Fibrinolytic States in a Patient With Congenital Deficiency of α2-Pinolgen Inhibitor

By Nobuo Aoki, Yoichi Sakata, Michio Matsuda, and Keiko Tateno

The fibrinolytic system of a patient with congenital deficiency of α2-proteinase (plasmin) inhibitor (α2PI) was studied. The patient's whole blood clot formed in vitro was lysed rapidly in several hours on incubation. This accelerated in vitro fibrinolysis was suppressed by an addition of purified α2PI. The degree of suppression was proportional to the amount of α2PI added. In spite of the accelerated in vitro fibrinolysis, the increases of fibrinogen/fibrin-related antigen and fragment A (the earliest plasmin-catalyzed degradation product of fibrinogen) were not detected when the blood was mixed with plasmin inhibitors immediately after drawing blood. It was also revealed by sodium dodecyl sulfate (SDS) gel electrophoresis that fibrinogen structure was not altered and plasminogen was in a native form. These results suggested that there was no increase in vivo fibrinogenolysis. When the patient's citrated plasma was incubated in vitro, no progressive degradation of fibrinogen was observed in SDS gel electrophoresis in spite of the accelerated conversion of native Glu-plasminogen to modified Lys-plasminogen in the patient's plasma. The conversion was most likely catalyzed by a trace amount of plasmin spontaneously generated during the incubation. When the patient's plasma was clotted by recalcification, the fibrin formed was rapidly lysed. This in vitro fibrinolytic activity seems to be dependent on plasminogen activator activity in plasma, since the incubation of plasma, which decreases plasma plasminogen activator activity, resulted in the decrease of the fibrinolytic activity. With an addition of a small amount of urokinase, fibrinolysis was extensive, but there was still a very low degree of fibrinogenolysis. These results suggest that α2PI is effective in inhibiting fibrinolysis, but other protease inhibitors in plasma such as α2-macroglobulin are ineffective in inhibiting fibrinolysis, although they are effective in inhibiting plasmin in the plasma milieu thereby preventing fibrinogenolysis. Administration of tranexamic acid to the patient corrected not only the patient's abnormal fibrinolytic system but also the hemorrhagic tendency.

The present study further elaborates on the fibrinolytic system of the patient with congenital deficiency of α2PI.

MATERIALS AND METHODS

Protein

α2PI was purified from human plasma by the method previously described. Native Glu-plasminogen, whose amino-terminal group is glutamic acid, was prepared from fresh plasma in the presence of 10 KIU/ml of aprotinin by affinity chromatography with lysine-Sepharose according to the method of Brockway and Castellino. Lys-plasminogen, whose amino-terminal group is lysine, was prepared from stored blood bank plasma by affinity chromatography followed by DEAE-Sephadex chromatography. Purified urokinase was a gift from Hitachi Kasei Comp., Tokyo, and its activity was expressed as international units.

Inhibitors

Aprotinin (bovine pancreas trypsin inhibitor) and tranexamic acid (trans-4-aminomethylcyclohexanecarboxylic acid) were purchased from Mochida Pharmaceutical Co., and Daiichi Pharmaceutical Co., respectively.

