November 1987:11B–5B

Molecular Mechanism of Action of Newer Thrombolytic Agents

DESIRE COLLEN, MD, PHD

Leuven, Belgium

Recombinant tissue-type plasminogen activator (rt-PA) and single chain urokinase-type plasminogen activator (scu-PA) are thrombolytic agents, characterized by a high but not absolute degree of fibrin specificity that is mediated through different molecular mechanisms. Both activators are still under clinical investigation but it has become apparent that their therapeutic dose in humans is high and associated with a variable degree of systemic activation of the fibrinolytic system and fibrinogen breakdown. Therefore, the quest for further improvement of agents and therapeutic schemes continues. Research is being pursued in this area along the following lines: 1) tissue-type plasminogen activator (t-PA) and single chain urokinase-type plasminogen activator in molar ratios of 4:1 to 1:4 do not act synergistically on thrombolysis in a plasma environment in vitro, but display significant synergism in animal models of thrombosis. In pilot studies in patients with coronary artery occlusion, rt-PA and scu-PA are markedly synergistic and efficient thrombolysis can be obtained with a fivefold lower combined dose than that of the separate agents. The combined dose does not seem to induce systemic fibrinogen breakdown. 2) Deletion mutants of rt-PA can be constructed with a significantly prolonged half-life in vivo, and a better thrombolytic potential after bolus intravenous injection. 3) Cleavage site-specific mutants of scu-PA that abolish the conversion to urokinase may have a higher fibrin specificity. The mutants constructed thus far, however, seem to have a lower specific thrombolytic activity. 4) Chimeric molecules obtained by fusion of cDNA encoding the NH_2-terminal region of t-PA, responsible for its fibrin affinity and cDNA encoding the COOH-terminal region of scu-PA, responsible for its enzymatic properties, combine both mechanisms of fibrin specificity, at least to some extent.

It is anticipated that some of these research lines will yield improved thrombolytic agents or therapeutic regimens.

(J Am Coll Cardiol 1987;10:11B–5B)

The fibrinolytic system, which is responsible for the removal of fibrin from the blood vessels, contains a proenzyme, plasminogen, which can be converted to the active enzyme plasmin by several different types of plasminogen activators. Inhibition of the fibrinolytic system can occur at the level of the plasminogen activators or at the level of plasmin.

Plasminogen Activators

Plasminogen activators are serine proteases with a high specificity for their natural substrate plasminogen. They convert plasminogen to plasmin by hydrolysis of the arginine 560-valine 561 peptide bond. Plasmin is responsible for the degradation of fibrin, but it has a poor substrate specificity and may also degrade several other plasma proteins including fibrinogen, factor V and factor VIII. Extensive activation of the fibrinolytic system, which commonly occurs during infusion of the thrombolytic agents streptokinase or urokinase, is, therefore, usually associated with a "systemic fibrinolytic state" characterized by plasminogen activation, depletion of alpha$_2$-antiplasmin (the circulating physiologic inhibitor of plasmin) and fibrinogen breakdown. Physiologic fibrinolysis, however, which is responsible for the removal of fibrin deposits from the vascular bed, is highly fibrin specific and not associated with systemic activation of the fibrinolytic system. This fibrin specificity of physiologic fibrinolysis results from specific molecular interactions among the reactants: plasminogen activator, plasminogen, fibrin and alpha$_2$-antiplasmin (1).

Classification of plasminogen activators. Two main classes of plasminogen activators have been identified; one related to the activator found in tissues (tissue-type plasminogen activator [t-PA]) and the other related to the activator found in urine (urokinase-type plasminogen activator [u-PA]). Urokinase-type plasminogen activator is normally obtained as a two chain molecule, but a single chain form (scu-PA) has also been isolated.

Thrombolytic agents are plasminogen activators that convert the inactive proenzyme plasminogen of the blood fi-

From the Center for Thrombosis and Vascular Research, University of Leuven, Leuven, Belgium.

Address for reprints: Desire Collen, MD, Center for Thrombosis and Vascular Research, K.U. Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

0735-1097/87/$3.50

brinolytic system to plasmin, a serine protease that degrades the fibrin of a blood clot. Thrombolytic agents can be classified into two main groups: 1) the "classic" thrombolytic agents, streptokinase and urokinase, and 2) the "new generation" thrombolytic agents, recombinant tissue-type plasminogen activator (rt-PA), single chain urokinase-type plasminogen activator (scu-PA) and acylated plasminogen streptokinase activator complex (APSAC).

