Severe Perinatal Thrombosis in Double and Triple Heterozygous Offspring of a Family Segregating Two Independent Protein S Mutations and a Protein C Mutation

By Caroline J. Formstone, Paula J. Hallam, Edward G.D. Tuddenham, Jennifer Voke, Mark Layton, Kypros Nicolaides, Ian M. Hann, and David N. Cooper

Molecular genetic and phenotypic analyses were performed in a highly unusual case of combined protein S and protein C deficiency manifesting in a family in which a child had died perinatally from renal vein thrombosis. Antenatal diagnosis in a second pregnancy was initially performed by indirect restriction fragment length polymorphism (RFLP) tracking using a neutral dimorphism within the Protein S gene and served to exclude severe protein S deficiency. An umbilical vein blood sample at 22 weeks gestation showed isolated protein C deficiency. This pregnancy proceeded to a full-term delivery without thrombotic complications. Molecular genetic analysis of the Protein C and Protein S genes segregating in the family then yielded one PROC gene lesion in the father and two Protein S gene lesions, one in each parent. These lesions were shown to segregate with the respective deficiency states through the family pedigree. Analysis of DNA from paraffin-embedded liver tissue taken from the deceased child showed the presence of both Protein S mutations, as well as the PROC mutation. Genotypic analysis of the second child showed a PROC mutation, but neither Protein S mutation consistent with its possession of normal protein S levels and a low/borderline protein C level. Antenatal diagnosis was then performed in a third pregnancy by direct mutation detection. However, although the fetus carried only the paternal PROC and Protein S gene lesions, the child developed renal thrombosis in utero. It may be that a further genetic lesion at a third locus still remains to be defined. Alternatively, the intrauterine development of thrombosis in this infant could have been caused, at least in part, by a transplacental thrombotic stimulus arising in the Protein S-deficient maternal circulation. This analysis may, therefore, serve as a warning against extrapolating too readily from genotype to phenotype in families with a complex thrombotic disorder.

The Protein C anticoagulant pathway represents an important control mechanism in hemostasis. Protein C, a vitamin K-dependent glycoprotein and zymogen of a serine protease, is activated at the endothelial cell surface by the thrombin/thrombomodulin complex and proteolytically cleaves and inactivates procoagulant factors Va and Vila. Protein S acts as a cofactor to activated protein C (APC) by enhancing the rate of inactivation of these factors. Protein S also serves to regulate hemostasis via an APC-independent mechanism. Approximately 60% of protein S in plasma is noncovalently complexed with C4b binding protein (C4bBP), while the remainder is unbound ('free'). The interaction of functional free protein S with C4bBP abolishes the anticoagulant activity of the former. The Protein C (PROC) gene resides on chromosome 2 and comprises 9 exons. The protein S (PROS) gene is located on chromosome 3, comprises 15 exons, and is closely linked to a highly homologous pseudogene. Hereditary deficiencies of both protein C and protein S are well-documented, and both are associated with an increased risk of venous thrombosis. Together they account for between 6% and 10% of families with hereditary thrombophilia. Two distinct types of hereditary protein C deficiency are recognized: type I, the most common, is characterized by a parallel reduction in protein C activity and antigen levels, whereas type II exhibits a greater reduction in activity than antigen. The classification of protein S deficiency is complicated by the presence of C4bBP-bound protein S in plasma. However, three types have been distinguished phenotypically: type I deficiency is characterized by reduced total and free antigen levels together with reduced anticoagulant activity. In type II deficiency, protein S activity is reduced, although total and free antigen levels are normal, whereas in type III deficiency, the total protein S antigen level is normal, but free protein S antigen and activity are reduced.

Estimates of the prevalence of clinically symptomatic deficiencies of protein C in the general population lie between 1:16000 and 1:36000, while the frequency of symptomatic protein S deficiency is 1:20000. Although most often found in the heterozygous state, severe homozygous or compound heterozygous deficiencies of both protein C and protein S and protein S deficiencies at the DNA level have been initiated. We describe here the clinical features together with the labora-
tory and molecular genetic analysis of a highly unusual family in which one PROC and two nonidentical PROS gene lesions are segregating.

