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A faster-acting and more potent form of tissue plasminogen activator

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ABSTRACT **Current treatment with tissue plasminogen activator (tPA) requires an intravenous infusion (1.5–3 h) because the clearance of tPA from the circulation is rapid ($t_{1/2}$ ≈ 6 min). We have developed a tPA variant, T103N,N117Q, KHRR(296–299)AAAA (TNK-tPA) that has substantially slower *in vivo* clearance (1.9 vs. 16.1 ml per min per kg for tPA in rabbits) and near-normal fibrin binding and plasma clot lysis activity (87% and 82% compared with wild-type tPA). TNK-tPA exhibits 80-fold higher resistance to plasminogen activator inhibitor 1 than tPA and 14-fold enhanced relative fibrin specificity. *In vitro*, TNK-tPA is 10-fold more effective at conserving fibrinogen in plasma compared to tPA. Arterial venous shunt models of fibrinolysis in rabbits indicate that TNK-tPA (by bolus) induces 50% lysis in one-third the time required by tPA (by infusion). TNK-tPA is 8- and 13-fold more potent in rabbits than tPA toward whole blood clots and platelet-enriched clots, respectively. TNK-tPA conserves fibrinogen and, because of its slower clearance and normal clot lysis activity, is effective as a thrombolytic agent when given as a bolus at a relatively low dose.**

Tissue plasminogen activator (tPA) is a multidomain serine protease of the plasminogen family (1). In the early 1980s, it was discovered that tPA is capable of fibrin-stimulated clot lysis (2) and that it could be used as an agent in the treatment of acute thrombotic disorders such as myocardial infarction (3, 4). Evolutionary pressures that shaped tPA conferred properties on the molecule that make it optimal for endogenous fibrinolysis. When tPA is used as a thrombolytic agent, additional properties may be desirable as well. Due to its rapid clearance from the circulation, tPA must be infused to achieve thrombolysis. Front-loaded dosing with increased initial concentrations of tPA have demonstrated more rapid and complete lysis compared to the standard infusion protocol (5, 6), and early patency is correlated with significantly improved survival rate (7). Bolus administration might further improve the lytic rate by quickly exposing the target clot to a higher concentration of the enzyme, but single bolus administration of wild-type (wt) tPA is not generally used in the clinic due to its clearance.

Many investigators have produced longer half-life versions of tPA that could be administered as a bolus (for reviews, see refs. 8 and 9). However, virtually all of the tPA variants with reduced clearance turned out to have significantly decreased fibrinolytic activities (10). There is a potential problem that must be addressed when considering bolus administration of a fully active tPA: when tPA is given as a bolus (or even as an infusion at high doses), the plasma levels of enzyme rapidly increase and plasminogen becomes activated systemically and on the surface of the clot. This systemic plasmin generation causes decreased levels of circulating plasminogen, fibrinogen, and α_2-antiplasmin. An undesirable consequence of systemic activation is bleeding that may be related to plasmin generation rather than fibrinogen depletion *per se*; both peripheral and intracranial hemorrhage are associated with systemic activation (11). One way to reduce systemic activation is to make tPA even more fibrin-specific—i.e., reduce its activity in the absence of clotted plasma. Systematic mutagenesis was applied to tPA with the hope of increasing the fibrin specificity of tPA. Fortunately, mutations in the protease domain were found that have this property (12); the best characterized example is a tetraalanine substitution at positions 296–299 [KHRR(296–299)AAAA (abbreviated as K)] (13, 14).

In our mutagenesis studies, we found variants that exhibit reduced plasma clearance of tPA sufficient to provide effective thrombolysis when the agent is administered as a bolus. It was an unexpected type of mutation, T103N (T) (which exhibits an additional glycosylation site on kringle 1), that produced a variant (T-tPA) with the most suitable pharmacokinetic profile. That mutation in combination with the tetraalanine substitution at positions 296–299 yielded a tPA variant (TK-tPA) with the desired clearance rate and enhanced fibrin specificity (15, 16). However, this combination variant still did not yield full *in vitro* or *in vivo* fibrinolytic activity when compared with wt tPA. In the present study, we show that through the appropriate combination of mutations at three distinct sites on tPA, it is possible to reduce the clearance while retaining full fibrinolytic activity. The key factor appears to be maintenance of fibrin affinity of the molecule that we achieved by an additional mutation, N117Q (N). The resultant variant (TNK-tPA) is substantially more potent than wt tPA.

