Endogenous VEGF Is Required for Visual Function: Evidence for a Survival Role on Müller Cells and Photoreceptors

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Abstract

Background: Vascular endothelial growth factor (VEGF) is well known for its role in normal and pathologic neovascularization. However, a growing body of evidence indicates that VEGF also acts on non-vascular cells, both developmentally as well as in the adult. In light of the widespread use of systemic and intraocular anti-VEGF therapies for the treatment of angiogenesis associated with tumor growth and wet macular degeneration, systematic investigation of the role of VEGF in the adult retina is critical.

Methods and Findings: Using immunohistochemistry and Lac-Z reporter mouse lines, we report that VEGF is produced by various cells in the adult mouse retina and that VEGFR2, the primary signaling receptor, is also widely expressed, with strong expression by Müller cells and photoreceptors. Systemic neutralization of VEGF was accomplished in mice by adenoviral expression of sFlt1. After 14 days of VEGF neutralization, there was no effect on the inner and outer retina vasculature, but a significant increase in apoptosis of cells in the inner and outer nuclear layers. By four weeks, the increase in neural cell death was associated with reduced thickness of the inner and outer nuclear layers and a decline in retinal function as measured by electrotretinograms. siRNA-based suppression of VEGF expression in a Müller cell line in vitro supports the existence of an autocrine role for VEGF in Müller cell survival. Similarly, the addition of exogenous VEGF to freshly isolated photoreceptor cells and outer-nuclear-layer explants demonstrated VEGF to be highly neuroprotective.

Conclusions: These results indicate an important role for endogenous VEGF in the maintenance and function of adult retina neuronal cells and indicate that anti-VEGF therapies should be administered with caution.

Introduction

The retina is one of the body’s most metabolically demanding tissues. To ensure adequate nutrient and oxygen delivery, the retina is supplied by two independent vascular beds, the inner retinal vasculature and outer choroidal circulation. These vascular beds have distinct morphologic properties; the inner retinal vasculature is the site of a blood-tissue barrier and is characterized by highly impermeable vessels, whereas the choriocapillaris consists of a highly fenestrated capillary plexus. Vascularization of the retina (reviewed in [1]) occurs late in gestation and is restricted to the inner retina. During development, vascular endothelial growth factor (VEGF) is expressed by astrocytes in the retinal ganglion cell layer (GCL), by cells of inner nuclear layer (INL), Müller cells, and retinal pigment epithelial cells (RPE) [2,3]. Targeted deletion of VEGF in the RPE results in failure of choroidal development and loss of visual function [4]. Whereas formation of the choriocapillaris appears to be independent of hypoxia [4], formation of the inner retinal vasculature requires induction of the hypoxia inducible factor-1α [5]. Hypoxia during retinal development suppresses VEGF production resulting in reduced vascular development [5], as is seen in retinopathy of prematurity [6]. Furthermore, normal retinal vascularization requires the coordinated expression of specific VEGF isoforms, which differ in their solubility and binding to VEGF receptors [7].

In addition to its role in developmental retinal vascularization, VEGF is upregulated in several ocular pathologies, including wet age related macular degeneration (AMD) [8] and proliferative diabetic retinopathy [9]. In AMD, injury to the RPE is thought to result in increased VEGF expression. Coupled with damage to Bruch’s membrane, this leads to proliferation of choroidal vessels, which invade the subretinal space [10]. In proliferative diabetic retinopathy, the neovascularization that arises from the inner retina is mediated by hypoxia-induced VEGF (reviewed in [11]).
The involvement of VEGF in AMD has led to the Food and Drug Administration approval of two intraocular anti-VEGF drugs, pegaptanib sodium (Macugen, OSI/Eyetech Pharmaceuticals, NY) [12], an aptamer which was reported to inhibit only VEGF165, and ranibizumab (Lucentis, Genentech) [13], a Fab fragment of the humanized monoclonal VEGF antibody bevacizumab (Avastin, Genentech) which neutralizes all VEGF isoforms. While the role of VEGF in developmental and pathologic retinal vascularization is well understood, its function in the normal adult is unclear. We and others have previously reported that VEGF is expressed in the adult retina by RPE [3] as well as by neuronal and glial cells [14,15,16,17]. Although initially thought to be endothelial-specific, VEGF has been shown to target a variety of non-vascular cells (reviewed in [18]) including, neural stem cells [19], ependymal cells [20] and neuronal cells of the central nervous system [21,22]. In addition, VEGF neutralization in humans via intraocular injection of Macugen and Avastin is associated with an increased incidence of retinal tears [23,24]. Similarly, preeclampsia, which is mediated in part by elevated circulating levels of a soluble form of VEGF receptor 1 called sFlt1 leading to systemic VEGF neutralization [25], is also associated with ocular symptoms including RPE lesions, choroidal ischemia and retinal detachments [26,27].

