Relationship between Protein C Antigen and Anticoagulant Activity during Oral Anticoagulation and in Selected Disease States

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Abstract

Protein C is a natural vitamin K-dependent plasma anticoagulant, deficiencies of which have been found in patients with recurrent thrombosis and warfarin-induced skin necrosis. To appreciate more fully the role of protein C in disease states and during oral anticoagulation, a new functional assay for protein C involving adsorption of plasma protein C on a Ca^{+2}-dependent monoclonal antibody, elution, quantitative activation, and assessment of plasma anticoagulant activity, has been developed. When oral anticoagulation is initiated, the anticoagulant activity of protein C decreases to a greater extent than either the amidolytic or immunologic levels. During stabilized warfarin treatment, there is no correlation between either amidolytic or antigenic levels and the functional protein C activity, suggesting that measurement of protein C anticoagulant activity may be necessary to reflect adequately the anticoagulant protection afforded by this protein. In contrast, there was a strong correlation between anticoagulant and amidolytic and immunologic levels in liver failure and disseminated intravascular coagulation. Two patients with thromboembolic disease have been identified who exhibit a marked decrease in anticoagulant activity, but who have normal immunologic and amidolytic levels. Thus, this assay permits assessment of protein C in individuals who have received anticoagulant treatment and identification of a new class of protein C-deficient individuals.

Introduction

Evidence is accumulating that protein C, a vitamin K-dependent plasma glycoprotein (1–3), plays a critical role in the hemostatic system. Upon activation in vivo by the thrombin–thrombomodulin complex (4–6), protein C is converted to a serine protease which exerts a potent anticoagulant effect by inactivating factors Va and VIIIa (7–9). Protein S, another vitamin K-dependent protein (10), is required for optimal expression of the anticoagulant activity of activated protein C, probably by facilitating its interaction with membrane surfaces (11, 12). Either total or partial deficiencies of protein C (13–21) and protein S (22–24) predispose to thrombosis. Homozygous protein C deficiency is associated with extensive thrombotic disease and death in infancy (13–17). Several reports have shown that patients with isolated partial deficiency of protein C experience recurrent

thrombotic episodes (18–21). The heterozygotes also appear to be at substantial risk of warfarin-induced skin necrosis, a condition characterized by diffuse thrombosis of small venules during the initial stages of oral anticoagulant therapy (25–27). When coupled with the observation that protein C antigen decreases rapidly when patients are placed on oral anticoagulants (28, 29), these observations raise questions about the best regimen for administering anticoagulants and the role of protein C in the initial stages of anticoagulant therapy. Measurements of protein C biologic function are required to determine the relative influence of different therapeutic regimens on the balance between the anticoagulant function of protein C and the procoagulant activity of the vitamin K-dependent clotting factors.

Four methods for measuring protein C functional activity have been previously described (30–34). Protein C is isolated from plasma by adsorption onto aluminum hydroxide (31) or barium citrate (30, 33) followed by activation with thrombin (30, 31) or with the thrombin–thrombomodulin complex (33). Alternatively, protein C is activated in plasma with the thrombin–thrombomodulin complex and then adsorbed with immobilized antiprotein C IgG (32). With the exception of one method that measures the ability of activated protein C to prolong the activated partial thromboplastin time (30), all the other methods measure its ability to cleave synthetic substrates. Each assay detects different features of protein C. Adsorption onto insoluble salts selects for the carboxylated forms of the vitamin K-dependent protein, whereas the method based on protein C activation in plasma with the thrombin–thrombomodulin complex and subsequent immunoadsorption (32) is not dependent on the extent of carboxylation. All the methods share relevant limitations. None of the assays differentiates between abnormal protein C molecules that cannot be converted to activated protein C and those that, once activated, cannot function as anticoagulants. The presence of heparin in the plasma samples hinders the reliable determination of protein C. Heparin adsorbs to barium citrate and aluminum hydroxide, and heparin induces rapid neutralization of thrombin bound to thrombomodulin by antithrombin III and enhances the inhibition of activated protein C by its inhibitor when the activation is performed in plasma.

The characterization in this laboratory of a Ca^{2+}-dependent monoclonal antibody to human protein C, which binds human protein C independently of the presence of γ-carboxy glutamic acid (Gla)[1] residues, offered an ideal tool for isolating protein C from human plasma. By employing this antibody, we have developed a new functional assay that allows the accurate and precise measurement and comparison of both the amidolytic and the anticoagulant activity of protein C. We present evidence that protein C isolated from plasma of patients on stabilized

warfarin treatment may be activated by purified thrombin–thrombomodulin to an extent and with a rate similar to those observed for protein C isolated from plasma of patients with comparably reduced levels of protein C antigen in plasma, but, once activated, protein C exerts little or no anticoagulant activity. We find that a similar abnormality of protein C can occur in certain patients with idiopathic recurrent deep-vein thrombosis. During the initiation of oral anticoagulant therapy, the anticoagulant potential of protein C drops rapidly, and this decrease is not reflected by either the antigenic levels or the amidolytic activities of the activated protein.