MATERIALS AND METHODS

tPA variants were constructed by oligonucleotide-directed mutagenesis as described (12). Expression and purification of the tPA variants was as described (15). Briefly, CHO cells were stably transfected with plasmids containing the tPA gene, amplified in the presence of methotrexate, and grown in serum-free medium for 6 days (17). The conditioned cell culture medium was concentrated and diafiltered (18), and the tPA variants were purified using lysine affinity chromatography. Quantitation of tPA and variants after purification was accomplished by a dual monoclonal assay sensitive to epitopes in the kringle 2 and the protease domains. The mutations evaluated in this study did not exhibit altered

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Abbreviations: tPA, tissue plasminogen activator; PAI_1, plasminogen activator inhibitor 1; wt, wild type. Mutations in tPA are abbreviated as follows: T, T103N; N, N117Q; K, KHRR(296–299)AAAA.

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Table 1. Pharmacokinetic and fibrinolytic activities, fibrin binding, fibrin specificity, and inhibitor resistance

tPA variant	Plasma clearance	Plasma clot lysis	Fibrin binding	Activity in plasma	Activity in plasma clot	Resistance to PAI
T	0.10	0.28	0.34	0.68	0.56	0.9
K	1.18	0.85	0.93	0.13	1.01	90
TK	0.16	0.34	0.33	0.13	0.65	90
TN	0.29	0.47	1.00	1.13	1.17	ND
NK	0.86	0.98	1.13	0.16	1.38	ND
TNK	0.12	0.82	0.87	0.06	0.85	80

Activities are normalized to those of wt tPA. tPA mutations: T, T103N; K, KHRR(296–299)AAAA; N, N117Q. *In vivo* plasma clearance in rabbits is expressed as a ratio of area under the curves for wt and variant tPA. Clot lysis is the normalized value for each sample in the plasma clot lysis assay. Fibrin binding results were determined with the variants in the two-chain (plasmin converted) form. Apparent K_d values for the variants were calculated at the fibrin concentration for half-maximal binding, which was normalized to that of wt tPA. Plasminogen activation activity of tPA variants in plasma normalized to that of wt tPA. Plasmin generation was determined by chromogenic assay using S2251 as substrate. Plasminogen activation activity of tPA variants in the presence of clotted plasma, normalized to that of wt tPA. PAI resistance is expressed as the second-order rate constant of wt tPA divided by those of the tPA variants. The rate constant for wt tPA was determined as $1.2 \times 10^7\ M^{-1}sec^{-1}$. ND, not done.

interactions with the monoclonal antibodies against wt tPA that were used in the assay.

Pharmacokinetic analysis of tPA variants was done in New Zealand White rabbits (weight, 3 kg; three rabbits per group) with a variation of previously described procedure (15). Each sample of tPA or variant (0.3 mg/kg of body weight) was administered by bolus injection via the ear vein and blood samples were drawn over a 2-h period. Blood samples (0.2 ml) were collected on EDTA with Phe-Pro-Arg chloromethyl ketone, processed to plasma, stored at −70°C, and quantified with an ELISA employing two monoclonal antibodies.

Fibrin binding, plasma clot lysis, and plasminogen activation assays were performed as described (12). The fibrin binding studies were done with ^{125}I-labeled Tyr-Pro-Arg-chloromethyl ketone as a specific reagent for iodinating tPA or variants (19). The second-order kinetic rate of tPA inhibition by plasminogen activator inhibitor 1 (PAI_1) was measured by following the loss of amidolytic activity over time as described (15). Methods for determining fibrinogen consumption (*in vitro*) and lysis of whole blood or platelet-enriched clots (*in vivo*) were as described in detail (16).