Given the expression of VEGF and VEGF receptor 2 (VEGFR2) in the adult retina, and the constitutive activation of the receptor, we hypothesized that VEGF plays a role in maintenance and function of the adult retina. We report that, in addition to the choriocapillaris and retinal vasculature, VEGFR2 is expressed by adult photoreceptor cells and Müller cells. While systemic neutralization of VEGF in the mouse did not lead to detectable changes in retinal vascular perfusion or permeability, there was significant cell death in the INL and outer nuclear layer (ONL) as well as loss of visual function. A perfusion or permeability, there was significant cell death in the INL and ONL as well as by the photoreceptors (ONL and inner segments) (Figure 1C, arrows). Consistent with the lac-Z staining, VEGFR2 expression, determined by immunohistochemistry, was observed throughout most of the neural retina (Figure 1D). Strong VEGFR2 immunoreactivity was noted in retinal microvasculature (Figure 1D, arrowhead). The comparison of VEGFR2 expression pattern with the Müller cell marker, cellular retinaldehyde binding-protein (CRALBP) [32] (Figure 1F), indicated expression of VEGFR2 by Müller cells. Müller cell nuclei are localized in the INL and they project processes that extend from the inner limiting membrane to the outer part of the ONL. Immunodetection of VEGFR1 revealed a punctuate staining of the inner and outer plexiform layers as well as of the inner segments (IS) of photoreceptors (Figure 1E). RT-PCR of adult rat retina and purified Müller cells revealed enrichment of RNA for VEGF as well as VEGFR1, VEGFR2 and neuropilin-1 (NPI) signals in Müller cells, suggesting that Müller cells are a significant source of VEGF and its receptors in the retina. The localization of VEGF receptors in the IS of the photoreceptors was confirmed by RT-PCR analysis of VEGFR1 and VEGFR2 on freshly isolated adult mouse photoreceptors (Figure 1H).

Examination of the activation status of VEGFR2, the main signaling receptor for VEGF, was accomplished by immunoprecipitation from pooled adult retina lysates, followed by immunoblotting for phosphorylated tyrosine. Results of this analysis indicated that VEGFR2 is constitutively activated in the adult retina (Figure 1I), an observation that is consistent with a role of VEGF in adult retina homeostasis.

Systemic VEGF neutralization does not affect retinal vasculature

To elucidate the function of VEGF in the adult retina, we neutralized VEGF systemically using an adenovirus expressing sFlt1 (Ad-sFlt1). sFlt1, a soluble form of VEGFR1 produced by alternative mRNA splicing, is a potent VEGF inhibitor [33]. As prior reports indicate a role for VEGF in maintenance of the microvasculature of various organs, including the trachea, pancreas and thyroid [34], we first sought to determine if systemic sFlt1-expression led to any changes in retinal vessels. Examination of retinal flat mounts from fluorescein-perfused mice expressing sFlt1 for 14 days revealed no gross changes in the retinal vasculature (Figure 2A). Collagen IV immunostaining of retinal sections from fluorescein-dextran perfused mice revealed normal perfusion in the retinas of mice that had expressed sFlt1 for 14 days compared to controls (Figure 2B and C). NG2 staining of retinal flat mounts from sFlt1-expressing mice showed no apparent changes in pericyte association to the retinal microvessels (Figure S2).

As it has been previously shown that systemic neutralization of VEGF leads to increased permeability in the lung and kidney of sFlt1-expressing rats [35] and in the brain of sFlt1-expressing mice [20], we sought to determine if systemic VEGF neutralization would affect the permeability of the retinal vasculature. The vessels of the retinal vasculature have properties similar to the blood brain barrier, and as such, are relatively impermeable [36]. Fluorescein angiography of the retina demonstrated no differences in fluorescein leakage between mice expressing Ad-null or Ad-sFlt1 for seven days (Figure 2D). Transmission electron microscopic examination of the retinal vasculature did not reveal structural
alterations in the inner retina capillaries or the choriocapillaris (Figure 3A and B). The capillaries of the GCL of sFlt1-expressing mice displayed normal morphology with well-defined extracellular space and basement membrane, few cytoplasmic vacuoles and normal tight junctions (Figure 3A). Similarly, there were no structural abnormalities of the RPE-choriocapillaris complex. The Bruch's membrane of sFlt1-expressing mice appeared normal with well defined layers and no apparent thickening. No sign of thrombosis could be detected (Figure 3B). The choriocapillaris retained their characteristic fenestrated phenotype (Figure 3C). Electron microscopic quantification of fenestrations in the choriocapillaris endothelium of mice expressing sFlt1 for 28 days showed no difference compared to the Ad-null infected controls (Figure 3D).

VEGF neutralization leads to neuroretinal cell apoptosis and loss of retinal function

Examination of retinal cryosections from mice expressing sFlt1 for 14 days revealed significant apoptosis in both the INL and ONL (Figure 4A–B). Retinal damage is generally associated with Muller cell activation that is marked by upregulation of GFAP [37]. However, there was no increase in GFAP expression in Muller cells of mice expressing sFlt1 for 14 days (Figure S3), suggesting that the observed cell death is not the consequence of any ongoing retinal injury. The increased cell death resulted in a significant reduction in the thickness of the INL and ONL by day 28 (Figure 5A).

The INL is comprised primarily of Muller and neural cells, including bipolar, horizontal and amacrine cells, whereas the ONL contains photoreceptor cell bodies. Electron micrographs revealed typical signs of apoptosis in various cells of the INL. Apoptotic Muller (Figure 5B) and amacrine cells (Figure 5C) could be identified based on their location and morphological characteristics [38]. Both cells showed chromatin condensation, membrane swelling and ruptured mitochondria (Figure 5B and C). Cell death was even more evident in the ONL where a high number of photoreceptor cell bodies displayed cellular shrinkage, and chromatin condensation (Figure 5D). The combination of

