Coronary spasm plays an important role in the pathogenesis of not only variant angina but also ischemic heart diseases in general, including other forms of angina pectoris, acute myocardial infarction, and sudden death.1–3 However, the precise mechanism(s) responsible for coronary spasm remains to be elucidated.

See p 2855

In the endothelium of both animals and humans, synthesis of nitric oxide (NO) from the amino acid l-arginine is catalyzed by the enzyme endothelial NO synthase (eNOS), and the continuously generated NO serves to maintain basal vascular tone.5,6 In patients with coronary spasm, the basal tone of the coronary arteries is increased, and they are hyperresponsive to the vasodilator action of nitroglycerin.7–9 Nitrovasodilators, including nitroglycerin, reduce vasomotor tone by way of their in vivo conversion to NO, and the hyperreactivity to nitrovasodilators seen in patients with coronary spasm is consistent with decreased endothelial release of NO.4,11

The effects of acetylcholine (ACh) on coronary arterial tone provide further evidence of decreased NO synthesis in patients with coronary spasm. We and others have shown that intracoronary injection of ACh elicits severe vasoconstriction in these patients, whereas ACh causes coronary vasodilation in subjects with healthy coronary arteries. ACh-induced vasodilatation is mediated by NO released from the endothelium. Therefore, it seems possible that the endothelium in the coronary arteries of patients with coronary spasm is dysfunctional and NO release in response to ACh is decreased. Indeed, we recently showed that basal, ACh-stimulated, and flow-dependent NO activities are decreased in both coronary and brachial arteries of the patients with coronary spasm.5,19,20

The prevalence of coronary spasm appears to be higher in the Japanese population than in whites, which suggests that genetic factors may be involved in its pathogenesis. We therefore hypothesized that eNOS may play an important role in the pathogenesis of coronary spasm and that there may be possible mutations of the eNOS gene in patients with coro-
coronary spasm. In this study, we examined possible mutations in the 5′-flanking region of the eNOS gene that may be associated with coronary spasm.

**Methods**

**Study Patients**

To search for possible mutations in the eNOS gene, we performed polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis in 11 patients (11 women; mean age, 49 years; range, 33 to 65 years) with coronary spasm and 9 control subjects with atypical chest pain (3 men and 6 women; mean age, 52 years; range, 35 to 70 years). We defined coronary spasm as an abnormal contraction of an epicardial coronary artery resulting in myocardial ischemia and not by an abnormal constrictor response to Ach. All 11 patients had experienced episodes of spontaneous angina associated with ST-segment changes. In this study, coronary spasm associated with ischemic ST-segment changes was angiographically documented during cardiac catheterization; spasm occurred spontaneously in 2 patients and was induced by intracoronary injection of Ach. All control subjects also underwent coronary angiography for evaluation of chest pain. They exhibited angiographically normal or nearly normal coronary arteries and did not show coronary spasm after intracoronary injection of Ach.

All subjects enrolled in the study gave informed consent. The study protocol was in agreement with the guidelines of the ethics committee at our institution.

**PCR-SSCP Analysis and Direct Sequencing**

Genomic DNA was prepared from blood leukocytes. PCR-SSCP analysis was carried out as reported previously. Briefly, on the basis of its known genomic structure, we divided the 5′-flanking region of the eNOS gene (nucleotide positions −1533 to +44) into 14 overlapping segments designated F1 to F14 (Figure 1). The sequences of the primers used in the PCR-SSCP analysis are shown in Table 1. Heat-denatured PCR products were separated by electrophoresis on polyacrylamide gels at 3 W for 16 to 18 hours under 3 gel conditions: 5% and 10% glycerol at 4°C and 5% glycerol at room temperature. The PCR products were then directly sequenced with an automated sequencer (ABI 373S, USA), and all DNA sequences were confirmed by reading of both DNA strands.

