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Pathophysiology and Mechanisms of Severe Retinopathy of Prematurity

M. Elizabeth Hartnett, MD¹

¹John A. Moran Eye Center, University of Utah, Salt Lake City, Utah

Abstract

Retinopathy of prematurity (ROP) affects only premature infants, but as premature births increase in many areas of the world, ROP has become a leading cause of childhood blindness. Blindness can occur from aberrant developmental angiogenesis that leads to fibrovascular retinal detachment. In order to treat severe ROP, it is important to study normal developmental angiogenesis and the stresses that activate pathologic signaling events and aberrant angiogenesis in ROP. Vascular endothelial growth factor (VEGF) signaling is important in both physiologic and pathologic developmental angiogenesis. Based on studies in animal models of oxygen-induced retinopathy (OIR), exogenous factors such as oxygen levels, “oxidative stress,” inflammation, and nutritional capacity have been linked to severe ROP through dysregulated signaling pathways involving hypoxia inducible factors and angiogenic factors like VEGF, oxidative species, and neuroprotective growth factors to cause “phases” of ROP. This translational science review will focus on studies performed in animal models of OIR representative of human ROP and highlight several areas: mechanisms for aberrant growth of blood vessels into the vitreous rather than into the retina through over activation of VEGF receptor 2 (VEGFR2) signaling, the importance of targeting different cells into the retina in order to inhibit aberrant angiogenesis and promote physiologic retinal vascular development, toxicity from broad and targeted inhibition of VEGF bioactivity, and the role of VEGF in neuroprotection in retinal development. Several future translational treatments are discussed, including considerations for targeted inhibition of VEGF signaling instead of broad intravitreal anti-VEGF treatment.

Background / Introduction

Retinopathy of prematurity (ROP) was described in 1942 by Terry¹ as “retrolental fibroplasia,” which likely represents the most severe form of ROP, stage 5. Earlier stages of ROP were not well described, because the Schepens/Pomerantzeff binocular indirect ophthalmoscope² had not been adopted universally to examine the peripheral retina. In order

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Address for Reprints: M. Elizabeth Hartnett, Corresponding Author, John A. Moran Eye Center, 65 Mario Capecchi Drive, Salt Lake City, Utah 84132.

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to understand potential causes of ROP, investigators exposed newborn animals, which vascularize their retinas postnatally, to conditions similar to what human premature infants then experienced. From the initial observation by Campbell, and later studies by Michaelson, Ashton and Patz, it became recognized that high oxygen at birth damaged fragile, newly formed retinal capillaries, causing vaso-oblivation. Once animals were removed from supplemental oxygen to ambient air, vaso-proliferation occurred at junctions of vascular and avascular retina. Thus, the two-phase hypothesis was developed, almost 30 years before the classification of human ROP into zones and stages. With advances in neonatal care including the ability to monitor and regulate oxygen and in funduscopy imaging of the peripheral retina in preterm infants prior to the development of stage 5 ROP, several changes in our understanding of ROP occurred. First, the hypothesis of ROP has been revised in that there is a delay in physiologic retinal vascular development and some hyperoxia-induced, vaso-attenuation in phase 1, followed by vaso-proliferation into the vitreous as intravitreal neovascularization (IVNV) in phase 2 (Figure 1).³ Second, it is recognized that the phenotype of ROP differs throughout the world in association with resources for prenatal care and oxygen regulation. Preterm infants of older gestational ages and larger birth weights than those screened in the US are now developing severe ROP in some regions with insufficient nutrition, neonatal or prenatal resources and care, and high, unregulated oxygen is used.^{4,6} Finally, heritable causes are recognized as important,⁶ but candidate gene studies often have been small and have not replicated findings potentially due to phenotypic variability.

The International Classification of ROP (ICROP) described stages and zones of ROP severity.⁷ Since human retinal vasculature is not complete until term birth, an infant born prematurely initially has incomplete vascular coverage of the retina. The zones of ROP define the area of retina covered by physiologic retinal vascularization. The stages often progress sequentially and describe the severity of disease. Stages 1 and 2 represent “early ROP,” and stage 3, the “vascular phase” in which intravitreal neovascularization (IVNV) occurs (Figure 1). Stages 4 and 5 ROP represent the “fibrovascular phase” with retinal detachment.⁸ Laser treatment for severe ROP, now described as type 1 ROP in the Early Treatment for Retinopathy of Prematurity Study (Table),⁹ can reduce the risk of a poor outcome in about 90% of eyes. In some infants, aggressive posterior ROP in which stage 3 and severe plus disease develops, without prior stages 1 or 2, in zone 1 or posterior zone 2.

