Endothelial Nitric Oxide Synthase Haplotype Associations in Biopsy-Proven Giant Cell Arteritis

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ABSTRACT. ***Objective***. To assess the influence of endothelial nitric oxide synthase (eNOS) polymorphisms in the susceptibility to giant cell arteritis (GCA).

Methods. We studied 57 patients with biopsy-proven GCA diagnosed at the Rheumatology Division of Hospital Xeral-Calde and 117 ethnically matched controls. Patients and controls were genotyped by PCR for a variable number tandem repeat polymorphism in intron 4, a T/C polymorphism at position –786 in the promoter region, and a polymorphism in exon 7 (298Glu/Asp or 5557G/T) of the eNOS gene.

Results. No differences in allele or genotype frequencies for individual polymorphisms were observed between patients with GCA and controls. However, when haplotype frequencies for the combination of the 3 eNOS polymorphisms were estimated, a significant increase in the frequency of haplotype C/1/T and a significant decrease in the frequency of haplotype C/1/G were observed in GCA patients compared to controls (p = 0.04, OR 1.8, 95% CI 1.0–3.3; p = 0.02, OR 0.3, 95% CI 0.1–0.8, respectively).

Conclusion. Significant differences in eNOS haplotype frequencies between GCA patients and controls may indicate a role for these polymorphisms in the susceptibility to this condition. (J Rheumatol 2003;30:2019–22)

Key Indexing Terms:
GIANT CELL ARTERITIS
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Giant cell (temporal) arteritis (GCA) is a vasculitis that involves large and medium size vessels, with predisposition to the extracranial branches of the carotid artery in the elderly[1-3].

Well documented reports of families of first-degree relatives with these conditions support a genetic component in their pathogenesis[4]. Well defined associations between GCA and genes that lie within the HLA class II region have been described[5]. Different genes may influence the phenotype and the outcome of this condition[6].

Raza, *et al* described a severe impairment of endothelium-dependent brachial artery vasodilatation in patients with primary systemic necrotizing vasculitis[7]. This is related to abnormal endothelial-mediated production of nitric oxide (NO). NO is the product of conversion of L-arginine to L-citrulline by a class of enzymes denoted NO synthases (NOS). Three isoforms of NOS have been identified: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), which is expressed constitutively on the endothelial cells lining the vasculature[8,9], and the inducible NOS (iNOS or NOS2) that is expressed only in response to certain inflammatory stimuli such as bacterial products, cytokines, and lipid mediators[8].

Endothelial-derived NO participates in several functions such as relaxing vascular smooth muscle cells, inhibition of platelet and leukocyte adhesion to vascular endothelium, inhibition of the vascular smooth muscle cell migration and growth, and limiting the oxidation of atherogenic low density lipoproteins. These actions suggest an atheroprotective role for endothelial NO in addition to its effect on vessel tone and blood pressure[8,9].

Several polymorphisms in the eNOS gene have been identified: a variable number tandem repeat (VNTR) polymorphism in intron 4, a T/C polymorphism at position –786 in the promoter region, and a polymorphism in exon 7 (298Glu/Asp or nt5557G/T) of the gene. These polymorphisms have been associated with many vascular diseases including hypertension, coronary artery disease or myocardial infarction, coronary spasm, cerebral vascular disease, various forms of renal disease, and deep vein thrombosis in different populations[10]. The polymorphism in exon 7 has also been associated with Behçet's disease in Italians[11].

Some investigators have suggested that GCA may share a common pathway with atherosclerosis[12-14]. Similarities in

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injury pathways between GCA and atherosclerosis have been recognized. Activated T cells and macrophages are increasingly appreciated as being active in the process of instability and rupture of atherosclerotic plaque[14]. GCA has been associated with an increased risk of atherosclerosis and aneurysm formation[15]. In Northern Sweden, increased mortality due to cardiovascular disease in patients with GCA has recently been described[16].

