STUDIES ON THE PATHOGENESIS OF AVASCULAR RETINA AND NEOVASCULARIZATION INTO THE VITREOUS IN PERIPHERAL SEVERE RETINOPATHY OF PREMATURITY (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

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ABSTRACT

Purpose: To study vascular endothelial growth factor (VEGF) regulation in the development of intravitreous neovascularization and peripheral avascular retina in peripheral severe retinopathy of prematurity (ROP).

Methods: The rat 50/10 model of ROP mimics zone II, stage 3 severe ROP and recreates fluctuations in transcutaneous oxygen levels in preterm infants. On postnatal (p) day ages p0, p8, p11-p14, and p18, retinas from the model or room-air (RA) age-matched pups were analyzed for mRNA of VEGF splice variants and receptors using real-time polymerase chain reaction or VEGF protein using enzyme-linked immunosorbent assay.

Results: On p14, when retinas were only 70% vascularized in the model but fully vascularized in RA, $VEGF_{164}$ expression was threefold greater in the model compared to RA. On p18, intravitreous neovascularization was associated with a 5-fold increase in $VEGF_{164}$ mRNA in the model compared to RA. By analysis of variance, $VEGF_{164}$ and VEGFR2 mRNAs were up-regulated in association with increasing developmental age ($P<.0001$ for both comparisons) or exposure to the model compared to RA ($P<.0001$ and $P=.0247$, respectively), whereas increasing developmental age was associated only with up-regulated $VEGF_{120}$ ($P=.0006$), $VEGF_{188}$ ($P=.0256$), and VEGFR1 ($P<.0001$) mRNAs. VEGF protein increased significantly in the model and on p14 and p18 compared to RA ($P<.0001$).

Conclusions: The model mimics contemporary severe ROP in the United States unlike other models of oxygen-induced retinopathy. Compared to RA retinas, VEGF significantly increased in association with avascular retina and intravitreous neovascularization. A hypothesis is proposed that VEGF up-regulation plays a role in the development of both important features.

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INTRODUCTION

Retinopathy of prematurity (ROP) affects about 16,000 preterm infants in the United States annually. Of these, 1,100 infants require treatment, and blindness is estimated to develop in 550.[1,2] ROP is one of the earliest and the most prevalent causes of visual impairment in children in the United States[3] and a leading cause of childhood blindness worldwide.[4]

Preterm birth results in incomplete retinal vascular development and areas of peripheral avascular retina. In ROP, changes at the junction of the vascularized retina and the peripheral avascular region can lead to the growth of blood vessels into the vitreous and later a fibrovascular tractional retinal detachment. An early hypothesis proposed by Michaelson,[5] Ashton and Cook, [6] and Patz and associates[7] posed that an angiogenic factor or factors were involved. Studies within the last decades have identified several factors, most notably vascular endothelial growth factor (VEGF).

Since the early description and understanding of ROP,[5-7] technological advances have permitted monitoring and regulation of oxygen to the preterm infant. Yet, most animal models now used to study the pathophysiology of severe ROP deliver very high and constant oxygen, no longer pertinent to most cases of severe ROP in locations that have implemented the technology to monitor and regulate oxygen. This thesis uses an animal model representative of peripheral severe ROP (rat 50/10 OIR model) to better understand the role of contemporary oxygen stresses on the expression of angiogenic factors in developmental angiogenesis, neovascularization into the vitreous, and peripheral avascular retina. In this thesis, specifically, the expression of VEGF and its receptors was determined in the rat 50/10 OIR model and in room air–raised pups at the same developmental ages. The following hypotheses were addressed: (1) that at the developmental age when retinal vascularization was incomplete in the 50/10 OIR model but complete in room air, VEGF would be relatively down-regulated in the 50/10 OIR model compared to room air, and (2) that when neovascularization into the vitreous occurred, VEGF would be up-regulated in the 50/10 OIR model compared to age-matched pups raised in room air. Based on the analyses and literature review, a refined hypothesis is presented to explain why blood vessels grow into the vitreous in severe ROP and not into the retina as occurs in natural retinal vascular development. If blood vessel growth were redirected from the vitreous into the retina in the preterm infant, this strategy would reduce blindness from ROP, improve blood flow and nutrition to the peripheral avascular retina, and potentially improve visual field.

The thesis first reviews what is currently known about ROP and its evolution and then presents a study to test the hypotheses above. Following the results, a refined hypothesis is presented and discussed.

CLINICAL ASPECTS OF ROP

Impact

Preterm births have increased 30% since 1981 and now account for almost 12.5% of all births in the United States.[2] In developing countries worldwide, ROP is now a leading cause of childhood blindness.[4] Besides the worthy goal of preventing blindness in even a

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few infants, there are also substantial benefits in reducing medical and societal costs associated with raising blind children to adulthood.[8]

Classification and Management of ROP

Although controversy exists regarding the exact mechanisms in the human infant, retinal vascular development is believed to begin adjacent to the optic nerve through a process of vasculogenesis, in which circulating angioblasts develop early retinal vessels in the region surrounding the optic nerve. From these initial blood vessels, angiogenesis then proceeds to extend the retinal vasculature to the ora serrata. Angiogenesis includes processes of proliferation and migration of endothelial cells toward a gradient and the recruitment of mural cells to form mature vascular structures. Most developmental retinal vascularization occurs through angiogenesis.[1] Based on the timing of occurrence and the extent of retinal vascular development, peripheral severe ROP appears to involve abnormalities in developmental angiogenesis. We define normal developmental angiogenesis as intraretinal vascularization, since the location of the blood vessels is within the retina, and distinguish it from pathologic neovascular growth outside the plane of the retina and into the vitreous (intravitreous neovascularization). Retinal vascular development is often incomplete in the preterm infant at birth and usually proceeds after birth. Until vascular development is mature, there is a risk of ROP developing. In the United States, infants born before 31 weeks gestation or smaller than 1,500 gm are screened for ROP beginning at 4 to 6 weeks chronologic age or 31 weeks postgestational age (see below), whichever is older.[9,10] Periodic examinations of preterm infants continue until mature retinal vascularization develops or until severe ROP develops and treatment is recommended. Most ROP resolves without requiring treatment, and less than 10% of infants develop severe characteristics, which increase the risks of retinal detachment and poor visual acuity (VA). Treatment guidelines have been developed based on the Cryotherapy for Retinopathy of Prematurity (CRYO-ROP)[11] and Early Treatment for Retinopathy of Prematurity (ETROP)[12] studies of the severe forms of ROP, threshold ROP and type 1 prethreshold ROP (Table). The terms for severe ROP—threshold and type 1 prethreshold—describe the risks of an untoward outcome, such as retinal detachment. In threshold disease, the risk approaches 50%,[11] whereas in type 1 prethreshold disease,[12] the risk approaches 15%. The multicenter study, CRYO-ROP,[11] recommended treatment guidelines for threshold disease, and the later multicenter study, ETROP, found benefit with treatment at an earlier, less severe level, type 1 prethreshold disease.[12] After treatment with laser or cryotherapy, close follow-up is important to determine if vitreoretinal traction and progressive retinal detachment develop. Should these characteristics develop, vitreoretinal surgery is considered to prevent macular detachment (stage 4 ROP) and total retinal detachment (stage 5 ROP, previously called retrolental fibroplasia).[1]

TABLE. DEFINITIONS OF THRESHOLD AND TYPE 1 PRETHRESHOLD RETINOPATHY OF PREMATURITY

THRESHOLD ROP (TESTED IN CRYO-ROP STUDY)*	TYPE 1 PRETHRESHOLD ROP (TESTED IN ETROP STUDY)*
Zone I or II, stage 3 (5 contiguous or 8 total clock hours with plus disease†)	Zone I, any stage with plus disease† Zone I, stage 3 without plus disease† Zone II, stage 2 or 3 with plus disease†

CRYO-ROP, Cryotherapy for Retinopathy of Prematurity; ETROP, Early Treatment for Retinopathy of Prematurity.
*In threshold disease, the risk approaches 50%,[11] whereas in type 1 prethreshold disease,[12] the risk approaches 15%.
†The ETROP study recognized plus disease as two quadrants of dilated and tortuous vessels, whereas the CRYO-ROP study defined plus disease as four quadrants of dilated and tortuous vessels.

The CRYO-ROP and ETROP studies found that postgestational age is correlated with the development of threshold ROP and type 1 prethreshold ROP.[1] Postgestational age is the sum of the gestational and chronologic ages expressed in weeks. For example, an infant born at 24 weeks gestation 16 weeks previously has a postgestational age of 40 weeks. The terms *corrected age, postconceptual age,* and *postmenstrual age* also are used, and there are some differences in the exact meanings. In this thesis, the term *postgestational age* is used.

ROP is classified by several parameters: zone, stage, plus disease, and extent of stage.[1]

ROP Zone. Three zones describe the extent of retinal vascular development (Figure 1). Zone I comprises a circle with the optic nerve at the center and the radius equal to twice the distance from the optic nerve to the fovea. Zone I, therefore, has the largest area of peripheral avascular retina. Zone II ROP comprises a circle with the optic nerve at the center and the radius equal to the distance from the optic nerve to the nasal ora serrata. Zone III is the remaining temporal crescent and carries the lowest risk of a poor outcome from severe ROP. ROP in the individual infant is described as the lowest zone into which intraretinal vascularization has extended or where a stage of ROP is present.

ROP Stage. The stage describes the ROP severity. Stages 1 through 3 ROP describe the retinal appearance between the vascular and peripheral avascular regions. Stage 1 is a line (Figure 2, left, Retcam [Clarity Medical Systems, Pleasanton, California] wide-angle fundus image), stage 2 is a ridge (Figure 2, right), and stage 3 is intravitreous neovascularization (Figure 3), a feature of severe

ROP. Stage 4 ROP describes a partial retinal detachment and can include tractional or serous components. Stage 5 ROP is a total retinal detachment. There is also a shift from neovascular to fibrovascular features, such as vitreous contraction, that occurs when stage 3 ROP evolves into tractional stage 4 ROP.[13]

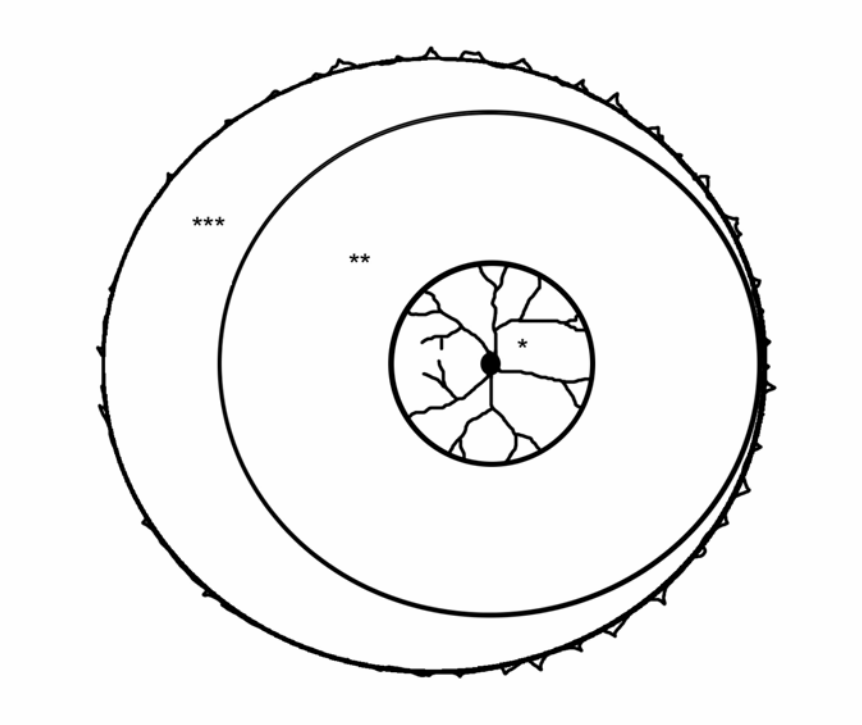

FIGURE 1

Artist diagram of a right eye showing the areas corresponding to ROP zones. The optic nerve and retinal vessels are drawn within zone I (*=zone I; **=zone II; ***=zone III).

