Identification of the Mechanism Responsible for the Increased Fibrin Specificity of TNK-Tissue Plasminogen Activator Relative to Tissue Plasminogen Activator*

(Received for publication, December 2, 1999, and in revised form, January 13, 2000)

Ronald J. Stewart‡‡, James C. Fredenburgh‡, Beverly A. Leslie‡, Bruce A. Keyt¶, Janice A. Rischke‡, and Jeffrey I. Weitz‡

From the ‡Hamilton Civic Hospitals Research Centre and McMaster University, Hamilton, Ontario L8V 1C3, Canada and ¶Genentech, Inc., South San Francisco, California 94080

TNK-tissue plasminogen activator (TNK-t-PA), a bioengineered variant of tissue-type plasminogen activator (t-PA), has a longer half-life than t-PA because the glycosylation site at amino acid 117 (N117Q, abbreviated N) has been shifted to amino acid 103 (T103N, abbreviated T) and is resistant to inactivation by plasminogen activator inhibitor 1 because of a tetra-alanine substitution in the protease domain (R296A/I297A/R298A/R299A, abbreviated K). TNK-t-PA is more fibrin-specific than t-PA for reasons that are poorly understood. Previously, we demonstrated that the fibrin specificity of t-PA is compromised because t-PA binds to (DD)E, the major degradation product of cross-linked fibrin, with an affinity similar to that for fibrin. To investigate the enhanced fibrin specificity of TNK-t-PA, we compared the kinetics of plasminogen activation for t-PA, TNK-, T-, K-, TK-, and NK-t-PA in the presence of fibrin, (DD)E or fibrinogen. Although the activators have similar catalytic efficiencies in the presence of fibrin, the catalytic efficiency of TNK-t-PA is 15-fold lower than that for t-PA in the presence of (DD)E or fibrinogen. The T and K mutations combine to produce this reduction via distinct mechanisms because T-containing variants have a higher $K_{\text{m}}$, whereas K-containing variants have a lower $k_{\text{cat}}$ than t-PA. These results are supported by data indicating that T-containing variants bind (DD)E and fibrinogen with lower affinities than t-PA, whereas the K and N mutations have no effect on binding. Reduced efficiency of plasminogen activation in the presence of (DD)E and fibrinogen but equivalent efficiency in the presence of fibrin explain why TNK-t-PA is more fibrin-specific than t-PA.

Tissue-type plasminogen activator (t-PA) is a trypsin-like serine protease that initiates fibrinolysis by converting plasminogen (Pg) to plasmin (P). Plasmin then solubilizes fibrin, yielding fibrin degradation products. Through a positive feedback mechanism, fibrin enhances its own degradation by stimulating t-PA-mediated Pg activation (2). To enhance this reaction, fibrin binds t-PA and Pg, thereby increasing the catalytic efficiency of t-PA-mediated Pg activation 1000-fold over that in the absence of fibrin (2–6). In contrast to the potent stimulatory effect of fibrin, fibrinogen (Fg) produces only modest enhancement in the catalytic efficiency of Pg activation by t-PA (2, 7, 8). Because it preferentially activates Pg in the presence of fibrin rather than Fg, t-PA is designated a fibrin-specific plasminogen activator.

Despite its apparent fibrin specificity, t-PA causes systemic fibrinogenolysis and $\alpha_2$-antiplasmin consumption when given to patients (9, 10). Recently, we demonstrated that the fibrin specificity of t-PA is compromised because (DD)E, the major degradation product of cross-linked fibrin (11), stimulates Pg activation by t-PA to a similar extent as fibrin (11–13). This occurs because, like fibrin, (DD)E also binds t-PA and Pg with high affinity, thereby enhancing their interaction (14). As a potent stimulator of t-PA-mediated activation of Pg, (DD)E generated during thrombus dissolution has the potential to induce systemic plasminemia (11, 15). These data suggest that the fibrin specificity of an activator not only depends on its relative stimulation by fibrin and Fg but also on the degree to which it is stimulated by (DD)E. Furthermore, activators that have reduced stimulation by (DD)E but retain activity in the presence of fibrin should be more fibrin-specific than t-PA.