To investigate genetic implications in the susceptibility to GCA and the possible role of the endothelial dysfunction in this vasculitis, we examined eNOS genetic polymorphisms in a series of patients with biopsy-proven GCA.

MATERIALS AND METHODS

Study population. The study group included patients diagnosed with biopsy-proven GCA (n = 57) at the Division of Rheumatology of Hospital Xeral-Calde, Lugo, Spain, and ethnically matched controls (n = 117) from the same area. All individuals were of Caucasian origin. The hospital is the only referral center for a mixed urban and rural population of nearly a quarter of a million people in the region[17,18].

Patients were included in this study if they had a positive temporal artery biopsy showing infiltration of mononuclear cells into the arterial wall with or without giant cells[17-20]. Patients were considered to have an associated diagnosis of polymyalgia rheumatica (PMR) if they also had marked aching and stiffness bilaterally with no other apparent cause in at least 2 of 3 regions: neck, shoulder girdle, and pelvic girdle[18].

Genotyping. *eNOS VNTR genotyping*. For each test 20 ng genomic DNA were amplified in a 10 µl final polymerase chain reaction (PCR) volume containing 5 pmoles of each primer (forward 5'-GGG AAC CTC AGC CCA GTA GTG AA-3'; reverse 5'-TCT CTT AGT GCT GTG GTC AC-3'), 200 µmol dNTPs, 10 × NH_4 buffer, and 0.6 units of *Taq* polymerase (Bioline, London, UK). The DNA was denatured at 95ºC for 2 min, and temperature cycling was set at 95ºC for 45 s, 58ºC for 45 s, and 72ºC for 45 s for 40 cycles, followed by a final extension at 72ºC for 5 min. The PCR product was visualized on a 2% agarose gel stained with ethidium bromide.

eNOS (–786). The PCR was carried out in a volume of 25 µl containing 100 ng genomic DNA, 10KCl buffer (Bioline), 3.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline), 5 pmol of each primer (forward 5'-GTG TAC CCC ACC TGC ATT CT-3'; reverse 5'-CCC AGC AAG GAT GTA GTG AC-3'), and 1 unit Taq DNA polymerase (Bioline) and 4 mM Betaine (Sigma, Poole, UK). The DNA was denatured at 95°C for 5 min, and temperature cycling was set at 35 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by a final extension at 72°C for 2 min. The PCR yielded a product of 282 bp. Analysis of the PCR product was performed by enzyme digestion using 4 units of *Msp*I (New England Biolab, Hitchin, UK) restriction enzyme. This resulted in products of 194 and 88 bp for allele T and 149, 88, and 45 bp for allele C. The digestion was incubated overnight at 37°C and the products of the digest were visualized on a 4% agarose gel stained with ethidium bromide.

eNOS (exon 7). The PCR was carried out in a volume of 25 µl containing 100 ng genomic DNA, 10NH_4 buffer (Bioline), 2 mM $MgCl_2$, 0.2 mM dNTPs (Bioline), 5 pmol of each primer (forward 5'-AAG GCA GGA GAC AGT GGA TGGA-3'; reverse 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3'), and 1 unit Taq DNA polymerase (Bioline) and 4 mM Betaine (Sigma). The DNA was denatured at 95°C for 5 min, and 32 cycles of 95°C for 45 s, 62°C for 45 s, and 72°C for 45 s, followed by a final extension at 72°C for 2 min. The PCR yielded a product of 248 bp. Analysis of the PCR product was performed by enzyme digestion using 4 units of *Ban*II (New England Biolab) restriction enzyme. The 248 bp PCR product was cleaved into 163 bp and 85 bp fragments in the presence of a G at nucleotide 894, which corresponds to wild-type Glu298, and 248 bp for allele T. The digestion was incubated overnight at 37°C and the products of the digest were visualized on a 3% agarose gel stained with ethidium bromide.

