Objective: To study the effects of oxygen fluctuations on rat vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGFR1), and VEGFR2 in a model of retinopathy of prematurity (ROP).

Methods: Retinas at several postnatal days (p) were analyzed for VEGF splice variants, VEGFR1 and VEGFR2 messenger RNAs (mRNAs) using real-time polymerase chain reaction or for VEGF protein using enzyme-linked immunosorbent assay.

Results: Older developmental age was associated with VEGFR1 (P < .001), VEGF120 (P < .001), and VEGF188 (P = .03) mRNA overexpression. Expression of VEGFR2 and VEGF164 mRNAs were associated with older age (P < .001) or exposure to the ROP model (P = .02 and P < .001, respectively). Expression of VEGF protein was greater at p14, when 30% avascular retina existed in the ROP model, compared with room air, when no avascular retina existed, and at p18, when intravitreal neovascularization existed in the model but not in room air (P < .001 for both).

Conclusions: Unlike models of oxygen-induced retinopathy that describe ROP before implementation of oxygen regulation, the ROP model re-creates oxygen stresses relevant to preterm infants with severe ROP today. Expression of VEGF164 and VEGFR2 mRNAs and VEGF protein were increased in association with the ROP model and older developmental age and at time points when not only intravitreous neovascularization but also avascular retina were present in the ROP model and not in room air.

Clinical Relevance: Regulation of VEGF may have a role in the development of avascular retina and intravitreous neovascularization in some forms of severe ROP.
levels, and poor postnatal growth. However, the mouse model may not represent many cases of severe ROP that occur in the United States today. First, since the initial description of ROP and recognition that high-oxygen exposure at birth have a role, technology has developed and been implemented to regulate and monitor oxygen exposure in preterm infants. Resuscitation in high oxygen is avoided if possible, and oxygen saturations are carefully monitored to keep them in the mid 80 to low 90 percentages, depending on the postgestational age of the infant (these saturations translate to arterial concentrations <70 mm Hg). In OIR models, including the mouse model, 75% inspired oxygen is used to induce capillary recession and constriction, and this level of inspired oxygen causes arterial oxygen levels higher than 300 mm Hg.

Second, in most OIR models, constant oxygen, rather than fluctuating oxygen, is used. However, oxygen levels in the preterm human infant fluctuate on a minute-to-minute frequency even when inspired oxygen is relatively constant, and the extremes of oxygen levels and the range of the fluctuations may have a role in human ROP. Third, in the human infant, development of the inner retinal vasculature to the ora serrata is complete at term, but preterm infants have incomplete vascularization of the retina at birth. With today’s ROP, there seems to be a delay in vascularization of avascular retina with areas of peripheral avascular retina and not the central vaso-obliterative that is a main feature in the mouse OIR model.

Molecular analyses of retinas of the mouse OIR model have reported reduced expression of several growth factors during the vaso-obliterative phase of avascular retina. Particularly notable is vascular endothelial growth factor (VEGF), an important factor in retinal vascular development and in pathologic angiogenesis. When the VEGF and other factors, including erythropoietin, insulin-like growth factor 1, or placental growth factor, were injected into the vitreous of an eye in the mouse OIR model before hyperoxia, central retinal avascularity was avoided if possible, and oxygen saturations are carefully monitored to keep them in the mid 80 to low 90 percentages, depending on the postgestational age of the infant (these saturations translate to arterial concentrations <70 mm Hg). In OIR models, including the mouse model, 75% inspired oxygen is used to induce capillary recession and constriction, and this level of inspired oxygen causes arterial oxygen levels higher than 300 mm Hg.

The model uses fluctuations in oxygen, a risk factor associated with severe ROP. Therefore, we measured retinal VEGF protein levels and the expression of VEGF splice variant and receptor messenger RNAs (mRNAs) in the model and in RA at the same developmental ages.

**METHODS**

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

**ROP MODEL**

Within 4 hours of birth at postnatal day 0 (p0), litters of 12 to 14 newborn Sprague-Dawley rat pups with their mothers (Charles River Laboratories International Inc, Wilmington, Massachusetts) were placed into an incubator (OxyCycler; Biospherix, New York, New York) to cycle inspired oxygen between 50% and 10% every 24 hours. Pups from other litters were used to supplement deficient litters. After 7 cycles of oxygen fluctuations at p14, pups were placed into RA for 4 days. Oxygen levels were monitored daily and were recalibrated as needed. Carbon dioxide in the cage was also monitored daily and was flushed from the system by maintaining sufficient gas flow and by adding soda lime if needed.