Whereas binding of t-PA to fibrin is predominantly mediated by its finger domain, high affinity t-PA binding to (DD)E is mediated solely by its second kringle domain (14, 16). Supporting the concept that interaction with (DD)E limits the fibrin specificity of t-PA, we demonstrated that vampire bat plasminogen activator, an agent homologous to t-PA but lacking a second kringle domain, is more fibrin-specific than t-PA because it fails to bind to (DD)E yet retains its finger-mediated interaction with fibrin (14, 17).

Recently, TNK-t-PA, a genetically engineered variant of t-PA, has been compared with t-PA for treatment of patients with acute myocardial infarction (18–20). Although the two agents produce similar efficacy rates, TNK-t-PA is easier to administer because it has a half-life 8.5-fold longer than that of t-PA (21). Consequently, TNK-t-PA can be given as a single intravenous bolus, whereas t-PA must be given by bolus followed by an infusion (22–25). The longer half-life of TNK-t-PA, relative to t-PA, is the result of two mutations introduced into the first kringle of t-PA: removal of the high mannose carbo-
glycosylation site within K1 is shifted 14 amino acids upstream. and protease domains. When mutations T and N are combined, the K2, and a protease domain (P). TNK-t-PA differs from t-PA in the K1 mutation by type-1 plasminogen activator inhibitor (26, 27). A third modification is introduced into the protease domain of position 103 with an asparagine (T103N, abbreviated T) (26). A glycosylation site by replacement of the threonine residue at glutamine (N117Q, abbreviated N) 2 and creation of a new hydrate at position 117 by substitution of asparagine with glutamine (N117Q, abbreviated N) 2 and creation of a new glycosylation site by replacement of the threonine residue at position 103 with an asparagine (T103N, abbreviated T) (26). A third modification is introduced into the protease domain of TNK-t-PA. By replacing the amino acids at positions 296–299 with alanine residues (K296A/H297A/R298A/R299A, abbreviated K), TNK-t-PA is rendered relatively resistant to inactivation by type-1 plasminogen activator inhibitor (26, 27).

In vivo studies indicate that despite producing equivalent thrombolyisis, TNK-t-PA causes less Fg and 2-antiplasmin consumption than t-PA (18, 19). The current study was undertaken to explore the mechanism responsible for the greater fibrin specificity of TNK-t-PA relative to t-PA. The fibrin specificity of TNK-t-PA and variants with only one or two modifications (T-, K-, KT-, and NK-t-PA; Fig. 1) were compared by incubating them in plasma in the absence or presence of a plasma clot and measuring Björklund generation as a sensitive index of fibrinogenolysis. Based on our previous work demonstrating that (DD)E-mediated stimulation of systemic plasminogen activation compromises the fibrin specificity of t-PA (12, 14, 28, 29), we examined the possibility that differences in fibrin specificity of the activators reflect the extent to which they are stimulated by (DD)E or Fg. To accomplish this, we compared the kinetics of Fg activation by t-PA to that of the TNK variants in the presence of fibrin, (DD)E, or Fg and determined the affinities of the activators for these fibrinogen derivatives.

2 Mutations in t-PA are abbreviated as follows: T, T103N; N, N117Q; K, K296A/H297A/R298A/R299A.
Characterization of the Fibrin Specificity of TNK-t-PA

no free α- or γ-chains are detected (12, 42).