Figure 1. VEGF signaling in the adult retina. (A) Sections of eyes from adult VEGF-lacZ mice stained for LacZ using x-gal (blue) revealed VEGF expression in the GCL (arrowheads) and INL layer (asterisks). (B) Flat-mounted retinas from adult VEGF-lacZ mice stained for lacZ (blue) and NG2 (brown) demonstrated VEGF expression by NG2-positive cells associated with microvessels (arrowhead) and by some astrocytes (arrow) in the GCL. (C) Sections of eyes from adult VEGFR2-lacZ mice stained for lacZ revealed expression of VEGFR2 in the GCL, in the INL (asterisks), and in the photoreceptors where a strong lacZ staining was observed in the inner segments (arrows). (D) Immunohistochemistry for VEGFR2 in sections of adult retina revealed expression in vascular cells (arrowhead), in Muller cells processes, and in the IS of the photoreceptors (arrows). (E) Immunohistochemistry for VEGF1 revealed a spotty expression in the IPL and OPL. VEGFR1 was also detected in the photoreceptor IS. (F) Staining of Muller cells using CRALBP revealed an expression pattern similar to VEGFR2 (compare with D). (G) Expression of VEGF and its receptors, VEGFR1 and VEGFR2, by Muller cells was confirmed by RT-PCR of RNA from adult rat retina and isolated rat Muller cells. (H) Expression of VEGFR1 and VEGFR2 in photoreceptors (PR) isolated from adult mouse retinas. (I) Immunoprecipitation (IP) of VEGFR2 from pooled adult mouse retinas, followed by immunoblotting for phosphorylated tyrosine revealed VEGFR2 expression (bottom panel) and activation (top panel) in the adult retina. As a control, lysates of porcine aortic endothelial (PAE) cells overexpressing VEGFR2 either untreated or stimulated with VEGF were immunoprecipitated and immunoblotted as described above. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, IS: inner segment, OS: outer segment. Scale bar is 100 μm.
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photoreceptor cell death and retraction of Müller cells processes (MP), which are normally closely associated with the photoreceptors, left large empty spaces containing cellular debris (Figure 5D).

Visual dysfunction resulting from the increased cell death observed in sFlt1 expressing mice was assessed using electroretinogram (ERG) recordings. The ERG results from the electric current created by light-induced activity of neuronal and glial cells [39]. ERG recording revealed a significant reduction of both a- and b-wave amplitude (Figure 5E). Reduction of more than half of the a-wave amplitude is indicative of a considerable loss of photoreceptor function and is consistent with our previous observations. The reduction of the b-wave is a sign of an altered photoreceptor post-synaptic cascade that reflects the increase in photoreceptor apoptosis. A possible contribution of bipolar cell dysfunction to the reduced b-wave could not be excluded.

Autocrine VEGF signaling plays a role in Müller cell survival

In light of the co-expression of VEGF and its receptors by Müller cells, we investigated whether the disruption of a VEGF autocrine pathway on Müller cells was mediating the increased cell death in the INL of sFlt1 expressing mice. Because of the technical difficulty to isolate sufficient numbers of differentiated Müller cells, we used the Müller cell line, MIO-M1. MIO-M1 is a spontaneously immortalized human Müller cell line that retains most of the characteristic Müller cell morphology, protein expression and function [40]. We used siRNA mRNA to alter autocrine VEGF signaling by inhibiting its autonomous expression. Transfection of siVEGF led to a 76% decrease in VEGF mRNA (Figure 6A) and a 94% reduction of secreted VEGF protein (from 1.63 ng/ml ± 0.01 to 0.1 ng/ml ± 0.01) at day 3 (Figure 6B). Inhibition of VEGF expression under serum-free conditions led to an increase in the number of TUNEL-positive MIO-M1 cells (Figure 6C). Quantification of apoptosis by FACS analysis revealed a doubling in the number of apoptotic cells in siVEGF-transfected cells compared to cells transfected with control siRNA (Figure 6D).

Apoptosis is tightly controlled by the balance between pro- and anti-apoptotic genes of the Bcl-2 family. Changes in the relative expression of the Bcl-2 family members in favor of pro-angiogenic genes will ultimately decide cell fate [41]. Therefore, we determined the expression level of the pro-apoptotic gene Bax and anti-apoptotic genes Bcl-2 and Bcl-xL by quantitative PCR. Inhibition of VEGF expression by MIO-M1 cells resulted in the up-regulation of the pro-apoptotic gene Bax (Figure 6E), leading to a significant increase in the Bax/Bcl-2 expression ratio (Figure 6F). No significant changes in Bcl-2 and Bcl-xL expression were detected (data not shown).

VEGF is a direct survival factor for photoreceptors

Based on our observation that photoreceptors express VEGF receptors, we used freshly isolated post-mitotic mouse photoreceptors to determine if VEGF could directly influence photoreceptor survival. Photoreceptor sheets were isolated by mechanical fractionation of flat-mounted retinas (Figure 5). Purity of the preparation was controlled by co-immunodetection of the photoreceptor marker, recoverin, and the Müller cell markers.
GFAP and glutamine synthetase (GS) (Figure S5). Isolated photoreceptor cells cultured in absence of serum died rapidly (as measured by Trypan blue exclusion) (Figure 7A). However, the addition of 10 ng/ml of VEGF165 was sufficient to rescue the photoreceptors, so that at 72 hr there were only 8.2% apoptotic cells in the presence of VEGF compared to 37.4% in the absence of VEGF (Figure 7B). This pro-survival function of VEGF was confirmed using photoreceptor explants in which 60 mm sections containing only the photoreceptor cell bodies and their inner segments were cultured in presence or absence of VEGF165. As observed with isolated photoreceptor cells, the absence of serum resulted in a time-dependent increase in apoptosis while the addition of VEGF led to significant protection against apoptosis (Figure 7C–D).

