Genetic Polymorphisms and Retinopathy of Prematurity

Richard W. I. Cooke,[1] *Jo A. Drury,*[1] *Roger Mountford,*[2] *and David Clark*[3]

Purpose. Retinopathy of prematurity (ROP) is a major problem among very preterm survivors of neonatal intensive care. Neovascularization of the retina is prominent in the proliferative stages of ROP and is under the control of several factors such as vascular endothelial growth factor (VEGF). This study was undertaken on the hypothesis that genetic polymorphisms of VEGF, transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF)-α would occur more frequently in preterm infants with progressive ROP than in those with mild or no disease.

Methods. The frequencies of VEGF -634 G→C, VEGF *936 C→T, TNF-α -308 G→A, and TGF-β -509 C→T were determined in DNA from 91 infants who had received treatment for threshold ROP and 97 comparison infants.

Results. The frequencies of the VEGF *936 C→T, TNF-α -308 G→A and TGF-β -509 C→T polymorphisms were similar in both groups. The distribution of alleles at VEGF -634 was significantly different between the two groups ($P = 0.03$). Homozygotes for the G allele, associated with higher VEGF production were twice as likely to have threshold ROP.

Conclusions. The progression of ROP to threshold ROP in very preterm infants may be influenced by genetic differences in VEGF production. Future efforts at prevention of threshold ROP may be directed toward blocking excess production of VEGF. (*Invest Ophthalmol Vis Sci.* 2004;45:1712–1715) DOI: 10.1167/iovs.03-1303

Retinopathy of prematurity (ROP) is a major problem among very preterm survivors of neonatal intensive care where disruption of normal retinal vascularization occurs due to premature birth. Threshold ROP occurs in approximately 5% of infants with a birthweight of less than 1250 g,[1] and despite treatment, approximately 10% to 15% of these children will become blind.[2] If visual acuity is considered, unfavorable outcomes are much higher. It is likely that factors contributing to normal vascularization during development are those involved in pathologic neovascularization.

The pathology of ROP can be separated into two phases: Phase 1 is hyperoxia-vasocessation, and phase 2 is hypoxia-vasoproliferation. The former occurs immediately after premature birth. Supplemental oxygen causes retinal hyperoxia, a downregulation of vascular endothelial growth factor (VEGF) and a consequent cessation of normal retinal vascularization.[3] Because of systemic factors and increasing metabolic demands, a shift to phase 2 occurs when a relative hypoxia develops that stimulates VEGF production, leading to renewed vascularization. Depending on local retinal responses, this can be normal vascularization or abnormal neovascularization.

Recent studies revealed the importance of VEGF in experimental animal models of ROP. VEGF expression was downregulated by hyperoxia in conjunction with cessation of normal growth and the loss of some of the developing vasculature in a mouse model of phase 1 ROP.[4]

Increased production of VEGF was demonstrated to play an important role in the proliferative phase of ROP.[5–7] Blockade of VEGF receptors effectively abolished retinal neovascularization in animal models.[8,9] Both retinal pigment epithelial cells and retinal glial cells release VEGF in response to hypoxia.

In humans, VEGF has been found to be elevated in vitreous fluid from eyes with active neovascularization compared with eyes with no neovascular disorders.[10] In addition, when the eyes from an infant treated with laser therapy for stage 3 ROP in one eye only were examined after death, there was no VEGF mRNA in photocoagulated areas and raised VEGF mRNA between laser scars. In the untreated eye, elevated VEGF mRNA was found in the peripheral (avascular) retina.[11] Conditions after preterm birth foster intense proliferation of vascular endothelium and glial cells at the junction of avascular and vascular portions of the retina, a process thought to result from liberation of angiogenic factors such as VEGF.

Many polymorphisms of the VEGF gene have been described, although most are relatively rare. Two common polymorphisms, -634 G→C—SNP identifier: rs2010963, OMIM identification number 192240.0001 (previously denoted +405 G→C, position relative to transcription start site)—in the 5′ untranslated region,[12] and *936 C→T in the 3′ untranslated region (GenBank accession number AF024710, nucleotide numbering relative to transcription initiation site; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD)[13] have been shown to decrease VEGF production in vitro and in vivo, respectively.

Transforming growth factor (TGF)-β1 has been shown to influence many cell- and growth-regulation events and is a prime candidate gene to investigate for conditions involving irregular or abnormal cell growth.[14] TGF-β1 is involved in the deposition of extracellular matrix (an essential process in new vessel formation), is upregulated in retinal ischemia patients and proliferative diabetic retinopathy, and probably plays an important role by stimulating angiogenesis and inhibiting the endothelial function in the eye.[15] Many polymorphisms have been described that alter activity. The TGF-β1 -509 C→T polymorphism has been extensively studied and shown to correlate with in vitro protein levels[16] (nucleotide numbering relative to transcription start site, GenBank accession number: J04431).

