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Vascular Endothelial Growth Factor Gene Polymorphisms Are Associated with Acute Renal Allograft Rejection

MAJID SHAHBAZI,[‡] ANTHONY A. FRYER,[†] VERA PRAVICA,[‡] IAIN J. BROGAN,[‡] HELEN M. RAMSAY,* IAN V. HUTCHINSON,[‡] and PAUL N. HARDEN*

*Departments of *Nephrology and [†]Biochemistry, North Staffordshire Hospital, Stoke-on-Trent, United Kingdom; and [‡]School of Biological Sciences, University of Manchester, Manchester, United Kingdom.*

Abstract. Acute rejection is a major cause of reduced survival of renal allografts. Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells and is expressed widely by renal tissue and T cells. VEGF influences adhesion and migration of leukocytes across the endothelium. This study investigates whether genetically determined variation in VEGF expression influences the development of renal allograft rejection. VEGF promoter polymorphisms were examined by using sequence-specific primer–PCR in 173 renal transplant recipients. Acute rejection occurred in 38.7%; median time to first rejection episode was 14 d. VEGF *in vitro* expression was investigated in stimulated leukocytes from 30 controls. The *−1154*G* and *−2578*C* alleles were associated with higher VEGF production. VEGF −1154 GG and GA genotypes were significantly associated with acute rejection risk at 3 mo (P = 0.004, odds ration [OR] = 6.8, 95% CI = 1.8 to 25 and P = 0.035, OR = 4.1, 95% CI = 1.1 to 15, respectively). Furthermore, VEGF −2578 CC and CA genotypes were associated with increased rejection risk (P = 0.005, OR = 4.1, 95% CI = 1.5 to 11.3 and P = 0.035, OR = 2.7, 95% CI = 1.1 to 7, respectively). These polymorphisms demonstrate linkage disequilibrium (P = 0.001). These data indicate that the *−1154*G* and *−2578*C* containing genotypes, encoding higher VEGF production, are strongly associated with acute rejection and may be useful markers of rejection risk.

The polypeptide VEGF is a potent regulator of normal and abnormal angiogenesis (1). Recent literature suggests that VEGF has several activities that may amplify acute inflammatory reactions. Overexpression of VEGF in skin results in increased vascular density, enhanced leukocyte adhesion, and tissue infiltration (2). Exposure of endothelial cells and macrophages to VEGF activates the transcription factor, NF-κB (3), which switches on synthesis of proinflammatory cytokines and chemokines (4). Monocytes stimulated with lipopolysaccharide express VEGF (5), and T lymphocytes produce VEGF both *in vitro* and when infiltrating solid tumors (6). The resultant increased expression of adhesion molecules (intracellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], and endothelial cell selectin [E-selectin]) increases leukocyte adhesion to endothelium and transmigration into the site of inflammation (4). Inhibition of VEGF by topically applied neutralizing antibody markedly suppresses acute rejection of rat corneal allografts, indicating a pivotal role for VEGF in the generation of rejection in this model (7).

The gene encoding VEGF is located on chromosome 6 and comprises a 14-kb coding region with 8 exons and 7 introns (8). Five promotor region polymorphisms have been identified by single-stranded conformational polymorphism analysis and sequencing (9). Acute rejection occurs in 20 to 40% of renal allograft recipients within 3 mo of transplantation, and is characterized by an acute inflammatory lymphocytic infiltrate (10). We propose that VEGF may be important in the generation of this inflammatory response, and that genetically controlled variation in VEGF production may influence susceptibility to acute allograft rejection. Accordingly, we have examined whether genotype is associated with *in vitro* VEGF production and rejection risk.