It is important to consider human retinal vascular development when studying what goes awry in ROP. Because of the difficulty in obtaining intact human preterm infant eyes, studies on human retinal vascular development have been limited, but reports indicate that the initial retinal vasculature develops through vasculogenesis in the posterior pole from precursor cells that migrate out of the deep retina and into inner layers.^{10,11} At approximately 15 weeks gestation¹¹ until at least 22 weeks gestation, these precursors become angioblasts and form the inner vascular plexus that extends to about zone 1. After 22 weeks gestation, when it is difficult to obtain fetal tissue, the ensuing development of the vascular plexi is based on studies done in other species and believed to occur through budding angiogenesis, that is, the proliferation and growth of blood vessels from existing blood vessels. In several species, astrocytes sense a “physiologic hypoxia”¹² and upregulate vascular endothelial growth factor (VEGF). Ensuing endothelial cells proliferate and migrate

toward the gradient of VEGF and thereby extend the inner vascular plexus toward the ora serrata. Angiogenesis is also important in the development of the deep retinal plexi. Besides astrocytes, glia, like Müller cells, and neurons, such as ganglion cells, are also important.^{13, 14, 15} The process is complex and requires interactions between different cell types and regulation of signaling pathways through several family members of VEGF and other pathways, including delta-like 4/notch and robo/slit, as examples, which regulate interactions between the sensing, endothelial tip cells and the proliferating stalk cells.¹⁶ Of all the factors involved in physiologic retinal vascular development, it is clear that VEGF is essential.

Animal Models to Study ROP

It is not safe to experiment on human preterm infant eyes because of risks of bleeding and retinal detachment. Therefore, models of oxygen-induced retinopathy (OIR) are performed in animals that vascularize their retinas postnatally in order to study disease mechanisms. Most OIR models recreate only some aspects of human ROP. All models have limitations, because they use newborn, instead of premature, animals. Newborn animals are healthy and do not have the comorbidities of human preterm infants, such as necrotizing enterocolitis, sepsis, bronchopulmonary dysplasia, shunting of oxygenated and deoxygenated blood, and immature lung development. Animals experience much higher arterial oxygen levels when given similar inspired oxygen levels as premature infants with these comorbidities. Neonatologists strive to avoid high oxygen in the perinatal period, but most animal models use high oxygen, making them less representative of human ROP. These are important considerations when choosing an OIR model to study a scientific question. The two most commonly used OIR models are in mouse and rat. Also important is the beagle OIR model. These species are not premature but complete retinal vascular development after term birth.

Mouse OIR Model

The use of transgenic mice makes the mouse OIR model most helpful to study mechanisms of high oxygen induced vascular loss followed by regrowth of vessels either into the retina or into the vitreous during relative hypoxia.¹⁷ However, there are a few ways in which the model does not represent human ROP. First, oxygen levels used do not recreate what human preterm infants experience. The arterial oxygen (PaO₂) in healthy newborn mice can approach very high levels (500 mm Hg) with 75% inspired oxygen, oxygen levels that are avoided in preterm infants. Day 7 pups placed into 75% constant inspired oxygen, experience vaso-obliteration of newly formed capillaries in the central retina and then are placed into room air and form intravitreal vascular buds at the junctions of vascular and avascular retina (Figure 2). Thus, the model is not similar to the phases of human ROP in that complete inner plexus vascularization has already occurred when the pups are placed into high oxygen, unlike the preterm infant whose retina is incompletely vascularized. Nonetheless, several signaling pathways important in human ROP have been identified using the mouse model. The model also may reflect aspects of ROP in the US and the UK in the 1940's or in places currently that lack resources to regulate oxygen and provide prenatal and perinatal care.⁵

