An Enzyme-Linked Immunosorbent Assay for the Detection of Plasminogen Activator-Induced Fribrin Clot Lysis in a Circulating Plasma System[1)]

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A technically easy and sensitive method for the evaluation of the fibrinolytic potency of plasminogen activator in a circulating plasma system was developed. A circulating apparatus was prepared by connecting silicone tubes with two separate chambers. Human plasma and plasminogen activator were allowed to circulate from one chamber through a peristaltic pump into another one which contained biotin-labeled fibrin clot. The extent of fibrinolysis was evaluated on streptavidin-coated microtiter plates by measuring the amount of the avidin-bound fibrin degradation products with peroxidase-linked antibody. The detection limit of the fibrinolytic products was 1.5 ng/ml. The present method dose not require any specialized equipment and is also applicable to a low-cost routine measurement of the thrombus selectivity of plasminogen activator.

Keywords tissue plasminogen activator; fibrinolysis; enzyme-linked immunosorbent assay; circulating plasma system

Considerable attention has recently been focused on the use of t-PA (EC 3.4.21.31) for the treatment of thrombotic disorders. t-PA exhibits several attractive features for this purpose, including demonstrable efficacy for coronary thrombolysis[2,3)] and preferential activation of fibrin-bound plasminogen resulting in clot selectivity.[4,5)] However, at doses required for the prompt lysis of coronary thrombi, t-PA, like urokinase, also results in a systemic fibrinolytic state and consequent hemorrhage.[3)] Several laboratories have embarked on efforts to endow t-PA with elevated fibrin selectivity and a longer half-life using both protein engineering and recombinant DNA methods.[6)] Accordingly, to evaluate the real fibrinolytic potency of these modified t-PAs, it is desirable that it be done in a circulating blood system. Therefore, for the evaluation of thrombolytic agents, most investigators have recently relied on the quantitation of plasmin-dependent cleavage of ^{125}I-labeled fibrin thrombus formed in animal veins[7,8)] or in a circulating plasma system.[9)] These assay methods are sensitive, but not convenient for low-cost routine laboratory measurement.

The avidin–biotin complex has recently become useful as an extremely versatile mediator in various bioanalytical applications.[10)] In the present study, using this principle, we developed a simple quantitative method for the detection of fibrin clot lysis in a circulating plasma system. The method involves the coating of microtiter plates with streptavidin and the addition of samples containing biotin-labeled FDP, followed by the determination of the bound protein with ELISA. We also describe the availability of this method for evaluating the fibrinolytic potency of t-PA.

Materials and Methods

Reagents The following reagents were commercially obtained: streptavidin and peroxidase-rabbit anti-sheep IgG (H + L) conjugate from Zymed Labs, Inc., San Francisco, CA; sheep anti-human fibrinogen IgG from Binding Site Ltd., Birmingham, UK; biotinyl-*N*-hydroxysuccinamide ester from Bethesda Research Labs, Inc., Gaithersburg, MD; human fibrinogen from KabiVitrum, Stockholm, Sweden; human plasmin from the Green Cross Co., Osaka, Japan; BSA and Tween 20 from Wako Pure Chemical Industries, Ltd., Osaka, Japan; *o*-phenylenediamine from Tokyo Kasei Kogyo Co., Tokyo, Japan; bovine thrombin and low molecular weight-type urokinase from Mochida Pharmaceutical Co., Tokyo, Japan. Recombinant human t-PA (TD-2061, 550000 IU/mg of protein) was kindly supplied by Daiichi Pharmaceutical Co., Tokyo, Japan.

Preparation of Biotin-Labeled Fibrinogen Human fibrinogen was dissolved in 20 mM sodium phosphate buffer (pH 7.2)/0.15 M NaCl (20 mM PBS) at a concentration of 10 mg/ml and rendered plasminogen-free by lysine-Sepharose affinity chromatography.[11)] The plasminogen-free fibrinogen (18.5 mg) was dissolved in 5 ml of 0.1 M $NaHCO_3$ (pH 8.4) and mixed with 200 μl of a biotinyl-*N*-hydroxysuccinimide ester solution (1 mg/ml in dimethylsulfoxide). The mixture was stirred at 25 °C for 2 h and dialyzed against 20 mM PBS at 4 °C overnight. The biotin-labeled fibrinogen was stored at −20 °C until use.

