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Accessibility
Levels of vascular endothelial growth factor-A165b (VEGF-A165b) are elevated in experimental glaucoma

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**Purpose:** Although ischemia has previously been suggested to contribute to the pathogenesis of glaucoma, neovascularization is not implicated in glaucoma. Because vascular endothelial growth factor-A (VEGF-A) is a key mediator in neovascularization response, we investigated the levels of the major pro-angiogenic (VEGF-A164) and anti-angiogenic VEGF-A subtypes (VEGF-A165b) in the retina during experimental glaucoma.

**Methods:** Glaucoma was induced unilaterally in rats by injecting 1.9 M hypertonic saline solution in the episcleral veins. The contralateral eye served as the control. The intraocular pressure (IOP) of each eye was measured via Tonopen in conscious rats. Eyes were enucleated either on the 5th or the 10th day of elevated IOP. Whole retinal lysates were separated by SDS–PAGE and transferred to PVDF membranes. Levels of VEGF-A164 and VEGF-A165b were analyzed by western blotting using specific antibodies. In a different group of rats, retinal ganglion cells were retrogradely labeled by injecting Fluorogold in the superior colliculus a week before the induction of glaucoma. After the eyes were enucleated on the fifth day of elevated IOP, posterior eye cups were sectioned using a cryostat. Levels and localization of VEGF-A164 and VEGF-A165b were examined in retinal sections by immunohistochemistry.

**Results:** VEGF-A164 levels remained unchanged between the control and glaucomatous retinas after five days (p=0.341) and 10 days of elevated IOP (p=0.117). The presence of the anti-angiogenic VEGF-A isoform has not been previously reported in the rat. An antibody specific to VEGF-A165b detected the anti-angiogenic protein in the rat retina. VEGF-A165b levels were significantly increased (2.33±0.44 fold, p=0.014) in the glaucomatous retinas compared to those in controls after five days of elevated IOP. VEGF-A165b levels were not different (p=0.864) between the control and glaucomatous retinas following 10 days of elevated IOP. Expression of both VEGF-A164 and VEGF-A165b were observed in the retinal ganglion cells (RGC) and inner nuclear layer (INL).

**Conclusions:** Five day elevation of IOP leads to an increase in the anti-angiogenic VEGF-A165b levels but not in the pro-angiogenic VEGF-A164 levels in the glaucomatous retina. VEGF-A165b levels return to baseline after 10 days of elevated IOP, and VEGF-A164 levels remain unchanged. We speculate that the short-term elevation of VEGF-A165b levels and/or the unchanged levels of VEGF-A164 contribute to the lack of neovascularization in the glaucomatous retina.

Glaucoma is a neurodegenerative disease of retinal ganglion cells (RGC) that leads to blindness. Although the most prominent risk factor for RGC death in glaucoma is elevated intraocular pressure (IOP), the sequence of events by which IOP causes RGC death still remains largely unknown. One possible mechanism that elevated IOP can induce abnormalities in blood flow in the glaucomatous eye. In open-angle glaucoma patients, abnormal vascular autoregulation has been observed in the inferior temporal retinal artery, the central retinal artery, the circulation of the optic nerve head, the choroid, and the perifoveal macular capillaries [1-8]. It has been suggested that dysregulation of blood flow may lead to decreased vascular perfusion in the retina and in the optic nerve head, resulting in an hypoxic response [9,10].

In the classical view of hypoxia, the ischemic tissue compensates for a decrease in oxygen levels by forming new blood vessels, a process known as neovascularization [11]. VEGF-A is a key mediator in neovascularization in ischemic retinopathies [12-14]. There are several VEGF-A isoforms expressed from a single gene via alternative splicing [15,16]. Among these, VEGF-A165 is the most abundantly expressed pro-angiogenic isoform in the retina [17]. More recently, anti-angiogenic sister isoforms of VEGF-A have also been identified [18-20]. For example, VEGF-A165b, an anti-angiogenic human VEGF-A isoform, has been shown to inhibit VEGF-A induced neovascularization in the mouse retina following ischemia [21].

There are only a few studies that have examined VEGF-A in glaucoma. VEGF levels were shown to be increased in the plasma of glaucoma patients when compared to that of healthy controls [22] and in the aqueous humor of glaucoma patients when compared to their plasma VEGF levels [23]. Despite these findings, neovascularization is not implicated...
in glaucoma, and the role of VEGF-A has not been examined in the glaucomatous retina.