Rat OIR Model

The most representative model of human ROP in the era of oxygen regulation is the rat OIR model, which has aspects of both vaso-attenuation centrally and delayed physiologic retinal vascularization peripherally¹⁸ (Figure 2). Shortly after birth, pups and dams are placed into a controlled oxygen environment that changes inspired oxygen levels from 50% to 10% every 24 hours for 14 days. This oxygen profile recreates transcutaneous arterial oxygen extremes similar to those in a human preterm infant with severe ROP.¹⁹ The notion of oxygen fluctuations, including intermittent episodes of hypoxia, has been associated with ROP.²⁰ However, the duration of the fluctuations in oxygenation in the rat model is 24 hours, whereas in the human preterm infant minute-to-minute fluctuations occur. The rat pups experience extrauterine growth restriction, a factor associated with severe ROP. The appearance of first delayed physiologic retinal vascular development followed by IVNV at the junction of vascular and avascular retina at day 18 is similar to type 1 severe ROP.⁹ Thus, the rat OIR model closely represents human preterm infants with severe ROP. The study of molecular mechanisms or potential treatments had been limited to pharmacologic manipulations in the rat, because the availability of transgenic rats is limited. Now, other techniques have been developed to permit study of molecular mechanisms in the rat. One example is the use of gene therapy to introduce short-hairpin RNAs (shRNAs) or genetic mutations to silence or knockout certain genes. Different viruses or viral vectors are used in gene therapy and include adeno-associated virus (AAV), adenovirus, or lentivirus, as examples. Several valuable aspects of a lentiviral vector are that it incorporates its gene cargo into the genome to allow stable transgene expression and have large cargo carrying capacity. Using lentivirus, cell specific promoters have been linked with shRNAs to target certain cell types in the retina and knockdown specific gene products in those cells only. This has been a novel and useful technique to determine the effects of angiogenic signaling in pathologic and physiologic retinal angiogenesis from knockdown of genes in specific retinal cells and to assess safety on transduced and other cells within the retina.^{14, 21, 22} In addition, techniques to delete genes have been developed that will permit gene knockout in many species besides mice in order to study molecular events in various models.

Beagle Oir Model

The beagle OIR model²³ is especially useful to translate drug doses from the puppy eye to the human preterm infant eye because of greater similarity in size between eyes of the puppy and preterm infant than between preterm infant and newborn rodent. The newborn beagle retina initially vascularizes through a process of vasculogenesis that is followed by angiogenesis similar to what occurs in premature human infant retina. However, the model uses very high oxygen to cause OIR, which differs from the pathogenesis of ROP in most premature infants. In the beagle model, newborn postnatal day 1 pups are placed into 100% oxygen for 4 days and then into ambient air to recreate the phases of OIR.²³

When comparing the phases of OIR (Figure 2) to what occurs in human ROP (Figure 1), it is helpful to clarify definitions.^{3, 8} Phase 1 in the rat OIR reflects the “early phase” of human ROP; i.e., delayed physiologic retinal vascular development. Phase 2 in rat and mouse models of OIR reflect vaso-proliferative IVNV similar to the “vascular phase” of human stage 3 ROP with plus disease. However, human ROP also has a third, “fibrovascular

phase,” in which retinal detachment occurs in stages 4 and 5 ROP, and few animal models develop this form of human ROP. However, the beagle OIR model shares some features seen in stage 4 ROP with retinal folding and dragging of vessels.²³

For clarity, the phases of OIR are described by the animal and phase. Phase 1 in mouse is vaso-oblivation and in rat is delayed physiologic retinal vascular development, and phase 2 is IVNV in both models. The phases of human ROP are described as “early” (delayed physiologic retinal vascular development and some vaso-attenuation),³ and “vascular” (IVNV) or “fibrovascular” (retinal detachment) (Figure 1).

Pathophysiology of Human Severe ROP

Most early investigations sought to understand causes of the vascular phase of human ROP by studying phase 2 OIR with IVNV, but several investigators^{24,25} strove to understand the early phase of human ROP by studying phase 1 OIR. The thinking was that in facilitating vascularization of avascular retina, there would be less hypoxia induced IVNV, and this line of thought aligned with clinical observations that infants with zone 1 ROP, compared to zone 2 ROP, were at greater risk of developing severe ROP and having poor outcomes.²⁶

Several exogenous stresses implicated in ROP, such as fluctuations in oxygenation, oxidative stress, nutritional factors and poor infant growth, activate inflammatory, oxidative and hypoxic signaling pathways.³ Studies of phase 2 OIR focused on induced angiogenic factors from these activated signaling pathways. As with most biologic processes, it has become recognized that interactions and crosstalk exist.

Hypoxia inducible factors

Hypoxia inducible factors (HIFs) are transcription factors that bind DNA at the hypoxia responsive element and enable transcription of a number of downstream genes that are angiogenic, including VEGF, angiopoietins, and erythropoietin, as examples. The classic mechanism involves hypoxia, which occurs in avascular retina once a newborn pup is removed from supplemental oxygen to ambient air. Hypoxia prevents HIFs from degradation by prolyl hydroxylases and thus allows them to translocate to the nucleus to cause angiogenic gene transcription.³ HIFs can also be stabilized through oxidative compounds or inflammatory cytokines, mediated through NFkB, which can lead to downstream angiogenic effector compounds, including succinate or RTP801. Using mouse and rat OIR models, investigators studied prolyl hydroxylase inhibitors to stabilize HIF and promote physiologic retinal vascular development in phase 1 OIR models.²⁷ Others found that administration of HIF-induced growth factors, including erythropoietin or VEGF, reduced avascular retina in phase 1 OIR.³ However, the concern with these strategies is that early and vascular phases of human ROP may not be sufficiently distinct in the individual preterm infant to determine a safe window of time to administer an angiogenic agonist in order to treat early ROP without causing vascular ROP.

