

Endothelial Nitric Oxide Synthase Gene Polymorphisms in Giant Cell Arteritis

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Objective. To examine potential associations of the Glu/Asp²⁹⁸ polymorphism in exon 7 and the 4a/b polymorphism in intron 4 of the endothelial nitric oxide synthase (eNOS) gene with susceptibility to and clinical expression of giant cell arteritis (GCA), particularly in patients with versus those without ischemic complications.

Methods. Ninety-one consecutive patients with biopsy-proven GCA, who were residents of Reggio Emilia, Italy, and 133 population-based controls from the same geographic area were genotyped by polymerase chain reaction and allele-specific oligonucleotide techniques for eNOS polymorphisms in exon 7 and intron 4. The patients were separated into 2 subgroups according to the presence or absence of ischemic complications (visual loss and/or jaw claudication and/or aortic arch syndrome).

Results. The distribution of the Glu/Asp²⁹⁸ genotype differed significantly between GCA patients and controls (corrected P [P_{corr}] = 0.003). Carriers of the Asp²⁹⁸ allele (Asp/Asp or Glu/Asp) were significantly more frequent among the GCA patients than among the controls (P_{corr} = 0.0002, odds ratio 3.3, 95% confidence interval 1.7–6.3). The distribution of the 4a/b genotype was similar in GCA patients and controls. No significant associations were found when GCA patients with and without ischemic complications were compared.

Conclusion. Our findings show that the Glu/Asp²⁹⁸ polymorphism of the eNOS gene is associated with GCA susceptibility.

Giant cell arteritis (GCA) is an inflammatory vasculopathy that preferentially involves medium-sized and large arteries. Ischemic complications such as visual loss, jaw claudication, central nervous system ischemia, and aortic arch syndrome are the predominant clinical manifestations in a subgroup of patients with GCA (1).

Constitutive endothelial nitric oxide synthase (eNOS) is expressed in the endothelium, where it produces nitric oxide (NO) from L-arginine (2,3). Several studies have suggested that the basal release of NO by endothelium mediates local vasodilation (4), antagonizes platelet aggregation (5), and inhibits vascular smooth muscle cell proliferation (6) and platelet and leukocyte adhesion (7–9). A genetically determined down-regulation of this important process could provide a mechanism for promoting vessel wall damage, and this may be implicated in the pathogenesis and clinical expression of GCA.

The Glu/Asp²⁹⁸ polymorphism in exon 7 and the 4a/b polymorphism in intron 4 are the most studied polymorphisms of the eNOS gene under conditions in which endothelial dysfunction plays a key pathogenic role, such as in coronary atherosclerosis, thrombosis, and other ischemic/thrombotic conditions (10–16). Interestingly, a functional role has been postulated for both polymorphisms (17–20).

The aim of this study was to assess the role of these 2 eNOS gene polymorphisms in the susceptibility to and clinical expression of GCA.

PATIENTS AND METHODS

Study population. We reviewed the computerized register of the Reggio Emilia Hospital pathology laboratory, which contains information on all temporal artery biopsies performed in Reggio Emilia between 1986 and 2000. The positive specimens were reviewed by a pathologist, and 112 patients with GCA residing in the Reggio Emilia area were identified. Of these, 91 patients could be contacted, and these

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Table 1. Demographic and clinical features of the 91 patients with biopsy-proven giant cell arteritis (GCA)*

Characteristic	All GCA patients (n = 91)	GCA patients with ischemic complications (n = 52)	GCA patients without ischemic complications (n = 39)	P
Male/female	20/71 (22.0/78.0)	10/42 (19.2/80.8)	10/29 (25.6/74.4)	NS
Age at onset of disease, mean \pm SD years	73.3 \pm 7.2	74.7 \pm 5.7	71.4 \pm 8.5	0.03
Headache	72 (79.1)	43 (82.7)	29 (74.4)	NS
Abnormalities of temporal arteries†	57 (62.6)	37 (71.2)	20 (51.3)	NS
Scalp tenderness	39 (42.9)	28 (53.8)	11 (28.2)	0.02
Jaw claudication	41 (45.1)	–	–	–
Visual symptoms	19 (20.9)	–	–	–
Visual loss	15 (16.5)	–	–	–
Systemic signs/symptoms‡	74 (81.3)	39 (75.0)	35 (89.7)	NS
Polymyalgia rheumatica	42 (46.2)	28 (53.8)	14 (35.9)	NS
ESR, mean \pm SD mm/hour§	93.0 \pm 30.4	92.4 \pm 29.2	93.9 \pm 32.5	NS
CRP, mean \pm SD mg/dl¶	9.3 \pm 6.2	9.3 \pm 6.0	9.3 \pm 6.4	NS

* Except where indicated otherwise, values are the no. (%) of patients. *P* values are for the comparison between GCA patients with ischemic complications and those without ischemic complications. NS = not significant.

