Determination of Functional Levels of Protein C, an Antithrombotic Protein, Using Thrombin–Thrombomodulin Complex

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Protein C is a vitamin K-dependent plasma protein. Activated protein C (protein C₆) is a potent anticoagulant functioning at the levels of factors V and VIII in the clotting cascade. Protein C₆ also promotes blood clot lysis, at least in part, by raising the levels of circulating plasminogen activator. Protein C can be activated relatively slowly by thrombin. However, an endothelial-cell-associated cofactor (thrombomodulin) has been described that enhances the rate of protein C activation by thrombin. When bound to thrombomodulin, thrombin no longer clots fibrinogen. The addition of a 1:1 molar complex of thrombin and thrombomodulin to protein C₆ in plasma results in the rapid, quantitative conversion of protein C to protein C₆ and is the basis of the functional protein C assay described here.

MATERIALS AND METHODS

Reagents

Bovine thrombin was isolated following activation of the purified prothrombin with purified factor Xa, factor Va, phospholipid, and calcium. Bovine antithrombin-III was isolated as described. Rabbit lung thrombomodulin was isolated as described. PR-6000 centrifuge (Damon/IEC, Needham Heights, MA) for 25 min. The precipitate was resuspended in 0.9% NaCl containing 0.2 M sodium citrate, 1 mM benzamidine, and 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) to a final volume of 2.6 liters. At 4°C, 288 ml 1 M BaCl₂ was added dropwise over a 2-hr period while stirring the plasma at 4°C. The barium citrate was then centrifuged at 3,600 g at 4°C in a PR-6000 centrifuge (Damon/IEC, Needham Heights, MA) for 25 min. The precipitate was resuspended in 0.9% NaCl containing 0.2 M sodium citrate, 1 mM benzamidine, and 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) to a final volume of 5 liters. At 4°C, finely ground ammonium sulfate was added to a 30% saturation. Following centrifugation, the precipitate was discarded. Ammonium sulfate was then added to 60% saturation. The precipitate was collected by centrifugation and resuspended in 150 ml 5 mM 2[N-morpholino] ethanesulfonic acid (MES) buffer, pH 6.0, containing 180 M NaCl and 1 M benzamidine. The sample was dialyzed 18 hr against two changes of the same buffer. The sample was then applied to a QAE Sephadex column (2.5 x 100 cm) (Sigma Chemical Co.), equilibrated with the dialysis buffer. The column was eluted with a linear NaCl gradient (from 180 M to 500 M NaCl of 2,000 ml total volume. Protein C in the eluate was assayed as previously described and fractions containing protein C pooled and dialyzed in 5 mM imidazole buffer, pH 6.0, containing 10 mM CaCl₂ and 1 mM benzamidine. After dialysis, the sample was applied to a 0.9 x 100 cm heparin-agarose column. The column was eluted with a linear NaCl梯度 of 2,000 ml total volume.

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gradient (0–500 mM) of 300 ml total volume in 5 mM imidazole buffer, pH 6.0, containing 10 mM CaCl₂ and 1 mM benzamidine. The protein C was eluted from the column and was stored at 4°C until used. Protein purity was judged by gel electrophoretic analysis (Fig. 1). Protein C fractions used were essentially free of contaminating protein. Minor contaminants in the protein C preparation may correspond to β-protein C as described by Kisel. Protein C samples were dialyzed against 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl immediately prior to use. Protein C was activated as previously described. Protein C concentration was calculated assuming E₁₀₀₀ = 14.5. The number of active sites/mole of activated protein C was determined with the active site titrant, p-nitrophenyl p'-guanidinobenzoate, as described by Chase and Shaw. The titration was performed in 0.1 M barbital buffer, pH 8.3, and nitrophenyl release was monitored at 405 nm in a Beckman DU8 spectrophotometer. The active-site-dependent hydrolysis of the titrant was determined by extrapolation to correct for spontaneous hydrolysis and deacylation of the enzyme. A molar extinction coefficient of 16,000 was used for p-nitrophenol. The activated protein C had 1.0 ± 0.05 mole active site/mole enzyme by this method.