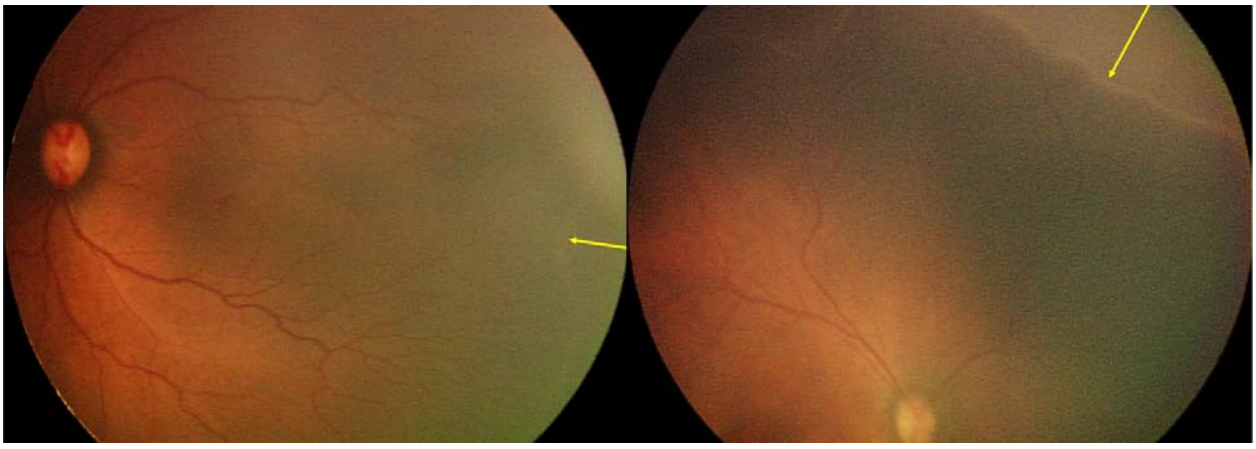

FIGURE 2

Retcam wide-angle image of the left eye of a preterm infant showing zone II, stage 1 ROP, identified with yellow arrow (left image) and of the right eye of a preterm infant showing zone II, stage 2 ROP, identified with yellow arrow (right image). Avascular retina is on the right of the arrows in both images.

Plus Disease. ROP also is characterized by dilated and tortuous vessels defined as plus disease. Plus disease is a feature of severe ROP (Figure 4).

Extent of Stage. In the CRYO-ROP study, the extent of the stage was used to characterize ROP severity and define the threshold at which the risk of retinal detachment approached 50%. However, the ETROP Study reported that the extent of the stage was less important than the features, plus disease and stage 3 ROP (Table).

The extent of ridge thickening or the presence of vitreous hemorrhage, plus disease, or fibrovascular changes between the retina and vitreous are important retinal features that predict progression to stage 4 ROP after laser treatment (Figure 5).[14,15]

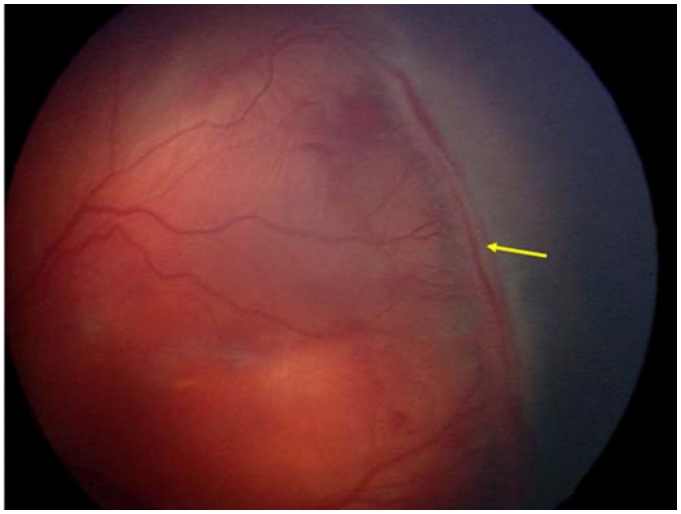

FIGURE 3

Retcam wide-angle image of the left eye of a preterm infant showing zone II, stage 3 ROP, identified with yellow arrow. Avascular retina is to the right of the arrow in the image.

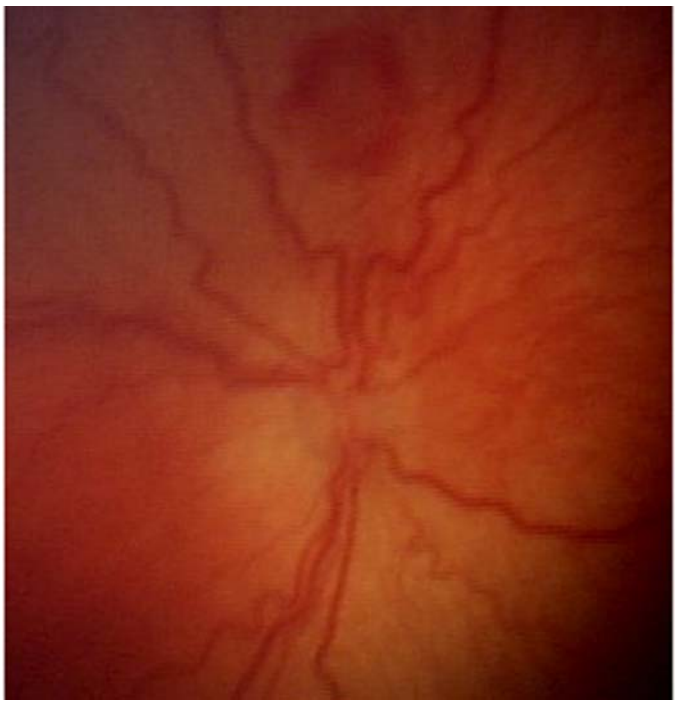

FIGURE 4

Retcam wide-angle image of the right eye of a preterm infant shows dilated and tortuous vessels from plus disease.

Therapy

The current approved treatment for severe ROP is laser or cryotherapy delivered in a nearly confluent pattern to the peripheral avascular retina. Laser is preferred to cryotherapy,[16] partly because laser causes less inflammation and breakdown of the blood-retinal barrier. Treatment can reduce the risk of untoward structural outcomes and poor vision. In the CRYO-ROP study, there was a 30% to 40% reduced risk of poor VA and adverse structural outcomes after treatment for threshold ROP.[1,11,17] In the ETROP study, which tested outcomes of laser treatment performed for less severe levels of ROP than threshold disease (Table), the risk of adverse structural outcomes at 2 years decreased from 15.4% to 9.1%.[18] If progressive stage 4 ROP develops, a lens-sparing vitrectomy releases the tractional components that cause retinal detachment.[1,19] In some cases, a scleral buckle is performed.

Several clinical trials are testing the effect of agents to inhibit the bioactivity of VEGF. These drugs are administered as intravitreous injections to preterm infants with severe ROP (see also "VEGF in Disease and Development" and the "Discussion" sections).

Types of Severe ROP

Features of severe ROP include plus disease and intravitreous neovascularization (ie, stage 3 ROP).[1] The peripheral avascular retina exists before the development of stage 3 ROP,[20] and the larger the area of peripheral avascular retina, the greater the risk of an adverse outcome from severe ROP.[21,22] Therefore, the presence of an avascular retina has been considered an important clinical feature in the development of severe ROP. In the United States today, most forms of severe ROP occur in zone II with stage 3 ROP and plus disease in at least two quadrants.[1] For this thesis, this form of severe ROP is referred to as *peripheral severe ROP*. In the CRYO-ROP study, most cases of severe ROP had peripheral severe ROP, but some zone I cases may have had aggressive posterior ROP.[17]

Aggressive posterior ROP is seen much less often than peripheral severe ROP.[23,24] Aggressive posterior ROP presents in zone I

with plus disease and flat neovascularization (Figure 6, left). This form of severe ROP affects infants of young postgestational ages and low birth weights and often presents at the first examination. Aggressive posterior ROP responds less well to laser and can progress to require surgical intervention, most often with lens-sparing vitrectomy for vitreous hemorrhage or retinal detachment.[23] Even with uncomplicated lens-sparing vitrectomy, late postoperative subretinal bleeding has been reported.[24] Aggressive posterior ROP has similarities to previous reports of Rush disease.[25]

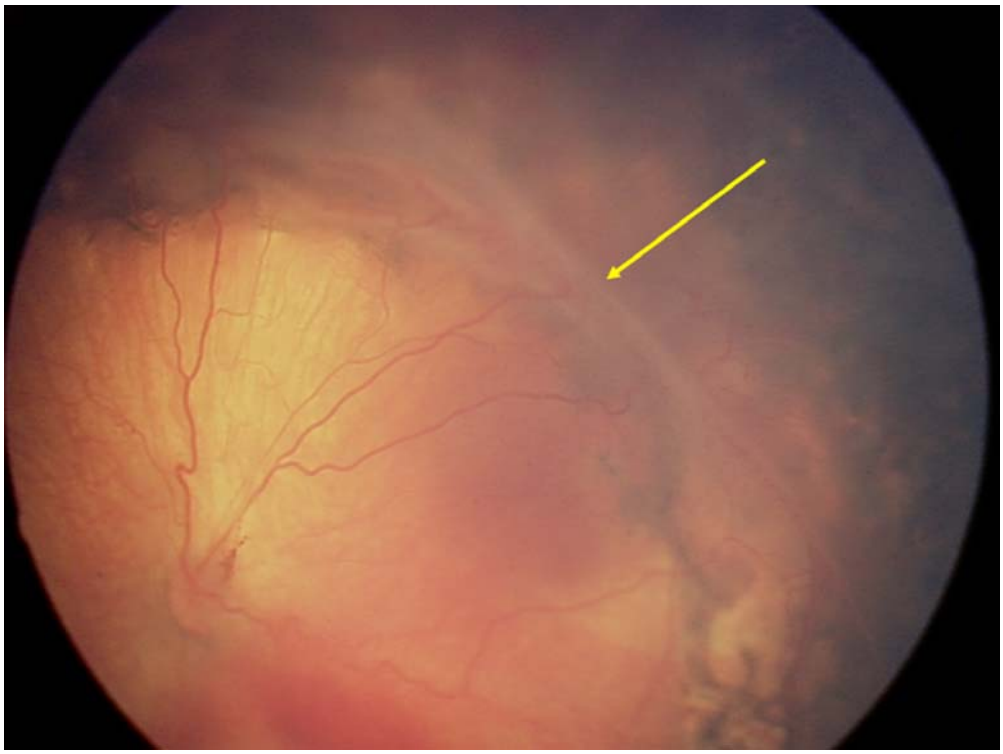

FIGURE 5

Retcam wide-angle image of the left eye of a preterm infant showing stage 4B ROP with fibrovascular contraction and a retinal detachment pulled into a peak, shown by yellow arrow. Pigmented laser spots in the avascular retina are to the right of the arrow in the image.

The appearance of stage 3 ROP differs between peripheral severe ROP and aggressive posterior ROP. In peripheral severe ROP, stage 3 is continuous, thickened, and elevated (Figure 3). There often is clear delineation among the intraretinal vascularization, stage 3 ROP, and the peripheral avascular retina. After laser treatment, regression of stage 3 ROP and flattening of the ridge often occur, and intraretinal vascularization can sometimes develop between laser spots into the avascular peripheral retina. However, in aggressive posterior ROP, stage 3 ROP appears lacy and is difficult to differentiate from normal intraretinal vascularization. Therefore, the junction between the vascular and avascular retina is not always distinct. The presence of hemorrhages provides a clue that flat intravitreous neovascularization may be present (Figure 6, right). Involution of flat intravitreous neovascularization after laser treatment may manifest areas of avascular retina, and new stage 3 ROP can develop at the junction of the vascular and newly identified avascular retina. Periodic examinations and possibly additional laser treatment are important.[26]

Stage 4 ROP also can differ in appearance based on whether it develops from peripheral severe ROP or aggressive posterior ROP. Whereas one or more features, such as clinically apparent thickening of the ridge, vitreous hemorrhage, or fibrovascular contraction at the ridge between the vascular and avascular retina, are the more common characteristics of peripheral severe ROP[14,15] (Figure 5), traction along the major arcades or around the optic nerve (Figure 7) also can develop in eyes with aggressive posterior ROP.

