Level of Protein C Determined by Combined Assays During Disseminated Intravascular Coagulation and Oral Anticoagulation

By Jun Mimuro, Yoichi Sakata, Kenji Wakabayashi, and Michio Matsuda

We have developed a variation of the solid-phase enzyme-linked immunosorbent assay (ELISA) to enable measurement of the activity and antigen levels of protein C (PC) in human plasma. With this assay it is possible to do both tests with the same sample and same microtiter plate coated with anti-PC monoclonal antibody (MCA)JTTC-4, which inhibited neither activation of PC nor activity of activated PC (APC). Even in patients undergoing heparin treatment for severe disseminated intravascular coagulation, there were no detectable differences between amidolytic activity and antigen levels of PC in patients’ plasma. In addition, there was a strong correlation between the immunologic levels of PC in patients’ plasma determined both by polyclonal ELISA using peroxidase-labeled immunopurified antiprotein C-IgG and those found with MCA ELISA using peroxidase-labeled MCAJTC-5, which does not bind to APC. In contrast, when oral anticoagulation therapy was started, immunologic levels of plasma PC estimated by peroxidase-labeled MCAJTC-1, a MCA that recognizes a γ-carboxyglutamic acid domain-related conformational change of PC induced by metal ions, decreased more rapidly than did either the PC level determined by polyclonal ELISA or the percent prothrombin time. This suggested that comparison of MCAJTC-1-recognized PC levels and prothrombin time may be necessary at the beginning of oral anticoagulation therapy to treat patients safely.

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Protein C (PC) is a vitamin K-dependent proenzyme of a serine protease that exerts an anticoagulant activity through selective inactivation of factor Va and VIIa. In addition, activated PC (APC) induces a fibrinolytic state when infused into dogs and decreases plasminogen activator inhibitor activity produced by cultured bovine aortic endothelial cells when cells are treated with APC.

Clinical evidence for PC’s role as a major antithrombotic factor arose from the observation that homozygous PC deficiency led to fatal neonatal purpura fulminans. Although many assays have been developed to measure PC activity and antigen levels, they all quantitate these two parameters separately.

Since this could be one reason that previous reports showed differences between PC activity and antigen levels in patients with disseminated intravascular coagulation (DIC), we developed a variation of ELISA to measure the levels of both PC activity and PC antigen in the same sample using one polystyrene plate coated with anti-PC MCA. By using anti-PC MCA specific to the Ca²⁺-induced PC conformation, the effects of warfarin treatment on the γ-carboxyglutamic acid (Gla) domain of PC were explored.

MATERIALS AND METHODS

Reagents. The following were purchased: essentially fatty acid-free bovine albumin; heparin sodium salt from porcine intestinal mucosa (170 μg/mg); horseradish peroxidase (type IV); Tween 20 (Sigma, St. Louis); solid-state lactoperoxidase-glucose oxidase; horseradish peroxidase-conjugated goat antimouse immunoglobulin (Bio-Rad, Richmond, CA); Sephadex G-25, Sephadex G-200, CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden); (DEAE diethyl aminoethyl)-cellulose (Whatman, Maidstone, Kent, England); 2,2′-azino-di(3-ethyl-benzthiazoline sulfonate); ABTS; (Kirkegaard & Perry, Gaithersburg, MD); H-D-Glu-Pro-Arg-p-nitroanilide (S2366) was a kind gift from Daichi Chemical (Tokyo). Thrombin was purified by the method of Lundblad et al. Human protein C was purified from citrated fresh human plasma as described by Suzuki et al. The synthetic peptide Asp-Pro-Glu-Asp-Gln-Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Cys was kindly given to us by Dr J. Stenflo, Malmo, Sweden. Thrombomodulin was purified from rabbit lung extract according to Esmon et al. Human antithrombin III was isolated as described previously. The extinction coefficients and mol wt used for calculating protein concentration were protein C, E₂₈₀nm = 14.5, 62,000; thrombin, E₂₈₆nm = 18.3, 37,000; mouse immunoglobulin, E₂₈₀nm = 15.5, 150,000. Monoclonal antibodies against the plasminogen binding domain of human protein C were prepared as described. Monoclonal antibodies (MoAbs) were produced according to the standard method as described previously using BALB/c mice and the mouse myeloma cell line P3-X63-Ag8-U1 (P3U1).

