Brief Genetics Report

Association of the VEGF Gene With Proliferative Diabetic Retinopathy But Not Proteinuria in Diabetes

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Diabetic retinopathy and nephropathy cause significant morbidity in patients with diabetes. Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability factor and is implicated in both of these diabetes complications. We previously reported transfection studies showing the VEGF −460 and VEGF +405 polymorphisms to increase basal VEGF promoter activity by 71% compared with the wild-type sequence. Therefore, we investigated the association of these VEGF polymorphisms with proliferative diabetic retinopathy and diabetic nephropathy. DNA was isolated from 267 U.K. Caucasians with diabetes, comprising 69 patients with proliferative retinopathy and 198 patients with other grades of retinopathy. The distribution of VEGF −460 genotype was significantly different between the proliferative retinopathy and nonproliferative retinopathy groups ($P = 0.027$); specifically, carriage of the VEGF −460C allele was associated with proliferative diabetic retinopathy (odds ratio 2.5 [95% CI 1.20–5.23]). The VEGF −460 genotype was predictive of retinopathy, even after controlling for blood pressure, glycemic control, duration of diabetes, and obesity ($P = 0.02$). The VEGF +405 genotype did not associate with proliferative retinopathy, and neither polymorphism was associated with diabetic nephropathy. The VEGF −460C polymorphism is a positive independent predictive factor for the development of proliferative diabetic retinopathy. Increased VEGF production from high-expressing haplotypes, including −460C, may promote neovascularization. *Diabetes* 53:861–864, 2004

Diabetes leads to specific microvascular complications of retinopathy, nephropathy, and neuropathy, as well as increased risk of atherosclerosis, which may reflect underlying endothelial dysfunction (1). The risk of developing these complications increases with poor glycemic control (2). Diabetic retinopathy is a major cause of new-onset blindness among diabetic adults and is characterized by increased vascular permeability, tissue ischemia, and neovascularization. Neovascularization of the retina carries a high risk of blindness as a result of vitreous hemorrhage and fibrosis. Vascular endothelial growth factor (VEGF) can stimulate angiogenesis, enhance collateral vessel formation, and increase the permeability of the microvasculature (3,4).

In diabetic retinopathy, VEGF plays a role in the neovascularization of proliferative retinopathy and in the breakdown of the blood-retinal barrier that is characterized by hyperpermeability of retinal vessels (5–8). VEGF levels have been found to be markedly elevated in the vitreous and aqueous fluids in the eyes of patients with proliferative diabetic retinopathy (9,10).

Diabetic nephropathy is the most common cause of end-stage renal failure in the western world, affecting almost 35% of patients with type 1 diabetes (11). VEGF is implicated in the development of diabetic nephropathy because neutralization with anti-VEGF antibodies in experimental models significantly reduces hyperfiltration, albuminuria, and glomerular hypertrophy (12,13). The VEGF −2549 insertion/deletion promoter polymorphism has also been associated with diabetic nephropathy, where constructs containing the 18-bp deletion had a 1.95-fold increase in transcriptional activity compared with those containing the insert (14).

The VEGF +405 5′ untranslated region polymorphism has been associated with diabetic retinopathy in Japanese type 2 diabetic patients (15). The C allele frequency was increased in patients with compared with those without retinopathy. Subgroup analysis showed that carriage of the C allele was particularly increased in patients with nonproliferative diabetic retinopathy but not in patients with proliferative diabetic retinopathy, as compared with patients without any retinopathy. The authors suggested that the +405 polymorphism was associated with the development of retinopathy rather than with neovascularization of the retina, i.e., proliferative retinopathy.

We recruited patients with diabetes for an association study between the VEGF gene and proliferative diabetic retinopathy and diabetic nephropathy. Taken together, 198 of the participants had no retinopathy, background retinopathy, maculopathy, or other grades of nonproliferative retinopathy. Sixty-nine volunteers had proliferative diabetic retinopathy (Table 1). Volunteers with proliferative retinopathy had a significantly higher incidence of microalbuminuria, had significantly raised creatinine and HbA_{1c} concentrations, and suffered with diabetes for

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VEGF, vascular endothelial growth factor.

