Complex Association of Protein C Gene Promoter Polymorphism With Circulating Protein C Levels and Thrombotic Risk

Martine Aiach, Viviane Nicaud, Martine Alhenc-Gelas, Sophie Gandrille, Emmanuel Arnaud, Jean Amiral, Louis Guize, Jean-Noël Fiessinger, Joseph Emmerich

Abstract—The allele and haplotype frequency of the –1654 C/T and –1641 A/G protein C (PC) gene promoter polymorphisms was determined and analyzed according to circulating PC concentrations in 394 healthy subjects aged 20 to 60 years. The CG haplotype was associated with a lower PC concentration in both homozygous and heterozygous subjects compared with noncarriers. The TA allele had the reverse effect, but only in homozygotes. The distribution of the CG and TA alleles was significantly different in 242 patients, aged 17 to 60 years, with venous thromboembolism. The CG allele increased the risk of thrombosis, with an OR of 1.39 (95% confidence interval (CI), 1.04 to 1.87). The TA allele was protective in subjects aged <45 years, with an OR of 0.68 (95% CI, 0.44 to 1.04). TA was also significantly associated with older age at the first thrombosis. This study confirms the link between the PC gene promoter and circulating PC levels, and suggests a complex effect on the risk of thrombosis. (Arterioscler Thromb Vasc Biol. 1999;19:1573-1576.)

Key Words: thrombosis ■ protein C ■ promoter ■ genetic risk
and their different combinations were determined in 394 control subjects and 242 patients with DVT.

Methods

Subjects
Healthy subjects, aged 20 to 60 years, were recruited between May and September 1996 from a health care center to which they had been referred for a routine checkup. None of these subjects had a history of arterial disease (stroke, myocardial infarction, angina, or peripheral vascular disease), venous thrombosis (pulmonary embolism or DVT), or known malignancy, as reported on a medical questionnaire.

Patients with venous thromboembolism (VTE) were recruited from the vascular medicine unit of a Paris hospital from November 1995 to June 1998 and were enrolled onto the study if they were younger than 61 and had experienced at least 1 episode of objectively diagnosed DVT (ultrasound ultrasonography or venography) and/or pulmonary embolism (perfusion and ventilation lung scan, conventional pulmonary angiography, or computed tomographic angiography). Blood sampling for DNA analysis, demographic data, and clinical characteristics recording were performed at the time of inclusion. The study was approved by the local ethics committee and all the subjects gave their informed consent.

Laboratory Investigations
Venous blood was collected onto 0.129 M trisodium citrate (1:10) and plasma was kept frozen until use.

PCantigen (PCag) was assayed by an immunoenzymatic method (Asserachrom Protein C, Diagnostica Stago). Control plasma was assayed within 3 months using a pool of plasma from 50 healthy men, aged 20 to 60, to construct the reference curve.

Molecular Biology
The PC gene promoter was analyzed after polymerase chain reaction amplification of the fragment of the promoter encompassing nucleotides –1738 to –1596 in the numbering system of Foster et al.9 Denaturing gradient gel electrophoresis (DGGE) of the amplified nucleotides –1738 to –1596 in the numbering system of Foster et al.9

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The factor V Arg506Gln mutation was identified using a previously described method. 14

Statistical Analysis
Data were analyzed using the SAS statistical software (SAS Institute Inc). Hardy-Weinberg equilibrium was tested by a χ² test with 2 degrees of freedom (df) separately in cases and controls. Allele frequencies were deduced from the genotype frequency.

The homogeneity of the results in men and women, and across age, was systematically tested by entering the corresponding interaction term. \( P<0.05 \) was considered significant.

Results
Table 1 shows the characteristics of the study population. Cases and controls were well-matched in terms of sex and age. As expected, the factor V Arg506Gln mutation was observed in 19.5% of cases and 3.8% of controls (\( P<0.001 \)). Women on oral contraception (a known risk factor for DVT) were more frequent among cases than among controls. The occurrence of thrombosis was associated with a known acquired risk factor in 59.5% of the cases. VTE was recurrent in 27.5% of the cases, and 40.1% of the cases had pulmonary embolism.