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Assay Methods

Concentrations of α2PI were determined by single radial immunodiffusion.8 Plasminogen was assayed by the caseolytic method previously described.9 Plasminogen antigen concentration was measured by single radial immunodiffusion.9 Spontaneous plasmin activity was assessed directly by adding plasmin-specific tripeptide substrate H-D-Val-Leu-Lys-p-nitroanilide (S-2251) (Kabi Diagnostica, Stockholm, Sweden) to the plasma according to the method of Latallo et al.9 The capacity of the plasma to neutralize plasmin (antiplasmin activity) was estimated by fibrinolysin assay, described previously.1 Fibrinogen concentration in plasma was determined by the method of Ratnoff and Menzies.25 Fibrin/fibrinogen-related antigen (FRA) were assessed by the hemagglutination-inhibition test of Merskey et al.32 Test samples were serially diluted for the test. When the maximum dilution giving inhibition (inhibition titer) was more than 100-fold only every 100-fold serial dilution of the sample was tested. The results were expressed as inhibition titers of test samples, and the sensitivity of the test was approximately 3 μg/ml of fibrinogen. Determination of fragment A (the earliest plasmin-catalyzed degradation product of fibrinogen) concentration in plasma was kindly performed by Dr. K. Takagi according to the method previously described.33 Fibrinogen was removed from plasma before the assay by heat precipitation at 60°C for 30 min. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed with 5% gel, according to the method of Weber and Osborn.24 Plasma fibrinogen subjected to SDS gel electrophoresis was obtained as follows. First, 0.2 ml of plasma was mixed with 0.4 ml of saline (0.15 M NaCl containing 0.02% tranexamic acid and 10 KIU/ml aprotinin) and heated at 56°C for 4 min. The precipitated fibrinogen was separated by centrifugation and resuspended in 5 ml of cold saline. This washing procedure was repeated three times. Subsequently, the washed precipitate was subjected to SDS gel electrophoresis after solubilization by heating at 56°C for 30 min with 0.2 ml of 4 M urea, 1% SDS, and 1% Dithiotreitol (Nakarai Chemicals Ltd., Tokyo) in 0.02 M phosphate buffer, pH 7.2.

RESULTS

In Vivo Fibrinolysis or Fibrinogenolysis

Blood drawn from the patient was immediately mixed with the aprotinin-tranexamic acid mixture (aprotinin 5000 KIU/ml, tranexamic acid 10 mg/ml) in a ratio of 10 μl of the mixture of 1 ml blood. Blood was then divided, and one portion immediately mixed with one-ninth volume of 3.8% sodium citrate to obtain citrated plasma. Fragment A concentration in the citrated plasma was 21.0 μg/ml, which is within the range of 2 SE from the normal mean 10.8 ± 5.5 μg/ml (mean ± SE, n = 20). Another portion of blood sample was incubated in a glass test tube at 37°C for 2 hr. The clot formed was removed by centrifugation, and the supernatant was titrated for FRA. FRA was less than 3 μg/ml.

Spontaneous In Vitro Fibrinolysis and Fibrinogenolysis

Blood drawn from the patient was divided into two portions. One portion was used for the study of fibrinolysis. Another portion was mixed immediately with one-ninth volume of 3.8% sodium citrate and used for the study of fibrinogenolysis. For the fibrinolysis study, 1 ml of blood was immediately placed in each glass test tube containing 20 μl of purified α2PI in various concentrations or buffered saline, and was incubated at 37°C for 7 hr. After the incubation, all the samples were centrifuged to remove the remaining clots. Sera thus obtained were titrated for FRA. The results are presented in Table 1 as indicators of fibrinolysis. Without an addition of α2PI, the clot was almost completely lysed and a large amount of FRA was detected. This accelerated in vitro fibrinolysis was suppressed by an addition of α2PI. The degree of the suppression was proportional to the amount of α2PI added, and the restoration of α2PI concentration to normal brought about the abolishment of accelerated in vitro fibrinolysis (Table 1).

For fibrinogenolysis study, 2 ml of citrated blood was placed in each glass test tube containing 40 μl of purified α2PI in various concentrations or buffered saline immediately after drawing blood from the patient, and was incubated at 37°C. After 7-hr incubation, 20 μl of the aprotinin-tranexamic acid mixture was added to each sample, and the all samples were centrifuged at 2000 g for 20 min to obtain plasma. To 0.3 ml each of plasma samples thus obtained, 15 μl of 0.5 M CaCl2 was added, and the samples were incubated at 37°C for 30 min. Clots formed were removed by centrifugation, and the supernatants were titrated by FRA. The rest of the samples were assayed for fibrinogen concentrations. These results are presented in Table 1 as indicators of fibrinogenolysis. Neither definite change of fibrinogen concentration nor increase of FRA was observed in any of these samples regardless of the amount of α2PI added.

