Synergism of thrombolytic agents in vivo

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ABSTRACT The existence of significant synergism between tissue-type plasminogen activator (t-PA) and single-chain urokinase-type plasminogen activator (scu-PA), and between t-PA and urokinase in thrombolysis in vivo is described. In a quantitative preparation of thrombolysis, consisting of rabbits in which a blood clot was induced in the jugular vein with 125I-labeled fibrin, intravenous infusion over 4 hr of t-PA, scu-PA, or urokinase in amounts of 0.5, 1.0, or 2.0 mg/kg body weight resulted in significant thrombolysis (30% to 60%). The simultaneous infusion of t-PA and scu-PA or of t-PA and urokinase had a significantly greater (p < .001) thrombolytic effect than could be anticipated on the basis of the added effects of each agents alone. However, no synergism was observed between scu-PA and urokinase. The observed $\alpha_2$-antiplasmin consumption and fibrinogen breakdown after urokinase at higher doses did not occur with the equivalent thrombolytic combinations of t-PA and urokinase. The combined use of synergic thrombolytic agents in patients may permit a significant reduction in total administered doses, probably with elimination of the systemic activation of the fibrinolytic system and the concomitant fibrinogen breakdown that is unavoidable with the currently used thrombolytic doses of each agent.


ONE APPROACH to the treatment of thromboembolic disease is the use of pharmacologic agents that activate the fibrinolytic system in blood. During activation of this system, the inactive zymogen plasminogen is converted to the active enzyme plasmin, a proteolytic enzyme with relatively low fibrin specificity. When plasmin circulates freely in the blood, it degrades a number of proteins, including fibrinogen and the blood coagulation factors V and VIII. Plasma does, however, contain a fast-acting plasmin inhibitor, $\alpha_2$-antiplasmin, which reacts extremely rapidly with free plasmin but only slowly with plasmin generated on the fibrin surface. Clot-specific fibrinolysis therefore requires generation of plasmin at the fibrin surface, out of reach of $\alpha_2$-antiplasmin.1

One promising approach to obtaining specific thrombolysis is the use of thrombolytic agents, the action of which is stimulated by the presence of fibrin. Indeed, streptokinase and urokinase, which have no specific affinity for fibrin, activate circulating and fibrin-bound plasminogen equally well. Plasmin formed in the circulation is immediately neutralized by $\alpha_2$-antiplasmin, and once the inhibitor is exhausted, several plasma proteins are then degraded by plasmin (fibrinogen, factor V, factor VIII). An overt systemic fibrinolytic state and very low fibrinogen levels may occasionally lead to major bleeding, although this is not a frequent complication when thrombolytic agents are only used over short time periods.

Two plasminogen activators with demonstrated fibrin specificity are tissue-type plasminogen activator (t-PA),1,2,* and single-chain urokinase-type plasminogen activator (scu-PA).3–6

Although both agents have shown promise in preliminary clinical studies for efficacious fibrin-specific thrombolysis, a certain degree of systemic fibrinolytic activation has been evident, as manifested by decreased fibrinogen and $\alpha_2$-antiplasmin levels in some patients. To optimize thrombolytic therapy in terms of both efficacy and safety some modifications may therefore still be required.

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Received April 1, 1986; revision accepted June 5, 1986

Dr. Stump is the recipient of Clinical Investigator Award HL 01176 of the National Heart, Lung, and Blood Institute.

*The nomenclature tissue-type plasminogen activator (t-PA, either in its single-chain form or two-chain form), single-chain urokinase-type plasminogen activator (scu-PA), and urokinase used is as adopted by the International Committee on Thrombosis and Haemostasis (Thromb Haemost 54: 893, 1985).
It has been recently shown that the mechanisms of action of t-PA and scu-PA are quite distinct. t-PA binds directly to the fibrin clot, where its activation of plasminogen is markedly enhanced.\textsuperscript{7} scu-PA, on the other hand, is capable of very efficient direct activation of plasminogen both in the presence and absence of fibrin. In plasma this activation process is strongly inhibited and is reversed only when fibrin is present, a process that occurs without direct binding of scu-PA to fibrin.\textsuperscript{8}

Because of the differing mechanisms of these agents, the possibility of synergistic action has been considered and investigated in vitro. With recombinant scu-PA and t-PA, only additive clot lysis effects could be demonstrated.\textsuperscript{8} More detailed studies using natural scu-PA from a human lung adenocarcinoma cell line\textsuperscript{6} have confirmed these earlier observations.\textsuperscript{*} However, because of the more pronounced fibrin-specific effects of scu-PA seen in vivo,\textsuperscript{10} and because of the recognition of additional factors in vivo that regulate the thrombolytic action of t-PA,\textsuperscript{11} we have explored further the possibility of synergism in a well-characterized animal preparation of jugular vein thrombosis.\textsuperscript{12}