The distribution of the PC gene promoter CG/TA/CA genotypes in the 394 controls is shown in Table 2. There was no significant deviation from Hardy-Weinberg equilibrium in the controls. CG was the most frequent allele and CA the least frequent.

| TABLE 1. Characteristics of Cases With Deep-Venous Thrombosis (DVT) and Controls |
|-----------------|--------------------|---------------|
|                  | Cases | Controls     |
| % Women          | 55.8 (3.2) | 49.5 (2.5) | 0.12 |
| Mean age         | 42.2 (11.3) | 43.0 (9.5) | 0.34 |
| % Oral contraception in women | 33.3 (4.1) | 19.0 (2.8) | 0.003 |
| % FV-G506 mutation | 19.5 (2.8) | 3.8 (1.0) | 0.001 |
| % DVT associated with acquired risk factors* | 59.5 (3.2) | ... | ... |
| % Recurrent DVT   | 27.5 (2.9) | ... | ... |
| % Pulmonary embolism | 40.1 (3.2) | ... | ... |
| Mean age at first DVT | 37.7 (12.2) | ... | ... |

SD in parentheses.

*Contraception, pregnancy, surgery, prolonged immobilization, or cancer.

Table 2 shows the distribution of the Poly-CG/TA/CA Genotypes and Allele Frequencies in Cases With DVT and Controls

| TABLE 2. Distribution of the Poly-CG/TA/CA Genotypes and Allele Frequencies in Cases With DVT and Controls |
|---------------------------------------------------------------|-----------------|-----------------|
|                  | Cases | Controls     |
|                  | n | % | n | % |
| CD-CG            | 56 | 23.2 | 69 | 17.5 |
| CG-TA            | 76 | 31.4 | 107 | 27.2 |
| CG-CA            | 46 | 19.0 | 69 | 17.5 |
| TA-TA            | 21 | 8.7 | 52 | 13.2 |
| TA-CA            | 33 | 13.6 | 75 | 19.0 |
| CA-CA            | 10 | 4.1 | 22 | 5.6 |
| Total            | 242 | 100.0 | 394 | 100.0 |
| CG allele frequency | 0.483 | 0.398 |
| TA allele frequency | 0.312 | 0.363 |
| CA allele frequency | 0.205 | 0.239 |

Difference in allele distribution between cases and controls \( P=0.012 \)
The effects were not significantly influenced by sex.

There was no significant deviation from Hardy-Weinberg equilibrium in for the PC promoter polymorphisms (Table 2). There was no association with 1 copy of PC homozygotes, heterozygotes, and noncarriers, respectively, after adjustment for age and sex (P<0.01). Conversely, the PC concentration was elevated in TA homozygotes (111.9%; 0.01), relative to heterozygous (104.4%) and noncarriers (104.5%). These effects were not significantly heterogeneous according to sex or age. The presence or absence of CA did not modify the PC concentration. These results confirm and extend a previous observation that the PC concentration is genetically determined in healthy individuals.11

Because the thrombotic risk correlates negatively with the PC concentration,4 we genotyped the 242 patients with VTE for the PC promoter polymorphisms (Table 2). There was no significant deviation from Hardy-Weinberg equilibrium in the cases. The CG frequency was higher in the cases than in the controls, and the TA frequency was higher in the controls than in the cases (P=0.012, inferring a significant effect of the CG and TA genotypes on the risk of DVT). The OR associated with 1 copy of CG was 1.39 (95% CI, 1.04 to 1.87; P<0.028), with no effect of age or sex, confirming that CG significantly increased the risk for thrombosis in this population. As shown in Table 4, although TA had no effect when subjects of all ages were included in the analysis, with an OR of 1 (95% CI, 0.72 to 1.37; P=0.98) for 1 copy of TA, the effect was significantly influenced by age (P<0.01): the TA allele tended to reduce the risk in subjects under 45 (median age), with an OR of 0.68 (95% CI, 0.44 to 1.06; P=0.09), whereas it tended to increase the risk in subjects over 45, with an OR at 1.56 (95% CI, 0.96 to 2.51; P=0.07).