Urokinase-Induced In Vitro Fibrinolysis and Fibrinogenolysis

Two-milliliters of citrated patient’s plasma was mixed with 40 μl of purified α2PI in various concentrations or its vehicle, and further mixed with 10 μl of 2000 U/ml urokinase to obtain approximately 10

<table>
<thead>
<tr>
<th>Table 1. Effect of Addition of α2PI on the Patient’s In Vitro Fibrinolysis</th>
<th>Fibrolysis</th>
<th>Fibrinogenolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2PI* (mg/100 ml in Sera)</td>
<td>FRA (titers)</td>
<td>FRA (titers)</td>
</tr>
<tr>
<td>0</td>
<td>800×</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>400×</td>
<td>0</td>
</tr>
<tr>
<td>0.6</td>
<td>300×</td>
<td>0</td>
</tr>
<tr>
<td>1.1</td>
<td>32×</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>16×</td>
<td>0</td>
</tr>
<tr>
<td>2.7</td>
<td>8×</td>
<td>0</td>
</tr>
<tr>
<td>5.3</td>
<td>2×</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The concentrations expressed are those in sera obtained in fibrinolytic studies after an addition of purified α2PI.
U/ml urokinase in plasma. Each sample was then divided, and one portion was subjected to fibrinolysis study after clotting with calcium, and another portion to fibrinogenolysis study by the same method as described above except that the incubation was only 2 hr instead of 7 hr. The results are presented in Table 2. The degree of fibrinolysis was inversely proportional to the concentration of a2PI, and fibrinolysis was extensive when a2PI was absent or at low concentrations. In contrast to fibrinolysis, the level of fibrinogenolysis was very low, and production of FRA after 2-hr incubation with urokinase was very small, even in the sample containing no a2PI.

**Fibrinogen Structure**

Gross structure of fibrinogen molecules was analyzed by SDS gel electrophoresis after reduction. Fibrinogen obtained from the patient’s fresh plasma or from the plasma incubated for 7 hr at 37°C exhibited patterns indistinguishable from those of normal control (Fig. 1), and no change of size of the polypeptide chains was detected.

**Plasminogen Activation During Clot Lysis**

Plasminogen activation during the patient’s in vitro clot lysis was assessed by measuring plasminogen activity and plasmin activity (amidolytic activity) spontaneously developed. To 2 ml of the patient’s fresh citrated plasma or from the plasma incubated for 7 hr at 37°C exhibited patterns indistinguishable from those of normal control (Fig. 1), and no change of size of the polypeptide chains was detected.

**Table 2. The Patient’s Urokinase-Induced In Vitro Fibrinolysis and Fibrinogenolysis: Effect of Addition of a2Pl**

<table>
<thead>
<tr>
<th>a2Pl* (mg/100 ml)</th>
<th>Fibrinolysis (FRA titers)</th>
<th>Fibrinogenolysis (FRA titers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>800 x</td>
<td>8 x</td>
</tr>
<tr>
<td>0.3</td>
<td>600 x</td>
<td>8 x</td>
</tr>
<tr>
<td>0.6</td>
<td>600 x</td>
<td>4 x</td>
</tr>
<tr>
<td>1.2</td>
<td>100 x</td>
<td>1 x</td>
</tr>
<tr>
<td>1.8</td>
<td>16 x</td>
<td>1 x</td>
</tr>
<tr>
<td>3.0</td>
<td>4 x</td>
<td>0</td>
</tr>
<tr>
<td>6.0</td>
<td>2 x</td>
<td>0</td>
</tr>
</tbody>
</table>

*The concentrations in plasma before addition of urokinase and calcium.

Conversion of Glu-Plasminogen to Lys-Plasminogen Upon Incubation of Plasma

Freshly prepared citrated plasma was divided into two portions. One portion (5 ml) was mixed immediately with 10 μl of aprotinin (5000 KIU/ml) and subjected to affinity chromatography with lysine-Sepharose to obtain purified plasminogen. The other portion (5 ml) was incubated at 37°C for 7 hr, mixed with 10 μl of aprotinin (5000 KIU/ml), and was also subjected to affinity chromatography with lysine-Sepharose to obtain purified plasminogen. Both plas-
minogen preparations were analyzed by SDS gel electrophoresis. Plasminogen obtained from fresh unincubated plasma was found to have the same mobility as that of Glu-plasminogen, whereas all the plasminogen obtained from the patient’s incubated plasma had the same mobility as that of Lys-plasminogen (Fig 2). In contrast, most of the plasminogen in normal plasma was found to remain as Glu-plasminogen after the incubation (Fig 2).

