Venous Thromboembolism Associated With Double Heterozygosity for R506Q Mutation of Factor V and for T298M Mutation of Protein C in a Large Family of a Previously Described Homozygous Protein C–Deficient Newborn With Massive Thrombosis

By Benjamin Brenner, Ariella Zivelin, Naomi Lanir, Judith S. Greengard, John H. Griffin, and Uri Seligsohn

It is remarkable that certain patients with heterozygous protein C (PC) deficiency manifest venous thromboembolism (VTE), whereas others, particularly those belonging to families with homozygous PC deficiency, remain asymptomatic. The goals of the present study of a family, in which the proband had homozygous PC deficiency, were to identify members with and without VTE, to determine the mutation causing PC deficiency, and to search for the R506Q mutation of factor V (FV) causing activated PC resistance. Heterozygosity for a T298M mutation in exon 9 of the PC gene was found in the father of the homozygous proband who died of massive thrombosis. Based on analysis of a three-dimensional molecular model of PC, we speculate that this mutation causes type I deficiency due to disruption of packing of hydrophobic side chains and loss of an H-bond between Q184 and T298. Forty-six family members were examined for the T298M mutation by polymerase chain reaction (PCR) amplification of exon 9 and restriction analysis using Mae I and for the FV R506Q mutation by PCR amplification of exon 10 and restriction analysis using Mnl I. VTE was observed in five of 11 members who were heterozygous for both PC and FV mutations. In contrast, VTE was not observed for the PC mutation in 13 heterozygotes who had normal FV, including the parents of the deceased proband, 10 heterozygotes for the FV mutation who had normal PC, and 12 individuals bearing neither mutation. These observations extend recent evidence of an increased thrombotic risk conferred by the coexistence of homozygous PC deficiency and heterozygous activated PC resistance and support the paradigm in which hereditary thrombophilia is often a multigenic disease.

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PROTEIN C (PC) is a vitamin K-dependent zymogen that on activation exerts an anticoagulant effect by selectively inactivating factors Va and VIIIa. Hereditary deficiency of PC can be manifested by thrombophilia in heterozygous carriers of the autosomal dominant PC deficiency and by purpura fulminans or massive thrombosis in homozygous neonates of the autosomal recessive PC deficiency type. It is remarkable that heterozygotes belonging to families with "autosomal recessive" PC deficiency have been reported to present with thromboembolism much less frequently than heterozygotes for the "autosomal dominant" type of PC deficiency. More than a decade ago, a study of a very large Arab family with massive thrombosis due to homozygous PC deficiency presenting in two neonates was published. At that time, except for a 20-year-old family member who had died earlier of pulmonary embolism probably related to Behçet's disease, none of 14 heterozygotes identified with PC deficiency belonging to this family had a history of thromboembolism. However, since 1984, five adult members of this family, aged 39 to 60 years, have presented with venous thromboembolism. It was the purpose of this study to characterize the mutation causing PC deficiency in this family and to search for additional hereditary defects that could have contributed to the manifestation of thrombosis in clinically affected individuals. The results show that the mutation that most often causes resistance to activated protein C (APC), namely R506Q in factor V, is present in the family and that only individuals who are heterozygous for both genetic defects have presented with thrombosis.

MATERIALS AND METHODS

Study group and blood processing. The study group consisted of 46 available members of a previously reported family with autosomal recessive PC deficiency. An updated clinical history was obtained and documentation of thrombotic events in symptomatic patients was compiled from their medical records. After informed consent was obtained, blood samples from family members were collected into two plastic tubes, one containing 1/10 volume of 3.8% sodium citrate for coagulation assays and the other containing 1/100 volume of 0.5 mol/L sodium EDTA for DNA extraction. After centrifugation (15 minutes at 1,600g), citrated plasma aliquots were stored at -70°C until analyzed. Genomic DNA was prepared by a standard technique.

Coagulation assays. The normal range (+2 SD to -2 SD of the mean) for each parameter examined was determined in 20 healthy men and women. PC antigen was determined by enzyme immunoassay, using a specific Asserachrom kit (Diagnostica Stago Asnieres, France). The normal range was 60 to 136 U/dL. PC activity was determined by a chromogenic assay, using the PC Stachrom kit (Diagnostica Stago). The assay was calibrated with a commercial calibration plasma (IL, Italy). The normal range was 78 to 146 U/dL. Antithrombin III activity was determined by chromogenic assay using Antithrombin III Asserachrom kit (Diagnostica Stago). The normal range was 78 to 128 U/dL. Total protein S antigen was determined by electroimmunoassay using the Asseraplote Protein S kit (Diagnostica Stago). After precipitation of protein S complexed to C4 binding protein in plasma with polyethylene glycol (PEG 5000), free protein S antigen in the supernatants was determined using electroimmunoassay. The normal range was 70 to 130 U/dL for total protein S and 65 to 130 U/dL for free protein S antigen.

