Endothelial Nitric Oxide Synthase Gene Polymorphism and Acute Myocardial Infarction

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Abstract—Recently a point mutation of guanine to thymine at nucleotide position 1917 in the endothelial nitric oxide synthase (eNOS) gene has been reported to be associated with coronary artery spasm. In addition, a significant association of the 4a/b polymorphism in intron 4 of the eNOS gene with coronary artery disease has been reported. However, the implications of these polymorphisms with respect to acute myocardial infarction (AMI) remain to be established. We conducted a case-control study of 226 patients with AMI and 357 healthy gender- and age-matched control subjects. In the former group, coronary angiograms were evaluated according to angiographic criteria based on the number of diseased vessels (≥75%) and the number of stenotic lesions (≥50%). Homozygosity for the Glu-Asp298 polymorphism existed in 5 of 226 patients with AMI (2.2%) but not in any of the 357 control subjects (P = .0085). However, when we evaluated the coronary angiograms of 226 case patients, there was no difference in the number of diseased vessels or the number of stenotic lesions between the patients with this homozygote and those without it. By contrast, there was no evidence of a significant increase in the risk of AMI or the severity of coronary atherosclerosis among individuals with the a/a genotype of the eNOS4a/b polymorphism. Our results imply that patients who are homozygous for the Glu-Asp298 polymorphism may be genetically predisposed to AMI; however, this mutation apparently is not related to the severity of coronary atherosclerosis. Further studies are needed to confirm our results and characterize the molecular mechanisms by which eNOS is involved in susceptibility to AMI. (Hypertension. 1998;32:521-526.)

Key Words: endothelium-derived relaxing factor n genes n myocardial infarction n atherosclerosis n angiography

Since the identification of nitric oxide (NO) as an important endothelium-derived relaxing factor, there has been an explosion of new information on the physiological and pathophysiological roles of NO. NO is synthesized from the amino acid L-arginine by a family of enzymes, referred to as NO synthase (NOS). Three distinct isofoms of NOS have been identified to date.1 The inducible NOS is expressed in vessel walls and macrophages by certain cytokines and endotoxin lipopolysaccharides in pathological conditions.2 The constituitive neuronal NOS is expressed in the central and peripheral nervous systems as well as in macula densa of kidney. It plays important roles in physiological3 and pathophysiological4 conditions. The constituitive endothelial NO synthase (eNOS) is expressed in the endothelium, where it produces NO from L-arginine. NO diffuses to the vascular smooth muscle cells, where it increases the concentration of cGMP by stimulating soluble guanylate cyclase, leading to vascular relaxation.5

Several studies suggest that the basal release of NO by the endothelium contributes to basal vascular tone5,6 and regulates blood flow and blood pressure. Recent reports have suggested a possible role of NO in the pathogenesis of coronary spasm.7 NO also inhibits the proliferation of smooth muscle cells.8 Furthermore, NO protects against platelet aggregation in vitro9 and in vivo10 and inhibits platelet adhesion to vascular endothelium.11 In addition, NO inhibits leukocyte adhesion to endothelium.12 All of these processes are important events during atherogenesis. Dysfunction of this important mechanism may promote atherogenesis by exposing the arterial wall to the direct vasoconstrictor effects of factors that mediate vasospasm and increase the risk of thrombosis, leading to acute myocardial infarction (AMI).

Among the reported polymorphisms of the eNOS gene, a significant association of the 4a/b polymorphism in intron 4 of the eNOS gene with coronary artery disease (CAD) has been reported.13 In addition, recent preliminary data indicate that the Glu-Asp298 polymorphism in exon 7 of the eNOS gene is associated with coronary spasm,14 although the implications of these polymorphisms with respect to AMI remain to be established.
Methods

Study Population
The Glu-Asp298 genotype of the eNOS gene was determined in 226 patients at first presentation of AMI and in 482 control subjects. We also determined the eNOS4a/b polymorphism in this population. Adult Japanese patients who satisfied the World Health Organization criteria for myocardial infarction were eligible for the study (185 men, mean age 61.5 years; 41 women, mean age 71.3 years). All enrolled patients had angiographically documented coronary artery narrowing, exceeding 75% luminal diameter, and underwent percutaneous transluminal coronary angioplasty, thrombolytic therapy, or both. A total of 482 control subjects (285 men, mean age 60.0 years; 197 women, mean age 60.5 years) who came to the Health Checkup Center of Nanasawa Rehabilitation Hospital for their regular checkup were enrolled in the study. They were genetically unrelated and were living in Kanagawa, Japan. The control individuals had no symptoms of CAD and had normal ECG results. Control subjects gave their informed consent to participate in this study. Because the gender distributions were different between the patient and control groups (P<0.0001) and the female control subjects were younger than female patients (P<0.0001), we recruited 357 control subjects who were matched to the case patients for gender and age for case-control comparisons (Tables 1 through 4).

