Vascular Endothelial Growth Factor Gene Polymorphisms in Behçet's Disease

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ABSTRACT. ***Objective.*** To evaluate potential associations of vascular endothelial growth factor (VEGF) gene polymorphisms with Behçet's disease (BD) and disease expression.

Methods. Case patients were 122 consecutive Italian patients with BD followed at the Rheumatology, Ophthalmology, and Neurology Units in Bologna, Ferrara, Milano, Palermo, Potenza, Prato, Reggio Emilia, and Trento over a 3-year period (1997-99) and who satisfied the International Study Group criteria for BD. Also selected as a control group were 200 healthy age and sex matched blood donors. All patients with BD and controls were genotyped by polymerase chain reaction and allele-specific oligonucleotide techniques for +936 C/T (rs3025039) and –634 C/G (rs2010963) mutations and for an 18 base pair (bp) insertion/deletion (I/D) polymorphism at –2549 of the the VEGF promoter region. *In vitro* release of VEGF by peripheral blood mononuclear cells (PBMC) was investigated by ELISA in healthy controls homozygous for the polymorphisms studied.

Results. The carriage rates of the alleles I and –634C were significantly more frequent in patients with BD than in healthy controls [p corr = 0.036, OR 1.8 (95% CI 1.1–2.9) and p corr = 0.05, OR 1.8 (95% CI 1.1–3.0), respectively]. While the distribution of allele +936T was similar in patients with BD and healthy controls, its frequency was significantly higher in BD patients with posterior uveitis/retinal vasculitis than in those without (p = 0.022, OR 2.4, 95% CI 1.1–5.0). Lipopolysaccharide-stimulated VEGF production from PBMC of healthy subjects was higher in II homozygous than in DD homozygous.

Conclusion. Our data indicate that carriers of –634C and I alleles are associated with susceptibility to developing BD. (J Rheumatol 2004;31:1785–9)

Key Indexing Terms:
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Behçet's disease (BD) is an inflammatory vasculopathy that involves the blood vessels of all sizes, including arteries, arterioles, veins, and venules. Vasculitis and thrombosis are important pathological features of BD[1,2].

Various abnormalities related to endothelial cell functions have been described in BD. Prostanoid synthesis in endothelial cells or in vessel walls is impaired in patients with BD[3,4], whereas plasma levels of thromboxane[3-5], von Willebrand factor antigen[6-8], and thrombomodulin[5,9,10] are higher than those of healthy controls. Endothelial cell-dependent vasodilatation is significantly impaired in patients with BD, as shown by high resolution ultrasound imaging[11]. Therefore, endothelial dysfunction may have a central role in the pathophysiology of BD.

Vascular endothelial growth factor (VEGF) may be implicated in the pathogenesis of vessel injury in BD. VEGF is not only a potent angiogenic factor, it also acts as a proinflammatory cytokine by increasing endothelial permeability and inducing adhesion molecules that bind leukocytes to endothelial cells[12,13]. Intercellular adhesion molecule-1 (ICAM-1) is strongly expressed in vascular endothelial cells

and perivascular inflammatory infiltrates in the immunopathologic studies of BD lesions[14,15].

VEGF appears to represent an attractive candidate susceptibility gene for BD, and its production may be partially subject to genetic control. Among healthy subjects, strong interindividual variations of VEGF plasma levels and VEGF production from stimulated peripheral blood mononuclear cells (PBMC) and VEGF gene expression have been reported[16]. +936 C/T mutation occurring in 3'-UTR[17] and –634 C/G mutation occurring in 5'UTR of the VEGF gene (erroneously reported as +405 polymorphism in a previous study)[18] have been found to regulate the VEGF plasma levels. Further, an 18 base pair (bp) insertion/deletion (I/D) polymorphism at –2549 of the VEGF promoter region has been described[19].

We evaluated potential associations of these 3 polymorphisms with BD susceptibility and with disease expression. In addition, we studied unstimulated and stimulated PBMC VEGF production in healthy subjects to assess possible functional relevance of the VEGF polymorphisms.

