The Mechanism of In Vitro Clot Lysis Induced by Vascular Plasminogen Activator

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The contribution of vascular plasminogen activator (v-PA) to the lysis of whole blood and plasma clots was investigated. v-PA released into the circulation after infusion of deamino-o-arginine vasopressin (DDAVP) was shown to bind quantitatively to plasma clots. Its apparent molecular weight, determined by the SDS-PAGE fibrin-agarose underlay method, was approximately 68,000 daltons, and its activity was quenched by antibodies against human tissue plasminogen activator (t-PA). Clots prepared from post-DDAVP plasma or post-DDAVP whole blood, rich in v-PA, did not lyse when incubated in imidazole buffer or normal plasma, as determined by the release of 125I from radiolabeled clots. However, clots made of v-PA-poor plasma or whole blood, incubated in v-PA-rich plasma, underwent substantial lysis. The concentration of PA in clots incubated in v-PA-rich plasma progressively increased in relation to the initial concentration of v-PA in the surrounding plasma. The results suggest that, at low concentrations of circulating v-PA, a hemostatic plug will lyse at a very low rate. However, when the v-PA concentration in the clot environment is increased, v-PA will accumulate progressively onto fibrin and induce thrombolysis.

IN BLOOD, lysis of fibrin is primarily due to the action of plasmin, a serine protease with high affinity for fibrin. The conversion of the zymogen, plasminogen, to its active form is brought about by different plasminogen activators (PA).1 Vascular plasminogen activator (v-PA) is produced by endothelial cells and is released into the circulation in response to a variety of stimuli.2-5 A derivative of vasopressin, deamino-d-arginine vasopressin (DDAVP), is increasingly used to investigate the extent of the release of v-PA in normal subjects and in patients suffering from recurrent venous thrombosis.6-8 v-PA has been purified from perfusates of the vascular tree of human cadavers9-11 and from plasma obtained after exercise12,13 or after venous occlusion.14 It is similar or identical to tissue plasminogen activator (t-PA). These two PAs have the same molecular weight and have strong affinities for fibrin.10,11,15,16 The activity of v-PA is quenched by anti-t-PA antibodies.2,2 The conversion of plasminogen to plasmin by v-PA and t-PA is greatly accelerated in the presence of fibrin, whereas fibrinogen is only effective at concentrations two to three orders of magnitude greater.17,18 Clot lysis induced by v-PA and t-PA has been studied in fibrin, plasma, and whole blood clots,19-22 but little attention has been paid to the effect of PA bound to fibrin versus the effect of PA in the surrounding milieu.

The aim of our work was to evaluate the lysis of whole blood clots induced by v-PA-rich plasma obtained after DDAVP administration and by v-PA-poor plasma. In particular, we wanted to compare the contribution to the lysis process of v-PA incorporated into the clot during coagulation with that of v-PA present in the surrounding plasma.

MATERIALS AND METHODS

CaCl2H2O, NaCl, and acetic acid were obtained from E. Merck, Darmstaidt, Germany. Imidazole, Tris, EACA, EDTA, K2HPO4, and fraction V bovine serum albumin were purchased from Fluka, Buchs, Switzerland; heparin (Liquemin) and thrombin (Topostasin) were from Roche, Basel, Switzerland. Thrombin was rendered plasminogen-poor by affinity chromatography on heparin-Sepharose 4B.24 Buffered citrate (citric acid/sodium citrate, 0.1 M, pH 4.5) was obtained from Behringwerke, Marburg, Germany; aprotinin (Trasylol) was from Bayer, Leverkusen, Germany. DEAE Affi-Gel Blue and all reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad, Richmond, CA. CNBr-activated Sepharose 4B and dextran (Macrodex, 6%) were from Pharmacia, Uppsala, Sweden. Bovine fibrinogen (fraction I, 80% clottable) was purchased from Sigma, St. Louis, MO. 125I-fibrinogen (Fibi-125 C, 0.3-0.4 mCi/mg 90% clottable) was purchased from Serono Laboratories, Saluggia, Italy. DDAVP was purchased from Ferring Pharmaceuticals, Malmö, Sweden. The imidazole buffer, pH 7.35, which was used for many experiments, contained 0.05 M imidazole and 0.14 M NaCl, unless specified otherwise. Clinical and highly purified high molecular weight urokinase (UK; 100,000 IU/mg) were a gift from Serono Laboratories, Coinsins, Switzerland, and were standardized against the international human urokinase reference preparation 66/46, donated by P. Gaffney of the National Institute for Biological Standards and Controls, London, UK. Pig heart t-PA was prepared according to methods previously described.24 Antibodies raised in goats against human t-PA from HeLa cells were kindly provided by W. D. Schleuning. Final concentrations are indicated in this article.
Subjects