Fibrinolysis in a Circulating Plasma System To examine the lysis of fibrin clot in continuously circulating plasma, an apparatus was prepared by connecting a silicone tube (3 × 600 mm i.d.) with two separate chambers, one of which was a 1.5-ml eppendorf microtest tube containing fibrin clot (Fig. 1). The fibrin clot was formed by adding 40 μl of a thrombin solution (100 NIH units/ml) to a mixture of citrated human plasma (250 μl) from normal volunteers (adult, male) and biotin-labeled fibrinogen (2.6 mg/ml, 10 μl). A solution (14 ml) of human plasma diluted 2:1 in 20 mM PBS/0.05% (v/v) Tween 20 (20 mM TPBS) was then circulated at a flow rate of 10 ml/min by using a peristaltic pump at 25 °C. t-PA was serially diluted (× 1/2) from 392000 to 3062.5 IU/ml with 20 mM TPBS, and 100 μl of each solution was added to the circulating system through another 7-ml chamber. At specified times, 20 μl of the circulating plasma were withdrawn, diluted with 180 μl

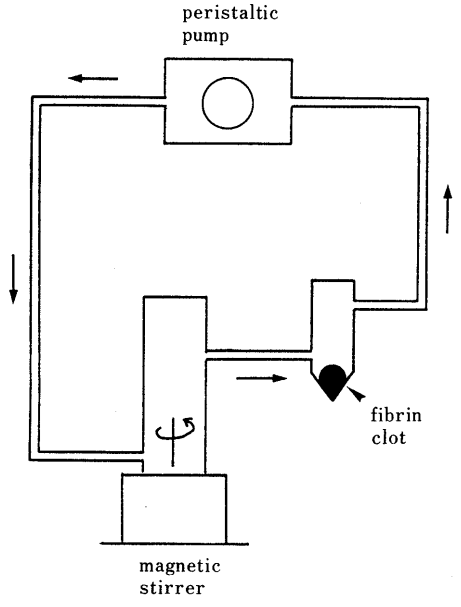

Fig. 1. Illustration of the Apparatus Used for Evaluating t-PA-Induced Fibrin Clot Lysis in a Circulating Plasma System

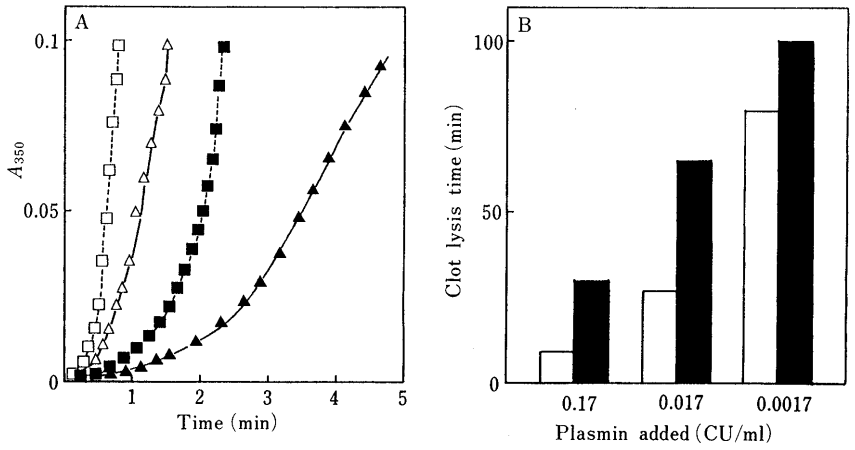

Fig. 2. Native and Biotin-Labeled Fibrinogens as Substrates for Thrombin and Plasmin

Panel A, fibrin polymer formation by thrombin. Each fibrinogen (0.85 mg/ml in 20 mM PBS) was clotted with 0.4 or 2 NIH units (U) of thrombin/ml, and the change in absorbance was monitored at 350 nm for 5 min. □, biotin-labeled fibrinogen plus 2 U of thrombin; ■, biotin-labeled fibrinogen plus 0.4 U of thrombin; △, native fibrinogen plus 2 U of thrombin; ▲, native fibrinogen plus 0.4 U of thrombin. Panel B, fibrin clot lysis by plasmin. In a plastic tube, 250 μl of each fibrinogen solution (1.7 mg/ml in 20 mM PBS) was clotted by adding 25 μl of thrombin (20 U/ml). Various amounts of plasmin were dissolved in 100 μl of 20 mM PBS and then overlaid on the clots. The lysis time was measured at 37 °C. Solid bars indicate the lysis times of native fibrin clots, while open bars indicate those of biotin-labeled fibrin clots. The data represent the mean of duplicate experiments.

of 20 mM TPBS, and used for determining the plasma concentration of biotin-labeled FDP.