TABLE 1
Clinical measurements in diabetic patients

	Nonproliferative retinopathy or no retinopathy	Proliferative retinopathy	*P*
n	198	69	
Sex (M/F)	1/1	1/0.76	NS
Diabetes type (1/2)	1/0.56	1/0.50	NS
Microalbuminuria (+/−)	1/1.93	1/0.53	<0.001
Systolic blood pressure (mmHg)	137 ± 17	141 ± 16	NS
Diastolic blood pressure (mmHg)	74 ± 11	76 ± 11	NS
HbA_{1c} (%)	8.7 ± 1.4	9.2 ± 1.4	0.026
Creatinine (mg/dl)	103 ± 24	129 ± 91	<0.001
BMI (kg/m^2)	27.7 ± 4.8	27.4 ± 4.7	NS
Age (years)	52.6 ± 16.1	51.1 ± 15.3	NS
Duration of diabetes (years)	20.6 ± 9.4	23.2 ± 9.2	0.037

Data are means ± SD or, for categorical variables, *n* or ratio. Comparisons between retinopathy groups are by unpaired Student's *t* test or χ^2 test. *P* values <0.05 are considered significant.

longer (Table 1). The alleles of the −460 VEGF polymorphism were determined and found to not differ from predicted Hardy-Weinburg equilibrium. We found that carriage of the −460C polymorphism differed significantly between the patients with proliferative diabetic retinopathy and the other subjects ($P = 0.027$) (Table 2). During logistic regression, the dependent variable was the presence or absence of proliferative diabetic retinopathy. We found a significant and independent predictive effect of the VEGF −460 polymorphism, with a *P* value of 0.02 (Table 3). Other significant factors were the presence of microalbuminuria and duration of diabetes (Table 3).

We have evidence from our previous studies that VEGF promoter haplotypes carrying the −460C allele have 71% greater promoter activity compared with those that do not have this allele (16,17). Therefore, we collapsed the analysis to carriage or not of VEGF −460C. This showed that carriage of the C allele was overrepresented in those with proliferative diabetic retinopathy compared with those with either no retinopathy or other grades of diabetic retinopathy (Table 2).

We have also performed analysis of the +405 VEGF polymorphism. We found that there was no association between carriage of this genotype and grade of diabetic retinopathy.

Of our population, 101 subjects had evidence of diabetic nephropathy. One hundred forty-seven individuals had no microalbuminuria and normal creatinine concentrations. Twenty individuals were not assessed because they had incomplete biochemical results. We found a highly significant difference in the systolic blood pressure between those with (142 ± 19 [means ± SD]) and without (134 ± 14) microalbuminuria ($P = 0.001$). There was no significant difference in diastolic blood pressure between the groups. There was, however, a marginal difference in BMI between those with (28.5 ± 5) and without (27 ± 4)

TABLE 2
Association of VEGF −460 genotype with proliferative diabetic retinopathy in patients with diabetes

Genotype	TT	CT	CC	CT or CC
Analysis by −460 genotype				
Proliferative retinopathy	10 (14)	43 (62)	16 (23)	
Nonproliferative retinopathy or no retinopathy	59 (30)	92 (46)	47 (24)	
$P = 0.027$				
Control subjects ($n = 23$)	28%	50%	22%	
Odds ratio (95% CI)	1	2.76 (1.29–5.90)	2.01 (0.83–4.83)	
Analysis by carriage of −460C				
Proliferative retinopathy	10 (14)			59 (86)
Nonproliferative retinopathy	59 (30)			139 (70)
$P = 0.016$				
Odds ratio (95% CI)	1	—	—	2.50 (1.20–5.23)
Analysis by type of diabetes: carriage of −460C				
Type 1 diabetes				
Proliferative retinopathy	7 (14)			42 (86)
Nonproliferative retinopathy	33 (27)			90 (73)
$P = 0.06$				
Odds ratio (95% CI)	1	—	—	2.2 (0.9–5.4)
Type 2 diabetes				
Proliferative retinopathy	3 (15)			17 (85)
Nonproliferative retinopathy	25 (35)			47 (65)
$P = 0.07$				
Odds ratio (95% CI)	1	—	—	3.0 (0.8–11.3)

Data are *n* (%) unless otherwise indicated.