MATERIALS AND METHODS

Family history. The first child, IV.1 (Fig 1), died at age 1 week from bilateral renal vein thrombosis. Although she showed no evidence of purpura fulminans, one kidney was fibrotic at postmortem with evidence of intrauterine thrombosis. The other showed evidence of recent infarction with renal vein thrombosis. Thrombosis was also present in the major cerebral veins. Pretreatment blood samples from IV.1 were not available for analysis. Several members of this English family had also experienced thrombotic manifestations and/or low levels of both protein C and protein S (Fig 1, Table I). I.2 experienced several episodes of phlebitis while II.6 suffered a deep vein thrombosis after surgery and has also experienced a pulmonary embolism. III.2, the propositus, experienced two iliofemoral thromboses as a teenager, the first at the age of 13 following traumatic injury sustained while playing football, the second at the age of 20, which was apparently spontaneous. He is currently on long-term warfarin prophylaxis. The mother of the deceased child (III.3) possessed levels of protein S consistent with an heterozygous type I deficiency state (Table 1). Measured protein C and S values for the father (II.2) (Table 1) were not useful because this individual had received anticoagulant therapy as he initially presented with clinical symptoms. His father (II.2) exhibited both protein C and protein S deficiency phenotypes (types II and III, respectively) (Table 1). Although maternal (III.3) protein C levels appeared normal, several of her immediate relatives (II.3, III.4, and III.5) possessed borderline normal protein C activity and/or antigen levels (Table 1).

During a second pregnancy (IV.2), fetal blood sampling (cordocentesis) at 22 weeks gestation showed protein S activity and free protein S antigen levels of 29 U/dL (normal range [NR], 23 to 30) and 33 U/dL (NR, 35 to 41), respectively (Table 2), both of which were normal for gestational age. Protein C activity, however, was lower than normal for gestational age (5 U/dL, NR, 9.5 to 13.5). The pregnancy was uneventful and a clinically normal baby was born at 39 weeks gestation. At birth, protein C activity was slightly low (8 U/dL, NR, 12 to 18), while total protein S antigen remained within the normal range (43 U/dL, NR, 30 to 50). Although protein S values were unchanged by 16 months of age, protein C antigen and activity levels were borderline normal (Table 2). It is unclear, however, why protein C activity and antigen levels are very similar in IV.2. This child is still healthy.

A third pregnancy (IV.3) was monitored very carefully. Chorionic villus sampling was performed at 14 weeks gestation. Delivery was by emergency Caesarean section at 38 weeks due to decreased fetal movement. Cord blood taken at the time of delivery contained a protein C activity level of 4 U/dL [NR, 24 to 38 weeks gestation], fetuses, and neonates, 8.1 to 22.9] and a borderline normal protein C antigen level of 17 U/dL [NR day 1 (full-term), 17 to 53]. Total and free protein S antigen levels were found to be 14 U/dL [NR day 1 (full-term), 17 to 53] and 2 U/dL [NR day 1 (full-term), 32 to 84], respectively, while protein S activity was 24 U/dL (NR, 55 to 119). A palpable kidney at 12 hours of age was investigated further by ultrasound imaging that showed left renal vein thrombosis, left adrenal hemorrhage, thrombosis in the inferior vena cava, and a thrombus in the bladder. Because protein C deficiency was felt to be his major problem, initial treatment was with intravenous fresh frozen plasma and heparin and then a few days later with protein C concentrate. His plasma protein C levels increased accordingly. A right atrial Hickman catheter was inserted at the age of 2 weeks for the regular administration of protein C concentrate and heparin. This was subsequently removed at the age of 6 weeks, protein C concentrate and heparin were withdrawn, and warfarin therapy commenced. The International Normalized Ratio is being maintained between 3.0 and 3.5, and it is planned to continue this treatment indefinitely. At the time of writing, at the age of 9 months, there have been no further thrombotic episodes. The child is thriving despite the functional loss of about 90% of his left kidney.