Methods

Reagents

Tris (Trizma base or Sigma 7-9), MOPS, heparin sodium salt from porcine intestinal mucosa (140 U/mg), Lubrol Px, rabbit brain cephalin, chymotrypsin, and bovine serum albumin (BSA) fraction V were obtained from Sigma Chemical Co. (St. Louis, MO); benzamidine hydrochloride hydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Bovine thrombin was isolated after activation of the purified prothrombin with purified factor Xa, Va, phospholipids, and calcium (35). Bovine factor Xa was purified and activated as described (36). Bovine antithrombin III (37) and rabbit lung thrombomodulin (6) were isolated as described. Human protein C was partially purified by quaternary aminoethyl adsorption and elution, $BaSO_4$ adsorption, and quaternary aminoethyl Sephadex chromatography (32). Protein C was separated from contaminating proteins by chromatography on a murine IgG monoclonal anti-human protein C antibody which requires Ca^{2+} for interaction with protein C. Removal of Ca^{2+} results in elution of protein C. A manuscript detailing the requirements and specificity of this antibody is in preparation. The monoclonal antibody, termed HPC4, was coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, CA) according to the instructions of the manufacturer. The final antibody concentration on the gel was 5 mg/ml. Activated protein C was obtained by incubating the purified human protein C (0.3–0.8 mg/ml) with bovine thrombin (5% wt/wt) for 3 h at 37°C in the presence of 2 mM EDTA. Gla-domainless protein C was prepared by limited proteolysis of human protein C with chymotrypsin (38). Immobilized thrombin–thrombomodulin complex was obtained by linking rabbit lung thrombomodulin to Affigel 10 via a non-inhibitory murine IgG monoclonal anti-rabbit thrombomodulin antibody. The antibody concentration on the gel was 5 mg/ml. After extensive washing, 5 ml of the gel was incubated with 0.7 mg of purified rabbit lung thrombomodulin diluted into 5 ml of Tris-buffered saline containing 0.01% Lubrol Px (wt/vol) for 1 h at 37°C on a rocker. Under these conditions 90–95% of the rabbit lung thrombomodulin was bound to the immobilized antibody. After discarding the supernatant, bovine thrombin (400 USP units, 168 μg) diluted in 5 ml of 20 mM Tris HCl, 0.1 M NaCl, 1 mg/ml BSA, pH 7.5, was added to the gel and incubated for 1 h at 37°C on a rocker. Residual thrombin activity in the supernatant was assessed by measuring hydrolysis of H-D-Phe-pipecolyl-Arg-p-nitroanalide (S-2238, Kabi, Stockholm, Sweden) in a model DU 7 Beckman spectrophotometer (Beckman Instruments, Inc., Irwin, CA). Less than 2% of the added thrombin was recovered. Protein C-depleted plasma was prepared by immunoadsorption of 40 ml of normal plasma through a 1.5 × 15-cm column of anti-human protein C goat IgG coupled to Bio-gel-agarose A-15 m, 50–100 mesh (Bio-Rad Laboratories) after cyanogen bromide activation (39) (~5 mg IgG bound per milliliter of agarose).

Protein C levels were measured immunologically using the Laurell rocket technique (40). Goat anti-human protein C IgG was incorporated into 1% agarose at a final concentration of 0.03 mg/ml to form the gel plates. The assay was sensitive to 10% normal plasma levels.

Conditions of the functional protein C assay

1 ml of citrated platelet-poor plasma was recalcified and heparinized by the addition of 100 μl of a buffer containing 1.0 M NaCl, 0.5 M Tris-HCl, 0.05 M benzamidine-HCl, 150 mM $CaCl_2$, 20 U/ml heparin, pH

7.5, and incubated with 100 μl of packed HPC4-Affigel 10 beads on a rocker at room temperature. After 1 h, the beads were removed by centrifugation in a Beckman Microfuge model B (Beckman Instruments, Inc., Palo Alto, CA) and, after discarding the supernatant, washed three times with a buffer containing 0.5 M NaCl, 20 mM Tris HCl, 2 mM $CaCl_2$, 5 mM benzamidine-HCl, 1 mg/ml BSA, 0.01% Lubrol Px, pH 7.5. Protein C was eluted by mixing the beads in 1 ml of a buffer composed of 0.1 M NaCl, 50 mM MOPS, 6 mM EDTA, 1 mg/ml BSA, pH 7.5, for 30 min at room temperature on a rocker. After centrifugation, 0.9 ml of the supernatant was collected and recalcified by the addition of 11 μl of 1 M $CaCl_2$ (12 mM final concentration). The mean recovery of protein C antigen, as assessed by measuring rockets in both the HPC4 eluates and plasma from 70 normal controls, was 82%, 95% confidence limits: 76–87%. Immunoprecipitable material was never detected in plasma supernatants after incubation with the HPC4-Affigel 10 beads. The extracted and recalcified protein C was subsequently activated by addition of 50 μl of sedimented thrombin–thrombomodulin complex with Affigel 10 beads (final thrombin–thrombomodulin complex concentrations, 100 nM) and by mixing on a rocker for 60 min at 37°C. The reaction was stopped by centrifuging the beads and removing the supernatant containing the activated protein C. After addition of 30 μg of bovine antithrombin III, the activated protein C was quantitated both according to amidolytic and anticoagulant activity. Antithrombin III had no influence on either the anticoagulant or the amidolytic assay. Fig. 1 shows the activation time-course of protein C extracted from normal pooled plasma and Fig. 2 shows an outline of the functional protein C assay.

Amidolytic assay. In a 1-cm cuvette, 50 μl of the activated sample was diluted with 400 μl of 0.1 M NaCl, 20 mM Tris HCl, 1 mg/ml BSA, pH 7.5. Substrate (50 μl of 2 mM S2238) was then added and the release of paranitroaniline was determined at 25°C by recording the change in A_{405} (absorbance at 405 nm) over 3 min in a Beckman DU7 spectrophotometer, which was programmed for kinetic analysis of the reaction. Five dilutions of protein C extracted from normal plasma and activated (5:5, 4:5, 3:5, 2:5, 1:5) were used to construct a calibration curve. Changes in A_{405} from triplicate determinations of the test sample were thus converted to protein C concentration (percent of normal plasma).

Anticoagulant assay. The factor Xa one-stage clotting time was performed to measure activated protein C (41). The assay was performed in the following manner: to 100 μl of normal pooled plasma at 37°C was added 100 μl of cephalin, 100 μl of purified bovine factor Xa, and 100 μl of 25 mM $CaCl_2$. The bovine factor Xa was diluted into 0.1 M NaCl, 0.02 M Tris HCl, 1 mg/ml BSA, pH 7.5, to give a 30-s clotting time (6 ng/ml). The protein C extracted from normal pooled plasma and activated (20 μl) was added to the incubated mixture prior to factor Xa. This level of activated protein C gives a 30–35-s prolongation of the clotting time. A standard curve was prepared using serial dilutions of the activated protein C down to 1:8 dilution. A linear relationship was obtained between the percent concentration of activated protein C and the prolongation of the clotting time. Triplicate determinations were run for each test sample and converted to protein C concentration (percentage of normal plasma). When bovine factor Xa was substituted for human factor Xa, virtually identical results were obtained in a series of 20 patients with protein C anticoagulant activity ranging from 20% to 150%. To determine the sensitivity of the assay, the normal plasma pool was serially diluted into protein C deficient plasma. Each dilution was then processed separately and assayed for amidolytic and anticoagulant activity as described above (Fig. 3). The intercept of the curves on the x-axis is not significantly different from zero. The detection limit for both assays was set at 5% of normal plasma with corresponding values of 1–2-s prolongations in the clotting assay and 1.5–2 × 10^{-3} (Delta) A_{405}/min in the chromogenic assay.

