Homozygous Protein C Deficiency: Description of a New Mutation and Successful Treatment with Low Molecular Weight Heparin

Paul Monagle1, Maureen Andrew1, Jacqueline Halton2, Richard Marlar3, Lawrence Weight

Correspondence to: Dr. M. Patricia Massicotte, Hamilton Civic Hospital Research Centre, Hamilton, Ontario, Canada; 2Dept. Pediatrics, Children’s Hospital of Eastern Ontario, Ottawa, Canada; 3Thrombosis Research Laboratory, Denver VA Medical Centre, Denver, Colorado, USA; 4Dept. Pediatrics, Janeway Child Health Centre, St. John’s, Newfoundland; 5Dept. Nuclear Medicine, Chedoke-McMaster Hospitals, Hamilton, Ontario, Canada

Summary

We present a kindred with a new mutation of the protein C gene, in which the proband had an unusual clinical presentation. The relationship between warfarin induced skin necrosis and level of anticoagulation was investigated. The pharmacokinetics of protein C concentrate was assessed to determine frequency of replacement therapy. The clinical and biochemical efficacy of therapy with low molecular weight heparin (LMWH) was assessed. The effect of long-term LMWH on bone density in the growing child was monitored using whole body densitometry.

Warfarin therapy required an INR of greater than 3.5 to avoid skin necrosis. If protein C replacement was to be used, doses of 100 U/kg/day would have been required to maintain protein C levels consistently at or above 0.20 U/ml. While receiving prophylactic therapy with LMWH for almost 3 years, there were no episodes of recurrent thrombosis, no skin necrosis and no bleeding. Biochemical markers of in vivo thrombin generation were suppressed and within the normal range. Bone density continued to increase at the normal rate throughout the treatment period.

LMWH is an effective form of long-term therapy for homozygous protein C deficient patients with measurable protein C levels.

Introduction

Protein C is a vitamin K dependent inhibitor of coagulation. An inherited deficiency of protein C is autosomal in nature and associated with an increased risk of venous thrombosis (1). Phenotypically two types have been described, type 1 in which both antigenic and functional levels are reduced, and type 2 in which functional levels are reduced to a greater extent than antigenic levels. There also appears to be both dominant and recessive forms of protein C deficiency. Heterozygous, clinically “overt” protein C deficiency (dominant) has a prevalence of 1/16000–1/36000 in the general population (2). An asymptomatic (recessive) “covert” form in which protein C activity levels are consistent with a heritable heterozygous deficiency state may occur in up to 1/200 of the general population (3). Homozygous type 1 protein C deficiency is rare with only 17 cases recorded in an international database of mutations (1) and only about 20 further kindreds reported in the literature (4). Most commonly, homozygotes present with purpura fulminans in the neonatal period. These infants have protein C levels that are less than 0.01 units/ml (U/ml). A small number of severe protein C deficiencies do not present with purpura fulminans at birth (5-14). Rather, they present with severe thromboembolic disease during childhood or early adult life. The protein C levels in these patients are usually measurable and range from 0.02 to 0.23 U/ml. Only 8 such patients have been confirmed by DNA analysis to be true homozygotes, as distinct from compound heterozygotes (8-10).

Management of homozygous protein C deficient patients is problematic (15). Although oral anticoagulants are commonly used, the target international normalized ratio (INR) must be kept quite high (3 to 4.5), to avoid skin necrosis. Patients with high INRs are at considerable risk for serious bleeding, such as into the central nervous system (16-18). Replacement therapy with protein C concentrate is expensive, requires intravenous access, and frequent infusions due to protein C’s short half life (19). On rare occasion, stanazol (20) and liver transplantation (21) have been described, each with their own limitations. Low molecular weight heparin (LMWH) has been used in a small number of patients who presented in adolescence or early adulthood with thromboembolic events (6). The long-term effects of LMWH on bone development in such patients has not been monitored with a sensitive test.