If ischemia contributes to the pathogenesis of glaucoma, why is there no neovascularization in glaucoma? To answer this apparent paradox, we investigated the levels of pro-angiogenic VEGF-A165 (the rat version of VEGF-A164) and anti-angiogenic VEGF-A165b (the rat version of VEGF-A165b) in normal and glaucomatous retinas after a short-term (five day) and an intermediate-term (10 day) elevation of IOP. Because of the lack of neovascularization in glaucoma, we hypothesized that the levels of VEGF-A165b but not VEGF-A164 would be increased in the glaucomatous retina.

METHODS

Subjects: Male rats (retired breeder Brown Norway; 300-450 g; n=16) were used for the study. Rats had ad libitum access to food and water during the study and were kept on a 12 h illumination cycle. All animal related procedures were performed in accordance with the statement for the use of animals in research released by the Association for Research in Vision and Ophthalmology.

Retrograde labeling of retinal ganglion cells: Rats (n=4) were anesthetized with an intraperitoneal injection of 1.5 mg/kg of acepromazine maleate, 7.5 mg/kg of xylazine, and 75 mg/kg of ketamine (Webster Veterinary Supply, Sterling, MA). Following shaving of the head, each rat was placed in a stereotaxic instrument. The skin covering the skull was incised along the midline using a surgical blade, and the skull was exposed and leveled. Next, for each hemisphere, a 30-gauge stainless steel needle was lowered into the superior colliculus at 5.3 mm posterior to the bregma, 1.5 mm lateral. The needle was advanced 4.8 mm ventral to the skull surface. Using a 1.0 ml syringe (Hamilton, Reno, NV), 2 μl of Fluorogold solution (3% in PBS with 10% DMSO; Fluorochrome, Denver, CO) was injected over 10 min into each hemisphere. Following the injections, the skin was sutured. Rats were allowed to recover for a week before glaucoma was induced experimentally.

Experimental induction of glaucoma: To elevate IOP, hypertonic saline solution (1.9 M) was unilaterally injected in the episcleral veins as described by Morrison and colleagues [24]. The contralateral eye of the rat served as the control. A maximum number of three injections that were two weeks apart were performed in the absence of IOP elevation. Rats that did not have an elevation of IOP after the third surgery were excluded from the study.

Intraocular pressure measurements: IOPs were measured with a TonoPen XL tonometer (Medtronic Ophthalmics, Jacksonville, FL) in conscious rats [25]. Measurements were taken between 10 AM and 2 PM. Before the first hypertonic saline injection, baseline IOPs for both eyes were measured for each rat. Following glaucoma inducing surgery, IOPs were measured three times a week. On each measurement day, an average of 15 readings was calculated for each eye. This study investigated a five day (n=6) and a 10 day elevation of IOP (n=6).

Tissue preparation: Rats were sacrificed by CO2 inhalation either after five days or 10 days of elevated IOP. For western blotting, retinas were isolated from eyes obtained after five days and 10 days of elevated IOP (six pairs each). Retinas were placed in 200 μl of 1 mM of EDTA/EGTA/DTT, 10 mM of Hepes (pH=7.6), 0.5% Igepal (Sigma Chemical Co., St. Louis, MO), 42 mM of KCl, 5 mM of MgCl2, 1 mM of PMSF, and a tablet of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). After retinas were sonicated and incubated for 15 min on ice, samples were spun at 21,000 rpm at 4 °C for 30 min. Retinal proteins were quantified by spectrophotometry using the Bio-Rad D, Protein Assay (Bio-Rad Laboratories, Hercules, CA).

For immunohistochemistry, four pairs of eyes that were enucleated after five days of elevated IOP were fixed with 4% paraformaldehyde for 20 min at room temperature. These eyes were previously back-labeled with Fluorogold. Next, the posterior eye cups were isolated and fixed with 4% paraformaldehyde for an additional 40 min at room temperature. After posterior eye cups were cryoprotected overnight in graded sucrose dilutions, they were placed in the optimal cutting temperature compound (Tissue-tek, Miles Diagnostik Division, Elkhart, IN) and were sectioned 16 μm thick using a cryostat.

