Endothelial Nitric Oxide Synthase Gene Is Associated With Diabetic Macular Edema in Type 2 Diabetes

Masakatsu Watanabe, MD1
Masatake Osaki, MD1
Nobuaki Takata, MD1
Tomoko Ohkubo, MD1
Susumu Kurihara, MD1
Hiroyuki Iizuka, MD2
Tatsuo Neda, MD1
Takuya Awata, MD1,2

OBJECTIVE — We examined the endothelial nitric oxide (eNOS) gene polymorphisms to assess its possible association with diabetic retinopathy and macular edema.

RESEARCH DESIGN AND METHODS — A total of 226 patients with type 2 diabetes and 186 healthy subjects were studied. Type 2 diabetic patients consisted of 110 patients without retinopathy, 46 patients with nonproliferative diabetic retinopathy, and 71 patients with proliferative diabetic retinopathy. Diabetic macular edema was present in 48 patients. Three polymorphisms of the eNOS gene were determined: T-786C in the promoter region, 27-bp repeat in intron 4, and Glu298Asp in exon 7.

RESULTS — Close linkage disequilibrium was observed between the T-786C polymorphism and the 27-bp repeat, which has been previously reported, but Glu298Asp was not in linkage disequilibrium with the other two polymorphisms. The eNOS gene polymorphisms were not significantly associated with the presence of retinopathy or with retinopathy severity or type 2 diabetes itself. However, by both association study and multiple logistic regression analysis, the T-786C and 27-bp repeat polymorphisms were significantly associated with a risk of developing macular edema with the −786C allele and the “a” allele increasing the risk.

CONCLUSIONS — The present study suggests that the eNOS gene is a novel genetic risk factor for diabetic macular edema. The eNOS gene polymorphisms may contribute to the development of macular edema by impairing basal eNOS expression and resulting in the breakdown of the blood-retina barrier.

Diabetes Care 27:2184–2190, 2004

© 2004 by the American Diabetes Association.
possible association of eNOS polymorphisms with DR has been examined in several studies, and a “b” allele of the 27-bp repeat was associated with a high risk of DR in type 1 diabetes (13,14). In the present study, we examined the eNOS polymorphisms to assess their possible association with DR and maculopathy. We found that eNOS gene variations contribute to the risk of diabetic maculopathy in type 2 diabetes.

**RESEARCH DESIGN AND METHODS** — We studied 226 type 2 diabetic patients and 184 healthy subjects; all were unrelated Japanese. Patients with type 2 diabetes were recruited from those admitted to the Saitama Medical School Hospital in Saitama prefecture, Japan (age range 15–86 years). Diagnosis and classification of diabetes was based on clinical features, laboratory data (including anti-GAD antibody, serum C-peptide levels, and urine C-peptide levels), and the guidelines of the Expert Committee Report of the American Diabetes Association (15). Healthy subjects were volunteers living in Saitama prefecture (age range 18–47 years). BMI was calculated as weight/height² (kg/m²). Blood pressure was measured by mercury sphygmomanometer. Fasting HbA1c, cholesterol, and triglyceride levels were measured with conventional methods. Informed consent was obtained from each individual, the study was approved by the Ethical Committee of Saitama Medical School, and all procedures were conducted in accordance with the principles of the Declaration of Helsinki.

**Ophthalmological evaluation**
All patients had a complete ophthalmological examination, including corrected visual acuity, slitlamp biomicroscopic examination with and without preset lens, funduscopic examination, and fundus photography. Funduscopic findings were determined and graded by trained retinal specialists (S.Y. and K.M.), based on a grading scale similar to international clinical DR/ME disease severity scale (http://www.aao.org/aao/education/library/international_DR.cfm). Proliferative DR was identified by the presence of neovascularization with or without vitreous hemorrhage. ME was determined by the presence of some retinal thickening or hard exudates in the posterior pole. Based on the severity grading, we performed fluorescein angiography of those patients with severe nonproliferative or proliferative DR to confirm the funduscopic findings.