MATERIALS AND METHODS

Study population. Case patients were consecutive patients with BD who were followed at rheumatology, ophthalmology, and neurology units located in Bologna, Ferrara, Milano, Palermo, Potenza, Prato, Reggio Emilia, and Trento, Italy, over a 3-year period (1997-99). Patients satisfied the International Study Group criteria for BD[20]. The cohort comprised 122 Italian patients with BD that were identified. The control group consisted of 200 sex and age matched healthy subjects who were unrelated volunteer blood donors. The median age of the controls was 32 years (range 20–44), of whom 50% were male. All study subjects were Caucasians who had resided in Italy for at least one generation. No ethnic differences were present between patients and controls; none were of Jewish background. Informed consent was obtained from patients and controls before inclusion in the study.

HLA class I typing. Serological HLA class I typing was performed by a standard microlymphocytotoxicity technique, using peripheral blood lymphocytes[21]. Out of the 122 Italian patients with BD, 98 were typed for HLA-B51 allele. The control group consisted of 130 healthy blood donors.

Molecular analysis of VEGF polymorphisms. DNA was extracted from whole peripheral blood of controls and patients with BD by a standard method with phenol, chloroform, and isoamyl alcohol. VEGF genotyping was determined by using polymerase chain reaction (PCR) followed by restriction analysis. Determination of 18 bp I/D (at –2549 of the promoter region), –634 C/G (rs2010963), and +936 C/T (rs3025039) polymorphisms was done as described[17-19,22].

The PCR products for VEGF–634 polymorphism were digested with restriction endonuclease Bsn FI (New England Biolabs, Beverly, MA, USA) and restriction fragments were analyzed on 2% agarose gel. The 304 bp C allele remained uncut while the G allele was cut into 2 fragments of 193 bp and 111 bp. The PCR products for VEGF+936 polymorphism were digested with restriction endonuclease Nla III (New England Biolabs), and restriction fragments were analyzed on 2% agarose gel. The 208 bp C allele remained uncut, while the T allele was cut into 2 fragments of 122 bp and 86 bp. The PCR products for VEGF 18 bp I/D were visualized on 2% agarose gel. The pattern shows 2 fragments: 234 bp I allele with 18 bp insertion and 216 bp D allele without insertion.

Measurement of VEGF peripheral production. PBMC from 13 control subjects GG homozygous, 14 CC homozygous, 19 DD homozygous, and 18 II homozygous (mean age 73 yrs; range 60–86) were isolated from the heparinized blood by centrifugation over Ficoll-Hypaque gradients (Sigma Chemical Co., St. Louis, MO, USA). After washing, cells were resuspended in RPMI 1640 medium (pH 7.2) (Sigma) containing 10% fetal calf serum (heat inactivated) (Mascia Brunelli, Milan, Italy), 2 mM glutamine (Sigma), and 200 µg/ml gentamycin (Bio Whittaker, Walkersville, MD, USA). PBMC were seeded at a concentration of 2×10^6/ml, 100 µl/well, in 96-well flat bottom plates and incubated with or without lipopolysaccharide (LPS, 10 µg/ml; Sigma) or anti-CD3 monoclonal antibody (obtained from the supernatants of hybridoma-secreting OKT3 Mab; ATCC, Rockville, MD, USA) at the peak stimulating concentration as assessed by preliminary experiments of blastogenesis (dose-response curve not shown). After 24 h incubation at 37°C in humidified atmosphere 5% CO_2, supernatants were collected, aliquoted, and stored at –80°C until analysis.

VEGF concentrations in sera and PBMC culture supernatants were evaluated by the immunoassay method utilizing a commercial ELISA kit following the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Statistical analysis. Statistical analysis was done using SPSS statistical package (SPSS Inc., Chicago, IL, USA). The frequencies of the alleles and genotypes in the patient and control groups were determined and were compared by the chi-square test. Mann-Whitney U test was used to compare variables between groups. Odds ratios were calculated, together with their 95% confidence intervals (95% CI). Corrected p values were calculated by multiplying p by the number of alleles or genotypes compared.

We performed power calculations for an unmatched case control study and estimated relative risk using Power and Sample Size calculation version 2.1.31 software. Linkage disequilibrium analysis was calculated using chi-square test for a 3 × 3 table.

