CONCISE REPORT

Calcium-Dependent Cysteine Protease Activity in the Sera of Patients With Thrombotic Thrombocytopenic Purpura

By William G. Murphy, Jane C. Moore, and John G. Kelton

Plasma and serum from patients with thrombotic thrombocytopenic purpura (TTP) can cause activation and aggregation of normal human platelets in vitro. It is possible that this platelet-activating factor contributes to the disease. In this report we describe studies designed to identify the platelet-activating factor in TTP. Platelet activation by sera from 15 patients with TTP was inhibited by leupeptin, iodoacetamide, and antipain but not by phenylmethylsulfonylfluoride, ε-aminocaproic acid, soybean trypsin inhibitor, aprotinin, and D-phenyllyl-1-prolyl-1-arginine chloromethyl ketone. These studies suggested that the platelet-activating factor in TTP serum was a cysteine protease.

We confirmed that a calcium-dependent cysteine protease (CDP) was present in the sera of each of the 15 patients when we used an assay based on the ability of CDP to proteolyse platelet membrane glycoprotein 1b (GP1b) and hence to abolish the ability of CDP-treated normal platelets to agglutinate in the presence of ristocetin and von Willebrand factor. This proteolytic activity was inhibited by EDTA, leupeptin, antipain, iodoacetamide, and by N-ethylmaleimide (NEM) but not by the serine protease inhibitors. Activity was detected in 15 of 15 patients with TTP before therapy was begun. In contrast, no activity was detected in the serum of any of five of the TTP patients tested in remission or in any of the sera from 36 patients with thrombocytopenia and 423 non-thrombocytopenic controls. To look for in vivo CDP activity in patients with TTP, we studied platelets from two patients with acute TTP (drawn into acid-citrate-dextrose, NEM, and leupeptin). These platelets showed a loss of GP1b from the platelet surface. Both patients were also studied in remission: GP1b on the platelet surface had returned to normal. These studies provide evidence that CDP is present in the sera of patients with TTP, that it is specific to this disease, and that it is active in vivo as well as in vitro. We postulate that a disorder of CDP homeostasis plays a major role in the pathophysiology of TTP.

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MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co, St Louis, except where stated otherwise. Platelet counts were measured on a Coulter S and platelet sizing was performed on a Coulter Channelizer (Coulter Electronics, Inc, Hialeah, FL). Fifteen patients with acute TTP were studied. All of these patients were tested at the initial presentation of their first episode of TTP before any plasma therapy had been given. Sera were available from five of these patients in remission of disease. Control sera for both the platelet release studies and ristocetin-induced agglutination inhibition studies were obtained from ten healthy volunteer donors, ten patients with idiopathic thrombocytopenic purpura, ten healthy females in
the third trimester of pregnancy, four patients with preeclampsia, eight patients with disseminated intravascular coagulation, two patients with systemic lupus erythematosus and thrombocytopenia, ten patients with thrombocytopenia after cardiopulmonary bypass, two patients with heparin-induced thrombocytopenia, one patient with a hemolytic transfusion reaction (anti-E), two patients with extensive tissue damage due to severe trauma, and eight randomly selected nonthrombocytopenic hospital controls. In addition, normal RBC were suspended in a 2% suspension in autologous normal serum and lysed by sonication and the resultant solution tested in the release assay and in the ristocetin agglutination assay. For the ristocetin-induced agglutination inhibition studies, 392 additional randomly selected hospital patients were included as controls. Sera were separated into 1-mL aliquots and stored at -70°C. Aliquots were thawed at 37°C on the day of testing.

Preparation of control CDP from platelet lysates. Fifty units of platelet concentrates were pooled, washed, and disrupted by nitrogen cavitation. CDP was purified as described. The proteolytic activity of the CDP preparation was determined by using a 4C-caseloinic acid assay. The purity of the preparation was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Studies of the effects of enzyme inhibitors on platelet 4C-serotonin release. Normal pooled platelets were loaded with 4C-serotonin (Amersham Corp, Oakville, Ontario) with an average specific activity of 1.67 x 10^4 pCi/10^9 platelets (n = 3). The ability of test or control serum to cause release of 4C-serotonin from labeled platelets was tested in a microtiter assay. The following reagents were tested for their ability to inhibit the release caused by test sera: phenylmethylsulphonylfluoride (PMSF), 1 mmol/L; D-phenylalanil

Evidence for in vivo activity of CDP in TTP. Platelets from a patient with acute TTP before plasma therapy (platelet count, 20 x 10^11/L) were separated from blood drawn into acid-citrate-dextrose (ACD) with NEM and leupeptin (1 mmol/L) and resuspended in calcium- and albumin-free Tyrode's buffer (pH 7.4) at a concentration of 120 x 10^9 platelets/L; these platelets were tested for their ability to agglutinate in the presence of ristocetin by using standard techniques. This assay was repeated when the patient entered remission.