Table 1. Phenotypic and Genotypic Data From Family Members

<table>
<thead>
<tr>
<th>Family Member</th>
<th>PC Activity (NR 73-121)</th>
<th>PC Antigen (NR 81-142)</th>
<th>PROC Mutation</th>
<th>PS Total (NR 77-100)</th>
<th>PS Free (NR 81-113)</th>
<th>PROS Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.2</td>
<td>87</td>
<td>128</td>
<td>NT</td>
<td>82</td>
<td>&lt;5</td>
<td>-</td>
</tr>
<tr>
<td>II.1</td>
<td>81</td>
<td>88</td>
<td>NT</td>
<td>134</td>
<td>58</td>
<td>NT</td>
</tr>
<tr>
<td>II.2</td>
<td>37</td>
<td>60</td>
<td>●</td>
<td>74</td>
<td>54</td>
<td>●</td>
</tr>
<tr>
<td>II.3</td>
<td>60</td>
<td>64</td>
<td>NT</td>
<td>106</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>II.4</td>
<td>82</td>
<td>109</td>
<td>NT</td>
<td>82</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>II.5</td>
<td>100</td>
<td>125</td>
<td>NT</td>
<td>94</td>
<td>127</td>
<td>NT</td>
</tr>
<tr>
<td>III.1</td>
<td>106</td>
<td>76</td>
<td>NT</td>
<td>114</td>
<td>82</td>
<td>NT</td>
</tr>
<tr>
<td>III.2</td>
<td>31†</td>
<td>16†</td>
<td>●</td>
<td>24†</td>
<td>&lt;10†</td>
<td>●</td>
</tr>
<tr>
<td>III.3</td>
<td>85</td>
<td>98</td>
<td>—</td>
<td>44</td>
<td>13</td>
<td>●</td>
</tr>
<tr>
<td>III.4</td>
<td>76</td>
<td>64</td>
<td>NT</td>
<td>58</td>
<td>31</td>
<td>NT</td>
</tr>
<tr>
<td>III.5</td>
<td>59</td>
<td>77</td>
<td>NT</td>
<td>39</td>
<td>4</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviations: ●, Arg 169 → Gin (PROC); ■, Met 570 → Thr (PROS); †, 2-bp deletion [codons 547/548] (PROS); NR, normal range for healthy adults; †, on warfarin treatment when tested; NT, not tested; —, no genetic lesion found.
(GIBCO-BRL, Middlesex, UK) were added to the dried pellet, and the mixture incubated at 55°C for 1 hour followed by 95°C for 10 minutes. A control tube containing no paraffin section was treated in a similar manner. The resulting solutions were used as templates for PCR amplification.

**PROS gene: Pro626 polymorphism analysis.** The alleles of the exon XV Pro626 polymorphism were determined at the level of both genomic DNA and mRNA (cDNA) by restriction fragment length polymorphism (RFLP) analysis as described.\(^{28}\)

**PCR/direct sequencing analysis of exon-containing DNA fragments.** Genomic DNA extraction from family members, DNA amplification, and PCR/direct sequencing were performed as described.\(^{28}\) The **PROS** gene exon VII and the **PROS** gene exon XIV from the deceased child (IV.1) were PCR amplified from paraffin-embedded liver tissue DNA in tubes washed previously in 10 mmol/L HCl and oven-dried. PCR amplification was performed as described.\(^{28}\) but following the recommendations of Woodward et al.\(^{28}\) 20 μL of a 1:10 dilution of both control and paraffin section samples were used for PCR amplification of **PROS** and **PROS** gene exons.

DNA samples from IV.2 and IV.3 were derived from chorionic villus samples, cord blood, and venipuncture after birth. Reverse-transcription (RT) PCR analysis for the **PROS** gene. Platelets from II.4 were separated from whole blood and RNA extracted.\(^{28}\) The specific reverse transcription of **PROS** mRNA and subsequent PCR amplification of cDNA fragments were performed as described.\(^{28}\)

Screening for APC resistance and factor V Leiden. Activated protein C (APC) resistance assays and screening for the common factor V Leiden lesion (CGA → CAA converting Arg 506 to Gln) were performed essentially as described.\(^{33}\) The **PROS** gene exon XIV in these family members was determined by PCR/direct sequencing of **PROS** exon XIV in these individuals (Fig 1, Table 1). Additional evidence for the Met570 → Thr substitution being the pathological lesion in the family was provided by sequence analysis of exon XIV from 50 controls (of Caucasian origin with no history of venous thrombosis), which failed to detect another example of the Met570 → Thr substitution. This mutation does not appear to be a common polymorphism. Moreover, its presence is associated with the clinical phenotype in the family through two generations (Fig 1). The maternal 2-bp deletion was detected in individuals III.4, III.5, and IV.1, but not in II.2 or IV.2. Thus, the deceased child (IV.1) possessed both paternal and maternal **PROS** gene lesions (Fig 1, Table 2).