Thrombolysis with the classic thrombolytic agents streptokinase and urokinase is generally associated with the generation of free circulating plasmin, which induces a "systemic fibrinolytic state" and extensive fibrinogen degradation. The new generation thrombolytic agents are assumed to be more clot selective as a result of molecular interactions that render them more effective at the fibrin surface than in the circulating blood. The mechanism of fibrin selectivity is, however, different for each activator.

Tissue-type plasminogen activator (t-PA). This protease is relatively, but not totally, inactive in the absence of fibrin, and fibrin strikingly enhances the activation rate of plasminogen by t-PA (2). Fibrin essentially increases the stability of the Michaelis complex by creating an additional interaction between t-PA and its substrate. The high affinity of t-PA for plasminogen in the presence of fibrin thus allows its preferential activation on the fibrin clot. The clot specificity of the activation of plasminogen by t-PA in vivo is, however, only relative and not absolute (3). The thrombolytic efficacy and fibrin specificity of natural and recombinant t-PA (rt-PA) has been demonstrated in animal models of pulmonary embolism, venous thrombosis and coronary artery thrombosis (for references see Ref. 4). In several multicenter clinical trials in patients with acute myocardial infarction, intravenous infusion of rt-PA resulted in coronary reperfusion in approximately 70% of patients, but was associated with an average decrease of the plasma fibrinogen level by 30 to 50% (5–7). At the high infusion rates currently used to obtain rapid coronary artery reperfusion in patients with acute myocardial infarction (40 to 90 mg over 1 hour), the plasma level of rt-PA is raised to approximately 1,000 times the physiologic concentration. Under these conditions systemic activation of the fibrinolytic system occurs to a variable degree in vivo (8).

Single chain urokinase plasminogen activator (scu-PA). This protease does not activate plasminogen in plasma because of competitive inhibition. The fibrin specificity of the activation of plasminogen by scu-PA is due to reversal by fibrin of this inhibited state (9). The detailed molecular interactions responsible for its unique action remain, however, to be more precisely defined. The fibrin specificity of thrombolysis with scu-PA is also only relative (10,11). Clot-specific thrombolysis by natural or recombinant scu-PA has been demonstrated in animal models of venous thrombosis and coronary artery thrombosis (for references see Ref. 4). In patients with acute myocardial infarction, intravenous infusion of 40 to 70 mg scu-PA over 1 hour resulted in coronary artery reperfusion in 75% of the patients but a pronounced decrease of fibrinogen was observed in 25% of the patients (10,11).

Anisoylated plasminogen SK (APSAC). APSAC is an inactive derivative of the plasminogen-streptokinase complex, obtained by acylation of the active center of plasminogen. It does not interact with plasminogen until it is reactivated by spontaneous deacylation. It was hoped that APSAC would have an affinity for fibrin and a certain fibrin selectivity due to reactivation after adsorption to the fibrin surface, but no significant fibrin selectivity of thrombolysis has been observed in humans (12).

New Developments in Fibrin-Specific Thrombolytic Therapy

With the development of the fibrin-specific thrombolytic agents rt-PA and scu-PA, many problems relating to the optimal use of these agents remain unsolved. In addition, it is becoming apparent that the fibrin specificity of these agents is not as pronounced in humans as was anticipated from several animal models. Therefore, the quest for thrombolytic agents with better fibrin selectivity continues. We are at present pursuing four main lines of research to obtain better agents or regimens for fibrin-specific thrombolytic therapy.

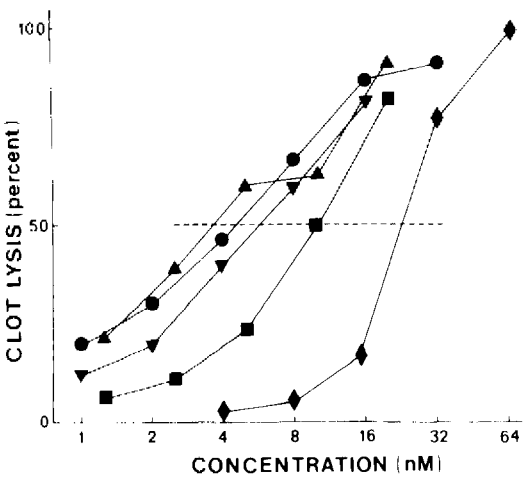
1. Synergism between t-PA and scu-PA. Because the mechanisms that regulate the fibrin specificity of t-PA and scu-PA are entirely different, combined infusions of both agents might act synergistically and be devoid of side effects on the hemostatic system. The mechanism of the fibrin specificity of thrombolytic agents is complex because it is regulated by molecular interactions between several components of the fibrinolytic system, such as between plasminogen and fibrin, between plasmin and alpha$_2$-antiplasmin, between t-PA and fibrin, between t-PA and plasminogen, between scu-PA and plasminogen, between scu-PA and a competitive inhibitor in plasma and between this scu-PA/inhibitor complex and fibrin. Furthermore, the demonstration of synergism between drugs cannot be based on a simple comparison of the effects of the agents when used in combination and the sum of their effects when used alone. Indeed, the correct method for analyzing drug interactions is more laborious and involved, as demonstrated by Berenbaum (13).

