T-786C Polymorphism in Endothelial NO Synthase Gene Affects Cerebral Circulation in Smokers
Possible Gene-Environmental Interaction
Shampa Nasreen, Toru Nabika, Hiroshi Shibata, Hidehiko Moriyama, Kazuya Yamashita, Junichi Masuda, Shotai Kobayashi

Abstract—Effects of smoking on white matter lesions, such as lacunar infarction and leukoaraiosis, are still controversial. We hypothesized that the endothelial NO synthase (eNOS) genotype was a modulating factor for the effect of smoking on cerebral circulation. We took a cross-sectional population from the participants of a health examination to study the effects of smoking and a single-nucleotide polymorphism in the eNOS gene, T-786C. Smokers and nonsmokers were defined as having a smoking index (cigarettes per day times years) of ≥200 and 0, respectively. One hundred sixty-six male nonsmokers and 344 male smokers were recruited. Cerebral blood flow was measured by the $^{133}$Xe inhalation method. Genotyping of T-786C was performed by using a newly developed allele-specific polymerase chain reaction. Smokers were exposed to greater oxidative stress, as estimated by urinary F$_2$-isoprostane excretion. In smokers, CC homozygotes of T-786C showed a significant decrease of cerebral blood flow (56.6 ± 13.3, 57.6 ± 11.5, and 44.0 ± 7.2 mL/min per 100 g tissue for TT, TC, and CC, respectively; $P$ = 0.03 by ANOVA) and a significant increase of cerebrovascular resistance, whereas the eNOS genotype did not affect these parameters in nonsmokers. This result indicated that the eNOS genotype could modify cerebrovascular circulation in a general population by potentiating the adverse effect of smoking. (Arterioscler Thromb Vasc Biol. 2002;22:605-610.)

Key Words: nitric oxide synthase | polymorphism | cross-sectional studies | cerebral circulation

Smoking is an established risk factor for cardiovascular diseases. However, the adverse effect of smoking is controversial in some areas of cerebrovascular disease, such as lacunar infarction and leucoaraisis, are still controversial.1–7 Such inconsistency may imply the importance of other environmental or genetic factors in modifying the effects of smoking. Because lacunar infarction and white matter changes are based on occlusive changes in small arteries perforating into the white matter and are characterized by decreased cerebral blood flow (CBF) and increased cerebrovascular resistance (CVR), the endothelial NO synthase (eNOS) gene is a good candidate for such a factor; eNOS constitutively produces NO, a potent vasodilator as well as an antitrophic factor for the arterial wall.8–11 Several reports have indicated that eNOS plays an essential role in the regulation of basal CBF in humans and in mice.9,10 Furthermore, because smoking antagonizes the action of NO through the induction of oxidative stress,12 it is logical to hypothesize that genetic alteration of eNOS activity may be a modifier of the effect of smoking on the cerebral circulation.

Recently, the eNOS gene was studied extensively for genetic polymorphisms to elucidate its genetic role in cardiovascular diseases. As a result, a single-nucleotide polymorphism (SNP) in the promoter region, T-786C, was found to modify the promoter activity in vitro.13 In addition, this SNP and a 27-bp repeat polymorphism in intron 4, called ecNOS4 a/b, were reported to influence eNOS mRNA and protein levels in vivo.14 Furthermore, because these 2 polymorphisms were in strong linkage disequilibrium in whites,15 the effects of ecNOS4 a/b seemed to be attributed at least partly to T-786C through the alteration of eNOS expression. These observations prompted us to study whether T-786C in the eNOS gene influenced the effect of smoking on the cerebral circulation. Results of the present study indicate that the interaction between this SNP and smoking affects CBF and CVR in subjects without any signs of cerebrovascular disorders. We also confirmed complete linkage disequilibrium between T-786C and ecNOS4 a/b in a Japanese population.

Methods

Subjects
Twelve hundred seventy-two consecutive participants (727 males and 545 females) who voluntarily visited the Shimane Institute of Laboratory Medicine (S.N., T.N., J.M.), Central Clinical Laboratory (H.S., H.M.), and the Third Department of Internal Medicine (K.Y., S.K.), Shimane Medical University, and the Shimane Institute of Health Science (T.N., K.Y., S.K.), Izumo, Japan.