Cord blood DNA was used to PCR amplify exon XIV from the second child (IV.2). Neither **PROS** gene lesion was detected in IV.2, an observation consistent with (1) the normal levels of total and free protein S noted in this child and (2) the absence of the paternal A allele demonstrated previously by antenatal Pro626 RFLP tracking analysis (see above). In the case of the third child (IV.3), antenatal screening for the presence of **PROS** gene mutations showed only the paternal Met570 → Thr mutation, the maternal **PROS** gene lesion being absent. Genotypic and phenotypic data obtained from the siblings in generation IV are summarized in Table 2.

It was previously demonstrated that individual III.3, who was heterozygous AG for the Pro626 **PROS** RFLP at the genomic DNA level, exhibited the loss of the A allele at the mRNA level ["allelic exclusion"].\(^{28}\) The A allele was in phase with the 2-bp **PROS** gene deletion identified in this individual. Loss of the **PROS** mRNA species bearing this mutation was consistent with her type I protein S deficiency state. The absence of the 2-bp deletion in II.4 cDNA (Fig 1) was also demonstrated by PCR/direct sequencing of platelet-derived cDNA from this individual. The demonstration of the loss of the A allele for the Pro626 RFLP by PCR/direct...
sequencing and RFLP analysis confirmed "allelic exclusion" in II.4.

Direct detection of the pathological lesion in the PROC gene. PCR/direct sequencing of all nine exons and splice junctions of the PROC genes of individual III.2 yielded only one deviation from the wild-type sequence: a heterozygous CGG → CAG transition in exon VII, which predicts an arginine to glutamine substitution at amino acid residue 169. The authenticity of this lesion was confirmed by sequencing the antisense DNA strand. This substitution was also found in individuals II.2, IV.2, IV.3, and the deceased child IV.1 (Fig 1) and, therefore, segregates with the protein C deficiency state through the family. By contrast, PCR/direct sequencing of all nine exons and splice junctions of the PROC genes of individual III.3 did not yield any deviation from the wild-type sequence.

Factor V Leiden screening. For individuals III.2, III.3, and IV.3, the presence of APC resistance was excluded by direct screening for the common factor V Leiden mutation and/or by means of the APC resistance assay.

DISCUSSION

Few families with a thrombotic diathesis and two distinct prothrombotic defects have been reported to date. However, the recent discovery of the common factor V Leiden variant (present in between 2% and 7% of individuals in European populations) has shown that combined deficiency states may not be as rare as was originally thought. Two cases of a triple deficiency have been reported from a single Canadian kindred: one individual was deficient in antithrombin III, protein C, and heparin cofactor II, and the other was deficient in antithrombin III, protein S, and heparin cofactor II. Such cases are, however, exceptionally rare, and none have been characterized at the DNA level. We report here the molecular genetic analysis of, and antenatal diagnosis in, a family with combined protein C and protein S deficiency in which three different gene defects are segregating.

The phenotypic data collected from family members indicated the complexity of antenatal diagnosis and the difficulty in counselling the parents of the deceased child in subsequent pregnancies. Since phenotypic data may be considered to be particularly unreliable in the case of fetal blood sampling owing to the low plasma protein levels at early gestational age, molecular genetic analysis was employed in the hope of improving diagnostic accuracy.