Serum and ascites immunoglobulin fractions used in this study were purified by DEAE-cellulose and protein C-sepharose affinity chromatography. Enzyme-coupled antibodies were prepared by coupling horseradish peroxidase using meta-periodate according to the method of Nakane and Kawai. Conjugated enzyme was separated from unconjugated immunoglobulin by gel filtration on a Sephadex G-200 column and was then stored at −20°C until use.

Immunoblotting technique. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels was carried out using a resolving gel of 10% acrylamide and a stacking gel of 4% according to the method of Laemmli. After fractionation by SDS-PAGE, the proteins in the gel were electroblotted (50 V, 1.5 hours) to nitrocellulose (Bio-Rad) using an electrophoretic apparatus (Matsuyoshi Industry, Tokyo) with a cooling system and were subjected to immunoblotting analysis as described previously.

Activation of protein C. PC (720 μg/mL) was activated with thrombin (13 μg/mL) and thrombomodulin (7 μg/mL) in 0.05 mol/L Tris-HCl, pH 7.4, 0.14 mol/L NaCl (TBS) containing 2.5 mmol/L Ca²⁺ at 25°C as described previously. APC was separated from thrombin, thrombomodulin, and residual PC by MAJTC-1 and MAJTC-5 immobilized on sepharose as described in Results.

From the Institute of Hematology, Jichi Medical School, Tochigi-Ken, Japan.

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Address reprint requests to Yoichi Sakata, Institute of Hematology, Jichi Medical School, Tochigi-Ken 329-04, Japan.
Radioiodination. PC and MoAbs were radioiodinated using lactoperoxidase-glucose oxidase and Na125I (19 Ci/mg) (New England Nuclear, Boston). The labeled PC and MCAJTC-1, MCAJTC-4, and polyclonal antibodies had specific activities of 9 × 104, 6 × 104, 6.6 × 104, 7.9 × 105 cpm/μg respectively.

Competition for MCAJTC-5 between protein C and synthetic activation peptide of protein C. Polyvinyl chloride microtiter plates were coated with MCAJTC-5 at 10 μg/mL in 0.05 mol/L carbonate buffer, pH 9.6, for 16 hours at 4°C. The plates were then washed seven times with TBS containing 1% bovine serum albumin. Complete coating of the well surface was accomplished by incubation with the same buffer for one hour at 25°C. After washing three times with TBS containing 0.05% Tween 20 (TBS-Tween), 125I-labeled PC was added to the well and the saturating level of PC was determined. Various concentrations of activation peptide and a constant saturating level of PC were added simultaneously to plates coated with MCAJTC-5 and were incubated for three hours at 37°C. After washing five times with TBS containing 0.05% Tween 20, the wells were removed and the radioactivity in each well was determined.

Binding studies. The dissociation constants (Kd) of 125I-labeled MCAJTC-4 for human PC and APC were assessed by a solid-phase assay, previously described by Frankel and Gerhard. Polyvinyl chloride microtiter plates (96 wells, Titer Tek; Flow Laboratories, McLean, VA) were coated with PC or APC dissolved at a concentration of 10 μg/mL in TBS for 16 hours at 4°C. After being washed five times with TBS containing 1% bovine serum albumin and 0.05% Tween 20, the plates were incubated with TBS containing 1% bovine serum albumin for two hours at 37°C. Various concentrations of 125I-labeled antibody, dissolved in TBS, were added to the coated wells and allowed to incubate for three hours at 37°C. The solution was removed by aspiration, and the wells were washed three times with TBS containing 1% bovine serum albumin and 0.05% Tween 20. After the final wash the wells were individually removed and subjected to γ counting. For each antibody concentration the results of four replicate wells were averaged. Subtracted from these counts were those from controls in which the same concentration of 125I-labeled antibody was incubated in wells fully coated with albumin. The level of the radioactivity found in each well was converted to bound antibody concentration, and the dissociation constants were calculated as previously described.17