Quantification of Plasminogen Activator-induced Fibrinogenolysis in the Absence or Presence of 125I-Labeled Plasma Clots—t-PA, TNK-t-PA, or a TNK-t-PA variant in concentrations ranging from 0 to 60 nM was incubated for 60 min at 37 °C in 500-μl aliquots of plasma in the absence of fibrinogen. A washed 125I-labeled plasma clot, and clot lysis and fibrinogenolysis were measured as follows. For clot lysis, at the end of the incubation period, clots were removed, washed once with 1 ml of Tris-buffered saline, and counted for residual radioactivity for 1 min. The extent of clot lysis was calculated by subtracting the residual radioactivity from the initial amount and expressing this value as a percentage of initial radioactivity. For fibrinogenolysis, aliquots of plasma were excited at a fixed wavelength (λem = 405 nm) and emission intensities were measured at the same wavelength. Excitation and emission slit widths were set to either 8 or 12 nm with a 1% attenuation emission filter (14). 5- or 10-μl aliquots of Fg or (DD)E (15 and 5 μM, respectively) were added to 2 ml of 0.1 μM active site blocked activator, and changes in scattering intensity were monitored. Control titrations were performed to determine the light scattering intensity of (DD)E or Fg alone.

Lysine Affinity of t-PA, TNK-t-PA, or Its Variants—To compare the affinity of the activators for lysine, changes in intrinsic fluorescence were monitored as each plasminogen activator was titrated with lysozyme or EACA (14). Additions of 10–20 μM of 20 μM t-lysin or EACA were made to a 2-mM solution containing 0.1 μM active site blocked activator. Intrinsic fluorescence was monitored with λex = 280 nm, λem = 340 nm with a 290-nm cut-off filter and slit widths set to 5 nm.

Data Analysis—For analysis of fibrin binding, the concentration of unbound protein in the clot supernatants was determined by comparing the fluorescence intensity with those of known concentrations of protein. The concentration of bound protein was then determined by calculating the difference between total and unbound protein concentrations. These values were divided by the Fg concentration to determine the number of moles of dEGR-labeled activator bound/mol of fibrin (B). For each point in the titration, values were plotted against the concentration of unbound protein, and Scatchard plots were constructed. Scatchard plots for the binding of t-PA, TNK-t-PA, and its variants to fibrin were curved downward, and these data are best fit to a two-site model by nonlinear regression analysis (Table Curve, Jandel Scientific, San Rafael, CA) according to the following expression.

\[ B = \frac{n_1 - L}{K_d + L} + \frac{n_2 - L}{K_d + L} \]  

(Eq. 1)

where L represents the concentration of unbound protein, \( n_1 \) and \( n_2 \) are the stoichiometries for each of the two sites, and \( K_{d1} \) and \( K_{d2} \) are their respective dissociation constants.

For analysis of solution-phase binding of PPACK-labeled activator to (DD)E or Fg, the emission intensity (I) of the incident beam after each addition of ligand was corrected for changes because of ligand scattering and dilution. Corrected values were compared with the emission intensity before the addition of ligand (I0), and these data, together with the total ligand concentration (L0), were fit by nonlinear regression analysis (Table Curve, Jandel Scientific) to the following equation.

\[ I/L_0 = 1 + \frac{\alpha}{2} \left( 1 + \frac{K_o + L_o}{n \cdot P_o} \right)^2 - 4 \cdot \frac{L_o}{n \cdot P_o} \]  

(Eq. 2)

where \( L_0 \) is the concentration of ligand added, \( P_o \) is the concentration of target protein, \( n \) is the stoichiometry, and \( \alpha \) is the maximum change in emission intensity. Using \( \alpha \) as a measure of 100% ligand bound, the amount of unbound ligand was determined after each addition of ligand, and Scatchard analysis was used to confirm the binding parameters derived from Equation 2.

RESULTS

**t-PA or TNK-t-PA-induced Clot Lysis**—To compare the thrombolytic activities of t-PA and TNK-t-PA, plasma clots prepared with trace amounts of 125I-labeled Fg were incubated in plasma for 1 h at 37 °C with either t-PA or TNK-t-PA in concentrations ranging from 0 to 60 nM. Using residual thrombus radioactivity as a measure of clot lysis, both activators produce a concentration dependent and saturable increase in clot lysis (Fig. 2). When added in equimolar concentrations, both activators produce a concentration dependent and saturable increase in clot lysis (Fig. 2). When added in equimolar concentrations, both activators produce a concentration dependent and saturable increase in clot lysis (Fig. 2).
in plasma in the absence of a plasma clot (Fig. 3A). Only with 60 μM t-PA, the highest concentration tested, does the Bβ1–42 concentration exceed 1% of total releasable Bβ1–42 (based on a plasma fibrinogen concentration of 9 μg/ml (45)). Presumably sufficient plasmin is generated at this t-PA concentration to overcome inhibition by α2-antiplasmin.

