Vascular Endothelial Growth Factor Gene Polymorphisms in Giant Cell Arteritis

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ABSTRACT. ***Objective.*** To examine potential associations of vascular endothelial growth factor (VEGF) gene polymorphisms with giant cell arteritis (GCA) and disease expression, in particular in patients with and without ischemic complications.

Methods. We enrolled 92 consecutive patients with biopsy-proven GCA residing in Reggio Emilia, Italy. Two hundred healthy blood donors from the same geographic area were selected as controls. All the GCA patients and controls were genotyped by polymerase chain reaction and allele-specific oligonucleotide techniques for 936 C/T and 634 C/G mutations and for an 18 bp insertion/deletion (I/D) polymorphism in the VEGF promoter region. *In vitro* release of VEGF by peripheral blood mononuclear cells (PBMC) was investigated by ELISA in controls homozygous for the polymorphisms studied.

Results. The carriage rates of the alleles I and C634 were significantly more frequent in GCA patients than in controls ($p = 0.025$, OR 1.9, 95% CI 1.1–3.1 and $p = 0.015$, OR 2.1, 95% CI 1.1–3.6, respectively). The distribution of allele T936 was similar in GCA patients and controls. No significant differences in the distribution of the polymorphisms studied were observed in patients with ischemic manifestations compared to those without ischemic manifestations. Lipopolysaccharide (LPS)-stimulated VEGF production by PBMC from controls was higher in II homozygous compared to DD homozygous patients.

Conclusion. Our data indicate that carriers of C634 and I alleles are associated with susceptibility to developing GCA. (J Rheumatol 2003;30:2160–4)

Key Indexing Terms:
GIANT CELL ARTERITIS VASCULAR ENDOTHELIAL GROWTH FACTOR
POLYMORPHISMS VEGF PRODUCTION ISCHEMIC MANIFESTATIONS

Giant cell arteritis (GCA) is an inflammatory vasculopathy that preferentially involves medium and large arteries. Ischemic manifestations such as visual loss, jaw claudication, central nervous system ischemia, and aortic arch syndrome are the predominant clinical findings in a subgroup of patients with GCA. These manifestations are related to luminal occlusion, mainly caused by the cooccurrence of intimal hyperplasia, neoangiogenesis, and the fragmentation of internal elastic laminae, which are closely correlated, probably reflecting shared regulatory pathways[1–4]. Vascular endothelial growth factor (VEGF) may play a pivotal role in mediating artery wall damage in GCA. Kaiser, *et al* have shown that tissue transcription of VEGF is correlated to the extent of neovascularization in the GCA arterial wall[5]. However, 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[6,7]. Cid, *et al* showed that inflammation-induced angiogenesis is the main site of leukocyte-endothelial cell interactions leading to the development of inflammatory infiltrates in GCA[8]. High concentrations of circulating VEGF have been documented in patients with active polymyalgia rheumatica (PMR)/GCA, and corticosteroid therapy reduces VEGF serum concentrations[9].

VEGF appears to represent an attractive candidate susceptibility gene for GCA 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[10]. A 936 C/T mutation occurring in 3'-UTR[11] and a 634 C/G mutation occurring in 5'-UTR of the

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VEGF gene[12] have been found to regulate the VEGF plasma levels. Further, an 18 bp insertion/deletion (I/D) polymorphism in the VEGF promoter region has been described[13].

We evaluated potential associations of these 3 polymorphisms with GCA susceptibility, and with disease expression in particular, comparing patients with and without ischemic manifestations. In addition, we studied unstimulated and stimulated PBMC VEGF production in controls to assess possible functional relevance of the VEGF polymorphisms.

MATERIALS AND METHODS

Patients. We reviewed the computerized pathology laboratory's register, which contains all temporal artery biopsies performed in Reggio Emilia between 1986 and 2000. The positive specimens were reviewed by a pathologist and 112 patients residing in the Reggio Emilia area were identified. Of these, 92 patients could be contacted and they were willing to participate in the study. The clinical findings at diagnosis and during the followup, the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) values at diagnosis, and the initial prednisone dosage were evaluated through interviews and by reviewing the medical records of the patients. The patients were divided in 2 subgroups according to the presence or absence of ischemic manifestations (visual loss and/or jaw claudication and/or aortic arch syndrome).

The control group was made up of 200 healthy unrelated blood donor volunteers residing in the same geographic area.

All patients and controls were Caucasians residing in the Reggio Emilia area for at least one generation.

The study was approved by the Ethics Committee of Reggio Emilia Hospital and informed consent was obtained from all patients or their relatives.