Amidolytic assay. Polyvinyl chloride microtiter plates were coated with MCAJTC-4 (10 μg/mL) in the same manner as in the competition study. After washing five times with TBS-Tween, purified PC, normal plasma, or patients' plasma in washing buffer (100 μL/well) were added to the coated wells and incubated for four hours at 37°C. The wells were then washed five times with TBS-Tween. A mixture of α-thrombin (4 ng/mL) and different concentrations of thrombomodulin, dissolved in TBS-Tween containing 1% bovine serum albumin and 5 mmol/L calcium chloride, was added to each well and incubated for various times at 37°C. After washing three times with TBS-Tween, antithrombin III (250 μg/mL), dissolved in TBS containing 1 U/mL heparin, was added to each well and incubated for 30 minutes at 25°C. H-D-Glu-Pro-Arg-Nitroanilide (S2366) was then placed in each well. Hydrolysis was performed at 25°C, and the absorbance at 405 nm was monitored on an ELISA analyzer ETY-96 (Toyo Sokki, Tokyo).

Solid-phase ELISA. Levels of PC antigen binding to MCAJTC-4-coated wells were determined before and after activation with thrombin-thrombomodulin complex. Before activation with thrombin-thrombomodulin, peroxidase-conjugated PCA, MCAJTC-5, or JTC-3 in TBS-Tween was added to each well in the absence or presence of 5 mmol/L CaCl2 and were then incubated at 4°C for 16 hours. At the end of this period, after washing three times with TBS-Tween with or without CaCl2, 200 μL of freshly mixed ABTS was placed in each well according to the manufacturer's directions. Hydrolysis was monitored at 25°C on an ELISA analyzer at 405 nm. For sequential determination of PC amidolytic activity and antigen levels bound in the same well, each well was washed three times with TBS-Tween after the APC amidolytic activity had been measured, and then horseradish peroxidase-conjugated antibody was added. Standard curves for each assay were constructed by using purified human PC dissolved in TBS containing 1% bovine serum albumin or either PC-deficient plasma or PC-immunodepleted plasma supplemented with purified human PC.

Collection of plasma samples. Blood was collected from anticoagulated veins into 0.1 vol of 3.8% sodium citrate and was centrifuged at 2,000 g for 20 minutes to prepare platelet-poor plasma (PPP). Pooled normal PPP was obtained from 20 healthy donors. All plasma was stored at −80°C until use. Single time-point plasma samples were taken from patients with DIC, which had been confirmed by marked reductions in platelet counts, fibrinogen, antithrombin III (except in acute promyelocytic leukemia), plasminogen, and α2-plasmin inhibitor levels, and prolonged partial thromboplastin and prothrombin times. All patients had elevated levels of fibrin degradation products and of fibrinopeptide A release, as measured by radioimmunoassay (RIA) described by Nosse1 et al.21 Patients with pre-existing liver disease or laboratory evidence of liver disease were eliminated from the DIC group in this study. Patients with laboratory evidence of DIC in association with acute leukemias (five acute promyelocytic, ten myeloid, and two lymphoid) and 16 other clinical conditions (septic, carcinomatous, or obstetric) were investigated. In the sequential study, four serial samples were taken over a period of time from five patients, including one associated with acute promyelocytic leukemia. Patients with severe chronic liver disease, except for hepatoma, had laboratory data consistent with liver failure (albumin <2 g/100 mL, cholinesterase <0.2 pH/hour, elevated transaminases, and prolonged prothrombin time). Plasma samples from previously described congenital PC-deficient patients17,22 and their families with no thrombosis for a period of 6 months were analyzed. Patients starting warfarin therapy due to deep-vein thrombosis or myocardial infarction were treated without a loading dose and received the same dose of warfarin for several days prior to testing.