Tumor necrosis factor (TNF)-α levels have been shown to be increased in many diseases, including proliferative diabetic

From the [1]Department of Child Health University of Liverpool, Neonatal Unit, and the [2]Regional Molecular Genetic Laboratory, Liverpool Women's Hospital, Liverpool, United Kingdom; and the [3]Department of Ophthalmology, University Hospital Aintree, Liverpool, United Kingdom.

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Corresponding author: Richard W. I. Cooke, Department of Child Health, University of Liverpool, Neonatal Unit, Liverpool Women's Hospital, Liverpool L8 7SS, UK.

TABLE 1. Genotype Distributions and Allele Frequencies for the Studied Polymorphisms

	VEGF −634 G→C		VEGF *936 C→T		TNF-α −308G→C		TGF-β −509 C→T	
Polymorphism	**ROP**	**Comparison**	**ROP**	**Comparison**	**ROP**	**Comparison**	**ROP**	**Comparison**
n/n (%)	43 (48)	30 (31.5)	64 (74)	65 (70)	55 (64)	59 (63)	41 (49)	47 (54)
M/n (%)	38 (43)	52 (55)	18 (21)	20 (21.5)	27 (31)	32 (34)	35 (41.5)	33 (38)
M/M (%)	8 (9)	13 (13.5)	4 (5)	8 (8.5)	4 (5)	3 (3)	8 (9.5)	7 (8)
P (Yates corrected χ^2)	0.03		0.6		1.0		0.6	
Allele frequency								
n	0.70	0.59	0.85	0.80	0.80	0.80	0.70	0.73
M	0.30	0.41	0.15	0.20	0.20	0.20	0.30	0.27
Odds ratio (95% CI)	2.0 (1.11–3.69)		1.27 (0.66–2.46)		1.05 (0.57–1.93)		0.81 (0.45–1.48)	

Yates corrected χ^2 and odds ratio carried out on n/n versus M/n + M/M for ROP versus comparison. n, common allele; M, rarer allele; n/n, homozygous common allele; M/n, heterozygous; M/M, homozygous rarer allele; 95% CI, Gart's 95% confidence interval.

retinopathy. Serum TNF-α was raised in nonproliferative diabetic retinopathy and proliferative diabetic retinopathy compared with diabetic and nondiabetic control subjects. TNF-α levels also correlate with progression of diabetic retinopathy.[17] The TNF-α −308 G→A polymorphism has been associated with increased disease susceptibility in cerebral malaria,[18] death of meningococcal disease,[19] and asthma.[20]

Whereas most very preterm infants acquire signs of mild ROP, only a few progress to severe and potentially blinding disease. Identification of genotypes predictive of a higher risk of disease progression could eventually alert neonatologists to at-risk cases and also provide a means to select infants who might benefit from prophylactic management with VEGF receptor blockade.

We hypothesized that these genetic polymorphisms of VEGF, TGF-β1, and TNF-α would occur more frequently in preterm infants with progressive ROP than in those with mild or no disease.

METHODS

The study was performed at Liverpool Women's Hospital, Neonatal Unit, and follow-up clinics, and at University Hospital Aintree, retinopathy follow-up clinics.

All infants born before 32 weeks' gestation or with a birth weight below 1500 g were screened for ROP by one experienced ophthalmologist (DC). Threshold ROP requiring treatment was defined as five continuous or eight cumulative clock hours of stage 3 disease in zone 1 or 2 in the presence of plus disease.[21]

Infants with a birth weight of less than 1500 g or less than 32 weeks gestation who had received treatment for ROP (stage 3+ or more) and were attending an ophthalmic follow-up clinic (DC), and similar infants who did not have threshold ROP attending a low-birth-weight follow-up clinic (RWIC) were recruited for the study. All were white. After written informed parental consent, a single buccal swab was collected from each infant by using a standardized technique. A specific anonymized pro forma was used to collect demographic and outcome data. Name, hospital number, and date of birth of the infant were not recorded. A unique study number was used to link patient data with genetic data. The study was approved by the local Pediatric Research Ethics Committee and was conducted in accordance with the guidelines in the Declaration of Helsinki.

Genotyping

DNA Preparation. Buccal cells in 5 mL sterile saline were centrifuged for 10 minutes at 4200 rpm. The supernatant was removed, and the pellet resuspended in 100 μL 50 mM sodium hydroxide. The suspension was heated to 95°C for 10 minutes before neutralization with 15 μL 1 M Tris/HCl (pH 8.0). After mixing and centrifugation the supernatant was used neat or diluted 1:5 in PCR reactions. DNA was stored at −40°C.

