Fibrin-Associated Plasminogen Activation in \( \alpha_2 \)-Plasmin Inhibitor Deficiency

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The clot formed from the plasma of a patient with congenital deficiency of \( \alpha_2 \)-plasmin inhibitor underwent a spontaneous extensive fibrinolysis. Radiolabeled fibrinogen was added to the plasma before clotting, and the whole process of the fibrinolysis was followed by measuring the release of radiolabels. Plasminogen activation was also followed by measuring the amidolytic activity that developed. There was an initial latent period, followed by an exponential increase of fibrinolytic activity. During the latent period, there was little or no release of radiolabels and no development of amidolytic activity. During the latent period, the clot was washed thoroughly to remove unbound proteins from fibrin and was incubated in buffered saline. The washed clot still underwent fibrinolysis, similar to the original plasma clot, suggesting that the plasminogen/plasminogen activators bound to fibrin during the initial latent period are responsible for fibrinolysis. The amount of plasminogen bound to fibrin during the latent period was close to the amount of plasminogen activated during the whole process of fibrinolysis. When the amount of plasminogen bound to fibrin was decreased by epsilon aminocaproic acid, the extent of fibrinolysis was decreased in parallel with the decrease of the amount of the bound plasminogen. This suggests that the amount of plasminogen bound to fibrin is one of the determinants of the rate of the fibrinolytic process.

\( \alpha_2 \)-PLASMIN INHIBITOR (\( \alpha_2 \)-PI) or \( \alpha_2 \)-antiplasmin is a proteinase inhibitor that efficiently inhibits plasmin-catalyzed fibrinolysis.\(^1\) Its congenital deficiency results in a severe hemorrhagic tendency\(^2,3\) that can be explained by a reduced resistance of hemostatic plugs to physiologically occurring endogenous fibrinolysis because of the deficiency of \( \alpha_2 \)-PI.\(^4,5\)

In our previous study, it was shown that the blood or plasma clot obtained from a patient with congenital deficiency of \( \alpha_2 \)-PI spontaneously underwent an extensive fibrinolysis, whereas no fibrinogenolysis occurred when blood plasma was not clotted.\(^6\) Two possible explanations were presented for the absence of fibrinogenolysis.\(^7\) One explanation involves the role exerted by \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)-M). Fibrinogen may be protected from plasmin degradation by \( \alpha_2 \)-M, which can effectively inhibit plasmin generated in a liquid phase, such as plasma,\(^6,8\) although it is ineffective for the inhibition of fibrinolysis.\(^9\) An additional explanation is that plasminogen is not readily activated by endogenous activator when fibrin is not formed.\(^10,11\)

In the present study, spontaneously occurring endogenous fibrinolysis observed in a patient with congenital deficiency of \( \alpha_2 \)-PI is shown to be caused by plasminogen activation, which takes place in close association with fibrin, that is, the activation of fibrin-bound plasminogen by fibrin-bound activators.

**MATERIALS AND METHODS**

**Plasma**

Blood was withdrawn from the antecubital veins of normal subjects or a patient with congenital deficiency of \( \alpha_2 \)-PI after obtaining informed consent, into 0.1 volume of 3.8% sodium citrate. The blood was then centrifuged at 2,000 \( g \) for 20 min to prepare platelet poor plasma.

**Fibrinogen**

Human fraction I-4, prepared according to the method of Blomback and Blomback,\(^12\) was used as the fibrinogen preparation after removing contaminating plasminogen and plasma fibronectin from the preparation with lysine-Sepharose\(^13\) and gelatin-Sepharose,\(^14\) respectively. The concentration was determined spectrophotometrically using \( A_{280}^\text{nm} = 15.1 \) at 280 nm,\(^15\) and the coagulable protein was \( >95\% \). Fibrinogen was dissolved in barbital-buffered saline (0.005 \( M \) barbital acetic acid-0.14 \( M \) NaCl, pH 7.4), and the concentration was adjusted to 2% (w/v) coagulable protein.

