Normalization of Markers of Coagulation Activation With a Purified Protein C Concentrate in Adults With Homozygous Protein C Deficiency

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Homozygous or doubly heterozygous protein-C deficiency can present at birth with purpura fulminans or later in life with venous thrombosis. Two homozygous patients who had previously sustained thrombotic episodes were investigated at a time when they were asymptomatic and not receiving antithrombotic therapy. The plasma levels of protein-C antigen and activity in both individuals were approximately 20% of normal. We administered a highly purified plasma-derived protein C concentrate to these individuals and monitored levels of several markers of in vivo coagulation activation. Assays for protein-C activation (activated protein C and protein C activation peptide) showed a sustained increase from reduced baseline levels, whereas thrombin generation (as measured by prothrombin fragment F1+2) gradually decreased over about 24 hours into the normal range. These investigations provide direct evidence that protein C is converted to activated protein C in vivo, and that the protein-C anticoagulant pathway is a tonically active mechanism in the regulation of hemostatic system activation in humans.

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Protein C is a 62,000 molecular weight glycoprotein that circulates as an inactive zymogen at a concentration of approximately 4 μg/mL. Thrombin is able to rapidly activate protein C after binding to thrombomodulin, a receptor present on vascular endothelial cells. This process results in the liberation of a dodecapeptide with a molecular weight of approximately 1,400. Once generated, activated protein C (APC) proteolytically inactivates factor VIIIa and factor Va and thereby inhibits the conversion of factor X to Xa and prothrombin to thrombin, respectively. Protein S enhances the binding of APC to phospholipid membranes and accelerates the inactivation of factor VIIIa and factor Va. Homozygous or doubly heterozygous protein-C deficiency can present at birth with purpura fulminans or later in life with venous thrombosis.

MATERIALS AND METHODS

Collection and processing of blood samples. Venipunctures were performed atraumatically with 21-gauge butterfly infusion sets using a two-syringe technique. Blood samples were drawn into plastic syringes preloaded with the appropriate solutions as described below. For all functional and immunologic assays of clotting factors, 3.8% (wt/vol) sodium citrate was used; the ratio of anticoagulant to blood was 0.1:0.9 (vol/vol). For assays of PCP, F1+2, and fibrinopeptide A (FPA), an anticoagulant containing a thrombin inhibitor, EDTA, and aprotinin was purchased from Byk-Sangtec (Dietzenbach, Germany); the ratio of anticoagulant to blood was 0.1:0.9 (vol/vol). For APC assays, an anticoagulant containing 0.3 mol/L benzamidine, 0.13 mol/L sodium citrate, 0.1 mol/L hepes, 0.02% (wt/vol) sodium azide, pH 6.8; the ratio of anticoagulant to blood was 0.1:0.9 (vol/vol). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 minutes at 1,600g and stored at -80°C before use.

Coagulation studies. Routine coagulation tests were performed by standard laboratory methods. Measurements of protein-C antigen and amidolytic activity and D-dimer were performed with commercial kits (Diagnostica Stago, Asnieres, France). A normal plasma pool was constructed by mixing equal volumes of plasma from greater than 30 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 and for thrombosis and were not taking any medications at the time of sample collection. The plasma concentrations of PCP and F1+2 were determined by double antibody radioimmunoassay (RIA), described in earlier reports from our laboratory.13-23 The FPA measurements were established by RIA with a kit provided by Byk-Sangtec.

The FPA measurements were established by RIA with a kit provided by Byk-Sangtec. Plasma APC levels were measured using an enzyme capture assay.24 The normal range for protein C measurements is 66% to 134% of a normal plasma pool. The mean plasma levels of APC, PCP, F1+2, FPA, and D-dimer in a control group of healthy individuals between the ages of 20 and 50 are 38.0 ± 3.3 pmol/L, 1.78 ± 0.77 pmol/L, 1.51 ± 0.68 nmol/L, 0.89 ± 0.35 nmol/L, and 115 ± 62 ng/mL, respectively.15,25

Protein C concentrate. A monoclonal antibody purified factor protein-C concentrate was manufactured from viral-inactivated, prothrombin-complex concentrate by Immuno AG (Vienna, Austria). This concentrate undergoes further viral inactivation by vapor heating. After reconstitution, the albumin-stabilized concentrate contains 125 U/mL of protein C with a specific activity of 14 U/mg. One unit is defined as the amount of protein C in 1 mL of pooled normal human plasma. The amount of F1+2 in 1 U of concentrate was 0.07 pmol.