PCR. PCR reactions (10 μL) were performed containing 5 μL master mix (Reddy Mix Custom PCR Master Mix; ABgene, Epsom, UK), 1 μL of 5 μM forward and reverse primer mix, 1.5 μL DNA, and 2.5 μL water. The mix for the VEGF −634 G→C polymorphism contained 2 μL betaine and 0.5 μL water. Thermocycling was performed for 35 cycles in a commercial system (Techne Progene; Techne, Cambridge, UK). Primers and PCR conditions for the VEGF *936 C→T,[13] VEGF −634 G→C[12] and TNF-α −308 G→A[22] polymorphisms were as previously described, with minor modifications.

SSCP Analysis. Single-strand conformation polymorphism (SSCP) analysis was used to identify the VEGF and TNF-α polymorphisms, with confirmation of genotyping by restriction fragment length polymorphism (RFLP) analysis.

PCR products were denatured in 2× formamide loading buffer, heated to 95°C for 3 to 5 minutes and snap cooled on ice before loading on cold native 49:1 polyacrylamide gels in a cold room at 35 to 40 mA. Ten percent gels were used for the TNF-α −308G→A and the VEGF *936 C→T polymorphisms, and 8% for the VEGF −634 G→C polymorphism. Visualization was achieved with silver staining.

RFLP Analysis. The TGF-β −509 C→T polymorphism was detected as described by Grainger et al.[16]

Statistical Analysis

The significance of differences in frequency of the alleles in the polymorphisms studied and the deviation from Hardy-Weinberg equilibrium were tested using a χ^2 test with the Yates correction. Odds ratios with the Gart 95% confidence interval were calculated.

Study size estimations were difficult to make with only limited information on the polymorphism frequencies in the preterm population studied and on their direction of clinical effect. However, we calculated that if the frequency of the wild-type alleles changed by 25% in the high-risk ROP group, we would have an 80% chance of detecting this difference ($P < 0.05$) with 70 infants in each group.

RESULTS

Children ($N = 188$) were recruited, 97 to the comparison group, and 91 to the ROP group. Median gestational age in weeks (range) was 26 (23–32) versus 25 (23–30) ($P < 0.0001$) and birthweight in grams was 920 (448–2302) versus 779 (440–1185) ($P < 0.0001$). Both were significantly higher in the comparison group. Thirteen eyes (7.6%) from 11 patients treated in the ROP group had no light perception.

Table 1 shows the genotype distributions for the polymorphisms by study group. Only the VEGF −634 G→C polymorphism distribution was statistically significant with a signifi-

cantly higher proportion of the low-VEGF producing allele being found in the comparison group. None of the children with eyes treated for ROP and poor structural outcome were homozygous for the VEGF −634 G→C low-producing allele.

When logistic regression analysis was performed with study group as the dependent variable, and gestational age and presence of the VEGF −634 G allele as independent variables, carriage of the VEGF −634 G allele remained an independent predictor of progression to threshold ROP ($P = 0.008$). The odds ratio was calculated, and homozygotes for the VEGF −634 G allele were twice as likely to develop threshold ROP.

There was no significant deviation from Hardy-Weinberg equilibrium for the TNF-α −308 G→A and TGF-β −509 C→T polymorphisms. The ROP group were also in Hardy-Weinberg equilibrium for the VEGF polymorphisms. However, there was a nonsignificant trend to deviation from Hardy-Weinberg equilibrium in the comparison group for the VEGF *936 C→T polymorphism ($P = 0.15$).

Discussion

We have demonstrated a significant difference in distribution of the VEGF −634 G→C genotypes in infants who were treated for threshold ROP compared with preterm infants with mild or no disease. This is an important finding as gestational age, birthweight, and duration of supplemental oxygen administration are associated with susceptibility to ROP, but are unable to distinguish progression to sight-threatening disease. Demographic factors can be combined with prethreshold ROP severity to calculate the risk of an unfavorable 3-month outcome using a risk analysis program[2] and from this a clinical algorithm has been developed. The clinical algorithm does not, however, take into account all the known risk factors and thus requires clinical judgment to be applied. The observed difference persisted when the difference in gestational age between the two groups was corrected for by logistic regression analysis.

