CONCISE REPORT

Plasma Protein S Deficiency in Familial Thrombotic Disease

By Hans Peter Schwarz, Michael Fischer, Pierre Hopmeier, Mary Ann Batard, and John H. Griffin

A family with a history of severe recurrent venous thromboembolic disease was studied to determine if a plasma protein deficiency could account for observed disease. Protein S levels in plasma were determined immunologically using the Laurell rocket technique. The propositus, his mother, his aunt, and his cousin who were clinically affected had 17% to 65% of the control levels of protein S antigen (normal range, 71% to 147%). Since three of these patients were receiving oral anticoagulant therapy, the ratios of protein S to prothrombin, factor X, and protein C in these patients were compared with values for a group of orally anticoagulated controls. These results suggested that protein S is half-normal in all family members with thrombotic disease. Other proteins known to be associated with familial thrombotic disease, including antithrombin III, plasminogen, fibrinogen, and protein C, were normal. Because plasma protein S serves as a cofactor for the anticoagulant activity of activated protein C and because protein C deficiency is associated with recurrent thrombotic disease, it is suggested that recurrent thrombotic disease in this family is the result of an inherited deficiency of protein S.

Protein S, a vitamin K-dependent plasma protein,1,2 increases the rate of inactivation of factor Va by activated protein C (APC) by enhancing the binding of APC to phospholipids and stimulating the inactivation of factor Va.3 APC is a potent inhibitor of coagulation in plasma because it inactivates factors V and VIII and is a profibrinolytic agent because it stimulates the release of plasminogen activator and neutralizes plasminogen activator inhibitor.4,8 Inherited protein C deficiency is associated with familial thromboembolic disease.9,11 In plasma immunologically depleted of protein S, the normal anticoagulant activity of APC is not readily apparent and reconstitution of such plasma with purified protein S restores anticoagulant activity of APC.12,13 Thus, the available information shows that protein S functions as a cofactor in vitro for the anticoagulant activity of APC, and therefore we hypothesized that an inherited deficiency in protein S would present as a congenital thrombophilia since protein C deficiency is associated with thrombotic disease.9,11 We report here a family with recurrent venous thromboembolic disease associated with an inherited deficiency of protein S.

METHODS

Purification of Human Protein S

Human protein S was purified from commercial factor IX concentrate (Proplex, Hyland Therapeutics, Travenol, Deerfield, Ill) by QAE-Sephadex and Blue Sepharose CL-6B chromatography (Pharmacia, Piscataway, NJ) as follows. Lyoophilized commercial factor IX concentrate (23 bottles) was dissolved in 245 mL of sterile deionized water and dialyzed against starting buffer (0.01 mol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L benzamidine·HCl, and 0.02% NaN3). After dialysis, the protein S pool was applied to a Blue Sepharose CL-6B column (2.5 cm × 10.5 cm), equilibrated in starting buffer, and washed at 15 mL/h with 315 mL of starting buffer. The protein S eluted in the void volume, while prothrombin adsorbed to the column. Fractions containing protein S, as judged by positive antigenic activity, were reduced and electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. As described previously,14 purified protein S appeared as two closely spaced bands with an apparent molecular weight of ~70,000 daltons. Protein concentration was determined spectrophotometrically using an extinction coefficient of 1.0 at 280 nm for human protein S,13 and confirmed by the method of Lowry et al.16 A limited amino terminal sequence determination of purified protein S showed the same terminal sequence as previously reported,14 ie, Ala-Asn-Ser-Leu-Leu-(Gla).

Goat antiserum to protein S was prepared by four successive weekly injections of 75 μg of purified protein S in multiple subcutaneous sites. The first two injections were in complete Freund's adjuvant while the subsequent two boosts were with incomplete adjuvant. Six months later, the goat was boosted with two successive weekly injections of 50 μg protein S in incomplete Freund's adjuvant and antiserum was obtained one week later. The antiserum was tested by double immunodiffusion and showed a line of identity between normal human plasma (NHP) and purified protein S. A second faint precipitation line was noted only with NHP and not with purified protein S. Upon a 50% dilution of NHP, this second line was not visible, while the line of identity to purified protein S remained.

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Antiprotein S Laurell plates were prepared using 1% agarose ("standard low m.", Bio-Rad, Richmond, Calif) and 0.33% antiserum. Electrophoresis was for 16 hours at 4 mAmp per plate in 0.081 mol/L Tris base, 0.024 mol/L Tricine, pH 8.6 with 0.3 mmol/L EDTA and NaCl. The plates were washed for at least four hours in 0.5 mol/L NaCl, rinsed in H2O for one hour, and then dried and stained using Coomassie Blue G-250. Each Laurell plate was calibrated with at least four dilutions of normal plasma pool (15 normals; dilutions, 100%, 50%, 25%, 12.5%) and each patient sample was analyzed at two dilutions on at least two plates. Antigen levels were calculated relative to the normal pool level that was defined as 100%. Under our conditions of electrophoresis, only one protein S rocket was observed in contrast to the reports by Dahlback that demonstrated two rockets, one for protein S and one for a complex of C4b-binding protein with protein S. Antigenic levels of protein C, factor X, and prothrombin were also measured using Laurell immunoelectrophoresis with Coomassie Blue G-250 staining as previously described.