RESULTS

The antigenic target for MCAJTC-4 and MCAJTC-5, two antibodies that have no effect on APC amidolytic activity, is the PC heavy chain, as has been described.17 As shown in Fig 1, MCAJTC-4 bound to both PC and APC, but MCAJTC-5 did not bind to APC. An experiment was designed to test whether 125I-labeled APC could bind to microtiter plates coated with MCAJTC-5. Prior to our binding experiments, the PC subsaturating level was determined using 125I-labeled PC. In the binding experiment, 125I-labeled PC was activated at 25°C with thrombin and thrombomodulin as described.17 After incubation for various time periods (Fig 2), samples were removed and mixed with antithrombin III to terminate the activation. One portion of each sample was used to determine APC activity against S2366, while the remainder was added to individual wells of a plate coated with MCAJTC-5 to observe binding of the radioactivity. Figure 2 shows that as APC amidolytic activity increased in each sample, the binding of radioactivity to the plate gradually decreased.

During the activation of human PC by thrombin-thrombomodulin complex, an Arg17-Leu19 bond is cleaved at the
Amino-terminal end of the protein's heavy chain, releasing a small dodecapeptide of mol wt 1,400.2 To determine whether the MCAJTC-5 had an epitope in the activation peptide region, a competitive binding assay was carried out using ¹²⁵I-labeled PC and a synthetic dodecapeptide.

The binding of ¹²⁵I-labeled PC to MCAJTC-5 was diminished by the presence of the synthetic dodecapeptide in a dose-dependent manner (Fig 3). However, a concentration of dodecapeptide higher than that of PC was necessary to prevent the binding of MCAJTC-5 to PC due to the lower affinity of MCAJTC-5 to dodecapeptide (Kd = 9.6 × 10⁻⁸ mol/L⁻¹).

Moreover, MCAJTC-5 inhibited the activation of PC both by thrombin and thrombin-thrombomodulin (data not shown). These results indicate that MCAJTC-5 recognizes the activation peptide region of PC. A similar MoAb against the PC activation peptide region was previously reported by Laurell et al.

The dissociation constants for ¹²⁵I-labeled MCAJTC-4 and MCAJTC-5 to human PC and APC were determined by the assay described in the Materials and Methods section. PC was activated by thrombin-thrombomodulin complex in the presence of Ca²⁺. After activation, the complex was removed by the application of the activation mixture to MCAJTC-1 immobilized on a column. PC and APC bound to this in the presence of Ca²⁺ and were eluted in its absence. Trace amounts of residual PC were removed from the eluate using MCAJTC-5 immobilized on a column. Pure APC was obtained in the breakthrough. Prior to the binding experiments were confirmed by a competition assay that the affinities of radiolabeled MCAJTC-4 and MCAJTC-5 for PC and APC were the same as those of the native nonlabeled MoAbs. The data were treated by a modified Scatchard plot, as described by Frankel and Gerhard. Each Sips heterogeneity index, α, for JTC-4 and JTC-5 toward PC and APC, when calculated from the data approximated 1.0. The dissociation constants for JTC-4 with PC (Kd = 6.80 × 10⁻⁹ M) and APC (Kd = 8.70 × 10⁻⁹ mol/L) were very close (Table 100).
COMBINED ASSAYS OF PROTEIN C

Table 1. Dissociation Constants for Anti-PC Antibodies to PC and APC

<table>
<thead>
<tr>
<th>IgG</th>
<th>Kd (M)</th>
<th>Kd (M)</th>
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<tr>
<td>JTC-4</td>
<td>6.86 x 10^-9</td>
<td>8.70 x 10^-9</td>
</tr>
<tr>
<td>JTC-5</td>
<td>5.10 x 10^-9</td>
<td>ND*</td>
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For determination of the dissociation constant (Kd), a variation of Scatchard analysis using 125I-labeled MoAb was performed as described in the Materials and Methods section.

* MCAJTC-5 did not bind to APC.

PC antigen levels were determined by ELISA in purified preparations and in PC-deficient plasma supplemented with PC using a combination of JTC-4 and horseradish peroxidase-coupled immunopurified anti-PC polyclonal antibody. A linear dose response curve of PC concentration against absorbance at 405 nm was obtained (data not shown). When a standard curve was constructed using plasma reconstituted by mixing normal and PC-immunodepleted plasmas, the results were also satisfactory (data now shown). Because the dissociation constants for MCAJTC-4 with PC and APC were very close, we explored the possibility of a combined assay system for PC antigen and PC function in plasma samples.