Materials and Methods

Patients

Unrelated white renal transplant recipients (n = 173; 90% of population) with functioning allografts were recruited at the North Staffordshire Hospital between May 1997 and June 1999. Local Hospital Ethics Committee approval and individual written informed consent was obtained. The standard initial immunosuppressive regimen since 1989 comprises prednisolone (20 mg/d), azathioprine (2 mg/kg), and cyclosporine (target trough level, 150 to 200 ng/ml). A single nephrologist collated demographic information, including age at transplantation, gender, number and date(s) of transplantation(s), immunosuppressant therapy, and HLA donor-recipient mismatch. Rejection episodes were defined by typical biopsy-proven appearances and/or an improvement in renal dysfunction with pulsed methylprednisolone. Acute rejection episodes were managed with methylprednisolone (500-mg pulses ×3); steroid-resistant rejection was treated with pulsed antithymocyte globulin or conversion to tacrolimus (since 1996). Patient demographics are shown in Table 1.

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Correspondence to Dr. Paul Harden, Department of Nephrology, North Staffordshire Hospital, Hartshill, Stoke-on-Trent, Staffordshire, UK. Phone: 0044-1782-554745; Fax: 0044-1782-620759; E-mail: pnharden@netscape.net

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Table 1. Patient demographics[a]

Mean age (± SD)	39.0 ± 15.3 yr
Gender	
male	114 (65.9%)
female	59 (34.1%)
Patients with rejection within 3 mo	67 (38.7%)[b]
Median time to rejection (range)	14 d (1 d to 8.1 yr)[c]
Median follow-up	8.3 yr
Proportion treated with ATG	11 (6.4%)
Donor	
cadaveric	162 (93.6%)
live	11 (6.4%)
No. of grafts	
1	147 (84.9%)
2	23 (13.3%)
3	2 (1.2%)
4	1 (0.6%)
HLA DR mismatch	
0	66 (38.2%)
1	65 (37.6%)
2	5 (2.9%)
unknown	37 (21.4)
Presenting pathology	
diabetes	7 (4.1%)
glomerulonephritis	56 (32.4%)
reflux	29 (16.8%)
dysplasia	8 (4.6%)
obstructive uropathy	10 (5.8%)
hereditary causes	22 (12.7%)
unknown etiology	41 (23.6%)

[a] ATG, antithymocyte globulin; DR, donor-recipient.
[b] 88.2% of rejections occurred within 3 mo.
[c] 9 subjects had a rejection episode between 3 mo and 8.1 yr.

Identification of VEGF Genotypes

Amplification refractory mutation system–PCR methodologies were developed for the single nucleotide substitutions at the positions −7 (185 bp), −1001 (198 bp), −1154 (203 bp), and −2578 (239 bp) in the VEGF gene as described previously (9,11). Briefly, blood leukocyte DNA was amplified in a 10-μl reaction comprising; PCR master mix (ABgene; ABgene Ltd., Epsom, UK), VEGF primers (5 μM) (9), human growth hormone primers (1 μM; internal control, 429 bp), 25 to 100 ng of DNA. Amplifications were performed by using annealing temperatures of 65°C (10 cycles) and 59°C (20 cycles). Amplified products were resolved in 2% agarose gels.

Relationship between VEGF Gene Polymorphism and In Vitro VEGF Production

Peripheral blood mononuclear cells (PBMC) from 30 healthy individuals were isolated by centrifugation over Histopaque 1083 (Sigma, Poole, UK) and cultured (1×10^6 ml^{-1}) in RPMI (Gibco Life Technologies, Paisley, UK) 1640 supplemented with 10% fetal calf serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin-streptomycin solution. Cells were stimulated with 100 ng/ml phorbol myristate acetate, 3 μg/ml lipopolysaccharide (*Escherichia coli* serotype 055:B5), and 1 ng/ml recombinant platelet derived growth factor–BB for 96 h at 37°C in 5% CO_2. VEGF levels were measured in culture supernatants by using a Quantikine immunoassay kit (R&D Systems, Oxon, UK) according to the manufacturer's instructions.

