Polymorphism in the Promoter of Lipopolysaccharide Receptor CD14 and Ischemic Cerebrovascular Disease

Daisuke Ito, MD; Mitsuru Murata, MD; Norio Tanahashi, MD; Hideki Sato, MD; Akira Sonoda, BS; Ikuo Saito, MD; Kiyota Watanabe, MD; Yasuo Fukuuchi, MD

Background and Purpose—A growing amount of evidence suggests that infectious and inflammatory processes may be involved in the initiation of arteriosclerosis, but the mechanisms are conceivably multifactorial and complex. Two European groups have recently demonstrated that a C(−260)→T polymorphism in the promoter of the CD14 lipopolysaccharide receptor may be a risk factor for coronary artery disease (CAD). The T allele of this polymorphism reportedly increases the expression of CD14 and may be involved in atherogenesis. In the present study we investigated a possible association between the C(−260)→T polymorphism in the CD14 promoter and the occurrence of symptomatic ischemic cerebrovascular disease (CVD).

Methods—Genotype frequencies of the C(−260)→T polymorphism in the CD14 promoter were determined in 235 patients with CVD, as confirmed by brain CT and/or MRI, and 309 age- and sex-matched control subjects.

Results—The distribution of genotypes was as follows: CVD patients, T/T 24.3%, C/T 53.2%, and C/C 22.6%; controls, T/T 26.9%, C/T 50.2%, and C/C 23.0%. There was no significant difference between the CD14 promoter genotypes of the CVD patients and the controls (χ²=0.601, P=0.741). We also measured the concentration of serum soluble CD14 and the density of membranous CD14 on monocytes in the CVD patients, but the polymorphism was not associated with either the concentration of soluble CD14 or the density of membranous CD14 (P=0.358, P=0.238, respectively).

Conclusions—Our results indicate that the C(−260)→T polymorphism in the CD14 promoter is not associated with an increased risk for CVD. (Stroke. 2000;31:2661-2664.)

Key Words: lipopolysaccharides ■ polymorphism (genetics) ■ risk factors ■ stroke

There are accumulating data indicating that infection may be linked to atherosclerotic disease.1–3 Although the true role of infection as a risk factor for atherosclerosis is unclear, several known mechanisms may play at least a partial role in this process. One of the most likely mechanisms involves lipopolysaccharide (LPS) and its receptor, CD14, both of which have been implicated in atherogenesis.3 LPS, a structural component of gram-negative bacteria, is bound in plasma by LPS binding protein.4 The LPS–LPS binding protein complex then binds to a glycosylphosphatidylinositol-anchored membrane protein, membranous CD14 (mCD14), on monocytes and macrophages and activates these cells. The activated phagocytes in turn secrete inflammatory cytokines through which LPS indirectly activates endothelial cells. Soluble CD14 (sCD14), which lacks a glycosylphosphatidylinositol anchor, can also be found in plasma. Endothelial cells and smooth muscle cells, lacking their own mCD14, are directly activated by LPS–sCD14 complex.5,6 Directly and indirectly activated endothelial cells express cell adhesion molecules and increased procoagulant activity, and they release free radicals, thereby mediating the initiation and development of atherosclerosis.

Two European groups have recently reported that genetic variation in the CD14 promoter may be a risk factor for coronary artery disease (CAD).7,8 Both groups, one in Germany and the other in the Czech Republic, identified a C(−260)→T nucleotide change in the promoter region of the CD14 gene.9 The German study7 of 2228 patients reported no significant association between this polymorphism and CAD within the total study group, but T homozygosity was associated with an increased risk of CAD in a subgroup with low coronary risk. The Czech study8 demonstrated that T allele frequency was significantly higher in myocardial infarction survivors and that the density of monocyte mCD14 was higher in T/T homozygotes than in other genotypes. Thus, the CD14 promoter genotype may affect inflammatory processes and be involved in atherogenesis, and it is therefore possible that this genotype might also be associated with other major forms of thrombotic disease, such as ischemic cerebrovascular disease.

The primary aim of this study was to determine whether the C(−260)→T polymorphism in the promoter of the CD14 gene is associated with symptomatic ischemic cerebrovascular disease (CVD).