Both t-PA and TNK-t-PA generate more Bβ1–42 in the presence of a clot than in its absence (Fig. 3B). The higher Bβ1–42 levels in the presence of a clot are not due to Bβ1–42 trapped within the clot because, in previous studies, we demonstrated that less than 10 μM Bβ1–42 is recovered when clots suspended in buffer are completely lysed by t-PA (12). At each activator concentration, t-PA causes more Bβ1–42 release in the presence of a clot than TNK-t-PA. Measurements of Fg consumption parallel Bβ1–42 results. With 15 μM activator, only 1% of the Fg is consumed in the presence of TNK-t-PA, whereas 29% of the Fg is consumed with t-PA (data not shown). These results reveal that, in the presence of a clot, TNK-t-PA produces less fibrinogenolysis than t-PA. Because both activators produce equivalent clot lysis but TNK-t-PA causes less Fg degradation, TNK-t-PA is more fibrin-specific than t-PA.

**Potentiation of t-PA or TNK-t-PA-mediated Plasminogen Activation by Fibrin, (DD)E, or Fg**—To explore the mechanism responsible for the increased fibrin specificity of TNK-t-PA relative to t-PA, we compared the ability of fibrin, (DD)E, or Fg to stimulate the activation of Glu-Pg by t-PA or TNK-t-PA. The rates of Glu-Pg activation in the presence of saturating concentrations of fibrin, (DD)E, or Fg (0.3, 0.3, and 2 μg/ml, respectively) were determined at concentrations of Glu-Pg ranging from 0 to 40 μg/ml and were compared with those measured in the absence of a cofactor. Cofactor concentrations were considered saturating when maximum catalytic efficiencies were achieved at Fg concentrations above 1 μg/ml, as determined in separate experiments. As illustrated in Fig. 4A, fibrin produces similar increases in the rates of Glu-Pg activation by t-PA and TNK-t-PA. In contrast, (DD)E markedly increases the rate of Glu-Pg activation by t-PA but not by TNK-t-PA (Fig. 4B). Fg, which moderately increases the rate of Glu-Pg activation by t-PA, has little effect on TNK-t-PA-mediated Fg activation (Fig. 4C). The observation that (DD)E and, to a lesser extent Fg, stimulate Glu-Pg activation by t-PA, but not TNK-t-PA, explains why TNK-t-PA is more fibrin-specific than t-PA.

**Fibrinogenolysis Induced by TNK Variants**—To begin to determine which mutation (or combination of mutations) in TNK-t-PA contributes to its increased fibrin specificity over t-PA, additional t-PA variants containing single (T- and K-t-PA) or double (TK- and NK-t-PA) mutations were examined (Fig. 1). All of the activators produce equivalent plasma clot lysis (data not shown). To compare the fibrinogenolysis induced by the TNK variants, 15 μg/ml t-PA, TNK-, T-, K-, TK-, or NK-t-PA was incubated in plasma in the absence or presence of a plasma clot for 1 h at 37 °C, and Bβ1–42 generation was used to compare the extent of Fg degradation. All of the activators generate more Bβ1–42 in the presence of a clot than in its absence. As illustrated in Fig. 5, t-PA causes the most Bβ1–42 generation in the presence of a clot. When compared with t-PA, T-t-PA produces 50% less Bβ1–42, K- and NK-t-PA produce 65% less Bβ1–42, and TK- and TNK-t-PA produce 80% less Bβ1–42. These data suggest that both the T and K mutations contribute to the enhanced fibrin specificity of TNK-t-PA and that their effects are additive.