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

For time point measurements, animals were humanely killed with pentobarbital (80 mg/kg via intraperitoneal injection) at the time of change in inspired oxygen level. Therefore, pups humanely killed at the start of even-numbered days up to p14 had just been exposed to 10% oxygen and those humanely killed on odd-numbered days to 50% oxygen. Pups humanely killed at p18 had been exposed to 7 cycles of oxygen fluctuations followed by 4 days in RA (eg, a p8 pup was starting the eighth day of life and had been exposed to 4 cycles of oxygen fluctuations and just finished 10% oxygen). For flat mounts, eyes were fixed in paraformaldehyde, 2%, for 2 hours. Retinas were isolated with ora serratas intact and were placed into a phosphate-buffered saline solution after the hyaloidal vessels and remaining vitreous were removed. By making 4 incisions 90° apart, the retinas were flattened and then placed onto microscope slides. For fresh tissue, eyes were not fixed in paraformaldehyde, 2%, and retinas were dissected without ora serratas. Tissue was frozen in modified radiomunoprecipitation assay buffer (20mM Tris base, 120mM 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-Aldrich Corp, St Louis, Missouri) and 1M orthovanadate (1:100) (Sigma-Aldrich Corp), then stored at −20°C for protein or in RNAlater (Applied Biosystems, Foster City, California) for RNA until analysis.

**TISSUE STAINING AND ANALYSIS OF FLAT MOUNTS**

To stain the vasculature, the flattened retinas were first permeabilized in ice-cold 70% vol/vol ethanol for 20 minutes and then in a combination of phosphate-buffered saline/1% Triton

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**REAL-TIME POLYMERASE CHAIN REACTION**

Samples were removed from RNAlater, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, California). DNA contamination was removed by using DNAfree (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). Approximately 200 ng of complementary DNA was analyzed per well by means of 1-step real-time polymerase chain reaction (PCR) using the TaqMan Master Mix with reverse transcriptase (RT) (3.7 U per reaction) (Applied Biosystems). Primers were specific for rat (annealing temperature, 60°C): VEGF120: forward, 5’-CCCAGGAGGATGTGCTGCT-3’; probe, 5’-GCTCACAGTGATTTCTGGC-3’; and reverse, TGCAGCATAGCAGATGTGAATGCAGACC; VEGF188: forward, 5’-GGCTTGTCACATTTTTCTGGC-3’; and reverse, GCACATAGGAGAAGATGAGCT-3’. The geometric mean of the cycle threshold automatically for each well, and each value was normalized to β-actin. Cycle threshold values were then calculated. 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 ROP model. The VEGFp0 at p0 was assigned a value of 1.0. The values for the 3 VEGF splice variants in the ROP model and in RA were related to this value and graphically represented in the figures. The VEGFR1 in RA at p0 was scaled to the value of 1.0 for comparisons of time points for RA and the ROP model for VEGF1. The VEGFR2 value at p0 was 59.6-fold greater than the VEGFR1 value. The VEGFR2 in RA at p0 was scaled to the value of 1.0 for graphical representation of time points for RA and the ROP model for VEGFR2. For statistical analysis, raw data were used.

**PROTEIN ANALYSIS**

The VEGF protein was analyzed using 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 radioimmunoprecipitation assay buffer were thawed, homogenized, and centrifuged (16 000g for 10 minutes at 4°C). Total protein was quantified using a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, California), modified from the Lowry assay. Supernatants were assessed 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. Often this required that whole litters be used for individual time points. For each time point, at least 3 retinas from different pups were analyzed from at least 2 different litters. Graphically represented are the mean fold changes relative to β-actin, with error bars representing standard errors. Although these normalized ratios were used for graphical representation in the figures, to avoid bias, the raw data were rescaled and statistically analyzed as described herein.

Initially, β-actin was analyzed by means of regression analysis of the ratio of each β-actin/VEGF splice variant or receptor mRNA for the cycle threshold 1 value against respective β-actin/VEGF splice variant or receptor mRNA for the cycle threshold 2 value, and the slope was found to be indistinguishable from 1.0, which is the ideal value. The geometric means of the product of 2 ratios were then determined for each time point and treatment, and this was the outcome analyzed. For the analysis of VEGF protein by means of 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 ROP 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 α-level adjustment for multiple comparisons among means accomplished using a method of simulation.

**RESULTS**

In p14 RA-raised pups, retinal vascularization of the inner capillary plexus had extended to the ora serrata.
Avascular retina was approximately 30% of the total retinal area at p14 and 25% at p18. No clock hours of IVNV were found at p14, and a median of 7.0 clock hours (mean [SE] avascular area, 3.2 [0.3] mm²) was present at p18 (Figure 1B). These findings are comparable with those reported in the literature.

