EXTENDED REPORT

Endothelial nitric oxide synthase gene polymorphisms in Behçet's disease and rheumatic diseases with vasculitis

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Objective: To assess potential associations between Korean Behçet's disease (BD) or other rheumatic diseases with vasculitis and two polymorphisms of the endothelial nitric oxide synthase (eNOS) gene, which include the Glu298Asp polymorphism in exon 7 and a variable number of tandem repeats (VNTR) polymorphism in intron 4.

Methods: 65 patients with BD, 27 with rheumatic diseases with vasculitis, and 80 controls were studied. Analyses of the Glu298Asp polymorphism in exon 7 and VNTR polymorphism in intron 4 of the eNOS gene were made by the polymerase chain reaction (PCR)-restriction fragment length polymorphism technique and PCR genotyping, respectively. Additionally, HLA-B51 typing was performed in the BD group and controls by a two step PCR sequence-specific primers method.

Results: Significant differences in Glu298Asp genotype frequencies were found between the BD or vasculitis groups and the controls (BD group *v* controls: $p_{corr} = 0.006$; vasculitis group *v* controls: $p < 0.001$). The Asp298 frequency was much higher in the BD and vasculitis groups than in the controls. Even after stratification of the BD group based on the results of HLA-B51 testing, a significant association of the Glu298Asp polymorphism was still found ($p = 0.002$, Mantel-Haenszel weighted odds ratio 4.3, 95% confidence interval 1.7 to 10.9). Distribution of the genotype frequencies in two eNOS gene polymorphisms was similar in connective tissue diseases-associated vasculitis and primary vasculitic syndromes. In contrast, distribution of alleles and genotypes of VNTR polymorphism did not differ between BD or vasculitis groups and the controls.

Conclusion: The Glu298Asp polymorphism in exon 7 of the eNOS gene seems to be a susceptibility gene for Korean BD and other rheumatic diseases.

Nitric oxide (NO) is synthesised, via L-arginine oxidation, by a family of nitric oxide synthases (NOS),[1 2] in which three isoforms have been identified: two constitutive, the neuronal NOS (nNOS, NOS-I) and endothelial (eNOS, NOS-III), and one inducible NOS (iNOS, NOS-II). The eNOS in the endothelium generates small amounts of NO in response to receptor stimulation (for example, acetylcholine, bradykinin) or shear stress, which contributes to regulate vascular tone. Under physiological conditions, NO does not cause tissue damage, because it is rapidly cleared by reaction with oxyhaemoglobin. In contrast, the iNOS induced by endotoxin and inflammatory cytokines in the infectious or inflammatory disorders results in very high NO production, which can destroy host tissues and modify the course of diseases.[3–7]

The eNOS gene is located on chromosome 7q35–36 and comprises 26 exons spanning 21 kb.[8] When a variant of this gene causes deficient NOS, disease processes may ensue. Many studies have been carried out to determine the relevance between DNA variants in the eNOS gene and vascular diseases. To date, a variety of vascular diseases, which include coronary artery disease (CAD) or myocardial infarction (MI), hypertension, stroke, and renal diseases, have been associated with the eNOS gene polymorphisms.[9–14] However, the results show interracial inconsistencies, and the specific genetic variants may be relevant only to restricted populations.[9]

The histological hallmark in Behçet's disease (BD) is known to be a vasculitis. Any sized vessel lesions such as small vessel vasculitis, large venous or arterial lesions can be involved, and venous lesions, including superficial thrombophlebitis and deep vein thrombosis, are a characteristic manifestation of the disease.[15 16] Although the definite pathogenic mechanism for the vascular lesions in BD remains unclear, endothelial dysfunction is thought to have an important role in the development of these lesions.[17–19] Recently, it was shown that brachial artery flow mediated dilatation was impaired in BD.[18] Because flow mediated dilatation is endothelium dependent and mediated largely by the release of endothelial NO,[20] the impairment of endothelium dependent, flow mediated dilatation suggests a decreased endothelial NO activity, which may contribute to vascular lesions in BD. In addition, a recent study reported that the Glu298Asp polymorphism of the eNOS gene was associated with BD susceptibility in an Italian population.[21]

On the other hand, various chronic inflammatory rheumatic disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), have higher cardiovascular mortality than age matched populations.[22–25] It has been reported that the endothelium dependent, flow mediated dilatation was also impaired in patients with SLE as well as patients with primary necrotising vasculitis, such as Wegener's granulomatosis, polyarteritis nodosa, Churg-Strauss syndrome, and Kawasaki disease.[26–28] However, to the best of our knowledge, studies on the association between polymorphisms of the eNOS gene and chronic rheumatic diseases with vasculitis have not been described.