Figure 4 shows the activation of PC bound to JTC-4 coated wells with a mixture containing 4 ng/mL of thrombin and different amounts of thrombomodulin. Since more than 10 ng/mL of thrombomodulin did not increase further the activation rate of PC and maximum activation was obtained after one hour of incubation at 37°C in the presence of 5 mmol/L CaCl2, these conditions were chosen for routine activation. After determination of the level of APC activity, plates were washed and the APC antigen level was measured.

As shown in Fig 5, the observed levels of PC and APC antigens correlated well, and the between-assay coefficient of variation was 2.5%. Furthermore, good correlation was observed between PC levels determined by this assay and by the Laurell rocket technique25 (data not shown). To determine whether this assay could detect both plasma PC activity and antigen levels, PC-deficient plasma supplemented with human PC was used to construct standard curves (Fig 6). A linear relationship between concentrations of PC added and of synthetic substrate cleaved was observed.

Since APC in plasma may exist as a complex with its specific inhibitor,26,27 the effect of plasma PC inhibitor (PCI) on the binding of APC to MCAJTC-4 was tested. To remove the effect of PC in normal plasma on the binding of APC to the plate, citrated and heparinized PC-deficient plasma were used, since these plasma inhibited APC activity in the same way as normal plasma, as previously reported by Comp et al.7 These plasma samples were preincubated for 60 minutes at 37°C with various concentrations of APC and were then added to wells coated with a constant amount of MCAJTC-4. The concentrations of APC and APC-PCI complex bound to MCAJTC-4 were determined by polyclonal ELISA (a combination of MCAJTC-4 and peroxidase-conjugated polyclonal anti-PC).

1), but 125I-labeled MCAJTC-5 showed no binding to solid-phase APC.

Fig 4. Activation of PC bound to MCAJTC-4 coated wells by thrombin (4 ng/mL) and O (O), 2 (■), 5 (▲), and 10 (▲) ng thrombomodulin/mL in the presence of 5 mmol/L CaCl2. APC was measured by hydrolysis of S2366.

Fig 5. Correlation between PC antigen levels determined by ELISA before and after activation. Experimental conditions were as described in the Materials and Methods section. Vertical bars represent SEM of nine measurements.

Fig 6. Effect of PC concentration on amidolytic activity. Purified human PC dissolved in TBS containing 1% bovine serum albumin (●) or PC-deficient plasma supplemented with purified PC (□) was added to wells of plates coated with MCAJTC-4. After washing, PC bound to the wells was activated by thrombin and thrombomodulin. Amidolytic activity of APC bound to the wells was measured as described in the Materials and Methods section.
Figure 8 illustrates the relationship of amidolytic activity to PC antigen levels after activation in controls and in the patient series using MCAJTC-4 and peroxidase-labeled polyclonal anti-PC IgG. The between-assay coefficient of variation was less than 2.3% when purified PC or PC-deficient plasma supplemented with purified PC was used.

There was a good correlation between the PC antigen levels and amidolytic activities in normal subjects, in patients with liver cirrhosis, and in warfarin-treated patients. In those with DIC, the correlation was also satisfactory, although PC levels were decreased (40 ± 21%, n = 27), except when DIC was associated with acute promyelocytic leukemia (90 ± 15%, n = 5). We also analyzed four serial samples from five cases of DIC with or without liver damage. Although PC activity and antigen levels varied according to the course of the DIC, there were no detectable discrepancies between the PC activity and antigen levels in each patient’s plasma (data included in Fig 8). Since heparinized normal plasma and PC-deficient plasma did not support the binding of 125I APC to MCAJTC-5 coated wells (data not shown), it was supposed that MCAJTC-5 recognized neither APC (Figs 2 and 3) nor APC-PCI complex.

