021 vs. 0.027 µL/min/mm Hg, $P = 0.003$) at 100 ng/mL PEDF.

CONCLUSIONS. Pigment epithelium-derived factor increased barrier function in both the in vitro and in situ models of the inner wall of SC. Modification of PEDF signaling in SC cells may be therapeutically exploited to increase outflow facility in people with ocular hypertension or decrease outflow facility in those with hypotony.

Keywords: aqueous humor, outflow facility, glaucoma, growth factors, trabecular meshwork, Schlemm's canal, VEGF, PEDF

Glaucoma is the second leading cause of blindness globally and is the leading cause of blindness in African Americans.¹ Glaucoma is characterized by loss of retinal ganglion cells and degeneration of the optic nerve head, associated with visual field loss. Elevated intraocular pressure (IOP) is the main causative risk factor for glaucoma, and thus the focus of treatment. Drugs that lower IOP are neuroprotective, preserving remaining vision.² However, current medical treatments for glaucoma (beta blockers, carbonic anhydrase inhibitors, alpha-2 receptor agonists, and prostaglandin F_{2α} receptor agonists) often do not sufficiently lower IOP and do not target the diseased tissue responsible for elevated IOP, the conventional outflow pathway.

The majority of glaucoma cases are classified as primary open-angle glaucoma (POAG). The outflow tissues in POAG appear grossly normal on examination.³ However, aqueous humor outflow resistance is often abnormal, which produces elevated IOP.⁴ Therefore, when IOP is elevated, the primary dysfunction likely resides in one or both of the two primary cell types, the trabecular meshwork (TM) and inner wall of Schlemm's canal (SC), that populate the conventional outflow pathway and generate resistance to outflow.

The SC inner wall has two diametrically opposed functions. First, it must allow aqueous humor to pass in a basal to apical direction, facilitating entry into the canal lumen. Second, the SC composes part of the blood-aqueous barrier along with the ciliary epithelium, the iris vascular endothelium, and the posterior iris epithelium. This barrier, formed by tight junctions between the continuous monolayer of endothelial cells that forms SC, prevents blood products from entering the eye if elevated episcleral venous pressure exceeds IOP.⁵ The factors that regulate the blood-aqueous barrier at the level of the SC are poorly understood.

Like the blood-brain barrier, the blood-retinal barrier restricts passage of molecules from the blood into the neuron-rich retina. This barrier consists of tight junctions between retinal pigment epithelial cells and between retinal endothelial cells. The barrier is regulated partly through a balance of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), which are antagonistic to one another.^{6,7} Vascular endothelial growth factor is a potent angiogenic factor that binds cell surface tyrosine kinase receptors, disassembles intercellular junctions, and induces endothelial proliferation.⁸ Pigment epithelium-derived factor is

FIGURE 1. Concentration-related effects of PEDF on net transendothelial electrical resistance of porcine angular aqueous plexus cell monolayers. Three primary cell lines (passages 2–4) were seeded at confluence onto Transwell filters and maintained for 1 week in culture before experimentation. Monolayers were then treated with serum-free media or media containing PEDF, and transendothelial electrical resistance (TEER) was measured after 48 hours (time of maximal effect). Shown are mean data at each concentration $\pm$ SD. *** $P < 0.001$.

a 50-kDa glycoprotein that is antiangiogenic and belongs to the serine protease inhibitor superfamily by sequence homology.⁹ Responses to PEDF can be mediated by multiple mechanisms. These include binding to a cell surface receptor called PEDF-R, which has phospholipase A2 activity¹⁰; activation of gamma-secretase and subsequent proteolysis of VEGF receptor-1 and -2^{6,11}; and competitive antagonism of the VEGF-R2 receptor.^{6,12} Levels of PEDF in monkey vitreous humor are maintained around 1 mg/mL.¹³