† Artery tenderness and/or decreases or absent temporal artery pulsation.

‡ Fever, anorexia, and weight loss.

§ The erythrocyte sedimentation rate (ESR) was available for 86 patients.

¶ The C-reactive protein (CRP) level was available for 66 patients.

individuals were willing to participate in the present study. The clinical findings at diagnosis and during the followup, the erythrocyte sedimentation rate (ESR) at diagnosis, the 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. Table 1 shows the clinical and demographic characteristics of the 91 patients enrolled in the study. The patients were separated into 2 subgroups according to the presence or absence of ischemic complications (visual loss and/or jaw claudication and/or aortic arch syndrome).

Control subjects were randomly recruited from the lists of patients who were under the care of the same public health service family physicians as the cases. Stratification of the group by age and sex was used to approximately match the controls with the cases according to their age and sex distribution. Control subjects with a medical history of vasculitis were excluded. At the end of this selection process, 133 control subjects were identified. The median age of the controls was 69 years (range 50–79 years), of whom 27.8% were male and 72.2% were female.

All of the study subjects were white, of Italian descent, and had resided in Italy for at least one generation. No ethnic differences were present between the patients and the controls. None of the study participants had a Jewish background.

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

DNA extraction and genotyping. Genomic DNA was extracted from samples of whole blood by a standard method, using phenol–chloroform–isoamyl alcohol (25:24:1) (21). Genotyping of Glu/Asp²⁹⁸ was carried out using a polymerase chain reaction (PCR) and restriction fragment-length polymorphism technique (12). PCR was performed at a total volume of 50 μ l: 250 ng of genomic DNA, 1.5 units of AmpliTaq DNA polymerase (Perkin Elmer, Emeryville, CA), 20 pmoles of each primer, 200 nmoles of each dNTP on a

Perkin Elmer 9600 Thermal Cycler under the following conditions: an initial denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds. The final extension step was prolonged to 5 minutes.

Genotyping of the polymorphism was investigated by amplification in exon 7 with 2 primers: 5'-AAGGCAGGAGACAGTGGATGGA-3' (sense) and 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' (antisense) followed by *Eco* 24I (*Ban* II) (MBI Fermentas, Vilnius, Lithuania) restriction endonuclease digestion for 2 hours at 37°C and resolution by 2% agarose gel electrophoresis. 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.

Genotyping of the variable number tandem repeat (VNTR) was executed by PCR in a total volume of 50 μ l: 250 ng of genomic DNA, 1.5 units of AmpliTaq DNA polymerase, 20 pmoles of each primer, 200 nmoles of each dNTP on a Perkin Elmer 9600 Thermal Cycler under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 1 minute, 54°C for 40 seconds, and 72°C for 1 minute. The final extension step was prolonged to 5 minutes. Genotyping of the VNTR was performed by amplification in intron 4 with 2 primers: 5'-GGGAACCTCAGC-CCAGTAGTGAA-3' (sense) (22) and 5'-TCTCTTAG-TGCTGTGGTCAC-3' (antisense) (10) and visualized by 2.5% agarose gel electrophoresis. The 454-bp product (allele b) was associated at the 5 tandem repeat, and 427 bp (allele a) was associated at the 4 tandem repeat.