Differences in the fluorescein angiographic appearances exist between peripheral severe ROP and aggressive posterior ROP.[27] Whereas vascular nonperfusion is present in the peripheral avascular region of peripheral severe ROP, both nonperfusion of the peripheral avascular region and sometimes central capillary nonperfusion between the retinal arterioles and veins have been reported in aggressive posterior ROP.[27,28] These distinctions help the interpretation of data from animal models of oxygen-induced retinopathy (OIR). However, before describing these, it is useful to consider the role of oxygen in the pathogenesis of ROP.

Role of Oxygen in ROP

In the 1940s, when ROP was first described, it was likely due, in part, to high unregulated oxygen levels at birth.[5-7] Blood oxygen levels were not measured until decades later. Therefore, the oxygen levels of infants who developed ROP in the 1940s can only be estimated at best. To better understand the cause of this initial wave of ROP, several animal models of OIR were developed[7,29] (see also "OIR Models"). The results of this early research led to improvements in oxygen monitoring and technology to avoid high unregulated oxygen levels at birth. With these implementations, the incidence of ROP decreased substantially. However, as infants of younger gestational ages and smaller birth weights survived, ROP again became more common. Today, with attempts to reduce high levels of inspired oxygen at birth, fluctuations in transcutaneous oxygen levels also are recognized as important in the development of severe ROP.[30-34] Fluctuations in inspired oxygen along with episodes of bradycardia and apnea of prematurity have been hypothesized to vary oxygen delivery to the developing preterm infant retina. In addition, there are conflicting reports about the role of supplemental oxygen in the development of severe ROP. The Supplemental Therapeutic Oxygen for Prethreshold Retinopathy of Prematurity (STOP-ROP) study found no adverse effect from supplemental oxygen,[35] and some investigators have reported beneficial effects from supplemental oxygen.[36] However, other investigators have reported that severe ROP is more common in infants who have had high oxygen saturation levels at older ages.[37,38] Thus, the pathogenesis of ROP appears complex and may involve not only high oxygen concentrations at birth, which most models of OIR mimic, but also current stresses of oxygen fluctuations and supplemental oxygen during the infant's course in the neonatal intensive care unit.

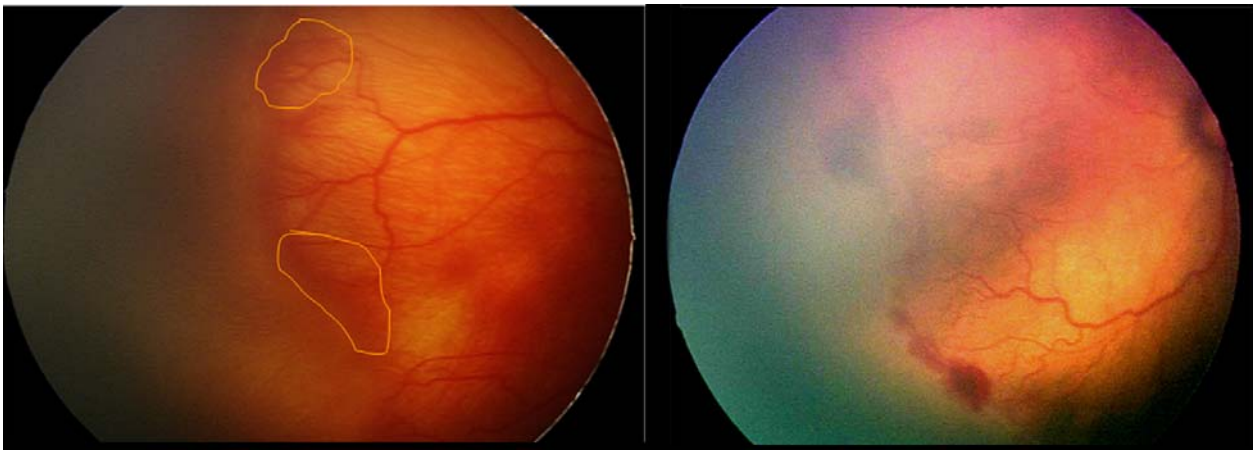

FIGURE 6

Retcam wide-angle image of the right eye of a preterm infant showing zone I, stage 3 ROP with outlined areas of flat neovascularization (left image) (some fine flat intravitreous neovascularization is not resolved with the Retcam) and of the right eye of a preterm infant showing zone I, stage 3 ROP with hemorrhage from underlying flat intravitreous neovascularization (right image).

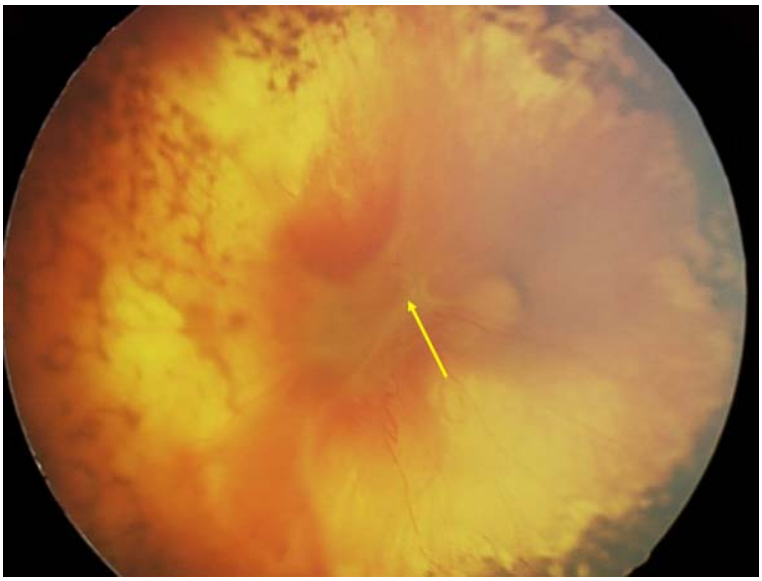

FIGURE 7

Retcam wide-angle image of the right eye of a preterm infant showing zone I ROP. The infant had severe intrauterine growth retardation and underwent laser for aggressive posterior ROP. Subsequently at 42 weeks postgestational age, fibrovascular contraction on the retinal surface and around the arcades is noted. Focus is on the elevated tractional retinal detachment (arrow), and the area of the attached retina appears out of focus.

PROPOSED BIOLOGIC MECHANISMS OF ROP

Clinical Pathology

Histopathologic analyses of clinical ROP specimens have shown a peripheral avascular area adjacent to vascularized retina. At the junction, Foos[39] described anterior proliferation of primitive mesenchymal spindle cells that preceded proliferating endothelial cells from which intravitreous neovascularization developed. The spindle cells did not label with factor VIII, desmin, or glial fibrillary acidic protein, suggesting they were not of an endothelial, pericytic, or glial origin. With the change from a vascularly active process in stage 3 ROP to a fibrovascular process in stage 4 ROP, mesenchymal tissue folded upon itself to form a retinal detachment.[39] These studies also highlighted, but from a pathophysiologic perspective, the importance of the clinical features, avascular retina and intravitreous neovascularization, in the development of stages 4 and 5 ROP.

Genetics

A retrospective analysis of monozygotic and dizygotic twins reported that 70% variance in the susceptibility of ROP was from genetic factors.[40] Although several candidates have been examined, the genetic picture appears complex. Some proteins within the wnt signaling pathway, including norrin, are candidates that have been studied based on their importance in retinal and vascular development[41,42] Mutations in the gene responsible for norrin cause Norrie disease, an X-linked condition associated with blindness from retinal vascular abnormalities and retinal detachment. Mutations in the Norrie disease gene were studied in ROP and have been reported to account for 3% of advanced cases.[43] Mutations within the cysteine knot configuration of the Norrie disease gene also were reported to be associated with severe retinal dysplasia in pediatric retinal diseases, including ROP, whereas other polymorphisms within the gene were associated with less severe vitreoretinopathies.[44] However, a study with a racially diverse population found no significant increase in the prevalence of polymorphisms in infants with severe ROP compared to control preterm infants with no or minimal ROP.[45] Mutations in the FZD4 receptor for norrin have also been associated with forms of severe ROP.[46] There is evidence that some polymorphisms in VEGF are associated with severe ROP,[47] whereas other evidence suggests opposite associations.[48] In addition, despite the finding that low serum insulinlike growth factor 1 (IGF-1) is associated with more severe ROP,[49-51] one study failed to show a relationship between a prevalent polymorphism in the IGF-1 receptor and the presence of ROP.[52] Another study reported associations between ROP and mutations in complement factor H, which regulates the alternative pathway of the complement system, endothelial per-arnt-sim [PAS] domain-containing protein 1 (EPAS1), which can transactivate VEGF receptor 1, and Indian hedgehog, which is important in development and angiogenesis.[53] Therefore, certain candidate genes have been reported in association with severe ROP, but there is not consistency in the literature. The disparities in studies reported suggest heterogeneity may be based on differences in samples of the study populations, phenotype of disease when reported, and discrepancies in how diagnoses were determined.

Animal Models

Much of the current understanding of the pathologic mechanisms in acute ROP is based on models of OIR. Most models expose newborn, full-term animals to high percentages of inspired oxygen, similar to what preterm infants were likely to have received in the 1940s before the ability to monitor and regulate oxygen levels.[28,54-57] Today, inspired oxygen and infant oxygen saturation are regulated. Therefore, the oxygen concentrations used in most models of OIR do not translate to blood oxygen levels in preterm infants who develop peripheral severe ROP today.

It must be noted that modeling the actual retinal tissue oxygenation that occurs in the preterm infant is currently not possible. There are several factors that do not translate well using available models of OIR. For example, the preterm infant has immature lungs and experiences shunting of blood. In addition, fetal hemoglobin has a different affinity for oxygen than does adult hemoglobin, and the amount of fetal to adult hemoglobin varies depending on postgestational age and whether blood transfusions have been given. Other cardiovascular and hematologic conditions, including sepsis and anemia of prematurity, may affect tissue oxygenation. However, these factors tend to reduce arterial and tissue oxygenation, whereas models that use high and constant levels of oxygen likely result in much higher oxygen concentrations.

OIR Models. Models of OIR take advantage of the fact that several animals (eg, cat, mouse, rat, beagle) undergo retinal vascular development after birth.[28,54-57] The most studied model of OIR is the mouse OIR model, because mechanisms of extreme oxygen stress and angiogenesis can be tested rigorously using genetically modified mice.[28,58] However, these models of OIR do not reflect the oxygen levels or stresses of preterm infants who develop severe ROP in the United States currently. First, these models use high levels of inspired oxygen that cause arterial oxygen levels to greatly exceed those recommended for preterm infants today. For example, in a normal rat exposed to inspired oxygen levels of 75%, the arterial oxygen level would be greater than 300 mm Hg.[59] Today, neonatologists strive for oxygen saturations in the mid 80% to low 90% range, depending on the postgestational age, and these saturations translate to arterial oxygen levels less than 60 mm Hg. In addition, most OIR models use constant oxygen followed by room air (RA) exposure, whereas infants who develop severe ROP in contemporary neonatal units that monitor and regulate oxygen reportedly have minute-to-minute fluctuations in transcutaneous oxygen levels.[30] Newly developed central retinal capillaries also are susceptible to high oxygen and constrict and recess, a process that has been termed vaso-obliteration in the mouse OIR model. However, there is no clinical correlate with vaso-obliteration in contemporary peripheral severe ROP. Finally, when animals are moved from high oxygen to RA, the nonperfused retina produces angiogenic factors that lead to budding of endothelial cells into the vitreous.[58] Thus, the models develop first central capillary nonperfusion followed by endothelial budding, appearances that are very different from what occurs in the development of peripheral severe ROP, the most common form of severe ROP in the contemporary

United States.