Normal blood was obtained from apparently healthy blood donors. Blood samples, obtained prior to and after DDAVP infusion (pre-DDAVP, post-DDAVP), were collected from 4 consenting human volunteers, aged 30–40 yr, who, after an overnight fast, had rested for 20 min in supine position. DDAVP (0.4 μg/kg body weight) was infused intravenously over a 5-min period. Ten minutes after the beginning of the DDAVP infusion, collection of 300–400 ml of blood was begun from the contralateral antecubital vein and lasted usually between 5 and 10 min.

Preparation of Plasma and Serum

Platelet-poor plasma was prepared from blood (9 parts) collected in buffered citrate, containing 50 mM EDTA (1 part, final pH 7.2), by centrifugation for 30 min at 2,500 g and 4°C. Serum was prepared from blood, collected in glass containers, or from plasma by addition of 10 U/ml of thrombin in the presence or absence of 15 mM CaCl₂. After incubation for 30 min at room temperature, the tubes were centrifuged for 30 min at 2,500 g and 4°C. For some experiments, aliquots of plasma and serum were stored at -70°C and thawed at 37°C for 10 min prior to use.

Fibrin Plate Assay

The fibrin plate assay was performed as previously described. For the determination of fibrinolytic activity, 30-μl test samples or PA standards were deposited in quadruplicate on the fibrin plates and incubated for 16 hr at 37°C. A linear correlation was obtained when the mean lysis diameter was plotted against the logarithm of the PA concentration (r = 0.99). Each experiment was performed at least in triplicate. Unless otherwise specified, all results are expressed in arbitrary t-PA units. One PA unit equals one international unit of urokinase for a lysis diameter of 15 mm (Fig. 1).

Euglobulin Preparation

An aliquot of 0.3 ml of plasma or serum was diluted 20-fold with ice-cold deionized water and the pH adjusted to 5.8–5.9 with 0.17 M acetic acid. After centrifugation at 800 g and 4°C for 5 min, the supernatant was discarded, and the tube walls were carefully dried with an absorbent paper. The euglobulin precipitate was redissolved in 0.3 ml of imidazole buffer and its activity tested on fibrin plates.

Binding of v-PA to Clots

Binding of v-PA to noncrosslinked plasma clots was studied by addition of thrombin (10 U/ml) to 0.5 ml of citrate-EDTA plasma. After centrifugation for 20 min at 4°C and 2,500 g, clots were squeezed on the tube walls with a plastic spatula and the supernatant saved. Clots were washed in physiologic saline and depolymerized in an initial volume of 0.017 M acetic acid at 4°C. Uptake of v-PA by preformed 0.2-ml normal plasma clots was evaluated by incubating clots under continuous end-to-end mixing for 0–30 min in 1.5 ml of normal or post-DDAVP plasma or of mixtures of these two. The activity of the resolubilized clots was assayed on fibrin plates.