RESULTS

Patients' Medical History

The propositus is a 40-year-old Caucasian male with a history of thromboembolic disease that began at the age of 30 in 1974. He developed right-sided pleuritic chest pain and dyspnea associated with pulmonary infiltrates and elevated hemidiaphragm of the right side. The diagnosis of pulmonary embolism was made. Other clinical symptoms indicated deep vein thrombosis in his right leg. The patient was treated with heparin and subsequently with oral anticoagulants for nine months. In 1976 he again developed iliofemoral thrombosis in his right leg complicated by bilateral pulmonary emboli. Clinical diagnosis was confirmed by venograms and perfusion lung scans. At the age of 34, he had a myocardial infarction. He has been hospitalized on numerous occasions with recurrent pulmonary emboli. Family history includes numerous episodes of thrombosis. The mother of the propositus, now aged 66, has suffered from thrombophlebitis since her young adulthood. A cousin of the propositus is a 30-year-old white male who developed deep vein thrombosis at the age of 27 and has been hospitalized several times because of calf vein thrombosis and multiple pulmonary emboli. The mother of this cousin, ie, the aunt of the propositus, had thrombophlebitis during pregnancy, but reported no other thrombotic problems. The maternal grandmother had recurrent thrombotic disease and died of pulmonary emboli. Laboratory studies of plasma from the propositus and from other clinically affected family members indicated normal levels of antithrombin III antigen and activity, plasminogen antigen and activity, clottable fibrinogen, and thrombin times. Normal values for protein C antigen in comparison to prothrombin and factor X antigen were found, implying normal protein C levels.

Protein S Measurements

Based on the Laurell rocket technique, the mean protein S antigen value of 30 normal adults was 97.5% of reference pooled normal plasma, and the observed values ranged from 71% to 147%. The statistical range for 30 normals (mean ± 2 SD) was from 61% to 134%. The plasma concentration of protein S antigen was determined to be 34 μg/mL in pooled normal human plasma.

After an initial determination of protein S antigen of the propositus as 23% of normal, a further study of the propositus and his family was undertaken. No evidence of liver disease or disseminated intravascular coagulation was apparent at the times of plasma collection. The aunt of the propositus had a protein S antigen level of 65% and normal values for prothrombin, factor X, and protein C (110%, 136%, and 85%, respectively). The severely affected family members were in a stable phase of oral anticoagulant treatment during our study and we were unwilling to stop this therapy because of the risk of thrombotic problems. Because protein S is a vitamin K-dependent plasma protein, its concentration is reduced during oral anticoagulant treatment. Therefore, we measured protein S antigen levels as well as other vitamin K-dependent factors in the propositus and his family members as well as in a reference group of control patients in a stable phase of oral anticoagulant therapy. Values of vitamin K-dependent proteins in patients receiving oral anticoagulant therapy are shown in Table 1. Ratios of protein S antigen to prothrombin antigen, factor X antigen, and protein C antigen were calculated for each individual, using data from Table 2, and are shown in Table 3. To diagnose an isolated protein S deficiency in the heterozygous state, the ratios of protein S to other vitamin K-dependent factors for patients were compared with the control group, as seen in Table 3. The validity of this approach to diagnose protein C deficiency has been previously suggested and confirmed. The ratios of protein S to prothrombin, factor X, and protein C for the propositus, his mother, and his cousin are all outside the normal range of reference anticoagulated

<table>
<thead>
<tr>
<th>Protein S</th>
<th>Prothrombin</th>
<th>Protein C</th>
<th>Factor X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (± 2 SD)</td>
<td>67 (± 29)</td>
<td>43 (± 13)</td>
<td>52 (± 16)</td>
</tr>
<tr>
<td>Observed range</td>
<td>37.6-96.4</td>
<td>30.5-56.1</td>
<td>35.9-67.5</td>
</tr>
</tbody>
</table>

The plasma samples from 37 randomly chosen patients receiving oral anticoagulant treatment were analyzed using the Laurell rocket technique. Prothrombin time values were in the therapeutic range, ie, between 1.5 and 2.5 times of control values. Values indicate the percentage of reference pooled normal plasma.
patients. These data suggest that the protein S antigen levels of the propositus, his mother, and his cousin are all outside the normal range. Protein S antigen levels of our patients were inferred by comparing the observed ratio of protein S with other vitamin K-dependent proteins to the ratio for 37 reference plasmas. Based on averaging the values for protein S/prothrombin, protein S/factor X, and protein S/protein C, the inferred values of protein S antigen for the propositus, his mother, and his cousin are 38%, 45%, and 30%, respectively. The asymptomatic 43-year-old sister and 37-year-old cousin of the propositus had normal values for protein S antigen (105% and 104%, respectively). Figure 1 depicts a family tree and the history of venous thrombosis associated with low levels of protein S.