Deviation from Hardy-Weinberg equilibrium suggests that one of the assumptions on which it is based is not met. This implies that there may be a selective advantage to a particular genotype in the preterm comparison population. The G allele appeared to be underrepresented in the comparison group. The allele frequency in the ROP group was similar to previously published frequencies.[12] Watson et al.[12] reported highest in vitro VEGF production for the VEGF −634 GG genotype, which, taken together with our data and the raised levels of VEGF observed in active stage 4 ROP[23] suggest that this genotype is associated with increased VEGF production, leading to abnormal neovascularization in the immature preterm eye. This view is also supported by reporter gene studies that showed that some VEGF haplotypes carrying the −634 G allele were associated with increased basal promoter activity and responsiveness to phorbol ester stimulation compared with the −634 C allele.[24] This increase in promoter activity and responsiveness seems to be dependent on colinearity with a series of other 5′ sequence polymorphisms.

The above data are in direct contrast with work in Japan by Awata et al.,[25] who described an increased risk of diabetic retinopathy in patients with the VEGF −634 CC genotype. They also reported higher fasting serum VEGF levels in healthy subjects with the CC genotype. It may be that, in the two different ethnic populations, the −634 G→C polymorphism is differentially linked to another polymorphic locus such as the −116 G→A substitution reported by Stevens et al.[24] (−1198 G→A relative to translational start site), and that it is this polymorphism that is responsible for the increased transcriptional activity observed and raised VEGF levels.

The polymorphisms studied were chosen because of evidence of functional relevance or associations with disease states.[12,13,22,26] However, we were unable to assess their functional relevance in this population because of the method of recruitment. Protein levels change over time, and children were recruited retrospectively at any time after diagnosis. This recruitment method may also have led to some selection bias in the groups due to early neonatal deaths.

The observed differences in allele frequencies were smaller than those used in our sample size estimation; therefore, we cannot rule out the possibility of a type II error (i.e., failure to detect a real effect correctly) in the population studied. Recruiting larger numbers of infants with threshold ROP within a reasonable time frame from a single center would be very difficult, although larger comparison groups could be obtained.

Less than 8% of eyes treated for threshold ROP had an unfavorable structural outcome, which is similar to rates reported in the STOP-ROP trial,[27] and to the early treatment group in the Early Treatment for Retinopathy of Prematurity Randomized Trial.[2] Functional outcome was not investigated because of the wide range of ages at the time of recruitment and the fact that visual acuity is due in part to non-ROP–related ophthalmologic and neurologic problems in premature infants.

An interesting development in VEGF research has been the discovery of its relationship with insulin-like growth factor (IGF)-1. It has been shown that lack of IGF-1 in knockout mice prevents normal vascular growth, despite adequate VEGF.[5] The same group have demonstrated an association between low serum IGF-1 and ROP in premature infants.[28] It seems that a background level of IGF-1 is required for VEGF signaling and that VEGF works synergistically with IGF-1. However, Simo et al.[29] found elevated IGF-1 and VEGF in the vitreous fluid of diabetic patients with proliferative diabetic retinopathy and found that IGF-1 was not related to proliferative diabetic retinopathy activity, whereas VEGF was raised in active proliferative diabetic retinopathy. The low levels of IGF-1 observed in infants that have severe ROP may simply have indicated sick immature infants most likely to experience complications. Local concentrations of growth factors in the retina are likely to be more important than systemic levels. It is possible that low IGF-1 is the trigger for phase I ROP and that VEGF genotype determines the amount of VEGF produced, variably resulting in normal vascular growth or the abnormal neovascularization in ROP. Given the considerable overlap in IGF-1 range observed in preterm infants,[28] it seems likely that genetic factors will also govern IGF-1 expression.

Shih et al.[30] found that selective VEGFR1 stimulation decreases hyperoxia-induced vaso-obliteration without vascular proliferation and neovascularization and concluded that these agents may be useful in the prevention-treatment of ROP. However, most of the support for the theory of vasoconstriction and vaso-obliteration is based on animal model work and extreme vasoconstriction of the retinal vessels has not been observed clinically since the introduction of modern ventilators and oxygenation monitoring.[31]

Another genetic factor that may be relevant is the Norrie disease gene. It has been estimated that the Norrie disease gene is responsible for approximately 3% of cases of advanced ROP, but current evidence is conflicting in different populations.[32–35] It has been suggested that in these cases ROP may have been a misdiagnosis; further work in this area is warranted. It seems unlikely, however, that it is a major factor in ROP.

Caution should be taken in the interpretation of genetic polymorphism association studies.[36] Further confirmation is required from larger studies, preferably measuring protein levels in association with genotyping. It is also important that the

ethnicity of the population be well defined, because many traits are race specific.

In conclusion, identification of genetic predisposing factors to diseases of prematurity such as ROP could help in the development of new and innovative treatments and allow targeting of these to a "high-risk" subgroup, reducing unnecessary exposure to potentially harmful therapies. The VEGF −634 G→C polymorphism may be one such factor.

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