Genotyping
DNA was extracted from peripheral leukocytes. For detection of Glu-Asp298 polymorphism of the eNOS gene, we used primer pairs to amplify a part of the eNOS gene containing exon 7 by polymerase chain reaction (PCR). Primer pairs for PCR were as follows: sense 5'-TCC CTG AGG AGG GCA TGA GGC T-3' and antisense 5'-TGA GGG TCA CAC AGG TTC CT-3'. Samples were amplified for 30 cycles, consisting of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute. The resulting 457-bp amplification product was incubated at 37°C for at least 20 hours with BanII (New England Biolabs Inc). The amplified fragments were digested by BanII into smaller fragments (137 and 320 bp). In the case of a G to T substitution at position 1917 of the eNOS gene, a BanII recognition site is lost. The restricted fragments were separated on 8% polyacrylamide gels with ethidium bromide staining (Figure, A). The PCR isoform typing results were checked in 10% of the samples by the direct sequencing of amplified DNA. All fragments that were not cleaved were also confirmed by direct sequencing (Figure, B) to avoid mistyping. In brief, after removal of dNTP and primers by columns, each sample was subjected to cycle sequencing using a dye terminator cycle-sequencing kit (Perkin-Elmer) according to the supplier's instructions. Electrophoresis was performed with the use of a DNA sequencer (model 310, version 2.1.1, Perkin-Elmer).

The eNOS4a/b gene polymorphism was detected by the method of Wang et al with minor modifications. Briefly, the DNA samples were subjected to amplification by PCR using primer pairs that flank the region of the 27-bp direct repeat in intron 4 of the eNOS gene. The amplified fragments were separated on 4% polyacrylamide gels with ethidium bromide staining. A total of 482 control subjects (285 men, mean age 60.0 years; 197 women, mean age 60.5 years) who came to the Health Checkup Center of Nanasawa Rehabilitation Hospital for their regular checkup were enrolled in the study. They were genetically unrelated and were living in Kanagawa, Japan. The control individuals had no symptoms of CAD and had normal ECG results. Control subjects gave their informed consent to participate in this study. Because the gender distributions were different between the patient and control groups (P<0.0001) and the female control subjects were younger than female patients (P<0.0001), we recruited 357 control subjects who were matched to the case patients for gender and age for case-control comparisons (Tables 1 through 4).

Clinical and Laboratory Measurements
All participants completed a standard questionnaire on personal medical history, family history, and smoking habits. The subject was considered to be a current daily smoker if she or he had regularly smoked for at least 20 hours with the restriction enzyme BanII. Homozygotes with a G to T substitution at position 1917 (T/T) showed a single band at 457 bp. Homozygotes with G at this position (G/G) showed 2 bands at 320 bp and 137 bp. Heterozygotes for this mutation (G/T) showed 3 bands at 457 bp, 320 bp, and 137 bp. B, eNOS exon 7 was amplified from genomic DNA of healthy control subjects (left) and patients with AMI (right) and sequenced directly, revealing a homozygous G to T transition at nucleotide position 1917.

Angiographic Criteria
The severity of CAD was evaluated from coronary angiograms on the basis of the number of diseased vessels with stenosis of ≥75% and the number of lesions with stenosis ≥50% as reported previously.

Statistical Analysis
All statistical analyses were conducted with use of the SPSS statistical package, version 6.1. Data are expressed as mean±SEM. The frequencies of the alleles and genotypes were compared between patient and control groups by the x2 test when appropriate. The distributions of genotype frequencies in control subjects, patients, or the overall study group were compared by the x2 test when the smallest of the 4 expected frequencies was <5. The distributions of gender (male or female), smoking, presence of hypertension, and diabetes mellitus among the 3 genotypes of patients were analyzed by construction of 3 x 2 contingency tables when the smallest of the 4 expected numbers was <5. The distributions of gender (male or female), smoking, presence of hypertension, and diabetes mellitus among the 3 genotypes of patients were analyzed by construction of 3 x 2 contingency tables and the x2 analysis. One-way ANOVA was used to analyze the relations between genotypes and the general characteristics or severity seen on coronary angiograms in the patient group.
Because the number of stenoses was not distributed normally, statistical tests were performed on square root–transformed stenoses.