**Effect of Fibrin, (DD)E, or Fg on the Kinetics of Glu-Pg Activation by TNK Variants**—To determine whether the increased fibrin specificity of the TNK variants reflects the degree to which the activators are stimulated by various cofactors, we compared the effect of fibrin, (DD)E or Fg on the
Characterization of the Fibrin Specificity of TNK-t-PA

**Fig. 4.** Potentiation of t-PA- and TNK-t-PA-mediated Glu-Pg activation by fibrin, (DD)E, or Fg. 0.5 μM TNK-t-PA (closed circles) or t-PA (closed circles) was incubated with Glu-Pg at the concentrations indicated, in the absence (open circles) or presence of 0.3 μM fibrin (A), 0.3 μM (DD)E (B), or 2 μM Fg (C). Plasmin formation was monitored using the plasmin-directed substrate S2251, and rates of plasmin formation were calculated. Fibrin increases the rates of t-PA- and TNK-t-PA-mediated plasmin formation to a similar extent. In contrast, (DD)E and Fg have less effect on the rate of plasmin formation by TNK-t-PA than by t-PA.

**Fig. 5.** Comparison of the fibrinogenolytic activity of TNK variants. An equimolar concentration (15 nM) of t-PA, TNK- T-, K-, TK-, or NK-t-PA was incubated in plasma for 1 h at 37 °C in the absence (closed bars) or presence (open bars) of a plasma clot, and the extent of fibrinogenolysis was determined by measuring plasma levels of B1–42. Each bar represents the mean ± S.E. of six experiments, each done in duplicate, for t-PA and TNK-t-PA, and four experiments, each done in duplicate, for T-, K-, TK-, and NK-t-PA. All activators produce more B1–42 in the presence of a plasma clot than in its absence. In the presence of a clot, t-PA generates 50% less B1–42 than t-PA, and K-t-PA and NK-t-PA both produce 65% less B1–42 than t-PA. Like T-t-PA, B1–42 production by TNK-t-PA is 80% less than that generated by t-PA.

The kinetic parameters of Glu-Pg activation by t-PA, TNK-, T-, K-, TK-, or NK-t-PA were determined with a constant amount of activator, and systematic variations in both the Pg and cofactor concentration. Data were fit to the Michaelis-Menten equation by nonlinear regression to determine the values of $k_{cat}$ and $K_M$. Cofactor concentrations were considered saturating when the catalytic efficiency ($k_{cat}/K_M$) reached a maximum. A maximum catalytic efficiency was achieved with both fibrin and (DD)E at concentrations ranging from 0.3 to 0.6 μM, whereas a Fg concentration of 1–5 μM was needed to reach the maximum catalytic efficiency. The results of these analyses are shown in Table I. Table II illustrates the relative catalytic efficiencies for t-PA, TNK-t-PA, and its variants in the presence of saturating amounts of fibrin, (DD)E, or Fg.

With fibrin, T-t-PA has the same catalytic efficiency as t-PA, whereas K-containing mutants have a catalytic efficiency about 1.5-fold lower than t-PA. In the presence of (DD)E, however, the catalytic efficiencies of all mutants are lower than that of t-PA; T-t-PA is 3-fold lower, K- and NK-t-PA are 8-fold lower, and TK- and TK-t-PA are 15-fold lower than t-PA. The T and K mutations contribute to the decreased catalytic efficiencies in the presence of (DD)E via distinct mechanisms because T-containing variants have a 2.3–3.2-fold higher $K_M$, whereas K-containing variants have a 5.2–6.5-fold lower $k_{cat}$ compared with t-PA (Table I). These data demonstrate that (DD)E stimulates t-PA to a similar extent as fibrin (fibrin/(DD)E ratio of 1; Table II). Because the catalytic efficiencies of the TNK variants are similar to that of t-PA in the presence of fibrin but markedly lower in the presence of (DD)E, the TNK variants are more fibrin-selective and have fibrin/(DD)E ratios greater than 1. By these criteria, TNK- and TK-t-PA are the most fibrin-selective, with ratios of 9.5 and 8.2, respectively. The catalytic efficiencies of all of the activators are approximately 10-fold lower in the presence of Fg than those in the presence of (DD)E (Table II). The hierarchy of fibrin specificity based on fibrin/Fg ratios, however, is similar to that based on fibrin/(DD)E ratios.