Inhibition of v-PA by Goat Antibodies Against Human t-PA and Human UK

Two-milliliter aliquots of normal goat serum, of goat anti-human high molecular weight urokinase (UK) or of goat anti-human t-PA serum were dialyzed against 20 mM potassium phosphate (pH 8.0) and passed over a 15-ml column of DEAE-Cellulose Blue B. The breakthrough peak contained the IgG fraction. Post-DDAVP citrate-EDTA plasma was clotted in the presence of aprotinin (100 KIU/ml). The clot was isolated, washed in physiologic saline, and depolymerized in 0.017 M acetic acid. The depolymerized and euglobulin made from the same plasma were incubated for 30 min at room temperature with an equal volume of physiologic saline containing 1% BSA and normal goat IgG or IgG directed against human t-PA or UK. The remaining fibrinolytic activity was assayed on fibrin plates.

SDS-PAGE Followed by Zymographic Detection on a Fibrin-Agarose Underlay

SDS-PAGE was performed according to Laemmli, using a 7.5% polyacrylamide separating gel of 12.7 x 14 x 0.15 cm and a 4% stacking gel. Solutions of pig heart t-PA (4 U/ml) and of depolymerized pre- and post-DDAVP plasma clots were mixed with sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.02% bromophenol blue. One hundred microliters of the mixture was applied. After 5 hr of electrophoresis at 25 mA/gel, the gels were washed for 1 hr with 2.5% Triton X-100. The fibrinolytic activity was zymographically revealed by placing the gels on a plasminogen-rich fibrin-agarose underlay for 6 hr at 37°C.

Whole Blood Clot Lysis

Normal or post-DDAVP blood was collected in plastic tubes containing an appropriate amount of 125I-fibrinogen to yield 125,000 cpm/ml of blood. After thorough and rapid mixing, 0.2-ml aliquots of blood were transferred into glass tubes. After incubation at 37°C for 1 hr, the clots were washed in 2 ml of 0.15 M NaCl at 4°C and transferred into plastic tubes containing 1.5 ml of normal plasma, post-DDAVP plasma, various mixtures of these two, or isotonic imidazole buffer containing 3% BSA. After incubation for 30, 60, 120, and 180 min at 37°C under continuous end-to-end mixing, the clots were washed again and their radioactivities measured. Results were expressed in percent of the total initial radioactivity, where 100% represents the sum of the activities of the clots, washing solutions, and medium in each experiment.

Plasma Clot Lysis

v-PA-rich (post-DDAVP) and v-PA-poor (pre-DDAVP) plasma were mixed with 125I-fibrinogen (125,000 cpm/ml plasma). Aliquots of 0.2 ml were transferred to glass tubes and clotted with thrombin (10 U/ml) in the presence of 15 mM CaCl₂ to ensure crosslinking of fibrin. After rapid and thorough mixing, the clots were incubated at
**RESULTS**

**Standard Curves of Various Plasminogen Activators Using the Fibrin Plate Assay**

Figure 1 demonstrates that straight and parallel curves were obtained when the logarithm of serial dilutions of pig heart t-PA, of euglobulins prepared from mixtures of post-DDAVP and normal plasma, or of serial dilutions of post-DDAVP plasma euglobulins were plotted against lysis zone diameters. Steeper calibration curves were obtained with urokinase. On the basis of these observations, all v-PA activities in this article were expressed in arbitrary pig heart t-PA units, designated as plasminogen activator (PA) units.

**Fibrinolytic Response to Intravenous Infusion of DDAVP in Human Volunteers and Characterization of Vascular Plasminogen Activator**

The response to DDAVP infusion in four volunteers was variable. Post-DDAVP plasma euglobulins prepared from citrate-EDTA plasma exhibited PA activities from 0.5 to 5 U/ml, corresponding to a tenfold (range 4–20) mean increase over baseline activity.

Using the SDS-PAGE fibrin-agarose underlay technique, the molecular weight of post-DDAVP plasminogen activator that had bound to a plasma clot was determined. The value of approximately 68,000 daltons was similar to that of pig heart t-PA (Fig. 2). Even after incubation times of up to 48 hr, the pre-DDAVP depolymerized plasma clots did not produce a lysis band at the 68-kd position. Figure 3 illustrates that the plasminogen activator released after infusion of DDAVP is immunologically related to human t-PA and was not quenched by goat anti-human high molecular weight UK IgG.