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TABLE 1. Demographic Data of the Studied Population

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, n</td>
<td>166</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>56.4±8.1</td>
<td>57.4±7.1</td>
<td>0.16</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.2±2.7</td>
<td>23.6±2.8</td>
<td>0.01</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>195.7±10.5</td>
<td>195.0±12.6</td>
<td>0.52</td>
</tr>
<tr>
<td>T-chol, mmol/L</td>
<td>5.38±0.91</td>
<td>5.23±0.94</td>
<td>0.08</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.31±0.36</td>
<td>1.36±0.40</td>
<td>0.20</td>
</tr>
<tr>
<td>TGs, mmol/L</td>
<td>1.39±0.78</td>
<td>1.57±1.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Uroinary IsoP, ng/mg creatinine†</td>
<td>1.11±0.63</td>
<td>2.11±1.25 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>CVR, mm Hg - min - mL⁻¹</td>
<td>1.74±0.37</td>
<td>1.77±0.44</td>
<td>0.46</td>
</tr>
<tr>
<td>CBF, mL - min⁻¹ - 100 g tissue⁻¹</td>
<td>57.3±11.1</td>
<td>56.5±13.0</td>
<td>0.51</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>27.7</td>
<td>32.8</td>
<td>0.24</td>
</tr>
<tr>
<td>DM, %</td>
<td>10.2</td>
<td>17.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>6.0</td>
<td>8.2</td>
<td>0.40</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; T-chol, total cholesterol; HDL-C, HDL cholesterol; TGs, triglycerides; and DM, diabetes mellitus. Values are mean±SD or as indicated.

*By Student t test or by χ² test.
†Urine samples of male participants followed up in 2000 (34 nonsmokers and 79 smokers) were used in the measurement.

Health Science for a health-screening examination between 1995 and 2000 were recruited into the present study. Although the participants were from a local population, they were not taken as a population-based cohort by the precise epidemiological definition. In the interview, participants were asked about histories of smoking, medication for hypertension, diabetes mellitus, and hypercholesterolemia. Participants were considered to have these diseases when medical doctors had already diagnosed them. Blood pressure or other biochemical markers, such as total cholesterol, HDL cholesterol, and triglyceride levels, were measured in plasma by using commercial kits after nitrate was reduced to nitrite (Cayman Chemical Co). With the use of commercial kits (Cayman Chemical Co), urinary F₂-isoprostane (IsoP) was measured in 113 consecutive male participants who had visited the institute in 2000. IsoP was considered a good biochemical marker of oxidative stress in vivo. Blood pressure was measured 3 times after at least 15 minutes of rest, just before the CBF measurement. The mean of the 3 measurements was taken as the representative blood pressure.

Periventricular hyperintensity (PVH) and brain infarction, including subcortical silent brain infarction, were monitored by MRI (0.2 T, Siemens). Diagnostic criteria for silent brain infarction and PVH have been described previously. CBV was measured by the 133 Xe inhalation method as described. CBV was calculated as mean arterial blood volume perfusing 100 g of brain tissue per minute. CBF measurements were performed in a quiet room with the subjects resting and their eyes closed. CVR was calculated as mean arterial blood pressure divided by mean CBF.