The respective frequencies of the CG, TA, and CA alleles were 39.8%, 36.3%, and 23.9% in our 394 French controls; 35.3%, 28.4%, and 32.4% in 102 British subjects12; and 36.4%, 30.7%, and 28.4% in 88 Dutch subjects.10 The CG and TA alleles were thus similar to those observed in other European populations.18 The distribution of the prothrombin 20210A allele (10.2% versus 2.8% in cases and controls, respectively), which has been found to increase the risk of venous thrombosis,17 was also similar to that seen in other European populations.18

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The clinical circumstances of the thromboses were then analyzed according to the PC promoter genotype. We observed an effect of the TA allele on age at the first thrombosis, which occurred at a mean (adjusted for sex) of 36.4 years in noncarriers, 38.8 years in heterozygotes and 44.2 years in homozygotes (P=0.07; assuming an additive effect of alleles, P=0.03). The CG allele had no such effect on age at onset.

The presence of acquired risk factors for thrombosis was also analyzed in CG and TA carriers. The CG allele was not significantly related to the type of thrombosis (spontaneous or acquired). In the younger patients (<45), acquired risk factors were less frequent among TA carriers: 57% in carriers versus 79% in noncarriers (P=0.008, 1 df). This effect, suggesting a protective effect of TA on development of thrombosis associated with acquired risk factors, was not observed in patients >45 years old.

No link was found between PC gene promoter polymorphism and the recurrence of thrombosis.

**Discussion**

We took advantage of the proximity of 2 frequent polymorphisms of the PC gene promoter to determine haplotypes with the DGGE technique in 636 subjects from Paris. Three hundred ninety-four of these subjects, aged 20 to 60 years, were recruited in a health center if they reported no history of venous or arterial thrombosis. Two hundred forty-two patients, aged 17 to 60 years, with confirmed VTE were recruited in a hospital vascular medicine unit. The controls and cases all lived in the Paris area, but there were no geographic or ethnic criteria for eligibility. It was thus important to check that the 2 populations were comparable with other European populations in terms of genetic risk factors for thrombosis. The frequency of the factor V mutation was 19.5% in the patients with thrombosis (cases) and 3.8% in the subjects without thrombosis (controls). These frequencies are similar to those observed in other studies.15,16 The distribution of the prothrombin 20210A allele (10.2% versus 2.8% in cases and controls, respectively), which has been found to increase the risk of venous thrombosis,17 was also similar to that seen in other European populations.18

The PC concentration was measured in the 394 controls using an immunoenzymatic assay, and the results confirmed the effect of the CG and TA alleles on PC gene expression. This effect had initially been established by comparing 40 individuals homozygous for the CG genotype to 28 individuals homozygous for the TA genotype.11 The results presented here show that not only homozygous individuals, but also heterozygous individuals, for the CG haplotype have lower PC concentrations than individuals who do not carry CG. Conversely, the TA haplotype increased the PC concentration, but only in homozygotes. As the risk of venous thrombosis correlates negatively with the PC concentration,4 a detrimen-

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**TABLE 3. Mean Protein C Antigen Level According to CG and TA Genotypes in Controls**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Mean (SEM)</th>
<th>n</th>
<th>Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarriers</td>
<td>149</td>
<td>111.5 (1.4)</td>
<td>160</td>
<td>104.5 (1.4)</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>176</td>
<td>106.0 (1.6)</td>
<td>182</td>
<td>104.4 (1.5)</td>
</tr>
<tr>
<td>Homozygotes</td>
<td>69</td>
<td>103.1 (2.5)</td>
<td>52</td>
<td>111.9 (2.7)</td>
</tr>
</tbody>
</table>

Means were adjusted for age and sex. These effects were not significantly heterogeneous across sex or age.