**Binding of Physiologic Plasminogen Activators onto Plasma Clots**

Figure 4 illustrates that redissolved noncrosslinked pre-DDAVP plasma clots contained approximately the same PA activity as the corresponding plasma euglobulins.
ulins. No activity was detectable in the serum euglobulins. In general, the activity recovered from post-DDAVP plasma clots was 50% higher than that of the corresponding plasma euglobulins. Because fibrin greatly accelerates the v-PA-induced conversion of plasminogen into plasmin, we questioned whether aprotinin, an inhibitor of the active site of plasmin, or EACA, an inhibitor preventing the binding of plasmin(ogen) to fibrin, had any effect on the activity recovered from the plasma clots. In the presence of these inhibitors, the activity recovered in the depolymerized post-DDAVP clot was the same as that in the corresponding plasma euglobulins (Fig. 5).

**Uptake of Plasminogen Activators From the Incubation Medium by Clots**

To determine if v-PA is taken up by clots from the surrounding medium, noncrosslinked normal plasma clots were incubated at 37°C in normal and v-PA-rich plasma. Figure 6 demonstrates that PA activity in the clot incubated in v-PA-rich plasma increased progressively from less than 0.1 U/ml to 3.9 U/ml during a 30-min incubation. No increase of PA activity was observed in clots that had been immersed in normal plasma.

There was good correlation \( r = 0.9 \) between v-PA concentration in the incubation milieu and the recovery of PA from noncrosslinked plasma clots (Fig. 7).

**Lysis of Noncrosslinked Plasma Clots and Effect of Calcium (Crosslinking) on Clot Lysis**

The crosslinking of fibrin and the covalent binding of \( \alpha_2 \)-antiplasmin to fibrin effected by factor XIIIa are dependent on the presence of free calcium ions.\(^{29}\) As this mechanism renders clots more resistant to the action of plasmin, we measured the lysis of post-DDAVP plasma clots prepared in the presence of various concentrations of calcium chloride, followed by incubation in post-DDAVP citrate-EDTA plasma. Lysis was complete if insufficient amounts of calcium ions were present during coagulation to allow the crosslinking process to take place. At \( \text{Ca}^{2+} \) concentrations corresponding to or surpassing those found in normal serum, plasma clot lysis was about 40% (Fig. 8).

**Effect of Surrounding Medium on the Lysis of Whole Blood Clots**

Figure 9 demonstrates that v-PA-rich whole blood clots incubated in normal plasma showed only 15% lysis after 3 hr, whereas v-PA-poor whole blood clots immersed in v-PA-rich plasma exhibited twice as much lysis \( (p < 0.01, \text{Student's } t \text{ test}) \). Lysis was absent in normal blood clots suspended in normal plasma or buffer and was highest (50%) in v-PA-rich clots.
VASCULAR PLASMINOGEN ACTIVATOR

There was good correlation ($r = 0.9$) between v-PA concentration in the incubation milieu and the degree of lysis of normal whole blood clots incubated in this milieu (Fig. 10).

Because cellular elements present in whole blood clots might contribute to clot lysis, we compared the lysis of post-DDAVP platelet- and leukocyte-poor crosslinked plasma clots and that of post-DDAVP whole blood clots incubated in imidazole buffer and normal and post-DDAVP citrate-EDTA plasma. In all three media, lysis of whole blood clots was slightly greater than that of crosslinked plasma clots (data not shown).