For detailed analyses, subjects with brain infarction, including silent brain infarction, or with high grade PVH (≥3, thick PVH surrounding the lateral ventricle with or without marked extension into the white matter) were excluded because of the possibility of secondary decrease of CBF under such pathological conditions. We then excluded female subjects because (1) only 15 smokers were found in the females and (2) a significant difference of CBF was observed between males and females (58.9±12.6 and 66.9±14.7 mL/min per 100 g tissue for male and female nonsmokers, respectively; P<0.001 by Student t test). From the 557 selected male subjects, we extracted 2 groups according to smoking status, as in our previous study: (1) the nonsmokers with a smoking index (measured as cigarettes per day times years) of 0 and (2) the definite smokers with a smoking index ≥200. This categorization was reasonable because the oxidative stress evaluated with the urinary IsoP was significantly different between the 2 groups (see Table 1). As a result, 510 male subjects (166 nonsmokers and 344 definite smokers) were included in further analysis. Forty-seven subjects were categorized as having a smoking index between 1 and 199. We excluded this population from the analysis because (1) only 1 CC homozygote of T-786C was found in this population, and (2) the effect of smoking was not clear in this population with a urinary IsoP of 1.49±0.68 ng/mg creatinine, which was not significantly different from that of the nonsmokers. Moreover, when we included these 47 subjects in either the definite smokers or nonsmokers, the results did not change (data not shown).

In addition to these subjects, 96 students (60 male and 36 female students) of Shimane Medical University who voluntarily participated in the study were genotyped as a reference population. Participants gave informed consent, and the study protocol was approved by the ethics committee of Shimane Medical University.

**Figure 1.** Allele-specific PCR for T-786C polymorphism in the eNOS gene. Top panel shows the primers used in the reaction and the expected PCR products. Underlining in the primer sequences indicates artificially introduced mismatches. Bottom panel shows a typical result of genotyping.

**eNOS Genotyping**

DNA was extracted from peripheral blood samples. Genotyping of eNOS4 a/b was performed as described previously. For genotyping of T-786C, a newly developed allele-specific polymerase chain reaction (PCR) was used (Figure 1). The oligonucleotide primers used in the reaction are listed in Figure 1. Artificial mismatches were achieved by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 20 seconds. The C and T alleles gave a 176-
and a 250-bp product, respectively, with a 387-bp common product (see Figure 1).

Statistical Analysis
Results are represented as the mean±SD. All statistical analyses were performed by using Statview (version 5.0, SAS Institute Inc). A difference was considered statistically significant at $P<0.05$.

Results
The demographic data of the studied populations are shown in Table 1. A slight difference in body mass index was observed between the definite smokers and the nonsmokers. Triglyceride levels were higher in the smokers. Urinary IsoP in the smokers was nearly double that in the nonsmokers, as reported previously.16 This observation indicated that the criterion for the definite smokers used in the present study was reasonable as far the level of oxidative stress was concerned. In spite of this, smoking status affected neither CBF nor CVR significantly, as indicated in Table 1.

In the subsequent analysis, we evaluated the effect of the T-786C genotype on smoking. In Table 2, genotype frequencies of T-786C are compared among the nonsmokers, the definite smokers, and the reference population. The 3 cohorts were in Hardy-Weinberg equilibrium, and their genotype frequencies did not differ significantly from one another ($\chi^2$ 2.23, df 4, $P=0.69$). This result suggested no apparent genetic stratification in the studied subjects. We used a mixed male and female population as a reference, although the studied populations consisted of males. Because the eNOS gene is located on chromosome 7, the eNOS genotype should be independent of sex. Actually, when the 502 female participants of the health examination were studied, the genotype frequencies were 1.2%, 22.1%, and 76.7% for CC, TC, and TT, respectively, which were not significantly different from those in the male population. In addition, ecNOS4 a/b was genotyped in 257 randomly selected subjects, showing complete linkage disequilibrium between ecNOS4 and T-786C; only 2 haplotypes, C-786/ecNOS4a and T-786/ecNOS4b, were identified in this population (data not shown). Because of this complete linkage disequilibrium, ecNOS4 a/b was not analyzed further.

When the effect of the T-786C genotype on MBP, CBF, and CVR was analyzed, CC homozygotes showed significantly lower CBF (56.6±13.3, 57.6±11.5, and 44.0±7.2 mL/min per 100 g tissue for TT, TC, and CC, respectively; $P=0.03$ by ANOVA) as well as higher CVR (1.76±0.44, 1.72±0.42, and 2.24±0.41 mm Hg·min·mL$^{-1}$ for TT, TC, and CC, respectively; $P=0.01$ by ANOVA) than did heterozygotes and TT homozygotes in the definite smokers (Figure 2). In contrast, there was no apparent difference of CVR or CBF among the 3 genotypes in the nonsmokers (Figure 2). MBP was not different among the 3 genotypes either in the smokers or in the nonsmokers.