**Allele-Specific Oligonucleotide Method**

The allele-specific oligonucleotide method was used to examine the frequency at which T→C, A→G, or T→A mutations appeared in the larger test population. The F7, F9, and F14 fragments were each amplified by PCR under the same conditions as were used for PCR-SSCP analysis. Each PCR product was blotted in duplicate onto nylon membranes. Hybridization was then done with radiolabeled oligonucleotide probes corresponding to either the normal gene sequence (5′-GGGTCAAGCCAGCCAGGAAA-3′, 5′-AGTTCTTGTGCTACAGG-3′, or 5′-GACAACAGACCCCCAAGCT-3′), which included T→C, A→G, and T→A, respectively, or the mutant gene sequence (5′-GGGTCAAGCAGCCAGGAAA-3′, 5′-AGTTCTTGTGCTACAGG-3′, or 5′-GACAACAGACCCCCAAGCT-3′), which included C→T, G→A, and A→G, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>F1 (210 bp)</th>
<th>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</th>
<th>F8 (220 bp)</th>
<th>5′-CCTCAGATGACACAGAACACTA 5′-ACCCATTCTAGCAGAGCCCA</th>
<th>F1 (210 bp)</th>
<th>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</th>
<th>F8 (220 bp)</th>
<th>5′-CCTCAGATGACACAGAACACTA 5′-ACCCATTCTAGCAGAGCCCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2 (241 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F9 (208 bp)</td>
<td>5′-TCATCTTGTAGAGGTCTCGAA 5′-TGATGCCCTGGTGGGAGCAT</td>
<td>F2 (241 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F9 (208 bp)</td>
<td>5′-TCATCTTGTAGAGGTCTCGAA 5′-TGATGCCCTGGTGGGAGCAT</td>
</tr>
<tr>
<td>F3 (211 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F10 (211 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F3 (211 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F10 (211 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
</tr>
<tr>
<td>F4 (188 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F11 (246 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F4 (188 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F11 (246 bp)</td>
<td>5′-TGGGCTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
</tr>
<tr>
<td>F5 (205 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F12 (253 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F5 (205 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F12 (253 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
</tr>
<tr>
<td>F6 (230 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F13 (278 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F6 (230 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F13 (278 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
</tr>
<tr>
<td>F7 (236 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F14 (221 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F7 (236 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
<td>F14 (221 bp)</td>
<td>5′-CCTAGTCTAGGCTTTAGAGGCTT 5′-CCGCTCTTCAGATGTGCCCAT</td>
</tr>
</tbody>
</table>

*Top of each pair is sense primer; bottom, antisense primer in each primer set. Base-pair length of amplified fragments in parentheses.*
Construction of eNOS Reporter Vectors
DNA fragments from the 5′-flanking region of the eNOS gene (nucleotide positions −1600 to +26), with and without the 3 previously described point mutations, were amplified by PCR with the genomic DNA. Upstream and downstream primers containing KpnI and XhoI sites, respectively, were synthesized, and the PCR products were digested and fused into the luciferase reporter gene vector PGV-B2 (Toyo Inc). The eNOS promoter/luciferase reporter gene plasmids containing either the normal sequence or all 3 mutations were designated PGV-eNOSwt and PGV-eNOSmt, respectively. To construct eNOS promoter/luciferase reporter gene plasmids, each with only 1 of the 3 mutations, the BsmI/XhoI DNA fragment (−855 to +44), BsmI/BsmI DNA fragment (−1333 to −855), or KpnI/BsmI DNA fragment (−1600 to −1333) were excised from PGV-eNOSwt and substituted for the corresponding fragments in PGV-eNOSmt. The eNOS promoter/luciferase reporter gene plasmids, containing either the T786→C, A952→G, or T1460→A mutations, were designated PGV-eNOSmt1, PGV-eNOSmt2, and PGV-eNOSmt3, respectively.

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were cultured in medium supplemented with 2% FBS at 37°C under an atmosphere of 5% CO2. Before transfection, the cells were transferred to 6-well dishes and allowed to grow until they were ∼80% confluent, at which time they were transfected. Cells were used for up to 3 passages.