Discussion

There is growing evidence that VEGF effects are not specific to the vasculature. A variety of non-vascular cells express VEGFR2, the primary VEGF signaling receptor, and VEGF has been shown to act on these cells to mediate proliferation, differentiation and/or survival (for review see [18]). Previous studies reported the expression of VEGFR2 by glial and neuronal cells of the retina [14,42,43,44] but VEGFR2 expression by photoreceptors has not been definitively established [43,45]. The combined use of VEGFR2-LacZ mice and immunohistochemistry for VEGFR2 allow us to unequivocally demonstrate the expression of VEGFR2 on Müller cells and photoreceptors. The wide expression of VEGF and VEGFR2 in adult retina and the finding that VEGFR2 is constitutively phosphorylated strongly indicates a role for VEGF
signaling in retina homeostasis. We therefore elected to assess the role of VEGF in the normal retina by systemic neutralization. Systemic blockade was selected over intravitreal injection because these studies are intended to assess the effect of sustained and long-term VEGF inhibition in adult mice.

The observation that Müller cells, which express both VEGF as well as its receptors, VEGFR1 and VEGFR2, undergo apoptosis in mice with systemic VEGF neutralization suggested an autocrine role for VEGF in Müller cells in vivo. This concept is strongly supported by the finding that siRNA suppression of VEGF expression in cultured Müller cells leads to a significant increase in cell death. Co-expression of VEGF and VEGFR2 has been observed in many tumor cells [46,47] but only in a few normal cell types, such as podocytes [48], skeletal muscle [49], aortic endothelium [50,51], and RPE [3]. However, a role for VEGF autocrine pathways in tissue homeostasis has only been demonstrated in vivo in the endothelium, where cell-autonomous signaling of VEGF appears to be required for endothelial cell survival [51].

Our data showed that VEGF autocrine signaling promotes Muller cell survival in serum-free conditions through the control of pro-apoptotic Bax gene expression. Interestingly, no changes in the level of expression of anti-apoptotic genes Bcl-2 and Bcl-XL were observed after alteration of VEGF autocrine signaling. Bax constitutes one of the main transcriptional targets of p53 in neuronal and glial cells [52] suggesting that VEGF autocrine signaling regulates Müller cell apoptosis through the control of p53 expression/activation and Bax expression.

The physical association between the photoreceptors that express VEGFR2 and the Müller cells that express VEGF points to a paracrine interaction in which Müller cells provide VEGF as a neurotrophic signal to photoreceptors. The dramatic increase in

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**Figure 5. Decreased retina thickness and loss of visual function in sFlt1 expressing mice.**

(A) Semi-thin epon sections from retinas of experimental mice expressing sFlt1 for 28 days showed substantial thinning of the INL (black double arrowheads line) and the ONL (white double arrowheads line) (n = 3 for Ad-null and n = 4 for Ad-sFlt1). (B–D) TEM of retinas from sFlt1 expressing mice at 28 days. (B–C) Micrographs taken in the INL region revealed apoptotic Müller cells (MC) and amacrine cells (A). Both cells displayed condensation of the chromatin, membrane swelling and rupture mitochondria (arrows). (D) In the ONL, a high percentage of photoreceptors appeared apoptotic (white asterisk). Cell death left numerous empty spaces containing membranous debris (arrowhead). Processes from the Müller cells that normally fill the intercellular space appeared shrunken (MP). (E) Scotopic ERG recordings of experimental mice 28 days post-infection using a flash intensity of +10 dB revealed a marked reduction of both a- and b-wave amplitudes in sFlt1-expressing mice (n = 6 for Ad-null and n = 10 for Ad-sFlt1). GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer, OPL: outer plexiform layer, Ch: choroid. Scale bars are 100 μm in A and 5 μm in B to D.

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apoptosis associated with visual dysfunction observed in mice expressing sFlt1 is consistent with this suggestion. Using purified photoreceptors, we were able to demonstrate a direct function of VEGF on photoreceptor survival independent of an effect on accessory cells. Müller cells act to support retinal neurons by providing structural support for the retina and regulating ion and neurotransmitter levels in the extracellular space [53]. In addition, they produce a variety of neurotrophic factors, including brain-derived neurotrophic factor (BDNF) [54], nerve growth factor (NGF) [55] and neurotrophins (NT-3 and NT-4/5) [56]. Our findings add VEGF to this list. Though retinal pigment epithelial cells (RPE) also produce VEGF in the adult [3], they are unlikely to be a primary source of VEGF for the photoreceptors because of their distance from the photoreceptor cell bodies. Moreover, RPE

Figure 6. Inhibition of the autocrine VEGF signaling in Müller cells leads to increased cell death. VEGF expression by M1-O1 Müller cells was inhibited by transfection of siRNA into sub-confluent cells. (A–B) Seventy-two hr following transfection of the control and VEGF siRNA, VEGF mRNA levels were determined by qPCR (A) and VEGF protein secretion was quantified by ELISA (B). siVEGF transfection led to a 75% inhibition of VEGF mRNA and more than 90% reduction of VEGF protein (n = 3). (C) Inhibition of VEGF was associated with an increase in TUNEL-positive cells after three days of culture in serum-free conditions (n = 4). (D) Quantification of cell death by FACS analysis demonstrated a doubling of the number of annexin-V positive apoptotic cells in siVEGF transfected cells compared to siControl (n = 3). (E) Increased apoptosis was accompanied by a significant up-regulation of the pro-apoptotic gene, bax, and by the increase of the Bax/Bcl-2 ratio (F). Scale bar is 100 µm.

