Roles for chloride ion and fibrinogen in the activation of [Glu¹]plasminogen in human plasma

(plasminogen activation by tissue plasminogen activator/ effectors of plasminogen activation/Cl⁻ inhibition of plasminogen activation/fibrinogen stimulation of plasminogen activation)

PATRICK J. GAFFNEY[†], TETSUMEI URANO[‡], VESNA S. DE SERRANO[‡], MAHER MAHMOUD-ALEXANDRONI^{*}, ALAN R. METZGER[‡], AND FRANCIS J. CASTELLINO^{‡§}

[†]Department of Chemistry, University of Notre Dame, Notre Dame, IN 46556; and ^{*}The National Institute for Biological Standards and Control, South Mimms, Herts, ENG 3QG, United Kingdom

Communicated by George B. Craig, Jr., January 21, 1988

ABSTRACT Using two-dimensional immunoelectrophoresis and an antibody to α_2 -antiplasmin, we assessed the plasmin generated in serum under different conditions as the plasmin- α_2 -antiplasmin complex. Activation in serum of human [Glu¹]plasminogen ([Glu¹]Pg) by recombinant tissue plasminogen activator was inhibited by the normal serum levels of Cl⁻ and was enhanced by physiological levels of fibrinogen in the presence or absence of Cl⁻. These results agree with the recognized ability of Cl⁻ to induce a conformation in [Glu¹]Pg less favorable for its activation than the conformation that results without Cl⁻. The enhancing effect of fibrinogen surpassed the inhibitory effect of Cl⁻ over a wide range of recombinant tissue plasminogen activator concentrations in physiological serum. Lesser inhibition by Cl⁻ was seen in a purified clot-lysis system, suggesting that [Glu¹]Pg conformation when attached to soluble fibrin matrix was less affected by the anion. The data regarding the roles of circulating fibrinogen and Cl⁻ in controlling the plasma level of activated [Glu¹]Pg have important implications in thrombolytic therapy with recombinant tissue plasminogen activator.

Tissue plasminogen activator (TPA) is regarded the major activator of plasminogen in human plasma and as such is an important defense via the fibrinolytic mechanism against fibrin deposition *in vivo*. A hypothetical mechanism by which TPA on the surface of fibrin causes dissolution of both preformed fibrin and forming fibrin has been proposed (1). This hypothesis, while explaining some phenomena seen during the classic hypercoagulable state known as disseminated intravascular coagulation, may not encompass all molecular events occurring during thrombotic episodes and use of TPA as a thrombolytic agent. Roles for Cl⁻ and fibrinogen have been implicated in human plasminogen ([Glu¹]Pg) activation in purified systems using streptokinase (2, 3), urokinase (4, 5), and TPA (6) as activators. These data show that Cl⁻, which exists in plasma at ≈ 90 mM, could have a significant inhibitory effect on [Glu¹]Pg activation and that the influence of fibrinogen on the initial rate of [Glu¹]Pg activation could be physiologically important during enhanced fibrinolysis *in vivo*. Although both effects have been shown in purified systems (2–4), this study was undertaken to assess the relevance of these effects on activation of [Glu¹]Pg to plasmin in its natural environment, plasma. The data suggest that anion and fibrinogen effects both need consideration when TPA is used as a thrombolytic agent; the influence of these effects on the degradation of circulating fibrinogen and the thrombus-specificity of TPA therapy must be weighed.

MATERIALS AND METHODS

Proteins. Affinity chromatography forms 1 and 2, differing in their carbohydrate content, of [Glu¹]Pg were purified by our modified procedure (7) of the Deutsch and Mertz (8) affinity chromatography technique. The lyophilized protein was dissolved in 10 mM 4-(2-hydroxyethyl)-piperazineethanesulfonic acid (Hepes)/NaOH, pH 7.4, and dialyzed against the same buffer. For determining the concentration of [Glu¹]Pg, an extinction coefficient (1%, 1 cm, 280 nm) of 17.0 and a M_r of 94,000 were used (9).

Recombinant TPA (rTPA) was a gift from Genentech (South San Francisco, CA) and was judged by reduced NaDodSO₄/PAGE to be $\approx 80\%$ single chain in form. The two-chain form of TPA was prepared for these studies. Single-chain TPA was incubated with Sepharose 4B-plasmin at a 10:1 molar ratio for 1 hr at room temperature, and complete conversion to two-chain TPA was identified by reduced NaDodSO₄/PAGE (9). The active-site concentration of two-chain TPA was determined by spectrofluorimetric titration with 4-methylumbelliferyl-*p*-guanidinobenzoate at 4°C (10).