**Thrombin**

Purified thrombin was prepared from a bovine thrombin preparation (Parke-Davis Co., Detroit, MI) according to the method of Lundblad.\(^16\)

**Plasminogen**

Native glutamyl-plasminogen (NH\(_2\)-terminal glutamic acid, Glu-plasminogen) was prepared from fresh human plasma in the presence of aprotinin (10 KIU/ml) (Mochida Pharm. Co., Tokyo, Japan) by affinity chromatography on lysine-Sepharose, followed by DEAE-Sephadex chromatography\(^17\) and by molecular exclusion chromatography on Ultrogel ACA 44 (LKB, Stockholm, Sweden).\(^18\)

**Other Reagents**

The plasmin substrate S-2251 (H-\(\beta\)-valyl-leucyl-lysine-p-nitroanilide dihydrochloride) was purchased from Kabi Diagnostica, Stockholm, Sweden. \( \varepsilon \)-Aminocaproic acid (EACA) was purchased from Wako Pure Chemical Inc. (Osaka, Japan).

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Supported in part by a Research Grant for Cardiovascular Diseases 55A-1 from the Ministry of Health and Welfare, and a research grant from the Ministry of Education, Japan.

Submitted November 16, 1982; accepted June 23, 1983.

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Coagulability was not changed by iodination, and the 251-labeled system, Aloka ARC-600 (Aloka Co., Tokyo). The labeled fibrinogen preparation had a specific radioactivity of 6.29 x 10^5 cpm/μg. Coagulability was not changed by iodination, and the 125I-labeled fibrinogen preparation had a specific radioactivity of 5.43 x 10^5 cpm/μg.

Radioiodination of Protein

Purified Glu-plasminogen and fibrinogen were radioiodinated by the solid-state lactoperoxidase method of David,23 using lactoperoxidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) and 125I-Na (18.5 Ci/ml) (New England Nuclear, Boston, MA). Radioactivity was counted by the Auto-Well Gamma System, Aloka ARC-600 (Aloka Co., Tokyo). The labeled fibrinogen preparation had a specific radioactivity of 6.29 x 10^5 cpm/μg. Coagulability was not changed by iodination, and the 125I-labeled fibrinogen preparation had a specific radioactivity of 5.43 x 10^5 cpm/μg.

Spontaneous In Vitro Fibrinolysis

One milliliter of α2Pl-deficient fresh plasma or normal plasma was mixed with 2 μl of radiolabeled fibrinogen. Four-hundred-microliter aliquots of this mixture were counted for radioactivity and were mixed with 10 μl of calcium chloride (0.5 M). The mixture was clotted with 10 μl of thrombin (100 U/ml) and incubated at 37°C for lysis. Aliquots of 25 μl were removed from the liquid phase at intervals for radioactive counting. Results were expressed as the percent release of radiolabel, which was calculated from the counts, applying a correction for the influence of repeated subsampling on the volume. These experiments were performed in duplicate. In some experiments, the clot was washed at 30 min after clotting and then suspended in buffered saline. The clot was first squeezed with a bamboo stick against the wall of the tube to express as much fluid as possible, washed 3 times by soaking it in 2 ml of Tris-buffered saline (Tris 0.05 M, NaCl 0.14 M, pH 7.4) containing 2% of human albumin (Miles Laboratories, Inc., Elkhart, IN) for 5 min at room temperature each time, and then finally suspended in 1 ml of buffered saline containing 2% albumin. Fibrinolysis was measured in the same way as described above.

Plasminogen Activation During Clot Lysis

Plasminogen activation during in vitro clot lysis of α2Pl-deficient plasma or during the incubation of normal plasma clot was followed by measuring the spontaneously generated plasmin activity (amidolytic activity). Two hundred microliters of α2Pl-deficient plasma was mixed with 5 μl of calcium chloride (0.5 M). The mixture was clotted with 5 μl of thrombin (100 U/ml) and incubated at 37°C for the amidolytic assay. 90 μl of Tris-buffered saline and 600 μl of 3 mM S-2251 were added to the plasma clot mixture after various lengths of time. The clot was immediately squeezed with a bamboo stick to express as much fluid as possible. This mixture, containing the suspended clot, was further incubated at 37°C. Exactly 3 min after the addition of S-2251, 100 μl of 50% acetic acid was added and mixed to stop the reaction. The absorbance of the liquid phase at 405 nm was measured, and the amidolytic activity was expressed as nanokatals per milliliter of the original mixture using an absorbance value of 10.5 at 405 nm for 1 mM p-nitroaniline. As a blank, acetic acid was added at the time of S-2251 addition. Fibrinogen consumption during the process of the clot lysis was assessed by a caseinolytic assay for plasminogen.24

