Increased Angiogenic Factors Associated With Peripheral Avascular Retina and Intravitreous Neovascularization

A Model of Retinopathy of Prematurity

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Objectives: To determine expression of vascular endothelial growth factor (VEGF), pigment epithelium–derived factor, and their respective receptors in retinas using a model of retinopathy of prematurity.

Methods: Retinas isolated from a 50/10 oxygen (inspired oxygen cycled between 50% oxygen and 10% oxygen every 24 hours)–induced rat model of retinopathy of prematurity (50/10 OIR model), and from room air–raised rat pups (RA) at birth, age 14 days (persistent peripheral avascular retina in the 50/10 OIR model and complete retinal vascularization in RA) and age 18 days (intravitreous neovascularization in the 50/10 OIR model) were analyzed for messenger RNA of VEGF164, neuropilin 1, neuropilin 2, VEGF receptor 1, VEGF receptor 2, pigment epithelium–derived factor, and pigment epithelium–derived factor receptor by real-time polymerase chain reaction.

Results: In the 50/10 OIR model compared with RA, fold changes in expression of VEGF164, neuropilin 1, and neuropilin 2 were significantly increased at ages 14 and 18 days. A trend for increased fold change was noted in expression of VEGF receptor 2 at age 14 days and a significant increase at age 18 days in the 50/10 OIR model compared with RA. Pigment epithelium–derived factor receptor was significantly increased at age 14 days in the 50/10 OIR model compared with RA.

Conclusion: Increased expression of VEGF164 and angiogenic receptors were found in association with both avascular retina at day 14 and intravitreous neovascularization at day 18 in a relevant model of retinopathy of prematurity.

Clinical Relevance: Increased VEGF and angiogenic receptors may have a role in the development of peripheral avascular retina and stage 3 retinopathy of prematurity.


INTERACTIONS BETWEEN NEURAL and vascular growth factors and receptors are important in ordered retinal vascularization.1 For example, the vascular endothelial growth factor (VEGF) family and signaling pathway are important in retinal vascular development2,3 and in neuronal survival.4,5 The neuropilins (NPs), originally described as receptors for proteins involved in neuronal axon guidance, can form complexes with VEGF receptors (VEGFRs) and act as coreceptors for VEGF-mediated angiogenic signaling.1 Pigment epithelium–derived factor (PEDF) is a neurotrophic factor6 and is involved in retinal vascular development.7 One way in which PEDF may be involved in normal vascularization is by downregulating VEGF expression and interfering with its binding to VEGFR2,8 believed to be the receptor most involved in angiogenic processes.9 However, both VEGF and PEDF interactions have been found in pathologic angiogenesis.

Because the VEGF signaling pathway and PEDF are important in physiologic and pathologic angiogenesis,2,3,7,8 we aimed to determine expression of these factors and their respective receptors in a model that is relevant to clinical retinopathy of prematurity (ROP). Previous investigations of the effects of oxygen stresses on expression of VEGF isoforms found that VEGF164 was the most prevalent isoform and was increased by successive fluctuations in oxygen level,10 a risk factor for modern-day ROP.11,12 In this study, we used the same 50/10 oxygen–induced rat model of ROP (50/10 OIR model) developed by Penn et al,13 which exposes rat pups to successive fluctuations in oxygen level. The model reproducibly and consistently develops peripheral avascular retina, similar to zone 2 ROP, followed by intravitreous neovascularization at the junction of vascular and avascular retina, similar to zone 2, stage 3 ROP. Previous investigations of ROP have used oxygen-induced retinopathy models.

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that expose animals to extreme constant hyperoxia to create vasoretraction of the central retinal capillaries. The most well known of these is the mouse model developed by Smith et al.\textsuperscript{14} During the obliterator phase, VEGF messenger RNA (mRNA) expression was downregulated in conjunction with arrest and loss of developing retinal vasculature.\textsuperscript{15} The newly formed capillaries can be protected from death by administering growth factors\textsuperscript{16-18} or nutrients\textsuperscript{19,20} before hypoxia. On return to room air, relative retinal hypoxia triggers signaling of VEGF and other angiogenic factors with capillary budding into the vitreous,\textsuperscript{21} and this proliferative phase can be prevented by administering agents to inhibit angiogenic factors such as VEGF.\textsuperscript{22}

We used a relevant model of zone 2, stage 3 ROP (50/10 OIR model) to measure mRNA of VEGF\textsubscript{164}, PEDF, and their respective receptors. We hypothesized (1) that angiogenic factors would be downregulated in the 50/10 OIR model at age 14 days (persistent peripheral avascular retina) compared with room air–raised rat pups (RA) at age 14 days (complete retinal vascularization) and (2) that angiogenic factors would be upregulated in the 50/10 OIR model at age 18 days (intravitreous neovascularization) compared with RA.