RESULTS

Table 1 shows the demographic and clinical characteristics of 122 Italian patients with BD.

Allele and genotype frequencies. Table 2 shows the allele and genotype frequencies of the 3 VEGF polymorphisms studied in patients with BD and healthy controls. As shown in Table 2, carriers of the I allele (II or ID) were significantly more frequent among patients with BD than healthy controls (odds ratio, OR, 1.8, 95% CI 1.1 to 2.9). Carriage rate of the allele –634C (percentage of subjects who were either heterozygous [–634C/–634G] or homozygous [–634C/–634C]) was higher in patients with BD compared to controls (OR 1.8, 95% CI 1.1–3.0). The distribution of allele +936T was not significantly different comparing patients with BD with healthy controls.

We investigated VEGF +936 C/T, –634 C/G and 18 bp I/D polymorphism associations with BD stratifying on HLA-B51. HLA-B51 allele was available in 98 Italian patients with BD. HLA-B51 was significantly higher in BD compared to healthy controls (58.2% vs 19.2%, p = 0.00001, OR 5.8, 95% CI 3.2–10.6). The significant association with I allele was preserved in HLA–B51+ patients (77.2% vs 58.0%, p = 0.008, p corr = 0.016, OR 2.5, 95% CI 1.2–4.8), but not in HLA-B51–. The association with the allele –634C was preserved in HLA-B51– patients (34.1% vs 18.6%, p = 0.025, p corr = 0.05, OR 2.3, 95% CI 1.1–4.7), but not in HLA-B51+.

The associations between 3 VEGF polymorphisms and BD clinical manifestations defined in Table 1 were evaluated in 122 Italian patients with BD, comparing patients

Table 1. Demographic and clinical features of 122 Italian patients with Behçet's disease.

	Number	Percentage
Female/male	55/67	45.1/54.9
Mean age at disease onset ± SD, yrs	29.7 ± 12	
Mean disease duration, yrs	11 ± 8	
Oral ulcers	122	100
Cutaneous lesions	102	83.6
Papulopustular lesions	69	55.6
Erythema nodosum	51	41.8
Genital ulcers	75	61.5
Epididymitis	6	4.9
Eye lesions	77	63.1
Anterior uveitis	31	25.4
Posterior uveitis and retinal vasculitis	62	50.8
Arthritis	52	42.6
Central nervous system involvement	24	19.7
Venous thrombosis*	39	32.0
Deep venous thrombosis	28	23.0
Positive pathergy test**	27	49
HLA-B51***	57	58.2

* Subcutaneous thrombophlebitis + deep vein thrombosis. ** Pathergy test was performed on 55 patients. *** HLA-B51 was performed on 98 patients.

with and without manifestations. The frequency of +936T allele was significantly higher in patients with BD with posterior uveitis/retinal vasculitis than in those without (21.0% vs 10.0%, p = 0.022, OR 2.4, 95% CI 1.1–5.0).

The frequencies of the I, –634C, and +936T alleles were not significantly different in patients with BD with deep vein thrombosis from those without (78.6% vs 69.1%, OR 1.6, 95% CI 0.6–4.5; 71.4% vs 77.7%, OR 0.7, 95% CI 0.3–1.9; 25.5% vs 29.8%, OR 0.8, 95% CI 0.3–2.1).

Given the sample sizes (122 cases and 200 controls) and the allele frequencies of the polymorphisms examined, we can exclude with 80% certainty a genetic relative risk of 1.4 at I/D polymorphism and 2.2 at +936 C/T polymorphism. Chi-square analysis indicated the studied polymorphisms were not in linkage disequilibrium.

VEGF production by PBMC cultures. The experiments were performed utilizing PBMC obtained from 13 control subjects –634G/–634G homozygous, 14 –634C/–634C homozygous, 19 DD homozygous, and 18 II homozygous. VEGF supernatant levels released in the presence of LPS stimulation were significantly higher in II homozygous than those in DD homozygous (Table 3). No significant differences in VEGF production in unstimulated and stimulated conditions were observed between –634G/–634G and –634C/–634C homozygous controls.