We present a family from a small island community with a previously undescribed protein C mutation. The proband is homozygote protein C deficient with functional levels of 0.07 U/ml. She presented with sepsis at birth, which was investigated. The relationship of oral anticoagulant induced skin necrosis to the intensity of anticoagulation, pharmacokinetics of protein C concentrate, data on the successful long-term management with LMWH and normal bone development while receiving LMWH.

Case Report

The index case presented in 1992 at nine years of age with a right popliteal deep venous thrombosis (DVT) confirmed with Doppler ultrasound and venography, 3 weeks following trauma to her right foot. The only significant past history was of recurrent urinary tract infections for which she was on Bactrim prophylaxis. She was treated with therapeutic doses of heparin for 5 days and subsequently with warfarin. A week after ceasing heparin, she developed multiple areas of skin necrosis. The INR at the time was 2.9. Warfarin was ceased and heparin recommenced. Investigations revealed a marked protein C deficiency which was confirmed with family studies. The largest area of skin necrosis on
her right leg required skin grafting which initially failed requiring immobilisation and regrafting. Four weeks later, while still receiving heparin, she developed further symptoms due to right common femoral and common iliac DVT shown by Doppler ultrasound and venogram. Intravenous streptokinase with concurrent heparin therapy, fresh frozen plasma and then protein C concentrate was administered for three days. Subsequently, heparin infusion and intermittent protein C therapy continued for a further 2 weeks until warfarin was recommenced. Over the next 12 months there were two further episodes of skin necrosis, each occurring during antibiotic therapy for concurrent infection. The INRs were 3.4 and 3.5 respectively. On both occasions warfarin was ceased and heparin therapy given with good resolution of symptoms. During the first episode, fresh frozen plasma was administered but resulted in pulmonary oedema. Protein C concentrate was then used. On each occasion warfarin therapy was recommenced and overlapped with heparin therapy.

In April of 1994, long-term therapy with LMWH was initiated and warfarin ceased. The dose of LMWH was 1 mg/kg (75 mg) subcutaneously twice daily (enoxaparin, Rhone-Poulenc Rorer) which was continued for 6 months. The dose of LMWH was then decreased to a prophylactic dose of 75 mg subcutaneously daily which has continued for 2 years and 10 months. There have been no further episodes of thrombosis nor skin necrosis. Her growth and development are normal for age.

The sibling of the proband is 3½ years younger. She has had no thrombotic events nor any episodes of skin necrosis, and had received no anticoagulant therapy prior to commencing prophylactic doses of LMWH in April of 1994. She remains asymptomatic while receiving 1 mg per kg (25 mg) of LMWH subcutaneously daily (enoxaparin, Rhone-Poulenc Rorer).

The mother of both children is 36 years old and has no history of thromboembolic disease, despite 3 pregnancies including one caesarian section, and having been on oral contraceptives at various times. The father is 37 years old and has no history of thromboembolic disease. A detailed family tree is provided in Fig. 1.

### Methods

#### Evaluation for Congenital Prethrombotic Disorders

The immediate family was evaluated for the presence of congenital prethrombotic disorders. Protein C was assessed using both a functional clotting assay (Diagnostico Stago, France) and an immunological assay (Laurell electrophoresis). Free protein S was measured by enzyme linked immunosorbent assay (ELISA) (Affinity biologicals, Hamilton, Ontario), and antithrombin by chromogenic assay (Chromogenix, Helena, Sweden). Heparin cofactor II was assayed by chromogenic assay (Diagnostico Stago, France). Factor V Leiden was assayed by direct DNA analysis. Fibrinogen was measured in a clot based assay (Clauss technique).

#### Protein C Gene Analysis

Blood samples from both parents, proband and sibling were collected into ethylenediamine tetra-acetic acid (EDTA) for analysis. All exons and splice junctions of Protein C were analyzed by sequencing, as described by Bertina (22).