Molecular analysis of VEGF polymorphisms. DNA was extracted from whole peripheral blood of healthy volunteers (controls) and GCA patients by a standard method with phenol, chloroform, and isoamyl alcohol. VEGF genotyping was determined by polymerase chain reaction (PCR) followed by restriction analysis. The primers for VEGF +634 polymorphism are 5'ATTTATTTTTGCTTGCCATT3' and 5'GTCTGTCTGTCTGTCCGTCA3'[12]; for VEGF +936 polymorphism 5'AAGGAAGAGGAGACTCTGCG3' and 5'TATGTGGGTGGGTGTGTCTA3'[11]; and for 18 bp I/D polymorphism 5'CCTGGAGCGTTTTGGTTAAA3' and 5'ATATAGGAAGCAGCTTGGAA3'[13]. PCR was carried out in a PE 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) in 50 ml reaction volume containing 100 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 200 mM each of dATP, dCTP, dGTP, dTTP (Amersham Pharmacia Biotech, Uppsala, Sweden), 2.5 mM $MgCl_2$, 0.5 mM each primer, and 1 U Taq DNA Polymerase (Perkin Elmer Cetus). Following an initial denaturation step (2 min at 95°C), samples were subjected to 35 cycles of 95°C for 30 s, 60°C for 30 s (VEGF +634), 67°C for 30 s (VEGF +936), 55°C for 30 s (18 bp I/D), 72°C for 30 s, with a final extension time of 5 min at 72°C.

The PCR products for VEGF +634 polymorphism were digested with restriction endonuclease Bsm 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 showed 2 fragments: a 234 bp I allele with 18 bp insertion and a 216 bp D allele without insertion.

Measurement of VEGF peripheral production. PBMC from 13 GG homozygous, 14 CC homozygous, 19 DD homozygous, and 18 II homozygous controls (mean age 73 yrs, range 60–86) were isolated from 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% heat inactivated fetal calf serum (FCS; Mascia Brunelli, Milan, Italy), 2 mM glutamine (Sigma), and 200 µg/ml gentamycin (BioWhittaker, 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 cells secreting OKT3 Mab, from American Type Culture Collection, Rockville, MD, USA) at the peak stimulating concentration as assessed by preliminary blastogenesis experiments (dose-response curve not shown).

After 24 h incubation at 37°C in a humidified atmosphere with 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 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 (SPSS Inc., Chicago, IL, USA). The frequencies of the alleles and genotypes in the patient and control groups were determined and compared by the chi-square test. The Mann-Whitney U test was used to compare variables between groups. Odds ratios (OR) were calculated, together with their 95% confidence intervals (CI). Corrected p values were calculated by multiplying p by the number of alleles compared.

RESULTS

Table 1 shows the demographic and clinical characteristics of the patients. Fifty-three patients had ischemic manifestations and 39 had none. Patients with ischemic manifestations were older at the onset of disease and had a higher frequency of abnormalities of temporal arteries and scalp tenderness compared to those without.

Allele and genotype frequencies. Table 2 shows the allele and genotype frequencies of the 3 VEGF polymorphisms studied in total GCA patients, GCA patients with and without ischemic manifestations, and controls.

As shown in Table 2, 29.3% of the patients were homozygous for the inserted version (II) and 42.4% were heterozygous, whereas among controls the distribution of these genotypes was 24% and 34%, respectively. Thus, carriers of the I allele (II or ID) were significantly more frequent in GCA patients than in healthy controls (71.7% vs 58.0%; p = 0.025, p_{corr} = 0.05, OR 1.9, 95% CI 1.1–3.1).

The carriage rate of allele C634 [percentage of subjects who were either heterozygous (C634/G634) or homozygous C634/C634)] was higher in GCA patients compared to controls (78.3% vs 64.0%; p = 0.015, p_{corr} = 0.030, OR 2.1, 95% CI 1.1–3.6). The distribution of allele T936 was not significantly different comparing total GCA patients with controls.

No significant difference in the distribution of the phenotypes studied was observed in patients with ischemic manifestations compared with those without ischemic manifestations (Table 2).

Table 1. Demographic and clinical features of 92 patients with biopsy-proven GCA.

