A Recombinant, Chimeric Enzyme With a Novel Mechanism of Action Leading to Greater Potency and Selectivity Than Tissue-Type Plasminogen Activator

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Background. Early intervention with thrombolytic agents has been shown unequivocally to reduce mortality after acute myocardial infarction. Presently used agents have disadvantages such as short half-life, immunogenicity, hypotension, and bleeding complications. Therefore, there is a need to develop improved thrombolytic drugs with novel mechanisms of action leading to improved properties.

Methods and Results. Hybrid plasminogen/tissue-type plasminogen activator (t-PA) complementary DNA was constructed and expressed in Chinese hamster ovary cells. The chimeric protein, comprising the fibrin-binding domains of plasminogen covalently linked to the catalytic domain of t-PA, was purified and evaluated in vitro and in vivo. The hybrid was inhibited rapidly in human and animal plasmas. The mediator of this rapid inhibition was shown to be $\alpha_2$-antiplasmin. The active center of the hybrid could be protected by reversible active center acylation with a novel inverse acylating agent, 4'-amidinophenyl-4-chloroanthranilic acid (AP-CLAN). An acylated (CLAN-) hybrid was cleared from the bloodstream of guinea pigs at 0.35±0.02 ml/min · kg$^{-1}$ compared with a clearance rate of 36±4 ml/min · kg$^{-1}$ for t-PA. The CLAN-plasminogen/t-PA hybrid was evaluated in a quantitative, “humanized” guinea pig pulmonary embolism model and shown to be approximately threefold more potent when given by bolus than an infusion of t-PA. Furthermore, the acylated hybrid was more fibrin selective than t-PA as determined by the relation between clot lysis and fibrinogen degradation.

Conclusions. An acylated, recombinant plasminogen/t-PA hybrid has sufficiently slow clearance to be administered by bolus and is more potent and fibrin selective than t-PA in vivo. (Circulation 1992;86:548–552)

Key Words • thrombolysis • hybrids • acylation • $\alpha_2$-antiplasmin

The treatment of acute myocardial infarction (AMI) has been revolutionized by the use of high-dose regimens of thrombolytic agents early after onset of clinical symptoms. Tissue-type plasminogen activator (t-PA, alteplase) has been the subject of well-publicized interest for the clinical management of AMI. However, t-PA shares with all other thrombolytic agents except anistreplase (Eminase, SmithKline Beecham) the disadvantage that it has a short plasma half-life and, therefore, is administered routinely by a complicated infusion protocol. We and several other groups have adopted a rational approach (site-directed mutagenesis) to identification of the features responsible for the rapid clearance of t-PA; however, none of the t-PA mutant proteins are cleared as slowly as Eminase. An alternative strategy is to create hybrid enzymes, but other groups have shown that construction of recombinant hybrid molecules containing the serine protease (B-) chain of t-PA is problematic; none of the chimeras reported show significant advantages over the parent molecule.

We previously have shown that it is possible to construct hybrid thrombolytic agents by nonrecombinant methods. Plasmin/t-PA hybrids constructed in this way had unique and interesting properties. Both plasmin/t-PA and plasm/urokinase-type plasminogen activator (u-PA) hybrids showed the key property of fibrin enhancement of plasmin catalytic activity, thus showing that specific fibrin-binding domains from t-PA were not required for the formation of ternary complexes among the activator, fibrin, and plasminogen. Like plasmin but unlike t-PA, the plasmin/t-PA hybrid was shown to be inhibited rapidly by $\alpha_2$-antiplasmin (AAP) in human and animal plasmas. If this rapid inhibition by AAP was blocked by reversible, active-center acylation, then the hybrid circulated for prolonged periods of time.

We were interested in constructing a recombinant, single-chain (sc) plasminogen/t-PA hybrid that would

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retain the unique properties of the nonrecombinant molecule. Furthermore, we wanted to determine the potency and selectivity of the recombinant molecule as a thrombolytic agent in vivo in active-center acylated forms.

**Methods**

**Synthesis of a Recombinant Plasminogen/t-PA Hybrid**

The precise methods used to construct the hybrids have been previously described. Briefly, plasminogen and t-PA complementary DNAs (cDNAs) were isolated from human liver (Clontech) and melanoma cell \(^1^9\) libraries. Appropriate restriction fragments comprising DNA encoding the plasminogen N-terminal preactivation and kringle domains and DNA encoding all of the t-PA C-terminal serine protease domain were ligated together using appropriate oligonucleotides to construct a sequence encoding GLu-P\(_{344}\)-plasminogen/Ser\(_{362}\)-Pro\(_{297}\)-t-PA.

