Coronary Artery Spasm and the Polymorphisms of the Endothelial Nitric Oxide Synthase Gene

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Background Coronary artery spasm plays an important role in the pathogenesis of vasospastic angina, and contributes to the development of several acute coronary syndromes. Endothelial nitric oxide synthase (eNOS) catalyzes the synthesis of nitric oxide, which regulates vascular tone, and may be related to coronary vasospasm. The present study investigated whether coronary spasm is related to particular polymorphisms of the eNOS gene.

Methods and Results Spasm provocation by serial infusions of acetylcholine was performed on 165 patients who were clinically suspected of having angina. In both study patients and healthy controls (n=400), genomic polymorphisms of the ecNOS gene were determined by using polymerase chain reaction. Quantitative luminal diameter measurements of the 3 major coronary arteries were initially obtained before and after acetylcholine injection, and then after isosorbide dinitrate injection, by using a computer-assisted analysis system. Logistic multiple regression analysis identified the a/a or a/b genotype in intron 4 of ecNOS (NOS4a: p=0.0431, odds ratio (OR) 2.43) and diabetes mellitus (p=0.0060, OR 4.88) as significant predictors of coronary spasm. In the patients with NOS4a, both the induced and spontaneous contractions were augmented.

Conclusion The present study results indicated that NOS4a could be a good marker for coronary artery spasm. (Circ J 2006; 70: 409-413)

Key Words: Acetylcholine; Coronary disease; Polymerase chain reaction; Quantitative coronary angiography

Coronary spasm plays an important role in the pathogenesis of vasospastic angina. It may also contribute to the development of other acute coronary syndromes. Acute myocardial infarction can occur at the site of atherosclerotic plaques that do not narrow the coronary artery lumen. Plaque rupture followed by thrombosis, and vasoconstriction at the site of the plaque, are involved in coronary artery occlusion. Coronary vasospasm has been suggested to occur as a result of reduced endothelium-dependent vasodilatation and hyperactivity of vascular smooth muscle cells. Kugiyama et al demonstrated a deficiency in the vasodilatory activity of endothelial nitric oxide (NO) in spastic arteries. Bioavailability of NO is biosynthesized from L-arginine by a family of NO synthases (NOSs). Endo-smooth muscle cells. Kugiyama et al demonstrated a dependent vasodilatation and hyperactivity of vascular deficiency in the vasodilatory activity of endothelial nitric oxide (NO) in spastic arteries. Bioavailability of NO is biosynthesized from L-arginine by a family of NO synthases (NOSs). Endothelial NO synthase (eNOS), or NOS3, is constitutively expressed in the endothelium and catalyzes the synthesis of NO that regulates vascular tone. Wang et al reported that a polymorphism of intron 4 of the ecNOS gene (27-bp tandem repeats: a allele (4 repeats, rare, NOS4a), b allele (5 repeats, common)) is associated with the smoking-dependent risk for coronary artery disease. The same research group also reported that these genotypes account for over 25% of the basal plasma NO production. Other polymorphisms, G894T (Glu298Asp) in exon 7 and T-786C in the 5' flanking region of the ecNOS gene, have been reported to be a risk factor for coronary artery disease, including coronary spasm. The aim of this study is to assess the relationship between the polymorphisms of ecNOS genes, especially NOS4a, and coronary vascular behavior at spasm provocation tests in detail, by using quantitative coronary angiography (QCA).

Methods

Study Patients
Of 1,094 Japanese patients who underwent coronary angiography at the Cardiovascular Institute Hospital, Tokyo, Japan, 165 patients were clinically suspected of having angina and did not have fixed stenoses in their coronary arteries. Spasm provocation by serial infusions of acetylcholine (Ach) was performed on these 165 patients according to the method of Yasue et al. A coronary arteriogram was obtained when ST segment changes and/or chest pain appeared or 1 min after each injection. When spasm was documented, 0.1 mg nitroglycerin (NTG) was injected into the right coronary artery and then 25 and 50 μg into the left coronary artery. A coronary arteriogram was obtained when ST segment changes and/or chest pain appeared or 1 min after each injection. When spasm was documented, 0.1 mg nitroglycerin (NTG) was injected into the left coronary artery and then 25 and 50 μg into the right coronary artery. A coronary arteriogram was obtained when ST segment changes and/or chest pain appeared or 1 min after each injection. When spasm was documented, 0.1 mg nitroglycerin (NTG) was injected into the coronary artery if the attack did not disappear within 2 min. A venous blood sample was obtained from the 165 patients. Blood samples were also obtained from 400 healthy subjects at the time of the health examination at Mitsui Memorial Hospital to determine the population incidence of polymorphisms of the ecNOS gene among...
Japanese people. Informed written consent was obtained from all subjects after the study was fully explained, and which was approved by the Ethics Committees of the Cardiovascular Institute Hospital and Mitsui Memorial Hospital. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Genetic Analysis