RFLP tracking was partially successful in that severe protein S deficiency was rapidly excluded in individual IV.2 during the second trimester. PCR/direct sequencing of PROC and PROS genes from family members was then employed to detect and characterize the underlying pathological lesions. Three mutations were found, one in the PROC gene and two in the PROS gene. The PROC mutation identified (Arg169 → Gln) has been reported at least three times previously in individuals with type II protein C deficiency. Its location within the Arg169-Leu170 activation site of protein C explains its association with a type II deficiency state. The detection and characterization of the PROS gene lesions has been reported previously. In that study, we confirmed the Authenticity of both lesions by sequencing the antisense DNA strand. In addition, no further deviations from the wild-type sequence were present in any of the other 14 exons and splice junctions of the PROS gene. The 2-hp PROS gene deletion generated an in-frame TAA termination codon just three amino acids C-terminal to the lesion, and this accounts for the type I protein S deficiency observed in maternal family members and for the allelic exclusion noted in II.4 and III.3. The paternal Met570Thr substitution occurred in a residue that is conserved in both rabbit and bovine protein S and in the homologous human protein, sex hormone binding globulin. The amino acid environment of Met570 appears hydrophobic, which suggests that this region is internal to the protein. The present study provided further support for the conclusion that Met570Thr is the pathological mutation by demonstrating its absence in 100 normal alleles. In addition, the presence of this PROS gene lesion is associated with the clinical phenotype in two generations of this kindred. We have previously suggested that the Met570Thr substitution may be associated with type III protein S deficiency. Total and free protein S antigen levels from individual IV.3 are also consistent with a type III deficiency. However, the biochemical characterization of an in vitro-expressed protein should establish whether the Met570Thr substitution is indeed a disease-causing mutation and possibly the pathological mechanism underlying this disorder.

The genotypic data from family members broadly confirmed our initial assumptions made regarding the number and nature of the gene defects and their pattern of inheritance. The deceased child's father (III.2) had correctly been predicted to be doubly heterozygous for mutations in both

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Weeks</th>
<th>PC Activity (UI/dL) (NR)</th>
<th>PC Antigen (UI/dL) (NR)</th>
<th>PROC Mutation</th>
<th>PS Activity (UI/dL) (NR)</th>
<th>PS Antigen (UI/dL) (NR)</th>
<th>PROS Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV.1</td>
<td>1 wk</td>
<td>23 (8.1-22.8)</td>
<td>23 (17-53)</td>
<td></td>
<td></td>
<td>28+ (+17-53) total</td>
<td></td>
</tr>
<tr>
<td>IV.2</td>
<td></td>
<td>22/40</td>
<td>5 (9.5-13.5)</td>
<td></td>
<td></td>
<td>29 (23-30) free</td>
<td>33 (35-41) free</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38/40 (term)</td>
<td>8 (21-65)</td>
<td></td>
<td></td>
<td>43 (20-64) total</td>
<td></td>
</tr>
<tr>
<td>IV.3</td>
<td>16 mo</td>
<td>66 (70-140)</td>
<td>67 (61-142)</td>
<td></td>
<td></td>
<td>88 (55-119) total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38/40 (term)</td>
<td>4 (8.1-22.3)</td>
<td></td>
<td></td>
<td>24 (55-119) free</td>
<td>14 (17-53) total</td>
</tr>
</tbody>
</table>

Abbreviations: ++, Phenotypic analysis performed posttransfusion of fresh plasma. No pretransfusion plasma available for phenotypic analysis; NR, normal ranges for gestational age/age of child.
PROC and PROS genes and her mother (III.3) to be heterozygous for a PROS mutation. The deceased child was shown to have possessed all three gene lesions segregating in this family and was thus compound heterozygous for protein S deficiency and heterozygous for protein C deficiency. This explanation for the clinical severity manifested by IV.1 suggested that predictions regarding clinical severity might be possible on the basis of genotype in other family members. This appears to be the case at least for the second child, IV.2, who is asymptomatic. She possesses the PROC gene lesion, but neither PROS mutation. Fetal and neonatal assays for IV.2 are consistent with the genotypic data, but her protein C activity level at age 16 months fell just outside the normal range for her age. Some considerable overlap between plasma protein C levels exhibited by heterozygotes and normals has been reported.\(^{43,44}\) and IV.2 may represent another example of this phenomenon.