Oxidative stress

Oxidative stress has been proposed in ROP because of the susceptibility of the phospholipid-rich retina to reactive oxygen species that can be generated in high or low oxygen. Repeated

oxygen fluctuations in the rat OIR model also leads to the generation of oxidative compounds. Although use of anti-oxidants, such as superoxide dismutase in liposomes²⁵ or apocynin,²⁴ reduced avascular retina in phase 1 of the rat OIR model, these substances did not reduce IVNV in phase 2 in the rat OIR model. In addition, human clinical trials that tested n-acetyl cysteine, vitamin E or lutein have not successfully or safely inhibited severe ROP, to date.^{15,28} These findings may reflect the complexities in oxidative signaling and that reactive oxygen species can be damaging or beneficial to the retina. Besides direct interaction with the phospholipids in retina, some species act as signaling effectors that promote physiologic or pathologic events. Nitric oxide (NO) can be activated by nitric oxide synthetases (NOS), including eNOS, and act as an endothelial relaxing agent in blood vessels, but in high oxygen NO can form nitro-oxidative forms like peroxynitrite that lead to microvascular degeneration in phase 1 OIR. Oxidative stress can activate VEGFR2 signaling that is needed in physiologic angiogenesis or over activate VEGFR2 signaling in phase 2 OIR. In the immunocompromised preterm infant, NADPH oxidase can generate reactive oxygen species (ROS) that defend against invading microorganisms. However, NADPH oxidase generated ROS can also cause endothelial cell injury and avascular retina in phase I OIR through activation of isoforms NOX1 or NOX2 or increase vasoproliferation in phase II OIR through activation of isoforms NOX1, NOX2 or through NOX4-induced activation of the transcription factor, STAT3, in endothelial cells.^{28,29} In contrast, activation of STAT3 in Müller cells inhibits the expression of erythropoietin and thus reduces angiogenesis in phase 1 OIR.³⁰ Although exogenous erythropoietin improves physiologic retinal vascularization in phase 1 OIR, it does not reduce phase 2 IVNV in the rat OIR model. Following along with this line of evidence, an intravitreal injection of a STAT3 inhibitor only reduces phase 2 IVNV compared to vehicle under conditions of supplemental oxygen. In the rat OIR model with supplemental oxygen, Müller cell STAT3 is not activated, but endothelial cell STAT3 is activated to mediate IVNV.³¹ In the rat OIR model without supplemental oxygen, endothelial cell and Müller cell STAT3 proteins are activated, and the angiogenic and angiostatic effects from activation of STAT3 in the two cell types counter one another. Broad inhibition of STAT3 with an intravitreal agent then does not appear to have an effect on phase II IVNV. These studies highlight the complexity of oxidative signaling pathways and subsequent biologic events, including angiogenesis, and also point to the importance in identifying signaling events in specific cells (Figure 3).

Extrauterine Growth Restriction and Nutritional effects

The roles of birth weight and postnatal growth in preterm infants have been recognized as important factors associated with ROP and in animal models of OIR.³² In human preterm infants, low IGF-1 was associated with extrauterine growth restriction, poor retinal vascular growth and later vasoproliferation.³³ Omega-3 fatty acids were also found to be important in reducing vasoproliferation in the mouse OIR in part by inhibiting TNFalpha and facilitating neuroprotection.³²

Genetic Variation

Besides environmental factors, 70% of the variance in ROP was reported secondary to heritable factors in a study of mono- and dizygotic preterm twins.⁶ Small candidate gene studies found several gene variants including those in the wnt pathway (*FZD4*, *LRP5*, *NDP*).

Variants of genes in the wnt pathway cause familial exudative vitreoretinopathy, which shares features of ROP but occurs in full-term infants. Other investigators found variants in *EPAS1* that transcribes erythropoietin, *SOD* that transcribes the antioxidant enzyme, superoxide dismutase, or *VEGF*. However, most studies involved small samples of infants with broad ranges in birth weights and gestational ages and were not controlled for multiple comparisons. In addition, interactions between genes and their function may be affected by other factors that are linked with ROP. Larger studies that control for multiple comparisons are needed.