Venous blood samples were collected in tubes containing Na2EDTA. Genomic DNA was extracted with the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The genomic polymorphisms (27-bp tandem repeats in intron 4, G894T in exon 7) of the eNOS gene were determined by using polymerase chain reaction (PCR) according to the methods previously described by Wang et al and Shimasaki et al, respectively.16,19

QCA

All medications except sublingual NTG were withdrawn at least 3 days prior to the spasm study. No study patient had taken NTG within 6 h of the study. The relative position among focal spot, patient, and height of the image tube was kept constant during the study. By using a computer-assisted analysis system (Cardio 500, Kontron Elektronik, Eching, Germany), quantitative luminal diameter measurements of the coronary artery were made by an observer who was blinded to the study subjects.8,25 The luminal diameter was measured at the middle segments between the side branches (anatomical landmarks) of the left anterior descending, left circumflex, and right coronary arteries. Both the mean diameter and the narrowest diameter of the 3 middle segments were determined before (control) and when ST segment changes and/or chest pain appeared or 1 min after Ach injection, and then after isosorbide dinitrate (ISDN; c,f,i) injection. Note that there may be a discrepancy between the clinical symptoms of coronary spasm and QCA measurements of the middle segments. Following acetylcholine injection, subtotal occlusion (h, arrow heads), and spasm induction, are seen at the distal RCA, while the middle segment (h, between the arrows), the site of QCA measurement, was constricted only partially.

Fig 1. Quantitative coronary angiography (QCA) measurements. Representative images of the coronary arteries of a patient who was suspected of having angina. The luminal diameters of the middle segments between the side branches (between the arrows), of the left anterior descending (a,b,c), left circumflex (d,e,f), and right coronary arteries (RCA; g,h,i), were measured. Both the mean diameter and the narrowest diameter of the 3 middle segments were determined before (Control; a,d,g) and after acetylcholine (b,e,h) injection, and then after isosorbide dinitrate (ISDN; c,f,i) injection. Note that there may be a discrepancy between the clinical symptoms of coronary spasm and QCA measurements of the middle segments. Following acetylcholine injection, subtotal occlusion (h, arrow heads), and spasm induction, are seen at the distal RCA, while the middle segment (h, between the arrows), the site of QCA measurement, was constricted only partially.
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Continuous variables. Analysis was performed with SPSS software (SPSS, Chicago, IL, USA). A 2-sided p value of less than 0.05 was considered to be statistical significance. Data are expressed as mean±SEM unless otherwise specified.

Results

The clinical characteristics of the 165 patients, who were suspected of having angina, are summarized in Table 1. In the Spasm-Positive group, patients were older, had higher body mass indexes, and a higher incidence of diabetes mellitus. The frequency (25%) of the a/a or a/b intron 4 genotype (NOS4a) in the Spasm-Positive group was significantly higher than that in the Spasm-Negative group (12.3%), which was equivalent to that of the healthy control group (16.6%). There were no significant differences in the frequency of the T allele of G894T in exon 7 among the Spasm-Positive group (TG + TT: 9.8%), Spasm-Negative group (TG + TT: 15.1%), and control group (TG + TT: 17.3%). Multiple logistic regression analysis with backward selection identified NOS4a (p=0.0431, odds ratio (OR) 2.43) and diabetes mellitus (p=0.0060, OR 4.88) as significant predictors of coronary spasm (Table 2). Factors that were not significant for prediction of spasm provocation included sex, age, BMI, T-C, HDL-C, TG, hypertension, current smoking, obesity, hyperlipidemia, hyperuricemia, Introns 4 NOS (aa/ab/bb), Exon7 NOS (GG/GT/TT), Hypertension, Current smoking, Diabetes mellitus, Obesity, Hyperlipidemia, Hyperuricemia, Introns 4 NOS (aa/ab/bb), Exon7 NOS (GG/GT/TT).

