Partial Protein S Gene Deletion in a Family With Hereditary Thrombophilia

By Hans K. Ploos van Amstel, Menno V. Huisman, Pieter H. Reitsma, Jan Wouter ten Cate, and Rogier M. Bertina

Familial thrombophilia, the hereditary predisposition to venous thromboembolic disease, is associated with a protein S deficiency in approximately 8% of the cases. Laboratory measurements of total protein S antigen in affected families have indicated that heterozygotes, ie, individuals carrying both a normal and a defective protein S gene, are severely at risk of developing venous thrombosis at a young age. The recent isolation of protein S cDNA has enabled us to start a search for genetic defects in the protein S gene of heterozygotes. Using Southern blotting on probands of six unrelated families with hereditary protein S deficiency, one proband was found to have a grossly abnormal gene pattern. The abnormality appears to involve at least the deletion of the middle portion of the protein S coding sequence. Family analysis showed that the defect cosegregates with the protein S deficiency. These data agree with the notion that hereditary thrombophilia associated with protein S deficiency is indeed directly the result of a defect in the protein S gene.

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The diagnosis of protein S deficiency has relied on the measurements of plasma total protein S antigen levels and the previous finding of the association between half normal plasma concentrations and the risk for thrombosis in some families with hereditary thrombophilia. No association between protein S deficiency and genetic defects in the protein S gene has been described yet. Here we report on a partial protein S gene deletion in a family with hereditary protein S deficiency. In this family only the heterozygotes for the gene defect suffered from recurrent thrombotic episodes, indicating that familial thrombophilia may indeed be the direct result of a defect in the protein S gene.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from Promega Biotec (Madison, WI). (α-32P)dCTP (3,000 Ci/mmol) and gene screen plus filters were obtained from New England Nuclear (Boston). The random priming kit was obtained from Boehringer Mannheim (Mannheim, West Germany). Ultra low gelling temperature agarose was purchased from Sigma Chemical Co (St Louis).

Protein S cDNA probes. Restriction fragments of human protein S partial cDNA clones were excised after electrophoresis from ultra low gelling temperature agarose gels. Labeling of the cDNA fragments was performed with a random priming kit using (α-32P)dCTP according to the instructions of the manufacturer. The cDNA fragments and their corresponding position to the protein S mRNA are depicted in Fig 1.

Southern blot analysis. High molecular weight DNA was isolated from peripheral blood leukocytes according to established procedures. After digestion with restriction enzymes the DNA...
fragments were separated on agarose gels and transferred to gene screen plus filters. The blots were prehybridized and hybridized according to the instructions of the manufacturer in 1 mol/L sodium chloride, 10% dextran sulfate, 50 mmol/L Tris-HCl (pH 7.5), and 1% sodium dodecyl sulfate (SDS) at 65°C. The filters were washed twice for five minutes at room temperature in 2 x SSC (300 mmol/L sodium chloride, 30 mmol/L sodiumcitrate) and twice for 30 minutes with 2 x SSC, 1% SDS at 65°C. A final wash step was performed for 30 minutes with 0.1 x SSC, 0.1% SDS at 65°C. The filters were autoradiographed at −70°C with an intensifying screen.

**Immunologic assays.** Total protein S, protein C, factor II, and factor X antigen levels were determined essentially as previously described20-22 and are given in Table 1. The identification of patients with an isolated protein S deficiency was performed as previously described.43

**Patients' medical history.** The propositus (II-2, Fig 2, Table 1) is a 37-year-old white man with a history of thrombotic disease since 1972. In that year, at the age of 22, he suffered from two episodes of documented deep venous thrombosis (DVT) of his left leg, within a period of 6 months. He was treated with oral anticoagulants for 1 year. Subsequently he suffered from recurrent episodes of superficial thrombophlebitis. In 1976, at the age of 26, he developed a left sided varicose ulcer. In 1986, at the age of 36, he was referred by his general practitioner to the Thrombosis Unit (Academic Medical Center, Amsterdam), for noninvasive testing because of clinically suspected DVT of his right leg. The identification of patients with an isolated protein S deficiency was performed as previously described.43

In November, 1986 he was admitted to the hospital because of progressive severe abdominal pain. Laparotomy was performed and revealed mesenteric vein thrombosis associated with necrosis of the distal part of the ileum, which was resected. The patient received maintenance therapy with oral anticoagulants and has been disease free since.

The histories of two other brothers (II-1 and II-4), now aged 38 and 31 and of his mother (I-2) and her family, are without thromboembolic events. Laboratory studies revealed normal values for antithrombin III activity and antigen, plasminogen activity and antigen, fibrinogen, and protein C activity and antigen (in compari-

### Table 1. Laboratory Values and Clinical Manifestations of Thrombotic Disease in Members of the Protein S Deficient Family

<table>
<thead>
<tr>
<th></th>
<th>PS Ag (%)</th>
<th>PC Ag (%)</th>
<th>FII Ag (%)</th>
<th>FX Ag (%)</th>
<th>STP</th>
<th>DVT</th>
<th>PE</th>
<th>Age at Onset (yr)</th>
<th>OAC</th>
</tr>
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<tbody>
<tr>
<td>I-1</td>
<td>56</td>
<td>84</td>
<td>82</td>
<td>108</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>29</td>
<td>−</td>
</tr>
<tr>
<td>I-2</td>
<td>122</td>
<td>105</td>
<td>108</td>
<td>82</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>II-1</td>
<td>107</td>
<td>89</td>
<td>98</td>
<td>100</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>II-2*</td>
<td>18</td>
<td>57</td>
<td>45</td>
<td>45</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>22</td>
<td>+</td>
</tr>
<tr>
<td>II-3</td>
<td>18</td>
<td>62</td>
<td>42</td>
<td>50</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>22</td>
<td>+</td>
</tr>
<tr>
<td>II-4</td>
<td>90</td>
<td>100</td>
<td>91</td>
<td>94</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</tr>
</tbody>
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**Abbreviations:** PS, protein S; PC, protein C; FII, factor II; FX, factor X; Ag, antigen; STP, superficial thrombophlebitis; DVT, deep venous thrombosis; PE, pulmonary embolism; OAC, treated with oral anticoagulants; ++, recurrent episodes.