Characteristics (%)	Total GCA Patients, n = 92	GCA With Ischemic Manifestations (A), n = 53	GCA Without Ischemic Manifestations (B), n = 39	p A vs B
Men/Women, n	21/71 (22.8/77.2)	11/42 (20.7/79.3)	10/29 (25.6/74.4)	NS
Age, onset of disease, yrs	73.4 ± 7.2	74.8 ± 5.7	71.4 ± 8.5	0.03
Headache	72 (78.3)	43 (81.1)	29 (74.4)	NS
Abnormalities of temporal arteries*	58 (63.0)	38 (71.7)	20 (51.3)	0.05
Scalp tenderness	39 (42.4)	28 (52.8)	11 (28.2)	0.02
Jaw claudication	41 (44.6)	—	—	
Visual symptoms	19 (20.7)	—	—	
Visual loss	16 (17.3)	—	—	
Systemic signs/symptoms**	75 (81.5)	40 (75.5)	35 (89.7)	NS
Polymyalgia rheumatica	42 (45.7)	28 (52.8)	14 (35.9)	NS
ESR, mm/h	93.2 ± 30.3	92.8 ± 29.0	93.9 ± 32.5	NS
CRP, mg/dl	9.3 ± 6.2	9.3 ± 6.0	9.3 ± 6.4	NS

Ischemic manifestations: patients with visual loss and/or jaw claudication and/or aortic arch syndrome. ESR was available in 87 patients; CRP was available in 66 patients. * Artery tenderness and/or decreases/absent temporal artery pulsation. ** Fever, anorexia, and weight loss.

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

Variable	Controls (A), n = 200	GCA (B), n = 92	GCA With Ischemic Manifestations (C), n = 53	GCA Without Ischemic Manifestations (D), n = 39	p A vs B	p C vs D
Alleles						
I	164/400 (41.0)	93/184 (50.5)	54/106 (50.9)	39/78 (50.0)	0.032 (p_{corr} 0.064)	NS
D	236/400 (59.0)	91/184 (49.5)	52/106 (49.1)	39/78 (50.0)		
T936	40/398 (10.1)	23/182 (12.6)	15/104 (14.4)	8/78 (10.3)	NS	NS
C936	358/398 (89.9)	159/182 (87.4)	89/104 (85.6)	70/78 (89.7)		
C634	156/400 (39.0)	91/184 (49.5)	48/106 (45.3)	43/78 (55.1)	0.019 (p_{corr} 0.038)	NS
G634	244/400 (61.0)	93/184 (50.5)	58/106 (54.7)	35/78 (44.9)		
Genotypes						
II	48/200 (24.0)	27/92 (29.3)	17/53 (32.1)	10/39 (25.6)		
ID	68/200 (34.0)	39/92 (42.4)	20/53 (37.7)	19/39 (48.7)	0.08 (p_{corr} 0.24)	NS
DD	84/200 (42.0)	26/92 (28.3)	16/53 (30.2)	10/39 (25.6)		
T936/T936	3/199 (1.5)	1/91 (1.1)	1/52 (1.9)	0/39		
T936/C936	34/199 (17.1)	21/91 (23.1)	13/52 (25.0)	8/39 (20.5)	NS	NS
C936/C936	162/169 (81.4)	69/91 (75.8)	38/52 (73.1)	31/39 (79.5)		
C634/C634	28/200 (14.0)	19/92 (20.7)	10/53 (18.9)	9/39 (23.1)		
C634/G634	100/200 (50.0)	53/92 (57.6)	28/53 (52.8)	25/39 (64.1)	0.039 (p_{corr} 0.117)	NS
G634/G634	72/200 (36.0)	20/92 (21.7)	15/53 (28.3)	5/39 (12.8)		

Ischemic manifestations: patients with visual loss and/or jaw claudication and/or aortic arch syndrome.

VEGF production by PBMC cultures. The experiments were performed utilizing PBMC obtained from 13 G634/G634 homozygous, 14 C634/C634 homozygous, 19 DD homozygous, and 18 II homozygous controls. VEGF supernatant levels released in the presence of LPS stimulation were significantly higher by PBMC from II homozygous compared to DD homozygous controls (Table 3).

No significant difference in VEGF production in unstimulated and stimulated conditions was observed between G634/G634 and C634/C634 homozygous controls.

DISCUSSION

GCA is a polygenic disease whose multiple genetic factors, in combination with not yet completely elucidated environmental risk factors, are probably important determinants of the GCA disease process and clinical expression[1-4]. The most studied genetic association is with genes of the HLA complex. As in rheumatoid arthritis, HLA-DRB1*04 and DRB1*01 alleles have been found to be associated to PMR/GCA[14,15]. We have recently found that G/R 241 polymorphism of intercellular adhesion molecule 1 (ICAM-1) was associated to PMR/GCA susceptibility[16].