Abbreviations: BD, Behçet's disease; CAD, coronary artery disease; CI, confidence interval; CTD, connective tissue disease; eNOS, endothelial nitric oxide synthase; MI, myocardial infarction; NO, nitric oxide; OR, odds ratio; PCR, polymerase chain reaction; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; VNTR, variable number of tandem repeats

Therefore we conducted a study for the potential associations between Korean BD or other rheumatic diseases with vasculitis and two commonly studied polymorphisms of the eNOS gene, which include the Glu298Asp polymorphism in exon 7 and a variable number of tandem (27 base pair (bp)) repeats (VNTR) polymorphism in intron 4.

PATIENTS AND METHODS

Subjects

The study groups included 65 patients with BD (22 men, 43 women) who fulfilled the criteria of the International Study Group,[29] 27 patients with rheumatic diseases with vasculitis (vasculitis group; 9 men, 18 women) who were classified according to internationally accepted criteria for each disease, as well as 80 healthy controls (30 men, 50 women). The mean (SD) ages of the BD group, vasculitis group, and controls were 38.6 (8.5) years, 35.0 (11.9) years, and 40.6 (11.6) years, respectively. All the subjects were ethnically homogenous Koreans who were unrelated.

The age at onset was defined as the time for which the patient had fulfilled the ISG criteria, which in the BD group was 32.5 (8.7) years. Table 1 summarises the clinical features of the patients with BD. In addition, the presence of one or more of the following clinical features during the course of the disease was regarded as a severe manifestation, as described in our previous study: posterior uveitis or retinal vasculitis, gastrointestinal ulcerations with bleeding or perforation, major organ involvement, or major vessel involvement.[30] On the other hand, the vasculitis group consisted of 17 patients with connective tissue diseases (CTD)-associated vasculitis (15 with SLE and 2 with RA) and 10 patients with primary vasculitic syndromes (3 with Takayasu's arteritis, 3 with Henoch-Schönlein purpura, 2 with polyarteritis nodosa, 1 with Wegener's granulomatosis, and 1 with Churg-Strauss syndrome). In the CTD-associated vasculitis, biopsy proven cases or patients with clinical evidence of vasculitis, such as digit gangrene or vasculitis affecting visceral organs, were included. Patients with hypertension, CAD, or end stage renal disease were excluded from this study. Informed consent was obtained from all the subjects.

DNA extraction

Genomic DNA was isolated from peripheral blood leucocytes by the Chelex extraction method.[31]

Analysis of the Glu298Asp polymorphism in exon 7 of the eNOS gene

The presence of Glu298Asp variants was determined in all the subjects by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. A set of primers (5′-AAGGCAGGAGACAGTGGATGGA-3′ and 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′) was used to amplify the 248 bp fragment including the Glu298Asp mutation site.[10] The amplified PCR products were digested with the restriction enzyme, *Ban*II, as recommended by the supplier (Roche Diagnostics, Mannheim, Germany). DNA fragments were separated by electrophoresis on 2% agarose gel. The Asp298 variant had no cutting site for *Ban*II by G to T substitution at nucleotide position 894 of the eNOS gene, so that the 248 bp PCR product was not cleaved into 163 bp and 85 bp fragments, in contrast with the wild-type Glu298 (fig 1).