**DISCUSSION**

To search for a laboratory diagnosis of protein S deficiency associated with inherited thrombotic disease, an immunologic assay for plasma protein S was developed. Based on the Laurell rocket technique, the level of protein S antigen in normal plasma is 34 μg/mL. This level of protein S is approximately that reported by Dahlbäck and Stenflo for total protein S antigen. Studies described here indicate that plasma levels of protein S antigen of approximately one third to one half of normal are associated with recurrent venous thrombosis in four members of the family, as depicted in Fig 1. Two asymptomatic family members have normal protein S antigen levels (Fig 1). The aunt of the propositus is not receiving oral anticoagulant treatment, has 65% protein S antigen, and is heterozygous for protein S deficiency. She has a history of venous thrombotic disease during pregnancy, but otherwise has not experienced the recurrent thromboses, including pulmonary emboli, that are reported by other clinically affected family members (Fig 1). It is suggested that the venous thrombotic disease in this family is the result of an inherited deficiency in protein S. This trait appears to be autosomal dominant.

The search for protein S deficiency associated with venous thrombosis was initiated based on the observations that protein S is a cofactor for the anticoagulant activity of activated protein C and that protein C deficiency is associated with venous thrombotic disease. The heterozygous deficiency state of protein C is associated with half-normal levels of protein C and a pronounced tendency toward venous thrombotic disease that often presents in early adulthood. The heterozygous deficiency state of protein S appears clinically to be very similar to the heterozygous deficiency of protein C, in that there is a remarkable history of recurring venous thrombotic disease beginning in young adulthood in the members of the family described here. The discovery of protein S deficiency associated with venous thrombosis that is clinically similar to protein C deficiency suggests that the cofactor activity of protein S observed in vitro may also be important for the in vivo activity of protein C as an anticoagulant enzyme. Since activated protein C is both anticoagulant and profibrinolytic, this suggests that the antithrombotic activity of activated protein C in vivo is based, at least in part, on its anticoagulant activity, since the clinical symptoms of protein C deficiency and protein S deficiency are so similar. The

**Table 2. Vitamin K-Dependent Protein Antigen Levels in Plasmas of Family Members With Recurrent Venous Thrombosis**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Propositus</th>
<th>Propositus' mother</th>
<th>Propositus' cousin</th>
<th>Propositus' aunt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein S</td>
<td>27</td>
<td>52</td>
<td>17</td>
<td>65</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>59</td>
<td>69</td>
<td>35</td>
<td>110</td>
</tr>
<tr>
<td>Protein C</td>
<td>48</td>
<td>86</td>
<td>44</td>
<td>85</td>
</tr>
<tr>
<td>Factor X</td>
<td>42</td>
<td>84</td>
<td>38</td>
<td>136</td>
</tr>
</tbody>
</table>

Values indicate the percentage of reference pooled normal plasma.

*Plasma was taken from each patient in the stable phase of oral anticoagulant therapy.

**Table 3. Ratios of Vitamin K-Dependent Proteins in 37 Reference Patients Receiving Oral Anticoagulant Treatment and in a Family With Thrombotic Disease**

<table>
<thead>
<tr>
<th>Protein S/Prothrombin</th>
<th>Protein S/Factor X</th>
<th>Protein S/Protein C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± 2 SD</td>
<td>1.57 ± 0.76</td>
<td>1.42 ± 0.78</td>
</tr>
<tr>
<td>Observed range</td>
<td>0.92 – 2.40</td>
<td>0.91 – 2.65</td>
</tr>
<tr>
<td>Propositus</td>
<td>0.46</td>
<td>0.64</td>
</tr>
<tr>
<td>Propositus’ mother</td>
<td>0.75</td>
<td>0.43</td>
</tr>
<tr>
<td>Propositus’ cousin</td>
<td>0.48</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Fig 1. Family tree of protein S-deficient family. The arrow denotes the propositus. Numbers indicate the percentage of protein S antigen. O, Females; ∆, males; †, deceased family members. O, ∆, Positive clinical history.
physiologic importance of the profibrinolytic activity of activated protein C remains to be demonstrated.7,8

Protein S deficiency joins the group of other plasma protein deficiencies associated with inherited thrombophilia. Other protein abnormalities or deficiencies associated with inherited venous thrombotic disease include protein C,9-11 plasminogen,19,20 fibrinogen,21,22 and antithrombin III.23-25 Further studies will be required to define the frequency of protein S deficiency in hereditary thrombotic disease and the importance of acquired protein S deficiency in other clinical conditions associated with acquired thrombotic disease.

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