Plasminogen Activator Inhibitor 4G Polymorphism Is Associated With Decreased Risk of Cerebrovascular Mortality in Older Women

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Background—A common 4G allele of a 4G/5G polymorphism in the promoter region of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with increased transcription of the PAI-1 protein, which may lead to decreased fibrinolysis. It has therefore been proposed as a candidate risk factor for myocardial infarction or stroke.

Methods and Results—We studied the relationship between PAI-1 4G/5G genotype and the risk of cardiovascular mortality in a prospective cohort study among 12 239 women initially aged between 52 and 67 years, with a maximum follow-up time of 18 years (153 732 follow-up years). PAI-1 4G/5G genotype was measured in DNA obtained from urine samples, which were collected at baseline, of 498 women who died of a cardiovascular disease and a random sample of 512 women from the same cohort who did not die of cardiovascular disease. The PAI-1 4G/5G genotype was not associated with risk of myocardial infarction or other cardiovascular mortality. However, PAI-1 4G4G homozygotes had a markedly reduced risk of cerebrovascular mortality compared with PAI-1 5G5G homozygotes: the relative risk was 0.4, with a 95% CI of 0.2 to 0.7, whereas the relative risk of cerebrovascular mortality in PAI-1 4G5G heterozygotes compared with PAI-1 5G5G homozygotes was 0.7, with a 95% CI of 0.4 to 1.1.

Conclusions—These findings are suggestive of an important contribution of PAI-1 in cerebrovascular pathology, probably via pathways other than fibrinolysis. PAI-1 may protect against destabilization of the atherosclerotic plaque, or it may inhibit neurotoxicity of tissue plasminogen activator in the brain. (Circulation. 2000;101:67-70.)

Key Words: plasminogen activators ■ cerebrovascular disorders ■ mortality

Increased plasma levels of tissue-type plasminogen activator (tPA) and its inhibitor, plasminogen activator inhibitor (PAI-1), are both associated with an increased risk of myocardial infarction1–4 and stroke.5 Although the relationship between these proteins and cardiovascular disease is evident, the interpretation is controversial. Plasma tPA and PAI-1 levels have opposite effects but are highly intercorrelated, and it is nearly impossible to separate these effects in epidemiological evaluations.1,2 Furthermore, plasma levels of tPA and PAI-1 show a marked circadian variation,6,7 and both parameters are highly dependent on other factors that are involved in cardiovascular disease, such as lipids, insulin, sex hormones, and inflammatory response (References 3, 8, and 9). To further complicate issues, plasminogen activation may on one hand protect against cardiovascular disease by fibrinolysis,10–12 whereas on the other hand, it may induce cardiovascular disease via destabilization of the atherosclerotic plaque13,14 or via brain damage by laminin degradation in brain tissue.15–17

Genetic markers have the advantage of not being affected by other risk factors of cardiovascular disease or by circadian variation. A common 4G polymorphism in the promoter region of PAI-1 is associated with increased PAI-1 transcription and is therefore an independent marker of increased plasminogen activation inhibition. Initially, it was thought that the PAI-1 4G allele was associated with an increased risk of myocardial infarction.18,19 However, all large studies reported no association between PAI-1 4G/5G genotype and myocardial infarction.4,20 In contrast, PAI-1 4G genotype may be inversely associated with morbidity of stroke.21

We had the opportunity to study the relationship between PAI-1 4G/5G genotype and both myocardial infarction and stroke mortality in a cohort study of 12 239 women initially aged 52 to 67 years who were followed up with regard to mortality for 16 to 18 years.22

Methods

Population

The cohort comprised 12 239 postmenopausal white women who were followed up with regard to vital status for 16 to 18 years as described previously.22 The study was approved by the Institutional
Risk Factors
At baseline, a questionnaire on cardiovascular risk factors, including medication, prescribed diets, presence of cardiovascular disease, and smoking, was completed. In addition, blood pressure, height (in meters), and weight (in kilograms) were measured. Women were classified as having diabetes mellitus if they reported use of insulin or oral blood glucose-lowering drugs or if they ate a diet designed for diabetic individuals. Women were defined as smokers if they reported current smoking. Body mass index (BMI; kg/m²) was calculated as weight (kg) divided by height squared (m²). Obesity was defined as BMI ≥30 kg/m². Hypertension was defined as systolic blood pressure >160 mm Hg and/or diastolic blood pressure >90 mm Hg and/or the use of antihypertensive medication.