### Results

#### Clinical Variables in Patients With AMI and Control Subjects

Table 1 compares clinical characteristics between the patients with AMI and the control subjects. As we recruited control subjects who were matched to case patients for gender and age, these variables were not different between patients with AMI and control subjects. As expected, in a study of myocardial infarction, patients with AMI had higher values of Chol/HDL-C and higher prevalence of hypertension and diabetes mellitus. There was a higher frequency of current smokers among patients with AMI, whereas the frequency of past smokers was lower than in the control group. The frequency of nonsmokers was significantly lower among patients with AMI than control subjects (4/226 patients vs 221/357 controls; \( \chi^2 = 60.8, P < 0.0001 \)).

### Distributions of Genotype and Allele Frequencies in eNOS Gene Variants in Patients With AMI and Control Subjects

#### Glu-Asp298 Polymorphism of eNOS Gene

A total of 226 patients with AMI and 357 healthy Japanese subjects were enrolled in the study. We also examined 18 subjects from 3 different families and confirmed that the Glu-Asp298 polymorphism of the eNOS gene is inherited in a simple mendelian fashion (data not shown). Representative genotyping results for subjects with each genotype are shown in panel A of the Figure. The genotype and allele frequency of the polymorphism in patients and control subjects are shown in Table 2. Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in control subjects, patients, or the overall study group. The allele frequencies of these eNOS genes were similar in these groups. However, when we assumed a recessive model of inheritance (ie, T/T versus G/T and G/G combined), the frequency of T/T homozygotes in patients with AMI was significantly higher than that in healthy control subjects (5 of 226 patients and none of 357 controls; \( P = 0.0085 \)).

### 4a/b Polymorphism of eNOS Gene

By contrast, the genotype distribution and allele frequency of 4a/b polymorphism were similar between patients with AMI and healthy control subjects (Table 3). When we assumed a recessive model of inheritance (ie, a/a versus b/a and b/b combined), the frequency of a/a homozygotes was not different between patients with AMI and control subjects (4 of 226 patients and 5 of 399 controls; \( P = 0.74 \)).

### Genotype Frequencies of Glu-Asp298 Polymorphism in Prespecified Study Subgroups

We analyzed the data by stratifying patients with AMI and control subjects according to age, gender, and other established risk factors for CAD (Table 4). In the subgroups of age \( > 60 \) years, male, body mass index (BMI) \( = 25 \) kg/m², without hypertension, and without diabetes mellitus, the frequencies of T/T homozygotes in patients with AMI were still significantly higher than those in control subjects.

### Relationship Between Demographic Characteristics and Severity of CAD and Genotypes in Patients With AMI

#### Glu-Asp298 Polymorphism of eNOS Gene

When clinical and laboratory values were compared among genotypes in the patients with AMI, no significant difference was noted (Table 5). When the severity of CAD determined...
by coronary angiogram was compared among genotypes, there were no differences in the number of diseased vessels or the number of stenotic lesions (Table 5).

4a/b Polymorphism of eNOS Gene

Similarly, there were no differences among genotypes in clinical and laboratory values (data not shown). There also were no differences among genotypes in the number of diseased vessels or the number of stenotic lesions (vessel: b/b 1.4±0.1, b/a 1.3±0.1, a/a 1.0±0.0, P=0.29; stenoses: b/b 2.0±0.1, b/a 1.8±0.1, a/a 1.7±0.3, P=0.33).

Discussion

We performed a case-control study of the endothelial NOS locus and found a significant association between homozygotes for Glu-Asp298 polymorphism and the occurrence of AMI. Lack of an increased risk of AMI in the eNOS GT heterozygotes suggests that the risk of AMI posed by the eNOS T allele is not dominantly expressed and that the increased risk is confined to eNOS TT homozygotes. To our knowledge, this is the first study to implicate Glu-Asp298 polymorphism of the eNOS gene as a genetic risk factor for AMI. Although Bonnardeaux et al20 reported no association between the eNOS gene and essential hypertension, the results of our study and a recent investigation by Wang et al13 suggest that the eNOS gene is related to CAD.

