Association of Retinal Vascular Endothelial Growth Factor With Avascular Retina in a Rat Model of Retinopathy of Prematurity

Steven J. Budd, MS; Hilary Thompson, PhD; M. Elizabeth Hartnett, MD

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 intravitreous 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 levels 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-obliteration 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-oblitierative 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, insulinlike 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 reduced. During the proliferative phase, expression of VEGF, erythropoietin, or insulinlike growth factor 1 was increased in association with intravitreal endothelial budding, and inhibition of growth factor bioactivity by several methods led to reduced endothelial budding. In the OIR model, the period between the 2 events is less than 10 days, whereas in preterm infants, severe ROP develops approximately 2 months or longer after birth. It is unknown what happens to the retinal growth factor concentration in the human infant during this interval.

To explore the effect of relevant oxygen stresses on the regulation of VEGF splice variants and receptors in association with important features of severe ROP seen today in the United States, we used a model developed by John Penn, that exposes rat pups to oxygen levels that produce arterial oxygen concentrations similar to transcutaneous oxygen levels reported in infants with severe ROP. The model also produces the characteristic appearance of severe ROP with peripheral avascular retina similar to zone II ROP, followed by retinal tortuosity similar to plus disease, and then IVNV at the junctions of vascular and avascular retina, similar to stage 3 ROP. 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; Biosphinx, 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 serrata 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 serttus. Tissue was frozen in modified radioimmunoprecipitation 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 orthovannadate [1:100] [Sigma–Aldrich Corp]), then stored at −20°C for protein or in RNA later (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...
X-100 for 30 minutes; they were then incubated with Alexa Fluor 568–conjugated Griffonia simplicifolia (Bandeiraea) isolectin B4 (5 µg/mL) (Molecular Probes, Eugene, Oregon) in a phosphate-buffered saline solution overnight at 4°C, as previously described. Images of the retinal blood vessels were captured using a Nikon 80i Research Upright Microscope with Surveyor/TurboScan software (Nikon Inc., Melville, New York) and were digitally stored for analysis.

Total retinal area, summed peripheral avascular retinal area, and areas of IVNV were computed in pixels using Image Tool v.3 (The University of Texas, San Antonio) and were converted to square millimeters (using a calibration bar on each image). The IVNV was defined as neovascularization growing into the vitreous at the junction of vascular and avascular retina. Images of the retinal blood vessels were captured using Adobe Photoshop (Adobe Systems Inc., San Jose, California), were assessed for the presence of IVNV, and were assigned a number (0-12) depending on the number of clock hours exhibiting IVNV. Areas were measured, summed, and expressed as a percentage of total retinal area for each eye. Measurements were performed by 2 independent masked reviewers. When discrepancies in measurements occurred, these were reviewed and a final consensus was determined.

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 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). 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’-GGCTTGTCACATTTTTCTGGC-3’; and reverse, 5’-GCACATAGGAGAGATGAGCT-3’; and reverse, 5’-CAGTGGACGCCTCCAGGATTT-3’; VEGF188: forward, 5’-TGCAGCATAGCAGATGTGAATGCAGACC; VEGF recep- tor 1 (VEGFR1): forward, 5’-GCACAGGTTTC TTGCGCTTTCGTTTTTTG; VEGF recep-
tor 2 (VEGFR2): forward, 5’-CGACATGAGAGATGAGCT-3’; and reverse, 5’-CTGCACAGTGGATTTC CAGGC-3’; and reverse, 5’-TCGAGAGTTTC CACAGTCGC-3’; and reverse, 5’-TGCAG CATAGCAGATGTGAATGCAGACC; VEGF recep-
tor 3: forward, 5’-GCACATGAGAGATGAGCT-3’; and reverse, 5’-CAGTGGACGCCTCCAGGATTT-3’; and reverse, 5’-ACCGGAGATTT TGCGGCTTTCGTTTTTG; VEGF receptor 1 (VEGFR1): forward, 5’-CCACCTC CATGTTT GAA G-3’; probe, 5’-AGTGGACGCCTCCAGGATTT-3’; and reverse, 5’-TACCAGCAGTCTCTGCTACCCTCC-3’; and reverse, 5’-TACCAGCAGTCTCTGCTACCCTCC-3’; and reverse, 5’-ATGCGAACGTCCA CAGGATTT-3’. Primers were made by the University of North Carolina’s Oligonucleotide Synthesis Core Facility (http://www.med.unc.edu/oligos/index.htm). Complementary DNA was mixed 1:1 with TaqMan Master Mix and primers. Rat β-actin was used as a control gene because its expression had previously been found to be stable under various oxygen stresses. Primers for rat β-actin were as follows: forward, TGCCCTAGCGTGTCAGGCTA; probe, CACTATCGCGCAATAGGGCGTTCC; and reverse, CAGGAGAAGGCTCGGAG.