RESULTS

T, N117Q (N), and K Variants of tPA. Combined site-directed mutagenesis, transient expression, and active-site-directed radiolabeling of tPA variants was used to evaluate the clearance of tPA variants in mice. Slow-clearing candidate molecules were selected for further biochemical analysis. The most effective long-half-life tPA variant contained the insertion of a glycosylation site in kringle 1 created by the Thr-103 → Asn substitution (T-tPA) (15). Interestingly, the carbohydrate at position 103 is of the complex type, and the carbohydrate at position 117 (normally high mannose) was also observed as complex carbohydrate in T-tPA (20). In another variant, the high mannose structure at position 117 was removed by a deglycosylation mutation, N-tPA. This variant exhibited a modest reduction in clearance in rabbits with minimal perturbation of fibrinolytic activity (21, 22). A combination variant (TN-tPA) was created with extraglycosylation and deglycosylation at positions 103 and 117, respectively, that effectively moved the glycosylation site on kringle 1 from position 117 to position 103.

Alanine-scanning mutagenesis was used to identify K, a mutation that has a much improved fibrin specificity and displays 8-fold less activity in the absence of fibrin while retaining full activity in the presence of fibrin. This mutation maps in a region of tPA that is crucial for interaction with the "fast-acting" inhibitor PAI_1 (23). Like previously described mutants in this region (38), K-tPA is ≈90-fold more resistant to inhibition by PAI_1 than wt tPA, as determined by the second-order inhibition rates (15). To create a variant of tPA with reduced clearance, enhanced fibrin-specificity, and PAI_1 resistance, the mutations at three loci, T103N, N117Q, and KHRR(296–299)AAAA, were combined in TNK-tPA.

Pharmacokinetic Analysis. The clearance of T-containing variants were compared to that of wt tPA in rabbits after bolus intravenous injections (Table 1). For TNK-tPA, the area under the curve was 8.4-fold greater than for wt tPA (Fig. 1); yielding a clearance of 1.9 ml per min per kg for TNK-tPA compared with 16.1 ml per min per kg for wt tPA. The data was fitted to biexponential and triexponential equations for TNK-tPA and wt tPA, respectively. Clearance of TNK-tPA exhibited an α and β phase with calculated half-life values of ≈9 and ≈31 min. wt tPA exhibited α, β, and γ phases of clearance with calculated half-life values of 1.3, 6, and 33 min, respectively. The rates of elimination (terminal phase of clearance) for the two molecules were equivalent as indicated by the similar slopes of the clearance curves after 2 h. However, the early phases of clearance were radically different. Fifteen minutes after injection, 50% of TNK-tPA remained in the circulation compared with only 1% of wt tPA remaining at the same time.

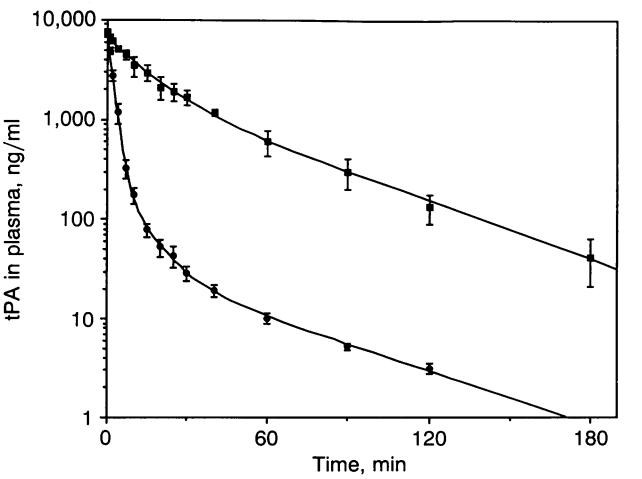

FIG. 1. Plasma clearance of TNK-tPA (■) and wt tPA (●). Bolus intravenous injections (0.3 mg/kg) were made in groups of rabbits (n = 3) and blood samples were collected at the times indicated. tPA antigen in plasma was assessed by a dual monoclonal ELISA (error bars indicate SD). Nonlinear curves were fitted to biexponential and triexponential equations for TNK-tPA and wt tPA, respectively.

Plasma Clot Lysis. Variants of tPA with reduced clearance were characterized with respect to fibrinolytic function by using a plasma-based clot lysis assay. In our experience, the most rigorous *in vitro* measure of fibrinolytic activity for a tPA variant is the rate of lysis of clotted plasma (in contrast to the lysis rate of a purified fibrin clot). In Table 1, a series of single and combination (double and triple loci) variants are compared for pharmacokinetic and *in vitro* fibrinolytic activities. All T-containing variants exhibited substantially reduced clearance; normalized values ranged from 10% to 30% of wt tPA clearance. However, the normalized plasma clot lysis activities of T-containing variants also displayed a wide range—from 28% (for T-tPA) to 81% (for TNK-tPA) compared with that of wt tPA. These data indicate that TNK-tPA has the combined properties of reduced clearance and conserved fibrinolytic activity (81% of wt tPA on a plasma-based clot).