Western blotting: Retinal proteins isolated after either five days or 10 days of elevated IOP were separated on Tris-HCl Ready-Gels (Bio-Rad Laboratories, Hercules, CA). Recombinant rat VEGF-A164 protein (25–250 ng; R&D Systems, Minneapolis, MN) was also loaded as a positive control in certain experiments. Proteins separated by SDS–PAGE were then transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA) for 1 h. After the membrane was blocked for 1 h at room temperature with 2% ECL Advance Blocking Agent (GE Healthcare, Piscataway, NJ) in Tris-buffered saline with Tween (TBS-T), it was incubated at 4 °C overnight with a primary antibody. The primary antibodies used in this study were as follows: rabbit polyclonal anti-VEGF (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-VEGF165B (1:1,000; Abcam, Cambridge, MA), and mouse monoclonal anti-α-tubulin (1:100,000; Sigma, Saint Louis, MO). The next day, membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. Goat anti-rabbit IgG (1:10,000–1:40,000; Jackson ImmunoResearch, West Grove, PA) and goat anti-mouse IgG (1:20,000–1:100,000; Jackson ImmunoResearch) were the secondary antibodies used in the study. Both of these antibodies had minimal cross-reaction to rat serum proteins. Next, membranes were processed with ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ) and
Levels of VEGF-A164 retina: The transparency to see the overlap of different fluorophores in a given section was achieved using a BX51 microscope to image the retinal sections for fluorescence. We used Alexa Fluor 594. We employed DPController 1.2.1.108 (Olympus) for Fluorogold and N41004 HQ Texas Red (Chroma) for each fluorophore. The excitation/emission filter cubes used for a given protein were 11006v2 Gold (Chroma, Rockingham, VT) respectively. Excitation/emission filter cubes used for a given protein were 11006v2 Gold (Chroma, Rockingham, VT) for Fluorogold and N41004 HQ Texas Red (Chroma) for Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1:500; Abcam). The staining was visualized using an Olympus BX51 microscope (Olympus, Center Valley, PA).

On our BX51 microscope, we used UPlanApo 0.70 NA 20X (Olympus) and UPlanApo 0.85 NA 40X (Olympus) objective lenses through a 10X ocular or camera lens to image our retinal sections for a total magnification of 200X or 400X, respectively. Excitation/emission filter cubes used for a given fluorophore were 11006v2 Gold (Chroma, Rockingham, VT) for Fluorogold and N41004 HQ Texas Red (Chroma) for Alexa Fluor 594. We used DPController 1.2.1.108 (Olympus) in conjunction with the DP70 color camera (Olympus) affixed to the BX51 to image the retinal sections for fluorescence. We then used Adobe Photoshop to layer the images and apply transparency to see the overlap of different fluorophores in a given section area.

**RESULTS**

Levels of VEGF-A164 do not change in the glaucomatous retina: Average peak IOP was (mean±SEM) 40.7±1.9 mmHg and 43.1±0.8 mmHg for the five-day (n=6) and 10-day (n=6) groups that were used in the western blot (WB) analysis, respectively. Average peak IOP was 43.4±0.8 mmHg for the other five-day group used for the immunohistochemistry (IHC) analysis (n=4).

In western blots, anti-VEGF antibody detected a 45 kDa band corresponding to the VEGF-A164 dimer in all retinas and in the positive control brain (Figure 1). VEGF-A164 levels remained unchanged between the control and glaucomatous retinas after five days (p=0.341, n=6; Figure 1A,C) and 10 days of elevated IOP (p=0.117, n=6; Figure 1B,C). Using this well-characterized antibody [26,27], the VEGF-A164 monomer was not detected in the retina or in the brain.

In VEGF-A164 IHC, there was some nonspecific staining in the blood vessels in the RGC layer and INL of the negative control retinas (Figure 2B,C). VEGF-A164 staining did not differ between the normal (Figure 2E) and glaucomatous retinas (Figure 2H). VEGF-A164 expression was localized to the RGC and to the cells in the INL of both groups (Figure 2E,H). In the RGC layer, VEGF-A164 staining colocalized with the retinal ganglion cell marker, Fluorogold (Figure 2F,I). Also, VEGF-A164 levels did not differ between the normal and glaucomatous retinas, which are consistent with the WB results.