Study Design
We used a nested case-referent approach. The case patients were all 608 women who died of cardiovascular disease, and the referents comprised a random sample of 618 of the cohort of 11,631 women who did not die of cardiovascular disease (sampling fraction 1:18.8). Urine samples of 59 cardiovascular cases and 49 referents were not collected at baseline or were lost during follow-up. DNA samples of 51 cardiovascular cases and 37 referents were not suitable for analysis. The final study group comprised 498 cardiovascular mortality cases and 512 women of the reference group.

Genotyping
DNA was isolated from 50-mL urine samples. A 221-bp fragment in the promoter region of the PAI-1 gene with the 4G/5G polymorphism was amplified by polymerase chain reaction (PCR) followed by dot blot and hybridization with antigen-specific oligonucleotides. The antigen-specific oligonucleotide for the 4G allele was 5'-P-ACACGTGGGGGAGTCAGC, and for 5G, it was 5'-P-ACACGTGGGGGAGTGACG. Mutation analysis was performed with samples blinded for case or referent status.

Data Analysis
Means and proportions of baseline cardiovascular risk factors were computed for the 3 PAI-1 4G/5G genotypes. The significance of mean difference was tested by ANOVA, and significance in proportions was tested by χ² statistics. Allele frequencies were calculated by the law of Hardy-Weinberg. The χ² goodness-of-fit test was used to determine whether the observed numbers of each genotype were in equilibrium.

A nested case-referent approach was used to estimate incidence rates (IRs) and rate ratios exactly as is done in a full-cohort analysis. Death due to other causes, loss of the patient to follow-up, and withdrawal from the study were considered censoring events. Incidence was assumed to follow a Poisson distribution. The 95% CIs were calculated with Huber’s method. Similarly, crude relative risks, IRs, and rate ratios were separately estimated for women who died of myocardial infarction (International Classification of Diseases [ICD] 410 to 414), cerebrovascular disease (ICD 430 to 438), and other cardiovascular disease (all remaining ICD codes between 390 and 459). The fraction of deaths that would not have occurred if women had the 4G4G genotype instead of 4G5G or 5G5G, analogous to the preventive fraction (PF) of Miettinen, was calculated as PFi = (IRi - IR0)/IR0 = 1 - IRRi. The relationship between PAI-1 4G5G genotype and cardiovascular mortality was also investigated in subgroups stratified on the basis of age (above or below the median), smoking (yes/no), obesity (yes/no), and hypertension (yes/no).

Results
The genotype distribution of the reference group was in Hardy-Weinberg equilibrium (χ² = 1.17; 1 df, P = NS). PAI-1 4G5G genotype was not associated with age, hypertension, diabetes mellitus, smoking, or BMI. Moreover, PAI-1 4G5G genotype was not associated with history of cardiovascular disease (Table 1).

The follow-up time of our cohort was 153,732 women-years: 47,052 years for women with the 4G4G genotype, 80,004 for women with the 4G5G genotype, and 26,676 for women with the 5G5G genotype. The estimated IRs for overall cardiovascular mortality and for fatal myocardial infarction and other cardiovascular mortality were similar among the PAI-1 genotypes (Table 2). The IR of cerebrovascular mortality for PAI-1 4G4G homozygotes was 4.5/1000 years (95% CI 2.6 to 6.4), which was lower than for PAI-1 5G5G homozygotes, who had an IR of 11.6/1000 years (95% CI 7.5 to 15.5). PAI-1 4G5G heterozygotes had an IR of 7.8/1000 years (95% CI 5.8 to 9.7), which was intermediate between both homozygotes. Adjustment for age at entry, smoking, hypertension, and obesity did not affect the association, nor did subgroup analysis in groups according to the presence of these risk factors (Table 3). The fraction of mortality cases that would not have occurred if women had the 4G4G genotype instead of 4G5G was 33%, and the fraction that would not have occurred if women had the 4G4G genotype instead of 5G5G was 61%. In other words, 26 fatal stroke events were attributable to 4G5G genotype and 13 to 5G5G genotype.