Binding of Plasminogen to Fibrin

Five microliters of the radioiodinated Glu-plasminogen was added to 2 ml of α2Pl-deficient plasma. Two-hundred-microliter aliquots of the mixture were counted for radioactivity by the Auto-Well Gamma system, Aloka ARC-451 (Aloka Co., Tokyo, Japan) and clotted with 10 μl of thrombin (50 U/ml) containing 0.5 M calcium chloride. After incubation at 37°C for 30 min, the clot formed was squeezed with a bamboo stick against the wall of the tube to express as much fluid as possible. The fibrin was then washed 3 times by soaking it and shaking it in 2 ml aliquots of 2 M NaCl, 0.05 M Tris-HCl buffer, pH 7.4, containing aprotinin (10 KIU/ml) and 2% human albumin (Miles Laboratories, Inc., Elkhart, IN), to wash out unbound material, for 5 min at room temperature each time. The amount of plasminogen bound to fibrin was calculated from the radioactivity remaining in the washed clot and the original radioactivity present in the clotting plasma, and it was expressed as a percentage of the total plasminogen present in the original plasma.

RESULTS

Fibrinolysis of the Plasma Clot

Spontaneous in vitro fibrinolysis of the α2Pl-deficient plasma clot was followed by the release of radioactivity from radiolabeled fibrin and by assaying for the amidolytic activity developed. There was no appreciable release of radioactivity during the initial half-hour period, after which the release exponentially increased until it reached a plateau level, where complete lysis occurred (Fig. I). There was a small amount of amidolytic activity (0.15 ± 0.002 nkat/ml, n = 5) in plasma before clotting, and this activity was not appreciably increased during the initial half-hour period after clotting (Fig. I). After this lag time, there was also an exponential increase of amidolytic activity until it reached a plateau, where fibrin was completely lysed (Fig. I). The plasminogen consumed during the whole process of fibrinolysis was 0.24 U/ml or 10% of the plasminogen present in the plasma on the average, which was calculated from the significant difference (p < 0.02) between the plasma plasminogen activity (2.39 ± 0.16 U/ml, n = 5) and the serum plasminogen

Fig. 1. Spontaneous fibrinolysis of the α2Pl-deficient plasma clot. The plasma containing radiolabeled fibrinogen was clotted by thrombin in the presence of calcium ions and incubated at 37°C. After various lengths of time, release of radiolabel (O) from the clot into the liquid phase was measured and expressed as a percentage of the total radioactivity (A). The amidolytic activity that developed (O) was also measured, using S-2251 as a substrate (B). Normal plasma clots were incubated as controls (●). n kat: Nanomoles of substrate hydrolyzed per second.
activity (2.15 ± 0.08 U/ml, n = 5) after complete fibrinolysis. No consumption of plasminogen was detected during the lag time. When the plasma was not clotted, no amidolytic activity was developed and no plasminogen was consumed. When normal plasma clot was incubated, only a small release of radioactivity and no increase of amidolytic activity were observed. The cumulative release of radioactivity was, at most, 10% at 8 hr of clot incubation, even if plasma was obtained from an individual who had taken a strenuous physical exercise or was obtained from a vein occluded for 10 min by tourniquet (Fig. 1). Physical exercises and venous occlusion are known to increase plasminogen activator content in blood.

**Fibrinolysis of the Washed Clots**

α2Pl-deficient plasma clots were squeezed and washed thoroughly to remove unbound material from fibrin at 30 min after clotting. These washed clots were then suspended in buffered saline, and fibrinolysis was observed. Fibrinolysis occurred similarly to the plasma clots (Fig. 2). When normal plasma clot was washed and suspended in buffered saline, little fibrinolysis was observed (Fig. 2), even if plasma was obtained after a strenuous physical exercise or after venous occlusion and contained an increased amount of plasminogen. When EACA was added to α2PI-deficient plasma before clotting in the same experiments, the extent of fibrinolysis decreased proportionally to the amount of EACA added (Fig. 3).

