Activation and Complexation of Protein C and Cleavage and Decrease of Protein S in Plasma of Patients With Intravascular Coagulation

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Activated protein C (APC) is inhibited by two major plasma inhibitors (PCIs). To find evidence for in vivo complexation of APC, immunoblotting studies were performed on plasmas of 85 patients with suspected disseminated intravascular coagulation (DIC). Samples from 62 of these patients contained 5% to 35% of protein C antigen in APC:inhibitor complexes, indicating that protein C activation and inhibition had occurred. In 24 normal plasmas, no detectable APC:PCI complexes were observed (<5%). Patients with higher levels of complexes had more abnormal coagulation test data for DIC. The major band of APC complexes detected by anti-protein C antibodies did not react with antibodies to the heparin-dependent protein C inhibitor (PCI-1) previously described. Rather, APC was complexed with another recently described plasma protein C inhibitor, PCI-2. Immunoblotting studies for protein S, the cofactor for APC, revealed that the majority of the DIC patient plasmas contained a higher than normal proportion of protein S in cleaved form, suggesting that protein S may have been proteolytically inactivated. Protein S total antigen levels were also found to be low in DIC patients, excluding those with malignancy. These studies support the hypothesis that the protein C pathway is activated during DIC.

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MATERIALS AND METHODS

Electrophoresis, immunoblotting and electroimmunoassay procedures, preparation of pooled normal human plasma (NHP) and individual normal plasmas, preparation of human protein C, protein S, PCI-1, and antibodies to each were previously described, except that the immunoblotting buffer was 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 1% casein (Sigma, St Louis), 0.02% NaN3, pH 7.4. Monoclonal antibody to protein C (C3) was used at 3 μg/mL, rabbit polyclonal antibody to PCI-1 was used at a dilution of 1:600, and goat polyclonal antibody to protein S was used at a dilution of 1:600. The monoclonal antibody (C3) is specific for the light chain of protein C, and it recognizes protein C, APC, and complexed APC with the same efficiency. Radiolabeled antigen (125I-protein C or 125I-protein S) in place of radiolabeled secondary antibody was used whenever possible in the detection step of the immunoblotting, in order to enhance specificity. Samples of rabbit antiserum to PCI-1 and mouse monoclonal antibody to PCI-1 were generous gifts from Dr Koji Suzie, Mie University School of Medicine, Tsu City, Japan, and Dr Johan Stenflo, University of Lund, Malmo, Sweden, respectively.

For immunoblotting studies of protein C antigen forms in plasma, 100 consecutively submitted plasma samples from 88 patients suspected by the attending physician of having DIC were collected in sodium citrate in the coagulation laboratory at University Hospital.

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University of Wisconsin, Madison. The plasmas were assayed for prothrombin time (normal range, 11.4 to 13.1 seconds), activated partial thromboplastin time (APTT) (normal range, 22.0 to 32.0 seconds), fibrin degradation products (FDP) levels (ThromboWellico test; Burroughs-Wellcome, Research Triangle Park, NC; normal range, <10 μg/mL), fibrinogen levels (normal range, 150 to 370 mg/dL), and platelets (normal range, 150 to 420 x 10^9/μL). These tests have been used as indicators for intravascular coagulation.27,28 Of the 88 patients, 11 were abnormal in all five of these tests, 22 were abnormal in four tests, 20 in three tests, 19 in two tests, and 13 in one test. Three were normal in five tests and were excluded from the study. The plasmas were frozen within four hours of blood collection at −80°C and later assayed for total protein C and protein S antigen by electroimmunoassay and for complexes APC antigen and cleaved protein S antigen by immunoblotting.27,31,39

