Serial Studies of Protein C and Its Plasma Inhibitor in Patients With Disseminated Intravascular Coagulation

By Richard A. Marlar, Janet Endres-Brooks, and Christine Miller

This study was undertaken to determine the levels of protein C antigen and activity and protein C inhibitor in sequential plasma samples of disseminated intravascular coagulation (DIC) patients. Our normal range for both protein C antigen and activity is 70 to 130 U/dL, and protein C inhibitor is 65 to 135 U/dL. A decreased level of protein C activity was found in 96% of the plasma samples from individuals with DIC; the protein C antigen was decreased in 73%. The inhibitor of protein C was decreased in all samples. Analysis of serial samples from patients with DIC reveals that protein C activity and antigen and protein C inhibitor decrease progressively during the initial stages of DIC and remain at a low level for 24 to 48 hours before gradually returning toward normal in nonfatal cases. The protein C activity decreases in parallel with protein C inhibitor and is lower than protein C antigen. In a fatal case of DIC, protein C activity and protein C inhibitor rapidly decreased to undetectable levels; however, protein C antigen was gradually decreasing but still detectable at time of death. In DIC, a discrepancy initially occurs between the activity and antigen of protein C, suggesting a complex with the inhibitor or other inactive forms of protein C. Protein C appears to play a major role in the control of DIC.

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MATERIALS AND METHODS

Collection of Plasma Samples

Venous blood was collected into 3.2% sodium citrate (9 parts blood:1 part anticoagulant) using either a vacutainer system or a 10-mL syringe and a 21-gauge butterfly needle. Plasma was obtained by centrifugation at 2,500 g for 20 minutes at 4°C. The normal plasma pool standard used in these studies was venous blood obtained from 50 normal, healthy donors (25 women and 25 men), using a two-syringe technique and processed as above. The patient plasma samples and the normal plasma pool were aliquoted, frozen, and stored at −80°C. DIC was determined based on clinical and laboratory findings.

DISSEMINATED intravascular coagulation (DIC) is a pathologic condition involving coagulation factors, platelets, vascular endothelial cells, fibrinolysis, and plasma inhibitors. This major breakdown of the hemostatic mechanism occurs when the procoagulant or thrombogenic factors outweigh the anticoagulant or control mechanisms. The generation of microthrombi can result in capillary and small vessel obstruction, tissue necrosis, and abnormal secondary bleeding.

Human protein C is the central protein in one of the major regulatory mechanisms of hemostasis. This system decreases the rate of thrombin formation by controlling factors V and VIII:C, both of which are involved in rate-limiting steps of coagulation. Protein C also functions as a profibrinolytic enzyme, increasing the rate of fibrin degradation. Patients with hereditary deficiencies of protein C having levels of 60% or less of normal can develop thromboembolic complications. Individuals born with homozygous protein C deficiency develop fatal neonatal purpura fulminans. Studies of protein C in DIC have reported that approximately 70% of the patients with DIC had decreased levels of protein C antigen. However, Rodeghiero et al did not find a significant decrease of protein C in their patients with DIC resulting from leukemia. These previous studies did not include serial protein C levels during the course of DIC, nor did they include measurements of protein C activity and protein C inhibitor.

This study was undertaken to extend the initial observations of decreased protein C antigen by evaluating serial samples from patients with DIC and to quantitate the levels of protein C activity and the plasma inhibitor of protein C in these samples.

Protein C Antigen Assay

Human protein C was purified as previously described, using commercial factor IX concentrate as starting material. Monospecific polyclonal antibodies to human protein C were made in New Zealand rabbits. No cross-reactivity with the other vitamin K-dependent proteins was found. IgG fraction was isolated by ammonium sulfate precipitation and diethylaminoethanol (DEAE) cellulose chromatography or by caprylic acid. A radiolabeled Laurell electroimmunnoassay was used to determine human protein C antigen in plasma samples. The log concentration of protein C in the plasma was linearly correlated to the height of the rockets.

Assay for Protein C Activity

Human protein C was assayed for its chromogenic activity by the method of Comp et al. This assay includes thrombin–thrombomodulin for activation of protein C. The removal of the activated protein C with antiprotein C IgG-Sepharose (5 mg IgG/mL of Sepharose). Assay of the activated protein C was performed with the 0.14 mmol/L chromogenic substrate S-2238 (Kabi, Stockholm) in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 0.02% sodium azide, pH 8.3. The correlation of the concentration of protein C activity to the change in optical density was linear.