**Expression, Purification, and Analysis of Recombinant Plasminogen/t-PA Hybrid**

The cDNA encoding the hybrid was incorporated into a mammalian expression vector \(^1^8\) that also included the dihydrofolate reductase (dhfr) gene. The vector was transfected into Dfr\(^-\) DXB11 CHO cells, and positive clones were selected by growth in selective media. Cultures were subjected to successive rounds of amplification with methotrexate, and stable clones were isolated. Amplified cultures were grown in serum-containing medium, washed, and then harvested for 3 or 4 days in serum-free medium. The hybrid was purified from serum-free harvest medium using metal chelate and lysine-Sepharose chromatography, essentially as described previously. The hybrid enzyme was analyzed by SDS-PAGE as described previously. Chain conversion was achieved by incubation with 0.0003 molar ratio of plasmin to activator at 37°C for 2 hours; GLu\(_{178}\) conversion was achieved by incubation with 0.003 molar ratio for 2 hours at 37°C.

**Inhibition of Hybrids in Human Plasma**

Hybrid (0.5 \(\mu g\)·mL\(^{-1}\)) was incubated in human plasma (pH 7.4) at 37°C under an atmosphere of 5% CO\(_2\)-95% O\(_2\). Aliquots were removed at intervals, and euglobulin fractions were prepared to remove inhibitors by addition of 18 vol acetic acid (0.01% vol/vol). After 30 minutes on ice, fractions were centrifuged (1,000 g at 4°C for 15 minutes), and precipitates were redisolved in 10 vol PBS-Tween (0.02 mol/l sodium phosphate, 0.15 M sodium chloride, and 0.01% Tween 80, pH 7.4). Serially diluted standards were added to plasma at 4°C and processed immediately at the same time as samples. Aliquots were assayed on human fibrin plates as described previously. For determination of the concentration of acylated hybrids, the fibrin plate assay method has a sufficiently long incubation time to allow complete deacylation of the materials, thus permitting measurement of the free enzyme. AAP-depleted plasma was prepared using rabbit anti-human AAP immunoglobulin G (IgG) coupled to CNBr-activated Sepharose. The IgG Sepharose was incubated with human plasma for 2 hours at room temperature and then removed by filtration on a Sinterglass filter under vacuum. AAP levels were depleted to <7% of untreated levels.

**Active-Center Acylation of Hybrid**

The acylation of the hybrid has been described in detail previously. Briefly, the hybrid was treated at 25°C with 4'-amidinophenyl-4-chloroanethranilic acid (AP-CLAN) in phosphate-buffered saline for 60 minutes until the amidolytic activity of the preparation had decreased to <2% of the original activity. The material was buffer-exchanged to remove excess acylating agent and then stored at -40°C.

**Plasma Clearance of Acylated Hybrid**

Plasminogen activators were administered to anesthetized guinea pigs as a single bolus injection via the jugular vein. At the times shown, citrated blood samples were collected from a cannula in the carotid artery. Fibrinolytic activity of the euglobulin fractions was assayed on human fibrin plates by reference to calibration curves for the appropriate activator prepared by serially diluting the enzyme into pooled predose plasma from the same group of guinea pigs. The calibration samples were processed using the same method and at the same time as the clearance samples. The plasma concentrations determined were expressed as percentage of the initial concentration expected, if the total dose administered was diluted into 45 ml plasma/kg body wt. Values are given as mean ± SEM of at least four observations. Pharmacokinetic parameters, quoted in the text, were derived with MODFIT.

**Thrombolytic Efficacy of Acylated Hybrid**

Thrombolysis was measured in a guinea pig pulmonary embolism model as described previously. Anesthetized guinea pigs were injected with heparin (100 units/kg) and human Lys\(_{378}\)plasminogen (3 mg/kg) via a cannulated jugular vein, followed 2 minutes later by a blood clot (5×1 mm) prepared from human fresh citrated whole blood mixed with \(^1^2\)I-labeled human fibrinogen. CLAN-hybrid was given as a bolus intravenous injection over 15 seconds. Ten percent of the total dose of t-PA was given by bolus, followed by an infusion of 50% over the first hour; then, the remaining 40% was infused over the second and third hours at the rate of 20% hr\(^{-1}\). The lungs were removed 4 hours after dosing, and residual radioactivity was determined. Clot lysis was calculated by expressing the difference between the initial and the residual clot as percentage of the initial clot count. Control animals, dosed with a bolus or an infusion of the vehicle, demonstrated 15–20% clot lysis.