DISCUSSION

BD is a polygenic disease whose multiple genetic factors, in combination with environmental risk factors that have not yet been completely elucidated, are probably of importance in determining disease susceptibility and clinical expression[1,2]. To date, the strongest genetic association identified in

Table 2. Allele and genotype frequencies (%) of I/D, 936 C/T, 634 C/G VEGF in patients and control subjects.

Variable	Healthy Control, n = 200	Behçet's Disease, n = 122	p (corrected p)	OR (95% CI)
Alleles				
I	164/400 (41.0)	114/244 (46.7)	NS	1.3 (0.9–1.7)
D	236/400 (59.0)	130/244 (53.3)		
+936T	40/398 (10.1)	38/244 (15.6)	0.046 (0.092)	1.7 (1.0–2.7)
+936C	358/398 (89.9)	206/244 (84.4)		
–634C	156/400 (39.0)	122/244 (50.0)	0.007 (0.014)	1.6 (1.1–2.2)
–634G	244/400 (61.0)	122/244 (50.0)		
Genotypes				
II	48/200 (24.0)	27/122 (22.1)		
ID	68/200 (34.0)	60/122 (49.2)	0.016 (0.048)	
DD	84/200 (42.0)	35/122 (28.7)		
+936T/+936T	3/199 (1.5)	3/122 (2.5)		
+936T/+936C	34/199 (17.1)	32/122 (26.2)	NS	
+936C/–936C	162/169 (81.4)	87/122 (71.3)		
–634C/–634C	28/200 (14.0)	29/122 (23.8)		
–634C/–634G	100/200 (50.0)	64/122 (52.2)	0.020 (0.060)	
–634G/–634G	72/200 (36.0)	29/122 (23.8)		
Carriage rate				
II+ID	116/200 (58.0)	87/122 (71.3)	0.018 (0.036)	1.8 (1.1–2.9)
DD	84/200 (42.0)	35/122 (28.7)		
+936T/+936 + 536T+936T/+936C	37/199 (18.6)	35/122 (28.7)	0.039 (0.078)	1.8 (1.0–3.0)
+936C/+936C	162/199 (81.4)	87/122 (71.3)		
–634C/–634C + –634C/–634G	128/200 (64.0)	93/122 (76.2)	0.025 (0.050)	1.8 (1.1–3.0)
–634G/–634G	72/200 (36.0)	29/122 (23.8)		

Table 3. VEGF production by peripheral blood mononuclear cells of healthy controls with G634/G634 and C634/C634 genotypes and with DD and II genotypes. VEGF values are expressed as mean ± standard deviation.

	G634/G634 Genotype, N = 13	C634/C634 Genotype, N = 14	p
VEGF released under unstimulated conditions, pg/ml	25.8 ± 36.8	0.9 ± 5.1	NS
VEGF released in presence of anti-CD3 stimulation, pg/ml	12.2 ± 22.0	3.8 ± 2.7	NS
VEGF released in presence of LPS stimulation, pg/ml	37.4 ± 91.7	16.6 ± 23.5	NS
	D/D Genotype, N = 19	I/I Genotype, N = 18	p
VEGF released under unstimulated conditions, pg/ml	14.8 ± 25.8	15.0 ± 22.0	NS
VEGF released in presence of anti-CD3 stimulation, pg/ml	5.9 ± 6.4	10.7 ± 19.5	NS
VEGF released in presence of LPS stimulation, pg/ml	13.8 ± 24.5	51.0 ± 89.5	0.05

BD has been with HLA-B51 and MICA-A6 alleles. However, the association with MICA-A6 is probably secondary to the strong linkage disequilibrium with HLA-B51[23]. Further, recent studies have found that R/G 241 and E/K 469 polymorphisms of *ICAM-1* gene and Glu/Asp 298 polymorphism of *eNOS* gene are associated with BD susceptibility[24-26].