Statistical analysis. Strength of association between GCA and alleles or genotypes of polymorphisms in the eNOS gene was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher exact analysis. The same methods were used to examine the strength of associations between GCA subgroups with or without PMR or visual manifestations and different alleles. Statistical significance was defined as $p \leq 0.05$. Calculations were performed with the Stata v6 statistical package.

Linkage disequilibrium and haplotype analysis. Estimated haplotype frequencies and testing for linkage disequilibrium between pairs of polymorphisms in the cases and controls were calculated using the EHPLUS program[21], which provides log-likelihood, chi-square, and number of degrees of freedom. To test for heterogeneity in haplotype frequencies between cases and controls, the likelihood ratio test is used. The program was used on the cases to obtain a set of haplotype frequency estimates, and the corresponding log-likelihood ($\ln L_{case}$). It was then repeated on the controls to obtain corresponding log-likelihood ($\ln L_{controls}$). Finally, it was performed on the entire sample to obtain ($\ln L_{combined}$). The test statistic $-2(n L_{case} + \ln L_{controls} - \ln L_{combined})$ is a chi-square with n – 1 degrees of freedom (when n is the number of haplotypes).

RESULTS

Twenty-seven of the 57 patients with biopsy-proven GCA had features of PMR. Visual ischemic manifestations were observed in 14 patients.

eNOS gene polymorphisms in GCA. eNOS gene polymorphisms including a VNTR polymorphism in intron 4, a T/C polymorphism at position –786 in the promoter region, and a polymorphism in exon 7 (298Glu/ASP) were examined in patients with GCA and controls (Table 1). No significant differences were observed. The allele and genotype frequencies were also analyzed in patients with GCA who developed PMR compared to patients with isolated GCA. However, no significant associations were observed (Table 1). Similarly, no association was observed when GCA patients were stratified by the presence of visual manifestations (data not shown).

eNOS haplotype analysis. Pairwise eNOS haplotypes were examined in GCA patients and controls. Significant linkage disequilibrium (LD) was detected between eNOS promoter and exon 7 polymorphisms in GCA patients and controls (chi-square 15.2, p = 0.001; chi-square 7.5, p = 0.05, respectively; Table 2). Chi-square analysis also indicated significant LD in GCA patients between eNOS intron 4 and exon 7 polymorphisms (chi-square 7.5, p = 0.05). This was not significant in controls. No significant difference in LD was observed between GCA patients and controls using log-likelihood analysis. Although an increase in the frequency of the promoter and exon 7 C/T haplotype was observed in GCA patients compared to controls, this failed to reach significance.

Haplotype frequencies for the combination of the 3 eNOS polymorphisms were estimated (Table 3). Chi-square analysis indicated significant LD among the 3 eNOS polymorphisms in GCA patients and controls (chi-square 24.8,

Table 1. Allele and genotype frequencies of eNOS gene polymorphisms in patients and controls.

Gene	Controls,	CGA, total,	GCA+PMR+,	GCA Alone,
eNOS (intron 4)	N = 98 (%)	N = 55 (%)	N = 26 (%)	N = 29 (%)
Genotype				
11	71 (72)	43 (78)	21 (81)	22 (76)
12	25 (26)	12 (22)	5 (19)	7 (24)
22	2 (2)	0 (0)	0 (0)	0 (0)
Allele				
1	167 (85)	98 (89)	47 (90)	51 (88)
2	29 (15)	12 (11)	5 (10)	7 (12)
eNOS (exon 7)	N = 97	N = 57	N = 27	N = 30
Genotype				
GG	35 (36)	15 (26)	8 (30)	7 (23)
GT	45 (46)	31 (54)	14 (52)	17 (57)
TT	17 (18)	11 (19)	5 (18)	6 (20)
Allele				
G	115 (59)	61 (54)	30 (56)	31 (52)
T	79 (41)	53 (46)	24 (44)	29 (48)
eNOS (–786)	N = 117	N = 55	N = 27	N = 28
Genotype				
TT	37 (32)	17 (31)	10 (37)	7 (25)
TC	58 (50)	27 (49)	12 (44)	15 (54)
CC	22 (19)	11 (20)	5 (19)	6 (21)
Allele				
T	132 (56)	61 (55)	32 (59)	29 (52)
C	102 (44)	49 (45)	22 (41)	27 (48)

No statistically significant differences were observed.

