The Clinical Spectrum of Heterozygous Protein C Deficiency in a Large New England Kindred

By Edwin G. Bovill, Kenneth A. Bauer, Joseph D. Dickerman, Peter Callas, and Barbara West

A family with a high incidence of venous thromboembolism was investigated. We performed medical evaluations on 184 of the 411 surviving members of the pedigree, which allowed assignment of individuals into positive, equivocal, or negative categories with respect to their clinical histories of thrombosis. Subjects with antigenic levels of protein C less than 66% of a normal plasma pool were classified as having protein C deficiency. Positive thrombotic histories were found in 13 of the 46 family members determined to be protein-C deficient and in only five of their 138 biochemically unaffected relatives. Statistical analysis of the association between thromboembolic disease and protein-C deficiency was strongly positive ($X^2 = 24.95, P < .0001$ with $n = 184$), indicating that heterozygous protein-C deficiency is an important independent risk factor for the development of thrombotic manifestations in this pedigree. However, the absence of thromboembolic manifestations in many of the protein-C deficient family members to date indicates that other, as yet undefined, factors must play an important role in the clinical expression of this disorder. 

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Protein C is a vitamin K-dependent glycoprotein of mol wt ~62,000 that circulates in plasma as an inactive zymogen. Thrombin is able to rapidly activate protein C when bound to thrombomodulin, an integral plasma membrane receptor that is present on vascular endothelial cells. Activated protein C (APC) inhibits the platelet-dependent conversion of prothrombin to thrombin via factor Va by inactivating factor VIIa and factor Va. Protein S, another vitamin K-dependent protein, enhances the binding of APC to phospholipid-containing membranes and accelerates the proteolytic inactivation of these two cofactors. The complementary component, C4b-binding protein, forms a complex with protein S and appears to be involved in regulating the function of the latter protein.

The first report of the association of heterozygous protein-C deficiency and a thrombotic tendency was by Griffin et al in 1981. The three affected family members had protein-C antigen levels of ~50% of normal and suffered their first thrombotic episodes at ~20 years of age. Since that time a number of families with a similar clinical picture have been described. This disorder is inherited in an autosomal dominant manner.

The importance of heterozygous protein-C deficiency as an independent risk factor for venous thromboembolism has been challenged by some investigators on the basis of two sets of clinical observation. First, several case reports have appeared describing a homozygous deficiency state in which newborns develop massive venous thrombosis and purpura fulminans in association with protein-C antigen levels less than 1% of normal. However, the heterozygous parents of these infants have only infrequently exhibited thrombotic manifestations in contrast to the patients from thrombophilic families with a partial deficiency of protein C. Second, a recent study by Miletich et al noted that the frequency of heterozygous protein-C deficiency is as high as one per 200 in a healthy adult population and that biochemically affected individuals had not yet exhibited thrombotic manifestations.

We have studied a large New England kindred in which a number of individuals have positive histories for thromboembolic events. The availability of many heterozygous protein-C deficient subjects and their biochemically unaffected relatives within a single kindred has allowed us to analyze the statistical association of this deficiency state with thrombotic symptoms. Such an analysis has not previously been possible because of the relatively small numbers of affected and unaffected individuals described in previously published family studies. The segregation of heterozygous protein-C deficiency in members of our family with positive histories over several generations provides compelling evidence that this biochemical defect can be an important risk factor for the development of thrombotic disease.

MATERIALS AND METHODS

Patient selection and clinical assessment. The study participants were drawn from a single large kindred that consisted of 411 members over six generations. A majority of the individuals studied resided in northern Vermont. All of the investigated subjects completed questionnaires and were personally interviewed as to their medical histories and the occurrence of thrombotic episodes. The patient's age as well as the presence of concurrent thromboembolic risk factors at the time of such events (eg, surgery, pregnancy, trauma, infection, birth control pills) was also sought. These evaluations took place at either the Clinical Research Center of the University of Vermont (Burlington), the North Country Hospital (Newport, VT), the Beth Israel Hospital (Boston), or the homes of family members. An effort was made to investigate as many consenting persons in the kindred as possible without regard to the presence or absence of a thrombotic history.

