Molecular Vision 2004; 10:837-44 <http://www.molvis.org/molvis/v10/a100>
Received August 25 2004 | Accepted 5 October 2004 | Published 8 November 2004

Effect of over-expression of pigment epithelium derived factor (PEDF) on developing retinal vasculature in the mouse

Wai T. Wong, Tonia S. Rex, Alberto Auricchio, Albert M. Maguire, Daniel Chung, Waixing Tang, Jean Bennett

F. M. Kirby Center for Molecular Ophthalmology, Department of Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA

Purpose: Pigment epithelium derived factor (PEDF) is a secreted protein with demonstrated anti-angiogenic properties, and with potential application for the treatment of neovascular disease. Delivery of pigment epithelium derived factor to the retina via virus mediated gene transfer has been shown to inhibit neovascularization in a number of experimental models. While pigment epithelium derived factor is endogenously expressed in the retina, its role in guiding normal vessel development and growth is not yet known. This study aimed to determine whether over-expression of pigment epithelium derived factor alters the normal pattern of retinal vessel development.

Methods: Neonatal (age postnatal day 2 (P2)) CD1 mice were injected subretinally unilaterally with AAV2/1.CMV.PEDF while contralateral eyes were injected subretinally with AAV2/1.CMV.EGFP as control. Cohorts of animals were sacrificed at P7 to P21 and the retinal vasculature was co-labeled through fluorescein-dextran perfusion and immunohistochemistry. Vascular size, localization, and structure were analyzed using light and confocal microscopy. Additional cohorts were use to obtain quantitative levels of pigment epithelium derived factor protein through ELISA.

Results: The extent of vessel growth from the optic disk to periphery over time (i.e., the radius of retinal vasculature), and the area of expansion of the neural retina were unaffected by over-expression of pigment epithelium derived factor to levels at least 3.5 fold higher than endogenous levels. The thicknesses of the various retinal layers were similar in AAV2/1.CMV.PEDF treated and control injected eyes. Three dimensional analysis of confocal images shows a slight delay in the rate of growth of vasculature into the deeper layers of the retina in pigment epithelium derived factor treated eyes compared to EGFP treated control eyes. However, the normal differentiation of vessels into arterioles, and venules, and the formation of a capillary network continued to occur, achieving normal and complete maturation of vascular structure by P21.

Conclusions: Over-expression of pigment epithelium derived factor in the developing retina exerted no marked or permanent effects on retinal vessel growth and differentiation. The findings are relevant to the safety of the potential therapeutic use of pigment epithelium derived factor in human retinal disease.

Pigment epithelium derived growth factor (PEDF) is a 418 amino acid, 50 kDa, secreted protein that is a member of the serpin superfamily [1]. Recent studies have demonstrated PEDF to be a protein with multiple biological activities. In addition to neurotrophic and neuroprotective properties, PEDF acts as a potent anti-angiogenic factor (reviewed by Tombran-Tink and Barnstable [2]). This anti-angiogenic property of PEDF has been well studied in the eye [3,4], where it is present endogenously in a variety of ocular tissues in both immature and adult animals [5,6].

The anti-angiogenic effects of PEDF have received much attention since this molecule has been found to inhibit the formation of aberrant new vessels in the animal models of pathological retinal neovascularization, including the laser photocoagulation model [7-9], the hyperoxia/hypoxia (retinopathy of prematurity (ROP)) model [7,10,11], and models in which neovascularization is induced by over-expression of stimulatory angiogenic factors, such as vascular endothelial growth factor (VEGF) [7,8]. The relevance of PEDF to human neovascular disease is also further suggested by studies showing that decreased levels of PEDF in the vitreous and ocular tissues are associated with proliferative diabetic retinopathy [12-14]; choroidal neovascularization in age related macular degeneration [15], and proliferative sickle cell retinopathy [16]. Together, these studies suggest that the loss of endogenous angiostatic activity through decreased levels of PEDF may play a role in the pathogenesis of neovascular ocular disease. As such, over-expression of PEDF may be a potential therapeutic modality in the treatment of neovascular disease in humans [17].

PEDF is expressed in the neural retina early in life in a developmentally regulated fashion in both mouse and human tissues [5,6]. Studies in the mouse show that the expression of PEDF in the ganglion cell layer is present near term (at embryonic day (E) 18.5 and P0) and increases in the first two postnatal weeks. Levels of PEDF then decrease slightly by adulthood [6]. This developmental onset/pattern of expression coincides with the timing of normal retinal vascular development in the mouse inner retina as characterized by Connolly et al. [18]. A three stage process has been described: in the

Correspondence to: Jean Bennett, F. M. Kirby Center for Molecular Ophthalmology, Department of Ophthalmology, Scheie Eye Institute, University of Pennsylvania, 310 Stellar-Chance Labs, 422 Curie Boulevard, Philadelphia, PA, 19104-6069; Phone: (215) 898-0915; FAX: (215) 573-7155; email: jebennet@mail.med.upenn.edu

first stage (P0-P10), there is radial growth of vessels from optic disk to the ora serrata in the presumptive nerve fiber layer; in the second stage (beginning at P4), vessels develop in the deeper retinal layer, while in the third stage (beginning at P7), vessel remodeling occurs to remodel an immature polygonal network of vessels into the final pattern of arterioles, venules, and capillaries, attaining an adult morphology by P14. The regulated expression of PEDF during the time of normal vasculature development suggests that it may play a developmental role. Transgenic mice deficient in PEDF have been reported to exhibit increased density of retinal microvessels, and a greater proportion of malpositioned vessels, indicating an endogenous role in regulating the amount of vasculogenesis taking place during development [19].