Table 1 The clinical features of 65 patients with Behçet's disease

Clinical features	Number of patients (%)
Oral ulcerations	65 (100)
Skin lesions	60 (92)
Genital ulcerations	55 (85)
Pathergy reactions	20 (31)
Ocular lesions	18 (28)
Arthritis	15 (23)
Intestinal lesions	12 (18)
Vascular lesions	12 (18)
Central nervous system lesions	5 (8)
HLA-B51	33 (51)
Epididymitis	1 (2)

Analysis of the VNTR polymorphism in intron 4 of the eNOS gene

Detection of VNTR polymorphism was performed in all the subjects by PCR genotyping. Two primers (5′-AGGCCCTATGGTAGTGCCTT-3′ and 5′-TCTCTTAGTGCTGT GGTCAC-3′), which were based on the sequences flanking VNTR in intron 4 of the eNOS gene, were used to amplify the corresponding DNA fragment.[12] The PCR products were separated by 2.5% agarose gel electrophoresis. The 420 bp wild-type product contained five 27 bp repeats (the ''b'' allele), and the 393 bp mutant type contained four 27 bp repeats (the ''a'' allele) (fig 2).

Analysis of HLA-B51 antigen

HLA-B51 typing was performed in the BD group and controls by a two step PCR sequence-specific primers method, as described in our previous study.[32]

Statistical analysis

The data were analysed using the SPSS statistical package program, version 10.0 for Windows (SPSS Inc, Chicago, IL, USA). The statistical significance was evaluated by χ^2 test or *t* test when indicated. Values of $p<0.05$ were considered significant, and these were corrected in certain cases by multiplying the values by the number of alleles investigated. The results were re-evaluated by multiple logistic regression analysis and the odds ratio (OR) was estimated where necessary. The association between the eNOS gene polymorphisms and BD, after stratification of the patients according to the results of HLA-B51 testing to exclude the

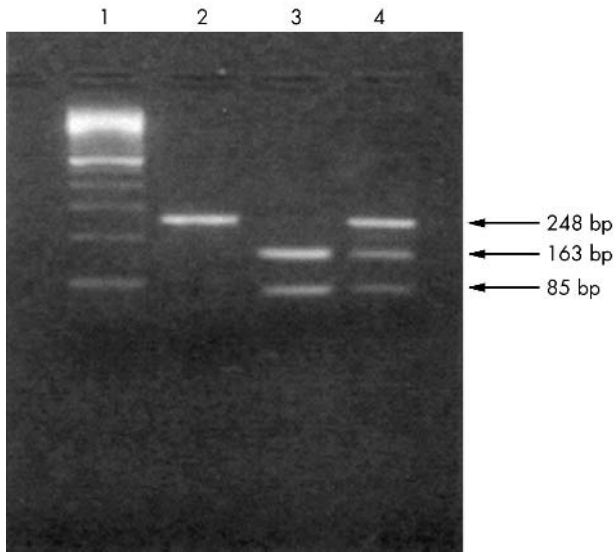

Figure 1 Analysis of the Glu298Asp polymorphism in exon 7 of the eNOS gene. Three kinds of restriction fragments are shown after digestion with *Ban*II: lane 1, size marker; lane 2, homozygote (Asp/Asp); lane 3, homozygote (Glu/Glu) lane 4, heterozygote (Glu/Asp).

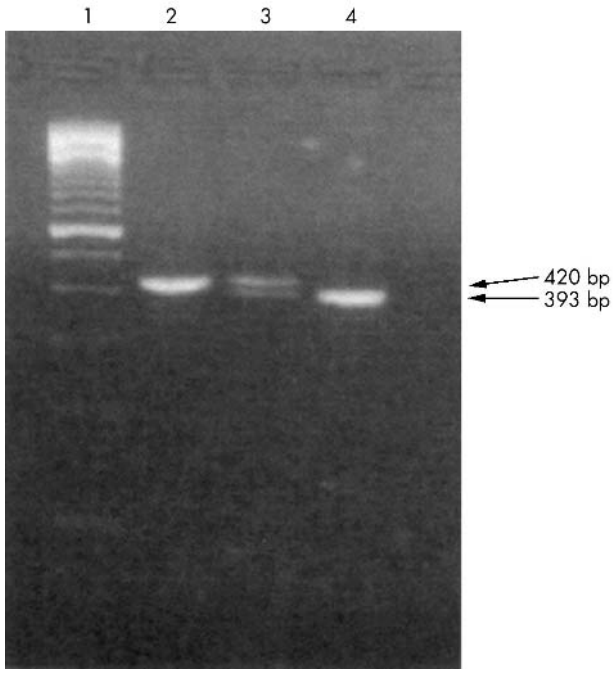

Figure 2 Analysis of the VNTR polymorphism in intron 4 of the eNOS gene. The band of 420 bp indicates five repeats and the band of 393 bp indicates four repeats of the 27 bp: lane 1, size marker; lane 2, wild type (4b/b); lane 3, four and five repeats heterozygote (4b/a); lane 4, four repeats homozygote (4a/a).