#### Pharmacokinetics of Protein C

To determine the effective recovery of protein C replacement, the proband was given intravenous protein C concentrate (Protein C concentrate, Immuno lot no. 88089311S) in doses of 40 and 100 units per kg (weight 78 kg; 3030 units and 8080 units respectively) over 15 minutes. Blood was taken at regular intervals for the following assays: protein C [Behring Helena kit, Behring Fibrinotimer], thrombin-antithrombin complexes (TAT) [Behring Elisa kit, Hoechst Canada] and prothrombin fragment 1.2 (F1.2) [Behring Elisa kit, Hoechst Canada]. The proband was receiving warfarin therapy with a stable INR of 3.0 to 4.0 during these studies.

### Table 1 Plasma protein C and Factor II levels for various INR values while on warfarin

<table>
<thead>
<tr>
<th>INR*</th>
<th>Factor II U/ml†</th>
<th>Protein C U/ml†</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3.4</td>
<td>0.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2.8</td>
<td>0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1.3</td>
<td>0.68</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*International Normalised Ratio; †units/ml.

![Fig. 1](image-url) Family tree showing common ancestral link of both parents. The affected member of this partnership is unknown (?), however the pattern of living ancestors testing positive confirm this as the most likely route of inheritance.

![Fig. 2](image-url) Plasma protein C levels after intravenous infusion of protein C concentrate to the proband plotted against time in hours. Doses infused on separate occasions were 40 (■), 40 (◆) and 100 (▲) U/kg.

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Pharmacokinetics of LMWH

Pharmacokinetics of 2 different LMWHs were measured in both the proband and her sister: Enoxaparin (Rhone-Poulenc Rorer) was injected subcutaneously via a subcutaneous catheter (Insuflon: Chronimed Inc.) at a dose of 1 mg/kg 12 h in the proband. The sibling was commenced on Enoxaparin at a dose of 1 mg/kg injected subcutaneously via an Insuflon catheter once daily. Blood samples were obtained to measure heparin concentrations on multiple occasions (anti-factor Xa levels, Stachrom kit, Diagnostica Stago), PF 1.2 and TATs. On a separate occasion, Clivarin (Knoll Pharma Inc.) was similarly tested at a dose of 100 U/kg once daily in the proband and 40 U/kg once daily in the sibling.

Bone Density Studies

Prior to commencement on LMWH, total bone mineral content (BMC) and total bone mineral density (BMD) were determined by dual energy x-ray absorptiometry (DXA), using a hologic QDR-1000 densitometer (Hologic incorporated, Waltham, MA). Serial assessments were performed over the 2 year and 10 month interval to detect early changes in bone mass caused by the LMWH. The results were compared with normal values obtained for Canadian children (23).

Results

Evaluation for Congenital Prethrombotic Disorders

The proband and sibling both have severe protein C deficiency with functional levels of 0.07 U/ml and antigenic levels less than 0.10 U/ml. The mother and father have functional protein C levels of 0.57 and 0.54 U/ml, respectively and antigenic levels of 0.64 and 0.52 U/ml respectively. All four subjects have normal levels of protein S, antithrombin, heparin cofactor II, and fibrinogen. In addition, all four subjects are normal for the Factor V Leiden mutation.

Protein C Gene Analysis

Only one mutation was found in all four family members when analysed by sequencing. Numbering is based on the Foster numbering system (24). Nucleotide 8892 has a T to G substitution which results in amino acid 393 Tyr changed to Asp. The two daughters are homozygous for this mutation, while both parents are heterozygous.

Warfarin Induced Skin Necrosis

Skin necrosis in the proband only occurred when the INR fell below 3.5. Table 1 provides the factor II levels at various INR values. The patient was clinically asymptomatic and on concurrent heparin or LMWH, at the time of these measurements.

Pharmacokinetics of Protein C

The maximum recovery of the infused protein C on 3 occasions was 43%, 67% and 77% respectively. The half life of protein C concentrate was 6-8 h. Following 40 U/kg, baseline protein C levels were reached by 18 h, and following 100 U/kg protein C levels remained above 20% at 24 h (Fig. 2).