Human fibrinogen was purchased from Kabivitrum (Stockholm). The material was reconstituted in water, passed over a column of Sepharose-lysine (7) to remove plasminogen, and dialyzed against 10 mM Hepes-NaOH, pH 7.4.

Antiserum to human α_2 -antiplasmin (APm) was raised in rabbits by repeated intramuscular injections of highly purified APm, supplied by D. Collen (Leuven, Belgium). The IgG fraction was isolated by (NH₄)₂SO₄ precipitation (30% wt/vol) from serum and dialyzed against phosphate-buffered saline.

Human thrombin and the thrombin-specific inhibitor, hirudin, were products of Sigma. Ancrod, a thrombin-like enzyme from the venom of *Agkistrodon Rhodostroma*, was supplied in a highly purified form by Revlon Health Care (Sussex, U.K.). This material was calibrated against the international reference standard for ancrod (74/581) supplied by the National Institute for Biological Standards and Control (London). Subsequently, this reagent will be referred to as arvin, and expressed in international units (IU)/ml.

[Glu¹]Pg Activation Assays in a Purified System. The assays were performed at 37°C in 10 mM Hepes-NaOH, pH 7.4, to which was added either 100 mM NaOAc or 100 mM NaCl. Assay components were the substrate, 0.5 μ M [Glu¹]Pg, D-Val-Leu-Lys-*p*-nitroanilide (S-2251) at a concentration of

Abbreviations: [Glu¹]Pg, human plasminogen containing Glu¹ at its NH₂ terminus; TPA, tissue plasminogen activator; rTPA, recombinant TPA; APm, α_2 -antiplasmin; Pm-APm, plasmin- α_2 -antiplasmin complex; APm_r, free APm; IU, international units.

[†]On study leave from the National Institute for Biological Standards and Control.

[§]To whom reprint requests should be addressed.

0.5 mM, and 1 μ M fibrinogen when desired to a final volume of 0.8 ml. The activation reaction was accelerated by addition of 4 nM (final concentration) two-chain rTPA.

Preparation of the Serum Samples. Normal fresh-frozen age-outdated human plasma was incubated with human thrombin at 5 IU/ml for 1 hr at 37°C. Normal serum was obtained by removal of the fibrin clot, and the residual activity of thrombin was inhibited with hirudin at 10 units/ml. This serum was dialyzed exhaustively against 10 mM Hepes-NaOH/100 mM NaCl, pH 7.4, or 10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4. Lyophilized fibrinogen (2 mg/ml, final concentration) was added to hirudin-treated serum to obtain fibrinogen-rich serum, and two aliquots were dialyzed against the above two buffers. Final concentrations of 1.25 nM, 2.5 nM, 5 nM, and 10 nM of two-chain rTPA were added to all four types of serum samples described above and incubated for 10 min at 37°C. All samples were quick-frozen in liquid N₂ until two-dimensional electrophoresis could be accomplished. Serum samples were also prepared from age-outdated human plasma by coagulation with arvin at 5 IU/ml for 2 hr at 37°C. This serum was then dialyzed against the two buffers described above. Results with serum from arvin-coagulated plasma were compared with those from thrombin-coagulated plasma.

Two-Dimensional Electrophoresis. Two-dimensional immunoelectrophoresis of the various serum samples was performed as described (11). Agarose gel (1%) in 30 mM sodium barbital/10 mM EDTA, pH 8.6, was used in both directions, while purified IgG at 24 μ g/ml of agarose solution was used in the second direction. Electrophoresis was run at 50 V for 2 hr in the first direction, and the second direction was run overnight using 5 V across the plate. The Apm antiserum detected both free Apm (Apm_f) and the plasmin-Apm (Pm-Apm) complex as separately migrating entities. The areas under the peaks of the stained plates were used to indicate the amounts of Apm_f and Pm-Apm, respectively.

Clot-Lysis Assay. The assay was conducted essentially as described elsewhere (12), with the following modifications. Final concentrations of 0.06 μ M [Glu¹]Pg, fibrinogen at 2.4 mg/ml, and the added rTPA were incubated with human thrombin at 4 IU/ml at 37°C in a buffer consisting of 10 mM Hepes-NaOH/100 mM NaCl, pH 7.4, or 10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4. The total clot-lysis time was measured for each rTPA concentration used.