John Penn[20] developed the rat 50/10 OIR model of peripheral severe ROP. This model exposes newborn rat pups to fluctuations in inspired oxygen between 50% and 10% every 24 hours for 14 days. The oxygen extremes in the rat 50/10 OIR model cause rat arterial oxygen levels[20] similar to the transcutaneous oxygen levels measured in preterm infants with peripheral severe ROP.[30] Rather than constant oxygen used in other models,[28,54,56,60] the 50/10 OIR model exposes pups to repeated fluctuations in oxygen, a risk factor for severe ROP.[30,33] As in peripheral severe ROP, in which fluorescein angiograms showed reduced peripheral retinal vascularization and perfusion with minimal central capillary nonperfusion (Lepore D, ARVO meeting, 2008, abstract), the rat 50/10 OIR model also has peripheral avascular retina and minimal central capillary nonperfusion.[28] The 50/10 OIR model reproducibly and consistently develops avascular retina analogous to human zone II ROP,[20,61] vessel tortuosity analogous to plus disease,[62] and subsequently intravitreous neovascularization analogous to stage 3 ROP.[61,63] These parameters can be measured and analyzed (Figure 8).

Molecular Mechanisms of Avascular Retina and Angiogenesis

Most hypotheses[60,64-66] of the pathophysiologic steps in severe ROP have been described using the mouse OIR model and other models of high oxygen-induced capillary recession and constriction, followed by relative hypoxia-induced angiogenesis[54-57] rather than the more relevant model of peripheral severe ROP. For example, using the mouse OIR model, Pierce and coworkers[67] found that high constant inspired oxygen at 75% led to reduced retinal VEGF expression in association with central capillary constriction and recession. When mouse pups then were placed into RA, relative tissue hypoxia in the newly developed central, nonperfused retina occurred, followed by overexpression of VEGF and endothelial budding above the internal limiting membrane (Figure 9).[58] Alon and coworkers[68] found that if VEGF was given during the period of hyperoxia, capillary constriction and recession decreased. Robinson and coworkers[69] reported that agents to inhibit VEGF, given during relative hypoxia, reduced endothelial budding into the vitreous. It also has been shown that other growth factors or nutrients (eg, IGF-1, IGF-1 binding protein-3, placental growth factor-1 arginyl-glutamine, omega 3 fatty acids)[70-74] interfere with the hyperoxia-induced capillary constriction and recession in the mouse OIR model. Conversely, inhibitors, including angiostatic factors,[75-77] can prevent capillary budding into the vitreous during the phase of relative hypoxia.

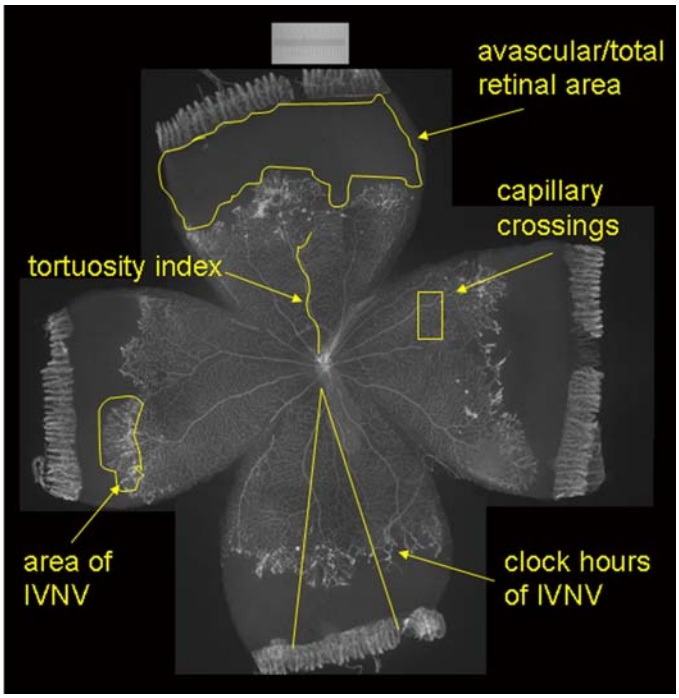

FIGURE 8

Lectin-stained retinal flat mount from rat on p18 after 14 days of oxygen fluctuations between 50% and 10%, then 4 days of room air exposure (rat 50/10 OIR model, see "Methods" section also). Peripheral avascular retina is between the ora serrata and vascularized retina. Quantitative measurements obtained using the model are shown in yellow. IVNV, intravitreous neovascularization.

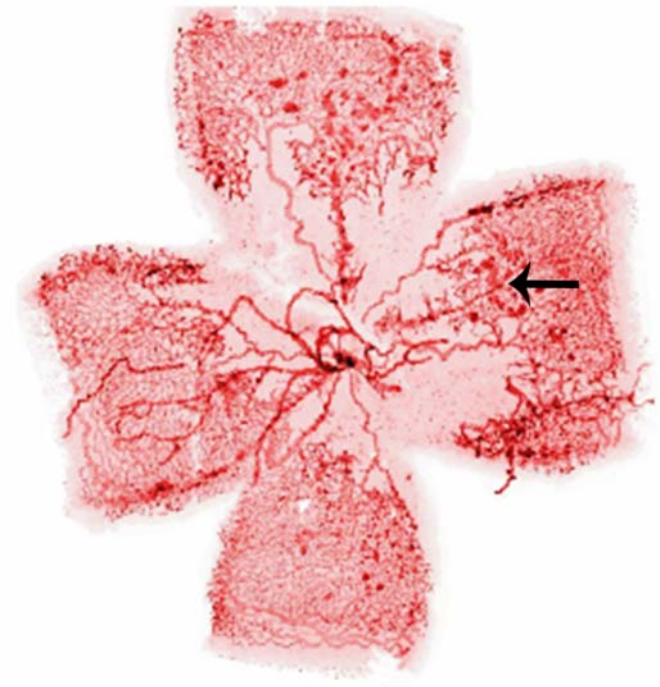

FIGURE 9

Retinal flat mount of mouse on p17 after 5 days of 75% oxygen from p7 to p12, then 5 days in room air from p12 to p17 (mouse OIR model). Capillaries and vessels are stained with Alexa Fluoro 594 conjugated isolectin and show central retinal capillary dropout with endothelial budding into the vitreous at the junctions of vascular and avascular retina. Some endothelial buds are identified by black arrow.

In both the rat 50/10 OIR model of peripheral severe ROP and the mouse OIR model, studies have reported that reactive-oxygen species (ROS), such as those produced by the enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase),

contribute to the development of the avascular retinal area[78] and the formation of intravitreous neovascularization.[79,80] Activated NADPH oxidase is the main source of superoxide radical from neutrophils[81] and can trigger angiogenesis[82] or apoptosis[83] in endothelial cells. It is believed that the determination of what signaling pathways are triggered may depend on the concentration and location of the ROS generated. Several investigators have shown that increased VEGF signaling causes intravitreous neovascularization in the 50/10 OIR model.[84,85] Inhibition of ROS through antioxidants, such as manganese superoxide dismutase[86] or vitamin E or C analogues,[87] also reduced the severity of retinopathy in the 50/10 OIR model. Pathways involving succinate[88] and prostaglandin E2[89] also have been reported as important.

VEGF in Disease and Development

VEGF. There are a number of angiogenic factors reported to be involved in OIR models. Examples include VEGFA, erythropoietin, IGF-1, angiopoietins, adrenomedullin, and hypoxia inducible factor-1α.[49,51,90] Of these, VEGFA is one of the most important in human retinal diseases associated with pathologic angiogenesis.[91-94] (VEGFA is the most widely studied member in the VEGF family and will be discussed and referred to as VEGF throughout this thesis.) VEGF protein increases in the ocular fluid of patients with intravitreous neovascularization, including ROP,[95,96] and VEGF mRNA was detected in the avascular retina of a human infant with stage 3 ROP.[97] Inhibition of VEGF bioactivity through several mechanisms also reduced angiogenesis in human choroidal and retinal diseases, including severe ROP, but most studies of ROP have lacked control groups.[98-101]

VEGF is up-regulated by hypoxia and ischemia.[92,93] Retinal hypoxia has been measured in the 50/10 OIR model in association with VEGF overexpression.[58,84] VEGF-triggered signaling led to intravitreous neovascularization in the 50/10 OIR model.[85] In the OIR and other models of retinal angiogenesis, inhibition of VEGF bioactivity reduced angiogenesis.[69,84,102-104] Thus, retinal hypoxia triggers VEGF signaling leading to intravitreous neovascularization, and inhibition of VEGF bioactivity reduces intravitreous neovascularization.

However, VEGF is also essential in retinal vascular development and as an endothelial and neuronal survival factor.[105-109] Most VEGF is expressed in the nerve fiber/ganglion cell layers, the outer nuclear layer in presumed Müller cells, the inner nuclear layer, and the retinal pigment epithelium (RPE).[58,103,110,111] In retinal vascular development, cells in the nerve fiber/ganglion cell region precede endothelial cell migration to the ora serrata.[112] These cells, astrocytes in cats[112] or angioblasts in dogs,[113] sense physiologic hypoxia and express VEGF. Coordination of several aspects of VEGF signaling is then essential for physiologic angiogenesis in normal vascular development. For example, the VEGF concentration regulates the rate of endothelial cell division.[114,115] In addition, presentation of VEGF, as in a gradient, regulates endothelial tip cells at the migrating front, and these tip cells provide directional guidance cues for migrating endothelial cells.[116] VEGF signaling also regulates the number and length of filopodia in endothelial tip cells in the 50/10 OIR model.[117] Other pathways, such as the delta-like ligand 4/Notch1 signaling pathway, regulate the VEGF-induced endothelial tip-to-stalk cell ratio at the junction of the vascular and avascular retina, which is important in permitting ordered developmental angiogenesis.[118,119]

Since VEGF can lead to pathologic neovascularization but is also important in physiologic retinal vascular development, it is important to study its role in ROP, which involves both processes. A single allele knockout to VEGF is lethal. Therefore, no genetic animal knockouts are viable to study VEGF in retinal vascular development or in models of OIR. One way to study different functions of VEGF is to study the role of the VEGF mRNA splice variants. There are at least five proangiogenic VEGF mRNA splice variants in humans and three in mice and rats that develop from alternative splicing of the parent gene. The most studied are (mouse/rat analogs in parentheses) $VEGF_{189}$ ($VEGF_{188}$), $VEGF_{121}$ ($VEGF_{120}$), and $VEGF_{165}$ ($VEGF_{164}$).[120] The splice variants have different biologic functions based on differences in heparin binding, which determine the gradient generated.[121-126] $VEGF_{164/165}$ is largely important in creating a gradient for retinal vascular development,[127] but it also is reported to lead to leukostasis, endothelial apoptosis, and avascular retina through inflammatory mechanisms in some models.[122] $VEGF_{120/121}$ is soluble, and $VEGF_{188/189}$ is believed to be involved in endothelial adhesion and migration.[127,128]

VEGF Receptors. Besides determining the role of VEGF splice variants in pathologic vs physiologic angiogenesis, it is also important to study the role of signaling through VEGF receptors. There are three known VEGF receptors and several coreceptors. The relationship of these receptors with VEGF family ligands is complex and incompletely understood. VEGF receptor (R)2 has lower affinity for VEGF than does VEGFR1,[129] and the VEGFR2 tyrosine kinase activity is strong and considered more involved in angiogenic processes.[130]

In embryogenesis, VEGFR1 acts to limit angiogenesis by trapping VEGF and preventing excessive signaling through VEGFR2.[114] Knockout of VEGFR1 or VEGFR2 is lethal. However, knockout of only the receptor tyrosine kinase of VEGFR1 (ie, the intracellular signaling domain of the receptor) produces viable offspring.[114] This VEGFR1 tyrosine kinase knockout permits VEGF to bind VEGFR1 but does not signal.[114] Thus, for viable offspring, it appears that some VEGF must be bound by VEGFR1 during development to limit the amount of VEGF available to signal through VEGFR2.