RESULTS

Complexed protein C antigen in plasmas of patients with intravascular coagulation. To see if APC:PCI complexes that were formed in plasma in vitro27,31,32 might be formed in vivo, 97 plasmas from 85 patients with DIC were analyzed using immunoblotting for protein C antigen. Previous studies demonstrated that the monoclonal antibody used recognizes complexed APC, free APC, and protein C with the same efficiency, so that the distribution of radioactivity in the immunoblot reflects the distribution of forms of protein C.27,31,40 The immunoblot in Fig 1 shows representative results for 21 of the plasma samples and the data showed that the majority of the patient plasmas contained one band of complexed protein C antigen that was similar in mobility to one of two bands obtained on Protac C treatment of normal plasma (band b). Sixty-seven of these untreated plasmas from 62 patients contained detectable levels (>5%) of protein C antigen in complexed form. The majority of these 62 patients' plasma contained low levels (<10%) of protein C antigen in complexed form, but 18 plasma samples from 16 patients contained 20% to 35% of antigen in complexed form. There was a weak inverse correlation (r = −.46) between percent protein C antigen in complexed form and the level of free protein C antigen. The plasma sample designated P-47 in Fig 1 contained two forms of complexed antigen that comigrate with the two forms (Fig 1, lane 2, bands a and b) that appear after activation of protein C and inhibition of APC in plasma. These two bands a and b are the two forms that appear after incubation of APC with plasma.27,31,32 We recently demonstrated that in normal plasma incubated with APC, band a contains APC:PCI-1 complexes, while band b contains APC:PCI-2 complexes.27,31 In normal plasmas from ten women and 14 men, no detectable APC:PCI complexes (<5%) were observed, and in control studies, freezing and thawing the plasma four times had no effect on the visual immunoblot pattern of protein C antigen, compared with the patterns of NHP and NHP incubated with APC (data not shown). In addition to these normal plasmas (see Materials and Methods), normal citrated plasmas from five females and five males were collected and frozen within three to four hours at −80°C. These were subjected to four freezethaws and immunoblotted. In all cases <5% of the radioactivity for protein C antigen was found in the region of complexed protein C antigen. In four separate experiments with NHP an average of 3% of the radioactivity was found in the region of complexed protein C antigen, and after very long film exposure a faint band that migrated like band b was visible in this region.

Comparison of in vivo and in vitro formed APC complexes. To see whether protein C could be activated in the DIC patient plasmas and then form normal APC:PCI complexes, the patient samples marked P-22 and P-47 in Fig 1 were treated with Protac C, incubated for one hour at 37°C, and then compared with normal plasma results in immunoblots (Fig 2). In each case, the major protein C antigen band shifted in mobility after Protac C treatment, migrating slightly slower like free APC (left panel). This suggests that before Protac C treatment, the major band consisted primarily of protein C, not APC. In plasma from patient P-47, Protac C treatment resulted in a small increase in complexed APC antigen, primarily in band b (APC:PCI-2). For the plasma of patient P-22, there was a larger increase in complexed protein C antigen after Protac C treatment with some increase in band b (APC:PCI-2) but most of this increase in band a (APC:PCI-1). The APC complexes formed in vitro in P-47 and P-22 plasmas had the same apparent mobility as the complexes formed in vivo.

The same patient samples that were immunoblotted for protein C antigen in the left panel of Fig 2 were also immunoblotted for PCI-1 antigen in the right panel of Fig 2. In this type of immunoblot, free PCI-1 appears as a doublet of low mobility near the top of the gel, and complexes with APC migrate with the mobility of band b.27,31 The band of unknown identity that has a mobility similar to protein C or APC is not due to protein C antigen, since the antibody does not recognize purified protein C or APC, and this band is not apparent when blots are developed with 125I-PCI-1 rather than with 125I-secondary antibody.27,31 The major band of APC complexes seen with the antibodies to protein C, apparently APC:PCI-2 (Fig 2, left panel, band b), was not detected in Fig 2, right panel by anti-PCI-1 antibodies in untreated or Protac C-treated plasmas. The untreated

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**Fig 1.** Immunoblot on nondenaturing 5% polyacrylamide gels of APC complexes in normal plasma and plasma of patients with DIC. Plasma samples (5 μL/Lane) from 21 different patients are shown. NHP in the second lane was incubated with 5 μL Protac for one hour at 37°C before electrophoresis. Detection was accomplished with monoclonal antibody to protein C, followed by 125I-protein C. P-22 and P-47 designate plasmas from two particular patients that were further studied (see Figs 2 and 3).
Correlation of APC complexes with other laboratory data for DIC patient plasmas. Table 1 summarizes laboratory and mortality data for the 16 patients for whom complex formation was most evident (ie, >20% of total protein C antigen in APC:PCI-2 complexes). The 16 patients with the markedly high levels of APC:PCI-2 complexes had higher mortality and more abnormal laboratory tests than the remaining patients. Of these 16 patient plasmas, three of the plasmas were from patients with severe infection, five from patients with solid tumors, three from patients with leukemia, two from patients with vascular disease, and three from patients with other diagnoses. Only one of these patients had evidence of liver disease. The patient with the two major forms of APC:PCI in plasma (designated P-47, Figs 1 and 2) suffered a perforated uterus on abortion. This patient had abnormal prothrombin time, fibrinogen level, and FDP levels.