Pigment epithelium-derived factor is also produced in the anterior eye by the ciliary epithelium and trabecular meshwork,^{14,15} and thus is a constituent of aqueous humor (3–300 ng/mL).^{16,17} While its role in retinal physiology is well characterized, the effects of PEDF on aqueous humor dynamics and SC function have not been studied. We hypothesized that PEDF participates in the regulation of conventional outflow facility by modifying SC barrier function. Using primary cultures of cells from porcine angular aqueous plexus (porcine equivalent of human SC) and human SC, we observed that PEDF significantly increased endothelial barrier function in a dose- and time-related manner. In perfused enucleated mouse eyes, we found that PEDF dose-dependently decreased outflow facility. Together, results suggest that PEDF modulates conventional outflow at the level of the inner wall of SC.

METHODS

Cell Culture and Transendothelial Electrical Resistance Measurements

Three strains of human SC cells (SC60, SC68, and SC55 isolated from postmortem enucleated eyes from human donors ages 58, 30, and 29 years, respectively) were used in the present study. Cells were isolated, characterized, and maintained in culture as previously described.¹⁸ For transendothelial electrical resistance (TEER) measurements, SC cells were plated at confluence at a density of 1×10^5 cells on Transwell filters (VWR, Atlanta, GA) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA).¹⁸ Cells were

maintained in culture for 2 weeks to allow cell monolayers to mature. Afterward, the filters were exposed to conditioned media from nonpigmented adult retinal pigment epithelial (RPE) cells at a 1:5 dilution in serum-free DMEM containing 0.2% lactalbumin hydrolysate (LH; Life Technologies) or conditioned media from pigmented fetal RPE media at a 1:5 dilution in serum-free DMEM containing 0.2% LH DMEM, or were treated with purified recombinant PEDF (1 µg/mL; Millipore, Billerica, MA) in serum-free DMEM containing 0.2% LH DMEM. Using an EndOhm chamber (World Precision Instruments, Stevenage, UK), TEER of the cell monolayers was determined just prior to treatment (time 0) and at 24 and 48 hours posttreatment.¹⁸

Three primary cell lines of porcine angular aqueous plexus (AAP) cells from different animals were isolated using the purimycin technique (passages 2–4) and characterized and maintained in culture as previously described.¹⁹ Cells were seeded at confluence on Transwell filters and maintained for 1 week in culture before experimentation. Monolayers were then treated with serum-free DMEM or media containing purified recombinant PEDF (0.3–3 µg/mL; Millipore), and TEER was measured just prior to treatment and 48 hours after treatment (time of maximal effect).

Ex Vivo Mouse Eye Perfusions

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were done using enucleated eyes from culled C57BL/6 mice procured from several Duke University laboratories.

C57BL/6 mice of either sex, approximately 12 weeks of age, were killed by euthanasia with carbon dioxide. Eyes were enucleated within 1 hour of death and perfused immediately or kept in PBS at 4°C for up to 4 hours. Enucleated eyes had a ring of muscle tissue that was used to secure the eye to a copper post in the perfusion chamber using ethyl cyanoacrylate. The setup and execution of experiments were as described previously,²⁰ with minor modifications. Under microscopic guidance, the anterior chamber of mouse eyes was cannulated with a 33-gauge beveled tip needle (World Precision Instruments). The needle was mounted on a micromanipulator and connected to a 50-µL glass syringe on a syringe pump (model 33; Harvard Apparatus, Holliston, MA) and a pressure transducer (model 142PC01G; Honeywell, Fort Washington, PA) via pressure tubing and T-junctions. The syringe pump was computer controlled, delivering a variable flow rate to the anterior chamber to maintain a desired IOP as measured by the in-line pressure transducer. Custom-written LabVIEW software (National Instruments, Newbury, UK) was used to vary the flow rate from the syringe pump to maintain the eye at a desired pressure.²⁰