Luciferase Reporter Gene Assays
Transient transfections were performed with TransIT-LT2 (Pan Vera Corp) according to the manufacturer’s instructions. The promoter/luciferase reporter gene (1.5 μg) was cotransfected with 1 μg β-actin-driven β-galactosidase reporter plasmid. After transfection, promoter activity was evaluated in HUVECs incubated for 48 hours under normoxic conditions. For the hypoxia-stimulation experiment, transfected HUVECs were exposed to normoxia for 24 hours and then exposed to hypoxia (1% O2, 5% CO2, and 94% N2) for an additional 24 hours. HUVECs were then pelleted, resuspended in reporter lysis buffer (LCβ-PGC: Toyo Inc), and centrifuged to remove cell debris. The extracts were then used for measurement of luciferase (20 μL) or β-galactosidase (10 μL) activities. Luciferase activity was measured in triplicate with a luminometer (Lumat LB 9507; Prof Dr Berthold, GmbH), and β-galactosidase activity was measured spectrophotometrically (at 410 nm). All data were normalized as relative light units/β-galactosidase activity.

Statistical Analysis
When the clinical characteristics of the study patients were considered, hypertension was operationally defined as blood pressures >140/95 mm Hg, and diabetes mellitus was defined as fasting blood glucose levels >140 mg/dL or >200 mg/dL in an oral glucose tolerance test.

Continuous variables were compared by 2-tailed unpaired t tests. Categorical variables were compared by χ2 analysis with Fisher’s exact probability. Odds ratios (approximating relative risk) were calculated as an index of the association of the eNOS genotype (normal homozygote, heterozygote, abnormal homozygote) with the phenotype of coronary spasm. For each odds ratio, we calculated a 2-tailed probability value and 95% CIs. The effects of the mutant allele were assumed to be either additive, dominant, or recessive; values for the additive effect were predicted by the Hardy-Weinberg equilibrium.

Multiple logistic regression analysis with forward stepwise selection (Wald) was performed with SPSS Advanced Statistics 6.1 for the Macintosh (SPSS Japan Inc). Independent variables were coded as the following dummy variables: genotype, 0 for normal homozygote and 1 for abnormal homozygotes or heterozygotes; sex, 0 for female and 1 for male; age, 0 for ≤60 years and 1 for >60 years; body mass index, 0 for <26 kg/m2 and 1 for ≥26 kg/m2; hypercholesterolemia, 0 for <240 mg/dL and 1 for ≥240 mg/dL; cigarette smoking, 0 for nonsmokers and 1 for smokers; hypertension, 0 for normotension and 1 for hypertension; and diabetes mellitus, 0 for absence and 1 for presence.

Promoter activities were assessed as a function of normalized luciferase activities in pairs of experiments and were compared by 2-tailed unpaired t tests. Statistical significance was defined as P<0.05.

Results
Detection and Identification of Mutations and Sequencing of the 5′-Flanking Region of the eNOS Gene
To identify possible mutations in the 5′-flanking region of the eNOS gene, we performed PCR-SSCP analysis on genomic DNA extracts from 11 typical patients with coronary spasm and 9 control subjects. In 4 of the 11 patients with coronary spasm, we discovered the presence of variant bands in PCR products of the F7, F9, and F14 fragments, but no variant bands were seen in the 9 control subjects (Figure 2). We subsequently sequenced both the substituted and unsubstituted fragments (Figure 3) and identified 3 point mutations: a T-to-C mutation at nucleotide position −786, an A-to-G mutation at nucleotide position −922, and a T-to-A mutation at nucleotide position −1468. These results were confirmed by complete sequencing of the eNOS gene from nucleotide position −1533 to +44. The sequence of the gene from subjects with variant bands (mutant-type) was virtually identical to that of the control subjects except for the 3 substitutions described above. Nucleotide positions are expressed relative to the previously described transcriptional initiation site.
Association of eNOS Gene Mutation With Coronary Spasm