Pharmacokinetics of Low Molecular Weight Heparin

a) Enoxaparine

Proband: The pharmacokinetics of therapeutic doses of Enoxaparine were measured over multiple days at the initiation of LMWH and on 2 other occasions 3 months later with similar results. Figure 3 provides the first pharmacokinetic response to Enoxaparine at a dose of 1 mg/kg subcutaneously Q12 h. The initial anti-factor Xa level reflected the cross over from standard heparin to LMWH. Peak anti-factor Xa levels occurred at 4 hours following administration with levels that ranged from 1.03 to 0.71 U/ml. Trough levels ranged from 0.7 to 0.45 U/ml. Plasma concentrations of TATs and PF 1.2 remained normal throughout treatment with LMWH (Fig. 3). A comparison of plasma concentrations of TATs in the proband showed that both warfarin and LMWH effectively suppressed TAT levels (Fig. 4).

Sibling: The pharmacokinetics of prophylactic doses of Enoxaparine were measured at the initiation of LMWH and on 2 other occasions 3 months later with similar results. Fig. 5 provides the first pharmacokinetic response. Peak anti-factor Xa levels occurred at 4 to 8 h following administration of enoxaparine with levels ranging from 0.89 to 0.63 U/ml. Trough levels at 24 h ranged from 0.01 to 0.11 U/ml.
Plasma concentrations of TATs were markedly increased (9.9 \, \text{mg/l}) prior to commencing LMWH and decreased to within normal range by 4 hours and remained at lower than original levels fluctuating between 2.8 and 6.7 \, \text{mg/l} (normal range < 4.0). PF 1.2 levels also fluctuated over the 24 h period (Fig. 5).

b) Clivarin

Pharmacokinetic evaluation of Clivarin gave similar result to Enoxaparin. In the proband once daily administration of 100 units per kg resulted in a rapid increase in anti-factor Xa levels to 1.18 U/ml within 2 h. The peak was 1.56 at 6 h post injection, and the trough at 24 h was 0.30 U/ml. TAT complexes remained within the normal range throughout the 24 h period. PF 1.2 were normal until the trough level at 24 h when they were 1.4 nMol (normal < 1.2 nMol). In the sibling, given 40 U/kg there was a similar rapid increase in anti-factor Xa levels to 0.73 U/ ml within 2 h. The level remained constant for 4 to 6 h. The trough level was 0.08 U/ml 24 h after the dose. The concentrations of PF 1.2 were normal during peak levels, but became slightly increased to 5 nMol at the trough level of anti-factor Xa activity.

Bone Density Studies

The initial and regular follow up results for lean body mass, body fat mass, BMC and BMD were plotted against age and compared to normal values for age (Fig. 6). The values showed that both the proband and sibling continued to have normal or above normal growth(weight) throughout the period of follow up. BMD growth slowed transiently during twice daily therapy with LMWH in the index case, however both BMC and BMD continue to follow normal centiles during the subsequent years of daily LMWH therapy. In the sibling BMC and BMD continue to follow normal centile growth.

Discussion

The new mutation described in this family highlights the heterogeneity of protein C deficiency, both at the molecular and clinical level. We have demonstrated the direct relationship between INR and warfarin induced skin necrosis, and described in vivo kinetics of protein C concentrate. Clinically, LMWH appears a safe, acceptable and effective
long-term alternative. This is supported by the biochemical and densitometry data.

The proband, sister and parents have the same novel mutation of the protein C gene (PROC) which was undoubtedly inherited through a common ancestral link over 4 generations ago. To date 132 different single base pair substitutions have been reported in the PROC gene, of which the majority are missense mutations (1). The site of our family's mutation is within the catalytic region of the protein (aspartic acid residues 185-419). Although point substitutions in the catalytic region usually give a type II phenotype (1), type I phenotypes, as seen in this family, have been documented (8). The responsible mechanism is probably impaired secretion of the protein, presumably due to charge related abnormal protein folding (1, 8). The latter may involve recognition of the protein C as abnormal by so-called “chaperone” proteins and hence removal from the endoplasmic reticulum or golgi apparatus (22).