Studies demonstrating the anti-angiogenic properties of PEDF have additionally shown the specificity of this effect; i.e., that PEDF induces the regression of neovascular vessels but preserves the normal, previously existing vessels [3]. This selectivity is important in considering PEDF as an anti-angiogenic agent for therapeutic use. Although studies in which PEDF was over-expressed in developing systems have not reported any gross disruption in the structure of the retina or the retinal vasculature [7,10,11], the effect of PEDF over-expression on developing vasculature has not yet been studied directly and systematically.

In the present study, we address the question of the effect, if any, that PEDF over-expression may exert on the development of retinal vasculature. Our approach involves virus mediated delivery of the gene encoding PEDF to the retina at an early stage of vascular development followed by documentation of the morphological effects on retinal vasculature development. Our study aims to establish if developing retinal vessels are sensitive to elevated PEDF levels, and in doing so, shed light on the endogenous role of PEDF. We also aim to address the specificity of PEDF on the inhibition of pathological neovascularization in a developmental setting and what implications this may have on the use of PEDF for the treatment of human retinal neovascular diseases.

METHODS

Recombinant adeno-asssociated virus (AAV): Recombinant AAV2/1 (AAV serotype 2 genome packaged within an AAV1 serotype capsid) vectors were selected for study as they result in rapid onset and high levels of transduction after subretinal injection. AAV2/1 vectors were produced as described elsewhere (20) using either a pAAV2-CMV-PEDF plasmid (containing human PEDF cDNA originally provided by Noel Bouck, Northwestern University, Chicago, IL) or a pAAV2-CMV-EGFP plasmid. Titers were determined using real-time PCR and quality control was performed as described [20].

Subretinal injection of viral vector into neonatal mice: Animal experiments were performed in compliance with institutional guidelines for the care and treatment of experimental animals. Cohorts of neonatal (P2) CD1 mice were anesthetized by hypothermia. Injections of viral particles into the subretinal space (temporal retina) were performed as described [21]. All injections were performed by the same investigator (AMM) in order to minimize variability. A total number of 18 animals in 4 different litters were injected. Each mouse was injected with $6x10^9$ particles of AAV in a 0.5-1 µl volume; for each animal, AAV.CMV.PEDF was injected into the right eye (experimental eye), while that containing AAV.CMV.EGFP was injected into the left eye (control eye).

Tissue preparation: Cohorts of animals were euthanized at P7 (1 animal), P8 (2 animals), P9 (1 animal), P10 (2 animals), P12 (6 animals), P13 (3 animals), P14 (2 animals), and P21 (1 animal) for tissue analyses. These timepoints were selected in order to evaluate the effects of transgene expression on the 3 stages of retinal vascular development [18]. The animals were perfused via an intracardiac route with fluorescein isothiocyanate-dextran (20 mg/ml, product number FD150S, Sigma Chemical Co., St. Louis, MO) [11], and eyes were immediately enucleated. Retinae were rapidly dissected free from the choroid and sclera, and whole mounted on a glass slide. They were fixed briefly (3-5 min) in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS). Whole retinae were mounted with Vectorshield mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Transgene expression: Expression of PEDF was verified by RT-PCR as described [11] in an additional cohort of animals. Tissue from these animals was harvested 7 days after injection, when levels of AAV2/1 induced transgene expression should have peaked [11,20]. Levels of PEDF protein were quantified through enzyme linked immunosorbent assay (ELISA) using modifications of methods described by Duh et al. [22]. Briefly, anterior segments (including lenses) were dissected away from the enucleated eyes and eyecups were homogenized in 50 µl saline. Protein concentrations were measured via the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). PEDF concentrations were measured using a two antibody sandwich ELISA. Assays were performed in 96 well immunoplates. Each well was coated with 100 µl of a solution containing 2 µg/ml of a mouse monoclonal anti-PEDF antibody (Chemicon, Temecula, CA) in 0.1 M $NaHCO_3$ for 16 h at 4°C. The plates were washed and blocked with PBS/10% fetal bovine serum (FBS). After washing, experimental samples or standard solutions of recombinant human PEDF ((range 1 to 1,000 ng/ml), Chemicon) were added and the plate incubated for 1.5 h at room temperature. After washing, 100 µl of rabbit anti-PEDF polyclonal antibody (Research Genetics, Huntsville, Al) was added and incubated for 1 h at room temperature. After washing, donkey anti-rabbit antibody conjugated with horseradish peroxidase (0.5 µg/ml in PBS/10% FBS; Jackson Immunoresearch, West Grove, PA) was added and incubated at 1 h at room temperature. The wells were washed and an ABTS substrate/peroxide mixture (Pierce, Rockford, IL) was added for 25 min. The reaction was stopped with 100 µl of 1% sodium dodecyl sulfate. The plate was read using a Wallac microplate reader. The limit of sensitivity of the assay was 1 ng/ml. The readings from each of 9 experimental, and 9 control eyes were averaged, and levels were compared to those in the standard curve. Results are expressed as ng PEDF per mg total protein [22]. EGFP expression was identified microscopically by identifi-

cation of EGFP specific fluorescence. Cell specificity of expression was identified by corresponding the immunohistochemical signal with the well defined retinal cell layers.