Each point mutation was examined in 174 patients and 161 control subjects by the allele-specific oligonucleotide method. This analysis revealed that the 3 mutations were always linked with each other (100% concordance); thus, the allele frequency was identical among the 3 mutations. The eNOS allele/T<sup>-786</sup>C, A<sup>-922</sup>G, and T<sup>-1468</sup>A homozygotes, heterozygotes, and normal homozygotes were present in 3 (2%), 48 (28%), and 123 (70%) of the 174 patients with coronary spasm, respectively. Conversely, the abnormal homozygotes, heterozygotes, and normal homozygotes were found in none (0%), 11 (7%), and 150 (93%) of the 161 control subjects, respectively. The frequencies of the genotypes were in agreement with those predicted by the Hardy-Weinberg equilibrium (P > 0.05). When the additive and dominant effect of the mutant eNOS allele was analyzed, the incidence of the abnormal allele was significantly higher in the coronary spasm group than in the control group (P < 0.0001; Table 2).

Clinical Characteristics of the Study Patients and Multiple Logistic Regression Analysis

The incidences of coronary risk factors, including age, sex, total cholesterol, hypertension, diabetes mellitus, body mass index, and cigarette smoking, were compared in the control and coronary spasm groups. The incidence of cigarette smoking was significantly higher in the coronary spasm group than in the control group (P = 0.0009), but there were no significant differences among the other risk factors for the 2 groups (Table 3). We then performed multiple logistic regression analysis with forward stepwise selection using all the clinical risk factors and the mutant allele of the eNOS gene. This analysis revealed that the most predictive independent risk factor for coronary spasm was the mutant allele (P = 0.0001), followed by cigarette smoking (P = 0.0093; Table 4).

Promoter Activities of the eNOS Gene

Luciferase reporter gene assays using the 5' flanking region of the eNOS gene containing the 3 mutations showed a

---

**TABLE 2. Frequencies of the Genotypes of the eNOS Gene**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 161)</th>
<th>Coronary Spasm (n = 174)</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS mutant allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&lt;sup&gt;-786&lt;/sup&gt;→C,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sup&gt;-922&lt;/sup&gt;→G,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&lt;sup&gt;-1468&lt;/sup&gt;→A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal homozygote</td>
<td>0/161 (0%)</td>
<td>3/174 (2%)</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Heterozygote</td>
<td>11/161 (7%)</td>
<td>48/174 (28%)</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Normal homozygote</td>
<td>150/161 (93%)</td>
<td>123/174 (70%)</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Additive effect</td>
<td>...</td>
<td>...</td>
<td>5.19 (1.85–17.60)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dominant effect</td>
<td>...</td>
<td>...</td>
<td>5.65 (2.82–14.66)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Recessive effect</td>
<td>...</td>
<td>...</td>
<td>Infinite</td>
<td>0.1798</td>
</tr>
</tbody>
</table>

Odds ratios (approximating relative risk) were calculated as a measure of the association of the eNOS genotype (abnormal homozygote, heterozygote, normal homozygote) with the phenotype of coronary spasm, with the effects of the abnormal allele assumed to be additive (abnormal allele vs normal allele), dominant (abnormal homozygote and heterozygote combined vs normal homozygote), and recessive (abnormal homozygote vs heterozygote and normal homozygote combined).