Table 2. Pairwise linkage disequilibrium in patients with GCA and controls.

eNOS	Controls		GCA Patients	
	Chi-square	p	Chi-square	p
Promoter + intron 4	1.23	0.7	0.1	0.9
Promoter + exon 7	7.5	0.05	15.2	0.001
Intron 4 + exon 7	6.3	0.09	7.5	0.05

p = 0.0008; chi-square 14.1, p = 0.05, respectively). No significant difference in LD was observed between GCA patients and controls using log-likelihood analysis. A significant increase in the frequency of haplotype C/1/T and a significant decrease in the frequency of haplotype C/1/G were observed in GCA patients compared to controls (p = 0.04, OR 1.8, 95% CI 1.0–3.3; p = 0.02, OR 0.3, 95% CI 0.1–0.8, respectively).

DISCUSSION

Endothelial dysfunction is an early step in the development of atherosclerosis. An endothelial dysfunction related to abnormal NO release in adults with primary systemic necrotizing vasculitis has been described[7]. This finding suggests that endothelial dysfunction due to vasculitis may be implicated in the premature arteriosclerosis observed in chronic inflammatory rheumatic diseases[7].

This study constitutes the first attempt to assess the impli-

Table 3. Estimated haplotype frequencies in GCA patients compared with controls.

eNOS Promoter	eNOS Intron 4	eNOS Exon 7	Haplotype Frequency	
			Controls	GCA Patients
T	1	G	0.365516	0.364628
T	1	T	0.160293	0.151137
T	2	G	0.056158	0.050264
T	2	T	0.000000	0.000008
C	1	G	0.134506*	0.059919*
C	1	T	0.208537**	0.311108**
C	2	G	0.074967	0.062924
C	2	T	0.000022	0.000011

* p = 0.02, OR 0.3, 95% CI 0.1–0.8; ** p = 0.04, OR 1.8, 95% CI 1.0–3.3.

cation of 3 polymorphisms in eNOS in the susceptibility to primary vasculitides, specifically in GCA. These 3 polymorphisms have been examined in case control association studies reporting association between eNOS polymorphisms and vascular diseases. The –786 T variant allele has been correlated with reduced eNOS promoter activity in human umbilical vein endothelial cells using *in vitro* luciferase reporter assays[22]. Polymorphisms of the eNOS gene were implicated in coronary atherosclerosis and thrombosis and in ischemic conditions[20,22-25]. In several functional studies of polymorphism in exon 7 it has been suggested that the T allele is associated with less NO production compared to the G allele as a result of reduced enzyme activity[10]. In scleroderma an association between the eNOS polymorphism (Glu/Asp) has recently been reported[26]. In addition, in Italians the polymorphism of exon 7 (Glu/Asp 298) was associated with susceptibility to Behçet's disease[11].

Although our data do not support the implication of a single individual allele or genotype, the significant differences in the haplotype frequencies for the combination of the 3 eNOS polymorphisms between Spanish patients with GCA and controls may suggest a role for these polymorphisms in susceptibility to GCA. In this regard, analysis for the 3 markers in the eNOS gene has shown that significant LD exists between them. Haplotype analysis has shown that C1T haplotype was significantly increased in GCA patients compared to the controls. The C1G haplotype was also significantly decreased, but 2 locus haplotypes were not found to be associated with GCA. As all these 3 polymorphisms were reported to be functional, it is possible that functional interaction exists among the 3 that determines overall eNOS activity in GCA-susceptible individuals.

Further replication is necessary with larger samples and in different populations to fully investigate the role of these polymorphisms in vasculitis.

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