Detection of R506Q mutation of the factor V gene. Detection of the factor V mutation of G to A at nucleotide 1691 in genomic DNA

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was essentially performed using the polymerase chain reaction (PCR) and primers, as previously described and using Mnl I digestion. A 206-bp DNA fragment of the factor V gene surrounding nucleotide 1691 was amplified by PCR using the following primers: F—5' CATACTACAGTGACGTGGAC 3' and R—5' TGGTTCCTTGAAGGAAATGC 3'. Digestion of this 206-bp fragment by Mnl I was essentially performed using the polymerase chain reaction (PCR) and subjected to complete nucleotide sequencing as previously described. Detection of C10738T PC mutation in DNA samples. A 1,235-bp genomic DNA fragment of exon 9 of the factor V gene was amplified by PCR and subjected to Mae III digestion. The digested normal allele yielded a doublet of approximately 430 and an additional band of 230 bp (a doublet of 60 bp and a band of 250 bp were not detectable). In the mutated allele, one Mae III restriction site was abolished and fragments of approximately 860 bp and 230 bp were observed instead. The primers of the PCR were: F—5' cagaagctt—GAAACCAACCAGCATCTACC 3' and R—5' gtcaagctt—GCTGGAAAGCTGGTCAAGCC 3'. Lower case letters represent bases not found in the PC sequence. The PCR was performed in 25 µL reaction mixtures consisting of the same ingredients used for detection of the factor V mutation, except for the PC specific primers. Thirty cycles of 94°C (60 seconds), 63°C (120 seconds), and 72°C (180 seconds) were performed. The amplified DNA product (6 µL) was digested with 1 U of Mae III (New England Biolab, Beverly, MA) at 37°C for 3 hours. The sample was then subjected to 4% Nusieve agarose electrophoresis (FMC, Rockland, ME) and the fragments visualized by ethidium bromide.

Characterization of the mutation responsible for PC deficiency. All nine exons of the PC gene in one heterozygous PC deficient family member were analyzed and subjected to complete nucleotide sequencing as previously described. Three mutations were found, two of which cause neutral polymorphisms (nt A655T and G10826A). A T298M heterozygous mutation in the PC gene due to nt C10738T was detected in this subject and subsequently in 24 of the 46 examined family members as shown in Fig 1. All 24 were heterozygotes for the T298M mutation and had plasma PC activity and antigen levels that were less than 2 SD below the normal mean or borderline low. None had reduced levels of antithrombin III activity, total protein S antigen, or free protein S antigen. Both parents (III-6 and III-7) of the newborns who died of purpura fulminans and massive thrombosis (IV-2, IV-4) (3) were heterozygous for the T298M PC mutation. It is likely, therefore, that both newborns, of whom one (IV-4) was shown to have PC antigen less than 1% of normal, were homozygous for the T298M mutation.

As depicted in Fig 1, heterozygosity for the R506Q mutation in the factor V gene was detected in 21 of the 46 family members examined. All five patients who presented with venous thrombosis and thromboembolism as adults (III-15, III-16, III-19, IV-16, and IV-19) were heterozygous for both the PC T298M mutation and the FV R506Q mutation. As can be seen in Table 1, thrombosis first manifested in these patients at an age range of 39 to 60 years. In all of them, the thrombotic events were spontaneous and have occurred after the description of the family. Deep vein thrombosis was observed in all of them, with recurrent events in three subjects and pulmonary emboli in two. Six additional patients with a mean age of 29 years (range, 7 to 50 years) were also heterozygous for both mutations (Fig 1) and so far have not presented with thrombosis. Similarly, no thrombotic episodes have been observed in 13 family members (mean age 37 years, range, 7 to 65 years) who were heterozygotes only for the PC T298M and in 10 subjects (mean age 37 years, range, 10 to 76 years) who were heterozygotes only for the FV R506Q mutation (Table 2).

**DISCUSSION**

The present study and our previous observations indicate that homozygous PC deficiency due to the T298M mutation is associated with an extremely severe thrombotic diathesis manifested soon after birth. Heterozygotes for the T298M
PC mutation in the described family, albeit having reduced levels of PC, have not presented with thrombosis unless they also carry the FV R506Q mutation, which is the most frequently identified hereditary risk factor for venous thrombosis.\(^7\text{-}^{10},15\) Previously, in a family in which heterozygotes for the PC T298M mutation were observed, evidence for associated thrombosis was equivocal\(^16\) and a possible coexistence of the FV R506Q mutation was not examined. It is, therefore, likely that PC deficiency stemming from the T298M mutation is recessive from a clinical standpoint.