**Plasmin Inhibitory Capacity of Plasma**

The capacity of the patient’s plasma to neutralize plasmin (antiplasmin activity) estimated by fibrinolytic method was 100 U/ml, which is within the range of 1 SD from the normal mean 83 ± 42 U/ml (mean ± SE, n = 12).

**Effects of Preincubation of Plasma on its In Vitro Fibrinolysis**

Citrated plasma freshly obtained from the patient was incubated at 37°C. At 0, 2, 3, and 4 hr of incubation, an aliquot was withdrawn and subjected to the euglobulin lysis time test as well as fibrinolysis study. For the fibrinolysis study, 1 ml of plasma was mixed with 25 μl of 0.5 M CaCl₂ in a glass test tube and incubated at 37°C for 7 hr. After 7-hr incubation, clots formed were removed by centrifugation and the supernatants were titrated for FRA. The results are presented in Table 3. Euglobulin lysis time was prolonged and FRA titers were decreased in proportion to the length of preincubation time of plasma before making euglobulin and plasma clots.

**Table 3. Effect of Plasma Preincubation on the Patient’s In Vitro Fibrinolysis**

<table>
<thead>
<tr>
<th>Plasma Preincubation (hr)</th>
<th>Euglobulin Lysis Time (min)</th>
<th>FRA (titers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>300x</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>200x</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>100x</td>
</tr>
<tr>
<td>4</td>
<td>130</td>
<td>32x</td>
</tr>
</tbody>
</table>

**Effects of Tranexamic Acid on the Patient’s Fibrinolysis**

Blood was mixed with tranexamic acid immediately after drawing blood in a ratio of 10 μl tranexamic acid (10%) to 1 ml blood, and incubated in a glass test tube at 37°C. The blood clot formed was not lysed, and FRA in the serum separated after 7-hr incubation was less than 3 μg/ml.

Five milliliters of 10% tranexamic acid was injected intravenously into the patient (body weight 45 kg). At 30 min after the injection, blood was drawn and in vitro fibrinolysis was tested. The blood clot was not lysed and FRA in the serum separated after 7 hr incubation was less than 3 μg/ml.

Five-hundred milligrams of tranexamic acid was given orally to the patient, and blood was drawn at 3 and 12 hr after the intake. The FRA titers of the sera separated after 7-hr incubation of these blood samples were only 8 times and 64 times, respectively.

The patient previously developed small purpura a few hours after venepunctures or incisions made for bleeding time test at the sites of the punctures or incisions. The development of purpura at these sites was abolished by oral intake of 1.5 g of tranexamic acid t.i.d.

**DISCUSSION**

The striking abnormality found in the laboratory examinations of the patient with congenital deficiency of α2PI was the spontaneous lysis of the blood clot formed in vitro. This abnormally increased fibrinolytic activity was solely ascribed to the deficiency of α2PI. As seen in Table 1, the accelerated lysis of the fibrin clot was completely corrected by an addition of α2PI to the blood to restore the inhibitor to a normal concentration. In spite of the accelerated in vitro fibrinolysis, no increased amounts of FRA and fragment A were found in the blood if the blood was mixed with inhibitors immediately after drawing to avoid in vitro fibrinolysis. This suggests that there was no accelerated degradation of fibrinogen in vivo in this patient. It is also apparent in the present study that fibrinogen in plasma was not appreciably degraded upon incubation in vitro (Table 1 and Fig. 1). In spite of an addition of urokinase, the degradation of fibrin-
The marked contrast observed between fibrinolysis and fibrinogenolysis in this patient may be explained by the differential inhibitory activity of α2-macroglobulin (α2M) on fibrinogenolysis and fibrinolysis. Concentrations of α2M and the other major plasmin inhibitors in plasma are normal in this patient and the capacity of the patient’s plasma to inhibit plasmin activity was well preserved. The affinity of α2M for plasmin is probably stronger than the affinity of plasmin for fibrinogen, and any plasmin generated in the patient’s plasma milieu may be readily inhibited by α2M and may fail to cause fibrinogenolysis. In contrast to this, α2M and probably other protease inhibitors as well are not able to inhibit fibrinolytic activity efficiently. This is in contrast to α2PI. When fibrin is formed, plasminogen and plasminogen activators are bound to formed fibrin, and plasminogen activation will take place on fibrin molecules. Thus, fibrinolysis may be dependent on the binding of plasminogen to fibrin. α2PI decreases the binding of plasminogen to fibrin, thus efficiently inhibiting fibrinolysis. Furthermore, α2PI is crosslinked to fibrin when blood is clotted, thus making the formed fibrin clot less susceptible to plasmin-catalyzed lysis. α2M and probably other protease inhibitors as well do not possess these properties and are thus unable to inhibit fibrinolytic activity efficiently. In this patient, plasminogen activation on fibrin molecules will take place freely without hindrance because of the lack of α2PI in the patient’s plasma and the inability of α2M and other inhibitors to inhibit fibrinolysis.