**Fibrin Selectivity of Acylated Hybrid**

Citrated blood samples (0.5 ml) obtained from the animals undergoing efficacy evaluation were mixed with the appropriate concentration of aprotinin to prevent proteolysis in vitro. Samples were cooled on ice and then centrifuged to prepare plasma, which was snap-frozen and stored at -40°C until assay. Fibrinogen was measured by the method of Claxton. Mean fibrinogen concentrations were expressed as a percentage of predose value. If a plasma sample was uncottable, this was included in the analysis by assuming a value of 25% of...
the predose level, the minimum concentration detectable in the assay.

Results

Biochemical Analysis of Plasminogen/t-PA Hybrid

As described in “Methods,” the hybrid comprised the preactivation peptide and five kringle domains of plasminogen (Glu1-Pro354) linked directly to the bridge region and B-chain domain of t-PA (Ser192-Pro217). When expressed in Chinese hamster ovary cells and purified rapidly, the material is recovered in the Glu1, sc form with M, of 100,000. However, it is possible (like t-PA) to cleave the molecule with plasmin between Arg275 and Ile276 (t-PA numbering) to form a Glu1, two-chain (tc) form. It is also possible (like plasminogen) to cleave off the preactivation peptide with prolonged plasmin treatment to produce a Lys78 tc form (Figure 1A). The simple, two-step affinity purification produced hybrid of >90% purity and >80% of the Glu1, sc form as estimated from the stained SDS–polyacrylamide gel. The specific activity of the hybrid is difficult to compare with that of t-PA because the hybrid is a different enzyme and, for example, shows fibrin plate concentration–response curves that are not parallel to those of t-PA. However, in a plasminogen activation (S2251) coupled assay, the hybrid gives a specific activity approximately 300,000 IU/mg protein in comparison with 550,000 IU/mg protein for t-PA. When the difference in molecular weight is considered, the hybrid has a specific activity per mole of 84% of that of t-PA. All of the following data were generated using the Glu1, sc form of the hybrid.

Inhibition of Hybrid by AAP

The rate of inhibition of the activity of the hybrid in human plasma was measured at 37°C. The hybrid was inhibited rapidly at 0.5 μg · ml⁻¹; at this concentration, it would be expected that any inhibition by plasminogen activator inhibitor-1 (PAI-1) would be negligible because of the low plasma levels of PAI-1. It was hypothesized that AAP might have been responsible for the rapid inhibition. Depletion of AAP in the plasma using specific anti-AAP antibody decreased the rate of inhibition substantially (Figure 1B). Furthermore, rapid inhibition could be restored by supplementation of plasma with a physiological concentration of AAP (data not shown). At low concentrations in a purified system, the hybrid is also inhibited by PAI-1 at approximately the same rate as t-PA.

Prevention of Inhibition of Hybrid by Reversible Active-Center Acylation

The mechanism of inhibition of plasmin by AAP is well understood. There is a primary, noncovalent interaction between the kringles of plasmin and AAP, followed by a secondary covalent interaction at the active site serine residue. We previously have used reversible acylation technology to temporarily mask the active site of thrombolytic agents, most notably in the synthesis of the acylated plasminogen/streptokinase complex, anistreplase.

We designed and synthesized a novel acylating agent, AP-CLAN, for the plasminogen/t-PA hybrid. The CLAN-derivative of the plasminogen/t-PA hybrid was
not inhibited in human plasma (Figure 1B). On deacetylation of the hybrid, in a buffer system, the material retained essentially 100% of the activity of the unacetylated form. In the deacetylated form, it is inhibitable in plasma by both PAI-1 and AAP (see above).

Clearance of CLAN-Plasminogen/t-PA Hybrid From the Circulation of Guinea Pigs

CLAN-hybrid was administered to anesthetized guinea pigs by bolus injection via the jugular vein. Plasma samples were then taken at various time intervals, euglobulin was precipitated, and fibrinolytic activity was measured in a functional fibrin plate assay that allows deacetylation of the hybrid in vitro. The data in Figure 1C show that the CLAN-hybrid is present for prolonged periods of time in the circulation of guinea pigs. Clearance rates of the acetylated hybrid were compared with those of native and acetylated t-PA given by bolus. The hybrid was cleared approximately 100-fold more slowly than either form of t-PA (0.35±0.02 compared with 36±4 ml/min·kg⁻¹ for native t-PA). For comparison, the clearance of anistreplase in the guinea pig is 0.6±0.1 ml/min·kg⁻¹. Similarly slow clearance rates of hybrid have been seen in rabbits and dogs (data not shown).