Attempts to use soluble thrombin–thrombomodulin complex as activator of protein C yielded nonlinear dose–response curves in the clotting assay described above, with upward deviations which were proportionately related to the final concentration of thrombomodulin in the clotting mixture and which hindered reliable assessment of the anticoagulant activity of activated protein C.

Reproducibility of the assay

Within- and between-day variations of the assays were determined with 12 replicate determinations of a normal plasma pool. The within-day coefficients of variation were 3% for both assays and the between-day coefficients of variation were 7% and 17% for the amidolytic and the clotting assay, respectively.

Factor VII and X assays

Factors VII and X were determined in one-stage clotting assays. Factor VII was assayed using activated rabbit brain thromboplastin (Dade Diagnostics, Inc., Aguarda, PR) and factor VII-deficient plasma (George King Biomedicals, Inc., Overland Park, KS). Factor X was assayed using Russell's viper venom in cephalin (Sigma Chemical Co.) and factor X- and VII-depleted plasma (Sigma Chemical Co.).

Patient population

Normal subjects and patients gave informed consent. Venous blood was collected in 0.13 M trisodium citrate. Platelet-poor plasma was obtained by centrifuging blood at 4,800 g for 15 min. Pooled normal plasma was composed of platelet-poor plasma from 15 healthy donors. All plasma was stored at −70°C. Normal controls ranged from 25 to 70 yr of age (mean age 41 yr). Patients on stabilized warfarin treatment were receiving the same dose of warfarin for at least 1 wk prior to testing. Patients starting oral anticoagulation because of deep-vein thrombosis were treated either with or without a loading dose of warfarin while on heparin therapy. The loading dose was administered for the first 2 d (30 and 20 mg of warfarin); on the third day patients received 10 mg of warfarin and the prothrombin time was measured. Subsequent dosages were adjusted according to the prothrombin time. Alternatively, 10 mg of warfarin was administered for the first 3 days.

Patients with severe chronic liver disease had laboratory data consistent with liver failure (albumin < 3 g/dl, γ-globulin > 2.5 g/dl, elevated transaminases, and prolonged prothrombin time) with or without biopsy-proven diagnosis. Patients with laboratory evidence of disseminated intravascular coagulation (DIC) secondary to different clinical conditions (obstetrical, posttraumatic, or infective), met diagnostic criteria indicated previously (42) (fibrin[ogen] degradation products > 20 μg/ml with markedly reduced platelet count and fibrinogen levels and prolonged prothrombin and partial thromboplastin times). Plasma samples from patients with a history of no underlying recurrent deep-vein thrombosis, which had been sent for protein C determination during a period of 1 yr, were screened for protein C deficiency with the assay described above. In general, more than one sample was received from each patient. None of the investigated patients had deficiencies of other clotting factors known to predispose to thrombosis (normal antithrombin III, plasminogen, fibrinogen, and protein S levels).

Statistical analysis

Unless specified, geometric means and 95% confidence limits of the mean were calculated after log transformation of the single values. The significance of the difference between means was tested with one- or two-way analysis of variance and the Student t test for unpaired or paired data. Linear regression and correlation coefficient were calculated with the least squares method.

Results

Functional protein C levels were determined in 70 apparently normal subjects (Fig. 4). Mean values were 99% in the clotting assay and 101% in the amidolytic assay. Corresponding protein C antigen mean level in plasma was 99%. Two-way analysis of variance showed that there were no significance differences among protein C levels tested with the three methods.

Mean functional protein C levels in 16 patients on stabilized warfarin treatment (prothrombin time 20±2.4 s [SEM]) were 9% in the clotting assay and 46% in the amidolytic assay, with
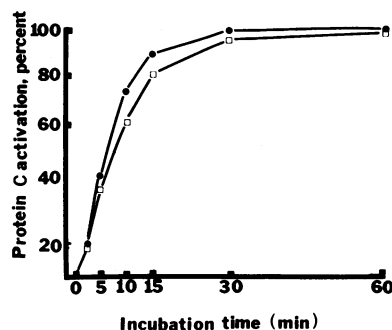

Figure 1. Time course of activation of protein C isolated from normal pooled plasma by immobilized thrombin–thrombomodulin complex. Conditions of protein C isolation and activation were the same as described for the assay. After recalcification and heparinization, 1.5 ml of normal pooled plasma was incubated for 1 h at room temperature with 100 μl of packed HPC4 Affigel 10 beads. After three washings, the protein was eluted over 30 min at room temperature in 1.5 ml of MOPS buffer containing 6 mM EDTA and 1 mg/ml BSA. After centrifugation, 1.4 ml of the supernatant was recalcified by the addition of 17 μl of 1 M CaCl$_2$ and incubated with 75 μl of packed thrombin–thrombomodulin complex with Affigel 10 beads at 37°C on a rocker (final thrombin–thrombomodulin complex concentration, 100 nM). At the indicated times, 200-μl aliquots were removed and activation was immediately stopped by centrifugation. Anticoagulant (□) and amidolytic (●) activated protein C activities were quantitated as described in Methods and expressed as percentage of the activity observed after 120 min of incubation.

a mean antigen level in plasma of 46%. 16 patients with severe chronic liver disease had mean protein C anticoagulant activity of 27% and mean protein C amidolytic activity of 30% with a mean plasma antigen value of 29%. 16 patients with laboratory evidence of DIC had a mean protein C anticoagulant activity of 29%, a mean protein C amidolytic activity of 34%, and a mean protein C antigen level in plasma of 34%. The ratio of functional protein C activity (amidolytic and anticoagulant) to protein C antigen was not significantly different from unity in controls and in patients with liver disease and DIC (Table I). However, patients on warfarin had reduced ratios of protein C anticoagulant activity to amidolytic activity and antigen levels ($P < 0.001$), with similar protein C amidolytic and antigenic levels.
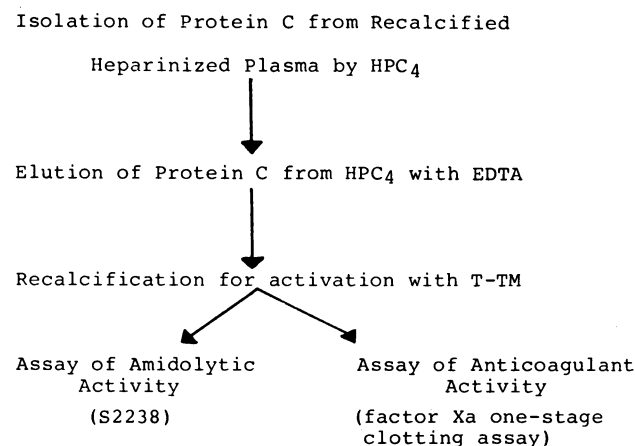
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Isolation of Protein C from Recalcified