**METHODS**

All animal studies complied with the University of North Carolina’s Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals). They were also in accord with the Association for Research in Vision in Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Visual Research.

**50/10 OIR MODEL**

We used a model of zone 2, stage 3 severe ROP (50/10 OIR).\textsuperscript{13} The model induces rat arterial oxygen levels\textsuperscript{13} that replicate extremes in transcutaneous oxygen levels of human preterm infants who develop severe ROP.\textsuperscript{13} Within 4 hours of birth, at postnatal age 0 days (p0), litters of 12 to 14 Sprague-Dawley rat pups with their mothers (Charles River, Wilmington, Massachusetts) were placed in an incubator (OxyCycler; Biospherix, New York, New York) to cycle inspired oxygen between 50\% oxygen and 10\% oxygen every 24 hours until p14. After 7 cycles of oxygen fluctuations, rat pups were placed in room air for 4 days. Oxygen levels were monitored daily and recalibrated as needed. Carbon dioxide in the cage was also monitored daily and flushed from the system by maintaining sufficient gas flow and by adding soda lime. The 50/10 OIR model develops vascular tortuosity at p12, peripheral avascular retina at p14 (Figure 1) (when development of retinal vascularization is completed in RA), and intravitreous neovascularization at p18 (Figure 2).\textsuperscript{23}

Compared with RA at the same time points after birth, it was previously shown that rat pups raised in an environment of oxygen cycling had increased areas of peripheral avascular retina.\textsuperscript{10} At p14, after 7 cycles of oxygen fluctuations in oxygen level, the percentage of avascular to total retinal area was 33\% in the 50/10 OIR model, whereas RA had fully vascularized retinas in which the inner capillary plexus covered the retina to the ora serrata.

**DISSECTION OF RETINAL TISSUE FOR mRNA ANALYSES**

Rat pups were euthanized using pentobarbital sodium (intraperitoneal injection of 80 mg/kg of body weight), and retinas were isolated using a modification of the technique by Chan-Ling.\textsuperscript{24} Retinas were dissected, without ora serratas, from at least 5 separate rat pups for each time point in RA or the 50/10 OIR model. Separate litters were used for each time point and condition to assure adequate litter size and consistency of features in the 50/10 OIR model. Replicate experiments were performed for each condition and time point. Tissue was frozen (RNA later; Applied Biosystems, Foster City, California) until analysis.
REAL-TIME POLYMERASE CHAIN REACTION

Retinas were homogenized in lysis buffer, and total RNA was extracted (RNeasy; Qiagen, Studio City, California). A kit (DNA-free, Applied Biosystems) was used to remove DNA contamination, and RNA quantity was determined. One microgram of RNA was reverse transcribed using a kit (High-Capacity cDNA Reverse Transcription, Applied Biosystems). The VEGF164 mRNA was reverse transcribed because it is the most prevalent of VEGF isoforms associated with intravitreous neovascularization in the 50/10 OIR model, is upregulated by repeated oxygen fluctuations, and is a ligand for NP1, which acts as a coreceptor with VEGFR2. Primers for the following were made by the University of North Carolina's Oligonucleotide Synthesis Core Facility (http://www.med.unc.edu/oiloni/index.htm): VEGF164 (5′GCCATACGAGAGATGAGCATCTG3′, 5′GCCAAGCTTTGATGGAGTCCCCTGAC Tamara), VEGFR1 (5′GCCACCTCATGTGAAAGGAGCACTG3′, 5′AGTCCAAGCAGCGTCCTGAC Tamara), VEGFR2 (5′CCGGTCTGGTTGGAGCCT3′, 5′GCCACCTCATGTGAAAGGAGCACTG3′). Fam-forward primers were designed to discriminate mRNA expression differences between p0 and p14 for each of 4 angiogenic receptors and coreceptors involved in VEGF signaling (VEGFR1, VEGFR2, NP1, and NP2). VEGF164 mRNA expression was noted in the 50/10 OIR model at 14 days (P<.001) and at 18 days (P=.06) (t test). 

and were expressed as fold increase over the p0 value for each growth factor or receptor, enabling comparisons between RA and 50/10 OIR.

STATISTICAL ANALYSIS

For real-time PCR analysis, samples from RA and the 50/10 OIR model were normalized to β-actin for each ligand or receptor group to allow comparisons of fold differences in expression within each group. t Test was used to analyze fold changes in mRNA expression of each ligand or receptor in RA vs the 50/10 OIR model at p14 and p18.