VEGF Signaling Pathway

Many laboratories have studied ROP using the mouse OIR model. This review focuses on the effects of stresses similar to what human preterm infants experience in the early and vascular phases of ROP and, therefore, reports mainly on studies that used the rat OIR model adapted to study molecular mechanisms.

VEGF is important in physiologic retinal vascular development and pathologic angiogenesis, and both processes occur in the preterm infant retina. Therefore, it is first important to determine the differences in VEGF signaling that lead to IVNV instead of physiologic retinal vascular development. It is helpful to review aspects of VEGF signaling. VEGF has different family members but much of the work on angiogenesis has involved VEGFA, heretofore referred to as VEGF for this review. VEGF activates different receptors. VEGF receptor 2 (VEGFR2) is activated in pathologic angiogenesis.

VEGFR1 can also be angiogenic, but in development binds VEGF with higher affinity than does VEGFR2 and can act as a decoy, preventing binding with VEGFR2. VEGFR3 is important in lymphangiogenesis and some in the regulation of angiogenesis. VEGF has different mRNA splice variants. Some of the translated forms are secreted and, therefore, have access to the vitreous, whereas others are cell-associated proteins that impact signaling locally and create a gradient for angiogenesis.¹⁶

A critical question in ROP, which involves both physiologic retinal vascular development and aberrant IVNV, is why does hypoxic retina in phase I ROP activate angiogenic signaling pathways that lead to blood vessel growth into the vitreous as IVNV rather than into the avascular retina to provide physiologic intraretinal vascular support? Several studies investigated why blood vessels grow into the vitreous rather than into the retina in phase II OIR.⁸ One possibility examined was whether VEGF concentration was greater in the vitreous than in the retina thereby drawing vascular growth toward the vitreous rather than into the retina. Evidence was not found to support this prediction. VEGF measured in the vitreous was more than 10-fold lower than in the retina at the time point when IVNV occurred in the rat OIR model. A limitation may have been the inability to measure local vitreous VEGF overlying IVNV compared to retinal VEGF anterior to IVNV.

Fluctuations in oxygenation are associated with ROP. Therefore, another study was done to determine whether repeated oxygen fluctuations, compared to hypoxia alone, altered the expression of VEGF splice variants to lead to different biologic outcomes. In the rat OIR model, repeated oxygen fluctuations increased the expression of retinal VEGF₁₆₄, an analog

to human VEGF₁₆₅, whereas hypoxia increased VEGF₁₂₀.³⁴ This finding suggested that VEGF₁₆₄ was more associated with pathologic features in phase II OIR. Another study reported that increased expression levels of VEGF₁₆₄ and VEGF receptor 2 (VEGFR2) were associated temporally with pathologic features in both phases 1 and 2 of the rat OIR model, whereas the other VEGF splice variants (VEGF₁₂₀ and VEGF₁₈₈) and VEGFR1 were associated with the control situation, physiologic retinal vascular development under ambient oxygen conditions. These studies support the thinking that VEGF₁₆₄ and VEGFR2 both may have roles in the features of phases 1 and 2 OIR and the early and vascular phases of human ROP.⁸

To study VEGF₁₆₄-VEGFR2 signaling on the OIR phases, different approaches were used to inhibit VEGFR2 signaling: a neutralizing antibody to rat VEGF₁₆₄ or a VEGFR2 kinase inhibitor.³⁵ Since VEGF is an angiogenic factor, inhibition of VEGFR2 activation was predicted to reduce not only IVNV but also physiologic retinal vascular development and therefore cause persistent avascular retina. At certain doses, each intervention reduced phase 2 IVNV but, surprisingly, did not inhibit physiologic retinal vascular development. These findings suggested that over activation of VEGFR2 signaling might both inhibit physiologic retinal vascular development and cause IVNV. Studying the VEGF signaling pathway in vivo is problematic, because a single allele knockout of VEGF or one of its splice variants or receptors is lethal. The investigators, therefore, used an embryonic stem cell model in which a knockout of VEGFR1 (*flt1*) caused VEGF to bind and over activate VEGFR2 and increase angiogenesis. Compared to control, over activation of VEGFR2 disordered angiogenesis and caused a pattern of growth similar to IVNV.³⁶ Physiologic vascularization was restored with a transgene of VEGFR1 containing a CD31 promoter specific to endothelial cells. The endothelial VEGFR1 thus trapped excessive VEGF and reduced its binding and activation of VEGFR2. This work demonstrated that over activated VEGFR2 in endothelial cells caused aberrant angiogenesis in vitro.