Inflammation of the arterial wall in GCA induces a series of structural changes, including the formation of new vasa vasorum. Ischemic manifestations in GCA are related to luminal occlusion, mainly caused by the cooccurrence of intimal hyperplasia, neoangiogenesis, and the fragmentation of internal elastic laminae[1-4]. VEGF may play a pivotal role in mediating artery wall damage in GCA. Kaiser, *et al* have shown that tissue transcription of VEGF is correlated to the

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

VEGF Production, pg/ml	G634/G634 Genotype, n = 13	C634/C634 Genotype, n = 14	p
Unstimulated	25.8 ± 36.8	0.9 ± 5.1	NS
After anti-CD3 stimulation	12.2 ± 22.0	3.8 ± 2.7	NS
After LPS stimulation	37.4 ± 91.7	16.6 ± 23.5	NS
	D/D Genotype, n = 19	I/I Genotype, n = 18	p
Unstimulated	14.8 ± 25.8	15.0 ± 22.0	NS
After anti-CD3 stimulation	5.9 ± 6.4	10.7 ± 19.5	NS
After LPS stimulation	13.8 ± 24.5	51.0 ± 89.5	0.05

extent of neovascularization in the GCA arterial wall[5]. Giant cells and CD68-positive macrophages at the media-intima junction were found to be the major cellular sources of VEGF[5]. Further, angiogenesis may have a proinflammatory role in GCA[6,7]. Adhesion molecules for leukocytes are strongly expressed in neovessels, which represent the main sites of recruitment of circulating leukocytes into vascular inflammatory lesions[8].

On the other hand, VEGF stimulating the formation of new vessels may play a compensatory role in preventing ischemic complications. Recently, Cid, *et al*, using *in vitro* and *in vivo* models, showed that a strong angiogenic response in temporal artery samples and strong angiogenic activity in serum was associated with a reduced prevalence of ischemic complications in GCA[17].

High concentrations of circulating VEGF have been documented in patients with active PMR/GCA[9]. Corticosteroid therapy reduces VEGF serum levels. Further, isolated PBMC from untreated PMR/GCA patients with active disease spontaneously secreted higher levels of VEGF than those from controls, and this was not modulated by the suppression of inflammation mediated by steroid therapy.

VEGF production may be partially subject to genetic control. Some of the VEGF polymorphisms have been associated with different VEGF production and they could be associated with differences in GCA susceptibility and clinical expression[11,12]. We found that the carriers of C634 allele are more susceptible to developing GCA. However, no differences in the frequency of these alleles were found in patients with compared to those without ischemic manifestations.

A functional role for C(-634)G polymorphism has been postulated by Watson, *et al*[12]. 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. Although the production of VEGF from PBMC in our study was higher in controls with GG compared to those with CC genotype, the differences were not significant. This inconsistency may be due to differences in the number of healthy controls studied. Watson, *et al*[12] studied 8 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. However, Awata, *et al*[18] found that fasting serum VEGF levels were significantly higher in controls with CC genotype than in those with other genotypes. The different origin of VEGF (leukocytes, aggregated platelets, and vascular endothelial cells in the Awata, *et al* study, activated leukocytes in Watson, *et al* and our studies)[12,18] may explain the contrasting results. Awata, *et al*[18] showed that the 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[19] as in GCA.

An 18-nucleotide insertion in the promoter region of the human VEGF gene has been described[13]. 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*[20] 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 in GCA patients than in 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.

VEGF angiogenic and proinflammatory activities may be the underlying rationale for the association between C(-634)G and 18 bp I/D polymorphisms and GCA. As shown by Cid, *et al*[8], inflammation-induced angiogenesis is the main site of leukocyte-endothelial cell interactions leading to the development of inflammatory infiltrates in GCA.

There is no clear evidence of the pathophysiologic VEGF effects on GCA clinical expression. VEGF in GCA may induce luminal occlusion, promoting GCA ischemic manifestations. On the other hand, VEGF stimulating the formation of new vessels may play a compensatory role in preventing ischemic manifestations. Therefore, increased VEGF production could promote or in contrast sometimes

protect against the development of ischemic manifestations in GCA. A strong angiogenic activity in serum was recently associated with a reduced prevalence of ischemic manifestations in GCA[17]. We found no difference in the frequencies of VEGF polymorphisms studied in GCA patients with and those without ischemic manifestations, although some of these polymorphisms are associated with higher VEGF production.

Our data indicate that carriers of C634 and I alleles are associated with susceptibility to developing GCA. These 2 polymorphisms may be associated with higher VEGF production. No significant difference in these alleles was found in GCA patients with compared to those without ischemic manifestations. VEGF plays an important role in the GCA inflammatory response, and genetically controlled variation in VEGF production may influence susceptibility to this condition.

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