Fibrin Binding, Fibrin Specificity, and Fibrinogen Conservation. As judged from results with an active site radiolabeling procedure (19), the fibrin binding properties of labeled tPA and T-containing variants differed (Fig. 2*A*). wt tPA and K-tPA displayed typical binding curves in response to increasing fibrin concentration with apparent dissociation constants of 0.21 and 0.26 μM, respectively. T-tPA and TK-tPA bound fibrin with ≈10-fold decreased affinity (apparent K_d, 2.5 and 1.8 μM, respectively) compared with that of wt tPA. However, restoration of normal fibrin affinity was observed in the combined extraglycosylation and deglycosylation mutants (Fig. 2*B*). TN-tPA and TNK-tPA have apparent K_d values for fibrin of 0.19 and 0.35 μM, respectively. These data indicate that variants with reduced fibrin affinity also exhibit reduced plasma clot lysis activity (Table 1). To achieve maximal fibrin binding, near normal clot lysis, and decreased clearance, the T mutation was combined with N and K mutations.

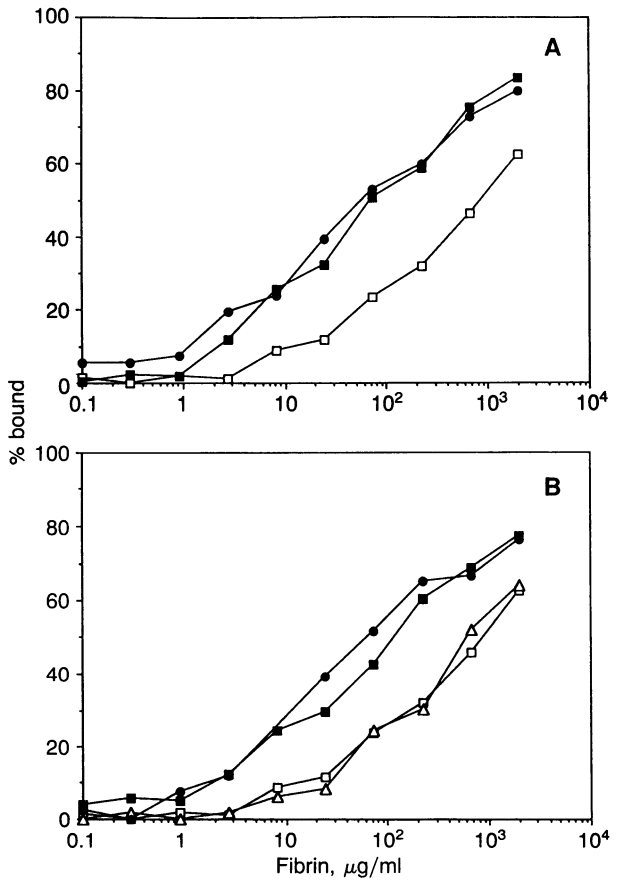

FIG. 2. Fibrin binding of tPA variants. Radiolabeled (^{125}I-labeled Tyr-Pro-Arg-chloromethyl ketone labeled) tPA or tPA variants (50 ng/ml) were incubated with human fibrinogen (100 ng/ml to 2 mg/ml) and human thrombin (0.1 unit/ml). After 1 h, the clots were centrifuged, and the bound radioactive tPA variant was assessed by γ counting. (*A*) Percent bound to fibrin for T-tPA (□), K-tPA (■), and wt tPA (●). (*B*) Fibrin binding of T-tPA (□), TK-tPA (△), TN-tPA (●), and TNK-tPA (■).