Protein S in DIC patient plasmas. Since protein S is the cofactor for APC, all plasmas of these patients with DIC were examined for evidence of alteration in protein S molecules and for total protein S antigen levels. Immunoblots for protein S antigen using SDS gels are seen in Fig 4. From the reduced samples (Fig 4), it can be seen that a small amount of protein S was cleaved in normal plasma (lane 1). This was found for all normal individual plasmas even when the blood was collected in citrate and protease inhibitors (4 mmol/L EDTA, 1 mmol/L p-amidino-phenyl-methyl-sulfonly-fluoride, 10 mmol/L benzamidine, 500 U/mL aprotinin, 200 μg/mL soybean trypsin inhibitor, 3 μg/mL leupeptin) and when immunoblotting was performed on plasma from freshly drawn blood (data not shown). Protein S contains two thrombin-sensitive sites, but protein S in plasma is difficult to cleave with thrombin, even at high thrombin concentrations (20 U/mL) and incubation for one hour at 37°C (data not shown).44 However, Protac C cleaves protein S to give the appearance of thrombin-cleaved protein S (Fig 4, lane 2). Preliminary tests suggest that Protac C-cleaved protein S, like thrombin-cleaved protein S, is inactive as a cofactor for APC. As seen in Fig 4, the majority, though not all, of the DIC patient plasmas contain higher than normal amounts of cleaved protein S. In control experiments, 23 individual normal plasmas and a pooled normal plasma sample were subjected to four cycles of freezing and thawing. In no case

Molecular weight of the APC complexes in DIC patients. Selected patient plasma samples with and without Protac C treatment were immunoblotted using denaturing sodium dodecyl sulfate (SDS) gels (Fig 3). For the six patient plasmas tested, bands of protein C antigen were visible at approximately molecular weight (mol wt) 110,000 before Protac C treatment, and the amount of the apparent APC:PCI complexes was increased after Protac C treatment. This suggests that the complexes formed in vivo as well as in vitro involve one or more inhibitors of approximately 50,000 mol wt covalently linked to APC. In other data not shown, a doublet of APC complexed in vitro was observed near 110,000 mol wt in 6% SDS-polyacrylamide gels.

Fig 2. Immunoblot using a 6% nondenaturing gel of Protac C-treated normal plasma and two DIC patient plasmas. Protac C treatment (indicated by +) and immunoblotting conditions were as in Fig 1, except that detection in the right panel was with rabbit polyclonal antibody to PCI-1, followed by 110-goat anti-rabbit IgG. P-47 and P-22 indicate plasmas of two patients with intravascular coagulation which were shown on the immunoblot in Fig 1.
was there a significant increase in cleaved protein S due to freezing and thawing (one sample shown in Fig 4 [NHP FT]). The last two lanes of the upper panel of Fig 4 contain nonreduced samples. Cleaved and intact protein S could not be distinguished in nonreduced samples, even with shorter film exposure. In the lower panel of Fig 4, reduced plasma samples were electrophoresed with closer spacing than in the upper panel. In the lower panel, the resolution was such that two forms of cleaved protein S could be distinguished in some samples. These two forms may represent protein S cleaved at one or two of the thrombin-sensitive sites.44

In order to assess whether protein S cleavage might have occurred after blood samples were taken, three types of experiments were performed. Three DIC patient samples were obtained in which blood was collected directly into sodium citrate containing protease inhibitors to make the final concentrations 1 mmol/L benzamidine, 10 μmol/L phenanthroline, and 333 U/mL aprotinin. Two of the three resulting plasmas were found to have abnormally high levels of cleaved protein S on immunoblots from reduced gels. In another experiment, a DIC plasma sample which contained approximately 80% of its protein S in cleaved form was incubated at 37°C for 20 minutes with added purified protein S. After immunoblotting, no increase in cleaved protein S was observed, relative to controls including a mixture of the two in 40 mmol/L benzamidine, which was not incubated. In a third experiment, seven DIC patient plasmas, which had not been thawed since the date of plasma preparation, were divided immediately after thawing. Half was made 40 mmol/L in benzamidine and aliquots were immediately prepared in SDS sample buffer for electrophoresis and immunoblotting. The other half did not have added benzamidine, and aliquots were incubated at 37°C for 20 minutes before electrophoresis and immunoblotting. Comparison of the nonincubated samples containing benzamidine with the incubated samples that did not contain benzamidine revealed that five of the seven plasmas contained abnormally high levels of cleaved protein S, but the amount cleaved was not increased after incubation in the absence of benzamidine. The results of these three experiments support the interpretation that most or all of the observed protein S cleavage occurred in vivo before the blood samples were taken.