RESULTS

Fig. 1 illustrates the enhancing effect of fibrinogen and the inhibiting effect of Cl⁻ on activation of [Glu¹]Pg by rTPA. Significant enhancement was seen at 1 μ M fibrinogen, and this effect was linear when plotted against (time)² in this coupled assay system, suggesting that the initial rate of activation of [Glu¹]Pg was linear under these conditions. When Cl⁻ was substituted for OAc⁻, an inhibition was noted. The enhancing effect of fibrinogen occurred in both Cl⁻ and OAc⁻.

To establish whether the effects seen were physiological, two aliquots of thrombin-derived serum were supplemented with physiological levels of fibrinogen and dialyzed against a 10 mM Hepes-NaOH buffer, containing either 100 mM NaOAc or 100 mM NaCl. Two additional aliquots of the same serum, without added fibrinogen, were also dialyzed against the aforementioned two buffers. Four types of serum were generated, designated serum fibrinogen/Cl⁻, serum Cl⁻, serum fibrinogen/OAc⁻, and serum OAc⁻. Each serum type was treated with various rTPA concentrations from 0 to 10 nM. The resultant plasmin at each rTPA level was assessed by two-dimensional immunoelectrophoresis with an antiserum to Apm used in the second dimension. Representative data, at 5 nM rTPA, are shown in the four immunoprofiles of

FIG. 1. The effects of Cl⁻ and fibrinogen on the activation of [Glu¹]Pg by two-chain rTPA. The amounts of human plasmin (HPm) generated as a function of activation time are illustrated. Assays were performed in 10 mM Hepes-NaOH/100 mM NaCl, pH 7.4, with 1 μ M fibrinogen (○) and without fibrinogen (●). Similar assays were carried out in 10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4, with 1 μ M fibrinogen (Δ) and without fibrinogen (▲).

Fig. 2. The absence of fibrinogen and the presence of Cl⁻ in serum clearly suppressed any major activation of [Glu¹]Pg as measured by formation of the Pm-Apm complex (C). Only a small level of activation of [Glu¹]Pg was seen at the highest rTPA concentration studied. The inhibitory effect of Cl⁻ in serum devoid of fibrinogen was significant (compare A with C and B with D), but not as evident as were the enhancing effects of fibrinogen in both Cl⁻ and OAc⁻ environments (compare profiles A, B, C, and D). The Pm-Apm/Apm_f ratio formed in each serum type was plotted against the rTPA concentration, and the results are illustrated in Fig. 3. At 5 nM rTPA with fibrinogen and OAc⁻, all reactive Apm was consumed in the Pm-Apm complex. This is compatible with a report that $\approx$ 25% of the Apm_f does not react with plasmin (13). Again, the data show that over a wide concentration range of rTPA the fibrinogen-enhancing effect outweighs the inhibitory effect of Cl⁻. This observation supports the data from the purified systems illustrated in Fig. 1.

The effects of both Cl⁻ and OAc⁻ environments on [Glu¹]Pg activation by rTPA in arvin-derived serum (data not shown), without added fibrinogen, were similar to those presented in Figs. 2 and 3. Thus, the coagulant used for sera preparation did not affect the results.

To distinguish between the ion effect in solution and at the fibrin-clot surface, various concentrations of rTPA were incorporated into a clot composed of the purified reagents—fibrinogen, thrombin, and [Glu¹]Pg. The buffer environments of Hepes-NaOH with 100 mM NaOAc or 100 mM NaCl were compared in typical clot-lysis-time assays (Fig. 4). The presence of Cl⁻ or OAc⁻ in the buffer had little effect on the clot-lysis times, at least at the rTPA concentrations investigated. This contrasts with these ion effects in solution shown in Figs. 1–3.

FIG. 2. Representative profiles by two-dimensional electrophoresis of APm (AP) and Pm-APm (PAP), in four types of serum at a concentration of rTPA of 5 nM. The antibody to APm was used in the second direction. (A) Normal serum dialyzed against 10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4. (B) Fibrinogen-enriched (2 mg/ml) serum dialyzed against the same buffer as in A. (C) Normal serum dialyzed against 10 mM Hepes-NaOH/100 mM NaCl, pH 7.4. (D) Fibrinogen-enriched (2 mg/ml) serum dialyzed against the same buffer as in C.