Informed consent. The protein-C infusion protocol was approved by the Institutional Review Board at Hotel Dieu (Paris, France). Written informed consent was obtained from the patients, and all clinical studies and informed consent procedures were also approved by the Committee on Clinical Investigations of the Beth Israel Hospital (Boston, MA).

Data analysis. For the PCP, F1+2, and FPA assays, estimation of relative immunoreactivity, computation of the slopes of the dose-response curves, and determinations of the various associated indices were obtained by a least-squares fit of the RIA results to a "four-parameter" model as described by Rodbard et al.26,27 For APC, a standard curve was generated by plotting the change in absorbance (ΔA605/time) of the chromogenic substrate, S-2366, versus standards of known APC concentration. Unknown plasma samples were run in duplicate, and the APC concentration was interpolated from the standard curve using the average of the two measurements.

RESULTS

We investigated 2 female patients, ages 58 (A) and 41 (B) with protein C deficiency who first presented with deep venous thrombosis at ages 45 and 24, respectively. Both sustained recurrences of deep venous thrombosis. However, these individuals had each experienced multiple episodes of coumarin-induced skin necrosis and neither patient was chronically receiving oral anticoagulants.28 Both had approximately 20% of the normal levels for protein-C antigen and amidolytic activity and normal levels of antithrombin III and protein S (total and free). Molecular analysis showed that the patients were homozygous for a single nucleotide alteration in the protein-C gene. There was an Ala to Thr transition at position 301 in the triplet coding of the protein-C gene of patient A, and a Gly to Ser transition at position 44 in patient B.28

Previous investigations of nonanticoagulated individuals with heterozygous protein-C deficiency showed reductions in plasma levels of PCP and APC and significant elevations in prothrombin fragment F1+2 concentrations as compared with normal age-matched controls. To show the normalization of these parameters of coagulation system activation in protein-C-deficient subjects, 2 adult patients with homozygous protein-C deficiency were hospitalized at Hotel Dieu (Paris, France) and were administered a highly purified concentrate of the zymogen. Patient A was chronically maintained on a single daily injection of low molecular-weight heparin, Fraxiparine 3,200 Institut Choay Units (Sanofi, Paris, France), which was discontinued 24 hours before the protein-C infusion. No heparin was detectable in the patient's plasma at the start of the study. Patient B was not receiving antithrombotic prophylaxis proximate to the date that the concentrate was administered.

After obtaining base-line blood studies, each subject received protein-C concentrate (80 U/kg body weight) for 5 minutes via a peripheral vein. No adverse clinical effects were observed. Blood samples were obtained by separate venipunctures at various time points from 30 minutes to 90 hours after the infusion. The plasma levels of fibrinogen, prothrombin, factor V, factor VII, factor VIII, and factor X did not change after administration of the concentrate (data not shown). Specimens were assayed for protein-C antigen and activity, PCP, APC, F1+2, FPA, and D-dimer (Tables 1 and 2). There was an immediate increase in the protein-C levels to greater than 200% of normal levels. The half-life of protein C in the circulation, determined from amidolytic activity measurements, was 12.2 hours in patient A and 15.7 hours in patient B.29 The peak concentrations of PCP and APC occurred at the earliest time point and gradually decreased to baseline levels as protein C was cleared from the circulation. At 24 hours, we noted that protein-C levels were still within the normal range, but the levels of circulating APC were already well below normal. We are uncertain of the basis for this observation, but in a complex biological system a number of factors modulate protein-C activation. These include the functional activity of thrombomodulin and the ability of thrombin and protein C to bind to the