In the adult, VEGFR1 may be involved in angiogenic processes and tumor metastasis and inflammation. Ligand binding to VEGFR1 by placental growth factor (PlGF) also can reduce capillary constriction and recession induced during hyperoxia in the mouse OIR model when PlGF is provided prior to hyperoxia.[70] Both VEGFR1 and VEGFR2 are expressed in endothelial cells. VEGFR2 also is expressed in RPE[107] and neuronal cells.[131,132] VEGFR1 is expressed in macrophages.[129] Determining the expression of VEGFR1 and R2 in development and in the 50/10 OIR model may be important to understand the roles of these receptors in normal and pathologic angiogenesis.

Other receptors include VEGFR3, which is involved in lymphangiogenesis, and the neuropilins, of which neuropilin-1 is a coreceptor for $VEGF_{164/165}$.[130] There is also a splice variant of VEGFR1, soluble flt-1 (s-flt-1), that can trap VEGF and may play a role

in the pathogenesis of preeclampsia.[133] Unlike the other receptors, which are cell associated, s-flt-1 can be released into the bloodstream.

PROPOSAL HYPOTHESES

Although other angiogenic agonists and inhibitors are involved in the pathogenesis of OIR or in physiologic retinal vascular development, VEGF has been reported to be important in both. In the models of high OIR, such as the mouse OIR model, hyperoxia down-regulates VEGF and is associated with capillary constriction and recession, leading to areas of central, nonperfused retina. Return to room air (RA) results in relative retinal hypoxia, and up-regulation of VEGF is associated with endothelial budding into the vitreous, initiating at the junctions of perfused and nonperfused retina. Although the mouse OIR and other models of high OIR have been used to describe events in ROP, they do not represent the severe ROP that develops in the United States today. Rather, the rat 50/10 OIR model better represents peripheral severe ROP (zone II, stage 3 ROP). However, little is known about the molecular mechanisms leading to the pathophysiology seen, especially the effects of oxygen fluctuations on VEGF regulation during the development of avascular retina and intravitreous neovascularization.

To better understand the role of oxygen fluctuations in the pathophysiology of intravitreous neovascularization and peripheral avascular retina in peripheral severe ROP, the mRNAs of VEGF splice variants and receptors in the rat 50/10 OIR model and in RA at the same developmental ages were measured. VEGF is a major factor involved in physiologic retinal vascular development and drives developmental angiogenesis.[112] Since developmental intraretinal neovascularization is incomplete in retinas of the 50/10 OIR model at the same time point when completed in RA, it is logical to postulate that VEGF would be decreased in the 50/10 OIR model compared to RA, similar to findings in other models of OIR (see "Molecular Mechanisms of Avascular Retina and Angiogenesis"). When intravitreous neovascularization develops in the 50/10 OIR model, but not in RA, it is logical to postulate that VEGF would be increased compared to RA. Therefore, the following hypotheses were addressed: (1) that on p14, the developmental age when retinal vascularization is incomplete in the 50/10 OIR model but complete in RA, VEGF would be relatively down-regulated in the 50/10 OIR model compared to RA, and (2) that on p18 when intravitreous neovascularization is present, VEGF would be up-regulated in the 50/10 OIR model compared to age-matched pups raised in RA. Based on the analyses and literature review, a hypothesis is presented to explain why blood vessels grow into the vitreous in severe ROP and not into the retina as that occurring in natural retinal vascular development.

METHODS AND MATERIALS

All animal studies complied with the Guide for the Care and Use of Laboratory Animals of the University of North Carolina Institute for Laboratory Animal Research and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research.

RAT 50/10 OIR MODEL OF PERIPHERAL SEVERE ROP

The model was described previously.[59] Within 4 hours of birth on p0, litters of 12 to 14 newborn Sprague-Dawley rat pups and their mothers (Charles River, Wilmington, Massachusetts) were placed into an Oxycycler incubator (Biospherix, New York, New York) to cycle inspired oxygen between 50% oxygen and 10% oxygen every 24 hours (Figure 10). Pups from other litters were used to supplement deficient litters. After seven cycles of oxygen fluctuations on p14, the pups then were placed into RA for 4 days.[59] Oxygen levels were monitored daily and recalibrated as needed. Carbon dioxide in the cage also was monitored daily and flushed from the system by maintaining sufficient gas-flow and by adding soda lime if needed.

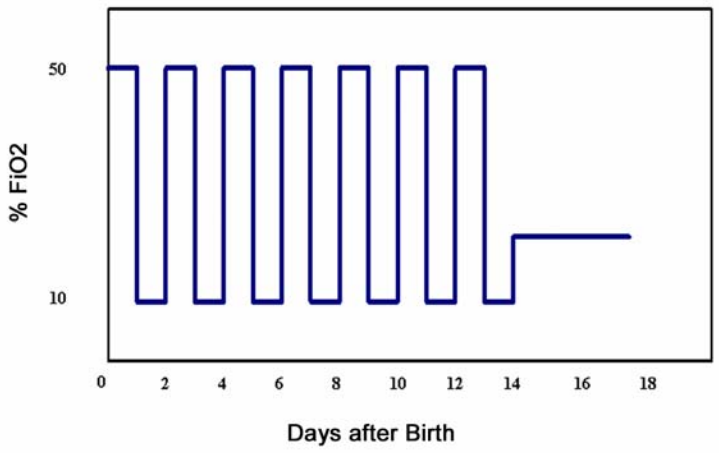

FIGURE 10

Representation of the rat 50/10 OIR model. Inspired oxygen (%FiO_2) on y-axis and postnatal day age on the x-axis. Changes in %FiO_2 occur every 24 hours beginning from birth with 50% FiO_2, then 10% FiO_2, until p14. Pups then are returned to room air (21% O_2) until analysis.

DISSECTION OF RETINAL TISSUE FOR FLAT MOUNTING AND mRNA AND PROTEIN ANALYSES

The methods were published previously[134] and are described below. For time point measurements, animals were euthanized with pentobarbital (80 mg/kg intraperitoneal injection) following the change in the inspired oxygen level. Therefore, pups euthanized on even-numbered days up to p14 had been exposed to 10% oxygen and on odd-numbered days to 50% oxygen. Pups euthanized on p18 had been exposed to seven cycles of oxygen fluctuations followed by 4 days in RA. For flat mounts, the eyes were fixed in 2% paraformaldehyde (PFA) for 2 hours. The retinas were isolated[134] with the ora serratas intact and placed into phosphate-buffered saline (PBS) after the hyaloidal vessels and remaining vitreous were removed. By making four incisions 90° apart, the retinas were flattened and placed on microscope slides. For fresh tissue, the eyes were not fixed in PFA and the retinas were dissected without ora serrata. Tissue was frozen in modified radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris base, 120 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10% glycerol) with a protease inhibitor cocktail (1:100, Sigma, St Louis, Missouri) and 1 M orthovanadate (1:100, Sigma), then stored at -20°C for protein or RNA*later* (Applied Biosystems, Foster City, California) for RNA until analysis.

Tissue Staining and Analysis of Flat Mounts

The methods have been published[85,117] and are described below. To stain the vasculature, the flattened retinas first were permeabilized in ice-cold 70% v/v ethanol for 20 minutes, then in PBS/1% Triton x-100 for 30 minutes, and then incubated with Alexa Fluor 568 conjugated *Giffonia simplicifolia* (Bandeiraea) isolectin B4 (5 μg/mL, Molecular Probes, OR) in PBS overnight at 4°C, as described previously.[117] Images of the retinal blood vessels were captured using a Nikon 80i Research Upright Microscope with Surveyor/Turboscan software and digitally stored for analysis.

The total retinal area, summed peripheral avascular retinal areas, and areas of intravitreous neovascularization were computed in pixels with Image Tool v.3 (University of Texas, San Antonio) and converted to square millimeters (using a calibration bar on each image). Intravitreous neovascularization was defined as neovascularization growing into the vitreous at the junction of the vascular and avascular retina.[61] Retinal flat mounts were divided into 12 clock hours of about equal area using Adobe Photoshop, assessed for the presence of intravitreous neovascularization,[84,85] and assigned a number (0-12) depending on the number of clock hours with intravitreous neovascularization. The areas of intravitreous neovascularization and avascular retina were measured, summed, and expressed as a percent of the total retinal area in each eye. Two independent masked reviewers performed the measurements. When discrepancies in measurements occurred, the flat mounts in question were reviewed and a final consensus was reached.

Real-time PCR

Samples were removed from RNAlater and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, California). DNA contamination was removed using DNA-free (Ambion, Austin, Texas), and RNA quantity was determined spectrophotometrically. One microgram of RNA from each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). About 200 ng of cDNA were analyzed per well by one-step real-time PCR using the TaqMan MasterMix with reverse transcriptase (3.7 U/reaction, Applied Biosystems). Primers were specific for rat (annealing temperature 60oC): VEGF164: forward 5′GCACATAGGAGAGATGAGCT 3′, probe 5′GCTCACAGTGATTTT CTGGC3′, reverse TGCAG CATAGCAGATGTGAATGCAGACC; VEGF120: forward 5′GCACATAGGAGA G ATGAGCT 3′, probe 5′GGCTTGTCACATTTTTCTGGC3′, reverse TGCAGCATAGCAGATGTGA ATGCAGACC; VEGF188: forward, 5′CAGTTCGAGGAAAGGGAAAG 3′, probe, 5′CAGTGAACGCTCCAGGATTT3′, reverse ACCGGGATTTC TTGCGCTTTCGTTTTTTG; VEGFR1: forward, 5′CCACCTCCATGTTTGAAGAC3′, probe, 5'AGTCCAGGTGAATC GCTTCA3′, reverse TACCAGCAGTCTGCTGACCTCCCC; and VEGFR2: forward 5′CTCCATCTTTTGGTGGGATG3′, probe 5′GCTGGTCTGGTTGGAGCCT3′, reverse AGGCCACAGACTCCCTGCTT TTACTG3, and were made by the Oligonucleotide Core facility of the University of North Carolina (http://www.med.unc.edu/olioli/index.htm). cDNA was mixed 1:1 with Taqman Universal Master Mix (Applied Biosystems) and primers. Rat β-actin was used as a control gene.135 Primers for rat β-actin were, forward, TGCCTGACGGTCAGGTCA, probe CACTATCGGCAATGAGCGGTTCCG, and reverse CAGGAAGGAAGGCTGGAAG. Duplicate reactions with a total volume of 16 μL were run for each sample and control using the AB 7500 PCR System. The 7500 System Software calculates cycle threshold (Ct) automatically for each well, and each value was normalized to β-actin. The Delta-Delta-Ct (DDCt) algorithm was used to calculate values. The p0 time point was within 4 hours of birth and was therefore the same time point for samples from RA pups and those in the 50/10 OIR model. VEGF188 at p0 was assigned a value of 1.0. The values for the three VEGF splice variants in the 50/10 OIR model and RA were related to this value to provide graphical representations. VEGFR1 RA at p0 was scaled to the value of 1.0 for graphical comparisons of time points for RA and the 50/10 OIR model for VEGFR1. The VEGFR2 value at p0 was 59.6-fold greater than VEGFR1. VEGFR2 in RA at p0 was scaled to the value of 1.0 for graphical comparisons of time points for RA and the 50/10 OIR model for VEGFR2. For statistical analysis, raw data were used.

Protein Analysis

VEGF protein was analyzed with an enzyme-linked immunosorbent assay (ELISA), which measures all VEGF splice variants. The most prevalent splice variants represent the greatest percentage in the ELISA value. Retinal samples frozen in RIPA buffer were thawed, homogenized, and centrifuged (16,000*g*, 10 minutes, 4°C). Total protein was quantified with a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, California, modified from the Lowry assay[136]). Supernatants were assayed without dilution in duplicate

using commercially available ELISA kits, raised against rat VEGF (R&D Systems, Minneapolis, Minnesota). The mean minimum detectable dose was 6.4 pg/mL for VEGF.

STATISTICAL ANALYSIS

To maintain the reproducibility of the ROP model, litters were never depleted below 12 pups.[137] Often this required that whole litters be used for individual time points. For each time point, at least five retinas from different pups were analyzed from at least two different litters. The mean -fold changes relative to β-actin with error bars representing standard errors are represented graphically. While these normalized ratios were used for graphical representation in the figures, to avoid bias, the raw data were rescaled and statistically analyzed as described below.