RESULTS

EXPRESSİON OF GROWTH FACTORS AND THEIR RECEPTORS IN RA

In RA, the retinal extent is fully covered by retinal vessels at p14. The VEGF164 mRNA expression increased 3-fold between p0 and p14 (Figure 3). The fold changes in expression differed between p0 and p14 for each of 4 angiogenic receptors and coreceptors involved in VEGF signaling (VEGFR1, VEGFR2, NP1, and NP2). Whereas VEGFR2 increased 5-fold (Figure 4), VEGFR1 increased approximately 42-fold (Figure 5). There was little change in expression of NP1 (Figure 6), and NP2 increased approximately 5-fold (Figure 7). The PEDF and PEDFR expression increased about 5- and 4-fold, respectively, between p0 and p14 (Figure 8 and Figure 9).

EXPRESSİON OF GROWTH FACTORS AND THEIR RECEPTORS AT P14 DURING PERSISTENT PERIPHERAL AVASCULAR RETINA IN THE 50/10 OIR MODEL

The 50/10 OIR model exposes animals to repeated fluctuations in hyperoxia and hypoxia and causes delayed
retinal vascularization,26 believed to contribute to peripheral avascular retina at p14, when RA have fully vascularized retinas. Because VEGF is important in developmental angiogenesis2,3 and because the 50/10 OIR model has incomplete retinal vascularization compared with RA,10 we anticipated that mRNA of VEGF164, VEGFRs, and coreceptors would have lower expression at p14 in the 50/10 OIR model compared with RA. However, we found that VEGF164 expression increased, with an almost 10-fold change between p0 and p14 in the 50/10 OIR model, and this represented a 3-fold increase in expression compared with RA (Figure 3). This finding also contrasts with results using the mouse oxygen-induced retinopathy model, in which VEGF expression was lower than that in room air during hyperoxia-induced vaso-obliteration.15

Compared with p0, all receptors had increased expression at p14 in the 50/10 OIR model, and all except VEGFR1 had increased fold change in expression compared with RA (Figures 4, 5, 6, and 7). The fold change in expression of NP1 and NP2 was significantly increased in the 50/10 OIR model compared with RA, whereas there was a trend for increased fold change in expression of VEGFR2 in the 50/10 OIR model. The fold changes in PEDF and PEDFR mRNA expression increased between p0 and p14, and there was a significantly increased fold change in PEDFR mRNA expression in the 50/10 OIR model compared with RA (Figure 9). There was little fold difference in PEDF mRNA expression in RA vs the 50/10 OIR model at p14 (Figure 8).

**EXPRESSION OF GROWTH FACTORS AND THEIR RECEPTORS AT p18 DURING INTRAVITREOUS NEOVASCULARIZATION IN THE 50/10 OIR MODEL**

The 50/10 OIR model rat pups are moved to a hyperoxic environment (from 10% oxygen to 21% oxygen [ie, room air]) at p14. At p18, intravitreous neovascularization develops at the junction of vascular and avascular retina. In association with intravitreous neovascularization at p18, we found greater fold changes in expression of VEGF164, VEGFR2, NP1, and NP2 compared with RA (Figures 3, 4, 6, and 7). The fold change in expression of VEGFR1 was similar to that in RA (Figure 5).

Because PEDF is an angiogenic inhibitor, we anticipated that PEDF and PEDFR would be reduced at p18 compared with RA. However, the fold changes in expression of PEDF and PEDFR at p18 were not significantly greater than those in RA (Figures 8 and 9).

**COMMENT**

In the 50/10 OIR model compared with RA, we found increased fold changes in mRNA of VEGF164, VEGFR2, and the NPs at p18 in association with intravitreous neo-
vascularization. This was anticipated because VEGF_{164}, VEGFR2, and the NPs are involved in angiogenic processes. However, at p14 in association with persistent peripheral avascular retina in the 50/10 OIR model, the fold change in mRNA expression of VEGF_{164} and both NPs (and a trend for increased VEGFR2) was greater than in RA, in which retinal vascularization was complete at p14. Furthermore, the fold change in VEGFR1 mRNA expression, believed to limit the amount of VEGF ligand present to signal through VEGFR2 during development, was not significantly elevated in the 50/10 OIR model vs RA. These were unexpected findings based on data from the mouse model of oxygen-induced retinopathy, in which VEGF was downregulated during “vaso-obliteration” and was upregulated during angiogenesis.