Assay for the Inhibitor of Protein C

The activity of protein C inhibitor was measured in plasma by a modification of the original chromogenic assay. Activated protein C concentrates were used as a source of activated protein C.
C (3 μg/mL, 40 μL) was incubated at 37 °C for 120 minutes with 40 μL of plasma. The maximum inhibition occurred prior to 120 minutes. No heparin or anti-prekallikrein antibodies were added to the assay. A 30 μL aliquot was mixed with 100 μL of antiprotein C IgG-Sepharose (5 mg IgG/mL of Sepharose) for five minutes. After centrifugation and removal of supernatant, the Sepharose was washed three times with 150 μL of 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, and 0.02% sodium azide, pH 8.3. After the final wash, 600 μL of 0.14 mmol/L S-2238 (Kabi) in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, and 0.02% sodium azide, pH 8.3 was added and incubated for 60 minutes at 37 °C. The optical density (OD) of the supernatant was then measured at 405 nm. There was a linear relationship between the OD and the concentration of protein C inhibitor in normal plasma.

Quality Control of Assays

For protein C activity and antigen and protein C inhibitor activity, the normal human plasma pool was arbitrarily defined as 100 U/dL or 100%, and each plasma sample was expressed as a percentage thereof or in units per deciliter. Each plasma sample was assayed at two different dilutions. For each assay described above, the within-assay variation and between-assay variation was 10% or less. For protein C activity and protein C inhibitor, the lowest limit of detection was 6 U/dL, and for protein C antigen, it was 3 U/dL.

Other Assays

Factor V and factor VIII:C were assayed by established procedures, using appropriate congenitally deficient plasma. Other assays such as PTT, fibrinogen, fibrin split products, and platelet count were performed in the hospital by standard procedures.

Patient Selection

The individual single time point plasma samples were taken from patients hospitalized with confirmed DIC. To confirm a diagnosis of DIC, attending physicians referred the samples to their hospital's coagulation laboratory. Laboratory diagnosis of DIC was confirmed by decreased fibrinogen and platelet count and a prolonged partial thromboplastin time. All patients had elevated fibrin split products. The initiating stimulus was determined in each patient and is categorized in Table 1. Patients with preexisting or known liver involvement were eliminated from this study.

Case Histories

Case A. A 7-year-old girl was admitted 12 hours after the start of "viral-like" symptoms including lethargy, headache, nausea, and diarrhea. She had petechiae on her abdomen, back, upper legs, and buttocks, but no overt bleeding was noted. The admitting laboratory results were as follows: prothrombin time (PT) 17.2 seconds (n = 11 to 13); activated partial thromboplastin time (APTT) 63.9 seconds (n = 25 to 35); thrombin time 19.6 seconds (n = 12.5 to 15); fibrinogen 85 mg/dL (n = 200 to 400); platelets 45,000/μL (n = 150,000 to 450,000); fibrin split products of >160, <320 (n = 10); and a positive fibrin monomer test. These screening tests, indicative of DIC, remained abnormal for the next four days, after which time many of the tests returned to normal. The patient was diagnosed as having meningococcemia and was treated only with large doses of antibiotics, hypotensive drugs, and support care. No plasma, plasma products, platelets, or heparin was given. Clinically, the patient recovered fully after eight days; laboratory results were within the normal range after six days.

Case B. A 56-year-old man diagnosed with acute myeloblastic leukemia, was hospitalized to undergo chemotherapy. After the second treatment, he was noted to have a prolonged PT (18.2 seconds; n = 11 to 13) and APTT (75 seconds; n = 25 to 35); however, no overt bleeding was noted. The chemotherapy treatment was stopped immediately, and a DIC screen was ordered. The laboratory findings showed very low levels of fibrinogen, platelets, and factor V, as well as elevated fibrin split products. These tests remained abnormal throughout the course of DIC. After further clinical deterioration, the patient died of cardiac arrest.

Case C. A 43-year-old woman with cancer of the pancreas was admitted to the hospital with clinical symptoms of DIC, which included ecchymoses, epistaxis, hematuria, and large areas of petechiae on the abdomen, chest, and upper arms. Laboratory findings confirmed the clinical diagnosis of DIC. Initial laboratory studies demonstrated normal levels of fibrinogen and antithrombin III, increased PT and thrombin time, normal APTT, decreased factor V, and mildly elevated fibrin split products. During the next 24 to 36 hours, markedly elevated fibrin split products and decreased platelets, fibrinogen, and factors V and VIII:C were found. The antithrombin III level decreased during the first 48 hours to a low of 31%. The patient was not given platelets or fresh frozen plasma. Intravenous (IV) heparin at 10 U/kg/h was administered for the duration of the DIC. After six days, the laboratory results showed a gradual return toward normal levels and the clinical symptoms disappeared. The patient fully recovered from this episode of DIC; however, three weeks later, a similar episode of DIC occurred, during which the patient died.