In the absence of a complete set of objective endpoints for the presence or absence of thromboembolic disease in all clinically suspected cases, we categorized the medical histories as positive, equivocal, or negative with respect to thrombotic manifestations.
based on the following criteria: (1) positive, hospitalized and treated for deep venous thrombosis (DVT), pulmonary embolism (PE), or other thromboembolic events; (2) evident hospitalization for DVT, PE, or other thrombotic events that were described by the patient as well as a history of superficial thrombophlebitis; (3) negative, no history of DVT, PE, or other thromboembolic events. Histories of myocardial infarction, cerebrovascular accidents, transient ischemic attacks, or peripheral vascular disease were also included in the present analysis. This study was approved by the Human Experimentation Committees of the University of Vermont College of Medicine and the Beth Israel Hospital.

Collection of plasma samples. Venipunctures were performed with 19- or 21-gauge butterfly infusion sets using a two-syringe technique. The blood was drawn into plastic syringes and immediately mixed with an anticoagulant solution of 3.8% (wt/vol) trisodium citrate. The ratio of anticoagulant to blood employed was 0.1:0.9 (vol/vol). After collection of blood samples, platelet-poor plasma (PPP) was obtained by centrifugation at 1,700 g at 4°C for 15 minutes. Aliquots of plasma were stored at −80°C prior to assay.

A normal plasma pool was constructed by combining equal volumes of plasma from 54 control subjects. This population consisted of healthy laboratory and medical personnel between the ages of 20 and 50 years, who gave a negative history for bleeding as well as thrombosis and who were not taking any medications at the time of sample collection.

Assays. The measurements of protein-C antigen were performed on 96-well microtiter plates (Nunc-immunoplate 1, USA/Scientific Plastics, Waltham, MA) using murine monoclonal antibody (MoAb) HPC-2. Human protein C purified by immunosorbent technique was employed in the standard curve. Radio labeling of protein C was carried out by the chloramine T method of Greenwood et al. The interassay coefficient of variation (CV) of this assay is 5%. Plasma protein-C levels were determined in the 54 normal individuals who contributed to the normal plasma pool. The mean protein-C antigen concentration was 52 ± SD 9 nmol/L (3.2 ± SD 0.56 μg/mL) with a reference interval from 34 to 69 nmol/L (2.1 to 4.3 μg/mL or 66% to 134% of normal). The results of this and the other assays employed in this study have been normalized to the mean value of the normal plasma pool and expressed as percent of the pool mean. Functional assays for protein C were performed employing the procedure of Sala et al. The plasma concentrations of total protein S, prothrombin, and antithrombin III were determined by previously described radioimmunoassays. The reference range and mean level for total protein S, prothrombin, and antithrombin III were 59% to 150% of normal and 100%, 72% to 147% of normal and 105%, and 67% to 125% of normal and 99%, respectively. The antithrombin-III-heparin cofactor activity in plasma samples was determined with an amidolytic assay reference range (78% to 115%), mean 100.

Laboratory diagnosis of protein-C deficiency. Criteria similar to those employed by Griffin et al and Bertina et al were used to define protein-C deficiency. In individuals who were not receiving oral anticoagulants, the deficiency state was diagnosed by the repeated finding of a protein-C antigen level below the lower limit of the normal range (66% of normal), while the concentration of prothrombin was within the normal range.

In family members undergoing stable anticoagulation with warfarin, protein-C deficiency was suspected when the immunologic levels of the zymogen were lower than the lower limit of the normal range at the corresponding intensity of treatment, and the ratios of their antigenic measurements of protein C to those of prothrombin were below the lower limit of the observed range for anticoagulated individuals without protein-C deficiency. The antigenic levels of protein C and prothrombin have been determined in 21 patients (mean age 47 years) maintained on chronic warfarin therapy for the prophylaxis of venous thrombosis or cardiac emboli secondary to either atrial fibrillation, ventricular aneurysm, mitral, or aortic valve replacement, etc. These individuals were all stably anticoagulated for a minimum of 3 months before evaluation, with prothrombin time (PT) ratios (PT of patient/PT of normal plasma pool) that ranged from 1.4 to 2.8. The mean antigenic levels of protein C and prothrombin in these individuals were 59% of normal (range 44 to 101) and 61% of normal (range 49 to 96), respectively. The mean ratio of the individual measurements of protein C to those of prothrombin was 0.92 (range 0.66 to 1.33). In a few instances a diagnosis of protein-C deficiency was assigned to stably anticoagulated individuals on warfarin with ratios slightly above the lower limit of 0.66, inasmuch as pedigree analysis indicated that they were obligate heterozygotes.