**Plasminogen Bound to Fibrin During the Initial Period of Clot Incubation**

After a 30-min incubation of α2PI-deficient plasma clot, the amount of plasminogen bound to fibrin was 8% ± 1.05% (n = 5) of the total plasminogen present in plasma. When EACA was added to plasma before clotting, the amount of plasminogen bound to fibrin was decreased proportionally to the amount of EACA added (Fig. 3).

**DISCUSSION**

When the α2Pl-deficient fresh plasma clot was incubated at 37°C, the clot was spontaneously lysed after several hours. Plots of the cumulative release of 125I-fibrin degradation products from the clots yielded sigmoidal curves, and there was a lag time lasting for more than half an hour (Fig. 1A). During the lag time, little or no activation of plasminogen was observed, since there was no appreciable generation of amidolytic activity (Fig. 1B), and there was no detectable consumption of plasminogen. During this initial latent period, the fibrin clot formed was thoroughly washed to remove unbound proteins and incubated in buffered saline. The washed clot was still lysed similarly to the plasma clot (Fig. 2). The amount of plasminogen bound to the washed clot was about 8% of the total plasminogen present in the plasma, and this amount was enough to lyse the clot in 8 hr. The amount of the bound plasminogen (8%) was close to the amount of plasminogen consumed (about 10%) during the whole process of plasma clot lysis, suggesting that most of the plasminogen activated during fibrinolysis was the
FIBRIN-ASSOCIATED PLASMINOGEN ACTIVATION

When the amount of fibrin-bound plasminogen was decreased by EACA, the rate of fibrinolysis was decreased in parallel with the decrease in the amount of bound plasminogen (Fig. 3). These observations indicate that spontaneous fibrinolysis observed in an α2Pl-deficient plasma clot is carried out by plasminogen and plasminogen activator(s) bound to fibrin during the initial latent period, and the extent or the rate of fibrinolysis is mainly determined by the amounts of these bound components.

When α2Pl-deficient plasma was incubated without formation of fibrin, no activation of plasminogen was observed in the previous study,12 nor in the present one. This indicates an important role for fibrin in the activation of plasminogen in the spontaneous fibrinolysis of the α2Pl-deficient plasma clot. Fibrin is known to greatly increase (up to 1,000-fold) the activation rate of plasminogen25 by binding both plasminogen and tissue-type (nonurokinase type) plasminogen activator to fibrin.26 Vascular plasminogen activator, which is believed to be secreted from vascular endothelial cells and to comprise the major portion of plasminogen activators present in circulating blood, is one of the tissue-type plasminogen activators and has a strong affinity for fibrin.26 Plasminogen activator responsible for spontaneous lysis of the α2Pl-deficient plasma clot is most likely vascular plasminogen activator, and gradual formation of the ternary complex of fibrin, plasminogen activator, and plasminogen25,27 may have taken place during the initial latent period before the burst of plasminogen activation occurs.

The fibrin-associated process of plasminogen activation observed in the α2Pl-deficient plasma clot is considered to be a physiologically occurring mechanism of fibrinolysis. In normal plasma, however, this physiologically occurring fibrinolytic process is blocked or retarded by α2Pl, which interferes with plasminogen binding to fibrin15,28-30 and inhibits plasmin formed in situ on fibrin molecules by being crosslinked to fibrin.13,31,32 In the present study, spontaneous fibrinolysis of normal plasma clot in vitro was slight, and no generation of amidolytic activity was detected (Figs. 1 and 2). It is conceivable, however, that a continued supply of tissue-type plasminogen activator and plasminogen in vivo from circulating blood to thrombi may overcome the inhibitory effect of α2Pl and bring about the dissolution of thrombi.

ACKNOWLEDGMENT

We are grateful to Dr. M. Kobakura for allowing us to obtain blood from the patient with congenital deficiency of α2Pl.

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