Investigators then determined whether or not VEGFR2 activation affected IVNV in the rat OIR model. Retinal flat mounts from the rat OIR model were colabeled with lectin to visualize the vasculature, and an anti-phospho-histone H3 label was used to identify mitoses of dividing vascular cells in anaphase (Figure 4). Two lines were drawn onto each mitotic figure in imaged retinal flat mounts. One line was between each pair of anti-phospho-histone, H3 labeled chromosomes at the cleavage plane set up by the dividing vascular cells. The other line was drawn along the long axis of the developing vessel. The angles between the two lines of all mitotic figures were measured. Angles at 90 degrees predicted elongation of developing vessels, whereas those 180 degrees apart predicted widening of the vessels. Mitotic cleavage planes having multiple different angles with the long axes of vessels predicted disordered angiogenesis. Two approaches to inhibit VEGF were then compared: a neutralizing antibody to rat VEGF₁₆₄ and a gene therapy approach using a lentivector to specifically target and knock down overexpressed VEGFA in Müller cells.^{21, 22} (Knockdown in Müller cells was chosen because VEGF splice variant expression levels had been localized to the inner nuclear layer, corresponding to the location of Müller cells,²¹ at time points preceding the development of phase 1 and 2 OIR in the rat.⁸) With each method to reduce VEGF bioactivity, doses were chosen that reduced VEGFR2 signaling and phase 2

IVNV compared to respective controls, but did not reduce physiologic retinal vascular development. In retinas treated with the targeted knockdown of Müller cell VEGFA²² or the intravitreal VEGF₁₆₄ antibody³⁷, “cleavage angles” predicted more ordered angiogenesis than in each respective control condition.

Together, these studies support the hypothesis that over activation of VEGFR2 disorders dividing endothelial cells, potentially allowing them to grow outside the plane of the retina in a pattern similar to IVNV. Inhibition of VEGFR2 signaling then would permit ordered, intraretinal vascularization (Figure 4). The investigators also tested an intravitreal neutralizing antibody to VEGF₁₆₄ against a control IgG antibody and found the anti-VEGF₁₆₄ antibody reduced tortuosity of arterioles measured with the ROPTool compared to control antibody.³⁷ This study provides evidence that VEGF signaling also plays a role in arteriolar tortuosity, as seen in human plus disease.

Subsequently, the Efficacy of Intravitreal Bevacizumab Treatment for Stage 3+ ROP (BEAT-ROP) study found that inhibition of VEGF with an antibody reduced IVNV and permitted ongoing physiologic retinal vascular development in some infants,³⁸ providing clinical evidence for the experimental findings that regulation of VEGF signaling orders disoriented developmental angiogenesis and may have a role in treating both phases 1 and 2 ROP. However, other infants treated with bevacizumab developed persistent avascular retina and later IVNV, sometimes at 60 weeks post-gestational age,³⁹ suggesting that individual doses of anti-VEGF agent and/or other factors are involved in physiologic retinal vascular development, at least in some phenotypes of ROP, and that it is important to study long-term effects from VEGF inhibition.

Therefore, a study was performed to test a later time point in the rat OIR model. An intravitreal neutralizing antibody to rat VEGF₁₆₄ at a dose that inhibited phase 2 IVNV was compared to an isotype goat IgG control. The anti-VEGF₁₆₄ antibody led to later recurrent IVNV in association with increased expression of other angiogenic compounds, including erythropoietin.⁴⁰ This study suggests that broad inhibition of VEGF may lead to rebound angiogenic effects potentially because it did not target the cell that overproduces VEGF. In addition the anti-VEGF₁₆₄ intravitreal antibody inhibited pup weight gain raising systemic safety concerns from broad intravitreal inhibition of VEGF bioactivity.

To knock down VEGF specifically in Müller cells that had been shown to express it, a lentivector gene therapy approach was used to introduce a cell-specific promoter and an shRNA to VEGFA in the rat OIR model.^{21,41} Compared to a control lentivector, the shRNA to Müller cell-VEGFA reduced retinal VEGF to levels in retinas of pups raised in room air and inhibited VEGFR2 signaling in endothelial cells.. Targeted knockdown of VEGFA was compared to its control lentivector and then to the experimental approach using an intravitreal antibody to VEGF₁₆₄ compared to its intravitreal IgG control. Both the VEGFA lentivector and VEGF₁₆₄ antibody caused the same fold reduction in IVNV areas compared to respective controls but did not affect the extent of physiologic retinal vascular development measured as vascularized to total retinal areas. However, the intravitreal anti-VEGF₁₆₄ antibody reduced capillary densities in the inner and deep retinal plexi, whereas the VEGFA lentivector did not.²² Thus, targeted knockdown of VEGFA in Müller cells

following repeated fluctuations in oxygenation appeared safer than broad intravitreal anti-VEGF₁₆₄ antibody. These studies support a line of thinking that intravitreal anti-VEGF₁₆₄ antibody reduces capillary support in the retinal plexi and leads to activation of angiogenic pathways that cause recurrent IVNV. The studies also support a cell-targeted approach to inhibit VEGF in ROP.