A previous mutation was described at nucleotide 8892 (1) resulting in the substitution of asparagine for tyrosine, both of which are neutral hydrophobic amino acids. In that case, the proband was heterozygote with antigenic levels of 0.47 and functional levels of 0.33 units/ml. She presented with thrombosis in adult life [personal communication: Dr. Seydewitz]. The mutation in our family results in the substitution of aspartic acid, a hydrophilic negatively charged amino acid, for the normal tyrosine, which should cause greater charge induced disruptions to protein folding. Heterozygotes in our family have remained asymptomatic without thromboembolic events despite the presence of additional prothrombotic risk factors in some members. The inconsistency of thrombotic events associated with protein C deficiency remains difficult to understand due to the broad spectrum of mutations, the dominant and recessive forms, and different clinical course of similar mutations (25).

Classically, homozygous protein C deficiency presents with neonatal purpura fulminans and plasma concentrations of protein C of less than 0.01 units/ml (26). However, a few cases of homozygous recessive type severe protein C deficiency with delayed clinical presentations (similar to our patient) have been reported (5-14). Usually there is no family history of thrombosis.

Our patient developed repeated episodes of severe skin necrosis, a well recognised complication of oral anticoagulation in protein C deficient patients (6), whenever the INR decreased below 3.5, in the absence of concurrent heparinization. Of the later presenting patients with severe protein C deficiency reported in the literature, treatment details are provided in sixteen (5-7, 9, 11-14). While receiving therapeutic amounts of warfarin, 3 (19%) developed recurrent thrombosis and 6 (38%) developed skin necrosis.

We evaluated the option of combining protein C replacement with a lower INR target range for warfarin. Daily protein C infusions would be necessary and likely require the placement of a central venous line (CVL) with the potential iatrogenic problems of CVL related thrombosis and infection (16). Therefore we decided to evaluate the role of therapeutic and prophylactic doses of LMWH in our patient and her sister. The decision to treat the asymptomatic sibling was made after careful discussion with the family. The severity of thrombosis and subsequent complications in the index case; the fact that the plasma protein C levels were identical, suggesting a high risk of similar disease in the sibling; and the families perceived ease of administration of once daily LMWH were important considerations which convinced us of the appropriateness of primary prophylactic therapy.

LMWH offers several advantages over warfarin or protein C replacement therapy in patients with protein C levels that are measurable but less than 0.20 U/ml. LMWH, unlike warfarin, does not reduce protein C levels, almost eliminating the risk of skin necrosis. Other advantages compared to warfarin include, probable reduced bleeding risk, the need for minimal monitoring, ease of administration. In comparison to protein C replacement, the need for a CVL and exposure to blood products is removed.

A possible adverse effect of LMWH therapy is osteoporosis. The previous cases of severe protein C deficiency treated with LMWH reported no evidence of osteoporosis however, sensitive tests were not performed (5, 6) and those patients were older. We used repeated sensitive measurements of bone density and with almost 3 years of follow-up, all measurements remain normal for age (27-31). Further follow-up in more children will be required before one can be certain of the safety of long-term LMWH, especially at therapeutic doses.

In summary this family presents a novel mutation of the PROC gene which is inherited in an autosomal recessive nature. Treatment with warfarin was complicated by recurrent skin necrosis when INR values were less than 3.5. Treatment with LMWH was clinically successful and markers of in vivo thrombin generation were suppressed. Subsequent prophylactic LMWH successfully prevented recurrent TE and any thrombosis in the asymptomatic sibling. After nearly 3 years of treatment they remain clinically well with normal parameters of bone density for age. Long-term prophylactic LMWH is well suited to severe protein C deficient patients with low circulating levels of protein C.

Acknowledgements

This work was supported by Project #7 of the Medical Research Council of Canada’s Group grant in Developmental Lung Biology. Dr. Maureen Andrew holds a career investigator award from the Heart and Stroke Foundation of Canada. Dr. M. Patricia Massicotte was a Research Fellow of the Heart and Stroke Foundation of Canada. The work by R. Marlar was supported in part by a grant from Veteran’s Affairs (RAM).

References