Levels of vascular endothelial growth factor protein: Levels of VEGF were measured by ELISA using the Quantikine M Murine VEGF Immunoassay Kit (R and D Systems Inc., Minneapolis, MN) according to manufacturer's directions. The statistical significance of differential findings between experimental and control groups was determined by an unpaired Student's t-test and considered significant if two tailed p values were less than 0.05.

Immunohistochemistry: In order to specifically label and visualize the endothelial cells of developing blood vessels, conjugates of isolectin from *Griffonia simplicifolia* were used [23]. We used a tetrameric type 1 isolectin IB4 (isolectin GS-IB4) that had been conjugated to Alexa Fluor 568 (Molecular Probes, I-21412, Eugene, OR). After perfusion with fluores-

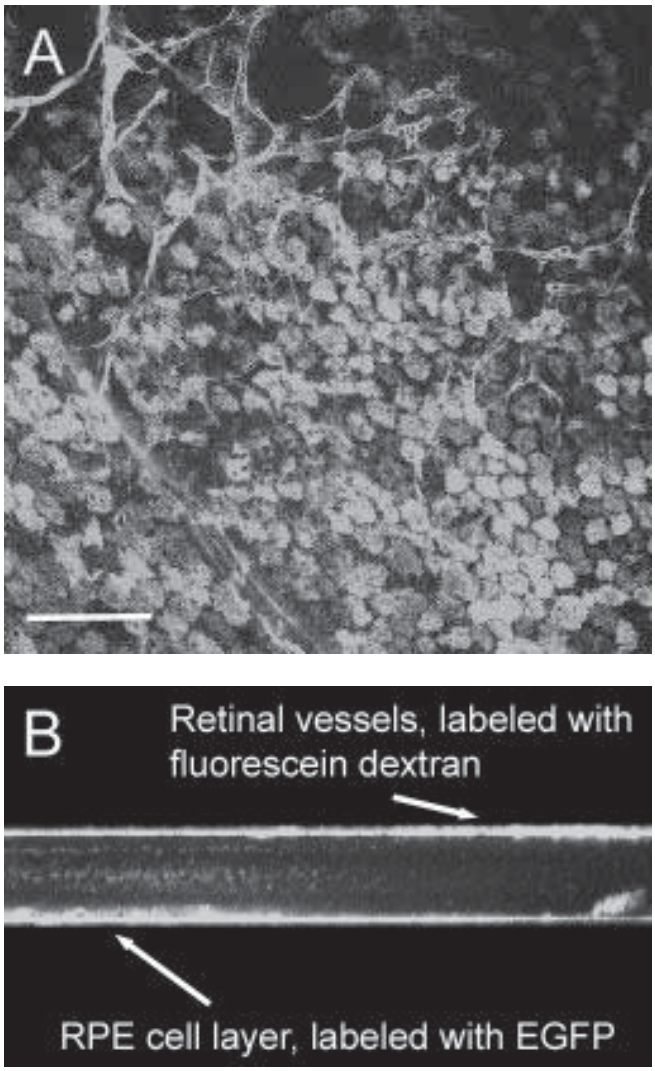

Figure 1. Robust expression of viral transgenes following subretinal injection AAV.. Confocal micrograph showing high levels of EGFP in the RPE cell layer of a P9 mouse that was injected subretinally with AAV2/1.CMV.EGFP at P2. **A**: An extended focus (merged z-stack) confocal image of this whole mount retina. The tissue was illuminated with the 488 nm line of the krypton-argon laser and the resulting emission viewed through a 522/35 nm filter. Retinal vessels are rendered visible by the perfusion of fluorescein dextran. Green fluorescence was clearly visualized in the cytoplasm of cells in the RPE. Scale bar represents 100 μm. **B**: The same z-stack of confocal images now viewed in cross-section. The fluorescent signal deep to the retina (indicated by the lower arrow) arises from the RPE layer. This layer is distinct from the fluorescence from the fluorescein labeled retinal vessels.