Interaction between smoking and T-786C genotype was further supported when the correlation between CBF and the smoking index was analyzed for each of the 3 genotypes separately. The inverse correlation was most evident for CC homozygotes among the 3 genotypes ($r=-0.55$ and $P=0.11$, $r=-0.10$ and $P=0.30$, and $r=-0.05$ and $P=0.32$ for CC, TC, and TT, respectively).

Although age was thought to be inversely correlated with CBF, we found no significant correlation between age and CBF in the nonsmokers ($r=-0.26$, $P=0.75$; data not shown), indicating that age, per se, did not have a major effect on CBF in the studied population. In the smokers, the mean age of CC homozygotes tended to be higher than that of the other 2 genotypes (63.6±10.8 years for CC, 56.8±6.7 years for TC, and 57.5±7.0 years for TT; $P=0.06$ by ANOVA). Even though age itself was not likely to influence CBF significantly, as indicated above, this difference of age among the 3 genotypes might affect the results. To address this possibility, CBF of subjects with the CC genotype was compared with CBF of TT and

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### Table 2. T-786C Genotype Frequencies in the Studied Populations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nonsmokers (N=166)</th>
<th>Smokers (N=344)</th>
<th>Reference (N=96)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT, n (%)</td>
<td>129 (77.7)</td>
<td>273 (79.4)</td>
<td>78 (81.3)</td>
</tr>
<tr>
<td>TC, n (%)</td>
<td>34 (20.5)</td>
<td>64 (18.6)</td>
<td>18 (18.8)</td>
</tr>
<tr>
<td>CC, n (%)</td>
<td>3 (1.8)</td>
<td>7 (2.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C</td>
<td>0.12</td>
<td>0.11</td>
<td>0.09</td>
</tr>
</tbody>
</table>

C indicates C allele frequency.
Effects of smoking and T-786C eNOS genotype on cerebral circulation, NOx, stable metabolites of NO, were measured in the subjects. Although NOx tended to be lower in smokers, no significant difference was obtained among the 3 genotypes (Figure 4).

Because this polymorphism seemed to influence the cerebral circulation, NOx, stable metabolites of NO, were measured in the subjects. Although NOx tended to be low in CC homozygotes in smokers and also in nonsmokers, no significant difference was obtained among the 3 genotypes (Figure 4).

Discussion

The major finding of the present study is that an interaction between smoking and the eNOS genotype could play an important role in the regulation of cerebral circulation. It should be emphasized that these observations were obtained under a setting of genetic epidemiology, which suggested that the effect of the eNOS genotype could actually influence the cerebral circulation in a general population.

We showed that the effect of T-786C on CVR and CBF was apparent only in smokers. This finding suggested that interaction between smoking and the eNOS genotype increased CVR. T-786C has recently been shown to affect the promoter activity of the eNOS gene; a luciferase promoter assay indicated that a construct with C-786 decreased the promoter activity by 50%.13 Meanwhile, smoking is known to induce oxidative stress, which is a potent suppressor of eNOS activity.14,29 Alternatively, such oxidative stress might promote the degradation of NO,30,31 Inasmuch as smokers showed greater urinary secretion of a biochemical marker for oxidative stress (see Results), smoking may cause a further decrease in the NO reservoir in CC homozygotes, resulting in the changes in cerebral circulation. This view was supported by the observation that the correlation between the smoking index and CBF was most evident in the CC homozygotes (see Results).

A similar interaction between smoking and the eNOS gene was observed in coronary artery disease; Wang et al demonstrated that the eNOS4 a allele, which was in strong linkage disequilibrium with the C-786 allele, was associated with coronary artery disease only in smokers. Nakayama et al33 showed that the risk of coronary spasm in those with the C-786 allele was greater in smokers than in nonsmokers. Furthermore, measuring the eNOS mRNA and protein levels in placenta, Wang et al showed that T-786C and ecNOS a/b polymorphisms influenced the eNOS expression only in smokers.14 These observations together with the present study strongly suggest that the eNOS gene polymorphism is a genetic factor potentiating the adverse effect of smoking.