Although patients with AMI had higher values of Chol/HDL-C and higher prevalence of hypertension, diabetes mellitus, and smoking status (both current and ex-smokers) than age- and gender-matched control subjects (Table 1), no evidence of increased frequency of eNOS TT homozygotes was found in high-risk subgroups in analyses stratified by well-established risk factors for CAD such as BMI, smoking status, Chol/HDL-C, hypertension, and diabetes mellitus (Table 4). Thus, it is not likely that our findings were due to selection bias.

In addition to established risk factors, genetic risk factors may have important roles in the pathogenesis of coronary atherosclerosis. Identification of these genetic risk factors is expected to enhance our understanding of the molecular basis for atherosclerosis. Case-control studies can detect weak susceptibility genes in polygenic diseases such as CAD. Using this approach, we and others have identified gene polymorphisms, including the M235T variant of angiotensinogen,21 the DD genotype of the angiotensin-converting

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**TABLE 4. Genotype Frequencies of Glu-Asp298 Polymorphism in Prespecified Study Subgroups**

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Genotype (GG + GT/TT)</th>
<th>Patients</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y ≥60 (n=218)</td>
<td></td>
<td>92/1</td>
<td>125/0</td>
<td>0.427</td>
</tr>
<tr>
<td>&gt;60 (n=365)</td>
<td></td>
<td>129/4</td>
<td>232/0</td>
<td>0.017*</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=470)</td>
<td></td>
<td>181/4</td>
<td>285/0</td>
<td>0.024*</td>
</tr>
<tr>
<td>Female (n=113)</td>
<td></td>
<td>40/1</td>
<td>72/0</td>
<td>0.362</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥25 (n=391)</td>
<td></td>
<td>147/4</td>
<td>240/0</td>
<td>0.022*</td>
</tr>
<tr>
<td>&gt;25 (n=192)</td>
<td></td>
<td>74/1</td>
<td>117/0</td>
<td>0.391</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or ex-smoker</td>
<td></td>
<td>180/4</td>
<td>196/0</td>
<td>0.054</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td></td>
<td>41/1</td>
<td>161/0</td>
<td>0.207</td>
</tr>
<tr>
<td>Chol/HDL-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.5 (n=304)</td>
<td></td>
<td>78/1</td>
<td>255/0</td>
<td>0.260</td>
</tr>
<tr>
<td>≥4.5 (n=279)</td>
<td></td>
<td>143/4</td>
<td>132/0</td>
<td>0.125</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With (n=291)</td>
<td></td>
<td>129/2</td>
<td>160/0</td>
<td>0.202</td>
</tr>
<tr>
<td>Without (n=292)</td>
<td></td>
<td>92/3</td>
<td>197/0</td>
<td>0.034*</td>
</tr>
<tr>
<td>No diabetes mellitus (n=455)</td>
<td></td>
<td>148/5</td>
<td>302/0</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

*P<0.05.

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**TABLE 5. General Characteristics and Severity of Coronary Atherosclerosis of Patients in Each Glu-Asp298 Genotype Subgroup**

<table>
<thead>
<tr>
<th>Variable</th>
<th>GG (n=189)</th>
<th>GT (n=32)</th>
<th>TT (n=5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>63.4±0.8</td>
<td>62.4±1.9</td>
<td>64.6±6.0</td>
<td>0.87</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>32/157</td>
<td>8/24</td>
<td>1/4</td>
<td>0.54</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.8±0.3</td>
<td>23.8±0.5</td>
<td>22.7±1.6</td>
<td>0.76</td>
</tr>
<tr>
<td>Nonsmoker/current and ex-smoker</td>
<td>51/138</td>
<td>11/21</td>
<td>1/4</td>
<td>0.58</td>
</tr>
<tr>
<td>Hypertension, with/without</td>
<td>106/83</td>
<td>23/9</td>
<td>2/3</td>
<td>0.18</td>
</tr>
<tr>
<td>Diabetes mellitus, with/without</td>
<td>63/126</td>
<td>10/22</td>
<td>0/5</td>
<td>0.29</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.94±0.07</td>
<td>5.15±0.19</td>
<td>4.54±0.31</td>
<td>0.33</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.23±0.05</td>
<td>1.35±0.12</td>
<td>1.38±0.25</td>
<td>0.55</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.03±0.02</td>
<td>1.15±0.42</td>
<td>1.01±0.23</td>
<td>0.18</td>
</tr>
<tr>
<td>Vessels</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
<td>1.2±0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>Stenosis</td>
<td>1.9±0.1</td>
<td>2.1±0.1</td>
<td>1.8±0.3</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM. Vessels indicates number of diseased vessels; Stenosis, square root of number of stenoses.
enzyme gene, 22 and the E4 allele of the apolipoprotein E gene, 21 as genetic risk factors for coronary atherosclerosis. In the present study, we report for the first time that a Glu-Asp298 polymorphism of the eNOS gene is also a genetic risk factor for AMI. However, since the TT genotype existed in only 5 of 226 patients (2.2%) with AMI, this genotype can explain only a small part of genetic susceptibility to AMI.