Preparation of Immunoabsorption Beads

A goat was injected with 1 mg human protein C in 1 ml Freund’s complete adjuvant subcutaneously on 3 occasions 1 wk apart. At the fourth week, 1 mg human protein C was injected in Freund’s incomplete adjuvant, one-half the dose being given subcutaneously and one half in the muscle of the rump. The animal was bled intravenously at weekly intervals thereafter for 5 wk. Goat IgG directed against human protein C was prepared. IgG was coupled to Bio-Gel agarose A-15 m, 50–100 mesh (Bio-Rad Laboratories, Richmond, VA) following cyanogen bromide activation. Approximately 5 mg IgG was bound per 1 ml agarose. The agarose was washed extensively with 4 M NaCl. To eliminate background amidolytic activity, immediately prior to use, the IgG-agarose was suspended in 20 mM Tris buffer, pH 7.4, and diisopropylfluorophosphosphate added to a final concentration of 1 mM, and the mixture was incubated for 30 min at 25°C. The IgG-agarose was then washed extensively with 150 mM NaCl in 20 mM Tris buffer, pH 7.4. For use in the assay, the beads were suspended in buffer at a ratio of 1 part beads to 2 parts buffer by vortexing immediately prior to addition to the assay reaction mixture. Immunoabsorption of normal plasma using equal volumes of IgG-agarose and plasma did not change the factor X or IX levels measured using one-step clotting assays, nor did such treatment alter the prothrombin time or partial thromboplastin time.

Immunoelectrophoretic Analysis

Protein C levels were measured immunologically using the Laurell rocket technique. Goat anti-human protein C IgG was used at 0.5% in 1% agarose gel plates. Human protein C supplemented barium-citrate-adsorbed human plasma was used to construct standard curves. The adsorbed plasma was prepared by adding 1 M BaCl₂ slowly to citrated plasma (80 ml BaCl₂/liter plasma) at 4°C. The precipitate was removed by centrifugation at 20,000 g for 10 min. The adsorbed plasma was dialyzed against 150 mM NaCl in 20 mM Tris buffer, pH 7.4, and frozen at –80°C prior to use.

Conditions of the Functional Protein C Assay

This functional assay is based on the activation of protein C in recalcified plasma by the thrombin-thrombomodulin complex. Direct quantitation of the activated protein C formed using a chromogenic substrate is not possible due to high background activity in the plasma. However, protein C, bound to goat IgG anti-human protein C retains the ability to cleave small synthetic substrates. Therefore, the protein C was recovered from the plasma using IgG-agarose prior to quantitation (Fig. 2).

All determinations were made using plasma made from blood collected in sodium citrate (9 parts blood to 1 part 3.8% sodium citrate, pH 5.5). Samples were collected and processed in plastic; blood was chilled to 4°C in melting ice immediately upon collection,
FUNCTIONAL LEVELS OF PROTEIN C

and centrifuged at 4°C. Plasma was assayed immediately or stored at -80°C before assay.

The activation of protein C in plasma was carried out at 25°C in the following manner. To 25 μl of citrated plasma in a 1.5-ml disposable micro test tube (Bio-Rad Laboratories, Richmond, VA) was added 75 μl of 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 8.3 mM CaCl2, 0.12 μg bovine thrombin (0.29 U), and 0.24 μg thrombomodulin. A control sample of plasma to which only buffer and CaCl2 were added was run simultaneously. The solution was mixed immediately and incubated at 25°C for 2 min. The level of thrombin–thrombomodulin used was selected to give rapid activation of the protein C in the dilute plasma (Fig. 3). The 2-min activation period was selected to give optimal protein C activation without formation of a fibrin clot in the recalcified plasma (Fig. 4).

Following the 2-min activation period, residual thrombin was inhibited by the addition of 50 μl of 800 μg/ml bovine antithrombin III, with immediate mixing. One minute later, 100 μl of goat IgG anti-human protein C-agarose beads were added to the mixture. The sample was then mixed, using a vortex mixture, for 2 sec at 1-min intervals for 6 min. The IgG-agarose was then washed by adding 1.2 ml of 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl. The contents of the tube were mixed for 1 min by repeated inversion of the tube and the precipitate by centrifugation in a Beckman Microfuge Model B (Beckman Instruments, Irvine, CA) for 10 sec. The supernatant was removed by aspiration, and the entire washing repeated 2 times.