possible confounding effect of HLA-B51, was estimated by calculation of the Mantel-Haenszel weighted OR.

RESULTS

Table 2 shows the genotype frequencies of the eNOS gene polymorphisms in each study group. There was a significant difference in the frequencies of the Glu298Asp genotypes in exon 7 between the BD group or vasculitis group and the controls (BD group *v* controls: $p = 0.002$, $p_{corr} = 0.006$; vasculitis group *v* controls: $p<0.001$). The frequencies of the Glu298Asp heterozygote (Glu/Asp) were much higher in the BD and vasculitis groups than in the controls. Of interest, the Glu298Asp homozygote (Asp/Asp) was found only in three patients of the vasculitis group. In contrast, the genotype frequencies of the VNTR polymorphism in intron 4 did not significantly differ between the BD group or vasculitis group and the controls.

Table 2 The genotype frequencies of eNOS gene polymorphisms in the Behçet's disease (BD) group (n = 65), vasculitis group (n = 27), and in controls (n = 80)

Genotype	Controls No (%)	BD group No (%)	Vasculitis group No (%)
27 bp repeats polymorphism			
4a/a	1 (1)	0	0
4b/a	17 (21)	13 (20)	4 (15)
4b/b	62 (78)	52 (80)*	23 (85)†
Glu298Asp polymorphism			
Asp/Asp	0	0	3 (11)
Glu/Asp	9 (11)	21 (32)	8 (30)
Glu/Glu	71 (89)	44 (68)‡	16 (59)§

For the 27 bp repeats polymorphism: *p = 0.604 between BD group and controls; †p = 0.356 between the vasculitis group and controls.
For the Glu298Asp polymorphism: ‡p = 0.002 (p_{corr} = 0.006) between the BD group and controls; §p<0.001 between the vasculitis group and controls.

Table 3 The allele frequencies of eNOS gene polymorphisms in the Behçet's disease (BD) group (n = 130), vasculitis group (n = 54), and in controls (n = 160)

Allele	Controls No (%)	BD group No (%)	Vasculitis group No (%)
27 bp repeats polymorphism			
4a	19 (12)	13 (10)	4 (7)
4b	141 (88)	117 (90)*	50 (93)†
Glu298Asp polymorphism			
Asp	9 (6)	21 (16)	14 (26)
Glu	151 (94)	109 (84)‡	40 (74)§

For the 27 bp repeats polymorphism: *p = 0.612 between the BD group and controls; †p = 0.453 between the vasculitis group and controls.
For the Glu298Asp polymorphism: ‡p = 0.003 (p_{corr} = 0.006) between the BD group and controls; §p<0.001 between the vasculitis group and controls.

Table 3 lists the allele frequencies of the eNOS gene polymorphisms. The distribution of the Glu298Asp alleles differed significantly between the BD group or vasculitis group and the controls (BD group *v* controls: $p = 0.003$, $p_{corr} = 0.006$; vasculitis group *v* controls: $p<0.001$). In addition, the frequencies of Asp298 were much higher in the BD and vasculitis groups than in the controls. Furthermore, the OR values for development of BD or rheumatic diseases with vasculitis in Koreans with Asp298 were 3.2 (95% confidence interval (CI) 1.4 to 7.3) or 5.9 (95% CI 2.4 to 14.5), respectively. However, in the case of the alleles of the VNTR polymorphism, no significant differences were found between the BD group or vasculitis group and the controls.