Eyes were perfused at four sequential pressure steps (4, 8, 15, and 20 mm Hg; equivalent to ~12, 16, 23, and 28 in vivo due to absence of episcleral venous pressure in enucleated eyes). The perfusion solution was Dulbecco's PBS including divalent cations and 5.5 mM glucose (DBG). Eyes were typically perfused for 20 minutes at each pressure in order to obtain approximately 10 minutes of stable flow data, and an average stable flow rate was calculated at each pressure step. Data were considered acceptable if a stable flow rate was achieved in at least three of the four pressure steps. Eyes were perfused in a closed humidified chamber at approximately 35°C, and careful attention was given to maintain hydration by covering the eye with a piece of Kimwipe (VWR) that was kept moist with drops of PBS. Heating of eyes during perfusion was accomplished by placement of the perfusion chamber (which contains a copper post at the center of a Lucite chamber) on a

FIGURE 2. Changes in electrical resistance of human SC monolayers treated with conditioned human retinal pigment epithelial media or purified PEDF. Three human SC cell strains were plated on Transwell filters at confluence. After 2 weeks, the filters were treated with (A) media conditioned by nonpigmented adult retinal pigment epithelial (RPE) cells (NP-CM) diluted 1:5 in 0.2% lactalbumin hydrolysate (LH) DMEM or (B) media conditioned by pigmented fetal RPE cells (P-CM) diluted 1:5 in 0.2% LH DMEM. The filters were tested at 0-, 24-, and 48-hour time points by transendothelial electrical resistance (TEER). (C) One SC cell strain was plated at confluence and then treated with 1 μ g/mL purified PEDF in 0.2% LH DMEM. TEER measurements were taken at 0, 24, and 48 hours. Shown are mean data at each time point $\pm$ SD. ** P < 0.01, *** P < 0.001.

heating pad. Selected eyes were fixed by removing the perfusion needle and immediately immersing the eye in 4% paraformaldehyde in isotonic saline.

We conducted three sets of perfusion experiments to measure the effects of a positive control (3,7-dithia PGE₁, PDA-205) and two concentrations of PEDF on conventional outflow facility in the mouse eye. Experiments were executed such that one eye was perfused with DBG containing drug and the contralateral eye with DBG containing drug vehicle. Eyes were perfused sequentially (one eye within an hour after enucleation, the contralateral eye ~3–4 hours after enucleation), where we randomized whether the left or right eye was experimental and whether the control or experimental eye was perfused first.

In the first set of experiments, we examined the effect of PDA (10 nM) on outflow facility. The PDA was dissolved in ethanol as a 10 mM stock solution and stored at -80°C . Since anterior chamber exchanges are not possible with the one-needle perfusion setup, experimental eyes were pretreated (perfused for 45 minutes at 8 mm Hg from a reservoir elevated 11 cm above the limbus) with 10 nM PDA in DBG. After pretreatment, the needle was placed in line with the syringe pump, which was then used to maintain designated pressure steps.

In the second and third set of experiments, we examined the effect of two different concentrations of PEDF (100 ng and 1 μ g/mL) on conventional outflow function. Recombinant PEDF (Millipore; 0.19 mg/mL in PBS) was diluted in DBG to make a stock solution of 1 μ g/mL. Experimental eyes were pretreated by perfusion as above with either 100 ng/mL or 1 μ g/mL PEDF in DBG. Control eyes were perfused with DBG alone, including pretreatment.

Outflow Facility Analysis

In order to calculate outflow facility (C), we used the modified Goldmann equation, $F = C(IOP) + F_u$ where F represents the flow rate at each pressure step (IOP) and F_u is an estimate of the pressure-independent outflow rate. Note that this equation is valid only when episcleral venous pressure is zero (as with ex vivo perfusion of enucleated eyes), F reaches equilibrium at each value of IOP , and C and F_u are independent of IOP . The values of C and F_u are calculated from the slope and intercept, respectively, of the best-fit linear regression.