    Heparinized Plasma by HPC4

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              ↓

Elution of Protein C from HPC4 with EDTA

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Recalcification for activation with T-TM

        ↙              ↘

Assay of Amidolytic        Assay of Anticoagulant
    Activity                    Activity
    (S2238)                 (factor Xa one-stage
                              clotting assay)
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Figure 2. Outline of the functional protein C assay T-TM, thrombin–thrombomodulin complex.
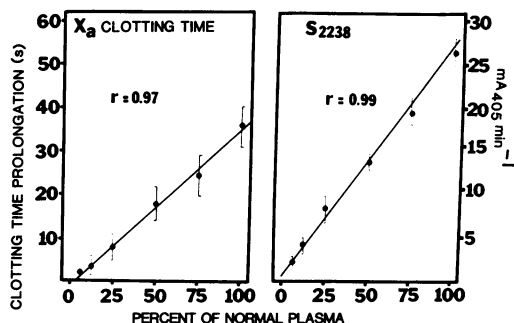
Figure 3. Dose–response curves were determined for protein C isolated from normal pooled plasma and activated (anticoagulant and amidolytic activity, mean and standard deviations of four different experiments). 1-ml mixtures of normal pooled plasma with protein C-depleted plasma (6.25%, 12.5%, 25%, 50%, 75%, and 100% normal pooled plasma concentrations) were independently processed in the functional assay as described in Methods. Regression and correlation coefficients were calculated on pooled data from four separate experiments. The intercept of both curves on the x-axis is not significantly different from zero.

To rule out the possibility that different values of protein C activity might partly reflect a different degree of adsorption of protein C to the monoclonal antibody in different plasmas, the recovery of protein C antigen was measured in controls and patients relative to the recovery of protein C from normal pool plasma. Similar mean relative recoveries of protein C, in no instance significantly different from unity, were observed (Table I). However, when in a different series of patients on warfarin (n = 23), rocket immunoelectrophoresis of plasma was performed in the absence of EDTA, the mean relative recovery of protein C was significantly, albeit slightly, lower than unity (0.81, P < 0.001), owing to overestimation of protein C antigen in
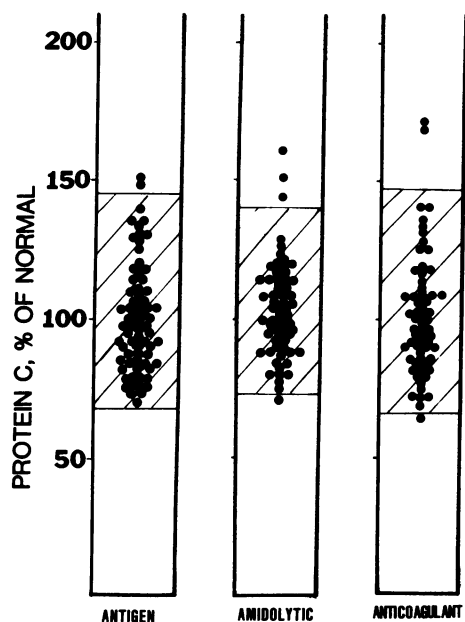

Figure 4. Distribution of plasma protein C antigen, protein C amidolytic activity, and protein C anticoagulant activity into apparently healthy subjects. Shadowed areas indicate ranges, calculated as mean±2 SD of log values prior to antilog transformation.

plasma (mean value 56%). In this series, protein C anticoagulant activity (mean value 11%) and amidolytic activity (mean value 46%) were not significantly different from the corresponding activities observed in the original series of patients on stabilized warfarin treatment (Table I). Fig. 5 shows the relationship of amidolytic to antigenic and of anticoagulant to amidolytic protein C levels in controls and in the patient series. In warfarin-treated patients, there was no correlation between the protein C anticoagulant and amidolytic activities (Fig. 5 B).

Removal of Gla residues from protein C has no effect on its activability by soluble thrombin–thrombomodulin complex (38). To estimate the in vitro activability of warfarin-induced protein C, the time course of protein C activation by the thrombin–thrombomodulin complex was monitored using the functional assay in three healthy individuals and in three patients on stabilized warfarin treatment. The rate of activation was compared to that observed for normal pooled plasma, a 1:1 mixture of pooled plasma with protein C-depleted plasma, and 3.5 µg/ml of purified human protein C in protein C depleted plasma (Table II). Virtually identical results were obtained for all the samples indicating comparable activation rates in normal and in warfarin-treated patients. To investigate whether warfarin-induced forms of protein C interfere with the anticoagulant activity of fully carboxylated protein C, resulting in a low ratio of anticoagulant to amidolytic activity, protein C was extracted and activated from three normal controls, three patients on stabilized warfarin treatment, and three patients with severe liver disease. Purified protein C and Gla-domainless protein C in protein C-depleted plasma (3.5 µg/ml) were also extracted and activated according to the procedure of the functional assay along with the protein C from normal pooled plasma. Mixtures of activated protein C from normal pooled plasma with increasing amounts (0.25–4.0 vol/vol) of activated protein C from the depleted plasmas reconstituted with the purified proteins and from the patients' and controls' plasmas were subsequently tested in the factor Xa one-stage assay. A close agreement between the clotting times observed and those expected on the basis of an additive effect was found for all mixtures (data not shown).