**Binding of t-PA, TNK-t-PA, or Its Variants to Fibrin—To characterize the binding of t-PA, TNK-t-PA, and its variants to fibrin, Scatchard plots were generated. The plots for all activators are nonlinear and indicate binding site heterogeneity (Fig. 6) (14, 16, 46). Each activator binds to fibrin via a high and low affinity site. High affinity binding is mediated by the finger domain, whereas low affinity binding is mediated by the second kringle domain of the activator (14, 16). The dissociation constant for high affinity binding of t-PA to fibrin is 0.05 μM, and that for low affinity binding is 2.6 μM (Table III). K- and NK-t-PA bind to fibrin with affinities similar to that of t-PA. $K_d$ values for the high and low affinity sites are 0.1 and 2.5 μM, respectively, for K-t-PA and 0.087 and 2.2 μM, respectively, for NK-t-PA, whereas T-containing mutants (TNK-, T-, and TK-t-PA) exhibit a 3-fold reduction in affinity for finger-dependent binding and a 5-fold lower affinity for kringle-dependent binding (Table III).

**Interactions of t-PA, TNK-t-PA, or Its Variants with (DD)E—**To determine whether differences in the catalytic efficiencies of plasminogen activation in the presence of (DD)E reflect changes in the affinity of the activators for (DD)E, the binding of each activator to (DD)E was measured by light scattering (14). Fig. 7A illustrates the relative scatter plots for the interactions of t-PA, TNK-, T-, K-, TK-, and NK-t-PA with (DD)E. With the conditions outlined under “Methods,” the scattering intensity of 0.1 μM PPACK-t-PA is 1.0 ($I_s$). At saturating levels of (DD)E, the maximum relative scattering intensity ($I_{scat}$) is 21; a value in good agreement with a calculated maximum relative scattering intensity of 22 if the stoichiometry is...
Characterization of the Fibrin Specificity of TNK-t-PA

10117

All values are presented as the means ± S.E. of at least three experiments.

| Activator | No cofactor | Dopamine (DD)E | Fibrin |
|-----------|-------------|----------------|--------|
|           | $k_{cat}/K_M \times 10^{-4}$ | $k_{cat}$ | $K_M$ | $k_{cat}$ | $K_M$ | $k_{cat}$ | $K_M$ |
| t-PA      | 0.02 ± 0.01 | 0.42 ± 0.03 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| TNK-t-PA  | 0.03 ± 0.02 | 0.45 ± 0.01 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| T         | 0.02 ± 0.03 | 0.43 ± 0.02 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| TK        | 0.03 ± 0.01 | 0.44 ± 0.01 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| K         | 0.02 ± 0.03 | 0.43 ± 0.02 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| NK        | 0.03 ± 0.01 | 0.44 ± 0.01 | 0.05 ± 0.02 | 2.8 ± 0.5 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |

**TABLE II**

Relative catalytic efficiencies of Glu-Fg activation by t-PA, TNK-t-PA, or its variants in the absence or presence of fibrin (DD)E or Fg

| Activator | (DD)E/Fg | Fibrin/DD/E | Fibrin/Fg |
|-----------|----------|-------------|-----------|
| t-PA      | 11.1     | 1.0         | 11.1      |
| TNK-t-PA  | 10.0     | 9.5         | 95.0      |
| T         | 9.5      | 3.2         | 30.0      |
| TK        | 11.6     | 8.2         | 94.7      |
| K         | 12.3     | 5.4         | 66.7      |
| NK        | 12.5     | 5.5         | 68.8      |

**DISCUSSION**

The fibrin specificity of plasminogen activators is traditionally defined as the ratio of stimulation of plasminogen activation that occurs in the presence of fibrin relative to that in the presence of Fg. However, this definition overlooks the fact that (DD)E compromises the fibrin specificity of t-PA by binding and stimulating t-PA to the same extent as fibrin (12–14, 48). Systemic plasmin generation triggered by (DD)E may explain why t-PA causes Fg and α2-antiplasmin consumption when given to patients.