Table 1 Clinical Parameters of Spasm-Positive and Spasm-Negative Subjects

<table>
<thead>
<tr>
<th></th>
<th>Spasm-positive (n=92)</th>
<th>Spasm-negative (n=73)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>61/31</td>
<td>49/24</td>
<td>0.912</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.0±7.4</td>
<td>56.7±10.2</td>
<td>0.022*</td>
</tr>
<tr>
<td>BMI</td>
<td>23.9±2.5</td>
<td>22.4±2.7</td>
<td>0.005*</td>
</tr>
<tr>
<td>T-C (mmol/L)</td>
<td>5.28±0.85</td>
<td>5.17±0.93</td>
<td>0.567</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.49±0.49</td>
<td>1.52±0.62</td>
<td>0.709</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.72±1.21</td>
<td>1.51±0.71</td>
<td>0.176</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35.9%</td>
<td>28.8%</td>
<td>0.344</td>
</tr>
<tr>
<td>Current smoking</td>
<td>57.6%</td>
<td>47.9%</td>
<td>0.220</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>21.7%</td>
<td>5.5%</td>
<td>0.002*</td>
</tr>
<tr>
<td>Obesity</td>
<td>14.1%</td>
<td>6.8%</td>
<td>0.124</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>25%</td>
<td>13.7%</td>
<td>0.065</td>
</tr>
<tr>
<td>Hyperuricemia</td>
<td>13%</td>
<td>8.2%</td>
<td>0.315</td>
</tr>
<tr>
<td>Intron 4 NOS (aa/ab/bb)</td>
<td>0/23/99</td>
<td>0/9/64</td>
<td>0.035**</td>
</tr>
<tr>
<td>Exon7 NOS (GG/GT/TT)</td>
<td>83/8/1</td>
<td>62/10/1</td>
<td>0.315</td>
</tr>
</tbody>
</table>

BMI, body mass index; T-C, serum total cholesterol; HDL-C, serum high density lipoprotein cholesterol; TG, triglycerides; †, aa + ab vs bb; ‡, GG vs GT + TT.

Table 2 Multiple Logistic Regression Analysis of Risk Factors for Coronary Spasm in the Acetylcholine Provocation Test

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% Confidence interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS4a</td>
<td>2.43</td>
<td>1.03–5.74</td>
<td>0.0431</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4.88</td>
<td>1.57–15.14</td>
<td>0.0060</td>
</tr>
</tbody>
</table>

NOS4a, 4 repeats in intron 4 of the endothelial nitric oxide synthase gene.

Fig 2. Summary of results of quantitative coronary angiography measurements. In each of the 165 patients, the luminal diameter of 3 middle segments was determined before (control) and after acetylcholine (Ach) injection, and after isosorbide dinitrate (ISDN) injection. Control/ISDN: Ratio of the luminal diameter under the control condition and that after ISDN injection. This parameter represents the degree of spontaneous spasm (basal coronary tone). Ach/ISDN: Ratio of the luminal diameter after Ach provocation and that after ISDN injection. This parameter represents the degree of induced (maximum) spasm. Mean: Mean of the ratios of diameters of the 3 middle coronary segments (right coronary arteries, left anterior descending, left circumflex). Min.: The smallest ratio of diameters among the 3 middle coronary segments. The solid bars represent data on the patients with a/a or a/b genotype. The dotted bars represent data on the patients with b/b genotype.

Fig 3. Serum nitrite/nitrate (NOx) concentrations. The serum NOx concentrations of the patients in all 3 genotype groups were similar. The NOx level of those with intron 4 a/a or a/b was 26.0±4.6 μmol/L, and the NOx level of those with intron 4 b/b was 20.4±2.1 μmol/L (p=0.29). The NOx level of those with exon 7 GG was 21.2±2.5 μmol/L, while the NOx level of those with exon 7 TG or TT was 20.6±4.9 μmol/L (p=0.92). The serum NOx concentration of the Spasm-Positive group (18.5±2.4 μmol/L) was slightly lower than that of the Spasm-Negative group (24.7±3.0 μmol/L); however, it did not reach statistical significance (p=0.10).
tation by logistic multiple regression analysis were: age (>60 years), male gender, body mass index (>26), hypertension, current smoking, hyperlipidemia including current lipid-lowering therapy, hyperuricemia and T allele of G894T in exon 7 of the ecNOS gene.

Fig 2 summarizes the vasoactive response of 3 major coronary arteries upon Ach infusion. In the patients who had the a/a or a/b genotype of intron 4, both the induced and spontaneous contractions, which are represented by the ratio of the luminal diameters following Ach or ISDN administration (Ach/ISDN) and the ratio of the luminal diameter in the control condition to that following ISDN administration (Control/ISDN), respectively, were augmented in comparison with those in the patients with the b/b genotype in intron 4. This phenomenon was seen in the most spastic segment as well as in all 3 coronary vessels. The final amount of Ach loaded in the provocation test was not different between groups.