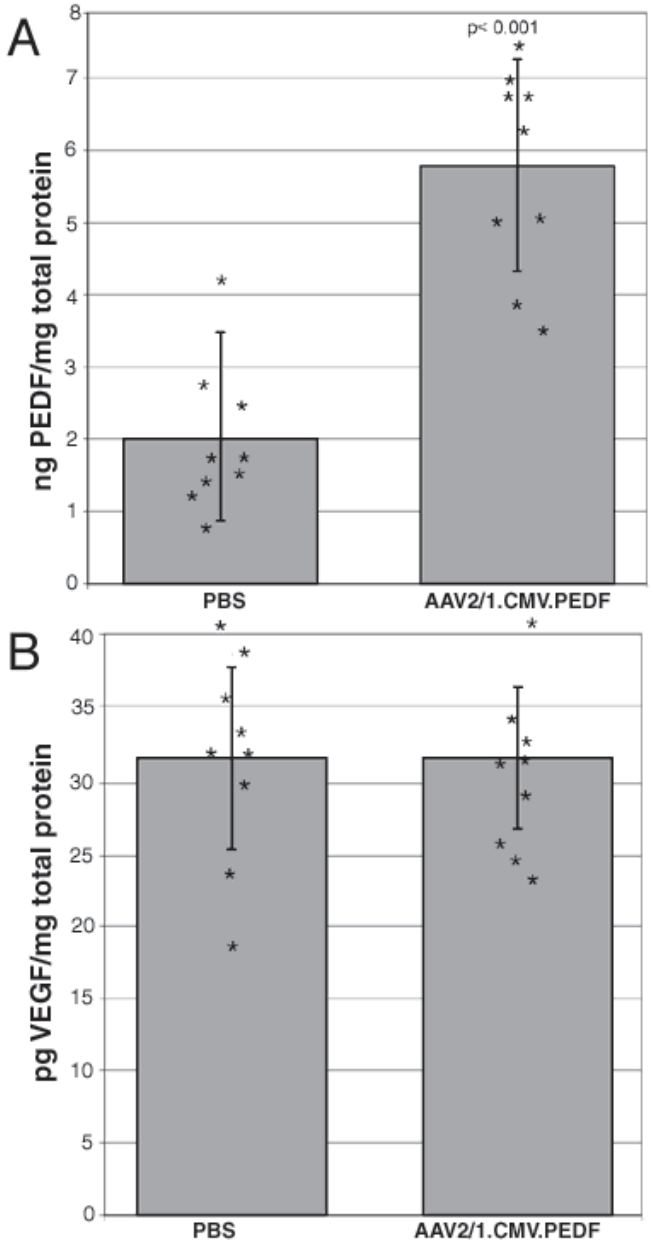

Figure 2. Significant increase in PEDF (but not VEGF) protein in AAV2/1.CMV.PEDF injected eyes. Levels of PEDF (**A**) and VEGF (**B**) protein were evaluated in cohorts of animals in which one eye had been injected with AAV2/1.CMV.PEDF and the contralateral eye injected as control. There was a significant increase in PEDF protein in AAV2/1.CMV.PEDF injected eyes whereas levels of VEGF did not change.

cein, whole retinae were fixed, washed, and incubated in conjugated GS-IB4, washed in phosphate buffer, and subsequently mounted on slides as described above.

Image analysis of retinal whole mounts: Fluorescein-perfused sections were visualized with fluorescence microscopy at low magnification and with fluorescence confocal microscopy at high magnification. Low magnification images were captured with a digital microscope camera (Axiocam, Carl Zeiss, Thornwood, NY) mounted on a Zeiss fluorescent dissecting microscope. Quantitative analyses of the images were performed using Axiovision software (Zeiss, Thornwood, NY). Length segments were measured in whole mount retinae in the calculations of average vascular radii and average retinal radii (see Results). Three to 9 individual measurements were made in different quadrants for each whole mount specimen. In addition, sections of the vascular tree were imaged at high magnification using confocal microscopy (Carl Zeiss laser scanning system; LSM 510). Stacks of images spanning the entire thickness of the vascular tree were obtained and 3 dimensional image analysis was performed using Zeiss LSM Image Browser image analysis software.

RESULTS

Expression of viral transgenes: Analyses of cohorts of animals injected with AAV2/1.CMV.PEDF at P2 showed high levels of expression within days of injection by RT-PCR [20]. Similarly, high levels of EGFP were noted in contralateral control injected eyes within 3 days after injection. Expression persists in a robust and stable fashion for the duration of the experiment (21 days for this study and for years in other studies; data not shown). As shown in Figure 1, transgene product was readily detectable in animals injected subretinally at P2 and then sacrificed and perfused with fluorescein dextran on P9. Confocal microscopy of whole mount retinal preparations demonstrates high levels of EGFP in retinal pigment epithelium (RPE) cells as evidenced by prominent fluorescence at 522 nm visible through a bandpass filter transmitting light with a window of 35 nm when the tissue was excited using the 488 nm line of the krypton-argon laser (Figure 1). Contralateral eyes injected with AAV.2/1.CMV.PEDF lacked EGFP induced fluorescence, as expected.

Levels of PEDF protein were evaluated by ELISA in cohorts of animals in which one eye had been injected with AAV2/1.CMV.PEDF and the contralateral eye injected as control. The average PEDF level in control eyes was 2.8±1.6 ng PEDF per mg total protein whereas that in AAV.2/1.CMV.PEDF injected eyes was 7.7±0.7 ng per mg total protein ($p<0.005$, Figure 2A).

ELISA analysis was also used to measure levels of VEGF protein. There was no significant difference between levels of VEGF in experimental or control eyes (Figure 2B).

Effect of PEDF over-expression on radial expansion in the developing retina: Whole mount retinae from animals aged P7-P14 that had been injected with experimental and control vectors were analyzed. We did not find a marked difference in the overall shape, size, and configuration in the vascular structures between treated and control eyes. There was also an absence of any asymmetry in the structure of vascular arbors corresponding to the location of the injection. The retinal whole mounts were of comparable area and thickness and showed normal lamination into cellular and plexiform layers. Figure 3 shows a composite picture consisting of images of whole mount retinae from the right (experimental) and left (control) eyes of a P10 animal in the series, demonstrating a qualitative similarity in vascular pattern between treated and control eyes.

In order to evaluate the effect of PEDF over-expression in a quantitative manner, we measured two parameters that reflected the overall radial growth of the vasculature and the neural retina: the average vascular radius, defined as the average distance measured from the optic nerve to the outermost edge of the expanding vascular tree, and the average retinal radius, defined as the average distance measured from the optic nerve to the outermost edge of the retina. An example of how these distances were measured is shown in Figure 3.