TABLE 3
Odds ratio adjusted by logistic regression for the association with proliferative diabetic retinopathy in patients with long-standing diabetes

Variable	Odds ratio (95% CI)	*P*
BMI	0.99 (0.90–1.08)	NS
Systolic blood pressure	1.00 (0.97–1.03)	NS
Diastolic blood pressure	1.03 (0.98–1.08)	NS
HbA_{1c}	1.11 (0.82–1.50)	NS
Creatinine	1.01 (1.00–1.02)	NS
Type of diabetes	1.23 (0.25–6.16)	NS
Microalbuminuria	3.29 (1.37–7.93)	0.008
Duration of diabetes	1.09 (1.02–1.15)	0.006
VEGF −460 C genotype	3.71 (1.23–11.24)	0.02

nephropathy ($P = 0.045$). There was no significant difference in current HbA_{1c} concentration. There was no significant association between either the −460 polymorphism or the +405 polymorphism and presence of microalbuminuria.

The VEGF gene has previously been identified as associated with the development of diabetic retinopathy in a Japanese population, although it was not an independent predictive factor for the development of proliferative diabetic retinopathy (15). The study by Awata et al. (15) differs from our study in a number of ways, including ethnicity, type of diabetes, and duration of diabetes.

In contrast to the study of Awata et al., which involved type 2 diabetic subjects, our study included both type 1 and type 2 diabetes. We felt this appropriate because development of diabetic retinopathy is thought to be a consequence of the hyperglycemic state rather than a result of the underlying cause of diabetes. Indeed, we found that there was no significant difference in the frequency of type 1 diabetes between the group with proliferative diabetic retinopathy and the group who did not have this complication, and logistic regression excluded type of diabetes as an independent risk factor in our population (Table 3). Subgroup analysis of retinopathy found a trend for association of −460C carriage and proliferative retinopathy for both types of diabetes (Table 2). In the previously reported Japanese study, all the patients had type 2 diabetes and there was a highly significant association between development of retinopathy and duration of disease. In the current study, all the patients had lived with a confirmed diagnosis of diabetes for at least 10 years or, in the case of those with type 2 diabetes and a duration of disease <10 years, had evidence of specific microvascular complications (either diabetic retinopathy or diabetic retinopathy and microalbuminuria) that suggested longer exposure to hyperglycemia. Therefore, patients in our study were enriched for the presence of microvascular complications. Despite this, duration of diabetes and HbA_{1c} were significantly higher in the proliferative disease group.

We have arbitrarily segregated those with the proliferative diabetic retinopathy spectrum from all other grades of retinopathy. Our hypothesis was that the VEGF gene would be associated with new vessel formation and, therefore, with proliferative diabetic retinopathy. We propose that the underlying mechanisms for background retinopathy and for diabetic maculopathy are different, although they may occur at different times within the same individual. Therefore, we grouped those individuals with background changes and those with all grades of maculopathy and nonproliferative retinopathy, with the exception of those with severe preproliferative disease treated with pan-retinal photocoagulation, together as nonproliferative diabetic retinopathy. In this way, we increased the power of the analysis by making a simple segregation of allele frequencies between the two groups. It is likely that some patients classified as nonproliferative will progess to proliferative in the future and that a small proportion of those treated with pan-retinal photocoagulation for severe preproliferative changes would not have developed neovascularization. Such unavoidable misclassification of a minority of cases tends to reduce the power of genetic analyses.

We have previously examined production of VEGF from stimulated peripheral blood mononuclear leukocytes in response to lipopolysaccharide stimulation. We reported that the highest VEGF production was observed for individuals homozygous for the G allele at +405 (17). Therefore, we also looked for association between the VEGF +405 polymorphism and development of proliferative diabetic retinopathy. We found no significant association between retinopathy status and the VEGF +405 polymorphism. Although there is linkage disequilibrium across the VEGF gene, carriage of −460C does not absolutely predict colinearity with +405G.