To gain information on the modification of the balance between the anticoagulant potential of protein C and the procoagulant potential of other vitamin K-dependent factors during the early phase of oral anticoagulation, the changes of protein C and of factor VII and X functional activities were followed in two small groups of four patients starting oral anticoagulation with or without a loading dose of warfarin. Blood samples were taken before (base line) and 4, 8, and 24 h, after the start of therapy, after which additional samples were taken daily for 6 d. Because levels before warfarin of all the considered parameters were similar in the two groups of patients, changes after warfarin were expressed as percentages of base-line values to decrease variability (Fig. 6). After 1 d of anticoagulation, mean protein C anticoagulant activity in the loading dose-treated group was 69±11% (SEM) of the mean activity in the nonloading dose-treated group. Accordingly, at 24 h, mean protein C antigen and amidolytic activity were 76±17% and 75±8% of the mean levels in the nonloading dose treated group. Corresponding mean factor VII and X levels in the loading dose-treated group were 32±13% and 93±15% of the respective levels in the nonloading dose-treated group. The mean half-disappearance times of protein C anticoagulant activity, factor VII, and factor X calculated with a semilog regression method after checking for linearity, were

Table I. Functional and Antigenic Protein C Levels in Controls and in the Patients' Populations

Ratio	Controls (n = 70)			Stabilized warfarin treatment (n = 16)		Chronic liver disease (n = 16)		DIC (n = 16)	
	Geom $\bar{x}$	95% CI	Range	Geom $\bar{x}$	95% CI	Geom $\bar{x}$	95% CI	Geom $\bar{x}$	95% CI
PC/anticoagulant activity (% of normal)	99	94–104	66–147	9	7–11*	27	18–39*	29	17–46*
PC/amidolytic activity (% of normal)	101	97–105	73–140	46	41–52*	30	22–41*	34	23–50*
PC/antigen (% of normal)	99	95–104	68–145	46	40–52*	29	20–42*	34	23–52*
PC/amidolytic–antigen	1.02	0.98–1.07	0.72–1.46	1.01	0.93–1.11	1.02	0.86–1.23	1.00	0.82–1.20
PC/anticoagulant–antigen	0.99	0.93–1.06	0.61–1.61	0.18	0.17–0.20*	0.90	0.79–1.02	0.88	0.77–1.02
PC/anticoagulant–amidolytic	0.98	0.94–1.01	0.74–1.30	0.18	0.13–0.25*	0.88	0.74–1.05	0.87	0.71–1.02
Relative PC/antigen recovery	1.01	0.96–1.07	0.68–1.51	1.03	0.27–1.22	0.95	0.79–1.16	0.90	0.75–1.22

The recovery of protein C antigen in the EDTA eluates from the monoclonal antiprotein C antibody (HPC4) relative to that observed in the eluate from normal pooled plasma was assessed as described in Methods. Ranges of the values obtained in normal controls were calculated as mean±2 SD of log values prior to antilog transformation. Abbreviations: PC, protein C; CI, confidence limit. * Significance of the difference of the means vs. normal controls (P < 0.001).

15.8 h (range 13–18.5), 9.9 h (6.4–12.1), and 52.6 h (23.1–55.4) in the loading dose-treated group and 18.8 h (15.2–32.8), 18.7 h (13.1–36.4), and 46.3 h (21.5–60.5) in the nonloading dose-treated group.

Table III shows the ratios of the percent changes of protein C anticoagulant activity to protein C antigenic and amidolytic levels and to factor VII and X activities during the first 3 d of oral anticoagulation in the two groups of patients. The results obtained are suggestive of a more rapid decrease of protein C in the loading dose-treated group. Factor VII and protein C ac-

tivities were reduced at the same rate when no loading dose of warfarin was administered, but factor VII activity was more rapidly depressed than protein C activity with the higher warfarin dosage. Because the pattern of disappearance of factor X activity was scarcely, if at all, influenced by the difference in the treatment schedules, the ratio of protein C to factor X levels at 24 and 48 h appeared lower with the loading dose. The small number of patients in each treatment group and the relevant within-group variability precluded the statistical evaluation of the results.

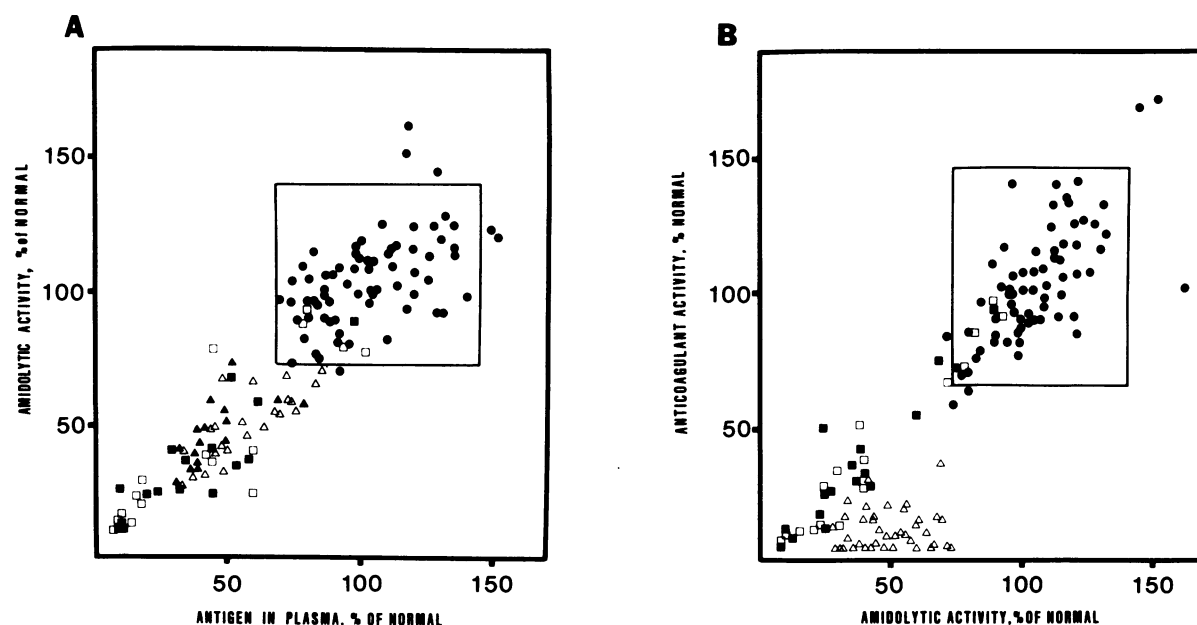To determine whether this assay could detect protein C vari-

Figure 5. (A) Relationship of protein C amidolytic activity to plasma protein C antigen levels in normal controls (●), patients with chronic liver disease (■), patients with DIC (□), and in two series of patients on stable warfarin therapy who had plasma antigen determinations performed either with (▲, n = 16) or without EDTA (△, n = 23) in the electrophoretic system. The boxed area (inset) represents the normal range for the two parameters. Correlation coefficients were: (●) r

= 0.56; (■) r = 0.88; (□) r = 0.85; (▲) r = 0.72; (△) r = 0.74. (B) Relationship of protein C anticoagulant to amidolytic activity in normal controls (●), patients with chronic liver disease (■), patients with DIC (□), and patients on stable warfarin therapy (△, n = 39). The boxed area (inset) represents the normal range. Correlation coefficients were: (●) r = 0.70; (■) r = 0.92; (□) r = 0.97; (△) r = 0.04 (NS).