We compared the average vascular radius between the experimental (PEDF injected) and control (EGFP injected) eye in 12 pairs of eyes (ages P7-P14). There was no marked difference in the overall size of the vascular tree in any of the 12 pairs (Figure 4A). When the average vascular radius is plotted on a graph with postnatal age as the horizontal axis (Figure 4B), the values in both treated and control eyes increased as a function of postnatal age and there was no significant statistical difference between the two growth curves. When the aver-

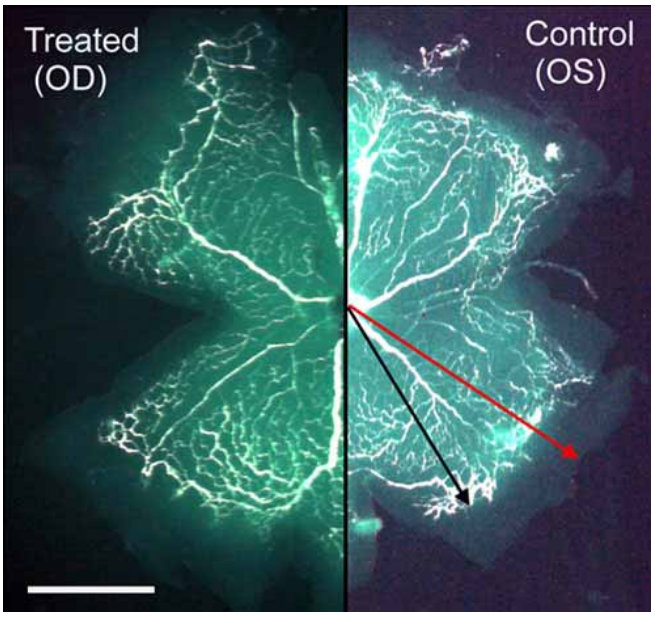

Figure 3. Effect of PEDF over-expression on radial growth of retinal vessels. Effect of PEDF over-expression on radial growth of retinal vessels. Composite fluorescent micrograph of a P10 whole mount retina, which had been injected subretinally at P2 with virus encoding PEDF in the right eye (OD, treated eye, left panel), and with virus encoding EGFP in the left eye (OS, control eye, right panel). Scale bar represents 1 mm. The red arrow indicates the distance measured in the calculation of the average vascular radius, the black arrow indicates that measured in the calculation of the average retinal radius.

age retinal radius was compared in the same way, there was again no significant difference between the growth curves in treated versus control eyes (Figure 4C). These data show that the over-expression of PEDF in the RPE layer during the time of early vascular development does not exert a significant effect on the symmetric outward radial extension of the vasculature. There is also no apparent effect on the radial expansion of the neural retina in the developing eyecup. In addition, the ratio between the average vascular radius to retinal radius was also not significantly different between the treated and control eyes (data not shown), even when measured separately at the 4 quadrants of the eyes.

Effect of PEDF Over-expression on Vessel Growth in Deeper Retinal Layers and on Vessel Differentiation: In order to examine the detailed structure of developing retinal vessels, we used 3 dimensional confocal microscopy to clearly characterize vascular structure at high magnification. We first labeled patent retinal vessels by intracardiac perfusion of fluorescein dextran. In order to visualize the structure of all vessels including nascent vessels that have not yet undergone cannulation to form patent lumina, we additionally labeled vascular endothelial cells immunohistochemically. Figure 4 shows an example of the detailed structure of the developing vasculature in en-face and cross-sectional views in representative eyes evaluated at P12. We observed that the vasculature of both experimental and control retinae undergoes differentiation into arterioles and venules; however, the rate of maturation of vessel structure from an immature polygonal pattern to the mature capillary structure was slightly delayed in retinas in which PEDF was over-expressed than in those of the control contralateral eyes (Figure 5A). The slight decrease in vessel differentiation was observed in 6 pairs of eyes. When cross-sectional images of the retinas were analyzed, we also observed a slightly decreased rate of vertical growth of vessels into the deeper levels of the retina in experimental compared to control samples. Fewer of the nascent vessels in the experimental retinae were observed to undergo cannulation and form patent lumina compared to the contralateral control eyes (Figure 5B). The decreased rate of vertical growth was observed in 3 separate pairs of eyes.

Effect of PEDF over-expression on final vasculature maturation: We examined the overall structure of vasculature trees when retinas over-expressing PEDF were allowed to fully develop and mature. Analyses of retinas isolated at P21 reveal that despite a slight delay in vertical growth and vessel differentiation at earlier ages (P12-P14), the vascular structure of PEDF treated retinas at P21 had undergone complete maturation into differentiated capillaries with normal stratification in deeper retinal layers (Figure 6). Similar results were observed in control (AAV2/1.CMV.EGFP injected) eyes (data not shown). Retinas were evaluated at P21, when retinal vasculature structure approximates that in the adult animal [18].