The generally asymptomatic nature of heterozygous PC deficiency was observed among more than 15,000 healthy blood donors surveyed in American and Scottish populations\(^17\text{-}^{19}\) and in heterozygotes belonging to families with the recessive type of PC deficiency.\(^5,18\) In contrast, however, identical PC gene mutations were independently observed in heterozygotes with thrombosis belonging to families demonstrating dominantly transmitted PC deficiency and in heterozygotes belonging to other families with recessive transmission of PC deficiency.\(^19\) These discrepancies set the stage for studies to look for additional genetic defects that contribute to an increased risk of thrombosis in heterozygotes from families with “dominant” PC deficiency. Indeed, Koellemann et al\(^20\) found that among family members who were heterozygotes for both PC deficiency and the factor V R506Q mutation, 73% presented with thrombosis, whereas among those who were heterozygotes for only PC deficiency, 31% presented with thrombosis.

A recent study showed that the risk of spontaneous thrombosis in heterozygotes for the factor V R506Q mutation increases with age.\(^21\) Our observations are consistent with this finding, as the patients who presented with thrombosis and were double heterozygotes for the R506Q mutation of factor V and the T298M mutation of PC were older than the individuals who have not manifested thrombosis and bore either or both mutations. Our finding of a clear segregation between symptomatic heterozygotes bearing both mutations and the asymptomatic heterozygotes bearing only one of the PC T298M or factor V R506Q mutations are also consistent with reports that heterozygosity for either defect is associated with only a fourfold to eightfold increase in the risk of venous thrombosis.\(^4,5,14\text{-}^{17},22\)

In contrast, double heterozygotes for factor V and PC mutations are at significant risk of recurrent spontaneous thrombosis during adulthood, suggesting that long-term anticoagulant therapy should be considered.

Taken together, our data and those of others suggest that certain PC mutations causing “recessive” deficiency confer a very low risk of thrombosis in the heterozygous state unless accompanied by another prothrombotic genetic defect as demonstrated in the described family. Other so-called “dominant” PC mutations may confer in the heterozygous state a significantly higher risk of thrombosis by themselves,\(^22\) and if associated with an additional genetic defect, the risk of thrombosis may be further enhanced.\(^20\) Once any type of PC deficiency is established, a search for the common factor V R506Q mutation in such patients is indicated, as Gandrille et al\(^23\) recently found that 14% of a group of 113 unrelated PC heterozygotes also had the factor V mutation.

The nature of the molecular defects in the mutated proteins found in this family has been recently clarified. Based on analysis of a computer graphics model of PC, it is predicted that mutation of T298 to M in PC would destabilize the structure of PC because the hydroxyl group of T298 is hydrogen-bonded to the side chain of Q184 in a hydrophobic pocket.\(^24\) In factor V, mutation of R506 to Q ablates normal proteolytic inactivation by APC.\(^7,25\text{-}^{27}\) Normally, an initial cleavage at Arg 506 in the factor Va heavy chain is the first event for normal inactivation by factor Va, followed by subsequent cleavage at Arg 306, which totally inactivates factor Va.\(^28\) In the R506Q-factor Va, APC cleaves Arg 306 and inactivates the mutant molecule ~10-fold more slowly than normal factor Va.\(^26,27\) Thus, the R506Q mutation is a mild risk factor, as APC inactivation, although retarded, can readily occur.

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Table 1. Thrombotic and Laboratory Manifestations in Symptomatic Patients Who Were Heterozygous for Both the PC T298M and the FV R506Q Mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age at Presentation</th>
<th>DVT†</th>
<th>PE†</th>
<th>Protein C (U/dL)</th>
<th>PS Ag (U/dL)</th>
<th>ATIII (U/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-15</td>
<td>F/55</td>
<td>1</td>
<td></td>
<td>70</td>
<td>70</td>
<td>140</td>
</tr>
<tr>
<td>III-16</td>
<td>F/60</td>
<td>3</td>
<td></td>
<td>59</td>
<td>72</td>
<td>145</td>
</tr>
<tr>
<td>III-19</td>
<td>F/60</td>
<td>1</td>
<td></td>
<td>59</td>
<td>70</td>
<td>145</td>
</tr>
<tr>
<td>IV-16</td>
<td>M/39</td>
<td>3</td>
<td>1</td>
<td>45</td>
<td>60</td>
<td>125</td>
</tr>
<tr>
<td>IV-19†</td>
<td>F/41</td>
<td>2</td>
<td>2</td>
<td>60</td>
<td>36</td>
<td>90</td>
</tr>
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</table>

Abbreviations: DVT, deep vein thrombosis; PE, pulmonary emboli.
* Numbers indicate number of episodes.
† Patient on oral anticoagulant therapy.

Table 2. Thrombosis and Heterozygosity for the R506Q Mutation of Factor V and for the T298M Mutation of PC

<table>
<thead>
<tr>
<th>Gene Mutation</th>
<th>No. of Patients</th>
<th>No. With Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>T298M</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>R506Q</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>T298M &amp; R506Q</td>
<td>11</td>
<td>5</td>
</tr>
</tbody>
</table>
In summary, genetic analysis of the PC and factor V mutations in this large family support the paradigm in which clinically significant venous thrombosis is often a multigenic disease.

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REFERENCES


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