Variants containing the K mutation, alone or in combination with other mutations, have increased fibrin specificity compared with that of wt tPA. Fibrin specificity was calculated as the ratio of plasminogen activation rate in the presence of fibrin vs. fibrinogen. The fibrin specificity ratio for K-containing variants was increased 25- to 35-fold compared with that of wt tPA. In addition, we used a more physiologic means of evaluating tPA variants—namely, determination of fibrin specificity in plasma—i.e., the ratio of plasminogen activation in the presence of a plasma clot compared with that in unclotted plasma (Table 1). Plasma clot specificity values for K-containing variants were 7.8, 5.0, 8.6, and 14 for K-tPA, TK-tPA, NK-tPA, and TNK-tPA, respectively. The increased fibrin (or plasma clot) specificity of TNK-tPA, which is a function of decreased plasminogen activation in the absence of a clot, resulted in greater fibrinogen conservation as determined by an *in vitro* assay with human plasma. An ≈10 fold greater concentration of TNK-tPA was required to consume 50% of the fibrinogen in plasma compared to wt tPA. The combination variants show that reduction of clearance can be accomplished without decreasing fibrinolytic activity of the molecule (as indicated by fibrin affinity and plasminogen activation). In fact, fibrin specificity can be increased concomitantly with the use of the K mutation.

Reduced Time to Lysis. The efficacy of tPA variants was evaluated in rabbits that had been fitted with an extracorporal arterial venous shunt containing a whole blood clot. Subsequently, wt tPA (0.18 mg/kg) was injected intravenously either as a bolus or by infusion for 90 min (Fig. 3). In both cases it induced similar initial rates of lysis (0.5% lysis per min). The lysis rate observed with bolus tPA decreased to 25% of the initial rate after 30 min, whereas lysis with an infusion of tPA persisted linearly (0.5% lysis per min)

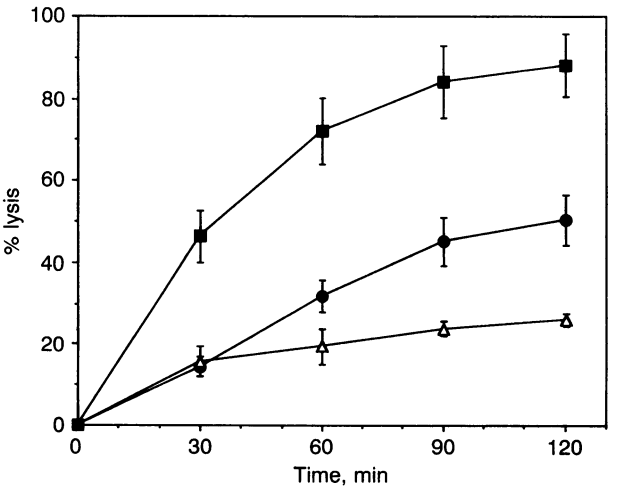

FIG. 3. Kinetics of fibrinolysis in rabbits. Lysis of whole blood clots in a rabbit arterial venous shunt was monitored for 2 h after the administration of wt tPA or TNK-tPA at 0.18 mg/kg of body weight. wt tPA was administered intravenously, either as a bolus (△) or as a continuous infusion (●) over 90 min with 15% of the total dose as a loading bolus. TNK-tPA was administered as a single intravenous bolus (■) over 30 sec. Each lysis vs. time curve represents the mean ± SD of five animals for TNK-tPA and wt tPA (bolus). For the infusion of wt tPA, the results represent the mean ± SD of five experiments with a total of 24 animals.

throughout the 90-min interval. The infusion of tPA was twice as effective as the bolus injection as determined by the percent of clot lysed after 2 h (26 ± 2% and 50 ± 6% for bolus and infusion, respectively). This is consistent with the clinical use of tPA by infusion to offset its rapid clearance. TNK-tPA was tested for efficacy at an equivalent dose (0.18 mg/kg) administered intravenously by bolus. The initial rate of lysis with TNK-tPA was 1.5%, a rate 3-fold greater than the rate with wt tPA (bolus TNK-tPA compared with infusion of tPA). After 2 h, the percent of clot lysed was 88 ± 8% with TNK-tPA. Thus a nearly 2-fold greater amount of lysis was induced by TNK-tPA by bolus compared with wt tPA administered by infusion, the optimal regimens for each. These results (Fig. 3) indicate that TNK-tPA induces 50% lysis 3 times faster than wt tPA does (35 compared with 120 min).