Histology

Following perfusions, selected eyes were fixed by immersion in 4% paraformaldehyde. Eyes were then transferred to 4% glutaraldehyde for 3 hours, bisected from cornea to the optic nerve with crystalline lens removed, and embedded in paraffin (65 degrees polymerization). Using a microtome (Leica Reichert, Buffalo Grove, IL), sections were taken at a thickness of 0.5 μ m and mounted on slides. A Zeiss microscope (Cambridge, MA, numerical aperture, 0.35, working distance: 70 mm, A plane Ph1) equipped with digital camera was used to capture photomicrographs.

Statistical Analysis

All data sets included in this study contained some missing data due to one eye of a pair failing to pass the stability criterion. To analyze data, we performed two statistical analyses: a Student's t -test (paired, two-tailed) that included only the subset of paired contralateral eyes and a Student's t -test (paired, two-tailed) that included the full set of eyes, using imputation in order to replace the missing data and thus equal sample sizes. More specifically, for pairs in which one of the eyes failed, we used the average flow rates at each pressure for the relevant group overall as the imputed data. The statistical significance threshold was taken to be a P value of 0.05.

RESULTS

To examine concentration-related effects of recombinant PEDF on SC canal barrier function, changes in TEER of porcine aqueous plexus cell monolayers (porcine equivalent of human SC) were measured after 48 hours of treatment with PEDF (0.3–3 μ g/mL). Figure 1 shows that the highest dose of PEDF tested significantly increased TEER by ~50%, from 24 to 35 $\text{Ohm}\cdot\text{cm}^2$ ($P = 0.0006$). The 1 μ g/mL concentration of PEDF was almost as effective, increasing TEER to 32 $\text{Ohm}\cdot\text{cm}^2$ ($P < 0.0001$). In contrast, TEER of cells treated with the lowest dose (300 ng/mL) was not different from control ($P = 0.66$). We observed that TEER of controls exposed to serum-free media was stable over the 48-hour evaluation period.

Using a second model system, time-related effects of endogenous and recombinant PEDF on barrier function of human SC cell monolayers were examined. Figure 2A shows

FIGURE 3. Raw perfusion data for paired enucleated mouse eyes $\pm$ PEDF. Each eye was perfused at four sequential pressures (black traces, 4, 8, 15, and 20 mm Hg) until stability in flow rate was reached for approximately 10 minutes at each pressure (blue traces). (A) Eye perfused with vehicle (DBG, 5.5 mM glucose in PBS). (B) Fellow eye perfused with PEDF (1 μ g/mL). Red regions of traces show data used to determine average flow at given pressures. Paired data shown are representative of 14 independent experiments.

that TEER of mature monolayers of human SC cells treated with serum-free media containing 20% conditioned media from nonpigmented adult RPE cells was indistinguishable from controls in serum-free media. In contrast to AAP cells, TEER of human SC cells decreased over time in serum-free media. Returning cells into media containing 10% FBS restored baseline TEER (data not shown). Remarkably, treatment with serum-free media containing 20% conditioned media from pigmented fetal RPE cells dramatically stabilized TEER of human SC cells over time. While there was no statistically significant difference in TEER at 24 hours, we observed a significant difference at 48 hours (Fig. 2B, $P = 0.0003$) such that cells treated with pigmented media remained at a stable TEER. In the current study, TEER for the untreated controls decreased by just over half of original values over the course of the experiment (48 hours). Similar effects were observed when cells were treated with serum-free media containing 1 μ g/mL purified PEDF. Figure 2C shows that recombinant PEDF treatment of human cell monolayers increased TEER by 75.9% at 24 hours ($P = 0.0021$), and by 147.3% at 48 hours ($P = 0.0013$), compared to control. Additionally, compared to baseline, PEDF-treated cells had a 24.4% increase in TEER at 24 hours ($P = 0.0099$) and a 14.6% increase at 48 hours ($P = 0.0132$).