Synergism of t-PA and scu-PA. Synergism is defined as a greater effect of a combination of agents than is expected from the effectiveness of its constituents. However, what is to be expected from a combination of agents is not necessarily the sum of the effects of its constituents when used separately. This would only apply if the dose-response curves of the drugs were linear, which is usually not the case with biologically active agents. Therefore, the effect of a combination of drugs can rarely be expected to be the sum of the effects of its constituents, and the assumption that it

should may lead to absurd conclusions (13). The proper way to compare different agents having the same effect and nonlinear dose-effect curves is to titrate them to the same end point (equi-effective dose). Synergism as pharmacologically defined will then occur when the same specified effect is obtained with combination of the two drugs in fractions of the equi-effective dose that are significantly <1. Furthermore, synergism of two agents like t-PA and scu-PA will be therapeutically useful only if it occurs with total amounts of the drugs, when used in combination, that are significantly smaller than the equi-effective dose of the most active agent when used alone.

In vitro studies. With these concepts in mind, we have performed a detailed study on the synergism of rt-PA and scu-PA in vitro using an iodine-125-fibrin–labeled plasma clot immersed in citrated plasma (14). Selected data that are most illustrative of the conclusions reached in that study are summarized in Figure 1. The equi-effective doses of rt-PA and scu-PA that induced 50% clot lysis in 2 hours were 4.5 ± 2.3 and 23 ± 4 n*M* (mean ± SD), respectively. With molar ratios of t-PA to scu-PA of 4:1, 1:1 and 1:4, 50% clot lysis in 2 hours was obtained with a total concentration of 3.8 ± 1.0, 5.5 ± 3.2 and 10 ± 4.6 n*M*, respectively. Because none of the combinations were found to be significantly more thrombolytic or significantly more fibrin specific than an equivalent amount of t-PA alone, we concluded that no (useful) synergism between t-PA and scu-PA can be demonstrated in vitro. Application of the formula of Berenbaum (13) to the data of Figure 1 yields sums of fractional equi-effective doses (50% clot lysis in 2 hours) which, for the ratios of t-PA to scu-PA of 4:1, 1:1 and 1:4, are 0.73 ± 0.20, 0.70 ± 0.19 and 0.79 ± 0.24 ($p > 0.05$).

Figure 1. In vitro clot lysis by tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (scu-PA) and mixtures thereof. The data represent percent clot lysis after 2 hours. (●) t-PA; (◆) scu-PA; (■) t-PA/scu-PA, molar ratio 1:4; (▼) t-PA/scu-PA, molar ratio 1:1; (▲) t-PA/scu-PA, molar ratio 4:1. (Redrawn, with permission, from Collen D et al. [14].)



The conclusions from our in vitro study (14) are in apparent conflict with those of several recent preliminary reports based on nearly identical in vitro findings (15–17). In those studies, however, the very marked nonlinearity of the dose-response curve of scu-PA or the lower specific activity of scu-PA as compared with t-PA, or both, was disregarded. At present only one of these studies has been published (18) and it claims that small amounts of t-PA combined with large amounts of scu-PA (molar ratios of 1:6 to 1:12) show a marked synergistic action. Apart from the fact that synergism was not evaluated by the equi-effective dose titration method (13), the best combination required a severalfold higher total amount of plasminogen activator than the equi-effective dose of t-PA alone. Consequently, the extrapolation from these in vitro findings that combination therapy in humans may have important clinical and economic implications appears to be unjustified.