**mRNAs OF VEGF RECEPTORS AND VEGF SPLICE VARIANTS**

We chose time points to determine expression levels preceding and including p14, when avascular retina was present in the ROP model but absent in RA-raised pups. We also analyzed for IVNV at p18. A previous study reported on VEGF protein concentration at time points after p14 and preceding the development of IVNV in the ROP model.

Expression of VEGFR1 mRNA increased many fold during development (Figure 2). For example, from p0 to p14, VEGFR1 mRNA increased 42-fold and from p0 to p18, 75-fold. Increased fold expression of VEGFR1 was significantly associated with older developmental age (P < .001) but not with whether pups had been placed into the ROP model or RA (P = .83). In contrast, the fold increase in VEGFR2 mRNA was relatively less than for VEGFR1 mRNA. For example, from p0 to p14, VEGFR2 mRNA increased approximately 5-fold and from p0 to p18, approximately 3-fold (Figure 3). Furthermore, there was a significant increase in expression associated with older developmental age (P < .001) and with exposure to the ROP model compared with RA (P = .02). There were no significant relationships in post hoc analyses.

We previously reported that VEGF164 was the most prevalent and that VEGF188 was the least prevalent splice variant in the ROP model using relative quantitative reverse transcription-PCR (RT-PCR). Using real-time PCR, we confirmed these findings. Because VEGF expression was reduced during the vaso-obliterative phase in the mouse OIR model, we predicted that the most prevalent VEGF splice variant, VEGF164, would have lower-fold expressions in the ROP model at the
There was a significant increase in expression of VEGF188 mRNA at p0 compared with p11 after hyperoxia in the ROP model (P < .001). There were no significant relationships in post hoc analyses. For graphical representations, the value of VEGF188 messenger RNA (mRNA) at p0 was scaled to 1.0, and fold expression of mRNAs of the splice variants at time points in RA and the ROP model were related to it. Error bars represent SE. Each time point had at least 5 retinas from different pups taken from at least 2 litters. mRNA indicates messenger RNA.

For VEGF164, the pattern of upregulation after hypoxia and downregulation after hyperoxia was also noted (Figure 5). However, only older developmental age was significantly associated with increased expression of VEGF120 (P < .001). There was no significant association with exposure to the ROP model compared with RA (P = .61). There was greater-fold VEGF188 expression at most time points in RA compared with the ROP model, and there was a significant association with increasing developmental age (P = .03) but not with exposure to the ROP model compared with RA (P = .60) (Figure 6). Post hoc testing revealed no significant meaningful relationships between RA and the ROP model at postnatal days analyzed or between sequential postnatal days in the ROP model for VEGF120 or VEGF188 mRNA expression.

VEGF PROTEIN CONCENTRATIONS IN RA AND THE ROP MODEL

Expression of VEGF protein was significantly associated with older developmental age or with exposure to
We previously reported that VEGF₁₆₄ expression, measured using relative quantitative RT-PCR, was increased after repeated fluctuations in oxygen but not after a single episode of 10% oxygen exposure, whereas VEGF₁₂₀ was up-regulated after an episode of hypoxia and not after repeated fluctuations in oxygen. Because fluctuations in transcutaneous oxygen are associated with increased risk of severe ROP, the data from these reports suggest that VEGF₁₆₄ may be the splice variant most associated with pathologic features in ROP. The VEGF₂ is believed to be the receptor most involved in angiogenic processes, whereas VEGF₁₁₀ is believed to trap VEGF and limit its signaling through VEGF₂ in development. The present data show that VEGF₂, but not VEGF₁₁₀, is significantly upregulated in association with the ROP model.

We also found that VEGF protein concentration was significantly greater in the ROP model than in RA at p14, when avascular retina existed in the ROP model, but vascularization of the inner retinal plexus had extended to the ora serrata in RA. These findings were unexpected and contrast with other models of OIR in which VEGF expression was reduced after hyperoxia-induced avascular retina and increased with relative hypoxia-induced endothelial cell proliferation into the vitreous. The early concept of hyperoxia-induced vaso-obliterration is clinically not observed in zone II, stage 3 severe ROP. Rather, there seems to be incomplete vascular development associated with preterm birth followed by a delay in peripheral retinal vascularization and then abnormal angiogenic patterning and proliferation at the junction of vascular and avascular retina. We found that VEGF was significantly increased in the ROP model when IVNV occurred. However, using the ROP model, these findings do not fit the biphasic hypothesis in that we found increased VEGF expression in association with avascular retina and IVNV.