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Subjects and Methods
We analyzed 235 unrelated Japanese patients with CVD and 309 age- and sex-matched control subjects. All CVD patients had visited the outpatient clinic of Keio University Hospital in Tokyo for regular follow-up examinations. We selected CVD patients aged ≤70 years at the onset of CVD. On the basis of the Classification of Cerebrovascular Diseases III report from the committee established by the National Institute of Neurological Disorders and Stroke,10 CVD patients who were diagnosed with atherothrombotic infarction, lacunar infarction, or transient ischemic attack were enrolled in this study. CVD patients with cardioembolic cerebral infarction and cerebral hemorrhage were not included in this group. We initially recruited 246 CVD patients who fulfilled the aforementioned criteria, but 11 subjects were excluded because of unwillingness to participate after an explanation of this study. Two hundred thirty-five CVD patients were finally enrolled in the present study. The mean interval between the onset of CVD and genotyping was 5.1±4.4 years. The control subjects consisted of employees of Keio University. The control subjects were recruited by first registering 603 subjects who came for a routine screening examination and gave their informed consent. We excluded 2 subjects with a history of CVD and 1 subject with a history of CAD from this study and ultimately selected 309 age- and sex-matched control subjects. There were 4 control subjects with hyperuricemia, 3 with postoperative gastric cancer, and 1 each with chronic nephritis, chronic hepatitis, idiopathic thrombocytopenic purpura, and postoperative bladder carcinoma. Written informed consent was obtained from all subjects after a full explanation of the study and a guarantee of total privacy. Brain CT and/or MRI was performed on all CVD patients within 2 days after the onset of CVD. MR angiography and/or extracranial duplex ultrasonography studies were available in >80% of the cases. Hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic pressure >90 mm Hg or current treatment with antihypertensive drugs. Smokers were defined as current smokers. Hypercholesterolemia was defined as a cholesterol level >220 mg/dL or current treatment with a cholesterol-lowering drug.

Polymorphism Analysis
Whole blood was collected into sodium citrate tubes. A direct DNA amplification kit (Shimadzu Co), which enabled us to amplify DNA from whole blood without DNA extraction steps, was used as described previously.11–13 Amplification of the 418-bp fragment of the CD14 promoter was performed with the 5′ primer 5′-CTAAGGCACTGAGGATCATCC-3′ and 3′ primer 5′-TAACTGAGGATCATCCC-3′ and 3′ primer 5′-CATGGTCGAATGCTTCCG-3′. A 0.5-μL volume of whole blood, 12.5 pmol of each primer, 200 μmol/L of each deoxynucleotide triphosphate, 5 μL of 5X Ampdirect-A, 5 μL of 5X Amp Addition-1, and 1.25 U Taq polymerase (TOYOBO Co) and water were added to the reaction to achieve a total volume of 25 μL. The polymerase chain reaction (PCR) consisted of 1 cycle of 15 minutes at 85°C and 4.5 minutes at 94°C; 42 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C; and 7 minutes at 72°C in a Gene Amp PCR system 2400 (Perkin Elmer). The PCR product (4 μL) was cleaved in appropriate buffer with 8 U of HaeIII restriction enzyme (New England Biolabs). The DNA fragments were separated by electrophoresis through a 2% agarose gel containing 0.5 μg/mL of ethidium bromide and visualized under UV light. Digestion of the PCR products yielded bands of 418 bp in TT homozygotes, 263 and 155 bp in CC homozygotes, and all 3 bands in the heterozygotes.

Biochemical Analysis
The serum sCD14 levels were determined with a commercially available enzyme-linked immunosorbent assay kit supplied by BCL. A total of 16 T/T homozygotes, 51 C/T heterozygotes, and 22 C/C homozygotes (mean±SD age, 65.3±8.4 years; 66 men) were randomly recruited from all CVD patients for this assay. The mean interval between the onset of CVD and blood sampling was 7.0±3.4 years.

Table 1. Clinical Characteristics of CVD Patients and Controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=309)</th>
<th>CVD Patients (n=235)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, %</td>
<td>77.2</td>
<td>77.9</td>
<td>NS</td>
</tr>
<tr>
<td>Age, mean±SD, y</td>
<td>58.6±4.4</td>
<td>58.3±7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>25.2</td>
<td>56.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>33.0</td>
<td>38.6</td>
<td>0.176</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>6.8</td>
<td>25.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>37.9</td>
<td>53.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*χ² tests were used to compare values of CVD patients and controls for all parameters except for age, which was compared by Student’s t test.

The density of mCD14 on the monocyte surface was measured by flow cytometry. A total of 10 T/T homozygotes, 10 C/T heterozygotes, and 10 C/C homozygotes (mean±SD age, 66.5±6.4; 19 men) were randomly selected from all CVD patients after genotyping. The mean interval between the onset of CVD and blood sampling was 5.6±2.8 years. Anti-CD14 (Leu-M3) monoclonal antibody, purchased from Becton Dickinson, directly conjugated with fluorescein isothiocyanate, was used in the analysis. Whole blood was analyzed with a FACScan (Becton Dickinson) flow cytometer. Distinct monocyte clusters were identified with a combination of CD14 monoclonal antibody intensity and forward light scatter. This display was used for gating the monocytes, and a minimum of 5000 cells per sample was analyzed.