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have been shown to secrete VEGF basally, presumably in support of the underlying fenestrated choriocapillaris [4]. In addition to supporting photoreceptors, Müller cells have been shown to maintain adult retinal ganglion cell (RGC) survival in vitro [57] and the fact that RGC express VEGFR2 [43] suggests that Müller cell-derived VEGF also plays a supporting role for RGCs. In support of this, it has been demonstrated that long-term VEGF (8 wk) blockade in the rat leads to RGC apoptosis [43].

The requirement of glial and neuronal cells for VEGF as a survival factor under normal conditions is one of the most intriguing findings of our study. Müller cells and neurons produce numerous neurotrophic factors to support the retina [58,59]. Dependence on VEGF signaling may be due to the high metabolic rates of retinal neurons and their extreme sensitivity to hypoxia [60]. As VEGF expression is tightly controlled by oxygen tension and glycemia level [61,62], VEGF would be the ideal candidate to support retina homeostasis during the early phase of an oxidative or ischemic insult. Indeed, VEGF has been shown to protect neuronal cells of the central nervous system (CNS) against apoptosis induced by serum deprivation or hypoxia [63]. The

**Figure 7. VEGF is a direct survival factor for photoreceptors.** (A) Photoreceptors isolated from adult C57BL/6J mice were cultured on laminin-coated wells in absence or presence of 10 ng/ml VEGF165 for up to 72 hr. Addition of 10 ng/ml of VEGF165 decreased the number of dead cells identified by trypan blue staining. (B) Quantification of the percentage of dead photoreceptors based on the trypan blue exclusion assay revealed a significant protection from apoptosis by VEGF165 (n = 9). (C) ONL explants containing only the photoreceptor nuclei and IS were cultured in basal medium ± 10 ng/ml of VEGF165 for 24, 48 and 72 hr. Apoptotic cells were detected by TUNEL staining. (D) Quantification of TUNEL-positive cells per sheet revealed a significant decrease in the number of apoptotic photoreceptors in presence of VEGF (n = 6).

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VEGF-dependent paracrine relationship between the Müller cell and photoreceptors that we have revealed here parallels the trophic association between Schwann cells and neurons in the CNS [64].

We have previously observed VEGF expression in virtually all vascularized adult tissues [50,65] and we and others have hypothesized that VEGF plays an important role in the maintenance of the adult vasculature [34,50]. In support of this, relatively short-term systemic VEGF neutralization has been shown to lead to the selective regression of some fenestrated microvessels [34], leading to the hypothesis that these fenestrated vessels are particularly dependent on VEGF for their survival. In fact, we have shown that nearly all fenestrated vessels, including for example the glomerulus of the kidney and the choroid plexus in the brain, lie in close proximity to epithelial cells that express high levels of VEGF [50]. Studies in vitro and in vivo [66,67] have demonstrated that ectopic application of VEGF can induce the formation of fenestrations. Interestingly, our analysis did not reveal an effect of systemic VEGF neutralization on the fenestrations of the choriocapillaris. A recent report in which intravitreal administration of Avastin in a primate model led to early loss of fenestration with rapid recovery in spite of the prolonged Avastin half-life [68], leads us to speculate that the loss of fenestrations is a transient effect that one might not expect to be detectable at this later (28 days post infection) time point.

Our observation of relatively low levels of VEGF in the innermost retinal layers and the fact that systemic VEGF expression does not influence the inner retinal vasculature, which is not fenestrated but is the site of the blood neural barrier, are consistent with the idea that high local VEGF levels reflect a particular dependence on VEGF, which renders them particularly sensitive to systemic VEGF blockade. Consistent with this notion, the microvasculature of the brain, which has relatively low levels of VEGF in the adult [50], was not influenced by 14 days of systemic VEGF inhibition [34]. In addition to the relatively low levels of local VEGF, the microvasculatures of the retina and brain are characterized by high ratio of pericyte to endothelial cells [69]. In light of the evidence that pericyte association with EC mediates vessel stabilization, it is reasonable to suggest that the microvasculature of the CNS is particularly stable. On the other hand, it is difficult to evaluate the extent of VEGF neutralization at the level of the retinal microvasculature by systemically expression of sFlt1. Based on our previous extensive use of endothelial cell-pericyte cocultures [70,71] and the documented close association between endothelial cells and pericytes in the retina [72,73], we suspect that the VEGF produced by pericytes to support retinal capillary endothelial cells (Figure 1D in this paper and [2]) may be relatively less accessible to soluble Flt-1 and thus may account for the lack of an effect of VEGF neutralization on the inner retinal vessels in the time frame of this study.

Our results have obvious implications for therapeutic use of anti-VEGF, particularly as it relates to the eye, as we observed that VEGF neutralization interferes with endogenous survival signals, leading to unexpected neural toxicity. On the other hand, however, since the adult retina expresses VEGF in the absence of active neovascularization, it is clear that the locally produced VEGF mediates neural protection without leading to vessel permeability or growth. The absence of active vessel proliferation/permeability in the normal retina in the presence of endogenous VEGF may be due to the dose, localization and/or the presence of opposing factors, such as TGF-β [74,75] or angiopoietin-1 [76]. However, it does indicate that, with appropriate delivery and/or dosing, VEGF may find use as a neural survival factor for degenerative retinal pathologies.