**Materials and methods**

**Thrombolytic agents.** t-PA was purified from the conditioned medium of a melanoma cell line in the absence of the plasmin inhibitor aprotinin\textsuperscript{13, 14} and scu-PA was purified from the conditioned medium of a lung adenocarcinoma cell line,\textsuperscript{6} as described elsewhere. t-PA was in the two-chain form with a specific activity of 500,000 IU/mg when calibrated against the first International Reference Preparation for t-PA.\textsuperscript{15} scu-PA was present as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with an apparent Mr of 54,000 under reducing conditions. Two-chain urokinase-type plasminogen activator was removed by benzamidine-Sepharose absorption to less than 1% as measured by activity with the chromogenic substrate Pyro-Glu-Gly-Arg-p-nitroanilide (S-2444). The preparation so treated had a specific activity in plasminogen-enriched bovine fibrin plates of 80,000 IU/mg. Both t-PA and scu-PA were essentially free of contaminating proteins.

Urinary urokinase (Winkinase), specific activity 100,000 IU/mg, was a gift from Dr. E. Murano, Bureau of Biologics, Bethesda, MD.

**Rabbit preparation of jugular vein thrombosis.** Experimental thrombolysis in rabbits with jugular vein thrombosis was carried out as previously described.\textsuperscript{13} In essence, an external jugular vein was exposed and cleared over a distance of 4 cm. Small side branches were ligated, a cannula was introduced in the main side branch (facial vein), and a woolen thread was introduced in the lumen. A 4 cm long vein segment was isolated between two vessel clamps and the volume of the segment was determined by saline injection via the catheter. The vein segment was then rinsed with a thrombin solution (100 NIH U/ml) and filled with a mixture of \textsuperscript{125}I-labeled fibrinogen and rabbit blood, avoiding injection of air bubbles. In all instances a clot formed quickly and was allowed to age for 30 min. The \textsuperscript{125}I content of the clot was determined from an isotope balance.\textsuperscript{12}

Thrombolysis was performed by intravenous infusion of solvent (controls), t-PA, scu-PA, or urokinase or simultaneous infusions of t-PA and scu-PA, of t-PA and urokinase, or of scu-PA and urokinase in a total volume of 20 ml, by a constant-rate infusion pump. The infusions were given via a contralateral marginal ear vein over 4 hr. Thirty minutes after the end of the infusion the thrombosed segment of the jugular vein was removed after careful suturing of both ends, and the residual radioactive material was measured. The extent of thrombolysis at completion of the infusion was calculated as the difference between the radioactivity originally incorporated in the clot and that remaining in the vein segment, and was expressed in percent of the original radioactivity. Three experiments were performed with each dose and combination of thrombolytic agents, and the results were expressed as mean ± SEM.

Two milliliter blood samples were drawn on citrate (final concentration 0.01M) before the start of the infusion and at hourly intervals for 270 min. These plasma samples were used for measurement of radioactivity, fibrinogen, and α2-antiplasmin levels as described.\textsuperscript{12}

**Results**

The thrombolytic effects of the three agents are shown in table 1. Systemic infusion of t-PA, scu-PA, or urokinase resulted in a dose-dependent degree of lysis as determined by comparison with controls infused with solvent only. At maximum doses, lysis was

<table>
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<tr>
<th>Dose (mg/kg)</th>
<th>t-PA</th>
<th>scu-PA</th>
<th>Urokinase</th>
<th>Thrombolysis (%)</th>
<th>Fibrinogen (% of baseline)</th>
<th>α2-Antiplasmin (% of baseline)</th>
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<td>25 ± 2</td>
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The data represent mean ± SEM of three experiments.

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**TABLE 1**

| Synergic effect of t-PA, scu-PA, and urokinase on thrombolysis in rabbits with jugular vein thrombosis

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*Laboratory Investigation—Thrombolysis*
57 ± 6% after t-PA (0.5 mg/kg), 31 ± 1% after scu-PA (1.0 mg/kg), and 44 ± 3% after urokinase (2.0 mg/kg). Control lysis was 9 ± 1%, quite comparable to that in this preparation in previous studies. Infusion of t-PA or scu-PA, even at maximum doses, did not induce systemic fibrinogen breakdown or α2-antiplasmin consumption, as shown in table 1. Urokinase, an agent that is not fibrin specific, when infused at a sufficiently high dose (2 mg/kg), caused extensive systemic fibrinolytic breakdown, with a decrease of fibrinogen to 37 ± 26% of the preinfusion value.

To study the effects on thrombolysis of these agents infused in combination, fractional doses of each were combined and the resulting dose-response curves were compared with the dose-response curves for each drug individually. The most pronounced synergic effect was seen when t-PA and scu-PA were combined: combination of a 20% dose of each (0.1 mg/kg and 0.2 mg/kg, respectively), i.e., a total fractional dose of 40%, resulted in 51 ± 10% lysis.

A similar analysis of combinations of t-PA and urokinase also showed synergism. Infusion of 20% fractional doses of each (40% total dose) resulted in 39 ± 7% lysis, whereas a 50% dose of t-PA alone resulted in 32 ± 1% lysis and a 50% dose of urokinase alone produced only 23 ± 1% lysis. There was no trend toward synergism observed at any combination of doses of scu-PA and urokinase.