Previously, using the ROP model, increased VEGF-VEGF₂ signaling was found to be associated first with arteriolar tortuosity and venous dilation, similar in appearance to human plus disease, followed by IVNV, and both were reduced by inhibiting the bioactivity of VEGF with an intravitreal injection of a neutralizing antibody to VEGF. Because VEGF is an angiogenic ago-
nist, IVNV and intraretinal vascularization would be anticipated to be reduced by an agent to inhibit VEGF bioactivity or signaling. However, neither a neutralizing antibody to VEGF\textsuperscript{28} nor a VEGFR2 tyrosine kinase inhibitor\textsuperscript{23} was found to interfere with ongoing intraretinal vascularization in the ROP model, although each intervention significantly reduced IVNV. Also, in a series of human infants with mostly zone II, stage 3 severe ROP, single injections of intravitreous bevacizumab were reported to cause regression of stage 3 ROP and permit ongoing retinal vascularization toward the ora serrata.\textsuperscript{36} These studies, along with the present data, suggest that excessive VEGF-VEGFR2 expression and signaling are associated with IVNV and greater avascular retinal area.

The processes of developmental and aberrant angiogenesis are complex, involving a variety of interacting factors. However, VEGF has been recognized as one of the most important in human retinal diseases associated with pathologic angiogenesis.\textsuperscript{37,38} Vascular endothelial growth factor is upregulated by hypoxia and ischemia\textsuperscript{39} and is increased in the serum and vitreous of patients with IVNV, including ROP.\textsuperscript{17,39} Also, in a human infant with stage 3 ROP, VEGF mRNA expression was detected in the avascular retina.\textsuperscript{40} Other pathways, including the deltalike ligand 4/Notch 1 signaling pathway, can regulate the numbers of VEGF-induced endothelial tip cells to stalk cells at the junction of vascular and avascular retina and permit developmental angiogenesis.\textsuperscript{41,42} Other angiogenic factors, such as angiopoietins, tumor necrosis factor, and hepatocyte growth factor\textsuperscript{43-45} have been reported to play a role in OIR. Inhibitors such as thrombin\textsuperscript{46} and pigment epithelium–derived factor\textsuperscript{47} may also be important. Interactions of growth factors and of other cells, such as pericytes, astrocytes, and other glia\textsuperscript{48,49} are important. Signaling events can affect endothelial tip cell filopodia number and length at the migrating front\textsuperscript{50} and affect the patterning of vessels.

Direct comparisons between mathematical fold changes in expression of mRNAs with biological outcomes do not take into account signaling cascades. Variability in measurements can also occur in the oxygen cycle and reflect the time for protein translation, posttranslational modification of protein, stability of mRNA, and sequestration of growth factor in extracellular matrix after mRNA upregulation. For the receptors, commercially available antibodies that recognize rat have not produced consistent results. Future studies of receptor protein and signaling will be important. Despite limitations, these data are useful to refine hypotheses on features of severe ROP seen today.

Expression of VEGF\textsubscript{164} mRNA was greater in RA than in the ROP model. This splice variant is cell associated, and its expression may reflect the vascular coverage of the retina. In fact, expression was increased in association with older developmental age but not with exposure to the ROP model. There was also upregulation after hypoxia, a finding reported previously using relative quantitative RT-PCR.\textsuperscript{31}

In conclusion, we used a model that represents zone II, stage 3 severe ROP as observed in countries such as the United States, where oxygen regulation and monitoring are implemented in neonatal intensive care units. We found that expressions of VEGF\textsubscript{164} and VEGFR2 were significantly increased in association with older developmental age and exposure to the ROP model compared with RA, whereas VEGF\textsubscript{120}, VEGF\textsubscript{188}, and VEGFR1 expressions were increased in association with older developmental age. The concentration of VEGF protein was greater in the ROP model at p14, when there was approximately 30% avascular retina, compared with in RA, when the inner vascular plexus had extended to the ora serrata. Additional studies are needed to explore the association of increased VEGF expression with avascular retina.

Although this study supports others in the literature that excessive VEGF signaling is involved in the pathologic features of zone II, stage 3 severe ROP, the following considerations should be made 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 a neuronal and an endothelial survival factor.\textsuperscript{51,52} Also, the development of the lung\textsuperscript{53} requires VEGF, and preterm infants are at risk for pulmonary disease. In addition, circulating drug levels are likely to be higher in infants than in adults because infants have relatively much smaller blood volumes than do adults compared with the differences between respective vitreous volumes.\textsuperscript{23} This study describes a model of zone II, stage 3 ROP and may not be relevant to other severe forms of ROP, such as aggressive posterior ROP. Finally, the Early Treatment for Retinopathy of Prematurity Trial reported that laser treatment of type 1 prethreshold ROP was associated with 90% success.\textsuperscript{35} Therefore, clinical trials are needed to test agents against the current standard of care.

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