Statistical Analyses

The two-tailed *t* test was used for comparisons of VEGF production. The Pearson χ^2 test was used to assess differences in the distribution of alleles and genotypes between groups (rejectors *versus* nonrejectors). Logistic regression was used to correct for imbalances in age and gender between cases and controls.

Results

Correlation between Polymorphisms and In Vitro VEGF Production

PBMC from −1154*G/G homozygous individuals produced significantly more VEGF than cells from −1154*A/A homozygotes (Figure 1). Similarly, VEGF production was significantly higher in cells from −2578*C/C homozygotes than those from −2578*A/A individuals (Figure 2). The two polymorphisms demonstrated linkage disequilibrium ($P < 0.0001$; $\chi^2_4 = 54.6$). No significant associations were identified for the −7 and −1001 polymorphisms.

Association of VEGF Polymorphism with Acute Rejection Risk

Table 2 shows VEGF genotype and allele frequencies in rejectors and nonrejectors within 3 mo of transplantation. Individuals with −1154*G/G demonstrated a 6.8-fold increased risk of rejection compared with those with the −1154*A/A genotype, with −1154*G/A subjects showing an intermediate risk. Indeed, only 3 (12.5%) of 24 individuals with −1154*A/A had an acute rejection episode compared with 33 (42.9%) of 77 of subjects with −1154*G/G. Similar results were obtained for individuals with −2578*C/C (odds ratio, 4.1) and −2578*C/A (odds ratio, 2.7) compared with the −2578*A/A genotype. These associations remained significant after correction for gender and age at transplantation (−1154*G/G, $P = 0.007$; −2578*C/C, $P = 0.008$). There were no significant associations between rejection risk and −7 or −1001 genotypes (data not shown).

Discussion

In this study, we showed that polymorphisms in the VEGF gene at position −1154*G/A and −2578*C/A are associated with an increased risk of acute renal allograft rejection. The −1154*G/G and −2578*C/C homozygotes carry the greatest risk, with a lesser proportionate risk associated with heterozygosity. We explored the functional importance of these VEGF polymorphisms and identified a parallel allele-specific increase in production of VEGF by stimulated PBMC from healthy volunteers (−1154*G/G and −2578*C/C homozygotes associated with the highest production of VEGF). Hence, renal allograft recipients with genotypes associated with increased production of VEGF have an increased frequency of allograft rejection.

VEGF is induced in the tissues by hypoxia, which is an inevitable consequence of the transplantation procedure. Recipient