In vivo studies. In vivo, however, in a jugular vein thrombosis model in the rabbit, significant synergism between t-PA and scu-PA and between t-PA and urokinase for thrombolysis was observed (19). When t-PA and scu-PA were infused in a molar ratio of approximately 1:3, the specific thrombolytic activity of the mixture was more than threefold higher than was anticipated on the basis of additive effects of both agents. Preliminary results in patients with acute myocardial infarction (20) suggest that rt-PA and scu-PA and rt-PA and urokinase act synergistically also in humans. Indeed, combinations of 10 mg rt-PA and 3 mg scu-PA produced coronary artery reperfusion in three patients with acute myocardial infarction and angiographically confirmed coronary artery occlusion. Combinations of 10 mg rt-PA and 300,000 IU urokinase induced reperfusions in three of four patients. Although these results are clearly of a preliminary nature and need to be confirmed in larger numbers of patients, we have more recently demonstrated that combinations of 10 mg rt-PA and 10 mg recombinant single chain urokinase-type plasminogen activator (rscu-PA) are indeed very effective and in addition produce coronary thrombolysis in the absence of systemic fibrinogen breakdown (21a).

This demonstration of marked in vivo synergism contrasts with the lack, or the at best marginal extent, of synergism observed in the in vitro clot lysis systems. Obviously, additional factors found only in vivo are involved. This is supported by the observation that the dose-response curve of in vivo thrombolysis with scu-PA is linear, whereas a clear threshold phenomenon of clot lysis in a plasma environment is observed in vitro (14,19).

In an effort to obtain more information on the mechanism of the in vivo synergism between thrombolytic agents, we have in the rabbit jugular vein thrombosis model (21) infused 1) rt-PA and scu-PA simultaneously over 4 hours; 2) rt-PA over 1 hour, then 15 minutes later scu-PA over 2 hours; and 3) scu-PA over 1 hour, then 15 minutes later rt-PA over 2 hours. Significant synergism on thrombolysis was ob-

served when rt-PA and scu-PA were infused simultaneously or when rt-PA was followed by scu-PA, but not when scu-PA was followed by rt-PA. This might suggest that "predigestion" of the clot by way of plasminogen activation with rt-PA or any other plasminogen activators renders it more sensitive to thrombolysis with scu-PA. However, injection of urokinase followed by scu-PA has no synergistic effect.

2. Mutants of rt-PA. Mutants of rt-PA, which have a higher fibrin affinity, a longer in vivo half-life or an improved fibrin specificity, may constitute potentially useful thrombolytic agents. Larsen et al. (22) recently found that specifically mutagenized rt-PA molecules can be obtained with a more than fivefold prolonged half-life in rats. The thrombolytic and pharmacokinetic properties of one of these variants were studied (23) in a canine model with copper coil-induced thrombosis of the left anterior descending coronary artery. The deletion mutant had a markedly slower disposition rate from plasma than did intact t-PA, which rendered it relatively more effective than natural t-PA after bolus injection.

3. Mutants of scu-PA. This protease differs markedly from urokinase with respect to fibrin-specific thrombolytic properties; therefore site-specific mutagenesis of scu-PA around the cleavage site might yield a more stable molecule. We (24) have recently constructed and characterized lysine 158 mutants of scu-PA in which lysine 158 was exchanged with glycine or with glutamic acid.

The mutant forms of scu-PA, in contrast to the natural scu-PA, could not be converted into an amidolytically active two chain form (tcu-PA) by plasmin and did not cause lysis of an iodine-125–fibrin labeled plasma clot immersed in citrated human plasma. However, in a purified system, both scu-PA-glycine 158 and scu-PA-glutamic acid 158 activate plasminogen after Michaelis-Menten kinetics, with a much lower affinity (K_m = 60 to 80 μM) but with a higher catalytic rate constant (k_2 = 0.01 s^{-1}) as compared with the wild type scu-PA (K_m = 1.0 μM, k_2 = 0.002 s^{-1}), indicating that conversion of scu-PA to tcu-PA is not a prerequisite for the activation of plasminogen. However, lysine-158 seems to be important for the stability of the Michaelis complex between scu-PA and plasminogen. A low molecular weight form of scu-PA with leucine 144 in the amino terminal position was found to be functionally indistinguishable from intact scu-PA (25). Low molecular weight scu-PA may therefore be preferable to intact scu-PA for the large scale production of this molecule by recombinant DNA technology.