Discussion
The results of our study do not support the presence of an association between PAI-1 4G/5G genotype and fatal myocardial infarction or total cardiovascular mortality in our cohort of 12,239 postmenopausal women. However, we found a significantly reduced risk of cerebrovascular mortality in PAI-1 4G4G homozygotes compared with PAI-1 5G5G homozygotes (IR ratio [IRR] 0.4; 95% CI 0.2 to 0.7) and a borderline significant

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**TABLE 1. Population Characteristics of PAI-1 4G/5G Genotypes**

<table>
<thead>
<tr>
<th>Population Characteristics</th>
<th>PAI-1 4G4G</th>
<th>PAI-1 4G5G</th>
<th>PAI-1 5G5G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at entry, y</td>
<td>59.8±3.9</td>
<td>57.4±4.2</td>
<td>59.4±4.2</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>114 (78.1)</td>
<td>97 (61.0)</td>
<td>188 (72.6)</td>
</tr>
<tr>
<td>History of CVD, %</td>
<td>16 (11.0)</td>
<td>5 (3.4)</td>
<td>36 (13.9)</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>21 (14.4)</td>
<td>5 (3.1)</td>
<td>33 (12.7)</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>44 (30.1)</td>
<td>46 (28.9)</td>
<td>93 (35.9)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.6±4.2</td>
<td>27.2±4.5</td>
<td>26.9±4.1</td>
</tr>
</tbody>
</table>

CVD indicates cardiovascular disease. Age and BMI are shown as mean±SD; all other values are shown as n (%).
TABLE 2. IRRs and IRRs of Mortality due to Myocardial Infarction, Cerebrovascular Disease, and Other Cardiovascular Disease

<table>
<thead>
<tr>
<th>PAI Genotype</th>
<th>4G4G</th>
<th>4G5G</th>
<th>5G5G*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up years</td>
<td>47 052</td>
<td>80 004</td>
<td>26 676</td>
</tr>
<tr>
<td>MI</td>
<td>74</td>
<td>114</td>
<td>37</td>
</tr>
<tr>
<td>No. of cases</td>
<td>15.7 (12.1–19.3)</td>
<td>14.2 (11.6–16.9)</td>
<td>13.9 (9.4–18.3)</td>
</tr>
<tr>
<td>IR</td>
<td>1.1 (0.7–1.8)</td>
<td>1.0 (0.7–1.6)</td>
<td>1</td>
</tr>
<tr>
<td>Stroke</td>
<td>21</td>
<td>62</td>
<td>31</td>
</tr>
<tr>
<td>No. of cases</td>
<td>4.5 (2.6–6.4)</td>
<td>7.8 (5.8–9.7)</td>
<td>11.6 (7.5–15.7)</td>
</tr>
<tr>
<td>IR</td>
<td>0.4 (0.2–0.7)†</td>
<td>0.7 (0.4–1.1)</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>51</td>
<td>83</td>
<td>25</td>
</tr>
<tr>
<td>No. of cases</td>
<td>10.8 (7.9–13.8)</td>
<td>10.4 (8.1–12.6)</td>
<td>9.4 (15.7–13.0)</td>
</tr>
<tr>
<td>IR</td>
<td>1.2 (0.7–2.0)</td>
<td>1.1 (0.7–1.8)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>146</td>
<td>259</td>
<td>93</td>
</tr>
<tr>
<td>No. of cases</td>
<td>31.0 (25.6–36.1)</td>
<td>32.4 (28.4–36.3)</td>
<td>34.9 (27.8–41.9)</td>
</tr>
<tr>
<td>IR</td>
<td>0.9 (0.6–1.3)</td>
<td>0.9 (0.7–1.3)</td>
<td>1</td>
</tr>
</tbody>
</table>

Genotype was determined in 498 case subjects and 512 referents. IR is shown per 10 000 years. Values in parentheses are 95% CIs.

*Reference group.
†P=0.002.

reduced risk compared with PAI-1 4G5G heterozygotes (IRR 0.7; 95% CI 0.4 to 1.1). We did not find interaction with other risk factors regarding the relation of PAI-1 4G/5G genotype and any form of cardiovascular mortality.

In early reports, it was suggested that PAI-1 4G genotype was associated with myocardial infarction. In these findings must be considered as spurious, because all large studies, including ours, did not confirm such an association. Other large studies on PAI-1 4G/5G genotype and stroke have not yet been reported. Additional support for the validity of our findings is provided by the relationship between plasma tPA levels and any form of cardiovascular mortality.