Anti-VEGF-A165b antibody does not recognize VEGF-A164: Because it had been predicted that VEGF-A164 and VEGF-A165b are highly homologous in amino acid sequence [18], we first investigated whether the anti-VEGF-A165b antibody would also recognize VEGF-A164. To test this possibility, we immunoblotted different concentrations (25 ng, 100 ng, and 250 ng) of the recombinant rat VEGF-A164 protein with the anti-VEGF-A165b antibody. Whereas the anti-VEGF-A165b antibody did not recognize the VEGF-A165b protein at any concentration, it recognized two bands around 22.4 and 45 kDa in a pair of control and glaucomatous retinas corresponding to the monomer and dimer forms of VEGF-A165b, respectively (Figure 3A). Next, we stripped the...
membrane and subsequently immunoblotted with the anti-VEGF antibody. A 22.4 kDa VEGF-A<sub>164</sub> monomer was detected at all concentrations (Figure 3B), confirming the presence of VEGF-A<sub>164</sub> recombinant protein in the same membrane. The dimer form was not observed with the recombinant rat VEGF-A<sub>164</sub> protein. The combination of these findings indicated that the anti-VEGF-A<sub>165b</sub> antibody does not recognize VEGF-A<sub>164</sub> and that VEGF-A<sub>165b</sub> is expressed in the rat retina.

**VEGF-A<sub>165b</sub> levels are increased in the glaucomatous retina:** Following five days of elevated IOP, the anti-VEGF-A<sub>165b</sub> antibody detected bands around 22.4 and 45 kDa in all retinas, which represent the monomer and dimer forms of VEGF-A<sub>165b</sub>, respectively (Figure 4A). Whereas VEGF-A<sub>165b</sub> dimer levels remained unchanged between the control and glaucomatous retinas (p=0.273, n=6), VEGF-A<sub>165b</sub> monomer levels were significantly increased in the glaucomatous retinas compared to those in controls (2.33±0.44 fold, p=0.014, n=6) (Figure 4A,B). However, following 10 days of elevated IOP, there was no change in levels for the VEGF-A<sub>165b</sub> dimer (p=0.483, n=6) or for the VEGF-A<sub>165b</sub> monomer (p=0.864, n=6) between the control and glaucomatous retinas.

**Figure 2.** Immunohistochemical analysis of VEGF-A<sub>164</sub> expression in the glaucomatous retina after five days of elevated IOP. **A-C:** Negative control. Some non-specific staining of blood vessels in the RGC and the INL was observed. **D-F:** VEGF-A<sub>164</sub> staining of the normal retina (n=4). VEGF-A<sub>164</sub> was present in the RGC and the INL. **G-I:** VEGF-A<sub>164</sub> staining of the glaucomatous retina (n=4). Staining was detected in the RGC and INL. VEGF-A<sub>164</sub> levels did not differ between the normal and glaucomatous retinas.

**Figure 3.** Anti-VEGF-A<sub>165b</sub> antibody does not recognize VEGF-A<sub>164</sub>. **A:** Incubation with VEGF-A<sub>165b</sub> antibody. This antibody recognizes the VEGF-A<sub>165b</sub> monomer (22.5 kDa) and dimer (45 kDa) in control and glaucomatous retinas (first two lanes). VEGF-A<sub>165b</sub> antibody does not recognize 25 ng, 100 ng, or 250 ng of VEGF-A<sub>164</sub> recombinant protein in the same membrane (last three lanes). **B:** Incubation of the same membrane with the anti-VEGF antibody after stripping. The anti-VEGF antibody recognizes VEGF-A<sub>164</sub> recombinant protein at all concentrations.
A positive control was the brain while the negative control was VEGF-
comparable levels in the control and glaucomatous retinas. The
IOP. Both 22.5 kDa and 45 kDa VEGF-A
densitometry readings in the retina following 10 days of elevated
expression following five days of elevated IOP. Retinal VEGF-A
monomer and dimer were detected at 22.5 and 45 kDa, respectively. B: Glaucomatous/control
eratio of normalized VEGF-A165b densitometry readings in the retina
following five days of elevated IOP. Expression of the 22.5 kDa
VEGF-A165b was increased significantly in the glaucomatous retinas
compared to the controls. C: VEGF-A165b expression following 10
days of elevated IOP. VEGF-A165b monomer and dimer were
observed at 22.5 and 45 kDa in the retina, respectively. D: Glaucomatous/control ratio of normalized VEGF-A165b
densitometry readings in the retina following 10 days of elevated
IOP. Both 22.5 kDa and 45 kDa VEGF-A165b were expressed at comparable levels in the control and glaucomatous retinas. The
positive control was the brain while the negative control was VEGF-
A164 recombinant protein. The loading control was α-tubulin.