The VEGF and PEDF are involved in pathologic angiogenesis and physiologic developmental retinal angiogenesis and are regulated by oxygen tension. Expression of VEGF is upregulated by hypoxia and expression of PEDF by hyperoxia. Pigment epithelium–derived factor is a potent angiogenic inhibitor, whereas VEGF is an agonist. Vascular endothelial growth factor receptor 2 is believed to be important in angiogenesis, whereas VEGFR1 is thought to bind and limit the amount of VEGF ligand that can signal through VEGFR2, permitting ordered developmental angiogenesis. It is increasingly recognized that the NPs are also important in angiogenic processes. Neurilpin 1 was initially described as a receptor for the semaphorins and as an axonal guide in the central nervous system. Akula et al° reported associations between VEGF, semaphorin 3A, vascular tortuosity, and electrophysiologic sensitivity variables of rods and the postreceptor retina in the 50/10 OIR model, suggesting that the postreceptor retina mediates vascular abnormalities in the model. We report herein that the NPs are upregulated in association with peripheral avascular retina and intravitreal neovascularization. Both NPs can function as receptors for several VEGF family members, including placental growth factor 1, VEGFB, and isoforms VEGF_{164/165} and VEGF_{145}. Less is known about PEDFR signaling. However, counter to the anticipated role of PEDF as an angiogenic inhibitor, PEDF knockout mice developed larger areas of vaso-obliteration following hyperoxia than wild-type littermates. Compared with RA, we found increased fold change in PEDFR mRNA expression at p14 associated with persistent peripheral avascular retina in the 50/10 OIR model. This finding may be important in the development of peripheral avascular retina because PEDF is an inhibitor of angiogenesis.

Arterial oxygen levels in the 50/10 OIR model are similar to transcutaneous oxygen levels measured in preterm infants who develop severe ROP; the model uses repeated oxygen fluctuations, a risk for ROP, rather than constant oxygen used in other models. In zone 2, stage 3 ROP, in which fluorescein angiograms demonstrate reduced peripheral retinal vascularization and perfusion with minimal central vascular retraction, there is peripheral avascular retina and minimal central obliteration in the 50/10 OIR model. The 50/10 OIR model reproducibly and consistently develops vessel tortuosity, which is analogous to plus disease, peripheral avascular retina at p14 (analogous to human zone 2 ROP), and subsequently intravitreal neovascularization at the junction of vascular and avascular retina (analogous to human stage 3 ROP). Other models of oxygen-induced retinopathy, including the mouse model, use oxygen levels higher than those in neonatal intensive care units in the United States and create central rather than peripheral avascular retina.

There are several possible explanations for our findings. At p14, VEGF expression may be increased as a result of previous exposure to 10% oxygen. We used real-time PCR to measure expression because it is the most quantitative method available. High VEGF mRNA expression is consistent with later development of intravitreal neovascularization at p18 but does not explain why blood vessel growth occurs into the vitreous rather than in the retina.
than into the retina. Loss of the physiologic gradient of VEGF in the retina may be why blood vessels grow into the vitreous. However, Geisen et al. measured vitreous VEGF and retinal VEGF in the 50/10 OIR model and found that vitreous levels were one-tenth those of retinal levels at p18, failing to provide support for a gradient of VEGF toward the vitreous and away from the retina. It is also possible that at p14 the model is moving toward a vasoproliferative phase. However, several studies found that inhibition of VEGF bioactivity using a neutralizing antibody or a VEGFR2 kinase inhibitor reduced angiogenesis into the vitreous (intravitreal neovascularization) but did not interfere with angiogenesis into the retina (intraretinal vascularization). Excessive signaling through VEGFR2 has been shown to interfere with developmental angiogenesis in other models. Increased signaling through VEGFRs may disorder and interfere with developmental angiogenesis. This might delay normal vascularization and contribute to peripheral avascular retina. However, because VEGF is also a neuronal survival factor, there is concern about possible systemic adverse effects of anti-VEGF agents that are absorbed into the bloodstream of the developing preterm infant.

Although measurement of mRNA expression by real-time PCR provides the most quantitative data, it does not always correspond to the concentration of translated proteins or activated proteins involved in signaling pathways. Further study in these areas is warranted.

In summary, expression of VEGF164 and its receptors and coreceptors was increased in the 50/10 OIR model at p14 in association with persistent peripheral avascular retina compared with that in RA at p14, when retinal vascularization was complete. We propose that overexpression of VEGF may interfere with retinal vascular development and contribute to peripheral avascular retina. We also found that VEGF164 was increased in the 50/10 OIR model at p18 in association with intravitreal neovascularization. Increased PEDFR expression in the 50/10 OIR model compared with RA may also have a role in contributing to peripheral avascular retina.

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