Analysis of data. Estimation of relative immunoreactivity, computation of the slopes of the dose-response curves, as well as determination of the various associated indices were obtained by a least-squares fit of the radioimmunoassay results to a “four parameter” model as described by Rodbard and Rodbard et al. Statistical analyses of data were conducted by standard techniques.

RESULTS

A kindred with a high incidence of venous thromboembolism has been investigated (Fig 1). A total of 184 out of 383 surviving family members were available for study. With one exception, these individuals were asymptomatic at the time of evaluation, and none had sustained an overt thrombotic episode in the previous 6 months.

The plasma concentrations of protein C and prothrombin were determined by immunoassay in each of the 184 tested members of the pedigree. Thirty-six of 173 nonanticoagulated members (mean age = 31 years, range 2 to 80 years) fulfilled the criteria for protein-C deficiency. The results of functional assays for protein-C activity in the plasma of these nonanticoagulated protein-C deficient subjects were similar to those measured by immunoassay (data not shown).

Miletich et al has recently addressed the problem of distinguishing whether an individual from a healthy population with a plasma protein-C antigen level of 55% to 65% of normal has the heterozygous deficiency state or is a low normal (ie, the person has a protein-C concentration that is at the lower end of the distribution of normal values). Of the 36 nonanticoagulated patients in our kindred who met the criteria for the heterozygous deficiency state, 29 had levels of protein C that were less than 55%. In addition, four of the seven nonanticoagulated protein-C deficient family members with levels greater than 55% had children with protein-C values less than 55%. Of the three remaining individuals, only one did not have a parent and grandparent from the kindred that was protein-C deficient. This subject could represent a low normal, a new mutation, or have inherited the deficiency state from his other parent.

Eleven family members were stably anticoagulated on warfarin at the time of analysis. Ten were classified as having heterozygous protein-C deficiency, bringing the total number of biochemically affected individuals in this kindred to 46. The chronic administration of oral anticoagulants substantially diminishes the immunologic levels of the vitamin K-dependent coagulation proteins from their pretreat-
The magnitude of this effect is dependent on the intensity of anticoagulant treatment. We used the criteria of Griffin et al. and Bertina et al. based on measurements of plasma samples from individuals on stable warfarin therapy from our own institution together with pedigree analysis to diagnose partial protein-C deficiency among the warfarin-treated members of the family.

Each subject was assigned to positive, equivocal, or negative categories with respect to their clinical histories of thrombosis (see "Methods"). Eighteen family members had positive thrombotic histories. Their clinical and laboratory values are summarized in Table 1. Thirteen of the eighteen with positive histories had a partial deficiency of protein C (mean age = 48 ± SD 18 years) while five did not (mean age = 60 ± SD 7 years). Of the 16 members with equivocal histories, five had a partial deficiency of protein C. Two of these subjects were chronically receiving warfarin, and both were protein-C deficient. Of the 150 members with negative histories, 28 met the criteria for heterozygous protein-C deficiency. In addition, family members reported that nine deceased obligate heterozygotes from this pedigree had had thromboembolic manifestations. None of the family members treated with oral anticoagulants had experienced skin necrosis.

The three cohorts with positive, equivocal, or negative thrombotic histories were then sorted into two groups based on whether or not the individuals met the criteria for the diagnosis of protein-C deficiency. A contingency table analysis was performed to test the hypothesis of independence between a positive, equivocal, or negative history of thromboembolic disease and a diagnosis of protein-C deficiency (Table 2). A highly significant relationship was observed between a positive history and the deficiency state ($X^2 = 24.95, P = 0.0001$ with $n = 184$). Our data indicates that a family member with protein-C deficiency has 7.8-fold times the risk of developing a positive thrombotic history as compared to a relative with a normal plasma level of the protein. A similar analysis was carried out to examine the association between positive histories of myocardial infarction, cerebrovascular accidents, transient ischemic attacks or peripheral vascular disease and protein-C deficiency. A significant relationship was not observed between these arterial disorders and the deficiency state (data not shown).