Determination of Biotin-Labeled FDP by ELISA Streptavidin was dissolved in 20 mM PBS at a concentration of 10 μg/ml, and 200 μl of the solution was incubated at 4 °C overnight in the wells of a polystyrene microtiter plate (Sumitomo Bakelite Co., Tokyo, Japan). The wells were washed 10 times with 200 μl of 20 mM TPBS and treated at 4 °C overnight with 200 μl of 20 mM PBS containing 1% (w/v) BSA. After the wells were washed exhaustively with 20 mM TPBS, the plate was stored at 4 °C until use. To each well was added 200 μl of the diluted plasma sample. After incubation at 25 °C for 2 h, the wells were washed 10 times with 200 μl of 20 mM TPBS. The wells were then filled with 200 μl of a sheep anti-human fibrinogen IgG solution (11 μg/ml in 20 mM TPBS) and incubated at 25 °C for 2 h. The wells were washed 10 times with 200 μl of 20 mM TPBS. To detect the bound sheep antibody, 200 μl of a solution of peroxidase-rabbit anti-sheep IgG conjugate diluted 1 : 3000 in 20 mM TPBS was added to each well, and it was incubated at 25 °C for 2 h. The wells were washed exhaustively with 20 mM TPBS, then the peroxidase reaction was started by the addition of 150 μl of 2 mM *o*-phenylenediamine in 0.2 M sodium phosphate buffer (pH 5.0)/0.1 M citrate/0.003% (v/v) H_2O_2. After incubation at 25 °C for 5 min, the reaction was stopped by the addition of 50 μl of 4 M H_2SO_4, and the absorbance at 492 nm was measured using a microplate reader (EIA Reader Model MPR-A4, Toyo Soda, Tokyo, Japan).

Protein Determination Protein concentration was determined by the method of Lowry *et al.*,[12] using BSA as the reference.

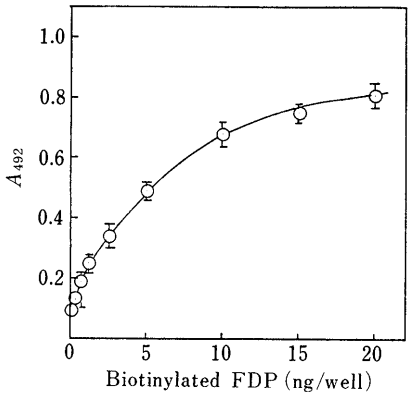

Fig. 3. Standard Curve for Biotin-Labeled FDP

One milliliter of a biotin-labeled fibrinogen solution (2.6 mg/ml in 20 mM PBS) was clotted with 100 μl of thrombin (100 NIH units/ml), and the clot formed was digested with 400 μl of plasmin (0.3 CU/ml). Dilutions were made in 20 mM TPBS to give final concentrations of 1.5—100 ng/ml. A 200-μl portion of each solution was used as the ELISA standard. Each point represents the mean ± S.D. of triplicate experiments.

Results and Discussion

Human fibrinogen was modified with a 10-fold molar exess of biotinyl-*N*-hydroxysuccinimide ester. As shown in Fig. 2A, the rate of thrombin-induced clot formation of biotin-labeled fibrinogen was faster than that of the native one. The labeled fibrin clot was also a good substrate for plasmin (Fig. 2B). Western blot analysis of the fibrin lysate with avidin-linked peroxidase indicated that the region of fragment D was preferentially biotinylated (data not shown). However, the overbiotinylation of fibrinogen with a 50-fold molar excess of the reagent resulted in a complete loss of the clot forming potency of this protein.

The ELISA was performed in three steps: combination of the coating of microtiter plates with streptavidin, binding of biotin-labeled FDP to the adsorbed avidin, and the quantification of the bound FDP with a peroxidase-linked antibody. The influence of the streptavidin concentration in the coating solution was studied using 4 different concentrations (5, 10, 20 and 50 μg/ml). The best response was obtained at a concentration of 10 μg/ml. The plate coated with 20 or 50 μg/ml streptavidin had a higher background than that with 10 μg/ml. On the other hand, the use of 5 μg/ml streptavidin gave only low reproducibility (data not shown). Therefore, using plates coated with 10 μg/ml streptavidin, a standard curve of the biotin-labeled FDP was constructed from 1.5 to 100 ng/ml (200 μl each was added to the streptavidin-coated well). As shown in Fig. 3, the labeled FDP gave a good correlation of absorbance *versus* the amount of protein. The background absorbance due to nonspecific binding was about 0.1 (optical density). The lowest amount of FDP detectable by this assay method was about 1.5 ng.