The same platelets were also assayed for glycocalcin content by SDS-PAGE (4% stacking gel over a 5% to 15% gradient). A quantity of 6.4 x 10^10 platelets were labeled with H and solubilized in 2% SDS with NE (6 mmol/L) and control platelets from a healthy donor were treated identically. The accuracy of the number of platelets solubilized and applied to the gel was confirmed by platelet sizing and by light and phase microscopy to rule out red cell fragment contamination.

We also assayed the platelet membrane GPIb content in a second patient with TTP by radioimmune precipitation using the monoclonal anti-GPIb antibody 6D1 (generously provided by Dr B. Collee, State University of New York at Stony Brook) as described. Platelets from acute disease (platelet count, 20 x 10^11/L) and remission after plasmapheresis (platelet count, 257 x 10^9/L) were collected from blood drawn into inhibitors as before. The platelets were frozen in dimethylsulfoxide and autologous plasma and tested together on the same day.

RESULTS

Characterization of the platelet-activating factor by the effect of enzyme inhibitors on platelet 4C-serotonin release. The sera from all 15 patients with TTP had platelet-activating activity as measured by the 4C-serotonin release assay (Table 1). No platelet-releasing activity was detected in any of the control sera except from one of the two patients with heparin-induced thrombocytopenia and from the patient with anti-E. Sera from five of the TTP patients in remission (after plasma therapy had been discontinued) had no platelet-activating activity. A preparation of CDP at a concentration of 1.5 caseinolytic U/mL also caused platelet aggregation and release; at a concentration of 70 caseinolytic U/mL the enzyme caused platelet lysis. Lysis (measured by 41 In release) did not occur at the lower concentration. SDS-PAGE analysis of the CDP preparation showed a major band at 76 kilodaltons (kD). Bands were also present at 118 kD and 64 kD, with some larger molecular weight proteins also present. The preparation was free of contaminating protease activity. The platelet release caused by the sera of patients with TTP during active disease and by the CDP preparation was consistently inhibited by leupeptin, IAAs, and antipain but was not inhibited by PPAC, PMSF, aprotinin, soybean trypsin inhibitor, or EACA (Table 1). Three of these patients who entered remission later relapsed.
CDP ACTIVITY IN PATIENTS WITH TTP

Table 1. Effects of Inhibitors on the Induction of $^{14}$C-Serotonin Release From Normal Platelets by Sera From 15 Patients With TTP

<table>
<thead>
<tr>
<th>Patients</th>
<th>PBS (pH 7.4)</th>
<th>PMSF (1 mmol/L)</th>
<th>PPAC (1 μmol/L)</th>
<th>Aprotinin (100 KIU/L)</th>
<th>EACA (0.2 μmol/L)</th>
<th>Soybean Trypsin Inhibitors (200 μg/ml)</th>
<th>IAA (0.5 μmol/L)</th>
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Concentrations are given as final concentrations. Inhibitors were incubated with test sera for ten minutes at 22°C before the addition of labeled platelets. Release for PBS is expressed as a percentage of the total possible release, ie, the amount of isotope taken up by the platelets. Release for the inhibitors is expressed as a percentage of the release in PBS. All tests were done in triplicate on two or more occasions. Serum from ten UP patients (1, 2, 3, 4, 5, 7, 8, 10, 11, 13) were incubated with platelets labeled with $^{111}$In as well as $^{14}$C-serotonin to determine whether there was any platelet lysis occurring. These sera caused less than 1% release of $^{14}$C.

Fig. 1. Effect of TTP serum and purified CDP with and without protease inhibitors on ristocetin-induced platelet agglutination. Protease inhibitors are (a) buffer, (b) PMSF, (c) PPAC, (d) EACA, (e) soybean trypsin inhibitor, (f) aprotinin, (g) IAA, (h) leupeptin, (i) NEM, (j) EDTA, and (k) a buffer control without added serum. Test sera, with and without inhibitors, were incubated with formalin-fixed normal platelets at 22°C for 80 minutes. The treated platelets were centrifuged and then resuspended in calcium- and albumin-free Tyrode’s buffer (pH 7.45) at a count of $300 \times 10^4$ platelets/L; agglutination of the platelets in the presence of ristocetin and normal plasma was recorded in an aggregometer.

Light Transmission

CDP

Increased Light Transmission

TTP SERUM

Increased Light Transmission

Evidence for in vivo activity of CDP in TTP. SDS-PAGE analysis of the platelets from a patient with TTP is shown in Fig 2A. The patient’s platelets show a marked loss of GP1b from the membrane. Other membrane proteins have

normal activity, which indicated that the inhibition of agglutination was due to an effect on the preincubated platelets.