Table II. Comparison of the Activation Rates of Protein C Extracted from Plasma of Three Normal Controls and Three Patients on Stable Warfarin Treatment

	Percent of protein C activation							Protein C levels		
	3.0 min	6.5 min	10 min	15 min	23 min	33 min	60 min	PC amidolytic	PC antigenic	PC anticoagulant
Normal pooled plasma	21	31	34	47	62	74	100	100	100	100
N	13	24	38	46	61	70	100	83	81	106
N	15	29	38	49	59	73	100	105	107	99
N	13	28	34	48	59	74	100	103	105	117
$\bar{x}\pm$SD	13±2	27±2	38±1	48±2	60±1	72±2		97±12	98±14	107±9
Normal pooled plasma 50%	12	24	34	44	54	73	100	54	52	58
W	10	26	34	42	59	70	100	56	49	10
W	16	29	32	44	56	76	100	52	50	10
W	12	31	36	47	59	71	100	40	38	16
$\bar{x}\pm$SD	13±3	29±3	34±2	44±3	58±2	72±3		49±8	46±7	12±3
3.5 µg/ml PC in PC depleted plasma	12	21	32	42	53	71	100	114	98	117

Conditions of protein C isolation and activation were essentially the same as described for the assay. After removal of aliquots for protein C antigen measurement, 1.5 ml of heparinized recalcified plasma was incubated for 1 h at room temperature with 100 µl of packed HPC4-Affigel 10 beads. After washing three times, the protein was eluted over 30 min at room temperature in 1.5 ml of MOPS buffer containing 6 mM EDTA and 1 mg/ml BSA. After centrifugation, 1.4 ml of the supernatant was recalcified by the addition of 17 µl of 1 M $CaCl_2$ and incubated with 50 µl of packed thrombin–thrombomodulin complex with Affigel 10 beads at 37°C on a rocker (final thrombin–thrombomodulin complex concentration, 64 nM). At the indicated times, 200-µl aliquots were removed and immediately centrifuged to separate the supernatant from the beads. Amidolytic activated protein C activity was quantitated with S2238 and expressed as percentage of the activity observed after 60 min of incubation with immobilized thrombin–thrombomodulin complex, at which time protein C anticoagulant activity was also measured. Abbreviations: N, normal; W, warfarin; PC, protein C.

ants, we assayed plasma samples from 109 patients with idiopathic recurrent deep-vein thrombosis. We have identified several unrelated protein C-deficient patients (Table IV). Prothrombin times and factor X levels were determined to rule out oral anticoagulation. Protein C anticoagulant, amidolytic, and antigen levels were comparably reduced in patients 1, 2, and 3, resulting in normal ratios of functional to antigenic levels. Patient 4 had protein C amidolytic and antigenic levels within the normal range but low anticoagulant activity. The ratio of the functional to the antigenic protein C levels was abnormal for both the amidolytic and the anticoagulant activity, but, in addition, there was also a reduced ratio of the anticoagulant to the amidolytic protein C activity. An isolated deficiency of protein C anticoagulant activity was observed in patient 5, with a normal ratio of amidolytic to antigenic protein C but with a low ratio of protein C anticoagulant to amidolytic activity.

Discussion

The protein C functional assay described in this paper allows virtually complete recovery of protein C independent of the vitamin K status of the patient and has the ability to discriminate between the amidolytic and the anticoagulant activity of activated protein C. The method is suitable for protein C determination both in plasma and in serum (data not shown) owing to the negligible protein C activation occurring when blood is clotted in vitro. The presence of heparin in the plasma samples does not interfere with the assay, thus allowing determination of protein C levels early in the hospital course, when heparin is being administered. This permits the accurate and precise measure-

ment of both the in vitro activatability and the anticoagulant potential of protein C either in patients on stabilized warfarin therapy or at the beginning of oral anticoagulation. Patients on stabilized warfarin therapy had mean protein C levels of 11% in the clotting assay and of 48% in the amidolytic assay. Their protein activated normally with purified thrombin–thrombomodulin complex and, once activated, did not inhibit the anticoagulant function of normal activated protein C. Thus the difference in amidolytic and anticoagulant activity cannot be attributed to inhibitory forms.

Functional levels of protein C in patients treated with oral anticoagulants have been reported to be lower (31, 33) or equal (32) to the antigenic levels according to the different isolation procedures employed. In two comparable series where the same isolation procedure was adopted but different systems were used for protein C quantitation, lower protein C levels were estimated by a clotting assay (30) than by an amidolytic assay (33). When considering that there was no correlation between anticoagulant and amidolytic protein C activities in our series, it can be concluded that the functional assays based on amidolytic methods do not reflect the biologic potential of protein C during oral anticoagulation.