Initially, β-actin was analyzed by regression analysis of the ratio of each β-actin/VEGF splice variant or receptor mRNA for the Ct1 value against respective β-actin/VEGF splice variant or receptor mRNA for the Ct2 value, and the slope was found to be indistinguishable from 1.0, which is the ideal value.[138] The geometric means of the product of the two ratios then were determined for each time point and treatment, and this was the outcome analyzed. For the analysis of VEGF protein by ELISA, the protein concentration was the outcome analyzed. A factorial analysis of variance with a completely randomized treatment arrangement was used to determine the significance of the factors, time point and treatment (RA vs 50/10 OIR model), and the interaction of time point and treatment. Post hoc testing of treatment and time point by treatment interaction means was accomplished using protected *t* tests on least squares means, with alpha level adjustment for multiple comparisons among means accomplished using a method of simulation.[139]

RESULTS

RETINAL FLAT MOUNTS OF PUPS RAISED IN RA OR IN THE 50/10 OIR MODEL

In p14 RA-raised pups, retinal vascularization of the inner capillary plexus extended to the ora serrata (Figure 11, left). There was no avascular retina or intravitreous neovascularization on p14 or p18 in retinas from RA-raised pups.

In the 50/10 OIR model, the avascular retina comprised about 30% of the total retinal area on p14 and 25% on p18. No clock hours of intravitreous neovascularization were found on p14, and a median of 7.0 clock hours (3.2 ± 0.3 mm^2 mean avascular area ± standard error) was present on p18 (Figure 11, right). These findings were comparable to those reported in the literature.[140]

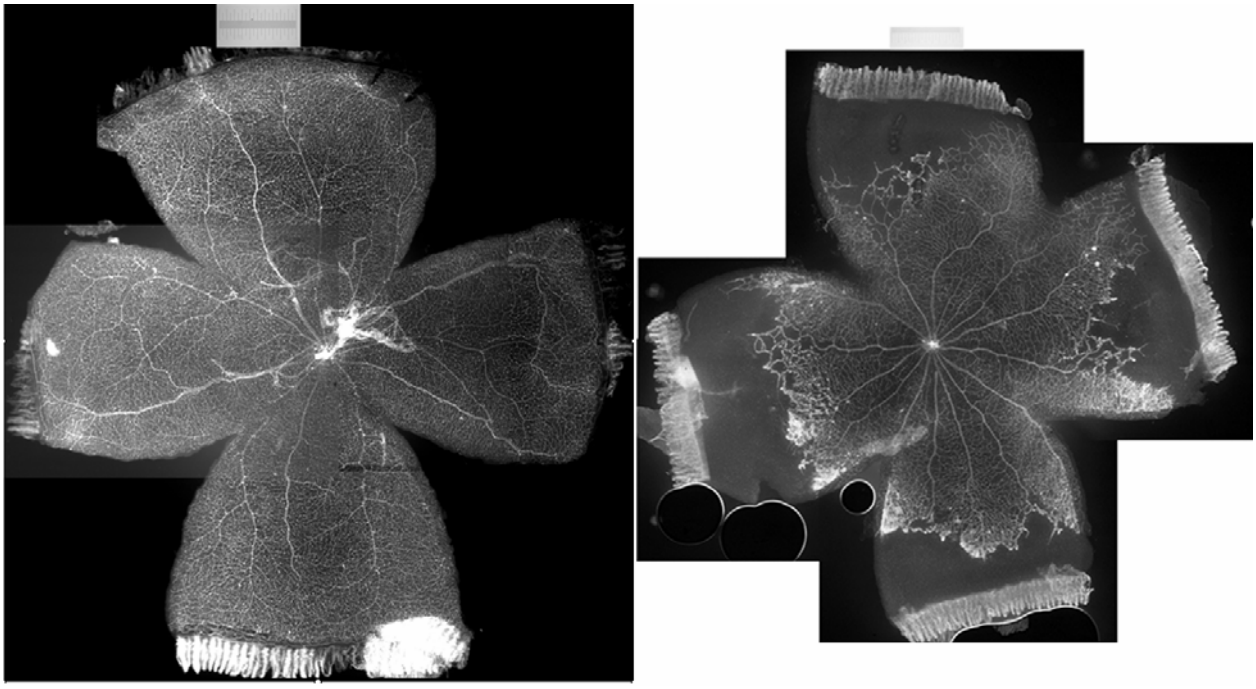

FIGURE 11

Lectin-stained retinal flat mount from rat on p14 raised in room air (RA) showing vascularization of the inner retinal plexus of vessels to the ora serrata (left image). Lectin-stained retinal flat mount from rat in 50/10 OIR model on p18 after seven cycles of 24 hours of 50% FiO_2 and 10% FiO_2 , then 4 days in RA showing intravitreous neovascularization at the junction of peripheral avascular retina and central vascularized retina similar to that in zone II, stage 3 ROP (right image). To flatten the retina, four relaxing incisions are made, creating a cloverleaf. The optic nerve is in the center and the ora serrata in the periphery. No macula is present in the rat.

mRNA OF VEGF RECEPTORS AND SPLICE VARIANTS AT DEVELOPMENTAL TIME POINTS IN THE 50/10 OIR MODEL AND RA

Time points were chosen that preceded and included p14 when avascular retina was present in the 50/10 OIR model but absent in RA-raised pups and on p18 when intravitreous neovascularization was present in the model and not in RA.

VEGFR1 mRNA increased many-fold during development (Figure 12). From p0 to p14, VEGFR1 mRNA increased 42-fold and from p0 to p18, 75-fold. Statistical analyses showed that increased expression of VEGFR1 was significantly (P<.0001) associated with older developmental age but not with whether pups had been exposed to the 50/10 OIR model or RA. The increase in VEGFR2 mRNA was relatively less than for VEGFR1 mRNA. From p0 to p14, VEGFR2 mRNA increased about 5-fold and from p0 to p18, about 3-fold (Figure 13). There was a significant increase in expression associated with older developmental age (P<.0001) and with exposure to the 50/10 OIR model compared to RA (P=.0247).

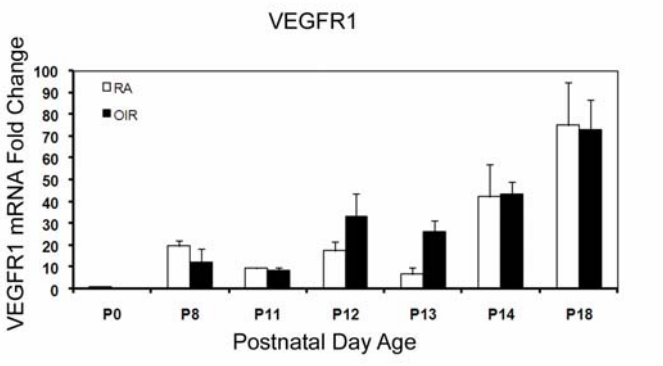

FIGURE 12

Real-time PCR values for VEGFR1 in retinas of rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur after hypoxia (10% inspired oxygen [FiO_2]) and p11 and p13 after hyperoxia (50% FiO_2). Following p14, pups are in RA (21% FiO_2). All values are normalized to β-actin and are compared to p0, which was assigned a value of 1.0 for graphic representation. Raw data were rescaled and statistically analyzed as described in the "Methods" section. Increased expression of VEGFR1 is significantly associated with older developmental age (P<.0001, ANOVA).

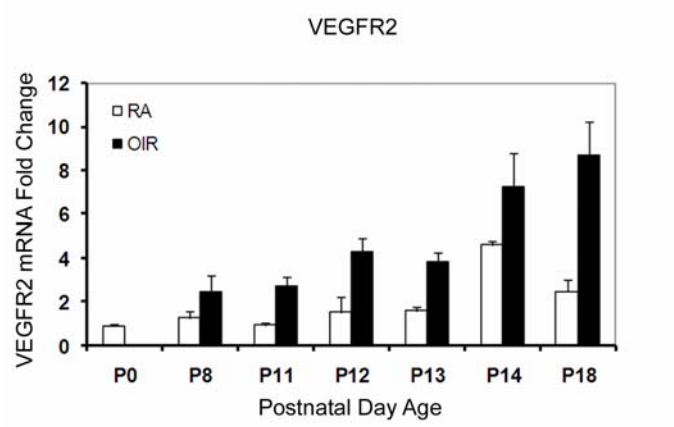

FIGURE 13

Real-time PCR values for VEGFR2 in retinas of rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur after hypoxia (10% inspired oxygen [FiO_2]) and p11 and p13 after hyperoxia (50% FiO_2). After p14, pups are in RA (21% FiO_2). All values are normalized to β-actin and are compared to p0, which is the same for RA and 50/10 OIR. All values are normalized to β-actin and are compared to p0, which was assigned a value of 1.0 for graphic representation. Raw data were rescaled and statistically analyzed as described in the "Methods" section. Increased expression of VEGFR2 is significantly associated with older developmental age (P<.0001, ANOVA) or with exposure to the 50/10 OIR model compared to RA (P=.0247, ANOVA).

As reported with relative quantitative PCR,[140] $VEGF_{164}$ was the most prevalent and $VEGF_{188}$ the least prevalent splice variant in the 50/10 OIR model determined using real-time PCR. $VEGF_{164}$ significantly increased in association with older developmental age (P<.0001) or with exposure to the 50/10 OIR model compared to RA (P<.0001). In contrast to that predicted, the expression of $VEGF_{164}$ mRNA was qualitatively greater in the 50/10 OIR model than in RA on p14 (Figure 14). In agreement with that predicted, on p18 when intravitreous neovascularization was present in the 50/10 OIR model, $VEGF_{164}$ expression was greater in the model than in RA. A pattern of $VEGF_{164}$ up-regulation was observed after hypoxic episodes (p12 and p14) and down-regulation after hyperoxia (p11 and p13). For $VEGF_{120}$, older developmental age was associated significantly (P=.0006) with increased $VEGF_{120}$ expression. However, there was no significant (P=.6142) association with exposure to the 50/10 OIR model compared to RA. A pattern of up-regulation following hypoxia and down-regulation after hyperoxia also was seen (Figure 15). $VEGF_{188}$ had greater expression at most time points in RA compared to the 50/10 OIR model, and there was a significant (P=.0256) association of older developmental age with increased $VEGF_{188}$ expression, but there was no significant (P=.5957) association with exposure to the 50/10 OIR model compared to RA (Figure 16).

PROTEIN CONCENTRATIONS OF VEGF IN RA AND 50/10 OIR

VEGF protein was associated significantly with older developmental age and previous exposure to the 50/10 OIR model compared to RA (P<.0001 for both comparisons). Although changes in protein concentration did not always correspond with mRNA expressions, VEGF protein shared a similar pattern of expression as the changes of $VEGF_{164}$ mRNA (Figure 17).

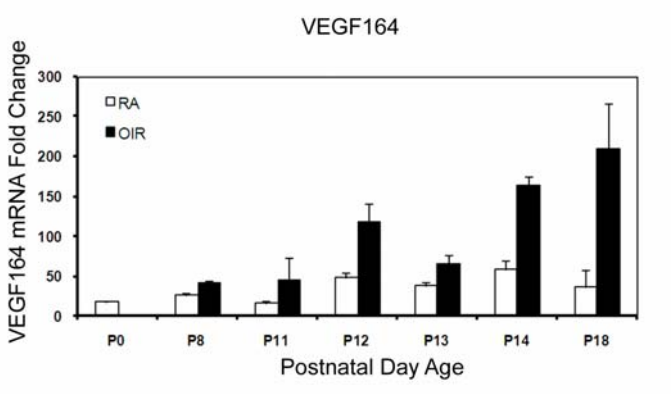

FIGURE 14

Real-time PCR values for VEGF splice variant, $VEGF_{164}$, in retinas of rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur immediately after hypoxia (10% FiO_2) and p11 and p13 after hyperoxia (50% FiO_2). After p14, pups are in RA (21% FiO_2). On p14, in RA the inner retina is vascularized to the ora serrata, whereas in the 50/10 OIR model, there is 30% avascular retina. All values are normalized to β-actin and are compared to p0. VEGF188 p0 RA mRNA was assigned a value of 1.0. The values for the three VEGF splice variants in the 50/10 OIR model and RA were related to this value to provide graphical representations. Raw data were rescaled and statistically analyzed as described in the "Methods" section. Increased expression of $VEGF_{164}$ is significantly associated with older developmental age (P<.0001, ANOVA) or with exposure to the 50/10 OIR model compared to RA (P<.0001, ANOVA). By post hoc testing, within the 50/10 OIR model, $VEGF_{164}$ mRNA was significantly (P=.0078) increased on p12 after hypoxia compared to p11 after hyperoxia.