In vitro fibrinolysis was extensive when the blood was allowed to clot immediately after drawing the blood. However, fibrinolysis became less marked in intensity when the blood was incubated for various lengths of time before clotting, and the degree of fibrinolysis was inversely proportional to the length of preincubation of plasma (Table 3). Euglobulin lysis time, which mainly reflects plasminogen activator activity, was prolonged in proportion to the length of plasma preincubation time (Table 3). These results may suggest that plasminogen activator activity present at the time of fibrin formation is critical for the lysis of the formed fibrin.

In this patient, it was observed that plasminogen activation during spontaneous in vitro fibrinolysis was surprisingly small. Plasminogen consumed by activation during the complete clot lysis was approximately 8%-10% of the original plasminogen when measured by caseinolytic method. Plasmin generated in this patient may be ultimately inhibited by α2M, yielding α2M-plasmin complex, since α2M is the most reactive plasmin inhibitor among the inhibitors in plasma other than α2PI. The presence of α2M-plasmin complex in the serum after complete clot lysis was suggested by the ability of the serum to hydrolyze plasmin specific tripeptide substrate, since α2M-plasmin complex is known to retain a certain extent of enzymatic activity towards low molecular weight substrates. Although a small but significant generation of plasmin was observed during spontaneous clot formation and lysis, no generation of plasmin was detected when plasma was not clotted. This enhancement of plasminogen activation in the presence of fibrin can probably be ascribed to a favorable microenvironment created on fibrin molecules by the adsorption concentration of plasminogen and plasminogen activators to fibrin.

The patient’s plasminogen was found to exist in vivo as native Glu-plasminogen, whose amino-terminal residue is glutamic acid. When the patient’s plasma was incubated at 37°C for 7 hr, all the plasminogen in plasma was converted to Lys-plasminogen with lysyl-amino terminus (Fig. 2). In the normal control plasma, plasminogen remained as Glu-plasminogen. This indicates that a small amount of plasmin, below the detection limits of our assay, generated in the patient’s plasma may have acted upon plasminogen to yield Lys-plasminogen before being neutralized by α2M, since plasmin is believed to catalyze the conversion of Glu-plasminogen to Lys-plasminogen. The amount of plasmin generated may have been sufficient to convert Glu-plasminogen to Lys-plasminogen but not enough to cause any appreciable structural change in fibrinogen (Fig. 1). In normal plasma, it is most likely that plasmin formed in plasma was immediately inhibited by α2PI and that there was little chance for plasmin to act on plasminogen. The appearance of Lys-plasminogen in the patient’s plasma might be contributory to the patient’s accelerated fibrinolysis, since Lys-plasminogen is more susceptible to activation than native Glu-plasminogen.

Synthetic fibrinolysis inhibitors, tranexamic acid, and its prototype 6-aminohexanoic acid, are known to inhibit fibrinolysis in a way similar to α2PI. In fact, tranexamic acid corrected the patient’s abnormal fibrinolytic state in vitro and in vivo. Intravenous or oral administration of the drug corrected not only the abnormal fibrinolysis but also the hemorrhagic tendency, which is caused by susceptibility of hemo- static plug to fibrinolysis because of α2PI deficiency.


Fibrinolytic states in a patient with congenital deficiency of alpha 2-plasmin inhibitor

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