A greater reduction of protein C anticoagulant activity than amidolytic or antigenic levels was anticipated based on previous studies with other vitamin K-dependent proteins (43). Preferential loss of anticoagulant activity probably reflects the inability of the partially carboxylated forms of the vitamin K-dependent factors to interact with membrane surfaces. Prothrombin is the best characterized of the vitamin K-dependent proteins with respect to the role of Gla residues in membrane interaction.
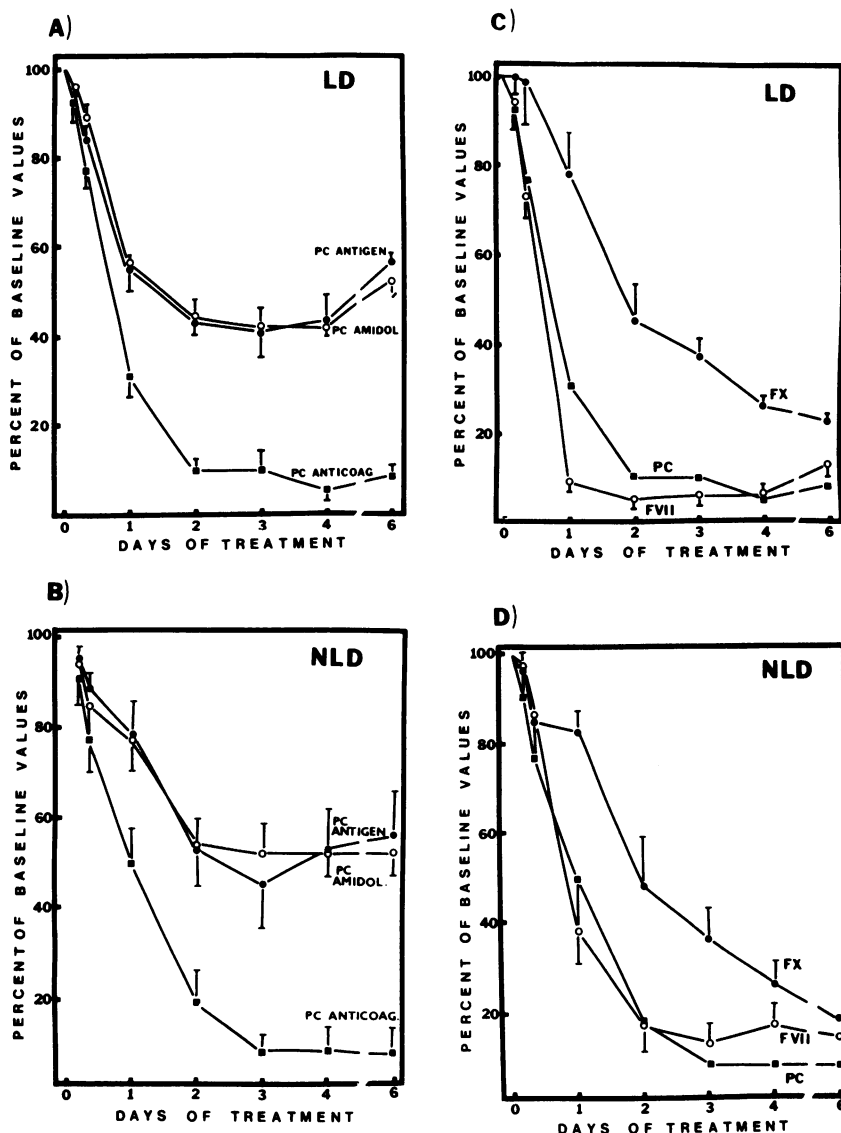
A) LD

PERCENT OF BASELINE VALUES

100 · 80 · 60 · 40 · 20

PC ANTIGEN
PC AMIDOL
PC ANTICOAG

0 1 2 3 4 6
DAYS OF TREATMENT

C) LD

PERCENT OF BASELINE VALUES

100 · 80 · 60 · 40 · 20

FX
PC
FVII

0 1 2 3 4 6
DAYS OF TREATMENT

B) NLD

PERCENT OF BASELINE VALUES

100 · 80 · 60 · 40 · 20

PC ANTIGEN
PC AMIDOL
PC ANTICOAG

0 1 2 3 4 6
DAYS OF TREATMENT

D) NLD

PERCENT OF BASELINE VALUES

100 · 80 · 60 · 40 · 20

FX
FVII
PC

0 1 2 3 4 6
DAYS OF TREATMENT

Figure 6. Changes of protein C (PC) anticoagulant activity, protein C amidolytic activity, and protein C antigen in plasma in two groups of four patients with deep-vein thrombosis, starting warfarin therapy either (*A*) with a loading dose (LD) or (*B*) without a loading dose (NLD). (*C* and *D*) Changes of the anticoagulant activity of protein C (PC) as compared to the changes of the procoagulant activities of factor VII (FVII) and factor X (FX) in the same two groups. Patients received either 30 mg of warfarin on day 1, 20 mg on day 2, and 10 mg on day 3, or 10 mg on each day for the first 3 d. Changes of all the parameters are expressed as percentages (mean±SEM) of the prewarfarin levels measured while patients were maintained on heparin infusion.

With prothrombin, the expression of coagulant activity appears directly related to membrane binding potential, and the loss of relatively few of the Gla residues during oral anticoagulation is sufficient to destroy the membrane binding potential (44). Although comparable studies with protein C are not available, the overall mechanism of the influence of oral anticoagulants on protein C function is likely similar to that of prothrombin. Previous studies demonstrated that the Gla-containing region of bovine activated protein C is required for expression of membrane-dependent anticoagulant activity, but is not required for amidolytic activity or for the proteolytic inactivation of factor Va (45). It is likely, therefore, that loss of membrane binding potential is responsible for loss of protein C anticoagulant activity during oral anticoagulation. The number of Gla residues in protein C required for expression of anticoagulant activity and membrane interaction remains uncertain.

The half-life of protein C has been estimated by immunologic methods in patients starting oral anticoagulation and has been found similar to that of factor VII (28, 29). This finding raised the hypothesis that a transient protein C deficiency state may contribute to the poor antithrombotic protection at the beginning

of oral anticoagulation (28, 29). Recently, the mechanism of warfarin-induced skin necrosis has been related to the congenital deficiency or protein C. Specifically, it has been proposed that the rapid decrease in protein C after the start of oral anticoagulation, relative to the decrease of most of the vitamin K-dependent clotting factors, gives rise to a transient hypercoagulable state (25–27). However, no information about the changes of protein C anticoagulant activity during the early phase of oral anticoagulation is available. We studied the changes of protein C in two small series of patients with deep-vein thrombosis treated with or without a loading dose of warfarin and compared them with the changes in the activity of factor VII and X. Although no statistical test was applied, the results suggest that the loading dose treatment resulted in more rapid disappearances of factor VII and of protein C antigen and activities than did the nonloading dose treatment. However, factor X levels were similarly influenced by either treatment schedule. Thus, when oral anticoagulants are administered with no loading dose, protein C and factor VII activities disappear at similar rates. With the loading dose, factor VII procoagulant activity disappears more rapidly than protein C anticoagulant activity. In contrast,