DISCUSSION

A large proportion of patients with idiopathic or recurrent deep vein thrombosis exhibit a low content of plasminogen activator in their vascular walls, prolonged euglobulin lysis times in the resting state, and/or a diminished release of v-PA from vessel walls after venous occlusion or after the administration of DDAVP. Peduzzi et al. have shown that patients with thrombosed retinal veins that did not recanalize after venous occlusion or after the administration of DDAVP,32 also exhibited a poor fibrinolytic response to venous stasis.33

Our studies attempt to clarify the contribution of circulating v-PA to clot lysis. The intravenous administration of 0.4 μg/kg body weight of DDAVP to apparently normal human volunteers induced a 4–20-fold increase of PA activity, confirming previous reports.5,6

The molecular weight of PA released after DDAVP infusion was approximately 68,000 daltons. PA activity released after DDAVP administration was blocked by goat anti-human t-PA IgG, but not by goat anti-UK or goat nonimmune IgG. These two observations confirm those of other investigators.7,9,10,14 The incomplete neutralization of PA activity in post-DDAVP plasma euglobulins or of redissolved clots might be due to the presence of minute amounts of non-v-PA (UK, kallikrein, plasmin) precipitated in euglobulins or entrapped in the clots, even after washing. Likewise, PA released after venous stasis was demonstrated to be indistinguishable from human t-PA,2 and it thus appears that PA released after DDAVP and after venous stasis are identical. As the systemic v-PA concentration attained after DDAVP infusion is similar to the local v-PA concentration in an occluded vein segment,5 post-DDAVP plasma can be used to study the contribution of v-PA to physiologic fibrinolysis.

v-PA bound quantitatively to noncrosslinked post-DDAVP plasma clots and could be recovered from washed clots, which were subsequently depolymerized; no activity was detectable in the corresponding serum. Furthermore, using the fibrin plate assay and the zymographic method, we were unable to detect v-PA activity in the sera of crosslinked clots prepared from post-DDAVP whole blood or plasma (data not shown). When noncrosslinked post-DDAVP clots were dissolved in acetic acid, the recovered PA activities were consistently 50% higher than those determined in the corresponding plasma euglobulins. This difference was abolished when aprotinin or EACA were added to the plasma prior to coagulation. Thus, it appears that the observed increase of PA activity eluted from the clot is either directly due to plasmin or plasmin mediated.

There was, in addition to the quantitative binding of v-PA to fibrin during clotting, a time- and concentration-dependent uptake of v-PA from the surrounding medium onto a preformed clot.

In spite of the binding of v-PA to the clot during coagulation, crosslinked post-DDAVP plasma or whole blood clots did not lyse appreciably during incubation in imidazole buffer or normal human plasma. This resistance to fibrinolysis results from the presence of Ca$^{2+}$ during clotting, which allows crosslinking of fibrin and of α2-antiplasmin to fibrin.29,34 However, when Ca$^{2+}$ concentrations were insufficient
to counteract the effect of EDTA, significant fibrinolysis did take place in noncrosslinked post-DDAVP plasma clots.

Normal crosslinked plasma clots or whole blood clots containing little or no v-PA showed significant lysis when incubated in post-DDAVP plasma, a milieu rich in v-PA. We were able to demonstrate that this phenomenon is explained by the progressive uptake of v-PA onto the clots. Indeed, there was good correlation between the concentration of v-PA in the incubation milieu on the one hand and lysis of whole blood clots, as well as uptake of v-PA by noncrosslinked plasma clots, on the other hand. Lysis of whole blood clots incubated in v-PA-rich plasma was somewhat greater than that of crosslinked platelet- and leukocyte-poor plasma clots suspended in the same incubation milieu, suggesting some participation of cellular elements in physiologic clot lysis. However, this difference was small.

These observations, extrapolated to thrombus formation in vivo, suggest that v-PA plays an important role in the regulation of the hemostatic-fibrinolytic balance. The presence of Ca\(^{2+}\) in the circulating blood allows crosslinking of fibrin and of \(\alpha_2\)-antiplasmin, which prevents premature lysis of a hemostatic plug. In the absence of stasis, release of v-PA from the endothelium will be small, and clot lysis will proceed at a slow speed. In the presence of venous stasis, as is the case when an occluding thrombus is formed, v-PA will normally be released from the upstream endothelial bed into the occluded segment and progressively lyse the thrombus.

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