To confirm that there was almost no discrepancy between PC activity and antigen levels in DIC patients, the patients’ PC antigen levels were also measured by ELISA using a combination of MCAJTC-4 and conjugate-labeled MCAJTC-5 (JTC-5 ELISA) or polyclonal anti-PC antibody (polyclonal ELISA). The between-assay coefficient of variation was 1.8% when purified PC or PC-deficient plasma supplemented with purified PC were used. As shown in Fig 9, there were no discrepancies between polyclonal ELISA and JTC-5 ELISA-determined PC antigen levels in samples from 40 patients with DIC.

It was suggested that MCAJTC-1 recognized a Gla domain-related conformational change induced by metal ions, evidenced by the fact that half-maximal binding was observed at a calcium concentration of 0.5 mmol/L, by the fact that the antibody, even in the presence of Ca2+, did not react with Gla domainless PC, and by the fact that Zn2+ and Tb3+ supported binding in essentially the same way.17 Since MCAJTC-4 did not interfere with the binding of MCAJTC-1 to PC in the presence of Ca2+,17 the amount of PC recognized by MCAJTC-1 in disease states and during warfarin therapy was measured using MCAJTC-4-coated
wells and peroxidase-conjugated MCAJTC-1 and was calculated from a standard curve as percent binding. The between-assay coefficient of variation was 2.3% when purified PC or PC-deficient plasma supplemented with purified PC were used. The ratio of PC antigen determined by JTC-1 ELISA to that measured by polyclonal ELISA in the presence of CA2+ was not significantly different from unity in normal controls and in patients with liver cirrhosis and DIC (Fig 10). However, as we previously reported,17 this ratio was reduced in warfarin-treated patients. Although PC, recognized by MCAJTC-1, might not be necessarily Gla domain-intact PC, to obtain a more convenient assay to measure Gla domain-intact PC in warfarin-treated individuals, the levels of PC in sequential plasma samples taken over a period of time from five patients treated with warfarin without a loading dose were measured by polyclonal, JTC-1, and JTC-5 ELISAs.

In all cases the levels of PC determined by JTC-1 ELISA decreased more rapidly than when measured by polyclonal or JTC-5 ELISA. Moreover, PC antigen levels determined by JTC-1 ELISA were reduced more rapidly than percent of prothrombin time (Fig 11).

DISCUSSION

Previous studies suggested that levels of PC antigen were low in clinical conditions associated with DIC, and increased clearance of in vivo activated PC has been suggested as an explanation. However, Radeghiero et al29 did not find a significant decrease in PC in their patients with DIC associated with acute leukemia. Our results are similar to theirs only in DIC cases associated with acute promyelocytic leukemia. Such DIC cases may be exceptional because these patients had normal PC and antithrombin III activity and antigen levels, although they had high levels of fibrinopeptide A release and α2-plasmin complex (data not shown). Marlar et al31 measured serial PC levels during DIC progression and observed that the PC activity decreased in DIC patients in parallel with PC inhibitor (PCI) and was lower than PC antigen levels. To determine chromogenic activity “after activation” with thrombin-thrombomodulin, they removed APC from the plasma by polyclonal anti-PC IgG-Sepharose. However, the dose of heparin used to treat DIC patients is enough to enhance the neutralization of APC activity by plasma PCI37 during activation with thrombin-thrombomodulin in vitro. Furthermore, other components may have been generated during DIC that influenced PC activation. In addition, they separately measured PC antigen levels in the plasma before activation by a radiolabeled Laurel electroimmunoassay.

On the other hand, D’Angelo et al38 initially removed PC from heparinized plasma in the presence of Ca2+ by using a Ca2+-dependent MCA directed against human PC, which bound to human PC independently of the presence of Gla residues. They then determined the activity of the removed PC. They neglected plasma APC and the APC-PCI complex because their Ca2+-dependent MCA recognized neither of them.