Materials and Methods

Animals

Adult VEGF-lacZ mice (from Andreas Nagy, University of Toronto, Canada) [28] and adult VEGFR2-lacZ mice [31] (generous gift of Victoria Bautch, UNC-Chapel Hill, NC) were used for localization of VEGF and VEGFR2, respectively. Adult C57BL/6J mice were used for analysis of VEGFR expression and subsequent VEGF-induced photoreceptor survival [JAX Laboratories]. For in vitro neutralization of VEGF, adult CD-1 mice 6-8 weeks old (Charles River Laboratories) were injected with either 1 × 10^9 viral particles (V.P.) Ad-CMV-null (control) or 2.5 × 10^9 V.P. Ad-CMV-sFlt1 (murine form consisting of the first three IgG repeats) [25,77] (Qi-Biogene) on day 0. sFlt1 expression was confirmed by ELISA (R&D Systems). Ad-CMV-null and Ad-CMV-sFlt1 did not contain an Ig Fc region. Absence of systemic immune response was confirmed by the absence of leukocyte and platelet activation (data not shown). Circulating levels of sFlt1 of ~200 ng/ml were measured on the plasma fraction seven days post-infection and were sustained for at least 21 days. Ad-null infected mice showed no detectable sFlt1. All animal protocols were approved by the Schepens Eye Research IACUC.

Fluorescein-dextran perfusion

Anesthetized mice were perfused through the aorta with fluorescein dextran 2 × 10^6 g/ml in 4% paraformaldehyde in PBS. Eyes were then removed, fixed in 4% paraformaldehyde at 4°C overnight and processed for flat-mount and cryosections.

Immunohistochemistry

For VEGF-lacZ and VEGFR2-lacZ staining, sections and flat-mounted retinas were stained for LacZ using the in situ β-galactosidase kit, according to the manufacturer’s instructions (Stratagene). Flat-mounted retina, cryosections and photoreceptor explants were incubated overnight at 4°C with rabbit anti-mouse VEGF-R2 T1014 (1:500, a gift from Dr. Brekken, University of Texas Southwestern Medical Center, Dallas, TX) [70,79], mAb anti-β-1 integrin (1:100, Santa Cruz), anti-γ, anti-VEGFR1 (1:100; Santa Cruz), rabbit anti-β1 integrin (1:100, Sigma), mAb anti-β1 integrin (1:250, Millipore), rabbit anti-VEGFR2-lacZ (1:50, Abcam), rabbit anti-VEGFR2 (1:500, Abcam), mAb anti-VEGFR2 (1:250, Chemicon), rabbit anti-recoverin (1:500, Chemicon). Secondary antibodies included biotinylated anti-rabbit or anti-mouse antibodies (Vector Laboratories) and Cy3-, Cy5- or FITC-conjugated antibody (Jackson ImmunoResearch Laboratories). mAb anti-β-galactosidase (1:250, Millipore, mAb anti-sulfated glycosaminoglycan (1:500, Chemicon), mAb anti-70 kDa (1:500, Chemicon). Secondary antibodies included biotinylated anti-rabbit or anti-mouse antibodies (Vector Laboratories) and Cy3-, Cy5- or FITC-conjugated antibody (Jackson ImmunoResearch Laboratories). Some antibodies were visualized using 3, 3-diaminobenzidine (ABC kit; Vector Laboratories). ECs were detected using FITC-conjugated Griffonia simplicifolia isoelectin B4 (Vector Laboratories). Cell nuclei were identified by DAPI labeling. Incubation using rabbit or mouse IgG as a primary antibody was conducted as a negative control.

Quantification of retinal vessel perfusion

Retinal vascular perfusion was measured by comparing the number of type IV collagen-positive vessels of the inner retina (GCL) to the number of FITC positive vessels on 3 serial cryosections separated by 150 μm.

Histology and electron microscopy

Tissues were prepared for TEM as previously described [20]. Ultrathin sections were treated with uranyl acetate and visualized using a Phillips 410 transmission electron microscope.
Quantification of INL and ONL thickness

Semi-thin sections were cut along the vertical meridian passing through the optic nerve and stained with Richardson’s stain for light microscopy. Three serial sections (100 μm apart) were quantified per animal. On each section, photographs were taken at 600 μm and 1800 μm from the optic nerve on each side. Three measurements were made per photographs. For each animal, the ONL and INL thickness was obtained by averaging all 36 measurements.

Quantification of choriocapillaris fenestrations

Endothelial fenestrations were quantified from electron micrographs of the peripheral region of the RPE-choroid (approximately 800 μm from the optic nerve) at a magnification of 10,400 ×. Only the RPE-side vessel wall was taken into consideration. Fenestrations were counted on 13–15 fields showing continuous choriocapillaris lumen with a length of 10 μm each.

VEGFR2 activation status

Adult mouse retinas were dissected, pooled, lysed, and protein concentration was determined using a BCA assay (BioRad). VEGFR2 immunoprecipitation and phospho-tyrosine blot was performed as previously described [3]. As a control, porcine aortic cells overexpressing human VEGFR2 (PAE-VEGFR2) were serum-starved overnight and incubated with 50 ng/ml VEGF165 (R&D Systems) for five min. Lysates were then collected and used for immunoprecipitation.

Fluorescein angiography

Fluorescein angiography was performed after intraperitoneal injection of 60 mg/kg of 25% fluorescein sodium (Akorn). Photographs were taken using the Topcon/Imagenet system (Topcon Medical System) with a preset 20D lens apposed to the fundus camera at regular time intervals (from 1 min to 4 min post IP injection). Fluorescein leakage was noted as diffuse opacity in the vitreous over-time.

TUNEL assay

Apoptotic cells were detected using the Dead End HRP kit (Promega) or the Roche In Situ Cell Death Detection TMR red kit (Roche Diagnostic) following the manufacturer’s instructions.