After removal of the supernatant from the final wash, the protein C bound to the agarose beads was assayed with the chromogenic substrate S-2238 (Kabi Diagnostica, Sweden). The assay mixture contained 600 μl 0.2 mM S-2238 buffered with 20 mM Tris, pH 7.4, containing 150 mM NaCl and 0.1% Lubrol PX (Sigma Chemical Co.). The micro test tube was then mixed on a Univixer (model 1305, Labline Biomedical Products, Inc., Melrose Park, IL) for 1 hr in a 37°C constant temperature room. At the end of the incubation period, the reaction was stopped with the addition of 100 μl M benzamidine HCl, followed by mixing. The immunoadsorption beads were removed by centrifugation in a microfuge, and the optical density of the supernatant was measured at 405 nm using a Beckman Model DU-5 spectrophotometer (Beckman Instruments, Irvine, CA). Specific chromogenic activity was 0.185A280/min/μg. Adsorption of the protein C on IgG agarose resulted in a 30% loss in specific chromogenic activity. The optical density of the sample blank (without thrombin–thrombomodulin in the reaction mix) was subtracted from that of the test tube and, in normal plasma, was approximately 25% of that seen in the sample tested with thrombin-thrombomodulin.

Barium-adsorbed human plasma supplemented with human protein C was used to construct standard curves (Fig. 5). A linear relationship between amounts of protein C added and the amount of synthetic substrate cleaved up to 10 μg protein C/ml plasma was observed.

Reproducibility of the Assay

Within-day and between-day variation of the assay was determined with 10 replicate determinations of a pooled normal plasma. The within-day value obtained was 5.3 ± 0.2 μg/ml (mean ± SD), and the between-day value was 5.3 ± 0.3 μg/ml.

Patient Populations

The study was approved by the University of Oklahoma Institutional Review Board, i.e., Human Experimentation Committee, and was in accordance with an assurance filed with and approved by the
Department of Health, Education, and Welfare. All patients gave informed consent. Patients receiving warfarin anticoagulation had been stabilized on a dose of warfarin at least 1 wk prior to testing. Normal volunteers ranged from 22 yr to 61 yr of age; the mean age was 35 yr. Patients with cirrhosis of the liver had a biopsy-proven diagnosis. Patients with intravascular clotting had platelet counts below 80,000, prothrombin times and partial thromboplastin times 5 sec or more above the normal ranges and fibrinogen levels of 120 mg/dl or less. Patients with disseminated intravascular coagulation (DIC) all had fibrin split products over 40 μg/ml.

Description of Patient

The functionally deficient patient was a 25-yr-old man with a history of severe recurrent venous thrombosis. At age 14, the patient had venographically documented deep femoral vein thrombosis with pulmonary emboli, documented by lung scan. The patient experienced right calf thrombosis at age 19 yr. There was no family history of thrombosis. Plasma samples were drawn from the patient while he was not receiving warfarin or heparin. The prothrombin time, partial thromboplastin time, partial thromboplastin, thrombin time, factor V and VIII levels, antithrombin-III level, and fibrin split products were all within normal limits.

RESULTS

Functional protein C levels were determined in 20 normal individuals (Fig. 6). The mean value was 4.8 ± 1.0 μg/ml (mean ± 2 SD). This is in agreement with the levels of protein C detected immunologically by Griffin et al.⁴ and Stenflo et al.¹⁹ The functional protein C levels in the 20 normal individuals agreed well with those determined in the same individuals by Laurell rocket immunoelectrophoresis (correlation coefficient r = 0.89, p < 0.01) (Figs. 7 and 8). Patients with cirrhosis of the liver had functional protein C levels ranging from 1.2 to 4.4 μg/ml, with a mean value of 2.5 μg/ml. Patients receiving warfarin anticoagulation had functional protein C levels between 2.0 μg/ml and 2.5 μg/ml, with a mean value of 2.6 μg/ml. In the cirrhotic patients and those on warfarin anticoagulation, there was a correlation between the functional protein levels and those measured by rocket electrophoresis (r = 0.80, p < 0.05). Four patients with intravascular coagulation were found to have functional protein C levels of less than 3.0 μg/ml. This is in agreement with the findings of Griffin et al., who observed decreased levels of immunologically detectable protein C in hospitalized patients with intravascular coagulation.

A patient with severe recurrent deep venous thrombosis with decreased functional protein C levels was identified using this functional protein C assay. The patient was examined at a time when he was not receiving heparin or warfarin. His clotting parameters were normal (see Materials and Methods). The patient's functional level of protein C was 1.7 μg/ml on one occasion and 1.9 μg/ml on a second occasion. The immunologic level was 4.8 μg/ml on both occasions. The ratios of functional activity to immunologically detected protein were 0.35 and 0.40. These ratios are both less than the normal functional activity/immunologic level ratios, ranging from 1.10 to 0.90, found in 20 normal individuals.