In order to test effects of PEDF in a model system containing intact conventional outflow tissues including the inner wall of SC, we perfused enucleated mouse eyes *in situ*. Cannulated eyes were perfused at constant pressure, where eyes were subjected to a series of four sequential pressure steps (4, 8, 15, and 20 mm Hg), and flow was measured. Figure 3 shows an example of raw perfusion data (pressure and flow recordings) for a set of paired mouse eyes, one perfused with vehicle (DBG, Fig. 3A) and the contralateral eye with PEDF (1 μ g/mL, Fig. 3B). Notice the dramatic reduction in flow in PEDF-perfused eyes at each pressure step.

All perfusion data for each experimental group were combined and compared by linear regression analysis of pressure–flow relationships. We examined effects of two concentrations of PEDF (100 ng/mL and 1 μ g/mL) and one concentration of PDA (10 nM), our positive control.²¹ The pressure–flow relationships for both experimental and control groups were linear over the pressures tested for each set of experiments, consistent with the modified Goldmann equation. Shown in Figure 4 are results from imputed paired experiments, where data were obtained from both eyes of the same mouse (experimental versus control). For example, Figure 4A shows that perfusion of eyes with 100 ng/mL PEDF caused a reduction in flow rate at each perfusion pressure. Conventional outflow facility (C , slope of the linear regression)

FIGURE 4. Pressure-flow relationships for enucleated mouse eye perfusions (imputed paired data). (A) Results (mean $\pm$ SD) from mouse eyes perfused with PEDF at 100 ng/mL or (B) PEDF at 1 μ g/mL concentration. (C) Data from mouse eyes perfused with EP4 receptor agonist (PDA-205) at 10 nM concentration (positive control). Slope of pressure-flow relationship equals estimate for outflow facility in presence or absence of drug.

decreased by $19.9 \pm 3.3\%$ (mean $\pm$ SD, $P = 0.003$, paired Student's *t*-test, $N = 11$ pairs) compared to that in paired contralateral eyes perfused without PEDF. We observed no statistical difference in the *y*-intercept (pressure independent outflow) of the linear regression in response to this dosage of PEDF ($P = 0.783$).

Data show that perfusion with 1 μ g/mL PEDF caused a greater reduction in flow rate at each perfusion pressure (Fig. 4B), compared to 100 ng/mL PEDF or vehicle control. Analyzing the slopes revealed that conventional outflow facility decreased by $43.7 \pm 19.1\%$ (mean $\pm$ SD, $P = 4.53 \times 10^{-5}$, paired Student's *t*-test, $N = 14$ pairs) compared to paired contralateral eyes perfused with vehicle. As before, we observed no statistical difference in the intercept of the linear

FIGURE 5. Relationship between outflow facility and PEDF concentration in perfused enucleated mouse eye pairs. Data show mean normalized outflow facility ($\pm$ SD) obtained from 11 pairs of mouse eyes perfused with the lower concentration (100 ng/mL) of PEDF and 14 pairs at the higher concentration (1 μ g/mL) compared to their contralateral eyes perfused with PBS. Data include both true pairs and imputed data. ** $P < 0.01$, *** $P < 0.001$.

regression in response to this dosage of PEDF ($P = 0.124$). A summary of all paired PEDF eyes perfused is shown graphically in Figure 5. Gross histological examination of eyes perfused with PEDF (1 μ g/mL) showed no obvious morphological differences of conventional outflow tissues from control (data not shown).

As a positive control, we perfused mouse eyes with an EP4 receptor agonist (PDA-205, 10 nM), observing an increase in flow rate at each perfusion pressure (Fig. 4C). Similar to data reported previously,²¹ PDA increased outflow facility by $99.6 \pm 20.4\%$ (mean $\pm$ SD, $P = 3.34 \times 10^{-5}$, paired Student's *t*-test, $N = 11$ pairs) compared to paired contralateral eyes perfused with vehicle. A summary of data obtained from all eyes perfused with PEDF or PDA is shown in the Table.