Statistical analysis. Statistical analysis was done using the SPSS statistical package (SPSS, Chicago, IL). The frequencies of the alleles and genotypes among the patients and the control group were determined and were compared by chi-square test. Odds ratios (OR) were calculated together with their 95% confidence interval (95% CI). Corrected *P* values

Table 2. Frequencies of the alleles, genotypes, and carriage rates of Glu/Asp²⁹⁸ and the variable number tandem repeat a/b in patients with GCA and controls*

Variable	Control subjects (n = 133)	GCA patients (n = 91)	<i>P</i> †	GCA patients with ischemic complications (n = 52)	GCA patients without ischemic complications (n = 39)	<i>P</i> ‡
Alleles						
Asp	101/266 (38.0)	89/182 (48.9)	0.02	50/104 (48.1)	39/78 (50.0)	NS
Glu	165/266 (62.0)	93/182 (51.1)		54/104 (51.9)	38/78 (50.0)	
4b	221/266 (83.1)	142/182 (78.0)	NS	80/104 (76.9)	62/78 (79.5)	NS
4a	45/266 (16.9)	40/182 (22.0)		24/104 (23.1)	16/78 (20.5)	
Genotypes						
Asp/Asp	19/133 (14.3)	13/91 (14.3)	0.001	8/52 (15.4)	5/39 (12.8)	NS
Asp/Glu	63/133 (47.4)	63/91 (69.2)		34/52 (65.4)	29/39 (74.4)	
Glu/Glu	51/133 (38.3)	15/91 (16.5)	NS	10/52 (19.2)	5/39 (12.8)	NS
4b/b	92/133 (69.2)	57/91 (62.6)		33/52 (63.5)	24/39 (61.5)	
4b/a	37/133 (27.8)	28/91 (30.8)	NS	14/52 (26.9)	14/39 (35.9)	NS
4a/a	4/133 (3.0)	6/91 (6.6)		5/52 (9.6)	1/39 (2.6)	
Carriage rate						
Asp/Asp + Glu/Asp	81/133 (60.9)	76/91 (83.5)	0.0001	42/52 (80.8)	34/39 (87.2)	NS
Glu/Glu	52/133 (39.1)	15/91 (16.5)		10/52 (19.2)	5/39 (12.8)	
4b/b + 4b/a	129/133 (97.0)	85/91 (93.4)	NS	47/52 (90.4)	38/39 (97.4)	NS
4a/a	4/133 (3.0)	6/91 (6.6)		5/52 (9.6)	1/39 (2.6)	

* Ischemic complications comprised visual loss and/or jaw claudication and/or aortic arch syndrome. See Table 1 for definitions.

† Comparison between GCA patients and control subjects. The corrected *P* values for each significant difference are 0.04, 0.003, and 0.0002, respectively.

‡ Comparison between GCA patients with ischemic complications and those without ischemic complications.

(*P*_{corr}) were calculated by multiplying *P* by the number of alleles compared.

RESULTS

Table 1 shows the clinical and demographic characteristics of the 91 patients with biopsy-proven GCA. The age at onset of disease was significantly higher in the patients with ischemic complications compared with those without ischemic complications. Scalp tenderness was significantly more frequent in patients with ischemic complications.

The allele and genotype frequencies of the eNOS 4a/b and Glu/Asp²⁹⁸ polymorphisms in GCA patients and in the control group are shown in Table 2. The distribution of the Glu/Asp²⁹⁸ genotype differed significantly between the GCA patients and the controls (*P* = 0.001, *P*_{corr} = 0.003). This distribution of the Glu/Asp²⁹⁸ genotype indicated that the difference in allele distribution was related to a reduced frequency of Glu/Glu homozygosity in the GCA patients compared with that in the controls, whereas Glu/Asp heterozygosity was higher in the GCA patients.

Allele Asp²⁹⁸ was significantly more frequent in the GCA patients than in the controls (*P* = 0.02, *P*_{corr} = 0.04, OR 1.6, 95% CI 1.1–2.3). Carriers of the Asp²⁹⁸ allele (Asp/Asp or Glu/Asp) were significantly more frequent among the GCA patients than among the

controls (*P* = 0.0001, *P*_{corr} = 0.0002, OR 3.3, 95% CI 1.7–6.3).

The distribution of allele and genotype frequencies of the 4a/b polymorphism did not differ significantly between the GCA patients and the controls.

The associations between the eNOS 4a/b and Glu/Asp²⁹⁸ polymorphisms and GCA clinical presentation were evaluated by comparing the 52 Italian GCA patients with ischemic complications with the 39 without ischemic complications. No significant associations were found with either of these 2 groups.

DISCUSSION

Although endothelial function has never been specifically investigated in patients with GCA, the elevated serum concentrations of indirect markers of vascular injury, such as von Willebrand factor (23), endothelin-1 (24), and intercellular adhesion molecule 1 (25), suggest the presence of endothelial dysfunction in patients with GCA.