RESULTS

The normal range of protein C activity and antigen is 70 to 130 U/dL. Protein C inhibitor has a normal range of 65 to 135 U/dL (Table 1). When plasma samples from 56 patients with DIC were tested, 54 of the 56 (96%) had decreased protein C activity, and 41 of these 56 (73%) individuals had decreased protein C antigen levels (Table 1). All had decreased levels of the inhibitor of activated protein C (Table 1). When these single point values were categorized by initiating stimulus, all major categories had a similar percentage of patients with decreased protein C antigen levels. All patients who died of DIC had decreased protein C activity, while 83% (20 of 24) had reduced levels of protein C.

Table 1. Protein C Activity and Antigen, and Activated Protein C Inhibitor Activity Levels in Patients With Confirmed Intravascular Coagulation Resulting From Various Underlying Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients</th>
<th>Protein C Activity*</th>
<th>Protein C Antigen*</th>
<th>Protein C Inhibitor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>83</td>
<td>70–130</td>
<td>70–130</td>
<td>65–135</td>
</tr>
<tr>
<td>Infection</td>
<td>27</td>
<td>&lt;6–60 (100%)</td>
<td>28–112 (78%)</td>
<td>15–60 (100%)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>18</td>
<td>&lt;6–120 (94%)</td>
<td>5–170 (72%)</td>
<td>&lt;6–30 (100%)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>6</td>
<td>15–75 (83%)</td>
<td>28–97 (50%)</td>
<td>10–60 (100%)</td>
</tr>
<tr>
<td>Obstetric complications</td>
<td>5</td>
<td>&lt;6–55 (100%)</td>
<td>16–80 (80%)</td>
<td>20–65 (100%)</td>
</tr>
</tbody>
</table>

*Values are expressed in units per deciliter. Parentheses show percentages of abnormal levels.
antigen. Of the patients who survived, 66% (21 of 32) also had decreased levels of protein C antigen. A true indication of the correlation of protein C levels to the severity or morbidity resulting from complications of DIC cannot be determined from single point samples. No correlation of low levels of protein C activity or antigen with the underlying causes of DIC was found. Protein C activity and antigen levels in samples from DIC patients were significantly lower than in normal controls as were the levels of protein C inhibitor.

In the sequential study of case A (Fig 1), protein C activity, protein C inhibitor, factor V, and factor VIII:C were abnormal at the time of admission. Each reached its lowest point 12 to 18 hours after the apparent onset of DIC. However, protein C antigen remained within the normal range during the first 24 hours after the apparent onset of DIC. It then became abnormal, reaching a trough at 36 hours, at which time its concentration correlated with the protein C activity. Both protein C activity and antigen remained at this low level for the next 52 hours. Protein C activity and antigen gradually increased, reaching normal levels at 155 hours. The inhibitor decreased to its lowest point by 24 hours, similar to protein C activity, and after 48 hours, it gradually increased, reaching normal levels at 126 hours.

Figure 2 illustrates the levels of protein C-related proteins in case B, a leukemic patient undergoing chemotherapy. In this instance, the DIC was very severe, with the patient dying 50 hours after onset. By the time DIC was detected in this patient, protein C activity, factor V, factor VIII:C, and the inhibitor to protein C were significantly decreased. By 26 hours, there was no detectable activity of protein C or inhibitor. On the other hand, protein C antigen was gradually decreasing at a steady rate, but remained in the normal range for the first 24 hours. Even after the administration of plasma and platelets, no increase in protein C activity, antigen, or inhibitor activity was noted. The protein C activity was undetectable, and protein C antigen was 40% in the last plasma sample obtained prior to the death of the patient.

In case C, the levels of protein C and its inhibitor were followed during the course of DIC in a patient with cancer of the pancreas (Fig 3). Protein C antigen decreased, but remained within the normal range during the first 36 hours after the apparent onset of DIC. The protein C antigen level continued to fall, becoming maximally reduced by 72 hours, and did not increase until 108 hours. Increasing levels of protein C antigen were found during the recovery phase, reaching normal levels at about 160 hours. Protein C activity was decreased at the time of admission, approximately 12 hours after onset of DIC. It reached its trough at 48 hours (24 hours prior to protein C antigen), and remained at this level until 96 hours. Protein C antigen and activity began their ascent toward normal levels simultaneously and proceeded at the same rate. The inhibitor to protein C was abnormal on the first sampling. It declined to the lowest point at 48 hours, then began increasing after 72 hours. Normal levels of inhibitor were reached 50 to 60 hours later. Factor V decreased initially and returned to normal levels by
during the DIC process. From our current work (Table 1)
cases. The activity of protein C and its inhibitor decreases
cannot accurately reflect the ongoing consumption of protein
of a functional assay for protein C. Because our work on
synthesis. We also noted a decrease in protein C activity as
They did observe decreased protein C antigen in their DIC
and their studies,'3"4 it can be concluded that 70% to 75% of
C antigen reduction in DIC based on single plasma samples
Griffin et al'3 and Mannucci and Vigano'4 observed protein
activity is decreased in many of these samples, sometimes
resulting in a discrepancy between antigen and activity. Griffin et al13 and Mannucci and Vigano14 observed protein
in DIC based on single plasma samples during the DIC process. From our current work (Table 1) and their studies,13,14 it can be concluded that 70% to 75% of
patients with DIC have abnormal levels of protein C antigen
if only single plasma samples are used. However, this does
not reflect the activity of protein C in these patients, nor do
these individual time points accurately reflect the overall
response of the protein C system during DIC.