Vasculitis is the pathological lesion underlying most of the clinical findings of BD. Inflammation affecting both arteries and veins in BD induces a series of structural changes, including vascular aneurysms and occlusions and thrombus formation. In one immunopathological study, Kobayashi, *et al* showed that the predominant lesion in vasculo-Behçet's is a neutrophilic vasculitis involving vasa vasorum[27]. Further, these authors observed that the number of vasa vasorum was significantly higher in patients with BD than in those with inflammatory aneurysms or Takayasu's arteritis, and that neutrophils and endothelial cells of the vasa vasorum are activated in BD.

VEGF stimulating the formation of new vasa vasorum may play a pivotal role in vessel wall damage in BD. Further, angiogenesis may have a proinflammatory role[12,13]. VEGF is able to upregulate endothelial adhesion molecule and chemokine expression. The expression of endothelial adhesion molecules for leukocytes in inflamed vessels, mediating the recruitment of circulating leukocytes into vascular inflammatory lesions, is an important immunopathogenic mechanism in vasculitis[28,29].

VEGF production may be partially subject to genetic control. Some VEGF polymorphisms have been associated with different VEGF production[17,18,30]; they could be associated with differences in BD susceptibility and clinical expression. We found that the carriers of –634C and allele I are more susceptible to developing BD. VEGF angiogenic and proinflammatory activities may be the underlying rationale for the association between C(–634)G and 18 bp I/D polymorphisms and BD.

A functional role for C(–634)G polymorphism has been postulated by Watson, *et al*[18]. These authors recently reported that LPS-stimulated VEGF production from PBMC varied depending on genotype, with the highest production observed for GG homozygotes and the lowest for CC homozygotes.

Awata, *et al*[30] showed that C allele was significantly associated with the presence of diabetic retinopathy. In this diabetic complication, angiogenic and proinflammatory VEGF activities seem to play an important role[31]. These authors found that fasting serum VEGF levels were significantly higher in healthy subjects with CC genotype than in those with other genotypes[30]. The different origins of VEGF (leukocytes, aggregated platelets, and vascular endothelial cells as observed by Awata, *et al*[30], and activated leukocytes by Watson, *et al*[18]) may explain the contrasting results.

Although the production of VEGF from PBMC in our study was higher in healthy subjects with GG genotype than in those with CC, the differences were not significant. The differing results of Watson, *et al* compared with ours may be due to differences in the number of healthy controls studied[18]. Watson, *et al* studied 8 healthy controls with GG genotype and 3 with CC genotype, while we studied 13 and 14 controls with GG and CC genotypes, respectively. Therefore, VEGF production from PBMC could not be influenced by C(–634)G genotype.

An 18 nucleotide insertion at –2549 in the promoter region of the human *VEGF* gene has been described[19]. This polymorphism has been found to be strictly associated with A/C 2578. Individuals with allele A at position –2578 also had an insertion of 18 nucleotides, whereas CC homozygotes did not contain this insertion.

Shahbazi, *et al*[32] showed that –2578C allele was associated with higher VEGF production and with acute renal rejection. We found that carriers of the I allele (II or ID) were significantly more frequent among patients with BD than healthy controls. This polymorphism seems to have functional relevance. We showed that LPS-stimulated VEGF production from PBMC was higher in II homozygotes compared to DD homozygotes.

An association between HLA-B51 and BD was also

observed in our Italian population. We investigated VEGF polymorphism associations with BD stratifying on HLA-B51. The association of BD with I and C634 alleles seemed to be dependent on the status of HLA-B51 allele. The I allele was significantly associated with BD only in HLA-B51+ patients, and –634C only in HLA-B51– patients. This should be interpreted with caution, however, since the sample size of HLA-B51 +/– patients after stratification was small.

We found no difference in the frequencies of VEGF polymorphisms in patients with BD with and without clinical manifestations, except for +936T allele, which was significantly more frequent in patients with BD with posterior uveitis/retinal vasculitis than in those without. The functional relevance of this association is unclear because subjects carrying this mutation have significantly lower VEGF plasma levels than subjects carrying the +936 CC genotype[17].

Our data indicate that carriers of –634C and allele I are associated with susceptibility to developing BD. These 2 polymorphisms may be associated with higher VEGF production. However, due to the limited power of the study, multicenter collaborations to recruit an adequate number of patients are required in order to confirm our data.

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