Thrombolytic Potency in Rabbits. The potency of TNK-tPA (by bolus) was compared with that of wt tPA (administered by infusion), in a dose–response experiment in rabbits with arterial venous shunts and whole blood clots. The extent of lysis after 2 h was evaluated with doses ranging from 6.7 to 180 μg/kg for TNK-tPA, and from 20 to 540 μg/kg for wt tPA (Fig. 4A). The percent of lysis was linearly correlated with the dose for both TNK-tPA and wt tPA. Maximal lysis was induced with TNK-tPA at 180 μg/kg (as shown in Fig. 3). The effective doses required to achieve 50% lysis were 21 and 160 μg/kg for TNK-tPA and wt tPA, respectively. This difference in ED_{50} corresponds to a 7.5-fold increase in potency of TNK-tPA compared with that of wt tPA, with respect to lysis of whole blood clots in the rabbit arterial venous shunt.

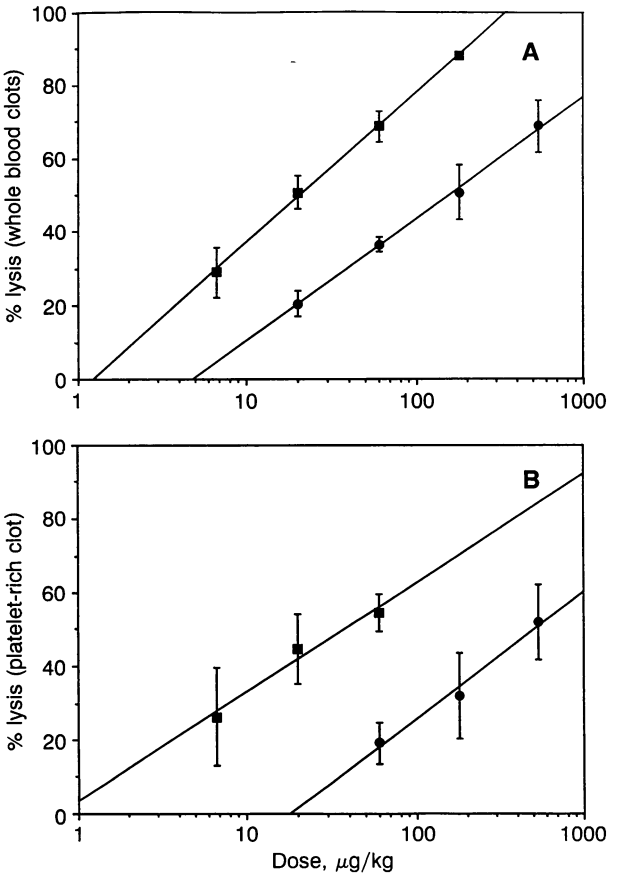

FIG. 4. Percentage of clot lysed in a rabbit arterial venous shunt after 2 h of treatment with various doses of TNK-tPA (■, bolus) or wt tPA (●, 90 min infusion with 15% as loading bolus). The clots were made from either whole rabbit blood (A) or platelet-enriched rabbit plasma (B). Each data point represents the mean ± SD of three experiments with five animals in each experiment. The relationship of lysis vs. logarithm of dose was fitted to a linear function. Relative potency was calculated by comparing the effective dose of wt tPA or TNK-tPA to achieve 50% lysis.

To define effects of TNK-tPA on clots more closely resembling coronary thrombi, platelet-rich clots were used (16). As previously noted, platelet-enriched clots are more resistant to lysis with wt tPA. Thus, three times more tPA is required to induce equivalent lysis of platelet-rich clots compared with whole blood clots. Both wt tPA and TNK-tPA yielded linear dose–response effects with platelet-rich clot lysis (Fig. 4B). The effective doses for 50% lysis were 37 and 500 μg/kg for TNK-tPA and wt tPA, respectively. Significantly, the TNK variant was 13.5-fold more potent in lysing platelet-rich clots *in vivo* compared with wt tPA. The observation that TNK-tPA lysed platelet-rich clots more effectively than whole blood clots (in contrast to the case with wt tPA) suggests that TNK-tPA, because of its relative PAI_1 resistance, may be less prone to inhibition by PAI_1 elaborated from activated platelets in platelet-rich clots. This concept is supported by the observation that the second-order rate constant for PAI_1 inhibition of TNK-tPA is $1.5 \pm 0.15 \times 10^5$ $M^{-1}\cdot sec^{-1}$, compared with 1.2×10^7 $M^{-1}\cdot sec^{-1}$ for wt tPA (see also ref. 23). Thus, TNK-tPA is 80-fold more resistant to inactivation by PAI_1 compared with wt tPA, potentially accounting for the greater efficacy of the variant in lysing platelet-rich clots *in vivo*.