**DISCUSSION**

We demonstrate in this report that VEGF-A165b is present in
the retina and localized primarily to the RGC layer and the
inner nuclear layer. Our findings for VEGF-A165b show a
distribution similar to that seen for VEGF-A164 in this report
and previous reports about VEGF-A164 by others [28,29].
Using back labeling techniques, we find that RGC express
VEGF-A165b. Our data show that VEGF-A165b levels are
increased early in the cause of experimental glaucoma but
return to baseline at a later time point. IHC results show that
this increase is primarily due to increased expression in the
RGC layer and in the INL.

Our results demonstrate that the levels for the pro-
angiogenic VEGF-A164 do not change in the glaucomatous
retina compared to control retinas in the rat after five days or
10 days of elevated IOP. Consistent with previous studies, we
observe that VEGF-A164 is expressed in the RGC and INL of
the retina [28,29].

VEGF-A165 is the most abundantly expressed pro-
angiogenic isoform in the retina [17]. Both VEGF-A165 and
VEGF-A165b mRNA are produced from the VEGF-A pre-
mRNA via alternative splicing [15,18]. VEGF-A165 and
VEGF-A165b share a 96.4% homology and differ only in the
last six amino acids in their amino acid sequence in humans
[18]. However, while VEGF-A165 is pro-angiogenic, VEGF-
A165b has an inhibitory effect on angiogenesis both in vitro and
in vivo [18,19]. For example, VEGF-A165b inhibits
neovascularization in the mouse retina following oxygen-
induced retinopathy [21]. More recently, other inhibitory
splice variants of VEGF-A have also been identified [19,20].
It has been suggested that the relative levels of the pro-
angiogenic and anti-angiogenic VEGF-A isoforms determine
whether angiogenesis will be stimulated or inhibited in a tissue
[20]. For instance, the expression of the pro-angiogenic
VEGF-A isoforms increases in the vitreous of human patients
with diabetic retinopathy whereas the expression of the anti-
angiogenic VEGF-A isoforms remains unchanged compared
to the normal vitreous [20]. Among the anti-angiogenic
VEGF-A isoforms, VEGF-A165b is observed to be the
dominant isoform [15,18].

What molecular mechanism is responsible for the
upregulation of VEGF-A165b mRNA in the glaucomatous
retina? Although the precise answer remains unknown,
proposed mechanisms include differential promoter selection,
alternate regulation of mRNA stability, and regulation of
alternative splicing [15,30-33]. In alternative splicing, as the
VEGF-A gene is being transcribed, the emerging pre-mRNA
is instantaneously processed by several RNA-binding proteins
and splice factors [15]. These proteins bind to the auxiliary
sequences on the pre-mRNA and determine which exons will
be spliced [34]. It is thought that several signal transduction
pathways, which are activated in response to changes in the
environment (e.g., receptor-mediated pathways, neuronal
activity, cellular stress-like hypoxia) affect alternative
splicing by altering the relative levels of RNA-binding proteins and splice factors or the localization of splice factors

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*Image 54x256 to 297x463*
within the cell [15,35,36]. More recently, microRNAs have also been shown to alter alternative splicing [37]. For example, in muscle and neuronal development, microRNAs lead to the inclusion of alternative exons by suppressing a repressor protein of alternative splicing [38,39]. In addition, transcriptional events may also affect the regulation of alternative splicing. For instance, the speed of RNA polymerase II can influence the choice of splice sites and recruitment of regulatory factors [40].

In conclusion, we report an increase in the retinal levels of the anti-angiogenic VEGF-A\textsubscript{165b} but not the pro-angiogenic VEGF-A\textsubscript{164} in our experimental glaucoma model. The combination of these findings suggests that the elevation of VEGF-A\textsubscript{165b} levels and/or the unchanged levels of VEGF-A\textsubscript{164} contribute to the lack of neovascularization in the retina in glaucoma.

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