---

**TABLE 3. Clinical Characteristics of the Study Patients for the Association Study**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 161)</th>
<th>Coronary Spasm (n = 174)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59±11</td>
<td>61±11</td>
<td>0.1419</td>
</tr>
<tr>
<td>Male/female, n (ratio)</td>
<td>91/70 (1.3)</td>
<td>90/84 (1.1)</td>
<td>0.3787</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>192±33</td>
<td>193±37</td>
<td>0.9567</td>
</tr>
<tr>
<td>Hypertension</td>
<td>48/160 (30%)</td>
<td>52/172 (30%)</td>
<td>0.9632</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>24/160 (15%)</td>
<td>24/172 (14%)</td>
<td>0.7864</td>
</tr>
<tr>
<td>Body mass index, kg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>23±3</td>
<td>23±3</td>
<td>0.9960</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>57/160 (37%)</td>
<td>92/171 (54%)</td>
<td>0.0009</td>
</tr>
</tbody>
</table>
During the initial screening of this study, we discovered 3 eNOSwt, transfected cell groups regardless of the construct (pPGV-24 hours, promoter activity was markedly augmented in all significant decrease (−22 ± 6%) in transcriptional activity compared with those that did not contain the mutations. To determine which mutation was responsible for the reduction in promoter activity, fragments of the 5′-flanking regions of the mutant eNOS, each containing only 1 of the 3 point mutations, were fused with the luciferase reporter gene. As shown in Figure 4, the T−786→C mutation reduced the promoter activity significantly (−52 ± 11%) in comparison with the normal sequence, whereas neither the A−922→G nor the T−1468→A mutation reduced promoter activity.

When transfected HUVECs were exposed to hypoxia for 24 hours, promoter activity was markedly increased in all transfected cell groups regardless of the construct (pPGV-eNOSwt, +110 ± 32%; pPGV-eNOSmt1, +38 ± 11%; pPGV-eNOSmt1. +69 ± 26%; pPGV-eNOSmt2, +102 ± 26%; and pPGV-eNOSmt3, +92 ± 58%). Moreover, pPGV-eNOSmt and pPGV-eNOSmt1 both exhibited substantial reductions (−50 ± 4% and −62 ± 11%, respectively) in transcriptional activity compared with pPGV-eNOSwt.

**Discussion**

During the initial screening of this study, we discovered 3 mutations (T−786→C, A−922→G, and T−1468→A) in the 5′-flanking region of the eNOS gene in the patients with coronary spasm. To further examine the relationship between these mutations and coronary spasm, we searched for the mutations in 174 patients with coronary spasm and 161 control subjects. We found that the mutations were always linked with each other and that they occurred more frequently in coronary spasm patients than in the control group. The distribution of the mutant allele was compatible with the Hardy-Weinberg equilibrium, indicating that the screening method was appropriate. Multiple logistic regression analysis of the mutant allele of the eNOS gene and other coronary risk factors revealed that the independent risk factor that best predicted the incidence of coronary spasm was the mutant allele of the eNOS gene.

To examine the extent to which each of the 3 point mutations modified eNOS gene expression, we performed luciferase reporter gene assays. This analysis revealed that only the T−786→C mutation suppressed eNOS gene transcription. We also showed that hypoxia increased eNOS promoter activity, which is in agreement with previous findings. The decrease in eNOS transcription is consistent with the notion that endothelial NO production is reduced in patients carrying the T−786→C mutation. Thus, the present results strongly suggest that the presence of the eNOS gene mutant allele reduces endothelial production of NO in the coronary arteries and predisposes the patients carrying the mutant allele to coronary spasm. NO is also known to suppress production of the potent vasoconstrictors endothelin and angiotensin II, which also induce vascular smooth muscle cell proliferation. Consequently, deficiency of NO production in vessels in patients with the mutant allele may also result in increased synthesis of these vasoconstrictors and smooth muscle cell proliferation, thereby leading to increased vessel reactivity. However, controversy still exists as to whether or not endothelial NO is deficient in patients with coronary spasm, and further studies are needed. There may be differences in the clinical characteristics of patients who are

**Table 4. Multiple Logistic Regression Analysis With Forward Stepwise Selection (Wald)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SEM</th>
<th>Wald</th>
<th>df</th>
<th>Significance</th>
<th>R</th>
<th>Exp(β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS mutant allele</td>
<td>1.6936</td>
<td>0.3577</td>
<td>22.4206</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>0.2133</td>
<td>5.4391</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>0.6173</td>
<td>0.2373</td>
<td>6.7647</td>
<td>1</td>
<td>0.0093</td>
<td>0.1030</td>
<td>1.8539</td>
</tr>
<tr>
<td>Constant</td>
<td>−0.4780</td>
<td>0.1623</td>
<td>8.6736</td>
<td>1</td>
<td>0.0032</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

eNOS mutant allele containing T−786→C, A−922→G, and T−1468→A mutations; abnormal homozygous carriers and heterozygous carriers combined.