DISCUSSION

The proposition that fibrin, via its affinity for both TPA (14) and plasminogen (15), can orchestrate the targeting of the TPA-mediated thrombolytic effect *in vivo* has not fully incorporated the effects of either Cl^- or fibrinogen concentrations in plasma. Because TPA has been heralded as a major advance in the treatment of occlusive vascular disease (16), addressing the roles that both Cl^- and fibrinogen play *in vivo* during thrombolytic therapy is appropriate because the exclusive specificity of TPA for thrombus fibrin is

debatable. Indeed, such specificity may not be fully desirable as plasmin-mediated formation of circulating plasma fibrinogen degradation products may have an advantageous circulatory effect via both preventing reformation of thrombi after therapy and producing a vasodilatory effect. Because the levels of circulatory rTPA during thrombolytic therapy regimens (16) ($\approx 1 \mu\text{g/ml}$) are far from physiological, the systemic activation of plasminogen and the subsequent degradation of fibrinogen may be affected by both circulating Cl^- and fibrinogen concentrations. The role of circulating Cl^- and fibrinogen as modulators of the fibrinolytic process must also be considered under the more physiological conditions associated with endogenously provoked thrombosis and hemorrhage.

Figs. 2 and 3 clearly show that in the physiological milieu of human serum, fibrinogen promotes activation of $[\text{Glu}^1]\text{Pg}$ by TPA, whereas Cl^- inhibits that reaction. One explanation may lie in the ability of Cl^- to conform the substrate $[\text{Glu}^1]\text{Pg}$ unfavorably for activation (5). Conversely, a lesser effect of Cl^- was seen during lysis of fibrin clots (Fig. 4), suggesting that this property of Cl^- relates more to molecular interactions in solution than to such interactions on the insoluble fibrin surface. Because these effects clearly occur in purified

FIG. 3. The ratios of Pm-APm/APm at different concentrations of rTPA. Ratios were calculated from the double-immunoelectrophoresis data (for representative photographs, see Fig. 2) and are plotted against the rTPA concentration. (A) Normal serum dialyzed against buffer A (10 mM Hepes-NaOH/100 mM NaCl, pH 7.4). (●) Fibrinogen (Fg)-rich (2 mg/ml) serum dialyzed against buffer A. (Δ) Normal serum dialyzed against buffer B (10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4). (○) Fibrinogen-enriched (2 mg/ml) serum dialyzed against buffer B.

FIG. 4. The effect of chloride ion on clot-lysis time. The logarithms of the clot-lysis times are plotted against concentrations of rTPA. Buffers were 10 mM Hepes-NaOH/100 mM NaOAc, pH 7.4 (○) and 10 mM Hepes-NaOH/100 mM NaCl, pH 7.4 (●).

systems with various activators (2–6) and in whole plasma and serum (this report), these findings appear relevant to physiological fibrinolysis and thrombolytic therapy with plasminogen activators such as urokinase, streptokinase, and rTPA.

A distinction needs to be made between (i) the mechanism(s) by which low levels of circulating TPA lyse the surface of forming fibers and convert them to fibrin degradation products and (ii) the mechanism by which high levels of rTPA destroy thrombi already established in the circulation. The roles of Cl^- and fibrinogen require investigation in both these cases. Zamarron *et al.* (17) showed that the requirement for exogenous TPA to lyse a preformed fibrin clot is far greater than that needed when TPA is incorporated into the fibrin clot. This difference is primarily due to the higher avidity of TPA for forming fibrin fibers, as opposed to the poorer affinity of TPA for preformed fibrin, particularly as binding sites for the latter may be inaccessible. Thus, small amounts of circulatory TPA, reportedly varying between 2–8 ng/ml of plasma (for review, see ref. 18), seem adequate to initiate plasminogen activation on the fibrin surface, leading to subsequent fibrin destruction. The ability of such low levels of rTPA to mediate the destruction of forming fibrin fibers in blood is partly supported by (i) the demonstration (19) that a small elevation of TPA levels in blood, using s.c. injections of rTPA in rats, protected against experimentally induced thrombosis and (ii) the suggestion that the fast-acting inhibitor of TPA (PAI) no longer inhibits TPA in the presence of forming fibrin (20). These two observations, in conjunction with both the stimulatory effect of forming fibers on the activation of plasminogen by TPA (14) and the Cl^- quenching effect (this report) in the fibrin(ogen)-free circulation, can, in part, explain how low levels of TPA mediate an effective defibrination process in blood.