Antenatal diagnosis was performed on a third child (IV.3) who was found to be heterozygous for the paternal PROS gene lesion, but lacked the maternal PROS gene lesion. That IV.3 also possessed the PROC gene lesion was only established after birth owing to the time required to detect and characterize this lesion. Since this child’s father (III.2) and grandfather (II.2) possessed both PROC and PROS gene lesions and were either clinically normal or only affected after childhood, it was reasoned that there should not be any thrombotic manifestations in the neonatal period. The inaccuracy of this prognosis rapidly became apparent when the child suffered severe early onset venous thrombosis.

Phenotypic data from individual IV.3, derived from cord blood and taken at the time of delivery, were consistent with his possession of the paternal PROS gene lesion (Table 2). His protein S activity was reduced to below 50% of the normal range for age (NR, 55 to 119). In addition, the protein S antigen levels (total and free) of IV.3 suggested that he manifested a similar type of protein S deficiency to his grandfather (II.2) [type III]. The protein C phenotype of this child (type II deficiency) was also similar to that of II.2. The presence of protein C deficiency in IV.3 was confirmed by the detection of the Arg169 → Gln mutation in his PROC gene.

Despite the wealth of clinical, phenotypic, and genetic data generated by this study, prognostic accuracy was still limited. One possible explanation for our inability to predict the clinical severity of individual IV.3 would be the presence of a second PROC gene lesion in both III.3 and IV.3. Although III.3 possessed normal levels of protein C activity and antigen, several of her relatives exhibited protein C levels, which were borderline normal (II.3, III.4, and III.5). However, sequencing of the PROC genes from both III.3 and IV.3 failed to detect another lesion.

The low protein C values apparent in II.3, III.4 and III.5 could in principle be attributable to another lesion in the PROC gene. There is no evidence to suggest, however, that the low protein C levels in II.3 are heritable. II.3 is unrelated to III.4, and III.5 who are full sisters and the remaining maternal family members (I.2, II.4, II.6, and III.3) exhibit normal protein C levels. No information is available on individual II.5 (Fig 1: father of III.4 and III.5). Since no PROC gene lesion was identified in III.3, further analysis of the PROC gene from individuals II.3, III.4, and III.5 was deemed unnecessary for the purposes of this diagnostic analysis. Alternatively their protein C deficiency phenotype may have been acquired.

The severe clinical phenotype manifested by IV.3 could also be explained by a further genetic lesion at a third locus (possibly as yet unknown); the presence of the relatively common factor V Leiden lesion was, however, excluded. Another possible explanation is that the doubly heterozygous fetus, IV.3, was being carried by a mother (III.3) who was herself known to be predisposed to thrombotic events by virtue of her possession of a PROS gene lesion. Exposure of the fetus to a potentially prothrombotic intrauterine environment might have served as a trigger for the early development of thrombotic symptoms.

Two further anomalies were encountered in our attempts to correlate genotype with phenotype in this family. Firstly, individual II.6 is clinically symptomatic yet her protein C and protein S levels are well within the normal range. This could conceivably be explained by the presence of another lesion in an as yet unidentified gene acting as an additional risk factor. Alternatively, her thrombosis may have been triggered by the acquired deficiency of an as yet unidentified anticoagulant factor. Secondly, while individual I.2 is clinically symptomatic (she also possesses a very low free protein S level and bears the PROS Pro626 A allele associated with the type 1 protein S deficiency state), we were unable to detect the 2-bp deletion within a sample of her lymphocyte DNA. The absence of the PROS gene deletion in I.2 may indicate de novo mutation in her germline giving rise to the lesion carried by her daughter II.4 and subsequently transmitted through the pedigree. Because neither phenotypic data nor blood samples were available from her father (I.1), the possibility that the lesion was passed down from him cannot be excluded. An intragenic recombination event occurring between the mutation and the polymorphic marker at codon 626 is unlikely, however, as these two sites are separated by only 556 bp.

Although highly unusual in terms of its complexity, this analysis and diagnosis should thus serve as a warning against extrapolating too readily from genotype to phenotype in kindreds with a multifactorial disorder, such as venous thrombosis. Clearly, the considerable clinical and phenotypic variability evident from this analysis must be taken into account in counselling families as complex as the one illustrated here.

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