**Figure 4. Effects of various promoter constructs and hypoxia on promoter activity of eNOS gene.** Promoter activity is expressed as luciferase activity/galactosidase activity. pPGV-eNOSwt indicates eNOS promoter construct containing normal sequence; pPGV-eNOSmt, eNOS promoter construct containing all 3-point mutations; and pPGV-eNOSmt1, pPGV-eNOSmt2, and pPGV-eNOSmt3, eNOS promoter constructs containing T−786→C, A−922→G, and T−1468→A mutations, respectively. *P<0.05 vs pPGV-eNOSwt under each condition. #P<0.05 vs each construct under normoxic conditions. Results are expressed as mean±SEM.
homozygous for the mutant eNOS allele and those who are heterozygous. We found 3 homozygous subjects among the 335 participants in this association study. These 3 patients suffered from severe coronary spasm, and 2 of the patients had also experienced acute myocardial infarction without organic stenosis. It is possible, therefore, that coronary spasm may be more severe and prolonged in homozygous subjects than in those who are heterozygous.

In 11 (7%) of the 161 control subjects carrying mutant eNOS alleles, coronary spasm could not be induced by intracoronary injection of ACh in those individuals, although vasoconstrictor responses to ACh were increased. Six of the subjects were smokers, and the histories of all 11 subjects indicated that they had experienced coronary spasm at some time. Because there is daily, monthly, and yearly as well as diurnal variation in the occurrence of coronary spasm,2-3,32 it is possible that these patients were not in the active phase of the ailment at the time of study. It is also possible that they may be destined to suffer from coronary spasm in the future, because they are relatively young (mean age, 50 years; range, 31 to 62 years).

We and others have shown that smoking impairs endothelium-dependent coronary arterial dilation in humans33-35 and that smoking is a major risk factor for coronary spasm.33,36 We obtained the same result in this study. Thus, both genetic and environmental factors are involved in the pathogenesis of coronary spasm. We also performed the association study in both the smoking and nonsmoking groups. Our preliminary analysis revealed that the T→C mutation is significantly associated with coronary spasm in each group (odds ratio of additive effect, 3.79 in the smoking group and 3.05 in the nonsmoking group).

In our recent study, we found a missense Glu298Asp variant in the eNOS gene and found that the variant was associated with coronary spasm.37 In the analyses of the frequencies of the T→C and Glu298Asp variants in >1000 DNA samples, there was no relationship in the linkage between the 2 eNOS variants. Recently, Wang et al38 reported that the smoking-dependent risk of ischemic heart disease is associated with a polymorphism in intron 4 of the eNOS gene. The relationship between the 3 mutations in the 5′-flanking region that we observed and the polymorphism in intron 4 of the eNOS gene remains to be elucidated. Thus, further analyses will be necessary to examine the other genetic risk factors for coronary spasm.

In conclusion, the present study demonstrates that 3 distinct point mutations occur in the 5′-flanking region of the eNOS gene (T→C, A→G, and T→A) and that these mutations are strongly associated with coronary spasm. Furthermore, we demonstrated that the T→C mutation substantially reduces promoter activity of the eNOS gene. Taken together, these findings strongly suggest that the T→C mutation in the eNOS gene compromises endothelial NO synthesis and predisposes the patients with the mutant allele to coronary spasm.

References


T-786→C Mutation in the 5'-Flanking Region of the Endothelial Nitric Oxide Synthase Gene Is Associated With Coronary Spasm
Masafumi Nakayama, Hirofumi Yasue, Michihiro Yoshimura, Yukio Shimasaki, Kiyotaka Kugiyama, Hisao Ogawa, Takeshi Motoyama, Yoshihiko Saito, Yoshihiro Ogawa, Yoshihiro Miyamoto and Kazuwa Nakao

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