Thrombolytic Potency of CLAN-Plasminogen/t-PA Hybrid In Vivo

The thrombolytic efficacy of the recombinant hybrid was determined using a “humanized” guinea pig pulmonary embolism model. This model has the advantage over several other animal models in that t-PA shows a similar degree of selectivity in terms of fibrinogen degradation, as is seen in patients undergoing treatment for AMI. Thus, it is possible to determine whether new agents are apparently more fibrin selective than t-PA. The data in Figure 2A show that at the 4-hour end point of the study, CLAN-hybrid, when administered as a single bolus, is approximately threefold more potent on a molar basis than t-PA given by a clinically relevant infusion regimen (using equal concentrations to give 50% clot lysis). This probably reflects the greater bioavailability of the hybrid. Plasma levels of hybrid were approximately 10-fold higher than those of t-PA at the start of the experiment, decreasing to approximately threefold higher at 3 hours.

Fibrin Selectivity of CLAN-Plasminogen/t-PA Hybrid In Vivo

Thrombolytic agents produce various degrees of disturbance to the normal hemostatic system by degradation of blood-clotting factors via the action of systemically generated plasmin. The relation between clot lysis and degradation of plasma fibrinogen is a measure of the fibrin selectivity of agents. Fibrinogen was measured using a clotting rate assay (Clauss) as described in “Methods.” The data in Figure 2B show that the CLAN-hybrid is more fibrin selective than equi bolus doses of t-PA, despite the fact that the hybrid was given by a single bolus injection and the t-PA was administered by an infusion regimen.

Discussion

We have shown that it is possible to design and produce a unique, recombinant plasminogen/t-PA hybrid that has acquired some desirable properties from each parent molecule. Plasmin is very rapidly inhibited by AAP, whereas t-PA interacts relatively slowly with this inhibitor. The normal hemostatic mechanism that controls the systemic activity of t-PA in the physiological setting is rapid inhibition by PAI-1. However, the small plasma reservoir of PAI-1 soon becomes exhausted at the very high pharmacological doses of t-PA used to treat patients with AMI, and activity of t-PA then is largely unopposed by rapid inhibitors. This is one of the reasons why t-PA loses its fibrin-selective activity in the clinical setting. By contrast, the activity of the plasminogen/t-PA hybrid is regulated by the much larger reservoir of AAP. To use the plasminogen/t-PA hybrid as a useful thrombolytic agent, it was desirable to delay this inhibition by using reversible active site acylation. In an acylated form, the hybrid then can circulate for prolonged periods of time to provide a reservoir of latent or proenzyme activity. The present results show that clearance of the CLAN-acylated form is approximately 100-fold slower than that of t-PA, permitting single-bolus administration. For bolus therapy, the administration of the total dose in a latent form may also lead to fewer side effects (e.g., hypotension) than bolus administration of large quantities of an active agent. This advantage has been seen in animal models when bolus acylated streptokinase/plasminogen complexes were compared with bolus streptokinase/plasmin.28 The potency of the CLAN-hybrid was shown to
be about threefold greater than that of t-PA in a small animal model of thrombolysis. The increase in potency presumably was related directly to the greater bioavailability of the acylated hybrid molecule. The blood levels of hybrid were approximately 10-fold higher than those of t-PA at the start of the experiment, decreasing to approximately threefold higher at 3 hours. The increase in potency is not a simple arithmetic function of the slower clearance rate for the following reasons: The hybrid was administered by bolus, whereas t-PA was given by infusion; the hybrid was administered in an acylated prodrg form, whereas t-PA was given in the native form; and the plasma inhibition rate of the (deacylated) hybrid at thrombolytic doses is much faster than that of t-PA.

The combination of rapid inhibition of native hybrid and the formation of a proenzyme species by active-center acylation provides a high degree of fibrin selectivity. On deacylation in the circulation, the activity of the hybrid will be quenched very rapidly by AAP. On deacylation, and when bound to fibrin in a clot or thrombus, the molecule will, like plasmin, be relatively protected from inhibition by AAP. This, together with the fibrin enhancement of the catalytic efficiency of these enzymes, will tend to localize plasminogen activation to the surface of fibrin rather than in the plasma milieu.

The acylated recombinant plasminogen/t-PA hybrid has particularly appealing properties for the treatment of thrombotic disease. We look forward to further evaluation in vivo of this type of interesting new molecule.

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