**TABLE 4. Odds Ratios (95% CI) for Deep–Vein Thrombosis Associated With CG and TA Alleles, all Ages**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n Cases</th>
<th>n Controls</th>
<th>n Cases</th>
<th>n Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarriers</td>
<td>64</td>
<td>149</td>
<td>112</td>
<td>160</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>122</td>
<td>176</td>
<td>109</td>
<td>182</td>
</tr>
<tr>
<td>Homozygotes</td>
<td>56</td>
<td>69</td>
<td>21</td>
<td>52</td>
</tr>
</tbody>
</table>

OR (95% CI) 1.39 (1.04 to 1.87) 1.00 (0.72 to 1.37) P=0.028 P=0.98

Heterogeneity of the ORs associated with one copy of CG across age: NS. Heterogeneity of the ORs associated with one copy of TA across age: P<0.01. The effects were not significantly influenced by sex.
nal effect of the CG allele could be expected. The CG allelic frequency was indeed significantly higher in patients with DVT than in controls (48.3% versus 39.8%). This indicates that individuals with the CG allele (1 copy, more so for 2 copies) have a higher risk of thrombosis than noncarriers. The risk of thrombosis was significantly increased (1.39-fold; 95% CI, 1.04 to 1.87), and the effect was homogeneous across age and sex.

The effect of the TA allele was more complex, being dependent on age. In patients under 45 (median age), the OR for thrombosis associated with 1 TA allele was 0.68 (95% CI, 0.44 to 1.06). This potentially protective effect was not observed after 45 years; on the contrary, the TA allele tended to increase the risk in this subgroup, with an OR of 1.56 (95% CI, 0.96 to 2.51).

A protective effect of TA is consistent with the finding of higher PC levels in TA control subjects. We cannot exclude a bias accounting for the fact that the TA protective effect was limited to the younger patients. There might be confounding factors in the older patients explaining the heterogeneity of the results, but the study of such factors requires a larger series of patients.

Also consistent with the protective effect of TA in younger adults is the observed link between TA and age at the first thrombosis. Thrombosis occurred later in TA carriers, with mean ages of 36.9, 39.5, and 44.6 years in noncarriers, heterozygotes, and homozygotes, respectively. No such effect was observed with the CG allele.

The last finding in this study was the link between the TA allele and known acquired risk factors for thrombosis. In the Leiden Thrombophilia Study, the distribution of acquired risk factors was not influenced by PC levels. It remains to be determined why the TA allele, which seemed to have a protective effect in subjects under 45 in our study, was more frequent in patients with spontaneous thrombosis than in those with circumstantial risk factors. The observation that TA mainly reduces the risk of thrombosis in patients under 45 with acquired risk factors suggests that it interacts with risk factors specific to younger people, such as trauma, oral contraception, and pregnancy. The patients with the TA allele and thrombosis might thus have unknown genetic abnormalities overwhelming the apparent protective effect of the TA allele.

The study of a large population of healthy subjects allowed us to confirm the link between the CG allele and lower PC concentrations, and to show the opposite effect of the TA allele in homozygotes. Carrying 1 copy of the CG allele increased the risk of thrombosis by 4% to 87% whereas the TA allele had a protective effect in subjects under 45. This illustrates the complexity of the genetic factors involved in the risk of venous thrombosis.

Acknowledgments

This work was supported by a grant from Programme Hospitalier de Recherche Clinique number AO94031 “Evaluation clinique et biologique du risque thrombotique”. We thank Marie-Laurence Aubry for her excellent technical assistance and José Bon-Deguingand for her excellent secretarial assistance.

References

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doi: 10.1161/01.ATV.19.6.1573
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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