Studies indicate that AMI results from 2 main processes: coronary atherosclerosis and the formation of a platelet aggregate at the site of a ruptured coronary atherosclerotic plaque. Coronary artery spasm may also play a part in the pathogenesis of AMI and sudden death. Our study provides no information about mechanisms by which Glu-Asp298 polymorphism of the eNOS gene predisposes patients to AMI. However, a recent study showed that N ω-nitro-L-arginine methyl ester–induced chronic inhibition of NO production accelerated neointima formation and impaired endothelial function in hypercholesterolemic rabbits. 23 Another report showed that chronic administration of L-arginine, the precursor of NO, improved endothelium-dependent vasorelaxation in a similar animal model, 24 indicating that continuous release of NO by endothelium inhibits the progression of atherosclerosis. NO plays important roles in inhibiting platelet activation and adhesion to the endothelium 25,26 as well as monocyte adhesion. In addition, a recent report suggests that there is a deficiency of both basal and stimulated NO activity in patients with coronary spastic angina, indicating pivotal roles of NO in the pathogenesis of coronary artery spasm, 2 which is now considered an early stage of coronary atherosclerosis. 26 Although the mechanism by which Glu-Asp298 polymorphism confers susceptibility to AMI is not clear, this polymorphism is probably not atherogenic, since the severity of coronary atherosclerosis, as assessed on the basis of coronary angiograms, was similar among the different genotypes. This is consistent with the recent report by Quyyumi et al, 27 who showed no correlation between the angiographic severity of coronary atherosclerosis and the magnitude of depression in basal NO activity.

A recent report by Wang et al 28 showed an association between the eNOS4a/a genotype and CAD. They also found that the eNOS4a/a genotype was associated with increased severity of coronary atherosclerosis, which is smoking dependent. In the present study, the genotype distribution of 4a/b polymorphism was similar in patients with AMI and healthy control subjects, and the severity of coronary atherosclerosis did not differ according to genotype in the patients with AMI, even when we analyzed the data by stratifying patients according to smoking status (nonsmokers, light current or ex-smokers, medium current or ex-smokers, or heavy current or ex-smokers; data not shown). One explanation of the discrepancy between our study and the previous one may be patient selection. Wang et al studied white patients with CAD who were referred to their hospital, whereas our patients were Japanese survivors of AMI who underwent coronary angiography at our hospital. Alternatively, the small sample size of patients with AMI may also explain the differences in the results of these studies.

Our study has several limitations. The first is the lack of functional studies. Whether the Glu-Asp298 polymorphism functionally underlies a mechanism leading to AMI should be determined. Second, the T allele of the Glu-Asp298 polymorphism and the 4a allele of the eNOS4a/b polymorphism both have an estimated frequency of only 0.1, and the association between Glu-Asp298 polymorphism and AMI is based on only 5 individuals who were homozygous for this polymorphism. Furthermore, this association is only valid under the presumption of a recessive gene effect. Thus, a larger sample should be examined to confirm the relation between these polymorphisms and AMI. Third, because our results are limited to the subgroup of survivors of AMI but not to the entire group of patients with CAD, these observations need further confirmation using prospective study design and in other subgroups of patients with CAD.

In summary, we have identified a new genetic risk factor for AMI. Our results imply that homozygosity for the Glu-Asp298 polymorphism of the eNOS gene may be involved in predisposition to AMI. However, this polymorphism can explain only a small part of genetic susceptibility to AMI. Further studies are needed to characterize the molecular mechanisms by which eNOS is involved in susceptibility to AMI.

Acknowledgments

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References


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