The present method was next applied to the assay of plasminogen activator-induced fibrin clot lysis in the circulating plasma system. A recombinant human t-PA was used as a standard. As shown in Fig. 4, the t-PA standard gave a dose- and time-dependent release of FDP. The detection limit with sufficient accuracy was about

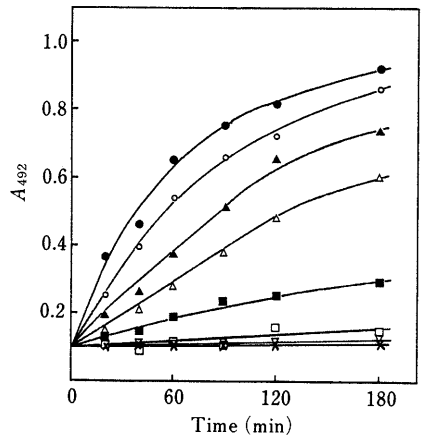

Fig. 4. Time Dependence of Fibrinolysis by Different Concentrations of Recombinant t-PA Standard

The t-PA concentrations in a circulating blood plasma were as follows: ●, 2800 IU/ml; ○, 1400 IU/ml; ▲, 700 IU/ml; △, 350 IU/ml; ■, 175 IU/ml; □, 87.5 IU/ml; ▽, 43.75 IU/ml; ×, 21.875 IU/ml. Experimental details were as described under Materials and Methods. Each point represents the mean of duplicate experiments.

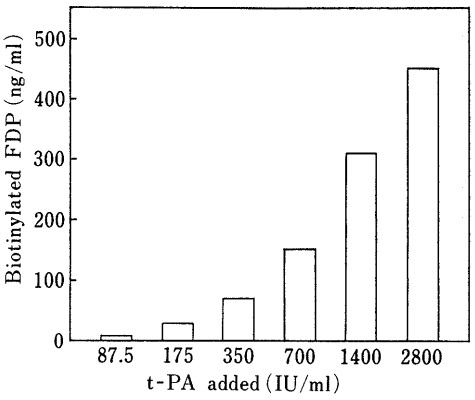

Fig. 5. Dose-Dependent Release of Biotin-Labeled FDP Induced by Recombinant t-PA Standard

The data show the plasma concentration of biotin-labeled FDP released by a 60-min circulation of each t-PA standard.

90 IU of t-PA/ml in the circulating plasma system. When the amount of the labeled FDP released by a 60-min circulation of each t-PA solution was calculated from the standard curve shown in Fig. 3, a good correlation was obtained between the t-PA dose and the labeled FDP concentration in the plasma (Fig. 5). The degree of clot lysis induced by a 120-min circulation of t-PA at 2800 IU/ml was about 50%. Among the customary methods for assaying t-PA activity, a typical one is the determination of plasma clot lysis in a test tube, where the clot is formed together with t-PA and its lysis time is measured. The lysis time of plasma clot containing 2800 IU of t-PA/ml by use of this customary method was 9.5 min. This suggests that t-PA could easily activate plasminogen in the plasma clot. On the other hand, the efficacy of t-PA on the fibrin clot lysis in our assay system and blood circulation *in vivo* largely depended on how effectively the used plasminogen activator could localize on the fibrin surface and activate the fibrin-bound plasminogen.[5] Comparison of t-PA and a non-selective fibrinolytic agent, urokinase, showed that the fibrinolytic potency of 2300 IU/ml urokinase on the plasma clot in a test tube was the same as that of 2800 IU/ml t-PA (lysis time, 9.5 min), whereas the potency of urokinase in our assay system was extremely lower than that of t-PA (2800 IU/ml t-PA, 450 ng/ml biotinylated FDP; 2300 IU/ml urokinase, 85 ng/ml biotinylated FDP). Therefore, the present results suggest that our assay method may offer a useful model system for the study of t-PA-related fibrin clot lysis in the vascular system. Another advantage of this method lies in its ease in elucidating the thrombus selectivity of various types of plasminogen activator expected to be used as a thrombolytic agent. Moreover, the present method dose not require radiolabeled chemicals and does not utilize any specialized equipment. Using this assay method, we are investigating the fibrinolytic potency of a newly synthesized conjugate type of t-PA.

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References and Notes

1) Abbreviations used: ELISA, enzyme-linked immunosorbent assay; t-PA, tissue plasminogen activator; DNA, deoxyribonucleic acid; FDP, fibrin degradation products; BSA, bovine serum albumin; Ig-G, immunoglobulin G; PBS, phosphate-buffered saline; TPBS, Tween 20 containing phosphate-buffered saline; IU, international unit; CU, casein unit.
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