Lack of Association Between Endothelial Nitric Oxide Synthase Polymorphisms and Henoch-Schönlein Purpura

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ABSTRACT. ***Objective.*** To assess the influence of endothelial nitric oxide synthase (eNOS) polymorphisms in the susceptibility and clinical expression of patients with cutaneous vasculitis fulfilling classification criteria for Henoch-Schönlein purpura (HSP).

Methods. Fifty patients from Northwest Spain with primary cutaneous vasculitis classified as HSP were studied. Patients and ethnically matched controls (n = 117) were genotyped by polymerase chain reaction techniques 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 any of the individual eNOS polymorphisms were observed between patients fulfilling HSP classification criteria and controls, or when patients were stratified for the presence of nephritis or joint or gastrointestinal manifestations. In the HSP group no linkage disequilibrium between these polymorphisms was found. No significant difference in haplotype frequencies was observed between patients and controls.

Conclusion. Our results do not support a role for these polymorphisms in the susceptibility to HSP. (J Rheumatol 2004;31:299–301)

Key Indexing Terms:
HENOCH-SCHÖNLEIN PURPURA
DISEASE SUSCEPTIBILITY
eNOS POLYMORPHISM
HAPLOTYPE ASSOCIATIONS

Henoch-Schönlein purpura (HSP) is the most common small blood vessel leukocytoclastic angiitis in children, and it is rare in adults[1]. Palpable purpura and joint, gastrointestinal (GI), and renal involvement are the most common manifestations[1]. Susceptibility to HSP and associated clinical heterogeneity in HSP may be conferred by a number of genetic loci. We have described that different genes may influence the phenotype and the outcome of this condition[2–6]. Abnormal endothelial-mediated production of nitric oxide (NO) has been observed in patients with primary systemic necrotizing vasculitis[7,8]. NO is the product of conversion of L-arginine to L-citrulline by a class of enzymes denoted NO synthases (NOS). Among them, endothelial NOS (eNOS) is expressed constitutively on the endothelial cells lining the vasculature[9]. Endothelial-derived NO participates in 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[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 the development of cardiovascular and cerebrovascular diseases in different populations[10,11].

In studying these polymorphisms in patients with biopsy-proven giant cell arteritis (GCA) from Northwest Spain, we observed significant differences in eNOS haplotype frequencies between patients and ethnically matched controls[12]. This observation may support a role for these polymorphisms in the susceptibility to primary vasculitides. We assessed whether these polymorphisms in the eNOS gene may also be implicated in the susceptibility to and clinical expression of cutaneous vasculitis that fulfilled classification criteria for HSP.

MATERIALS AND METHODS

Study population. Patients classified as having HSP were recruited from the

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Divisions of Pediatrics and Rheumatology of the Hospital Xeral-Calde, Lugo, Spain. Clinical characteristics of these Caucasoid patients (n = 50; 39 younger than 21 yrs) have been reported[2]. All patients presented with palpable purpura involving the legs. GI bleeding and/or bowel angina was found in 40 patients. Thirty-seven patients had arthralgias and/or arthritis. All 32 patients with renal manifestations had hematuria (> 10 red blood cells/high power field). Antinuclear cytoplasmic antibody (ANCA) tests were negative in the 6 adults on whom they were tested. Ethnically matched controls (n = 117), all blood donors, were from the same region.

Inclusion criteria. As described[2], in adults a skin biopsy showing leukocytoclastic vasculitis was always required. In children, a diagnosis of cutaneous vasculitis was considered if they had typical nonthrombocytopenic symmetric palpable purpura involving the lower extremities and other conditions such as connective tissue disease and infections had been excluded[2]. Patients with primary cutaneous vasculitis were classified as having HSP according to the criteria proposed by Michel, *et al*[13].

Genotyping. As described[12], patients and controls were genotyped by polymerase chain reaction (PCR) techniques for a variable-number tandem-repeat polymorphism in intron 4, a T/C polymorphism at position –786 in the promoter region, and single nucleotide polymorphisms in exon 7 (298Glu/Asp or 5557G/T) of the eNOS gene.

eNOS VNTR genotyping. For each test 20 ng genomic DNA were amplified in a 10 µl final 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 dNTP, 10 × NH_4 buffer, and 0.6 units of Taq polymerase (Bioline, London, UK). The DNA was denatured at 95°C for 2 min, and temperature cycling was set at 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s for 40 cycles followed by a final extension at 72°C 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 of genomic DNA, 10 KCl buffer (Bioline), 3.5 mM $MgCl_2$, 0.2 mM dNTP (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 of 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 restriction enzyme (New England Biolabs, Hitchin, UK). 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 reaction products were visualized on a 4% agarose gel stained with ethidium bromide.

eNOS (exon 7). PCR was carried out in a volume of 25 µl containing 100 ng genomic DNA, 10 × NH_4 buffer (Bioline), 2 mM $MgCl_2$, 0.2 mM dNTP (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 of 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 restriction enzyme (New England Biolabs). The 248 bp 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 were visualized on 3% agarose gel stained with ethidium bromide.