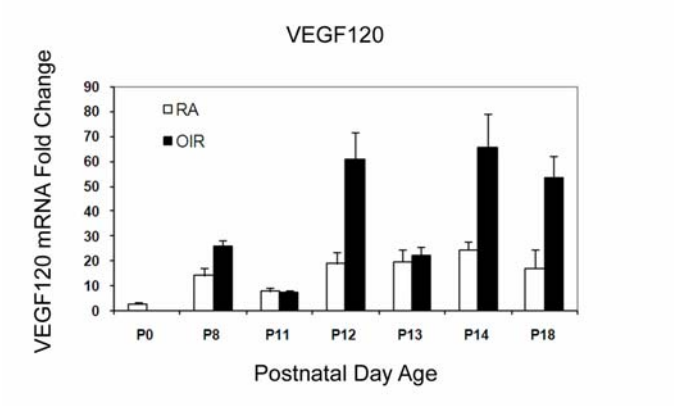

FIGURE 15

Real-time PCR values for VEGF splice variant, $VEGF_{120}$, in retinas of rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur immediately after hypoxia (10% FiO_2) and p11 and p13 after hyperoxia (50% FiO_2). After p14, pups are in RA (21% FiO_2). On p14, in RA the inner retina is vascularized to the ora serrata, whereas in the 50/10 OIR model, there is 30% avascular retina. All values are normalized to β-actin and are compared to p0. $VEGF_{188}$ p0 RA mRNA was assigned a value of 1.0. The values for the three VEGF splice variants in the 50/10 OIR model and RA were related to this value to provide graphical representations. Raw data were rescaled and statistically analyzed as described in the "Methods" section. Increased expression of $VEGF_{120}$ is significantly associated with older developmental age (P=.0006, ANOVA).

POST HOC TESTING OF VEGF SPLICE VARIANTS AND PROTEIN

To address the hypotheses concerning VEGF regulation in avascular retina on p14 and intravitreous neovascularization on p18, post hoc testing of the interaction between treatment (50/10 OIR model compared to RA) and developmental age was performed for VEGF splice variant and receptor mRNAs and VEGF protein. Relevant relationships between the model and RA were examined at different postnatal ages.

Post hoc analyses of mRNAs did not show significant differences in any splice variant mRNA between the model and RA at the same postnatal ages. Within the 50/10 OIR model, $VEGF_{164}$ mRNA significantly (P=.0078) increased on p12 after hypoxia compared to p11 after hyperoxia. For VEGF receptor mRNAs, there were no significant relationships seen in post hoc testing.

Post hoc analyses of VEGF protein showed several significant relationships. Compared to RA at the same time points, VEGF significantly increased in the 50/10 OIR model on p14 (P<.0001) while 30% avascular retina was present and on p18 when intravitreous neovascularization was present (P<.0001). In addition, VEGF significantly increased in the 50/10 OIR model on p8 (P<.0001), p12 (P =.0017), and p13 (P <.0001) compared to the same time points in RA.

INTERPRETATION OF DATA AND HYPOTHESES

$VEGF_{164}$ was overexpressed significantly in association with older developmental age or exposure to the 50/10 OIR model compared to RA, whereas $VEGF_{120}$ and $VEGF_{188}$ were overexpressed significantly in association with older developmental age and not with exposure to the 50/10 OIR model. VEGFR1 expression increased significantly in association with older developmental age but not with exposure to the 50/10 OIR model, whereas VEGFR2 expression increased significantly in association with older developmental age or exposure to the 50/10 OIR model. These association data led to the hypothesis that $VEGF_{164}$ and VEGFR2 are important in the development of features of peripheral severe ROP.

Since increased VEGF drives developmental angiogenesis, it was logical to propose that when avascular retina persisted in the 50/10 OIR model but vascularization was complete in RA, VEGF would be reduced compared to RA. However, the opposite was

found. Contrary to the prediction that VEGF would be down-regulated in incompletely vascularized retinas in the 50/10 OIR model, VEGF increased significantly (P<.0001) compared to fully vascularized retinas from RA age-matched pups. In support of the prediction that VEGF would be up-regulated with intravitreous neovascularization, VEGF increased significantly (P<.0001) on p18 in the 50/10 OIR model when peak intravitreous neovascularization occurred.

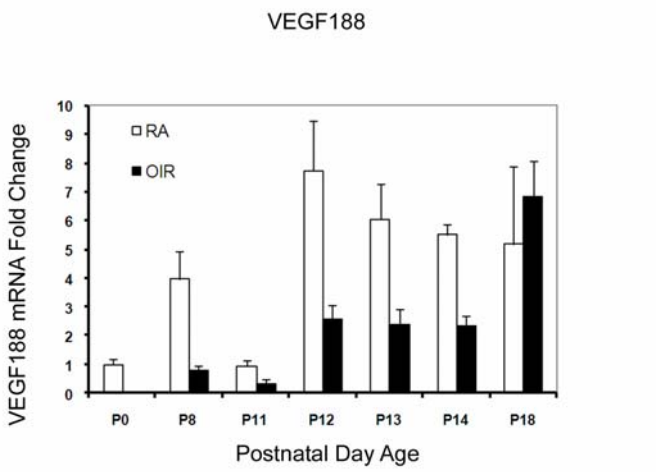

FIGURE 16

Real-time PCR values for VEGF splice variant, $VEGF_{188}$, in retinas of rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur immediately after hypoxia (10% FiO_2) and p11 and p13 after hyperoxia (50% FiO_2). After p14, pups are in RA (21% FiO_2). On p14, in RA the inner retina is vascularized to the ora serrata, whereas in the 50/10 OIR model, there is 30% avascular retina. All values are normalized to β-actin and are compared to p0. VEGF188 p0 RA mRNA was assigned a value of 1.0. The values for the three VEGF splice variants in the 50/10 OIR model and RA were related to this value to provide graphical representations. Raw data were rescaled and statistically analyzed as described in the "Methods" section. Increased expression of $VEGF_{188}$ is significantly associated with older developmental age (P=.0256, ANOVA).

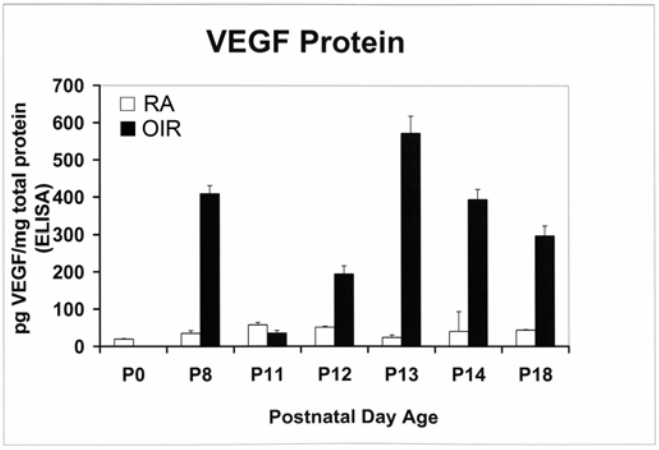

FIGURE 17

ELISA measurements of retinal VEGF from rat pups from selected days from p0 through p18 in room air (RA) or the 50/10 OIR model. In the 50/10 OIR model, p8, p12, and p14 occur immediately after hypoxia (10% FiO_2) and p11 and p13 after hyperoxia (50% FiO_2). After p14, pups are in RA (21% FiO_2). On p14, in RA the inner retina is vascularized to the ora serrata, whereas in the 50/10 OIR model, there is 30% avascular retina. VEGF is significantly associated with older developmental age or with previous exposure to the 50/10 OIR model compared to RA (P<.0001, ANOVA). By post hoc testing, the VEGF concentration is significantly greater in the 50/10 OIR model compared to RA at p8, p13, p14, p18 (P<.0001), and p12 (P=.0017).

DISCUSSION

VEGF is an important growth factor involved in retinal vascular development and development of pathologic retinovascular diseases, including ROP. The understanding of the pathogenesis of ROP has been based, in large part, on studies using animal models of OIR. However, most of those models were developed to reflect the oxygen profiles of preterm infants in the 1940s before the ability to regulate and monitor oxygen. In addition, the models are based on high oxygen causing vaso-obliteration, which is not seen in human infants with severe ROP. Based on one of the most widely used models, the mouse OIR model, ROP pathophysiology has been described in two phases: first, a decrease in growth factors, including VEGF, during hyperoxia-induced vaso-obliteration, and second, an increase in growth factors and VEGF during vasoproliferation. However, data using the rat 50/10 OIR model, which mimics peripheral severe ROP and shares oxygen profiles with preterm infants who have severe ROP, do not support the vaso-obliteration–vasoproliferation hypothesis. A revised hypothesis is presented below to explain why blood vessels grow into the vitreous (intravitreous neovascularization) rather than into the retina.

In normal retinal vascular development, precursor cells (astrocytes or angioblasts, depending on the species) migrate in front of endothelial cells that develop into intraretinal blood vessels.[112,141] The precursor cells sense hypoxia in the peripheral avascular retina and up-regulate VEGF in response to hypoxia. The increased retinal VEGF concentration induces endothelial cell proliferation and provides a chemotactic gradient to attract endothelial cells to migrate toward the ora serrata. Vascularization of the inner retina continues in this manner and is complete in the inner plexus by about p14 in the rat.[140,142] In retinal vascular diseases associated with intravitreous neovascularization, substantial evidence supports VEGF overexpression as an important cause. However, it is unclear why increased retinal VEGF would cause blood vessels to grow outside the retinal plane into the vitreous rather than into the retina

toward the ora serrata as during physiologic retinal vascular development. Gerhardt and coworkers[116] showed that high VEGF concentrations injected into the vitreous caused endothelial tip cells to point filopodia toward the vitreous. The filopodia are believed to provide guidance cues for migrating endothelial cells. These observations led to the hypothesis that the chemotactic force of the vitreal VEGF attracts endothelial cells to grow away from the retina and into the vitreous in diseases with intravitreous neovascularization. However, in the 50/10 OIR model, the retinal concentration of intraretinal VEGF was at least 10-fold greater than that in the vitreous at the time when intravitreous neovascularization was greatest,[84] and this finding fails to support the above hypothesis.

Another possible reason retinal endothelial cells do not grow into avascular retina in the presence of VEGF overexpression is that the avascular retina may be permanently injured and unable to support vascular growth. This seems unlikely in ROP, because intraretinal vascularization often proceeds toward the ora serrata when ROP regresses. Vascularization of avascular retina upon regression of intravitreous neovascularization also occurs in all OIR models.

It is also possible that blood vessel growth into the vitreous is easier than into the retina because the vitreous has more extracellular space than the retina. This hypothesis remains viable. Vessel growth often requires receptors or proteins, such as integrins. The study of integrins in the context of normal or pathologic vitreous states is largely unexplored.

The 50/10 OIR model reproducibly first develops areas of peripheral avascular retina and later intravitreous neovascularization at the junction of vascular and avascular retina. In the 50/10 OIR model, VEGF increased on p14 in association with 30% peripheral avascular retina compared to the same developmental time point as in RA-raised pup retina in which no peripheral avascular retina was present. Geisen and coworkers[84] provided evidence that an intravitreous injection of neutralizing antibody to $VEGF_{164}$ administered on p12 at a dose that reduced VEGFR2 signaling, inhibited intravitreous neovascularization in the 50/10 OIR model without adversely affecting intraretinal vascularization. In addition, Budd and associates[117] found that a receptor tyrosine kinase inhibitor of VEGFR2 significantly inhibited intravitreous neovascularization in the 50/10 OIR model and did not interfere with intraretinal vascularization. Finally, in a series of human infants with zone II, stage 3 severe ROP, single injections of intravitreous bevacizumab caused regression of stage 3 ROP and permitted ongoing retinal vascularization toward the ora serrata.[99] These studies suggested that excessive VEGF-VEGFR2 expression and/or signaling are associated with both intravitreous neovascularization and avascular retinal areas and that reducing VEGF signaling to more "physiologic" levels may not interfere with ongoing intraretinal vascularization.