**Genotyping of the eNOS gene polymorphisms**
The human eNOS gene consists of 26 exons (16). We identified three polymorphisms of the eNOS gene: a T-786C in the promoter region, a 27-bp repeat in intron 4, and a Glu298Asp in exon 7. T-786C and Glu298Asp are “single nucleotide polymorphisms” (SNPs), and the 27-bp repeat is a “variable number of tandem repeats” polymorphism. This latter gene’s two alleles are “a” and “b”: the “a” allele has four repeats and the “b” allele has five repeats. The T-786C and Glu298Asp polymorphisms were determined by PCR–restriction fragment–length polymorphism analysis. The PCR primers for T-786C were 5’-TGGCCTGAATGCTGGAGACTGTA-3’ (forward) and 5’-AATTGGGGGACACAAAAAGAGCA-3’ (reverse). The T-786C allele results in the gain of an MspI site. After digestion by MspI, PCR products were electrophoresed on a 4% agarose gel and visualized by ethidium bromide staining. The Glu298Asp polymorphism was genotyped using PCR–restriction fragment–length polymorphism as described by Miyamoto et al. (6). The 27-bp repeat polymorphism was determined using PCR amplification with oligonucleotide primers, as described by Wang et al. (17). The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Statistics**
Distribution of genotypes and alleles was compared by χ² test or Fisher’s exact test. Linkage disequilibrium was assessed using the SNPAlyze V3.1 (Dynacom, Yokohama, Japan) (18). Continuous clinical data were compared using unpaired Student’s t test, and categorical clinical data were compared using Fisher’s exact test. Multivariate logistic regression analyses were performed to assess the independent role of the eNOS genotype and other variables, including sex, age at onset, duration of diabetes, systolic blood pressure (SBP), HbA1c level, cholesterol level, and treatment of diabetes (categorical variable: insulin therapy or no insulin therapy). We used StatView version 5.0 for these tests. Statistical significance was defined as P < 0.05.

**RESULTS** — Among the 226 type 2 diabetic patients studied, 109 patients had no evidence of retinopathy, and the remaining 117 had DR that could be classified into one of four categories, as defined by their manifestation of either nonproliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) and by the absence or presence of ME (Table 1). Table 1 shows the clinical characteristics of the diabetic patients included in the study. Among the 46 patients who had NPDR, seven showed signs of ME, and of the 71 patients with PDR, 41 also had ME. Compared with diabetic patients without DR, patients in the ME− PDR group were younger at diabetes diagnosis, and patients with DR had a longer duration of disease. Patients in the ME+ PDR group had significantly elevated SBP and cholesterol levels when compared with DR− patients. And finally, patients with DR had a significantly higher prevalence of insulin therapy than patients without DR.

eNOS polymorphisms and DR
Close linkage disequilibrium was observed between the T-786C polymorphism and the 27-bp repeat, as has been previously reported (10,19); however, Glu298Asp was not in linkage disequilibrium with the other two polymorphisms. We compared the frequency of genotypes and alleles of the three polymorphisms across the DR− (no diabetic retinopathy), DR− All, NPDR, and PDR groups (Table 2). There were no significant differences in these genotype and allele frequencies between any groups versus the DR− patient results. Thus, these eNOS polymorphisms were not associated with the presence of DR or with DR severity. Furthermore, genotype and allele frequencies of the eNOS polymorphisms in 204 healthy subjects were not significantly different from the frequencies in the total type 2 diabetic patients (data not shown), suggesting that these eNOS polymorphisms were not associated with type 2 diabetes itself. This result was not in accord with the recent report by Monti et al. (20).
eNOS gene and diabetic macular edema

We compared the frequency of genotypes and alleles between ME- DR and ME+ DR groups (Table 3). Although there was no significant difference in the frequency of Glu298Asp genotypes and alleles between the two groups of patients, the T-786C allele was significantly increased in patients with ME+ DR compared with patients with ME- DR. In addition, the genotype distribution was significantly different between these two groups. The association of the “a” allele with ME was a bit stronger than that seen for the −786C allele (P = 0.006 and 0.029, respectively).