Constitutive eNOS is expressed in the endothelium, where it produces NO from L-arginine (2,3). Reduced activity of NO, the major endothelium-derived vasodilator, may lead to vasoconstriction, platelet aggregation, and monocyte adhesion, which separately or together may promote vascular disease in patients with

GCA or other vasculitis. Intima hyperplasia and inflammatory changes of the arterial wall are the main causes of luminal obstruction and tissue ischemia in GCA (26). The process of neocapillarization in GCA leads to the appearance of microvessels in the media and the hyperplastic intima (27), and eNOS is highly expressed on the endothelial cells lining these neovessels (28). NO derived from eNOS is critical for the regulation of leukocyte-endothelial cell interaction in these areas. A reduced NO production is associated with increased leukocyte adhesion, which perpetuates the vasculitic reaction. Thus, a genetically determined down-regulation of the expression and activity of eNOS may be implicated in the pathophysiology of GCA.

Endothelial dysfunction, as evaluated by endothelium-dependent vasodilation studies, occurs early in the development of atherosclerosis, even before the formation of plaques, and it is considered to be the initiating event in atherosclerosis (29). An important part of this dysfunction in atherosclerosis is related to a decrease in NOS activity. The eNOS gene could be a candidate gene for the development of atherosclerosis, and it has been extensively studied in this condition. The Glu/Asp²⁹⁸ polymorphism in exon 7 and the 4a/b polymorphism in intron 4 of the eNOS gene are the most studied polymorphisms of this gene in coronary atherosclerosis, thrombosis, and other thrombotic/ischemic conditions (10–16). Asp²⁹⁸ and/or 4a polymorphisms have been found to be associated with angiographic evidence of coronary artery disease and myocardial infarction (10–15).

The 894 G/T substitution in exon 7 results in a glutamate or aspartate, respectively, at position 298 in the eNOS protein. Because glutamate and aspartate are conservative substitutions, it has been postulated that the polymorphism serves as a marker for a functional effect elsewhere in the eNOS gene or in its vicinity. Tesouro et al showed that the eNOS gene with a polymorphism at nucleotide 894 generates protein products with differing susceptibility to cleavage, suggesting that this polymorphism has a functional effect on the eNOS protein (17). These authors demonstrated that the Asp²⁹⁸ variant is more susceptible to proteolytic cleavage than is the eNOS Glu²⁹⁸, and this might contribute to abnormally low NO generation in carriers of the Asp variant. Recently, Veldman et al demonstrated that the presence of an Asp allele of the eNOS Glu/Asp²⁹⁸ polymorphism is associated with a reduced basal NO production (20). If the Asp²⁹⁸ polymorphism is associated with altered NO synthesis, this could provide a mechanism for promoting vessel wall damage not only

in atherosclerosis but also in vasculitis. A recent study from our group showed that the Asp²⁹⁸ polymorphism of the eNOS gene is associated with Behçet's disease (30), a vasculitis in which endothelial NO activity was found to be impaired and NO production to be decreased (31).

We have studied Glu/Asp²⁹⁸ and 4a/b polymorphisms in Italian patients with GCA and have found that whereas Asp²⁹⁸ was associated with susceptibility to GCA, no association with the 4a/b polymorphism was evident. However, the significance of this association is tempered by the lack of a consistent susceptibility among the Asp-homozygote individuals. The only association was with heterozygotes. Replication studies in other populations as well as studies that evaluate whether Glu/Asp²⁹⁸ polymorphisms affect eNOS expression in temporal artery biopsy specimens must be performed before definitive conclusions can be drawn.

In our study, we also evaluated whether these polymorphisms were associated with the presence of ischemic complications (visual loss and/or jaw claudication and/or aortic arch syndrome). When patients with and without these manifestations were compared, no associations were found.

In conclusion, the Asp²⁹⁸ polymorphism of the eNOS gene was found to be associated with GCA. Although this finding requires further confirmation in other populations, it is potentially important because it implies that a genetically determined endothelial dysfunction may predispose individuals to this vasculitis. Interestingly, pilot studies have found that low-dose aspirin and the anti-tumor necrosis factor α antibody infliximab may improve the efficacy of corticosteroid treatment of GCA (32,33). In addition, 2 recent studies have demonstrated improved endothelial function using these 2 drugs (34,35). As a result, drugs that improve endothelial function, in particular those which by the up-regulation of eNOS expression increase NO production, could have a potential therapeutic effect in GCA.

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