| Table 1. Coagulation Parameters in Patient A With Homozygous Protein C Deficiency After Protein C Concentrate Administration at a Dose of 80 U/kg Body Weight |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | PC Ag (% of normal) | PC Act (% of normal) | PCP (pmol/L) | APC (nmol/L) | F1+2 (nmol/L) | FPA (nmol/L) |
| Preinfusion      | 18              | 18              | 0             | 5.8           | 2.15           | 4.82           |
| 30 min           | 295             | 267             | 2.89          | 65.0          | 1.92           | 2.12           |
| 1 h              | 235             | 248             | 61.7          | 1.84          | 1.82           |
| 2 h              | 210             | 227             | 53.3          | 1.90          | 1.47           |
| 4.5 h            | 185             | 201             | 40.0          | 1.95          | 4.11           |
| 6.5 h            | 162             | 179             | 41.7          | 1.81          | 1.15           |
| 11 h             | 155             | 123             | 29.2          | 1.34          | 1.47           |
| 24 h             | 115             | 88              | 15.0          | 1.12          | 2.45           |
| 30 h             | 100             | 63              | 13.3          | 1.20          | 1.73           |
| 44 h             | 42              | 43              | 1.00          | 1.01          | 4.84           |
| 68 h             | 27              | 28              |               | 1.50          |               |

Abbreviations: PC, protein C; Ag, antigen; Act, activity; —, plasma was not obtained at the time point for this assay.
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qualitatively followed a pattern similar to patient B were disproportionately increased as compared to the F1+2 levels at the termination of the study were increasing but did not reach the preinfusion measurements. The D-dimer levels returned towards the preinfusion values at the termination of the study and were 1,650 ng/mL in patient A and 1,800 ng/mL in patient B. Thus, it is likely that normalization of APC and/or thrombin generation results in the suppression of fibrinolytic mechanism activity as measured by the D-dimer assay.

To evaluate whether protein-C activation products in the concentrate might have affected the results of our study, the amounts of PCP and APC in the infused drug were quantified. We determined that 1 U of concentrate contained 0.004 pmol of PCP. Therefore, an infusion of 80 U/kg body weight to a 60-kg patient results in the administration of 19 pmol of PCP. Assuming that distribution of the peptide into the extravascular space does not occur immediately after the infusion, the plasma PCP level would be expected to increase by 8 pmol/L. However, the peptide is rapidly cleared from the circulation with a half-life of approximately 5 minutes. It is also highly probable that the peptide does indeed distribute extravascularly. Thus, at 30 minutes after drug administration, the infused PCP would provide a negligible contribution to the observed increase of 2 to 3 pmol/L in plasma peptide concentration.

For APC, we found that 1 U of concentrate contained 0.072 pmol. Using the same assumptions for APC as for PCP, the plasma level of the enzyme would increase 144 pmol/L immediately after the infusion. However, APC is neutralized by several protease inhibitors in blood and has a half-life of only roughly 15 minutes. The inhibitors of APC include protein-C inhibitor, α1-antitrypsin, α2-antiplasmin, α2-macroglobulin, and plasminogen-activator inhibitor-1. Thus, in the samples obtained from our patients at 30 minutes, the APC in the product might contribute as much as 18 to 36 pmol/L of the observed 40 to 60 pmol/L increment in APC concentration as compared with baseline. Based on these considerations, we conclude that the elevations in PCP and APC levels, which were sustained for many hours after protein-C concentrate administration, result from the in vivo activation of the infused zymogen by thrombin bound to vascular thrombomodulin.

**DISCUSSION**

Patients with homozygous or doubly heterozygous protein-C deficiency that is not associated with purpura fulminans generally have protein-C levels of 10% to 20% of normal in the absence of oral anticoagulant therapy. In the present study, we investigated 2 such homozygotes with antigenic and functional protein-C levels of approximately 20% of the normal levels. Assays for protein-C activation and thrombin generation were used to monitor coagulation activation after the administration of 80 U/kg body weight purified protein-C concentrate. This dose was chosen to...
maintain protein-C levels at a minimum level of 100% of normal for a 24-hour period.

After infusion with the concentrate, the levels of PCP and APC showed a sustained increase from baseline levels, whereas indices of thrombin generation decreased into the normal range. The concentrations of prothrombin fragment F₁+₂ normalized gradually over a period of nearly 24 hours into the normal range, whereas FPA levels declined substantially in the earliest postinfusion samples. Therefore, these infusion studies provide direct evidence that protein C is converted to APC in vivo and that the protein-C anticoagulant pathway is a tonically active mechanism in the regulation of hemostatic system activation in normal individuals.