The study was conducted among nonhospitalized subjects, and data on cardiovascular end points were obtained from hospital records or general practitioner diagnosis. We expect a degree of misclassification between ischemic and hemorrhagic fatal stroke because the diagnosis was not routinely confirmed by CT scan or MRI. It is therefore not possible to make a reliable distinction between risk of ischemic or hemorrhagic fatal stroke. However, it does not seem likely that the protective relationship between PAI-1 4G genotype and the risk of total stroke mortality reflected an effect on hemorrhage only, because the incidence of hemorrhagic stroke among women in the Dutch population is only 20% of total stroke and maximally 35% for fatal stroke. We do not expect important misclassifi-

![TABLE 3. IRRs (95% CI) of Cardiovascular Mortality in Subgroups of Women Younger/Older Than the Median Age and in Subgroups of Smoking/Not Smoking](image-url)

<table>
<thead>
<tr>
<th>PAI-1 4G4G vs 5G5G</th>
<th>Age ≤56.5 y</th>
<th>Age &gt;56.5 y</th>
<th>Smoking</th>
<th>Not Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI</td>
<td>0.9 (0.5–1.8)</td>
<td>1.3 (0.7–2.5)</td>
<td>0.9 (0.4–2.1)</td>
<td>1.2 (0.7–2.2)</td>
</tr>
<tr>
<td>Stroke</td>
<td>0.3 (0.1–0.8)</td>
<td>0.4 (0.2–0.9)</td>
<td>0.4 (0.1–1.2)</td>
<td>0.4 (0.2–0.8)</td>
</tr>
<tr>
<td>Other</td>
<td>1.5 (0.6–3.8)</td>
<td>1.0 (0.5–2.0)</td>
<td>1.4 (0.6–3.5)</td>
<td>1.0 (0.5–2.0)</td>
</tr>
<tr>
<td>Total</td>
<td>0.8 (0.5–1.4)</td>
<td>0.9 (0.5–1.5)</td>
<td>0.9 (0.5–1.7)</td>
<td>0.9 (0.6–1.4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PAI-1 4G5G vs 5G5G</th>
<th>Age ≤56.5 y</th>
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<th>Not Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI</td>
<td>0.8 (0.4–1.5)</td>
<td>1.3 (0.7–2.4)</td>
<td>1.2 (0.6–2.5)</td>
<td>1.0 (0.6–1.6)</td>
</tr>
<tr>
<td>Stroke</td>
<td>0.8 (0.4–1.8)</td>
<td>0.6 (0.3–1.1)</td>
<td>0.7 (0.3–1.7)</td>
<td>0.7 (0.4–1.2)</td>
</tr>
<tr>
<td>Other</td>
<td>1.2 (0.5–2.8)</td>
<td>1.1 (0.5–2.1)</td>
<td>1.3 (0.6–3.0)</td>
<td>1.0 (0.5–1.9)</td>
</tr>
<tr>
<td>Total</td>
<td>0.9 (0.5–1.4)</td>
<td>1.0 (0.6–1.6)</td>
<td>1.1 (0.6–1.9)</td>
<td>0.9 (0.6–1.3)</td>
</tr>
</tbody>
</table>
cations to have occurred between cerebrovascular events and myocardial infarction or other cardiovascular mortality.

The explanation of the reduced risk of stroke for PAI-1 4G4G homozygotes remains speculative but may be found in a vascular pathological pathway that does not involve fibrinolysis. Increased tPA levels and activity have been observed in and around the atherosclerotic plaque.13 tPA and urokinase plasminogen activator (uPA) are the activators of plasmin, which then activates matrix metalloproteinases (MMPs). There is good evidence to suggest that MMPs are responsible for the degradation of the fibrous cap of the atherosclerotic plaque, which results in rupture, leading to an occlusive infarction.13,14 The increased PAI-1 expression in the atherosclerotic plaque may inhibit tPA and uPA and therefore protect the fibrous cap against degradation by MMPs and subsequently against rupture.13,14

An alternative explanation may be that increased PAI-1 expression is associated with reduced plasminogen activation in brain tissue and therefore protects against laminin degradation and brain cell death.15,16 Excessive tPA has been associated with increased size of nonthrombotic ischemic infarction in mice.17 Moreover, tPA supplementation enhanced infarct size both in mice lacking the gene coding for tPA and in mice with normal genotype.17

In conclusion, a significantly lower mortality rate from stroke was found for women with the PAI-1 4G4G genotype compared with women with the PAI-1 5G5G genotype. Our findings are consistent with previously reported data that also indicated a protective association with PAI-1 4G genotype. These findings are suggestive of an important contribution of tPA and PAI-1 in cerebrovascular pathology, probably via pathways other than fibrinolysis, such as destabilization of the atherosclerotic plaque13,14 or via a neurotoxic effect of tPA.15,17

References

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