Systemic fibrinolytic activation was not observed (table 1) after any of the combinations tested, including the highest concentrations of urokinase with t-PA at which significant synergism was observed.

**Statistical analysis of the thrombolysis data.** The percent thrombolysis versus the total fractional amount of thrombolytic agents infused (fraction of 0.5 mg/kg for t-PA, 1.0 mg/kg for scu-PA, and 2.0 mg/kg for urokinase) were fitted by linear regression analysis (figures 1 and 3). In all instances highly significant correlations were obtained. The slopes of the regression lines of data obtained with simultaneous infusions were then compared with the mean value of the slopes of the regression lines of the values obtained with each agent alone. Simultaneous infusion of t-PA and scu-PA yielded a regression line with a slope of 106 (percent thrombolysis with 1.0 fractional amount), as compared with slopes of 47 for t-PA and 21 for scu-PA (figure 1). This difference was highly significant (t = 4.2, p = .0002). Likewise, the slope of the regression line obtained with t-PA and urokinase (figure 2) was 74, which was significantly higher than the mean value of the slope for t-PA (47) and for urokinase (38) (t = 2.8, p = .007). The combined infusion of scu-PA and urokinase (figure 3) yielded a slope of 38, which was not significantly different from the mean values of the slopes for scu-PA and for urokinase (t = 1.2, p = .24). We therefore conclude that combined infusions of t-PA and scu-PA and of t-PA and urokinase have a highly significant synergic effect on thrombolysis in vivo that is not observed with the combination of scu-PA and urokinase.

**Discussion**

This study provides the first demonstration of synergistic fibrinolysis in vivo mediated by combinations of
plasminogen activators. The combined use of t-PA and scu-PA or of t-PA and urokinase produced significantly greater lysis than could be explained on the basis of their additive effects. The demonstration of synergism in vivo contrasts with the lack of synergism in a clot lysis system in vitro, which makes the understanding and further investigation of this effect more difficult. Obviously, additional factors found only in vivo are involved. This is further supported by the observation that the dose-response curve with respect to thrombolysis in vivo with scu-PA is linear, whereas a clear threshold phenomenon is associated with clot lysis in a plasma environment in vitro. Thus, animal preparations will probably be required to define the synergistic mode of action.

The present study was carried out with natural t-PA and scu-PA obtained from conditioned cell culture media. In view of the demonstrated equivalence in thrombolytic properties of melanoma t-PA and recombinant t-PA and of cell culture scu-PA and recombinant scu-PA, it can be anticipated that the phenomenon of synergism will also hold for these latter agents, which are being developed for therapeutic use in man. We have also performed experiments with two-chain t-PA only because of the established equivalence, in terms of thrombolysis, of one-chain and two-chain t-PA in the present experimental preparation.

Two of the more important goals of thrombolytic therapy are optimal efficacy and minimal toxicity. To these ends, the impact of the findings of this study on thrombolytic therapy in man could potentially be great. Indeed, to achieve maximally efficient throm-

FIGURE 2. Dose-response data for thrombolysis with t-PA, urokinase, and combinations of t-PA and urokinase. The amount infused is expressed as a fraction of 0.5 mg/kg for t-PA and of 2.0 mg/kg for urokinase. The data representing percent thrombolysis vs amount infused were fitted by linear regression analysis, yielding the following correlation coefficients and probabilities: for t-PA: \( r = .96 \) and \( p < .0001 \); for urokinase: \( r = .96 \) and \( p < .0001 \); and for the combination of t-PA and urokinase: \( r = .90 \) and \( p = .0001 \).

FIGURE 3. Dose-response data for thrombolysis with scu-PA, urokinase, and combinations of scu-PA and urokinase. The amount infused is expressed as a fraction of 1.0 mg/kg for scu-PA and of 2.0 mg/kg for urokinase. The data representing percent thrombolysis vs amount infused were fitted by linear regression analysis, yielding the following correlation coefficients and probabilities: for scu-PA: \( r = .94 \) and \( p < .0001 \); for urokinase: \( r = .96 \) and \( p < .0001 \); and for the combination of scu-PA and urokinase: \( r = .87 \) and \( p = .0003 \).
bolysis with each of these agents, relatively large amounts of material are needed. Their infusion at these doses in patients is unavoidably associated with moderate (with t-PA and scu-PA) to extensive (with urokinase) systemic activation of the fibrinolytic system and fibrinogen breakdown.\(^6\)\(^\text{19}\) Provided the present observations can be extrapolated to man, the combined use of synergic thrombolytic agents may allow significant reduction of total doses, with resultant elimination of systemic fibrinolytic activation and its undesirable breakdown of the hemostatic system.

We are grateful to Mr. J. Geboers, Division of Epidemiology of the School of Public Health, University of Leuven, Belgium, for his expert help and advice with the statistical analysis.

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Synergism of thrombolytic agents in vivo.
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Circulation. 1986;74:838-842
doi: 10.1161/01.CIR.74.4.838

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