Statistical analysis. Strength of association between HSP and eNOS alleles or genotypes was estimated using odds ratios and 95% confidence intervals. Levels of significance were determined using contingency tables by either chi-square or Fisher exact analysis. Statistical significance was defined as $p < 0.05$. Estimated haplotype frequencies and testing for linkage disequilibrium (LD) between pairs of polymorphisms in cases and controls were calculated using the EHPLUS program, which provides log likelihood, chi-square, and the number of degrees of freedom.

RESULTS

eNOS gene polymorphisms in HSP. No significant differences in the allele and genotype distributions were observed between patients and controls (Table 1). The allele and genotype frequencies were also analyzed in patients who developed nephritis compared to those without this complication. However, no significant association was observed (Table 1). No difference according to the age of onset of the disease (children versus adults) was found. Similarly, no association was observed when HSP patients were stratified by the presence of joint or GI manifestations (data not shown).

eNOS haplotype analysis in HSP. No significant LD between these polymorphisms was observed in the patient group. Also, no significant differences in pairwise LD were observed between patients and controls using log likelihood analysis. A mild increase in the frequency of promoter and exon 7 polymorphism T/G haplotype was observed in HSP patients compared to controls (47% vs 40%). No LD was observed in HSP patients when the estimated frequencies for the combination of these 3 polymorphisms were examined. Similarly, no significant differences in haplotype frequency were observed between patients and controls (data not shown).

DISCUSSION

This study was the first to assess the potential implication of 3 polymorphisms in eNOS in the susceptibility to patients with cutaneous vasculitis who fulfilled classification criteria for HSP. Besides the haplotype association with GCA[12], an association between eNOS polymorphisms and coronary atherosclerosis and ischemic cerebrovascular disease has been described[10,11]. The polymorphism of the exon 7 (Glu/Asp 298) has also been associated with susceptibility to Behçet's disease and scleroderma[14,15].

However, given the sample sizes (50 cases, 117 controls) and the allele frequencies, we can exclude a genetic effect of these 3 eNOS polymorphisms in the susceptibility for cutaneous vasculitis that fulfilled classification criteria for HSP in Northwest Spain. Differences of pathogenic mechanisms between small and large blood vessel vasculitis may explain the different results in terms of association of these 3 eNOS polymorphisms in HSP and GCA. In addition, the different ethnic origins of the world populations may explain differences in terms of genetic susceptibility to autoimmune diseases. This might imply different pathogenic mechanisms for the development of HSP in different populations. Additional studies in patients with different genetic backgrounds are required to determine the role of these polymorphisms in the pathogenesis of HSP.

Table 1. Allele and genotype frequencies (%) of eNOS gene polymorphisms in patients with HSP. No statistically significant differences were observed between HSP patients and controls and between HSP patients with and without nephritis.

Gene	Controls	HSP (total)	With Nephritis	Without Nephritis
eNOS (intron 4)	(n = 98)	(n = 49)	(n = 32)	(n = 17)
Genotype				
11	71 (72)	39 (80)	25 (78)	14 (82)
12	25 (26)	9 (18)	6 (19)	3 (18)
22	2 (2)	1 (2)	1 (3)	0 (0)
Allele				
1	167 (85)	87 (89)	56 (88)	31 (91)
2	29 (15)	11 (11)	8 (12)	3 (9)
eNOS (exon 7)	(n = 97)	(n = 45)	(n = 28)	(n = 17)
Genotype				
GG	35 (36)	22 (49)	12 (43)	10 (59)
GT	45 (46)	16 (36)	11 (39)	5 (29)
TT	17 (18)	7 (16)	5 (18)	2 (12)
Allele				
G	115 (59)	60 (67)	35 (63)	25 (74)
T	79 (41)	30 (33)	21 (37)	9 (26)
eNOS (–786)	(n = 117)	(n = 49)	(n = 31)	(n = 18)
Genotype				
TT	37 (32)	19 (39)	11 (35)	8 (44)
TC	58 (50)	20 (41)	13 (42)	7 (39)
CC	22 (19)	10 (20)	7 (23)	3 (17)
Allele				
T	132 (56)	58 (59)	35 (56)	23 (64)
C	102 (44)	40 (41)	27 (44)	13 (36)

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