The serum NOx concentrations of the patients in all 3 genotype groups were similar (Fig 3). The NOx level of those with intron 4 a/a or a/b was 26.0±4.6 μmol/L, and the NOx level of those with intron 4 b/b was 20.4±2.1 μmol/L (p=0.29). The NOx level of those with exon 7 GG was 21.2±2.5 μmol/L, while the NOx level of those with exon 7 TG or TT was 20.6±4.9 μmol/L (p=0.92). The serum NOx concentration of the Spasm-Positive group (18.5±2.4 μmol/L) was slightly lower than that of the Spasm-Negative group (24.7±3.0 μmol/L); however, it did not reach statistical significance (p=0.10).

Discussion
A detailed QCA analysis demonstrated that the coronary arteries of patients with intron 4 a/a or a/b genotype showed significantly higher basal tone and greater spastic reaction against Ach than patients with intron 4 b/b genotype. The finding that this spastic response was seen not only in the most spastic segment but also in 2 other coronary vessels indicates that this response occurred at a localized segment as well as in the entire coronary tree. The precise mechanism through which coronary artery spasm occurs remains to be elucidated. Endothelial dysfunction has been considered to play an important role in coronary artery spasm. Previous studies demonstrated that the diameter of spastic coronary arteries is smaller at baseline, but comparable with the diameter of control arteries after NTG injection. This indicates that the basal tone in the spastic arteries is increased. Kugiyama et al demonstrated that upon intracoronary infusion of N-monomethyl-L-arginine, an inhibitor of NOS, the control coronary arteries constricted, but the spasm coronary arteries did not. These results suggest that deficient NO release is an important factor in the pathogenesis of coronary artery spasm. The NOx concentration of the Spasm-Positive group in the present study was slightly lower than that of the Spasm-Negative group. This result is consistent with the reports mentioned above. There is still controversy as to the relationship between the genomic polymorphism of intron 4 of the ecNOS gene and the plasma NOx level.

In the present study, the serum NOx concentrations of the patients in all genotype groups were similar. Coronary vasospasm is caused by hyperactivity of vascular smooth muscle cells as well as reduced endothelium-dependent vasodilatation. It has not been elucidated whether the genomic polymorphism of intron 4 of the ecNOS gene is related to either of these factors. In association studies between particular genes and traits, positive association may also occur if the allele does not cause the trait but is in linkage disequilibrium with the actual cause.

Yoshimura et al demonstrated that the ecNOS4a allele was only significantly linked to the T-786C mutation (p<0.0001) that affected the promoter activities of ecNOS. In other series of our genomic study using microsatellite markers, we did not find any link between ecNOS4a and microsatellite markers tested within 2 Mb of ecNOS (data not shown). Therefore, we can speculate that ecNOS4a polymorphism is closely associated with T-786C mutation, or that both ecNOS4a and T-786C are associated with the true cause of the disease.

Yoshimura et al also reported that a polymorphism in exon 7 of the ecNOS gene (G894T or Glu298Asp) is associated with coronary artery spasm. In the present study, however, the frequency of the T allele in exon 7 in the Spasm-Positive and Spasm-Negative groups did not differ. This may be partly because of a difference in the study populations. Although the Japanese are thought to be racially homogeneous, the frequency of the TG + TT genotype in the control groups of studies conducted in different regions of Japan differs. In studies conducted in the western part of Japan, the frequency of the TG + TT genotype was reported to be 9.6% (Kyoto) and 11.7% (Kumamoto), while in studies conducted in the eastern part of Japan, the frequency was 17.4% (Yokohama) and 17.3% (our data: Tokyo). However, these frequencies of the TG + TT genotype in Japan are much lower than that of 60.2% in the UK.

It is well known that diabetes and impaired glucose tolerance are related to endothelial damage and then to functional abnormality of vascular tone including spasm. Smoking is also known to cause endothelial dysfunction, but the mechanism(s) is still unclear. Because both diabetes/impaired glucose tolerance and smoking are associated with endothelial dysfunction, both could be a risk factor for vasospastic angina. Although Sugishii and Takatsu reported that smoking appeared to be a major risk factor for vasospastic angina without significant coronary narrowing, current smoking was not identified as a significant predictor of coronary spasm in the present study using logistic multiple regression analysis. This discrepancy may be related to inaccurate information on smoking habits (duration and cessation), and the difference in the drug used for spasm provocation.

The limitation of the present study is that we could not determine T-786C polymorphism because we only had the documented permission of genotyping a/b of intron 4 and G894T for ecNOS.

In conclusion, the present study results indicated that the ecNOS4a genotype could be a good marker for coronary artery spasm. Further investigation is still necessary to understand the relationship between the actual cause of coronary artery spasm and the genomic structure of the ecNOS gene.

Acknowledgement
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References