Contingency table analysis was also carried out after excluding the nine patients who were chronically anticoagulated with warfarin. A significant association between a positive thrombotic history and protein-C deficiency was still observed ($P = 0.03$).

**Fig 1. Pedigree of family.**

*Pedigree: Protein C Deficient Kindred*

- **I-1** - Protein C deficiency identified
- **I-2** - Family history of thrombosis
- **I-3** - Positive clinical history of thrombosis
- **I-4** - Negative clinical history of thrombosis
- **II-1** - Protein C deficient, no history of thrombosis
- **II-2** - Protein C deficiency and positive clinical history
- **II-3** - Negative screen
- **III-1** - Died of thrombosis

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for accounts lung scans). These findings were sufficient evidence to classify them as protein-C deficient.

The early age of onset of thrombosis in the individuals with protein-C deficiency should be noted. In addition, by the age of 62 years all surviving protein-C deficient individuals had no thrombotic histories compared to those with protein-C deficient family members, the mean antigenic plasma levels of antithrombin III were similar to those in their 138 biochemically unaffected relatives, 100% of normal ± SD 16 ± 18.7. The levels of antithrombin III in the protein-C deficient individuals with (n = 13) and without (n = 33) positive histories were compared. Functional levels of antithrombin III in these two groups were compared by the generalized Wilcoxon test with P < .0001.

Table 2. Contingency Table Analysis of Protein C Deficiency v History of Thromboembolic Disease

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X² = 24.95 (P < .0001)

Abbreviations: DVT, deep venous thrombosis; (R), recurrent; PE, pulmonary embolism; PVT, portal vein thrombosis; W, currently on stable warfarin therapy.

Figure 2 shows a graph of the percentage of family members without protein-C deficiency who are free of positive thrombotic histories compared to those with protein-C deficiency. A Kaplan-Meier plot was used for this analysis with the event defined as the age of onset of the first thromboembolic episode. The two curves were significantly different by the generalized Wilcoxon test with P < .0001. The early age of onset of thrombosis in the individuals with protein-C deficiency should be noted. In addition, by the age of 62 years all surviving protein-C deficient individuals had sustained a thromboembolic event.

We observed a median age of 25 years in the protein-C deficient individuals with negative histories of thrombosis in contrast to a median age of 34 years in those with a positive history. Seventy percent of those with negative histories were under 34 years of age.

As hereditary deficiencies of antithrombin III or total protein S have also been correlated with a thrombotic diathesis, the plasma levels of these two proteins were determined in the members of the kindred. In the 46 protein-C deficient family members, the mean antigenic plasma levels of antithrombin III were similar to those in their 138 biochemically unaffected relatives, 100% of normal ± SD 16 ± 18.7. The levels of antithrombin III in the protein-C deficient individuals with (n = 13) and without (n = 33) positive histories were compared. Functional levels of antithrombin III in these two groups were compared by the generalized Wilcoxon test with P < .0001.

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*Risk factor present during patient’s first thromboembolic event. BCP = birth control pills, Preg = pregnancy

†Confirmatory diagnostic tests for venous thromboembolic disease were performed in 70% of patients (eg, venography and/or ventilation perfusion lung scans).

‡This individual had an acute DVT during pregnancy when this sample was drawn. She was not on warfarin but was receiving heparin, which likely accounts for the low ATIII levels. Her diagnosis was confirmed by the fact that she has two children with levels of protein C less than 55%.

§These two individuals have ratios just above the upper limit for protein-C deficient individuals on warfarin; however, they each have nonanticoagulated offspring with less than 55% protein-C levels. These findings were sufficient evidence to classify them as protein-C deficient.