Since a number of patients with thrombosis receive heparin anticoagulation, the effect of heparin on the assay was examined (Fig. 9). At heparin levels above...
0.2 U/ml, there was a marked decrease in measured protein C activity. Attempts at neutralization of heparin by the addition of protamine sulfate were not successful; at concentrations of protamine sulfate above 5 μg/ml, the protein C measured was significantly reduced. The use of protamine sulfate bound to agarose (5 mg/ml agarose) to preadsorb heparin in plasma also proved unsuccessful; no protein C activity could be measured after preincubation with the protamine sulfate-agarose. Treatment of plasma with ECTEOLA-cellulose and commercially prepared triethylaminoethyl cellulose tablets also resulted in a total loss of detectable protein C by either the functional or immunologic assay.

The facility with which activated protein C could be recovered and assayed from plasma on the antibody-agarose suggested that the immunoabsorption method might prove useful in monitoring the rate of activated protein C inhibition in plasma. Activated protein C was added to plasma of normal individuals (Fig. 10) and the disappearance of activated protein C followed over 40 min by binding the residual activated protein C to goat IgG anti-protein C-agarose and quantitating the bound activated protein C using the chromogenic substrate S-2238. In each normal plasma, the activated protein C was progressively inhibited over the 40-min incubation period. The inhibition profiles obtained were similar to those of Marlar and Griffin, who assayed the residual activated protein C in dilute plasma using the same substrate. However, the half-disappearance time observed by Marlar and Griffin in normal individuals ranged from 5 to 18 min, whereas the inhibition of activated protein C, as measured by the immunoabsorption technique, showed half-disappearance times of 13–24 min (range of 10 normal individuals). The shorter half-disappearance time of 5–18 min was obtained by Marlar and Griffin in the presence of 35 U heparin/ml. Although antithrombin-III and heparin have little or no effect on the activity of activated protein C in a purified system, we wished to determine if heparin would enhance the inhibition of activated protein C in plasma as measured by the immunoabsorption technique. The results are shown in Fig. 11. The addition of 2U heparin/ml plasma resulted in a 2–3-fold increase in the rate of activated protein C inhibition over the first 20 min of the reaction. Increasing concentrations of heparin were tested in a similar manner (Fig. 12), and maximal enhancement of activated protein C inhibition was observed at concentrations of 7.5 U/ml and above.

**DISCUSSION**

The functional protein C assay we describe provides a rapid, reproducible method of measuring protein C in normal and patient plasma. The assay takes advantage of the alteration of thrombin substrate specificity brought about by the binding of thrombin to thrombomodulin. Thrombin bound to thrombomodulin clots
molecules in the plasma are being activated. If protein C molecules are present in these plasmas, which are incompletely carboxylated or totally lacking \( \gamma \)-carboxyglutamic acid residues, these forms are being activated. The activation of these warfarin forms of protein is compatible with the findings of Esmon et al.\textsuperscript{21} that modified protein C, which has had the \( \gamma \)-carboxyglutamic acid region removed by chymotrypsin cleavage, is converted to the activated form by the soluble thrombin–thrombomodulin complex with activation parameters identical to native protein C. Development of an assay to distinguish between native and modified forms of protein C will require modification of the method we have outlined.

The measurement of activated protein C inhibition by the immunoadsorption method has several advantages over the assay of the activated protein in dilute plasma previously described. First, at the time the residual protein C is measured, other plasma proteins and inhibitors have been removed by washing. For example, there is no need to add antikallikrein antibodies to the assay to reduce background activity. Secondly, heparin is not required in the assay. This is of importance since we, in agreement with Canfield and Kisiel,\textsuperscript{22} find that heparin significantly increases the rate of activated protein C inhibition in plasma. This effect of heparin on activated protein C inhibition may have implications concerning the use of heparin in disease states where protein C activation may be occurring, such as disseminated intravascular coagulation. These data indicate that heparin, like warfarin, may inhibit both procoagulant and anticoagulant pathways.

Immunoadsorption of activated protein C from plasma may provide a useful alternative method for the study of activated protein C inhibition by its plasma protease inhibitor(s). Available methods for the assay of activated protein C inhibition in plasma are complex and have led to two different conclusions about the role of the activated protein C inhibitor in human disease. Recently, Marlar and Griffin\textsuperscript{20} suggested that a deficiency of the activated protein C inhibitor may be responsible for factor V/VIII deficiency, and these observations were confirmed by Giddings et al.\textsuperscript{23} Using somewhat different assay conditions, Canfield and Kisiel\textsuperscript{13} observed normal levels of activated protein C inhibitory activity in the factor-V/VIII-deficient plasmas they studied. The use of the immunoadsorption assay described here may prove helpful in resolving this apparent discrepancy.

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