4. Hybrids of t-PA and scu-PA. On the basis of the hypothesis that the fibrin selectivity of t-PA and scu-PA are localized in different structural domains, we recently (26) produced a hybrid molecule that has both the fibrin affinity

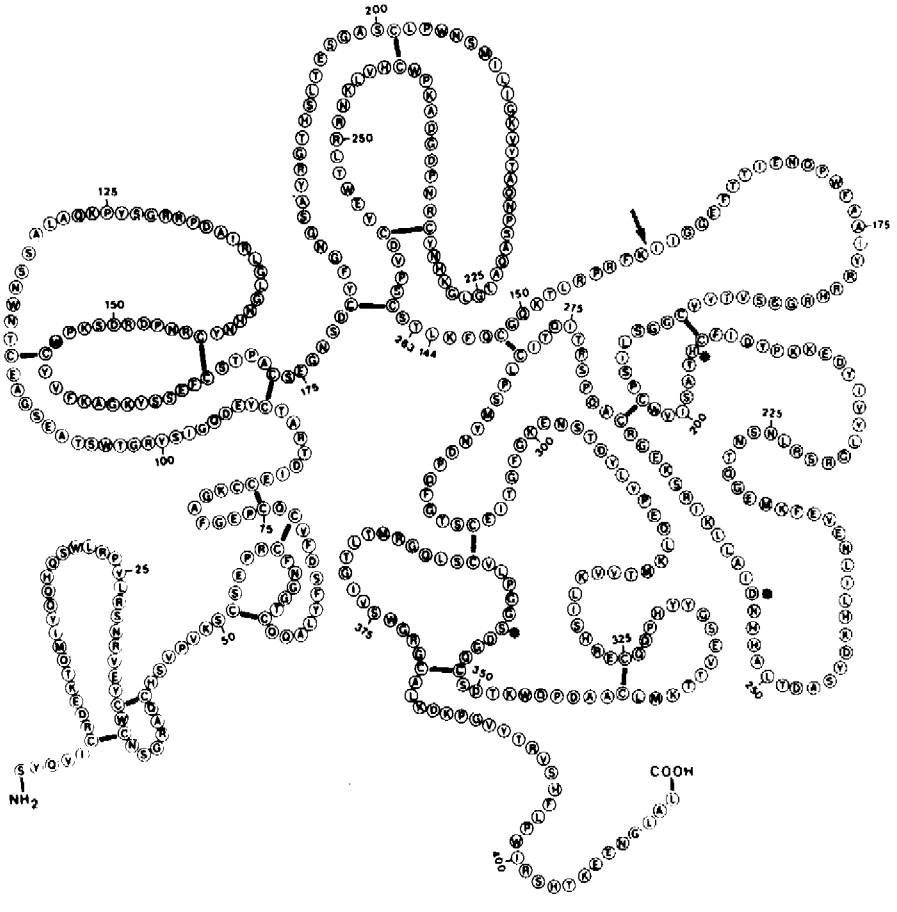


Figure 2. Schematic representation of the fusion protein consisting of amino acids 1 to 263 of tissue-type plasminogen activator and amino acids 144 to 411 of urokinase-type plasminogen activator.

of t-PA and the enzymatic properties of scu-PA. This hybrid consists of amino acids serine-1 through threonine 263 of rt-PA fused to amino acids leucine-144 through leucine-411 of scu-PA (Fig. 2). The protein has an approximate M_r of 70,000 and, on immunoblotting, reacts with rabbit antisera developed against human t-PA and human u-PA. The urokinase-like amidolytic activity (S-2444) of the protein is only 320 IU/mg but increases to 43,000 IU/mg after treatment with plasmin, which results in conversion of the single chain molecule (t-PA/scu-PA) to a two chain molecule (t-PA/tcu-PA).

Both proteins activate plasminogen directly with Michaelis constant (K_m) 1.5 μM and catalytic rate constant (k_2) 0.0058 s^{-1} for t-PA/scu-PA and with K_m = 80 μM and $k_2 = 5.6\ s^{-1}$ for t-PA/tcu-PA, and both bind specifically to fibrin, although more weakly than t-PA. In an in vitro system composed of a whole human ^{125}I-fibrin labeled plasma clot immersed in human plasma, the t-PA/tcu-PA hybrid has a higher fibrin selectivity of clot lysis than does tcu-PA, but this difference was not evident between t-PA/scu-PA and scu-PA. It remains to be investigated whether these t-PA/u-PA hybrid proteins, which combine the fibrin affinity of t-PA with the enzymatic properties of u-PA (either scu-PA or tcu-PA), produce improved fibrin-mediated plasminogen activation in vivo.

Conclusions. We believe that some of these research lines will yield "new generation" thrombolytic agents or therapeutic regimens that are superior to rt-PA or scu-PA, or both, in terms of specific thrombolytic activity and fibrin specificity.

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