The frequency of HLA-B51 antigen was significantly higher in the BD groups than in the controls (50.8% *v* 23.8%, $p = 0.001$, OR 3.3, 95% CI 1.6 to 6.7). Even after stratification of the BD patients according to the results of HLA-B51 testing, the Glu298Asp polymorphism still correlated significantly with BD ($p = 0.002$, χ^2 8.7, Mantel-Haenszel weighted OR 4.3, 95% CI 1.7 to 10.9). In addition, the distribution of genotype frequencies in the Glu298Asp and VNTR gene polymorphisms was similar between CTD-associated vasculitis and primary vasculitic syndromes (table 4).

In the associations between the two eNOS gene polymorphisms and clinical features presented in table 1, the frequencies of Glu298Asp and VNTR variant genotypes were

Table 4 The genotype frequencies of eNOS gene polymorphisms in primary vasculitic syndromes (n = 10) and vasculitis associated with connective tissue diseases (n = 17)

Genotype	Primary vasculitic syndromes No (%)	Connective tissue disease associated vasculitis No (%)
27 bp repeats polymorphism		
4a/a	0	0
4b/a	1 (10)	3 (18)
4b/b	9 (90)	14 (82)*
Glu298Asp polymorphism		
Asp/Asp	1 (10)	2 (12)
Glu/Asp	3 (30)	5 (29)
Glu/Glu	6 (60)	10 (59)†

For the 27 bp repeats polymorphism: *p = 1.0 between primary vasculitic syndromes and vasculitis associated with connective tissue disease.
For the Glu298Asp polymorphism: †p = 0.916 between primary vasculitic syndromes and vasculitis associated with connective tissue disease.

significantly higher only in patients with BD with vascular lesions ($p = 0.038$; $p = 0.033$, respectively), but the significance was lost after multiple logistic regression analysis using the Enter method with other clinical variables of BD (data not shown). On the other hand, the mean (SD) age at onset in patients with BD with Glu298Asp or VNTR variant genotypes was comparable with that in the patients without variant genotypes (Glu298Asp: 32.8 (8.2) years *v* 32.4 (9.0) years; VNTR: 32.0 (8.8) years *v* 32.7 (8.7) years). In addition, severe manifestations were found in 24 patients in the BD group. Although the frequencies of Glu298Asp or VNTR variant genotypes in the patients with severe manifestations were higher than those in the patients without these manifestations, they did not reach significance (Glu298Asp: 41.7% *v* 26.8%, $p>0.05$; VNTR: 29.2% *v* 14.6%, $p>0.05$).

DISCUSSION

A number of polymorphisms have been identified in the promoter region, exons, and introns of the eNOS gene, but the findings have shown interracial inconsistencies. Until now, the most studied eNOS gene polymorphisms have included the Glu298Asp polymorphism in exon 7 and VNTR polymorphism in intron 4, both of which may contribute to the development of various vascular diseases, including CAD or MI, coronary spasm, hypertension, stroke, and renal diseases.[9] In our study the Glu298Asp polymorphism appeared to be a susceptibility marker for Korean BD and other rheumatic diseases with vasculitis. Whereas our study showed that the OR (95% CI) values of Asp298 were 3.2 (1.4 to 7.3) for BD and 5.9 (2.4 to 14.5) for rheumatic diseases with vasculitis, a previous Italian study showed that this value for BD was 2.1 (1.5 to 3.3).[21] In our study groups the frequencies of homozygote variants of the eNOS gene polymorphisms—that is, Asp/Asp and 4a/a, were very low, which was also found in other studies,[10–13] with the exception of one.[21]

Previously, it was proposed that two polymorphisms of the eNOS gene, the Glu298Asp polymorphism in exon 7 and VNTR polymorphism in intron 4, might be associated with the altered function of this gene.[33–37] Such functional DNA variants in the eNOS gene may lead to a change in eNOS expression and enzymatic activity.[9] On the other hand, recent studies have shown that endothelium dependent, flow mediated vasodilatation, mainly mediated by the release of endothelial NO,[20] was impaired in BD, SLE, and primary necrotising vasculitic syndromes.[18 26–28] Thus, it seems reasonable to assume that this altered eNOS expression resulting from the gene polymorphisms reduces the release of endothelial NO, which in turn contributes to the endothelial dysfunction seen in BD and other rheumatic diseases with vasculitis. Furthermore, the impaired production of basal NO and associated endothelial dysfunction may predispose to thrombosis or atherosclerosis related disorders.[9 38] This proposed pathogenic mechanism appears to be supported by our study, which showed a significant correlation between the Glu298Asp polymorphism of the eNOS gene and BD or rheumatic diseases with vasculitis. In future studies it will be necessary to investigate the association between eNOS gene polymorphisms and endothelium dependent, flow mediated vasodilatation.