In this study, the 2 homozygous protein-C deficient patients had elevated baseline levels of F₁+₂ and FPA. Coagulation activation has been studied previously in asymptomatic individuals with heterozygous deficiencies of protein C or protein S. The plasma protein C and protein S concentrations are approximately 50% of normal in these patients. In nonanticoagulated persons with these disorders, the F₁+₂ levels are significantly increased as compared with age-matched controls.17,43 Approximately one third of patients have levels greater than the upper limit of normal controls (defined as the mean + 2 standard deviations). Metabolic turnover studies with ¹³¹I-F₁+₂ indicate that elevated concentrations of the fragment are not because of diminished clearance.17 Fibrinopeptide-A levels are elevated in approximately 20% of the subjects.17,43

Protein-C activation as measured by the PCP or APC assay is reduced to about 50% of normal levels in asymptomatic persons with heterozygous protein C deficiency.17,24 Based on the present data, we surmise that the reduced levels of protein-C activation and excessive thrombin generation in such individuals would normalize in response to protein-C repletion.

It is interesting to contrast the basal state measurements of thrombin generation in patients with defects in the protein-C anticoagulant pathway with those in thrombophilic patients with defective function of the heparan sulfate-antithrombin III mechanism. The enzymatic activities of coagulation serine proteases, including thrombin, factor Xa, and factor IXa, are inhibited by this mechanism, resulting in the formation of stable enzyme-inhibitor complexes. In asymptomatic patients with heterozygous antithrombin III deficiency, it was initially reported that F₁+₂ levels are frequently increased as compared with age-matched unaffected siblings.30 Subsequently, it was shown that the high concentrations of the fragment resulted from an in vitro anticoagulant effect resulting from the action of low amounts of heparin in the presence of reduced blood levels of antithrombin III.45 In patients with heterozygous antithrombin-III deficiency, repletion studies with purified antithrombin-III concentrate did not result in a decline in plasma F₁+₂ levels.46 A recent study of 26 antithrombin-III-deficient subjects from Italy did not show significant elevations in plasma F₁+₂ or fibrinopeptide-A levels.48 In contrast to these studies, a cross-sectional study of heterozygous antithrombin-III-deficient persons from a large Canadian kindred with a functional deficiency of the inhibitor (Antithrombin-III Hamilton, a type-II mutation having diminished serine protease reactivity) found significantly higher F₁+₂ values in affected family members, though the majority had levels that were within the normal range.46 Therefore, overall, the present evidence indicates that patients with heterozygous antithrombin-III deficiency do not have excessive thrombin generation in the basal state as measured by the F₁+₂ assay which is attributable to a deficiency of this inhibitor.

There have been cases of individuals with homozygous type II antithrombin III deficiency (because of mutations in the heparin binding domain of the inhibitor),37 but the F₁+₂ levels in these patients have not been reported. Antithrombin III repletion studies in these severely deficient patients, similar to those described here in homozygous protein-C-deficient subjects, would permit a more complete assessment of whether the heparan sulfate-antithrombin III mechanism is involved in regulating the basal level of thrombin generation in vivo.

Prospective studies with objective endpoints will be required to determine whether low basal rates of protein C activation or elevated basal rates of thrombin generation will be useful in predicting the subsequent development of thrombosis. Our previous studies show that the basal activity of the hemostatic mechanism is mainly attributable to factor VII- or tissue factor pathway function, which is responsible for the continuous generation of factor IXa, factor Xa, and thrombin.48-50 However, the generated factor Xa is unable to convert factor X to factor Xa, thereby indicating that the intrinsic pathway is dormant with regard to the generation of factor Xa or thrombin. The conversion of a prethrombotic state to a thrombotic event probably results from small increases in the generation rate of procoagulant enzymes that exceed the inhibitory threshold of an individual’s endogenous anticoagulant mechanisms and from the sequestration of these proteases on specialized cell surfaces.

Thus, patients with elevated basal F₁+₂ and FPA measurements because of hereditary deficiencies of protein C or protein S seem poised to respond in a hypersensitive manner to environmental stimuli and generate additional thrombin via the extrinsic pathway. This free thrombin could then be used to ignite the dormant intrinsic cascade, ultimately resulting in the generation of even larger amounts of free thrombin and the development of thrombosis.

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