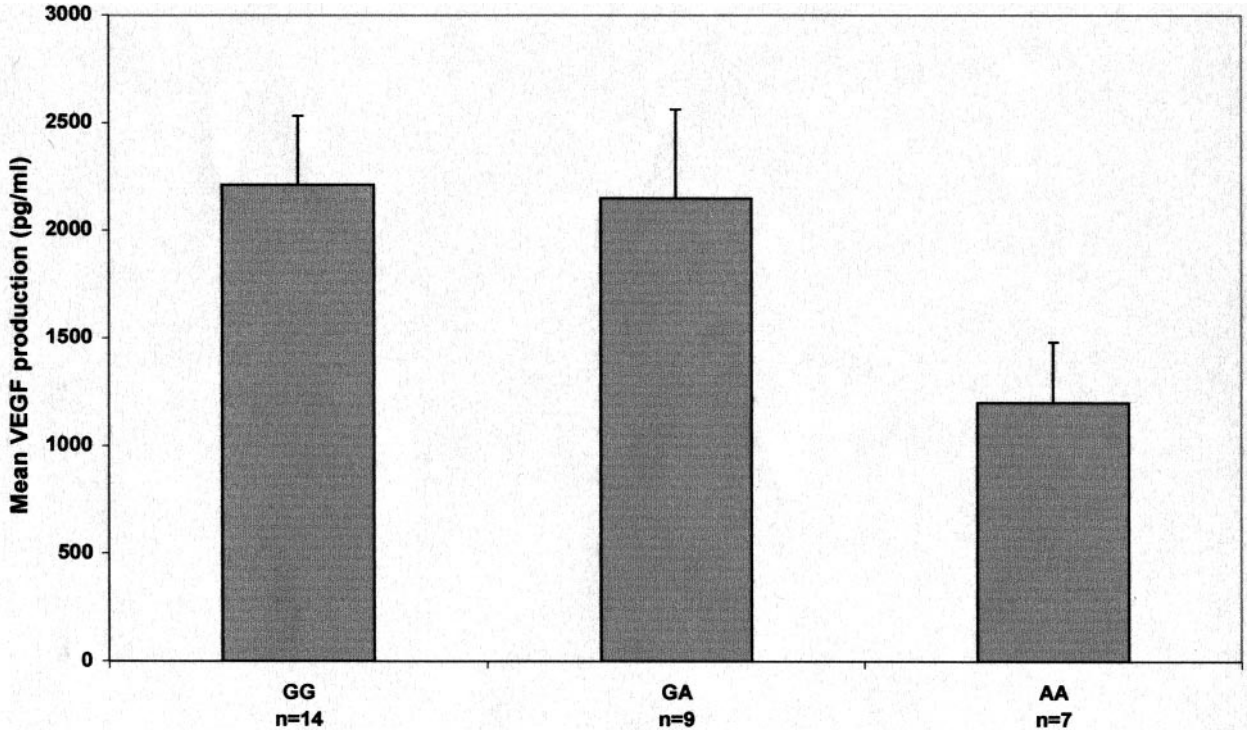

Figure 1. Association between vascular endothelial growth factor (VEGF) −1154 genotype and VEGF production. Figure shows mean ± SE VEGF production from peripheral blood mononuclear cells (PBMC) from subjects with the −1154 AA, GA, and GG genotypes. AA *versus* GG, $P = 0.024$; AA *versus* GA, $P = 0.054$; GG *versus* GA, $P = 0.896$.