All of the plasmas of the patients with DIC were analyzed for total protein S antigen level by Laurell electroimmunoassay, with the data shown in Fig 5. The samples were divided into several broad categories by diagnosis and FDP levels, and a mean and standard deviation were determined for each group, either including or excluding patients with evidence of liver disease who might be expected to have lower levels of protein S. Whether or not the patients with liver disease were included, the mean protein S antigen level was low in each group, except for those patients with malignancy for whom the values were normal.

**DISCUSSION**

Immunoblotting studies showed that the forms of APC:PCI complexes formed in plasma in vitro also arise in vivo. In plasma samples from 62 of 85 patients with DIC, some protein C antigen comigrated on nondenaturing and on SDS gel immunoblots with APC:PCI complexes that were formed in vitro in normal plasma. In nondenaturing gels.
decreased with DIC. The studies here demonstrating functional activities of protein C and its inhibitors would fall due to activation of protein C and subsequent complexation with inhibitors in vivo in patients with DIC. Moreover, the protein C activity to antigen ratios for patients with significant levels of APC:PCI complexes would be reduced. Protein C activation peptide has also been detected in DIC.47

The major band of APC complexes in plasma of patients with DIC did not react with three different antibodies to PCI-1 (Fig 2 and text), although a minor band of APC complexes was visualized in some plasmas with these antibodies. This suggests that a second PCI (PCI-2) in plasma exists that participates in the regulation of APC during DIC. The existence of a second PCI in plasma has recently been suggested in other studies.27–31 Free PCI-1 antigen was apparently low in eight of nine plasmas tested here and it is possible that APC:PCI-1 complexes were formed and cleared from circulation before the blood samples were taken. The relative rates of formation of APC:PCI-1 and APC:PCI-2 complexes in plasma in vitro are approximately equal in the absence of heparin although addition of CaCl2 and MgCl2 causes a slight decrease in APC:PCI-1 complexes.27 However, neither the relative rates of formation nor the relative rates of clearance in vivo can be inferred at this time. It is also possible that the major role of PCI-1 or PCI-2 in vivo is other than the inactivation of APC and that the relative rates of enzyme:inhibitor complex formation and clearance are significantly modulated by other enzymes or by heparin-like substances in vivo.

Plasmas of the majority of the patients with DIC contained a higher than normal proportion of protein S antigen in cleaved form (Fig 4). This suggests that during DIC protein S may participate in the regulation of hemostasis and thrombosis and protein S may be regulated by proteolytic inactivation. The identity of the protease(s) responsible for cleavage of protein S in vivo is uncertain. Although thrombin cleaves and inactivates purified protein S,46–48 high thrombin concentrations are required, and calcium ions are inhibitory.46–49 A platelet membrane protease has recently been reported to cleave platelet-bound protein S.50 The finding here that protein S levels are low in plasmas of patients with DIC (Fig 5) is apparently not in agreement with other studies.51 The variable findings may depend on the number of patients with malignancy who are included in various studies, since we found the protein S antigen levels in those patients to be normal. Also, some differences may occur because of the effect of high C4b-binding protein levels (which some of these patients may have) on assay results by electromunoassay v by radioimmunoassay. However, it is possible that in patients with low levels of protein S, the cleaved protein S may have been partially cleared from circulation.

That APC and its cofactor protein S may play physiologically important roles as regulators of intravascular coagulation is suggested by the recent demonstration that APC prevents the coagulopathy as well as the fatal outcome associated with E coli-induced shock in a baboon model of septicemia16 and inhibits platelet-dependent thrombosis in a baboon arterial model.18 Moreover, infants with a hereditary homozygous deficiency of protein C develop intravascular
coagulation shortly after birth that is fatal when untreated, and heterozygous deficiency of protein S in some families is associated with thromboembolic disease. Thus, the implication of studies reported here that protein C is activated and inhibited by plasma PCI(s) in vivo and that protein S is cleaved and decreased during DIC is consistent with the hypothesis that APC and protein S are physiologically important regulators of intravascular coagulation. The data here also support the hypothesis that the recently discovered PCI-2 (see other article by Heeb et al in this issue) participates in the in vivo regulation of APC. PCI-2 was later isolated and identified as ε1-antitrypsin.

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