DISCUSSION

Using three complementary models from three different species, we demonstrate for the first time that PEDF significantly impacts the behavior of cells that regulate conventional outflow facility. We observed that PEDF increases transendothelial resistance of cultured porcine AAP cells and human SC cell monolayers. Pigment epithelium-derived factor decreased outflow facility in perfused enucleated mouse eyes by 44% at 1 μ g/mL concentration, the greatest acute increase in resistance for any substance reported to date. Taken together, results suggest that PEDF, as a constituent of aqueous humor, participates in the regulation of conventional outflow function.

Differentiated, pigmented RPE cell cultures secrete many proteins produced by RPE in vivo, including PEDF. Consistent with its role in supporting neighboring cells, including retinal endothelia, we observed that conditioned media from differentiated RPE stabilized barrier function of human SC cells while undifferentiated, nonpigmented RPE cells had no effect. Previous studies have demonstrated that 1-cm primary cultures of human fetal RPE secrete PEDF from the apical surface at a rate of approximately 30 ng/mL/h.⁷ By ELISA (ChemiKine; Millipore), we measured PEDF levels to be 3.38 ng/mL in our media supplemented with fetal pigmented RPE media and were unable to detect PEDF in the conditioned media from nonpigmented RPE cells. Concentrations of PEDF in aqueous humor in primate and human eyes have been estimated to range from 0.3 to 0.9 μ g/mL.^{13,17} However, it is likely that

TABLE. Summary of Ex Vivo Mouse Eye Perfusion Data

	Outflow Facility	SD		Outflow Facility	SD	<i>n</i>	<i>P</i>
PDA 10 nM			Control				
Paired	0.040	0.010		0.019	0.009	4	0.025*
Imputed paired	0.043	0.009		0.022	0.007	7	$3.34 \times 10^{-5***}$
PEDF 100 ng/mL			Control				
Paired	0.022	0.003		0.026	0.002	4	0.192
Imputed paired	0.021	0.004		0.027	0.002	7	0.003**
PEDF 1 µg/mL			Control				
Paired	0.013	0.008		0.027	0.008	6	0.002**
Imputed paired	0.016	0.007		0.029	0.008	8	$4.53 \times 10^{-5***}$

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. *P* values are for paired and imputed paired Student's *t*-tests (equal sample sizes).

multiple metabolic and pharmacokinetic factors regulate the bioavailability of PEDF in the posterior and anterior segments of the eye. In future studies, it will be interesting to determine how these factors interact to regulate ocular barriers.

Due to the presence and prominence of a true SC, ex vivo perfusion of mice represents an excellent model to study the role of SC's inner wall in outflow regulation.²⁰ Like that in humans, the mouse conventional outflow pathway has a linear pressure-flow relationship, does not "wash out" (decrease outflow resistance with time of perfusion), and is supported by ciliary muscle tension that keeps the SC lumen open.^{20,21} The mouse outflow pathway has a proportionally large SC lumen and inner wall compared to the human, suggesting that the inner wall plays a prominent role in outflow resistance generation.

A growing number of substances have been tested for effects on conventional outflow function; however, none have acutely decreased outflow facility as much as PEDF (by ~44%). Similar acute effects on outflow facility were observed with sphingosine 1-phosphate (S1P), decreasing outflow facility in humans by 36%²² and mice by 39%.²³ Interestingly, antagonizing S1P2 receptors increased outflow facility, suggesting an active role for endogenous mediators that control barrier function. It will be interesting to see whether antagonism of PEDF signaling increases outflow facility as well.