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of antithrombin III were also determined with a heparin cofactor assay. No significant differences in these parameters were observed between the two groups of patients (data not shown). Bertina et al.28 has previously reported that immunologic levels of total protein S are reduced in stably anticoagulated patients on warfarin. Several of our protein-C deficient patients were on oral anticoagulants, which complicates our analysis of the level of PS in the various populations. Table 1 demonstrates that PS levels were reduced below our normal range in only two of nine individuals on warfarin. With the exception of one nonprotein-C deficient individual, protein-S deficiency was not observed in any other members of the kindred. This one subject has a protein-S level just below the lower limit of normal and had no history of thromboembolic disease.

**DISCUSSION**

The importance of protein C as one of the regulators of hemostasis has been substantiated by the identification of a clinical disorder in which newborns present with massive venous thrombosis and purpura fulminans in association with protein-C antigen levels less than 1% of normal.12,14-16 This disease is phenotypically expressed in an autosomal recessive manner, and such homozygous patients were frequently members of inbred kindreds in which both parents were heterozygous for protein-C deficiency.

Recent studies have appeared describing families in which heterozygous protein-C deficiency is associated with a thrombotic diathesis.9-11 Investigators from the Netherlands and France have reported on 65 patients derived from 25 unrelated pedigrees, and approximately 70% of these individuals had a history of deep venous thrombosis or pulmonary embolism.11,29

It has been observed, however, that the clinical expression of thrombotic disease in patients with heterozygous protein-C deficiency is highly variable. Many of the heterozygous relatives of infants with homozygous protein-C deficiency are asymptomatic,12,14-16 and 1 in 200 to 300 healthy blood donors have low protein-C levels without any history of overt thrombotic manifestations.13 This latter observation has led Miletich et al.11 to suggest that an additional genetic defect in association with heterozygous protein-C deficiency leads to the expression of a thrombophilic state. Rigorous analysis of the importance of heterozygous protein-C deficiency as an independent risk factor for venous thrombembolism has not previously been possible due to the relatively small number of affected and unaffected members available for study in enough generations of a kindred.

In the present investigation we have evaluated 184 individuals from a single kindred with partial protein-C deficiency without incurring selection bias with regard to the presence of the deficiency state or a history of thrombotic manifestations. The subjects with positive thrombotic histories had sustained major episodes of venous thrombembolic disease that frequently were recurrent. Positive thrombotic histories were found in 13 of the 46 family members determined to be protein C deficient and in only five of their 138 biochemically unaffected relatives. Statistical analysis of the association between thromboembolic disease and protein-C deficiency was strongly positive, indicating that heterozygous protein-C deficiency is an important independent risk factor for the development of thrombotic disease in this pedigree.

The percentage of family members free of positive histories of thrombosis up to a given age was analyzed in the protein-C deficient subjects and their biochemically unaffected relatives by Kaplan-Meier analysis (Fig 2). The data illustrate that a number of affected individuals developed disease at a relatively early age, and the median age of onset of such events was 50 years. Broekmans and Bertina29 had previously noted that the median age of onset of thromboembolic events in protein-C deficient patients was 30 years of age. It should be noted that this analysis pooled members of unrelated families and is also subject to the potential bias of selecting those individuals for study who have exhibited the most profound thrombotic diatheses. Indeed it is evident from our pedigree that a selection bias might have occurred had we elected to analyze only certain segments of the family, as some branches demonstrated a relatively close relationship between thrombosis and heterozygous protein-C deficiency while others did not.

Despite the positive association between venous thromboembolic disease and partial protein-C deficiency, it is apparent that there is marked intrafamiliial heterogeneity in the expression of clinical manifestations among biochemically affected family members. In an attempt to elucidate other biological factors that might influence the phenotypic manifestations of this disorder, we determined the levels of antithrombin III and total protein S in the members of this kindred. The results of these two assays were not significantly different among the various groups with positive, equivocal, or negative histories of thrombosis. However, a variety of other molecular defects can also be suggested to explain the predisposition of certain individuals with protein-C deficiency to the development of thrombotic disease.30 These include decreased production of thrombomodulin or heparan sulfate by endothelial cells, excessive exposure of tissue factor by endothelial cells or white blood cells, and/or diminished activity of the fibrinolytic mechanism. At the present time we have little knowledge about the influence of such factors on the clinical expression of thrombotic disease in patients with heterozygous protein-C deficiency.

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