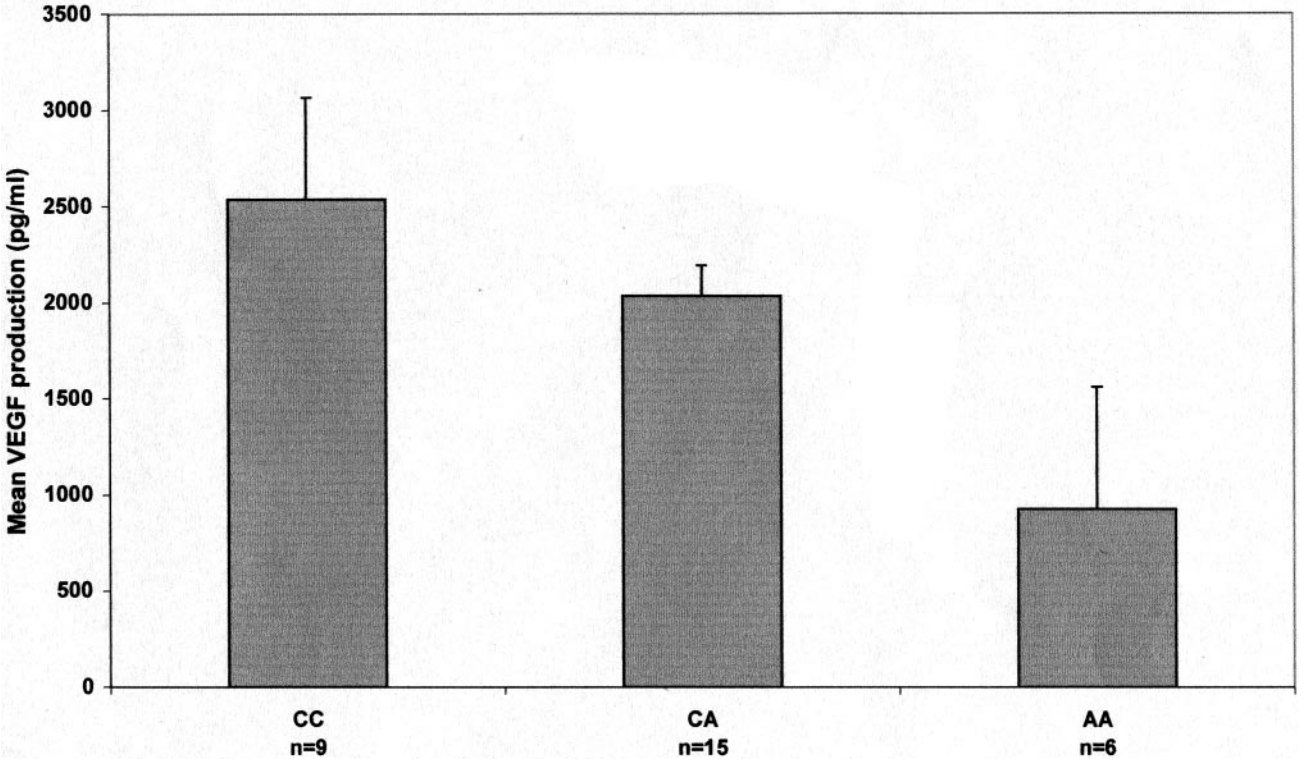

Figure 2. Association between VEGF −2578 genotype and VEGF production. Figure shows mean ± SE VEGF production from PBMC from subjects with the −2578 CC, CA, and AA genotypes. AA *versus* CC, $P = 0.042$; AA *versus* CA, $P = 0.090$; CC *versus* CA, $P = 0.330$.

Table 2. VEGF allele and genotype frequencies in rejectors *versus* nonrejectors at 3 mo

	Nonrejectors (%)	Rejectors (%)	*P*	Odds Ratio	95% CI
−1154 G → A					
genotype					
G/G	34 (34.7)	33 (54.1)	0.004	6.8	1.8 to 25.0
G/A	43 (43.9)	25 (41.0)	0.035	4.1	1.1 to 15.0
A/A	21 (21.4)	3 (4.9)	reference		
allele					
G	111 (56.5)	91 (74.6)	0.001	2.2	1.4 to 3.7
A	85 (43.4)	31 (25.4)	reference		
−2578 C → A					
genotype					
C/C	24 (23.3)	24 (37.5)	0.005	4.1	1.5 to 11.3
A/C	50 (48.5)	33 (51.6)	0.035	2.7	1.1 to 7.0
A/A	29 (28.2)	7 (10.9)	reference		
allele					
C	98 (47.6)	81 (63.3)	0.005	1.9	1.2 to 3.0
A	108 (52.4)	47 (36.7)	reference		

neutrophils and macrophages infiltrate the allograft after reperfusion and may produce VEGF. Allorecognition proceeds with the infiltration of recipient T cells and further production of VEGF. Protocol biopsy studies have demonstrated the ubiquitous presence of subclinical leukocyte infiltration in functioning renal allografts with stable renal function (12). Increased VEGF production promotes enhanced endothelial permeability and augmented leukocyte migration into the allograft, which may promote a clinically recognized rejection episode.

The role of donor VEGF genotype in this study is not known. In our studies on tumor necrosis factor–α genotype, recipient rather than donor genotype was associated with acute rejection risk (13). Unfortunately, there were insufficient numbers of patients with biopsy-proven chronic rejection in this study to test associations with VEGF genotypes. This analysis would be extremely interesting, because there is increased expression of VEGF protein in the renal interstitium in chronic renal allograft rejection (14).

In summary, the recently described polymorphisms at positions −1154 and −2578 of the VEGF gene are associated with both increased expression of VEGF and risk of renal allograft rejection. This relationship requires more detailed study, because VEGF may potentially be one of a small number of critical growth factors and cytokines in the pathogenesis of acute allograft rejection.

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