However, they could find no discrepancies in the ratio of amidolytic activity in the removed PC to Laurell rocket-determined plasma PC antigen in both controls and DIC patients. In contrast to their separate assays, the combined assay system for PC antigen and activity levels described in this paper provides a suitable method for measuring the specific activity (ratio of amidolytic activity to antigen level) of PC in normal and patient plasma. Before activation we adsorbed PC from plasma to the plate coated with MCAJTC-4, which binds PC, APC, and APC-PCI almost equally. After activation of adsorbed PC, thrombin-thrombomodulin was neutralized and washed away. Then the amidolytic and antigen levels of PC just adsorbed on the

\[
y = 1.05x - 0.73 \\
g = 0.96 \text{ (n=41)}
\]

\[
y = 1.01x - 2.54 \\
g = 0.93 \text{ (n=49)}
\]

\[
y = 0.53x - 0.59 \\
g = 0.68 \text{ (n=13)}
\]

Fig 10. Correlation between polyclonal ELISA and JTC-1 ELISA. Plates were coated with MCAJTC-4. Plasma samples were added to the wells and incubated for three hours at 37°C. After washing, the amount of bound PC was measured in the presence of 5 mmol/L CaCl₂ using polyclonal ELISA and peroxidase-conjugated JTC-1 (JTC-1 ELISA). Symbols are the same as in Fig 8.

Fig 11. Changes in PC antigen level compared to changes of prothrombin time (○) in plasma of patients with deep vein thrombosis (dotted line) or myocardial infarction (solid line) starting warfarin therapy (10 mg/d) without loading dose. Plasma PC antigen level was measured by polyclonal ELISA (□), JTC-5 ELISA (△), or JTC-1 ELISA (○) in the presence of Ca2+.
plate were determined successively. Our results were basically the same as those reported by D’Angelo.

To confirm our results the levels of PC antigen in plasma were determined by polyclonal ELISA without activation (MCAJTC-4-polycyonal anti-PC) and were compared with those determined by JTC-5 ELISA (MCAJTC-4-MCAJTC-5), which was not able to measure APC or APC-PCI. PC levels correlated well in both methods (Fig. 7).

Given that the half-life of APC has previously been reported to average 10 to 15 minutes and that of PC activation peptide five minutes,22 APC concentrations generated in the systemic circulation of patients with DIC might be as high as 0.02% to 2% of the total circulating zymogen. Our results and previous observations strongly suggest that PC levels are low and APC levels are undetectable in the circulating plasma of individuals with DIC. This may be due to rapid clearance of APC from the plasma by the reticuloendothelial system or due to binding of APC to endothelial cells or other surfaces. However, we cannot deny that there may be detectable APC-PCI complex in the plasma of patients with liver disease-associated DIC due to a decrease in clearance. We also developed the PC assay to enable the assessment of levels of Glα domain-incomplete PC in plasma of patients. This could be achieved by comparison of PC levels determined by JTC-1 ELISA and polyclonal ELISA or JTC-5 ELISA.

Previous studies demonstrated that the Glα domain of PC was essential for optimal PC activation on the endothelial cells and was required for expression of membrane-dependent anticoagulant activity. Recently it has been proposed that the rapid decrease in PC levels after the start of warfarin therapy, relative to the decreases in concentration of most other vitamin K-dependent clotting factors, gives rise to a transient PC deficiency state, resulting in a transient hypercoagulable condition.21-24

The levels of PC determined by JTC-1 ELISA may not necessarily correlate with PC anticoagulant activity, since the number of Glα residues in PC required for expression of anticoagulant activity and membrane interaction is not clear. However, the decrease in PC levels measured by JTC-1 ELISA was more rapid than that of percent prothrombin time and of PC levels measured by JTC-5 or polyclonal ELISA (Fig 11). Although no statistical test was applied, these results suggest that comparison of the decrease in PC levels measured by JTC-1 ELISA with that of percent prothrombin time would be useful in recognizing a transient hypercoagulable state in patients treated by oral anticoagulant therapy. Following these observations we treated patients with minidoses of heparin at the beginning of oral anticoagulation therapy, even in cases of nonloading dose treatment. However, since heparin also enhances inhibition of APC by PCI, further studies are needed, especially to treat congenital or acquired PC-deficient patients with warfarin.

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Level of protein C determined by combined assays during disseminated intravascular coagulation and oral anticoagulation

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