Table III. Changes in the Mean Ratio of Protein C Anticoagulant Activity to Protein C Antigen, Protein C Amidolytic Activity, Factor VII Activity, and Factor X Activity in Patients Starting Oral Anticoagulation with or without a Loading Dose of Warfarin

Ratio		Time (h)				
		4	8	24	48	72
PC/anticoagulant–antigen	NLD	0.96±0.08	0.88±0.06	0.62±0.03	0.30±0.09	0.17±0.04
	LD	1.00±0.07	0.93±0.06	0.57±0.03	0.24±0.04	0.25±0.11
PC/anticoagulant–amidolytic	NLD	0.97±0.09	0.93±0.07	0.65±0.11	0.32±0.13	0.15±0.05
	LD	1.00±0.99	0.99±0.07	0.56±0.07	0.23±0.04	0.25±0.13
PC/anticoagulant–FVII	NLD	0.93±0.08	0.91±0.09	1.31±0.09	1.00±0.10	0.66±0.14
	LD	1.00±0.09	1.07±0.07	4.22±1.23	2.03±0.24	1.83±0.43
PC/anticoagulant–FX	NLD	0.98±0.15	0.91±0.05	0.61±0.13	0.38±0.13	0.26±0.10
	LD	0.90±0.05	0.81±0.09	0.44±0.11	0.18±0.04	0.29±0.14

Protein C anticoagulant, amidolytic and antigenic levels, and factor VII and X procoagulant activity were measured in the two groups of four patients while on heparin infusion, before warfarin administration. Because levels before warfarin were similar for all the parameters, changes after warfarin were expressed as percentage of base-line values and the ratios of the parameters calculated at corresponding times (mean±SEM). Abbreviations: PC, protein C; FVII, factor VII; FX, factor X; NLD, no loading dose; LD, loading dose.

protein C anticoagulant activity is more rapidly reduced, relative to factor X activity, with the loading than with the nonloading dose. Because a full antithrombotic effect has not been demonstrable until 7–10 d of oral anticoagulation (46), it is likely that factor VII procoagulant activity, which is reduced after 24–48 h, is not sufficient by itself to alter the thrombotic risk in patients starting oral anticoagulation. If factor X levels more closely reflect the antithrombotic effect of warfarin, the clinical extrapolation of our findings is that patients with protein C deficiency starting oral anticoagulation may benefit from lower dosages of warfarin than those generally used. Very small doses of long-acting oral anticoagulants (1–3 mg/d) apparently resulted in successful management of one patient with severe protein C deficiency and repeated episodes of acenocoumalone-induced skin necrosis (27).

Patients with severe chronic liver disease had comparably reduced levels of protein C antigen and activity, but, at variance with warfarin-treated patients, there was no discrepancy between the anticoagulant and the amidolytic activities. It has been previously shown that in liver disease protein C antigen levels are low (30, 32, 33) and negatively correlated with the ability to synthesize proteins (47); the finding of a parallel reduction of functional and antigenic protein C levels in this series is consistent with an impaired synthesis of a normally functioning protein.

Protein C levels are low or unmeasurable in clinical conditions associated with disseminated intravascular coagulation (30, 42, 48, 49). Although decreased synthesis due to concomitant liver damage has been suggested as an explanation for this finding (42, 48, 49), increased clearance of in vivo activated protein C cannot be ruled out as an alternative mechanism. Increased plasma levels of protein C activation peptide in DIC have been recently reported, along with the suggestion that, in this condition, activated protein C concentrations might be as high as 0.02–2% of the total circulating zymogen (50). This suggested to us that, although the monoclonal antibody (HPC4) does not recognize activated protein C nor activated protein C bound to its specific inhibitor (51) (data not shown), the new assay may provide reliable assessment of protein C function also in patients with DIC. This hypothesis was confirmed by the observation in 16 patients with laboratory evidence of DIC of virtually identical

Table IV. Functional and Antigenic Levels of Protein C in Five Unrelated Patients with Recurrent Deep-Vein Thrombosis

	Normal range	Patient				
		1	2	3	4	5
PC/anticoagulant activity (% of normal)	66–147	51	56	34	53	35
PC/amidolytic activity (% of normal)	73–140	64	59	38	97	79
PC/antigen in plasma (% of normal)	68–145	69	54	51	151	100
Relative PC/antigen recovery	0.66–1.51	1.09	1.02	1.31	1.00	0.95
PC/amidolytic–antigen	0.72–1.46	0.93	0.09	0.75	0.64	0.79
PC/anticoagulant–amidolytic	0.65–1.54	0.80	0.95	0.89	0.55	0.44
Factor X (% of normal)		125	115	134	125	93

Plasma samples from 109 patients with recurrent deep-vein thrombosis and normal antithrombin III, plasminogen, and fibrinogen levels were examined. Factor X activity was determined to exclude oral anticoagulation. With the exception of patient 4, for whom only one sample was available, all the values represent the mean of triplicate measurements performed on two different plasma samples. Ranges of the values obtained in controls were calculated as for Table I. Abbreviation: PC, protein C.

protein C levels as assessed by both functional and antigenic measurements and of a relative recovery of protein C antigen not significantly different from that observed in normal controls.

Finally, the functional test has proven useful to recognize a new type of congenital deficiency of protein C. Besides patients with reduced protein C antigen in plasma, patients with normal antigen but reduced amidolytic levels of protein C have been reported in the literature (31, 32, 34, 52). Upon examination of plasma samples from 109 patients suffering from recurrent idiopathic deep-vein thrombosis, we have found five unrelated individuals presenting different abnormalities of protein C. Three subjects had comparably reduced levels of protein C functional activity and antigen, indicating impaired synthesis of a normal molecule. Patient 4 had a reduced ratio of amidolytic to antigenic levels, but, in addition, a definitely lower anticoagulant than amidolytic activity. Patient 5 had an isolated reduction of protein C anticoagulant activity, with normal amidolytic and antigenic levels. Further studies are needed to characterize the abnormality more fully, which can be hypothesized as an impaired interaction of activated protein C with either protein S, phospholipids, or the physiologic substrates.

Acknowledgments

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