To obtain further evidence for the effects of smoking and eNOS genotype, we measured plasma NOx in our population. The results indicated that although NOx tended to be lower in CC homozygotes, there was no significant difference in levels between smokers and nonsmokers or among the 3 genotypes of eNOS T-786C. We found large variances in plasma NOx levels in our population. Plasma NOx originated from dietary intake as well as from endogenous synthesis.32 Therefore, overnight fasting has been used to reduce the influence of dietary NOx in most clinical and population-based studies33–35 because plasma NOx levels after 12 hours of fasting were indicated to be mainly reflective of endogenous NO production.33,34 However, the results of the present study implied that the noise from dietary intake might still perturb the NOx measurement after overnight fasting in our population. Consistent with this, several studies pointed out that at least 48 hours on a low nitrate diet was necessary to exclude the influence of dietary NOx.36,37 Accordingly, stricter study designs may be required to clarify the genetic effects of the eNOS gene on plasma NOx levels accurately in our population. In spite of such limitations, the trend of lower NOx
observed in CC homozygotes seems consistent with a previous study showing a significant effect of ecNOS4 on plasma NOx levels in another Japanese population.35

White matter changes (or leukoaraiosis) are thought to be closely related to vascular dementia.38 In this pathological condition, decreased CBF and hyalized thickening of cerebral arterioles were constantly observed.20,21,38 It is thought that diffuse arteriopathy based on long-lasting hypertension interacting with other unknown environmental and genetic factors resulted in increased CVR, hypoperfusion and, finally, demyelination in the white matter to establish such pathological conditions.38,39 In the present study, we indicated that the combination of smoking and the eNOS genotype could influence CBF and CVR even in the subjects without any pathological white matter changes. Given the pathophysiological roles of NO in the cerebral circulation,4–11 this observation implies that eNOS is a logical candidate for a genetic risk factor that accelerates vascular dementia, especially in smokers. Because the importance of genetic factors in the development of white matter changes has been pointed out,38,40 it is of interest to study the effect of eNOS genotype on the progression of leukoaraiosis or vascular dementia under a case-control and a prospective study design.

There are some limitations of the present study. CBF and CVR are likely affected by multiple genetic and environmental factors. Therefore, one should be aware of confounding factors influencing the interpretation of the results. It may be best to apply multiple regression analysis to these parameters to take account of confounding factors. However, this is difficult to do in the present study because (1) the smoking index did have a normal distribution because of the large number of the nonsmokers (ie, smoking index 0), (2) the genotype was a categorical datum that was not easy to include in a regression analysis, and (3) the small number of CC homozygotes did not allow for multivariate analysis, even though we genotyped >1000 subjects. Because of this, we applied a univariate analysis with careful consideration of possible confounding factors. The largest possible confounding factors were sex, white matter changes, and age. We excluded the former 2 and indicated that age, per se, was not likely to have large effects on CBF in our population (see Results).

In the interview, we did not take down information about being a current smoker or exsmoker. Because an in vivo study has suggested that the cessation of smoking results in a rapid decrease of oxidative stress estimated with IsoP,41 current smoking status, not past smoking status, might have a major effect on the cerebral circulation. However, we could not exclude the possibility of chronic effects of smoking on the cerebral circulation being due to secondary pathological changes in the cerebral arteries caused by a long-lasting decrease of NO activity.11,42 In this context, it is necessary to examine whether the cessation of smoking can restore CBF in CC homozygotes.

We focused on the 2 polymorphisms, T-786C and ecNOS a/b, in the present study because they have been repeatedly shown to alter eNOS function or NO metabolism.13,14,35 We did not include Glu298Asp in the study because this SNP was not likely to have a functional role in eNOS activity.14,43 However, because several studies have suggested an association of this SNP with cerebrovascular disorders,44 it may be interesting to determine whether it has significant effects on the cerebral circulation in a separate study.

Acknowledgments

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References

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