Electroretinography

Mouse ERG was assessed 28 days post adenovirus infection on dark-adapted mice using a UTAS-E3000 recording system (LKC, Technologies, Inc.). After pupil dilatation with 1% tropicamide, each mouse was placed in front of a Ganzfeld bowl (UTAS-E3000; LKC Technologies). ERG responses to a series of increasing-intensity light flashes: ±0, +10 and +20-dB were averaged over 10 separate flashes per light intensity.

Cell culture

The human Muller cell line, MIO-M1 [a gift from G.A. Limb, Institute of Ophthalmology and Moorfields Eye Hospital, London, UK], was maintained in culture at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Gibco) supplemented with 2 mmol/L glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 10% fetal bovine serum (Sigma). Cells were used from passage P16 to P25. MIO-M1 apoptosis was quantified using the Vybrant Apoptosis Assay #2 (Molecular Probes) followed by flow cytometry using a FACSCAN flow cytometer.

siRNA mediated gene silencing of VEGF

Inhibition of VEGF expression was achieved using an ONTARGETplus SMARTpool containing four pooled siRNA duplexes (Dharmacon). An unrelated control siRNA pool that lacks identity with known gene targets was used as a control for non-sequence-specific effects. MIO-M1 cells were transfected with siRNA using Dharmafect 1. VEGF in the cell culture supernatant was determined using a Quantikine human VEGF Immunoassay kit (R&D Systems).

Reverse Transcription-PCR Analysis

Total RNA was isolated from tissue and cells using RNA-Be solution (IsoText Diagnostic Inc.). Muller cells were isolated from adult rat retinas by density gradient centrifugation as described previously [80] and total RNA isolated using the RNeasy kit (Qiagen). RNA was reverse-transcribed using Superscript III (Invitrogen). Standard PCR was performed with 1 U Taq DNA polymerase (Roche Diagnostics) and 0.2 μM of appropriate primer pair (Table 1). qPCR reactions were performed using the SYBR Green Master mix and the ABI Prism 9700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. For primer sequence, see Table 2.

Isolation of photoreceptor sheets

Sheets of photoreceptors were isolated from adult C57BL/6j mice as described previously [81,82]. Retinas from 6–8 week old C57BL/6j mice were dissected and flattened by making 4 radial cuts, then placed on a 20% gelatin block secured to a vibratome chuck. Starting at the vitreal surface, sequential sections were cut until the photoreceptor layer was reached then a 200 μm thick section, containing the ONL and IS, was collected. In some experiments the 60 μm thick sections were cultured as ONL explants in the presence or absence of 10 ng/ml VEGF165 (R&D Systems) and apoptotic cells per section were quantified by TUNEL assay.

Trypan blue exclusion assay

Photoreceptor cells collected by enzymatic digestion (papain) of vibratome-isolated photoreceptor sheets were cultured in 96 well laminin-coated plates (Sigma) at 37°C under 5% CO₂ in neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 1% pen/strp (Sigma), 1% glutamine (Sigma) and 0.2% nystatin (Sigma), in the presence or absence of 10 ng/ml VEGF165 (R&D Systems, obtained from the NIH-NCI Preclinical Repository). Twenty-four, 48 or 72 hrs post-plating, 10 μl of trypan blue dye was added for 5 min and the numbers of stained (dead) and unstained (live) cells were counted.

Statistical analysis

Values are expressed as mean±SD (unless specified); statistical analysis was performed using an unpaired Student t test (***, P<0.001, **: P<0.01, *: P<0.05, ns: P>0.05).

Online supplemental material

Fig. S1 shows VEGF-expressing astrocytes, characterized by GFAP and β-gal staining, in adult VEGF-LacZ retina flat-mount. Fig. S2 demonstrates normal pericyte coverage of retinal microvessels 14 days after Ad-sFlt1 infection. Fig. S3 demonstrates the absence of GFAP up-regulation in Muller cells after Ad-sFlt1 infection. Fig. S4 shows the successive steps of photoreceptor sheets isolation by vibratome sectioning. Fig. S5 demonstrates the purity of the photoreceptor explants which stain positive for

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**Online supplemental material**

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were dissected, flat mounted and stained for the pericyte marker, VEGF-expressing cells were detected on retina flat-mounts of adult VEGF-lacZ mice by -gal staining. Co-staining with GFAP revealed a high number of astrocytes, with processes wrapping the retina vessels, expressing VEGF as shown by the blue staining of their cell bodies (black arrowheads). Some lacZ-positive GFAP-negative cells, presumably pericytes, are observed apposed to the vessel wall (white arrows). Scale bar are 100 μm.

**Supporting Information**

**Figure S1** Expression of VEGF by astrocytes in adult retina. VEGF expressing cells were detected on retina flat-mounds of adult VEGF-lacZ mice by -gal staining. Co-staining with GFAP revealed a high number of astrocytes, with processes wrapping the retina vessels, expressing VEGF as shown by the blue staining of their cell bodies (black arrowheads). Some lacZ-positive GFAP-negative cells, presumably pericytes, are observed apposed to the vessel wall (white arrows). Scale bar are 100 μm.

**Figure S2** Normal pericyte coverage in microvessels of sFlt1 expressing mice. Mice expressing Ad-null or Ad-sFlt1 for 14 days were perfused with h.m.w fluorescein dextran (green), the retinas treated mice. Mice expressing Ad-null or Ad-sFlt1 for 14 days were stained for the intermediate filament protein, glial fibrillary acidic protein (GFAP). Upregulation of GFAP in the Muller cells endfeet and processes is associated with an ongoing injury response. However, GFAP appeared normally restricted to the astrocytes in the GCL of both Ad-null and Ad-sFlt1 mice. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer. Scale is bar 100 μm.