In direct contrast to our work and to that of others,13,14
Rodeghiero et al15 observed no decrease in protein C antigen
in DIC resulting from leukemia without liver involvement.
They did observe decreased protein C antigen in their DIC
patients with liver disease, suggesting a decrease of protein C
synthesis. We also noted a decrease in protein C activity as
well as a decrease in factors X and VII in DIC patients with
liver disease. However, in DIC patients without liver disease,
the protein C activity was decreased compared to the levels of
factors VII and V. These observations are limited by the lack
of a functional assay for protein C. Because our work on
individual patients with DIC suggests a discrepancy between
protein C antigen and activity, we feel an antigen assay
cannot accurately reflect the ongoing consumption of protein
C. The discrepancy in activity reflects the presence of protein
C inhibitor complex or some other form of inactive protein
C.

Based on serial studies, protein C activity and protein C
inhibitor decrease progressively during the initial stages of
DIC and gradually return toward normal in the nonfatal
cases. The activity of protein C and its inhibitor decreases
immediately and in parallel with the protein C substrates,
factors V and VIII:C. Protein C antigen decreases at a slower
rate, remaining significantly higher than the activity and
inhibitor for a period of time (usually 24 to 36 hours). This
slow decrease of protein C antigen may reflect measurement
of inactive protein C. Both protein C and protein C inhibitor
remain at their lowest level for approximately 24 to 48 hours,
with each subsequently rising toward normal levels at similar
rates. Activated protein C inhibitor rises from the trough at a
rate parallel to but approximately 24 to 36 hours ahead of the
protein C. Factors V and VIII:C increase even sooner than
both protein C and protein C inhibitor. In fatal DIC (Fig 2),
protein C activity and protein C inhibitor rapidly (12 to 24
hours) decrease to undetectable levels. In a similar parallel
fashion, rapid decreases in factors V and VIII:C activity are
seen. The decline of protein C antigen appears much slower
than that of its activity. In fatal DIC (Fig 2), protein C
antigen was 40% at the time of death, whereas protein C
activity and protein C inhibitor were undetectable. The
absence of protein C activity, protein C inhibitor, and factors
V and VIII:C may not be compatible with life when the
coagulation system is systemically activated as in DIC.

Protein C and its plasma inhibitor may play an important
role in helping to control the DIC process. In DIC, thrombin
is generated. Aside from cleaving fibrinogen and performing
its other procoagulant functions, some of the excess thrombin
binds to thrombomodulin on the endothelial cell surface,
leading to increased levels of activated protein C in plasma.
The activated protein C inactivates factors Va and VIII:C.13,14
This negative feedback mechanism has the potential to slow
down the formation of excess thrombin and stop the DIC
process. The inhibitor of activated protein C slowly removes
the increased levels of activated protein C,18 thus regulating
the protein C system. Once the generation of excess thrombin
is decreased by the action of activated protein C and other
regulatory mechanisms, the coagulation process can return
to normal. In addition, the protein C system may induce
fibrinolytic activity13 to facilitate clearance of excess thrombi and generating fibrin split products. If activated
protein C is being consumed too rapidly, the regulatory
ability of the protein C system is sharply reduced, resulting
in uncontrollable thrombosis.
Protein C appears to play a major role in the control of the DIC process. Our data show that protein C decreases during DIC, which could mean the loss of a control mechanism necessary to halt intravascular coagulation process. Ulutin and co-workers have shown that infusion of tissue factor induces DIC and fibrinogen consumption in animals, whereas the concomitant infusion of activated protein C and tissue factor protected them from DIC and maintained significantly higher levels of fibrinogen. More studies with protein C and its plasma inhibitor in an animal model of DIC are needed. Administration of a protein C concentrate during the initial stages of DIC could help maintain the regulatory control of the protein C system, thereby halting the DIC process.

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