*Proband.
son with prothrombin and factor X antigen) for the propositus as well as the other affected family members.

RESULTS

DNA samples from ten unrelated normal individuals and from six unrelated protein S deficient patients (identified according to the criteria described in reference 36) were digested with the restriction enzymes EcoRI, MspI, HindIII, and PvuII and analyzed by Southern blotting. Protein S cDNA fragments spanning the complete coding and 3'-untranslated region were used as probes (probes A and B in Fig 1). Of the six protein S deficient patients, one (proband II-2, Fig 2, Table 1) showed an abnormal hybridization pattern for all four enzymes used, indicating a gross alteration in the protein S gene. In Fig 3 the MspI digest is shown for proband II-2 (Table 1, Fig 2) and for one unrelated normal subject. The patient shows an additional 4 kb MspI fragment. This fragment was never observed while surveying 50 additional normal individuals, which excludes the possibility of a frequent neutral gene polymorphism.

The organization of the protein S gene is still unknown. However, to get some insight into the mutational events causing the defect, the blot was reprobed with restriction fragments of the coding region of the protein S cDNA (probes A, C, and D, Fig 1). In Fig 3 the data are shown for the MspI digest of patient II-2 and a normal subject. Hybridization with probe A visualizes the additional 4 kb fragment. This fragment was also detected after hybridization with probe C. The protein S cDNA contains an MspI restriction site located between probe A and C.3435 The observation of a shared hybridizing MspI fragment between probe A and C, therefore suggests that in some way the normal organization of the protein S gene has been interrupted. The reduced signals of the hybridizing 9.0 kb, 2.5 kb, and 0.7 kb MspI fragments as compared with normal (Fig 3), point to a deletional event in which a part of the coding region of the protein S gene is involved. This was confirmed by hybridization with the 5'-region of probe C (Fig 1, probe D); the 4 kb MspI fragment was not observed, indicating that the band resulted from the deletion of at least a major part of the exons encompassed by probe D (Fig 3).

Analysis of the DNA from the other members of the family (Table 1, Fig 2) showed that for all enzymes (viz, MspI, HindIII, EcoRI, PvuII) the additional hybridizing fragment cosegregated with protein S deficiency. In Fig 4, the segregation pattern is shown for PvuII digested DNA. The affected members I-1, II-2 and II-3 show the aberrant hybridization pattern. They all have an additional 14 kb PvuII fragment, that is absent in the unaffected members.

DISCUSSION

DNA from six unrelated patients with an inherited protein S deficiency was examined to search for genetic defects in

![Fig 3. MspI digest of a normal subject (N) and of the protein S deficient proband II-2 (P). Hybridization was performed with the protein S cDNA probes (see Fig 1) as indicated at the bottom. The arrows indicate for the patient the additional fragment and the fragments with a reduced intensity. Molecular weights of the fragments are given in kilobases.](image)

![Fig 4. PvuII digest of DNA from the members of the protein S deficient family (see Fig 2). The Southern blot was hybridized with probes A and B combined. The arrows indicate the position of the additional hybridizing fragment. Molecular weight markers are given in kilobases.](image)
the protein S gene. Southern blot analysis revealed one proband with gross alterations in the protein S gene. After digestion with four different enzymes, viz. EcoRI, HindIII, MspI, and PvuII, hybridization patterns were observed that deviated from normal. No differences were found in the protein S genes of the five other protein S deficient patients, indicating that if these genes were defective, the changes were subtle (e.g., point mutations) and remain undetectable on Southern blotting. Similar observations have been made for protein C deficiency.39

A more detailed analysis of the gene abnormality using restriction fragments covering various parts of the coding region of human protein S cDNA indicates that a large part of the middle portion of the protein S gene has been deleted.

Family analysis showed the cosegregation of the additional hybridizing fragment, which is characteristic for the defective allele, with heterozygosity for protein S deficiency. The data establish that in this family, protein S deficiency is the result of a defect in the protein S gene itself and not indirectly due to a defect in some regulatory gene.

Furthermore, it is evident that in this particular family, thrombosis occurred only in members heterozygous for the protein S gene defect, which provides further evidence for the notion that at least in the investigated members of this family protein S deficiency and familial thrombosis are causally related.30-33 Earlier we reported the presence of two protein S genes, both located on chromosome 3, per haploid genome.35

It is unknown whether both genes take part in the synthesis of plasma protein S or that one gene has been silenced during evolution. Therefore, the establishment of a causative link between the observed partial gene deletion and low plasma protein S levels, awaits elucidation of the organization of the protein S locus. However, in this family the protein S gene defect can be used as a genetic marker for inherited protein S deficiency, thereby supplementing the diagnosis based on plasma total protein S antigen levels.

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