Statistical Analysis
The differences in the frequencies of the CD14 genotypes and alleles and other risk factors were analyzed by the χ² test. Mean age in the 2 groups was compared by Student’s t test. Multiple logistic regression methods were used to control for possible confounding factors. The relationships between mCD14 densities, serum sCD14 levels, and CD14 genotypes were tested by 1-way ANOVA. Associations and differences with probability value <0.05 were considered significant. All statistical analyses were performed with the use of Statview software (version 5.0 for Windows, SAS Institute).

Results
A total of 235 CVD patients and 309 control subjects were recruited for this study. Table 1 summarizes the clinical features of the CVD patients and the control subjects studied. There were no significant differences in age or sex between the 2 groups. The risk factors hypertension, diabetes mellitus, and smoking were significantly more common in the CVD patients.

The distributions of genotypes and the allelic frequencies of the polymorphism in the CD14 promoter in the control and CVD groups are shown in Table 2. Among the CVD patients, 24.3% were T/T, 53.2% were C/T, and 22.6% were C/C. This genotype distribution was not significantly different from the distribution in the control group (T/T, 26.9%; C/T, 50.2%; C/C, 23.0%). (χ²=0.601, P=0.741). T allele frequencies were also comparable between CVD patients and control subjects (50.9% and 51.9%, respectively). This was confirmed by the results of multiple logistic regression analysis with the established risk factors hypertension, hypercholesterolemia, diabetes mellitus, and smoking (χ²=0.692, P=0.405). In further calculations, subgroups were formed as low-risk groups (excluding subjects with hypertension, hypercholesterolemia, diabetes mellitus, or smoking). The CD14 promoter genotype was not related to CVD in any of
the subgroups (data not shown). As shown in Table 2, analysis by CVD subtype (atherothrombotic infarction, lacunar infarction, transient ischemic attack) also failed to yield any difference in genotype distribution compared with the control group.

We also measured the serum concentration of sCD14 and the density of mCD14 on monocytes in the CVD patients (Table 3). Although the sCD14 concentration tended to be slightly higher in the TT genotype patients, there were no significant differences in either sCD14 concentrations or the density of mCD14 between any of the genotypes.

### Discussion

The present study is the first to examine the relationship between CVD and the CD14 promoter polymorphism. The Japanese population has a slightly higher T allele frequency than European populations.\(^7,8\) Our study demonstrated that the CD14 promoter polymorphism is not associated with CVD, even in low-risk subjects. Although the number of subjects in the CVD subtypes was relatively small, no differences in genotype distribution were found in the analysis by CVD subtypes either. In addition, there were no significant correlations between the genotypes of the CVD patients and the sCD14 concentration in their serum or the density of mCD14 on their monocytes.

A Czech group\(^6\) demonstrated that the T allele was associated with a higher density of mCD14 on monocytes in healthy volunteers. They suggested that the C\((-260)\)→T change in the promoter region affects the level of CD14 gene expression. Their findings are in clear contrast to the results of our own studies. Several reasons may account for these conflicting results. First, the genetic background of Japanese and European populations is different. The T allele in the CD14 promoter may be in linkage disequilibrium, with other mutations involved in the expression or function of CD14 in Europeans but not in Japanese. Second, the choice of subjects for measurement of mCD14 was different in the 2 studies. The Czech group recruited healthy volunteers, whereas our subjects were selected from CVD patients. Although we excluded CVD patients with acute CVD and other acute illnesses from the analysis of mCD14 and sCD14, chronic thrombotic disease in itself may affect CD14 gene expression.

Third, the age of the subjects was another important difference between the 2 studies. The Czech group selected young subjects (aged 20 to 30 years), whereas the mean ages for measurement of sCD14 and mCD14 in our study were 65.5±8.4 and 66.5±6.4 years, respectively. It is possible that the level of expression of CD14 changes with age.

The most important finding in our study was that a polymorphism in the CD14 promoter is not associated with the occurrence of CVD. However, several points should be borne in mind when one interprets the results of the present study. All CVD patients in this study were selected from patients who had visited the outpatient clinic for a regular checkup, and they were therefore cerebrovascular attack survivors. Therefore, both selection and survival bias cannot be avoided in this disease-association study, and it is likely that early mortality of CVD in patients could lead to underestimation of the incidence of this polymorphism. Moreover, patients with cardioembolic cerebral infarction were not included in the present study because the primary aim of our study was to examine atherogenesis of the cerebrovascular system. Therefore, we cannot exclude the possibility that this polymorphism could be related to pathogenesis of cardioembolic cerebral infarction. Finally, our study refers to the association between this polymorphism and CVD only in the Japanese population. The relevance of this polymorphism should be investigated in other populations and by prospective and family studies. Nevertheless, our findings suggested that the C\((-260)\)→T polymorphism in the CD14 promoter may have less effect on the occurrence of CVD than on that of CAD.

### Acknowledgments

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