DISCUSSION

Creating slow clearing variants of tPA for use as single bolus thrombolytic agents has been the goal of many mutagenesis efforts (8, 9). Treatment of acute myocardial infarction with a bolus of tPA is likely to be more convenient and might well lead to shorter times to reperfusion. Although many efforts at constructing slow-clearing forms of tPA have been successful, most of these molecules are deficient in fibrinolytic activity. The mutations in TNK-tPA that induce slower clearance (T and N) change the position of glycosylation and the complexity of the carbohydrate modification in kringle 1. Earlier studies have examined the effects of extraglycosylation within the growth factor domain (24, 25). Although the substitution variant, Y67N-tPA, had slower clearance, the fibrin binding and plasma clot lysis activities were substantially reduced (ref. 24 and D. L. Higgins and B.A.K., unpublished results). Domain deletion and alanine-scanning mutagenesis of tPA indicated that determinants of clearance are localized within the K1 domain; however, neither of the strategies that have been used resulted in tPA variants with the appropriate clearance and activity profiles. T-tPA exhibited significantly reduced clearance with some loss of fibrin binding and clot lysis activity. The T mutation resulted in an initially unexpected conversion of the N117 site from a high mannose to a complex carbohydrate site (20). Complex carbohydrate at N117 has been observed with des-finger and/or growth factor domain deletion variants (26). N variants of tPA typically exhibit a 2-fold reduction in clearance compared with that of wt tPA (21, 22) due to the loss of high mannose carbohydrate that results in decreased clearance mediated by the mannose receptor (27, 28). Therefore, the 8-fold reduced clearance of TNK-tPA cannot be entirely due to the loss of high mannose carbohydrate. The carbohydrate site at position 103 may be interfering with the binding of T-containing variants to one or more of the multiple receptors implicated in tPA clearance (29–31).

Molecular modeling of the tPA kringle 1 structure, based on the x-ray-determined structure of tPA K2 (32), suggested possible steric hindrance of two glycosylation sites on the same kringle domain. Amino acid sequence comparison of kringle-containing proteins suggested that glycosylation at position 103 was relatively conservative with respect to the kringle structure; analogous N-linked glycosylation sites are

observed in kringle 1 of prothrombins from different species (bovine and rat) and kringle 4 of hepatocyte growth factors (human and rat) (33–36). However, the combination of glycosylation at positions 103 and 117 has not been observed in nature. We constructed variants of tPA with the same number of glycosylation sites as that in wt tPA. By combining extraglycosylation (T) with deglycosylation (N), the location of the N-linked carbohydrate was moved from positions 117 to 103 within kringle 1. The carbohydrate at position 103, in the presence or absence of position 117 glycosylation, is of the complex type. To our knowledge, TNK is the first tPA variant with significantly reduced clearance, normal fibrin binding, and full *in vitro* fibrinolytic activity. As such, the decreased clearance results in a commensurate increase in thrombolytic potency *in vivo*, which may allow for a reduction in tPA dosage. These properties suggest that TNK-tPA can be administered as a bolus instead of by infusion and that it may induce recanalization remarkably rapidly (37).

The K mutation in TNK-tPA increases fibrin specificity such that even 10-fold greater concentrations of the tPA variant in plasma do not increase fibrinogenolysis *in vitro* compared with that induced by wt tPA (15). In addition to the increased fibrin specificity, the K mutation confers almost two orders of magnitude of increased resistance to PAI_1. The importance of this property is yet to be determined. The concentration of active circulating PAI_1 in normal human plasma is much lower than the concentrations of tPA induced therapeutically. However, the concentration of active PAI_1 in a platelet-rich clot may be much greater than that in plasma (39). The PAI_1 resistance of TNK-tPA may be responsible for its high lysis of platelet-rich clots compared with whole blood clots (16). In concert, our results show that TNK-tPA exhibits reduced clearance, comparable fibrin binding and fibrinolytic activity, increased fibrin specificity, and increased resistance to PAI_1 compared with wt tPA, all of which may constitute significant advantages for clinically induced thrombolysis.

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