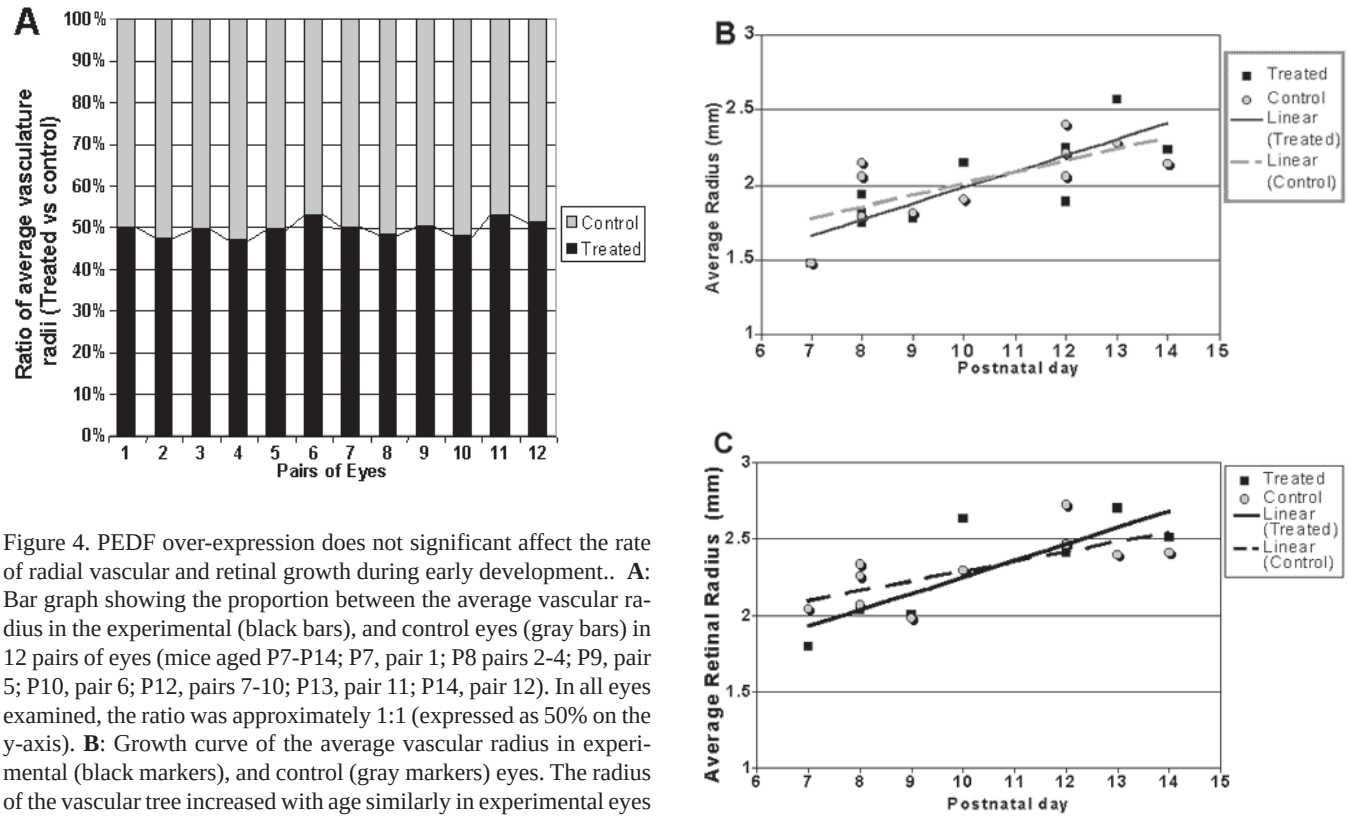

Figure 4. PEDF over-expression does not significant affect the rate of radial vascular and retinal growth during early development.. **A**: Bar graph showing the proportion between the average vascular radius in the experimental (black bars), and control eyes (gray bars) in 12 pairs of eyes (mice aged P7-P14; P7, pair 1; P8 pairs 2-4; P9, pair 5; P10, pair 6; P12, pairs 7-10; P13, pair 11; P14, pair 12). In all eyes examined, the ratio was approximately 1:1 (expressed as 50% on the y-axis). **B**: Growth curve of the average vascular radius in experimental (black markers), and control (gray markers) eyes. The radius of the vascular tree increased with age similarly in experimental eyes and control eyes (Student's t-test, 2 tailed; p=0.89). PEDF over-expression does not significantly inhibit the rate of radial vascular expansion. **C**: Growth curve of the average retinal radius in experimental (black markers) and control (gray markers) eyes. The radius of the neural retina increased with age similarly in experimental eyes and control eyes (Student's t-test, 2 tailed; p=0.80). PEDF over-expression does not significantly inhibit the rate of radial retinal growth.