Since VEGF is neuroprotective and VEGF₁₆₄ was associated with pathologic features in the rat OIR model,⁸ investigators used the lentivector gene therapy approach to knockdown Müller cell VEGFA or the splice variant, VEGF₁₆₄, compared to a control lentivector containing a shRNA to the non-mammalian gene luciferase.¹⁴ Pup weight gain was not adversely affected by either experimental condition. Both the VEGFA and VEGF₁₆₄ lentivector shRNAs significantly reduced IVNV compared to control, but only the VEGF₁₆₄ knockdown maintained inhibition at a later time point in the model. Also, targeted Müller cell knockdown of VEGFA, but not of VEGF₁₆₄, increased cell death and thinned the outer nuclear layer, suggesting that targeting Müller cell VEGF₁₆₄ may be safer than targeting VEGFA. However, longer-term studies on structure and function are needed. Taken together, these studies raise concern about the safety of even targeted knockdown of VEGFA and support investigation of other treatment strategies.

Clinical or Translational Implications

The BEAT-ROP study suggests that VEGF inhibition with bevacizumab may alter angiogenic pathophysiology, but follow up studies also suggest that the treatment has a broader effect on the overall biochemistry of ROP and may account for some of the late failures. Experimental studies show that inhibition of VEGF using intravitreal antibodies, VEGFR2 inhibitors, or targeted knock down of overexpressed VEGF or VEGF₁₆₄ in Müller cells may reduce IVNV and permit physiologic retinal vascular development. Broad inhibition of VEGF signaling in multiple cell types such as what occurs with an intravitreal anti-VEGF antibody can lead to recurrent IVNV and systemic toxicity shown by reduced body weight gain.⁴⁰ Even targeted knockdown of Müller cell-derived VEGF experimentally may lead to retinal neuronal death. Other studies have shown the VEGFtrap to inhibit retinal vascularization²³ and retinal neural function.⁴² The intravitreal aptamer, pegaptanib, did not inhibit severe ROP (verbal communication from Mike Trese, 2014), but many questions exist including the mechanism of action of the aptamer, the timing when delivered, the dose used and lack of specificity in targeting Müller cells. Pegaptanib is being studied for ROP; therefore, experimental studies are needed to determine long-term safety as well as efficacy of VEGF₁₆₄ knockdown. Although gene therapy or subretinal injections are not recommended in premature infant eyes, studies to regulate VEGFR2 signaling in endothelial cells and preserve the neuroprotective effects of VEGFR2 signaling in the developing retina seem warranted based on experimental evidence.

The American Academy of Ophthalmology and the American Academy of Pediatrics provided guidelines for the use of anti-VEGF agents in ROP.⁴³ Still, more information on appropriate dose, type of agent and long-term safety is needed. It is not reasonable to make assumptions that the systemic effects from an intravitreal drug in the preterm infant will be similar to those in adults. A single intravitreal injection of anti-VEGF treatment appears to

change the natural history of ROP with occurrences reported almost 4 months later and reduced systemic VEGF for at least 2 weeks.³⁹ The reduction of systemic VEGF may have implications in developing organs, including lung, brain and kidney. A preterm infant's vitreous volume is about 1 mL and blood volume is ~120 mL, whereas an adult's is approximately 4 mLs and blood volume over 5000 mLs. Therefore, there is less dilution of an intravitreal drug that enters the preterm infant blood stream compared to the adult. In the US, an infant with severe ROP is often younger and smaller (with less blood volume) than an infant with severe ROP in countries lacking optimal resources for prenatal care. Therefore, the safety profiles from studies in the US and throughout the world may not be comparable. Anti-angiogenic treatment may need to be individualized based on eye and infant size. It is important to monitor body weight gain and not only birth weight, vascular coverage, persistent avascular retina, and recurrence of IVNV as safety parameters in infants. However, these outcomes alone may be insufficient based on outer nuclear layer thinning and cell death after experimental targeted knockdown of VEGFA.¹⁴