Therefore, a new hypothesis is proposed that VEGF overexpression and excessive signaling through VEGFR2 disorders developmental angiogenesis and thereby delays retinal vascular development in severe ROP. The disordered angiogenesis initially causes persistent peripheral avascular retina, but with more disordering of endothelial cell divisions outside the plane of the retina and growth into the vitreous, manifests as intravitreous neovascularization. Based on previous studies, excessive signaling through VEGFR2 interferes with developmental angiogenesis in an embryonic stem cell model.[143] In angiogenesis, endothelial cells undergo mitosis forming a cleavage plane that subsequently divides the cell into two daughter cells. The orientation of the cleavage planes to the long axis of the vessel predicts the direction of migrating daughter cells, such that a cleavage plane perpendicular to the long axis of the developing vessel leads to vessel elongation and a plane parallel to the axis results in vessel widening.[143] However, if the orientation of the cleavage planes of proliferating cells is disordered (Figure 18), the cells develop an irregular growth pattern similar to that seen in intravitreous neovascularization. There is evidence to support this concept from the *flt-1*$^{-/-}$ embryonic stem cell model. The *flt-1*$^{-/-}$ model is the murine knockout of VEGFR1. VEGFR1 is believed to trap VEGF during development[129] and regulate the amount of VEGF that signals through VEGFR2. Therefore, in the *flt-1*$^{-/-}$ model, VEGF binds VEGFR2 rather than VEGFR1. The result is increased VEGF-VEGFR2 signaling. Cleavage planes of dividing endothelial cells in the *flt-1*$^{-/-}$ embryonic stem cell model were disoriented, and ordered angiogenesis was restored with a soluble *flt-1* transgene that targeted endothelial cells.[143] The gene rescue experiment restored a "VEGF trap" to reduce VEGF signaling through VEGFR2. This embryonic stem cell study provides support that excessive VEGF-VEGFR2 signaling can cause disordered angiogenesis. (It should be noted that a single allele knockout of VEGF or a knockout of the receptors is lethal. Therefore, to study the signaling pathways, it is necessary to use a nonviable method, such as an embryonic stem cell model.)

In vivo studies using the rat 50/10 OIR model also provided evidence that increased VEGF-VEGFR2 signaling alters the orientation of dividing vascular cells in major retinal vessels, causing a change from perpendicular, favoring vessel elongation, to parallel, favoring vessel dilation. Tortuosity also increased. The pattern of dilation and tortuosity decreased with an intravitreous injection of a neutralizing antibody to VEGF[144] at a dose that inhibited VEGFR2 signaling.[84] Thus, in an in vivo system, increased VEGF signaling through the VEGFR2 altered vascular cell cleavage plane orientation in major vessels. This study also provided evidence for a VEGF-associated mechanism in ROP plus disease.

In the models of high OIR, such as the mouse OIR model, return to RA represents a decrease in oxygen exposure from 75% to 21% oxygen, a relative hypoxia, and increased VEGF expression in association with intravitreous budding of endothelial cells. However, in the 50/10 OIR model, return to RA represents an increase in oxygen exposure from 10% to 21% and yet was associated with intravitreous neovascularization on p18. These findings suggested that additional events or stresses, other than reduced inspired oxygen, may be involved. Increased oxygen demand occurs as the hyaloid regresses and the photoreceptors elongate and mature.[145] Evidence implicates the photoreceptors in creating oxygen demand during development that results in features of severe retinopathy seen in ROP models, including the 50/10 OIR model.[146,147] In addition, in young rat pups, increases in inspired oxygen do not increase choroidal oxygen concentration[148] unlike in adult rats, and this may reduce the potential ability of the choroid to meet an increasing oxygen demand.

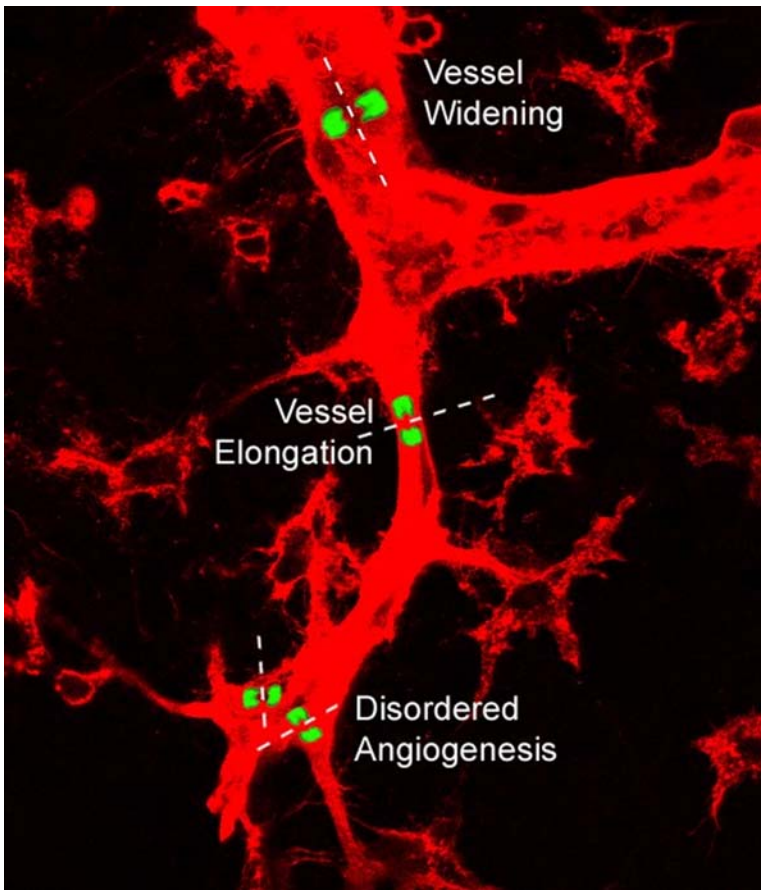

FIGURE 18

Lectin-stained retinal flat mount of 50/10 OIR model at the junction of vascular and avascular retina magnified to show endothelial tip cells and filopodia. Artist rendition of phosphohistone H3 stained mitoses of vascular cells and cleavage plane axes is superimposed over major axes of vessels. The orientation of the cleavage plane axis to the long axis of the vessel predicts whether the vessel is elongated, widened, or disordered as in intravitreous neovascularization.

Further studies are needed to test the hypotheses presented in this thesis. In future studies, it will be important to study the role of various angiogenic inhibitors in the 50/10 OIR model and ROP. Pigment epithelial growth factor (PEDF), a potent angiogenic inhibitor, is also important in retinal vascular development and therefore an important consideration.[149] However, PEDF is increased by hyperoxia rather than hypoxia. Therefore, it seems unlikely that its expression would increase in the avascular, hypoxic retina.[60,79,150] Other angiogenic agonists may be involved,[49,51,90] including those regulated by hypoxia through hypoxia inducible factor or EPAS1[53] (eg, erythropoietin, adrenomedullin), those involved in development such as IGF-1, and factors stimulated during inflammation, such as cytokines, tumor necrosis factor alpha, or prostaglandins.[66,89] Inhibitors, such as thrombospondin-1,[151] angiostatin, and endostatin,[77] and cell-cell interactions of endothelial cells with pericytes, astrocytes, or other glia[152,153] are important. Regulation of endothelial filopodial number and length at the migrating front of vascular cells in the retina[117] also is important and can affect the patterning of vessels.[129]

$VEGF_{188}$ had the lowest expression of the VEGF splice variants and reduced expression in the 50/10 OIR model compared to RA at all time points except p18. Its expression was increased in association with older developmental age, but its mRNA expression levels were not affected significantly by the 50/10 OIR model. $VEGF_{188}$ is heparin bound and described as cell associated. The apparent, but insignificant, reduced expression in the 50/10 OIR model compared to RA may be explained in part by a smaller area of retinal vascularization in the model than in RA at the same developmental ages and, therefore, fewer endothelial cells with which $VEGF_{188}$ can associate.

Measurement of mRNA was important to assess rapid changes from fluctuations in inspired oxygen in the 50/10 OIR model. Measurement of protein is also important, since there can be differences in the stability of mRNA based on the oxygen level. This may explain some discrepancies between mRNA and protein measurements and the significance found in post hoc statistical analysis of VEGF protein. Direct comparisons between mathematical changes in expression with biologic outcomes fail to consider signaling cascades and other factors involved in endothelial and other cells. A useful determination of VEGF signaling is the measurement of the activation of VEGF receptors. For rat retina, the commercially available antibodies to measure phosphorylation of the tyrosine

residues important in signaling in VEGFR1 and R2 by Western blot have not yielded consistent and reliable data. Future studies will be important to determine protein levels and activation of signaling through the receptors.

CLINICAL OBSERVATIONS AND RELEVANCE

The following is presented to integrate the results of this thesis with clinical observations in ROP. A preterm infant is born with avascular peripheral retina because of incomplete retinal vascular development. At the time of birth, reduction in growth factors previously supplied by the maternal circulation may slow retinal vascular development. However, most severe ROP develops several months later, and it is unknown if severe ROP coincides with the infant's ability to produce endogenous factors. In the 50/10 OIR model, a representative model of severe ROP today, VEGF and VEGFR2 increased in association with repeated fluctuations in inspired oxygen and may lead to increased VEGF-VEGFR2 signaling. If this translates to the infant, excessive VEGF-VEGFR2 signaling may disorder developmental angiogenesis by affecting the orientation of the cleavage planes of dividing vascular cells at the junction of vascular and avascular retina. The disordered angiogenesis then may interfere with retinal vascular development and manifest initially as persistent avascular retina. As more dividing endothelial cells have disordered growth and divide outside the plane of the retina, intravitreous neovascularization may become apparent. The avascular retina, along with a regressing hyaloidal circulation, reduces oxygen supply to the developing retina. Oxygen demand and metabolic activity are increased as photoreceptors elongate and mature. Furthermore, the choroidal circulation may be unable to increase the oxygen concentration even with increased inspired oxygen as found by Cringle and coworkers[148] in rats on p15. Thus, a cycle is created that if unbroken may lead to the features of peripheral severe ROP. When VEGF signaling is restored to physiologic levels, ordered intraretinal vascularization can proceed again to the ora serrata, such as seen in a few clinical series that tested anti-VEGF agents in severe ROP.[99,154] The intravitreous neovascularization may involute because of insufficient growth factor support to the endothelial cells.[105,108,109] If increased VEGF-VEGFR2 signaling occurs again, new ROP can develop in the more peripheral retina representing episodes of the cycle repeated, interrupted, and then repeated. This pattern is evident in clinical ROP.

Although excessive VEGF signaling may be involved in the pathology of peripheral severe ROP, and restoration to more physiologic levels may reduce stage 3 ROP and permit vascularization of the avascular retina, the following should be considered before using anti-VEGF agents in the preterm infant eye. Inhibiting VEGF bioactivity in the preterm infant undergoing retinal and neurologic development may have adverse consequences, because VEGF is both a neuronal and an endothelial survival factor.[109,155,156] VEGF is also important in renal and lung development.[157] Circulating drug levels from intravitreous injections will likely be higher in infants than adults because of higher vitreous volume to blood volume ratios.[61] This thesis describes a model of peripheral severe ROP (zone II, stage 3 ROP) and may not be relevant to aggressive posterior ROP or possibly to other, less common forms of severe ROP yet to be fully understood.[65] Finally, laser treatment for type 1 prethreshold ROP led to reduced adverse outcomes in about 90% of cases in the ETROP study.[12] Therefore, to assess the efficacy of any intervention, a clinical trial with suitable controls is needed.

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