Duplicate reactions with a total volume of 16 µL were run for each sample and control using the Applied Biosystems 7500 PCR System (Applied Biosystems). The Applied Biosystems 7500 PCR software calculates 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 VEGFR1. 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 percentages 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 bicinechonic acid protein assay kit (Bio-Rad, Hercules, California), modified from the Lowry assay. 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. 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 the 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...
There was no avascular retina or IVNV at p14 or p18 in RA-raised pups. In the ROP model, 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.33

mRNAs OF VEGF RECEPTORS AND VEGF SPICE 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 study29 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 posthoc analyses.

We previously reported that VEGF_164 was the most prevalent and that VEGF_188 was the least prevalent splice variant in the ROP model using relative quantitative reverse transcription-PCR (RT-PCR).33 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, VEGF_164, would have lower-fold expressions in the ROP model at the
compared with age-matched RA-raised pups (\(P < .001\)) and with exposure to the ROP model compared with RA (\(P < .001\)). Because VEGF is upregulated in hypoxia and downregulated in hyperoxia,\(^{34}\) we anticipated that the pattern of VEGF\(_{164}\) expression would show greater-fold expression after 24 hours in hypoxia (p12 and p14) and lower-fold expression after 24 hours in hyperoxia (p11 and p13). As predicted, the pattern of VEGF\(_{164}\) expression varied based on whether hypoxia or hyperoxia had occurred in the ROP model, and in post hoc analyses, VEGF\(_{164}\) mRNA expression was increased at p12 after hypoxia compared with p11 after hyperoxia in the ROP model (\(P < .001\)) (Figure 4). For VEGF\(_{120}\), 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 VEGF\(_{120}\) (\(P < .001\)). There was no significant association with exposure to the ROP model compared with RA (\(P = .61\)). There was greater-fold VEGF\(_{188}\) 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 VEGF\(_{120}\) or VEGF\(_{188}\) 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
the ROP model compared with RA (P < .001 for both). The VEGF protein shared some similarities in the pattern of expression as fold changes in VEGF164 mRNA (Figure 7). In post hoc testing, there were significant increases in VEGF protein expression in the ROP model compared with RA at p14 and p18 (P < .001) and at p8, p12, and p13 (Figure 7).

**COMMENT**

We analyzed retinal mRNA expression in rats of the VEGF splice variants VEGFR1 and VEGFR2 and of VEGF protein at different developmental time points in RA and ROP models that uses oxygen extremes and fluctuations relevant to human preterm infants who develop severe ROP in countries that have implemented oxygen regulation and monitoring. This model also has a retinal appearance similar to that of human zone II, stage 3 ROP. We found that increased expression of VEGF164 was significantly associated with older developmental age or exposure to the ROP model compared with RA, whereas increased expression of VEGF120 or VEGF188 was significantly associated with older developmental age and not with exposure to the ROP model. Expression of VEGF164 was significantly increased in association with older developmental age but not with exposure to the ROP model, whereas VEGF188 expression was significantly increased in association with older developmental age or with exposure to the ROP model.

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 VEGFR2 is believed to be the receptor most involved in angiogenic processes, whereas VEGFR1 is believed to trap VEGF and limit its signaling through VEGFR2 in development. The present data show that VEGFR2, but not VEGFR1, 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 compared 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-oblation 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-VEGFR 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 nor a VEGFR2 tyrosine kinase inhibitor 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 intravitreal bevacizumab were reported to cause regression of stage 3 ROP and permit ongoing retinal vascularization toward the ora serrata. 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. Vascular endothelial growth factor is upregulated by hypoxia and ischemia and is increased in the serum and vitreous of patients with IVNV, including ROP. Also, in a human infant with stage 3 ROP, VEGF mRNA expression was detected in the avascular retina. 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. Other angiogenic factors, including erythropoietin, insulinlike growth factor 1, hypoxia-inducible factor-1α, angiopoietins, tumor necrosis factor, and hepatocyte growth factor have been reported to play a role in OIR. Inhibitors such as thrombospondin-1 and pigment epithelium–derived factor may also be important. Interactions of growth factors and of other cells, such as pericytes, astrocytes, and other glia are important. Signaling events can affect endothelial tip cell filopodia number and length at the migrating front 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 VEGF164 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.

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 VEGF164 and VEGFR2 were significantly increased in association with older developmental age and exposure to the ROP model compared with RA, whereas VEGF120, VEGF188, 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. Also, the development of the lung 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. 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. Therefore, clinical trials are needed to test agents against the current standard of care.

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Correspondence: M. Elizabeth Hartnett, MD, University of Utah, Department of Ophthalmology, John Moran Eye Center, 65 Mario Capecchi, Dr, Salt Lake City, UT 84132 (me.hartnett@hsc.utah.edu).

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