New Study Directions

Erythropoietin Derivatives

Besides anti-VEGF agents, there is renewed interest in erythropoietin derivatives for their neuroprotective effects. However, some experimental evidence suggests erythropoietin may increase the risk of severe ROP.⁴⁴ Darbepoietin, a form of erythropoietin, neither increased nor reduced the risk of severe ROP in one trial, although numbers were low.⁴⁵ Erythropoietin binds the erythropoietin receptor (EPOR), which forms a homodimer and activates the JAK/STAT pathway in hematopoiesis. Activated EPOR can also bind the beta common receptor to form a “tissue protective factor”, which is protective in models of stroke and inflammation. Certain forms of erythropoietin preferentially bind the tissue protective factor and are being studied in ROP. In the rat OIR model, the beta common receptor was not highly expressed, whereas VEGF increased the expression and activation of EPOR and led to an interaction between activated EPOR and VEGFR2, which over activated STAT3 in endothelial cells to cause phase 2 IVNV. This report supports the idea that EPOR is important in phase 2 IVNV and can be activated by erythropoietin or VEGF.⁴⁶

IGF-1/IGF-1BP3 and omega 3 fatty acids

A clinical trial on IGF-1/IGF-1BP3 is underway in Europe to test its role on infant growth, increasing physiologic retinal vascular development to reduce early avascular retina, and prevent the vascular phase of ROP. To reduce the potential of causing vasoproliferation, attempts are being made to only replenish IGF-1 to levels that would be normal in preterm infants at low risk of developing severe ROP.

Nutrition

Omega-3 fatty acids can reduce phase 1 and phase 2 in the mouse OIR model and are being investigated in human infants. WINROP, an algorithm originally based on IGF-1 but simplified to include only weight gain, is being studied to identify infants at the greatest risk of severe ROP.⁴⁷ The hope is that this strategy will reduce the burden in screening for ROP, which is increasing world-wide.

Anti-oxidants

Oxidative signaling is important to ROP phases, but as experimental models demonstrate, the picture is complex and outcomes following activation of factors can depend on the cell type within the retina. Clinical trials testing certain anti-oxidants (lutein, vitamin E, n-acetyl cysteine) have not safely or effectively vascularized ROP.

Other avenues of study

Experimental evidence suggests that neural guidance molecules, such as the semaphorins that repel neurons during development may also guide capillaries in physiologic retinal vascular development and pathologic conditions, like IVNV.¹⁵ Inflammatory mediators and the prostaglandin pathways have been studied in experimental models of ROP.⁴⁸ Plasmin is being tested for stage 4 or 5 ROP in a clinical trial. However, for early and vascular phases of human ROP, plasmin breaks down extracellular matrix into components important for physiologic retinal vascular development.⁴⁹ Beta adrenergic inhibition has been suggested to reduce severe ROP, but beta-adrenergic agonism also can be anti-angiogenic.⁵⁰ More study is needed.

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Figure 1.

Human ROP: Human ROP is classified by zone, stage, and the presence of plus disease. To facilitate comparing phases of ROP (delayed physiologic retinal vascular development and vaso-proliferation) with experimental studies, ROP can be divided into early ROP, which comprises delayed physiologic retinal vascular development and stages 1 and 2 ROP; vascular ROP, which comprises stage 3 ROP and in severe ROP, plus disease; and fibrovascular ROP, which comprises stages 4 or 5 ROP with partial or total retinal detachment, respectively.

**Drawing by James Gilman, CRA, FOPS

Phases of Oxygen Reduced Retinopathy (OIR)

Figure 2. OIR models

Models of Mouse and Rat OIR showing oxygen profiles, phases 1 and 2 OIR and retinal flat mounts stained with lectin to visualize the vasculature.

Pathways Involved in Disordered Development Angiogenesis

Figure 3.

Diagram of activated signaling pathways leading to the phases of human ROP based on experimental methods in the rat OIR model. Over activation of VEGFR2 can cause both phases of OIR and differential effects from STAT3 signaling based on the cell activated. Also targeted inhibition of VEGF in Müller cells can cause cell death and thinning of the outer nuclear layer.

Disordered Angiogenesis

Figure 4.

a. Intravitreal Neovascularization (IVNV) grows aberrantly into the vitreous instead of into the retina.

**Drawing by James Gilman, CRA, FOPS

b. Endothelial cells grow into the vitreous as IVNV rather than into the retina as intraretinal blood vessels. The angle between the cleavage plane of dividing daughter cells and the long axis of the vessel predicts whether the vessel will be elongated or widened. One line of evidence shows that over activated VEGFR2 signaling disorders divisions of endothelial cells and permits their access to the vitreous cavity and diverts them from growing into the retina.

**Drawing by James Gilman, CRA, FOPS