**Logistic regression analysis**

We further assessed the relationship between eNOS polymorphisms and DR or ME by multivariate logistic regression analysis, including the patients’ clinical features and the genetic polymorphisms. The Glu298Asp genotypes were not associated with both DR and ME by logistic regression analysis. However, the 27-bp repeat genotype was not significantly associated with an increased risk of developing retinopathy overall, although a longer duration of diabetes, insulin therapy, and a higher SBP showed a significantly increased risk of retinopathy (Table 4), also, the T-786C genotype, which was in strong linkage disequilibrium with the 27-bp repeat, was not significantly associated with DR. However, in addition to higher SBP, lower HbA1c levels, and insulin therapy, the 27-bp repeat genotypes had a significant increased risk of developing ME (P = 0.001) (Table 4), with the presence of the “a” allele increasing the risk. The T-786C genotypes was also associated with an increased risk of developing ME, with the −786C allele...
CONCLUSIONS — We first evaluated the possible association of the eNOS gene with DR. Previously, several candidate genes were reported to be associated with DR. In particular, the gene for aldose reductase has been extensively examined, and dinucleotide polymorphism at the 5'-region and C–106T polymorphism were found to be associated with DR (21,22). Recently, we found that a common polymorphism, C–634G, in the 5’-untranslated region of the vascular endothelial growth factor (VEGF) gene was significantly associated with the presence of DR (23). Subsequently, it was suggested that the C–634G polymorphism was functionally significant (24) and that the VEGF gene polymorphisms, including the C–634G polymorphism, were also associated with amyotrophic lateral sclerosis (24) and giant cell arteritis (25). The possible associations of the eNOS gene with diabetic microvascular complications have also been studied; however, these associations remain inconsistent as follows. Some studies (9,10), but not all (11,12), reported that the “a” allele of the eNOS 27-bp repeat polymorphism was positively associated with DN. In contrast, Taverna et al. (13) reported that the “b” allele and the bb genotype were associated with severe DR. Similarly, Frost et al. (14) reported that the bb genotype of the 27-bp repeat was associated with DR. However, Neugebauer et al. (9) found no association of the 27-bp repeat with DR, and Tavakkoly-Bazzaz et al. (26) recently reported that the bb genotype of the 786C allele, which was in linkage disequilibrium with the “a” allele, was significantly associated with DR. In the present study, the present study, the presence or absence of ME was defined by the international criteria, and ME was present in 7 (15.2%) of 46 patients with NPDR and 41 (57.7%) of 71 patients with PDR. By both association study and multiple logistic regression analysis, we found that the T–786C and 27-bp repeat polymorphisms were significantly associated with a risk of ME development, with the −786C and “a” alleles increasing the risk. Few reports have analyzed the susceptibility genes for ME; Santos et al. (27) reported an association of apolipoprotein E gene with ME in a small population (36 patients with type 2 diabetes), and Kumaramanickavel et al. (28) reported an association of aldose reductase gene with PDR and ME in 214 patients with type 2 diabetes.

Because ME is an important cause of visual impairment and may occur at any stage of DR, it is important to elucidate risk factors that contribute to the progression of ME. In the present study, the presence or absence of ME was defined by the international criteria, and ME was present in 7 (15.2%) of 46 patients with NPDR and 41 (57.7%) of 71 patients with PDR. By both association study and multiple logistic regression analysis, we found that the T–786C and 27-bp repeat polymorphisms were significantly associated with a risk of ME development, with the −786C and “a” alleles increasing the risk. Few reports have analyzed the susceptibility genes for ME; Santos et al. (27) reported an association of apolipoprotein E gene with ME in a small population (36 patients with type 2 diabetes), and Kumaramanickavel et al. (28) reported an association of aldose reductase gene with PDR and ME in 214 patients with type 2 diabetes.

Table 3—Genotype and allele distribution of eNOS gene polymorphisms in type 2 diabetic patients with ME− DR and those with ME+ DR

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ME− DR</th>
<th>ME+ DR</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>T−786C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>87.0%</td>
<td>70.8%</td>
<td>69</td>
</tr>
<tr>
<td>TC</td>
<td>13.0%</td>
<td>27.1%</td>
<td>48</td>
</tr>
<tr>
<td>CC</td>
<td>0.0%</td>
<td>2.1%</td>
<td>48</td>
</tr>
<tr>
<td>27-bp repeat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bb</td>
<td>87.0%</td>
<td>64.6%</td>
<td>69</td>
</tr>
<tr>
<td>ab</td>
<td>13.0%</td>
<td>33.3%</td>
<td>69</td>
</tr>
<tr>
<td>aa</td>
<td>0.0%</td>
<td>2.1%</td>
<td>69</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GluGlu</td>
<td>84.1%</td>
<td>85.4%</td>
<td>69</td>
</tr>
<tr>
<td>GluAsp</td>
<td>15.9%</td>
<td>14.6%</td>
<td>69</td>
</tr>
<tr>
<td>AspAsp</td>
<td>0.0%</td>
<td>0.0%</td>
<td>69</td>
</tr>
</tbody>
</table>

P values are ME− DR vs. ME+ DR.

Table 4—Odds ratios adjusted by logistic regression analysis for the association with DR and ME among type 2 diabetic patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>DR vs. no DR</th>
<th>ME vs. no ME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.87 (0.46–1.65)</td>
<td>0.676</td>
</tr>
<tr>
<td>Age at onset</td>
<td>1.02 (0.99–1.04)</td>
<td>0.385</td>
</tr>
<tr>
<td>Duration of disease</td>
<td>1.11 (1.05–1.16)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP</td>
<td>1.02 (1.00–1.03)</td>
<td>0.047</td>
</tr>
<tr>
<td>Hba1c</td>
<td>1.02 (0.88–1.19)</td>
<td>0.799</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.00 (1.00–1.01)</td>
<td>0.294</td>
</tr>
<tr>
<td>Insulin therapy</td>
<td>2.46 (1.27–4.76)</td>
<td>0.008</td>
</tr>
<tr>
<td>cNOS 27-bp repeat genotype*</td>
<td>0.88 (0.47–1.66)</td>
<td>0.689</td>
</tr>
</tbody>
</table>