The acute effects of S1P and PEDF are in contrast to the decrease in outflow facility observed with chronic exposure of outflow tissues to TGF-β2 or corticosteroids. Perfusion of human eyes with TGF-β2 reduces outflow facility by 27% after a week, increasing accumulation of extracellular material under the inner wall of SC.²⁴ Similarly, dexamethasone treatment decreases outflow facility in both human and mouse eyes over a week's time, impacting extracellular matrix material accumulation, thickened juxtacanalicular tissue, and decreased intratrabecular spaces (Overby DR, unpublished observations, 2013).²⁵ Interestingly, dexamethasone treatment of TM cells induces PEDF secretion by 7-fold after 8 days.¹⁵ Since TM cells secrete PEDF, the local concentration of PEDF at the inner wall of SC is likely higher than concentrations in aqueous humor sampled upstream by paracentesis. Thus, dexamethasone effects on outflow facility (and thus IOP) are likely in part due to increased PEDF secretion at the level of the inner wall. Hence, corticosteroid treatment of human SC monolayers indeed increases barrier function, decreasing hydraulic conductivity of cell monolayers by 3-fold.²⁶

Physiologic levels of PEDF reported in aqueous humor are quite variable. For example, Ogata et al.¹⁶ reported that mean PEDF level in human aqueous was 860 ± 40 ng/mL, which is close to the concentration of PEDF we tested, and observed effects on outflow facility in situ. The authors went on to show that eyes with advanced glaucoma had a lower mean level of

PEDF (0.46 µg/mL) compared to cataract controls (0.86 µg/mL), suggesting that secretion of PEDF is decreased in glaucoma in attempt to compensate for decreased outflow function.²⁷ In another study, the level in aqueous humor from monkeys was 300 ng/mL.¹³ On the other end of the spectrum, Shin et al.²⁸ reported that mean PEDF level in human aqueous was 3.08 ± 1.89 ng/mL. It has also been shown in both humans¹⁶ and rats²⁹ that levels of PEDF in aqueous humor decrease with age. The variation in reported amounts is likely due to differences in the sensitivity and methodology of techniques used by different laboratories. In our hands and those of others, the detection of PEDF is sensitive to temperature, storage conditions, and protein binding, masking PEDF epitopes available for ELISA detection (Rogers ME and Stamer WD, unpublished observations, 2013; Becerra SP, written communication, 2013).

In conclusion, these studies demonstrate that PEDF, in physiologic concentrations, produces the greatest acute increase in aqueous outflow resistance of any biological substance that is currently known. Such findings argue for a role for PEDF, in conjunction with other agents, as a homeostatic modulator of normal aqueous humor outflow in the mammalian eye, which is primarily responsible for tightly controlling IOP throughout life. Just as PEDF may contribute to normal homeostasis, it may also play a role in pathological states, like glaucoma, especially considering its potency and effect on IOP. For example, cataract surgery in children increases risk of glaucoma in later years, almost exclusively in cases in which the entire lens and its capsule have been removed, a condition known as aphakia.³⁰ Modern procedures replace the cataractous lens with an intraocular lens (pseudophakia), which maintains the barrier between the vitreous and aqueous spaces and appears to markedly reduce the risk of glaucoma. Asrani and colleagues³⁰ hypothesized that biologically active substances present in the vitreous cavity enter the anterior chamber after the lens and capsule have been removed, increasing aqueous outflow resistance. Similarly, Chang³¹ observed that open-angle glaucoma risk significantly increases following uncomplicated pars plana vitrectomy (PPV) surgery in adults, where the vitreous humor is surgically removed. The risk of glaucoma is particularly increased where cataract surgery has been performed prior to, during, or after vitrectomy.³² These authors hypothesized that elevated pO₂ levels may be responsible; however, another explanation is chronic exposure of outflow tissues to increased levels of biologically active agents like PEDF arising from the posterior segment of the eye. Consistent with this idea is evidence that protein concentrations differ between the vitreous and aqueous humor in the eye, which may result from regional demands to maintain normal eye physiology.^{33,34} Further study of the role of PEDF signaling in health and disease is warranted,

particularly related to evaluating its therapeutic potential to modify outflow facility in those with ocular hypertension or hypotension.

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