Diabetic nephropathy is another microvascular complication of long-standing diabetes with abnormalities centered on the renal glomerulus. The earliest accessible manifestation of this development is the presence of albumin in the urine. We did not find any association between VEGF polymorphisms at either −460 or +405 with the presence of microalbuminuria.

In conclusion, we present evidence that the human VEGF gene is an independent risk factor for the development of proliferative diabetic retinopathy in patients with long-standing diabetes. VEGF production is known to be stimulated by high glucose levels, advanced glycosylation end products, IGF-I, angiotensin II, and hypoxia, all of which are present in the retinal microvascular bed (8,18). The most plausible mechanism is that the −460C VEGF allele is linked to a promoter haplotype with increased activity, either under basal conditions or in response to these stimuli. We found that the VEGF genotype was predictive even after controlling for variables such as systolic blood pressure. This is the second time that the VEGF gene has been implicated in diabetic retinopathy; on this occasion, we make an association with the proliferative diabetic retinopathy spectrum, whereas in the earlier study, the association was with development of any grade of retinopathy. We propose that in the future individuals at increased risk of developing proliferative diabetic retinopathy may be identified genetically and offered enhanced screening or possibly novel interventions targeting VEGF action.

RESEARCH DESIGN AND METHODS

A total of 268 U.K. Caucasian diabetic patients were recruited from the Manchester Diabetes Centre. Diagnosis of diabetes was based on the guidelines of the Expert Committee report of the American Diabetes Association. All patients had either a confirmed diagnosis of diabetes for >10 years or

evidence of specific diabetes complications. There was an excess of type 1 diabetic patients, as the type 2 diabetic patients were typically under hospital review because of poor glycemic control or the presence of complications (Table 1). The presence and type of diabetic retinopathy were determined by an independent, masked ophthalmologist and classified as either proliferative retinopathy or all other grades of retinopathy. Nonproliferative diabetic retinopathy included signs of microaneurysm, intraretinal hemorrhage, the presence of exudates, and macula edema. Proliferative diabetic retinopathy includes neovascularization of the optic disk, neovascularization elsewhere, fibrovascular proliferation, and severe nonproliferative diabetic retinopathy treated as neovascularization by pan-retinal photocoagulation. This last group has an 80% risk of progression to high-risk proliferative retinopathy over a 5-year follow-up, and photocoagulation is the standard of care in our institution (19,20). As such, photocoagulation alters the natural history of the disease, then these patients are classed with the proliferative retinopathy group.

Diabetic nephropathy was diagnosed by the presence of microalbuminuria or by microalbuminuria and raised serum creatinine concentration in the presence of diabetic retinopathy and in the absence of other causes such as infection.

Informed consent was given by each participant, and the study was approved by the Central Manchester Research Ethics Committee and conducted in accordance with the principles of the Declaration of Helsinki.

Screening of VEGF gene polymorphisms. Genomic DNA was extracted from the peripheral blood of each individual. Genotyping for the −460 and +405 polymorphisms was performed using Taqman-based assays. For the −460 polymorphism, the forward primer was 5′ GAGAGTGAGGACGTGTGT GTCTGT 3′, the reverse primer was 5′ CAGATCTATTGGAATCCTGGAGTGA 3′, the C allele probe was 5′FAM TGAGGGCGTTGGAG MGB 3′, and the T allele probe was 5′ VIC TTGAGGGTGTTGGAGC MGB 3′. The VEGF +405 assay was purchased as an assay from Applied Biosystems (SNP ID rs2010963, catalog no. C 8311614 10).

Statistics. Distribution of genotypes and alleles was compared using χ^2 test or Fisher's exact test. Continuous clinical data were compared by unpaired students *t* test. Logistic regression analysis was performed to assess the independent role of the VEGF genotype and other variables, including BMI, systolic blood pressure, diastolic blood pressure, HbA_{1c} concentration, duration of diabetes, and presence of microalbuminuria.

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