*bb = 0, ab = 1, aa = 2.
ME. In agreement with previous studies, we observed that a patient with higher SBP had a significant increased risk of developing ME (3). Although Uwabo et al. (29) reported an association of the 27-bp repeat polymorphism with essential hypertension, we did not observe a significant association of the eNOS polymorphism with SBP (Spearman’s rank-order correlation coefficients; data not shown), suggesting that the association of the eNOS polymorphisms with ME does not come from its association with hypertension. In the present study, patients with lower Hba1c levels had a significantly increased risk of ME. This result apparently contradicts the observation of the Diabetes Control and Complication Trial that intensive treatment of diabetes to achieve near-normal blood glucose levels resulted in a 23% risk reduction in ME (30). However, the current Hba1c levels in the present study cannot be regarded as the patients’ long-term glycemic control. Supporting this notion, insulin therapy, which could reflect patients’ lower insulin secretion capacity and previous poor glycemic control, was also associated with an increased risk of DR and ME development, although insulin therapy itself may exacerbate BRB breakdown (31). One possible explanation for the relationship between lower Hba1c levels and ME development in the present study may be so-called early worsening, subsequent to a rapid improvement of glycemic control (32).

There have been several reports suggesting that the T-786C polymorphism is functional. Nakayama et al. (7) reported that, using luciferase reporter gene assays, the −786 T-to-C substitution resulted in a significant reduction in eNOS gene promoter activity. Furthermore, eNOS mRNA levels in placenta carrying the −786C allele substitution were significantly lower than levels in placenta without the substitution, and serum NO metabolite levels among individuals carrying the −786C allele were significantly lower than among those without the −786C allele (33). Miyamoto et al. (33) found that these effects might depend upon the fact that the −786C allele can bind the replication protein A1, which acts as a gene repressor protein. Ohtoshi et al. (34) also reported that plasma NO metabolite levels in the −786C allele carriers were significantly lower than in those without it in diabetic patients. The 27-bp repeat polymorphism, which is in close linkage disequilibrium with the T-786C polymorphism, may also be functional. The “a” allele has been significantly associated with lower plasma NO metabolite levels (35). Moreover, it was demonstrated that the 27-bp repeat could bind nuclear proteins as an enhancer/repressor to promote/suppress the transcription efficiency (36). Taken together, although full agreement has not been obtained, the T-786C and the 27-bp repeat polymorphisms are likely to be functional in a fashion that the eNOS gene expression is decreased in both the −786C allele carriers and the “a” allele carriers. Recent publications, which assessed possible associations of the T-786C or the 27-bp repeat polymorphism with vascular disease (37,38) or endothelial dysfunction (39,40) also confirmed functional significance of these polymorphisms.

Several lines of evidence suggest that impaired eNOS expression in the retina could contribute to the development of ME, in which the breakdown of the BRB would have an important role. Although high levels of NO produced by iNOS in pathological processes, such as immune response or inflammation, can be detrimental to the body, physiological concentrations of NO, maintained by eNOS, have a protective effect on the vascular endothelium (5). Thus, eNOS impairment may accelerate damage to endothelial cells caused by hyperglycemia and hypertension, leading to breakdown of the inner BRB (which is maintained by endothelial cells). Furthermore, impaired eNOS activity can induce deregulation of vascular tone and can result in hypoxia, leading to VEGF-induced vascular permeability. Impaired basal NO production may also permit leukocyte adhesion to the endothelium (41), which would result in the breakdown of the BRB and capillary nonperfusion (42). Alternatively, impaired eNOS activity may directly increase microvascular permeability (43,44). Interestingly, eNOS is localized to the Muller cells (45), which are known to be important in maintenance of the BRB (2).

In conclusion, we have found that the eNOS gene polymorphisms, T-786C in the promoter region, and 27-bp repeat in intron 4 were associated with ME in Japanese patients with type 2 diabetes. Together with the previous studies, it is suggested that defective eNOS expression in the retina, relevant to the polymorphisms, may contribute to the development of ME. Our present study was cross-sectional and comprised exclusively Japanese patients and volunteers with a medium sample size. Large, prospective studies are necessary to confirm our findings.

Acknowledgments—This work was supported in part by a grant from the Japanese Ministry of Education, Culture, Sports and Science and Technology; Maruki Memorial Special Scholarship B; and a grant to Saitama Medical School Research Center for Genomic Medicine.

References

9. Neugebauer S, Baba T, Watanabe T: Association of the nitric oxide synthase gene polymorphism with an increased risk for progression to diabetic nephropathy in