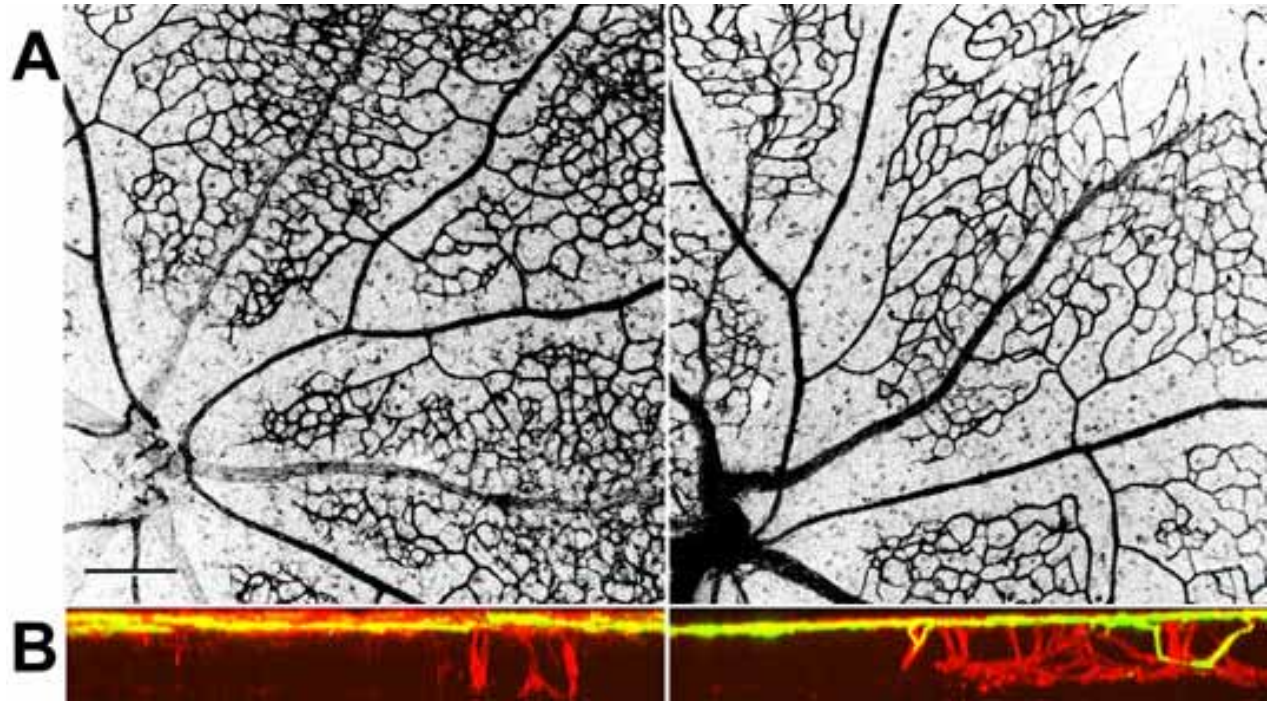

Figure 5. PEDF over-expression slows the rate of vessel differentiation and growth of vessels into the deeper layers of the retina. **A**: Extended focus confocal images of the structure of vascular trees in whole mount retinae from a P12 animal injected subretinally at P2. Vascular structures are visualized by immunohistochemistry with isolectin GS-IB4, which specifically labels endothelial cells. The retina injected with AAV2/1.CMV.PEDF (experimental eye) is shown on the left, and the fellow eye injected with AAV2/1.CMV.EGFP (control eye) is shown on the right. In both retinae, there is differentiation of vessels into arterioles (thinner vessels) and venules (thicker vessels). However, in the experimental eyes (left), the presumptive capillary structure retains a denser, more immature, polygonal vascular pattern compared to the control eyes (right). In the control eyes, there is a slightly sparser, more differentiated branching structure. **B**: The confocal cross-section reconstructions in the same retinae corresponding to those shown in the above in **A**. The yellow signal shows the distribution of fluorescein dextran after intracardiac injection, indicating the distribution of patent vessels in the vascular tree. The red signal shows the pattern of GS-IB4 labeling, indicating the structure of both patent vessels as well as nascent vessels that have not yet undergone cannulation. The experimental eyes (left) show a sparser distribution of nascent vessels in the deeper layers of the retina; many of these vessels have not yet undergone cannulation. However, in the control contralateral eyes, there is a greater number of vessels growing into the deeper layers with some vessels having already undergone cannulation.

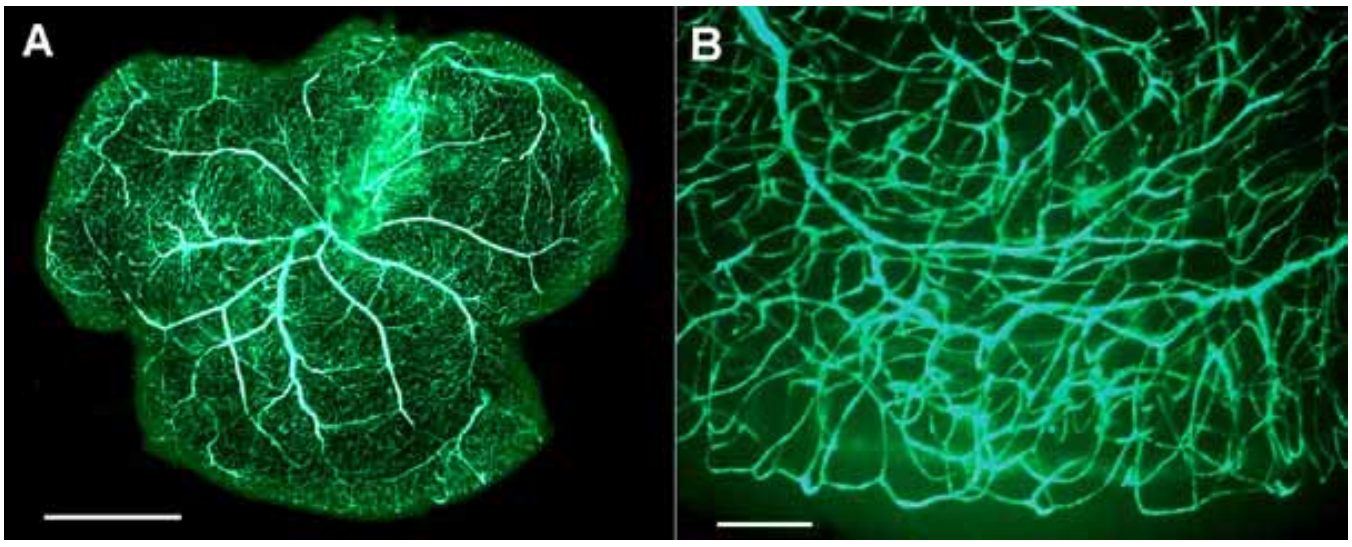

Figure 6. PEDF over-expression does not prevent eventual vessel development and differentiation from taking place. **A**: Whole mount retina from a P21 retina injected with AAV2/1.CMV.PEDF at P2. Scale bar represents 1 mm. **B**: High magnification of the same whole mount showing details in capillary structure. Scale bar represents 100 μm. Experimental retinas over-expressing PEDF continue to develop, attaining a mature capillary structure and normal stratification in deeper layers of the retina by P21.