Preincubation of TTP sera with EDTA, IAA, NEM, antipain, and leupeptin resulted in a loss of the inhibition of ristocetin-induced platelet agglutination in all 15 patients. Preincubation with PMSF, PPAC, aprotinin, EACA, or soybean trypsin inhibitor had no effect (Fig 1). Plasmin, 5 U/mL, also inhibited ristocetin-induced platelet agglutination; in contrast to TTP sera and CDP, plasmin was inhibited by PMSF, EACA, aprotinin, soybean trypsin inhibitor, antipain, and leupeptin but not by IAA or NEM. No change was seen in the platelet counts or in the distribution of the platelet size of the fixed normal platelets before and after exposure to TTP sera or CDP, which indicated that fragmentation of the platelets was not occurring.

Confirmation of the presence of CDP activity in the serum of patients with TTP. Formalin-fixed normal platelets preincubated with sera from all 15 TTP patients during periods of active disease and preincubated with purified CDP lost their ability to agglutinate in the presence of ristocetin and normal plasma (Fig 1). None of the control sera or the five TTP remission sera prevented the platelets from agglutinating. Nonpretreated formalin-fixed platelets added to the mixture of nonagglutinating pretreated platelets, normal plasma, and ristocetin in the aggregometer agglutinated normally, which indicated that the inhibition of agglutination was due to an effect on the preincubated platelets.
factor could participate in the pathogenesis of this disease. The studies described in this report suggest that this factor is CDP. We have demonstrated the presence of CDP in the sera of patients with TTP in several ways. First, we studied the effect of enzyme inhibitors on the platelet release reaction induced by TTP sera. Cysteine protease inhibitors but not serine protease inhibitors consistently inhibited platelet release. We then confirmed that CDP activity was present in TTP sera by demonstrating that TTP sera possessed the ability to proteolyze GP1b and that this proteolytic activity was calcium dependent and was sensitive to inhibition by cysteine protease inhibitors but not by serine protease inhibitors. The CDP activity was highly specific to TTP: we detected no activity in 459 control sera, including 316 patients with thrombocytopenia of various causes and more than 400 randomly selected hospital patients. Further, no activity was present in the sera of five of the TTP patients in remission, although it recurred in three patients upon relapse.

Platelets from two patients with TTP (from blood drawn directly into CDP inhibitors) showed a loss of GP1b from the platelet membrane during active disease. Platelets (tested in one patient) also showed a loss of the ability to agglutinate in the presence of ristocetin, comparable to that seen in Bernard-Soulier platelets. These observations are consistent with the enzyme-induced loss of glycopocalcin from the platelet membrane and suggest that CDP is active in vivo in patients with TTP.

CDP is a cytosolic enzyme found normally in several tissues including platelets.11 Its exact physiological role is uncertain, though it is known to be capable of proteolysis of a number of proteins including platelet membrane glycoproteins.4 It has been demonstrated previously4 and we have confirmed that the enzyme has platelet-activating activity. The pathway of aggregation is via platelet GPIIb/IIIa but not by anti-GP1b monoclonal antibody. It is likely, therefore, that platelet aggregation is the major in vivo effect of CDP on the platelet in TTP and that the effect on GP1b is a secondary one.

It is uncertain how our current studies showing CDP activity in TTP relate to previous observations. Several investigators have implicated vWF as an active or passive participant in TTP. Abnormally large multimers of vWF are present in the circulation of convalescent patients with recurrent TTP.12,13 Because the large multimers disappear during acute episodes of the illness, it has been postulated that vWF directly participates in the intravascular platelet clumping that characterizes TTP. In addition, we have shown that the platelet-aggregating activity of TTP can be enhanced in vitro by the addition of a preparation rich in large multimers of vWF.3 And yet, CDP has been demonstrated to cleave vWF, thereby resulting in the loss of the largest multimers.14 Therefore, it is possible that the abnormalities of vWF in TTP patients represent an epiphenomenon and are a consequence of CDP activity. However, these issues must await further investigations for their resolution.

On the basis of the findings described in these studies, we also been lost from the platelets. The patient's platelets did not agglutinate in the presence of ristocetin (Fig 2B). After remission, the patient's platelets agglutinated normally.

Radioimmune precipitation analysis of platelets from a second patient studied during active disease and in remission also showed a decrease of detectable GP1b on the platelets during active disease and a return to normal levels during remission (data not shown).

**DISCUSSION**

TTP is characterized by disseminated platelet thrombi within the circulation; it is possible that a platelet-activating...
postulate that platelet aggregation in TTP is generated through a disturbance of CDP homeostasis that leads to the presence of enzyme activity in the serum. Our studies also indicate that inhibition of ristocetin-induced platelet agglutination is a sensitive and specific assay for TTP.

ACKNOWLEDGMENT

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REFERENCES

Calcium-dependent cysteine protease activity in the sera of patients with thrombotic thrombocytopenic purpura

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