It is known that up to 25% of patients with BD may have systemic venous thrombosis.[39] Endothelial dysfunction is thought to play an important part in the development of thrombosis in these patients.[17–19] On the other hand, the incidence of MI has been estimated to be at least 50-fold higher in young patients with SLE than in age matched controls,[22] and occlusive coronary disease in these patients may result from atherosclerosis, thrombosis, or vasculitis.[25] Moreover, Shimasaki *et al* demonstrated that the Glu298Asp polymorphism of the eNOS gene was a significant risk factor for MI, independently of the traditional CAD risk factors, such as hypertension, smoking, hyperlipidaemia, and diabetes mellitus.[10] Although only patients with SLE who had vasculitis were included in the current study, we showed that the frequencies of Glu298Asp variant genotypes were increased to the same extent in patients with CTD-associated vasculitis and in patients with primary vasculitic syndromes. However, published reports of controlled studies for cardiovascular mortality or CAD in rheumatic diseases other than SLE are few. As far as we are aware, this is the first study to correlate the eNOS gene polymorphisms with rheumatic diseases with vasculitis.

Although the exact pathogenesis for BD is not completely understood, it has been suggested that the disease is triggered in genetically susceptible people by environmental factors, such as infectious agents. To date, HLA-B51 has been implicated as the candidate gene showing the strongest association with BD.[15 32 40] Recently, Salvarani *et al* showed that the Glu298Asp polymorphism of the eNOS gene was another susceptibility gene for BD that was independent of HLA-B51 in Italian populations. The association between the polymorphisms of this gene and clinical manifestations of BD was not found in their study.[21] Our findings are similar to those obtained in the Italian study, and the initial statistical significance between eNOS gene polymorphisms and patients with BD with vascular lesions was lost when multiple logistic regression analysis was performed. In addition, no significant associations were found between these polymorphisms and the age at onset or severe manifestations in the BD group.

Excessive NO production occurs during various rheumatic diseases, including SLE, RA, Sjögren's syndrome, vasculitis, and osteoarthritis.[3] On the other hand, there have been conflicting reports about serum NO concentrations in patients with BD. Some authors have reported significantly higher NO levels in patients with active disease than in patients with inactive disease or control subjects.[41–43] Other researchers have described the opposite and argued that decreased NO levels in the active stage might be attributable to the rapid transformation of NO to peroxynitrites.[44 45] In any case, we believe that the increased NO levels found in a variety of rheumatic diseases, such as BD and CTD, result from the induction of iNOS expression by inflammatory stimuli, irrespective of the physiological NO production by eNOS.

In summary, the Glu298Asp polymorphism appeared to be a susceptibility gene for Korean BD and other rheumatic diseases, including CTD-associated vasculitis and primary vasculitic syndromes. Because the association between eNOS gene polymorphisms and vascular diseases varies according to race, this study should be carried out in other ethnic populations. Because only patients with SLE who manifested vasculitis were recruited in the current study, further studies are warranted in other patients with SLE who have no clinical evidence of vasculitis. Moreover, it will be necessary to clarify whether there is an association between eNOS gene polymorphisms and other inflammatory rheumatic diseases without vasculitis, such as ankylosing spondylitis. On the other hand, replacement therapy to alleviate the NO or eNOS deficit has been considered in disease states where the effective levels of endothelial NO are decreased.[6] In this respect, L-arginine, which is a precursor of NO, has been shown to improve endothelium dependent vasodilatation in hypercholesterolaemic humans.[46] Therapeutic modalities that restore deficiencies in NO or eNOS may be considered in the treatment of BD or rheumatic diseases with vasculitis.

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