DISCUSSION

Although previous studies in which PEDF protein or DNA encoding PEDF have been delivered to the retina at early developmental timepoints have not documented any gross disruptions in vasculature development (7,10,11,23), the present study is the first to directly examine the effect of over-expressing PEDF on the fine temporal-spatial aspects of retinal blood vessel development. This is important as the balance in PEDF levels with levels of other pro- or anti-angiogenic factors (such as VEGF) may account the normal vascular development of the eye [5,6]. Alterations of the PEDF:VEGF ratio may account for the pathological neovascularization seen in disease states such as diabetic retinopathy and choroidal neovascularization [7-16].

Our results indicate that over-expression of PEDF up to 3.5 fold in the RPE cell layer during vascular development does not exert any statistically significant effect on the rate of normal radial expansion of retinal vessels from center to periphery. There is also no significant effect on the rate of radial expansion of the neural retina. Various retinal cell layers were similar in thickness in experimental and control eyes providing further evidence that the neurotrophic activity of PEDF did not cause gross alterations in the outcome of the program of differentiation of retinal progenitor cells. The only effects detected in this study are confined to the slightly decreased rate of vessel growth into the deeper layers of the retina, and a decreased rate of maturation of nascent vessels from an immature polygonal architecture to the mature capillary structure. However, these differences disappear further along in development and retinas over-expressing PEDF display a normal mature vasculature tree at P21 that is similar morphologically to that of the control contralateral eye.

Our results indicate that while PEDF may play an endogenous developmental role as a neurotrophic factor in the retina, it is unlikely that the normal expansion and differentiation of the developing retinal vascular structure is permissively or quantitatively regulated by developmentally changing levels of PEDF. The slight transient delay in vertical vessel growth and differentiation indicates that while developing vessels may not be completely insensitive to PEDF, high levels of PEDF do not prevent normal and complete maturation of vessel growth from taking place. The absence of a significant effect seen here may arise from the selective action of PEDF that is confined to neovascular vessels. Over-expression of PEDF did not result in any significant alteration in levels of a known pro-angiogenic protein, VEGF. While it is possible that the over-expression of PEDF to levels higher than is possible using the present method may induce a more appreciable effect on developing vessels (and on VEGF levels), the level of expression using the present method is sufficient to significantly inhibit pathological neovascularization [11] without inducing developmental arrest. At present, the only viral vectors capable of transducing retinal cells and resulting in rapid onset of high levels of transgene expression are adenovirus, lentivirus and AAV2/1 [20]. We chose AAV2/1 due to its favorable characteristics with respect to inflammatory response and the ability to purify the virus from cellular contaminants. AAV2/1 targets RPE cells predominantly upon subretinal injection (and does not transduce retinal cells after intravitreal injection). PEDF is secreted by transduced RPE cells and is capable of diffusing across the retina as shown by its inhibitory effects on inner retinal neovascularization [11].

Is there an endogenous role of PEDF in regulating some aspect of retinal vessel development? Doll et al. [19] have reported that mice lacking PEDF develop retinas with increased microvascular density and aberrantly positioned vessels. It is possible that the endogenous levels of PEDF may suppress an abnormal proliferation of vessels. It is also possible that the regulatory effect of endogenous PEDF, in conjunction with additional signaling molecules, may not be so much to regulate per se the nature and extent of normal development, but more to confine vessel growth to permissive strata of the retina and prohibit vessels from crossing tissue boundaries (e.g., into the vitreous or across Bruch's membrane). A similar idea has been proposed for the inhibitory effect of cell growth mediated by PEDF in relation to the invasion of osteosaroma [24].

Experimentally induced high levels of PEDF in adult animals have been effective in suppressing neovascularization specifically without deleteriously affecting the structure of existing vessels [10]. The present study indicates that this specificity may be similarly confined to neovascular vessels in developing retinas. As such, these results lend support to the use of PEDF in a therapeutic context. In young animals, up to 3.5 fold increased levels of this protein do not cause significant changes in retinal neuronal cell differentiation patterns. Further, increased levels of PEDF do not significantly impair full maturation of the retinal vasculature. Finally, the general lack of toxicity of over-expression of PEDF at such a dynamic developmental timepoint implies that PEDF will not have significant toxic effect on the stable retinal vasculature present in the adult retina. These studies lend support to the notion that PEDF gene therapy appears to be safer than expected from the perspective that it does not interfere with normal developmental vascularization of the retina.

ACKNOWLEDGEMENTS

This study was funded by grants from the Juvenile Diabetes Research Foundation (JDRF grant 4-1999-840), a Foundation Fighting Blindness Center Grant, a Research to Prevent Blindness Lew Wasserman Merit Award (JB), the Macula Vision Foundation, the National Institutes of Health (NIDDK P30 DK47757; R01 EY12156), the Paul and Evanina Mackall Trust, and the F. M. Kirby Foundation.

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The print version of this article was created on 8 Nov 2004. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.