Defibrination occurs during disseminated intravascular coagulation, and, indeed, probably during all minor local and disseminated expressions of hypercoagulability. Thus, we propose that Cl^- inhibits some stimulatory-activation capacity of fibrinogen in the circulation and that such an effect is decreased when TPA is targeted toward the circulating fibrin. Similar mechanisms could occur during the use of higher concentrations of rTPA ($\approx 1 \mu\text{g}/\text{ml}$) during current regimens of thrombolytic therapy (16, 21). However, in this case, the target fibrin is emeshed in a thrombus, which, in the severe clinical expression of thrombosis, may allow little perfusion. The *in vitro* data of Zamarron *et al.* (17) predicted the need for large circulating levels of TPA for lysis at the clot surface. While the plasma Cl^- level should inhibit some of the fibrinogen-enhanced activation of $[\text{Glu}^1]\text{Pg}$ by rTPA, clinical reports indicate a considerable loss of fibrinogen (i.e., fibrinogenolysis) during high-dosage therapy with TPA (21). This observation is supported by the stimulation of serum $[\text{Glu}^1]\text{Pg}$ activation by fibrinogen at high rTPA levels (Fig. 3) in contrast to the less significant inhibitory effect of Cl^- .

Circulating forms of fibrinogen are thus suggested to be clinically important stimulators of systemic fibrinogenolysis during this type of rTPA therapy. However, critical to the ultimate success of rTPA therapy at high dosage (1 mg/kg of body weight during the first 30 min) may be the diminution of the Cl^- inhibition of $[\text{Glu}^1]\text{Pg}$ activation at the clot surface, as compared with the effect of TPA on $[\text{Glu}^1]\text{Pg}$ in the circulation. While this mechanism was probably not the intent in designing rTPA regimens, we again emphasize that fibrin degradation products, many of which are small peptides, may have a homeostatic vasodilatory effect rather than any damaging hemorrhagic consequence.

These studies were supported by Grant HL-13423 from the National Institutes of Health.

- Collen, D. (1980) *Thromb. Haemostasis* **43**, 77–89.
- Chibber, B. A. K., Radek, J. M., Morris, J. P. & Castellino, F. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1237–1241.
- Chibber, B. A. K., Morris, J. P. & Castellino, F. J. (1985) *Biochemistry* **24**, 3429–3434.
- Urano, T., Chibber, B. A. K. & Castellino, F. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4031–4034.
- Urano, T., Sator de Serrano, V., Chibber, B. A. K. & Castellino, F. J. (1987) *J. Biol. Chem.* **262**, 15959–15964.
- Castellino, F. J., Urano, T., Chibber, B. A. K. & de Serrano, V. S. (1988) in *Fibrinolysis: Current Prospects*, eds. Gaffney, P. J., Castellino, F. J., Plow, E. & Takada, A. (Libbey, London), pp. 27–33.
- Brockway, W. J. & Castellino, F. J. (1972) *Arch. Biochem. Biophys.* **151**, 194–199.
- Deutsch, D. & Mertz, E. (1970) *Science* **170**, 195–196.
- Violand, B. N., Byrne, R. & Castellino, F. J. (1978) *J. Biol. Chem.* **253**, 5395–5401.
- Urano, T., Urano, S. & Castellino, F. J. (1988) *Biochem. Biophys. Res. Commun.* **150**, 45–51.
- Gaffney, P. J., Lane, D. A. & Brasher, M. (1975) *J. Clin. Sci. Mol. Med.* **49**, 149–156.
- Gaffney, P. J. & Curtis, A. D. (1987) *Thromb. Haemostasis* **58**, in press.
- Clemmensen, I., Thorsen, S., Mullertz, S. & Peterson, L. C. (1981) *Eur. J. Biochem.* **120**, 105–112.
- Hoylaerts, M., Rijken, D. C., Lijnen, H. R. & Collen, D. (1982) *J. Biol. Chem.* **257**, 2912–2919.
- Thorsen, S. (1975) *Biochim. Biophys. Acta* **393**, 55–65.
- TIMI Study Group (1975) *N. Engl. J. Med.* **312**, 932–936.
- Zamarron, C., Lijnen, H. R. & Collen, D. (1984) *Thromb. Res.* **35**, 335–345.
- Gaffney, P. J., Creighton, L. & Mahmoud-Alexandroni, M. (1988) in *Fibrinolysis: Current Prospects*, eds. Gaffney, P. J., Castellino, F. J., Plow, E. & Takada, A. (Libbey, London), pp. 129–140.
- Garcia-Frade, L. J., Poole, S., Hanley, S., Creighton, L. J., Curtis, A. D. & Gaffney, P. J. (1986) *Thromb. Haemostasis* **56**, 299–301.
- Gaffney, P. J., Creighton, L., Pring, J. B. & Mahmoud, M. (1985) *Thromb. Haemostasis* **54**, 270 (abstr.).
- European Cooperative Trial (1985) *Lancet* **ii**, 965–969.