**Figure S3** Absence of glial activation in the retina of sFlt1 treated mice. Mice expressing Ad-null or Ad-sFlt1 for 14 days were perfused with h.m.w fluorescein dextran (green), the retinas were dissected, flat mounted and stained for the pericyte marker, NG2 (red). No changes in the association of pericytes with the retinal microvessels were observed (arrows). Scale bar is 50 μm.

**Figure S4** Isolation of photoreceptors sheets. Adult mouse retinas were flat-mounted onto a 20% gelatin block and sectioned along the horizontal plane until the ONL was reached. (A) Photograph of a gelatin block stained by hematoxylin and eosin (H&E) showing that only the photoreceptors (ONL, IS and OS) remain after the inner layers are sectionned. (B) H&E staining of an isolated photoreceptor sheet that will be subsequently used for ONL explant or photoreceptor cell culture. OS: outer segment; IS, inner segment; ONL, outer nuclear layer. Scale bar is 100 μm in A and 50 μm in B.

**Figure S5** Verification of the photoreceptor explant purity. ONL explants were co-stained for the photoreceptor marker, recoverin, and the Muller cell markers, GFAP and GS. All cells in the ONL explants were positive for recoverin, confirming the presence of photoreceptors. No GFAP- or GS-positive cells were detected. Some remnants of Muller cell basal processes could be observed (arrows), demonstrating the purity of the photoreceptor sheets. Positive control for GFAP and GS staining consisting of full retina sections were included (data not shown). Scale bar is 20 μm.

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**Author Contributions**

Conceived and designed the experiments: MSG. Performed the experiments: MSG. Analyzed the data: MSG. Wrote the paper: MSG. Oversaw all aspects of the work, collaborated on study design, interpretation of results and manuscript preparation: PAD. Responsible for all aspects of the study: MSG. Contributed materially to all figures except Figure 7 and Supplemental Figures 4 and 5: MSG. Analyzed all the data, assembled the figures and wrote the first draft of the manuscript: MSG. Developed and

**Table 1. RT-PCR primers.**

| Gene          | primer forward (5'–3') | primer reverse (5'–3') | Product size |
|---------------|------------------------|------------------------|--------------|
| Mouse VEGFR1  | gagagctcataaagcagcgcagagt | cagctttcatcagaggtgcg | 456 bp       |
| Mouse VEGFR2  | tacaccaagagcatggtggtgtg | ctgggtttccatcggatacttc | 499 bp       |
| Mouse Neuropilin-1 | tcagagacccatcagagagttg | ttgcatacctgtgccaac | 619 bp       |
| Mouse Neuropilin-2 | agactcaccacatcattcaggg | tgcacacctgtgccaac | 421 bp       |
| Mouse GAPDH   | gtggcaaaagtgataggttggtgc | gatgtagaccccttgggcc | 291 bp       |
| Rat VEGFR1    | caaagggtcctacgctgttc | cctccatgcagacatttcc | 240 bp       |
| Rat VEGFR2    | gcacaaaggggaactggaagaqa | ctcctacgcctggtgctgtc | 537 bp       |
| Rat VEGF      | gctctctggggtcagttgac | acgacgaatctgcgctgtga | 145 bp       |
| Rat Neuropilin-1 | ccacagagagccacaccatt | tgacccctcggtaccacca | 333 bp       |
| Rat GAPDH     | ggtcatcctcgtgctgagaac | tgcgttctctcagaccaaat | 294 bp       |
| Human VEGFR1  | caaagggcacagaggtggagt | gatgtagctttacctccttgt | 498 bp       |
| Human VEGF2   | gagggctctctggtgatttg | tgcagcagctgaccgctgttg | 709 bp       |
| Human GAPDH   | caaatcctgagcaggctca | ggagtgggtgtgcgctgtga | 715 bp       |

doi:10.1371/journal.pone.0003554.t001

**Table 2. qPCR primers.**

| Gene          | primer forward (5'–3') | primer reverse (5'–3') |
|---------------|------------------------|------------------------|
| VEGF          | ggggcaagatcatcaggaatgt | attggagtagagtacgtcgg |
| Bax           | aagtgcagcaggttgcctgcgc | gcccacaaagtgctgcttgcc |
| Bcl-2         | cccccacagagctgtgctttg | ccacactgtgaccccttg |
| Bcl-xl        | tggagtaactcttggggtcagtc | agcagcagctgcctgacagg |
| GAPDH         | cccatcatactctccagaga | cagcggcaccattgatttg |

doi:10.1371/journal.pone.0003554.t002
characterized the sFlt model and assisted in characterization of the vascular phenotype (Figures 2–5): ASRM. Collaborated with mouse work and participated in the vascular analysis (Figures 2–5): TEW. Developed the method for isolation of photoreceptor sheets and is responsible for Figure 7 and Supplemental Figures 4 and 5: BAT. Carried out the analysis of retinal function via electoretinograms (Figure 5): ES. Participated in immunohistochemistry, tissue culture and RT-PCR: TK. Conducted studies on VEGF expression in the inner retina and contributed to Figure 1: DCD. Oversaw the development and characterization of photoreceptor sheets: MJY. Contributed to Figure 7 and Supplemental Figures 4 and 5: MJY.

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