TNK-t-PA, a genetically modified variant of t-PA, is as effective as t-PA for treatment of patients with acute myocardial infarction (18–21). However, TNK-t-PA is more convenient to use because its longer half-life permits single bolus administration, whereas t-PA must be given as a bolus followed by an infusion. TNK-t-PA is more fibrin-specific than t-PA and causes less Fg and α2-antiplasmin consumption (18, 19). This phenomenon could explain why the need for red blood cell transfusion was lower in patients treated with TNK-t-PA than it was in those given t-PA (20).

The mechanism responsible for the enhanced fibrin specificity of TNK-t-PA, relative to t-PA, is unclear. Keyt et al. (26)
Characterization of the Fibrin Specificity of TNK-t-PA

Table III

Dissociation constants for the binding of t-PA, TNK-t-PA, or its variants to fibrin, (DD)E, Fg, or lysine.

| Ligand | t-PA | TNK | T | TK | K | NK |
|--------|------|-----|---|----|---|----|
| Fibrin* | 0.050 ± 0.016 | 0.15 ± 0.02 | 0.16 ± 0.01 | 0.18 ± 0.03 | 0.10 ± 0.01 | 0.087 ± 0.012 |
| (DD)E | 2.6 ± 0.26 | 15 ± 3 | 13 ± 2 | 13 ± 3 | 2.5 ± 0.2 | 2.2 ± 0.1 |
| Fg | 0.80 ± 0.12 | 3.2 ± 0.2 | 4.0 ± 0.2 | 3.8 ± 0.4 | 0.90 ± 0.11 | 0.75 ± 0.01 |
| Lysine | 230 ± 10 | 340 ± 23 | 460 ± 26 | 510 ± 10 | 180 ± 9 | 150 ± 8 |

* Two dissociation constants are indicated for fibrin binding because activators bind to fibrin via a high affinity site and a low affinity site.

Figure 7. Binding of t-PA, TNK-t-PA, or its variants to (DD)E or Fg. To measure binding to (DD)E (A), (DD)E was titrated into 0.1 μM active site blocked activator, and scattering intensities obtained in the presence of (DD)E (I) were compared with those obtained in its absence (I). Solid lines represent nonlinear regression analysis of the data. T-containing activators (T-, TK-, and TNK-t-PA) bind to (DD)E with lower affinity than activators without this mutation (K-, NK-, and t-PA), indicating that the T mutation compromises activator binding to (DD)E. Binding to Fg (B), which was quantified using the same technique, yields similar results with T-containing activators (T-, TK-, and TNK-t-PA) exhibiting lower affinity for Fg than K-, NK-, and t-PA.

Table III demonstrates that TNK-t-PA has less enzymatic activity in the presence of Fg than t-PA, although both agents have similar activities in the presence of fibrin. Presumably differences in the presence of Fg reflect less stimulation of TNK-t-PA than t-PA. However, these studies fail to consider the potential influence of (DD)E. The current study was undertaken to explore the mechanism for the greater fibrin specificity of TNK-t-PA relative to t-PA and to identify the responsible mutations so that structure-function relationships could be better defined.

Over a range of activator concentrations that approximates those achieved after intravenous bolus injection (18, 21), t-PA and TNK-t-PA produce equivalent clot lysis (Fig. 2), results that are in agreement with clinical trial data indicating that TNK-t-PA and t-PA are equally effective (18–20). In contrast, in the presence of a plasma clot, TNK-t-PA induces less Bj1-42 generation than t-PA (Fig. 3B). Because our previous studies revealed that clot-derived (DD)E is responsible for the increase in Fg consumption (12), we hypothesized that TNK-t-PA would be less responsive to (DD)E than t-PA.

To test this hypothesis, we determined the kinetics of Pg activation by t-PA or TNK-t-PA in the presence of fibrin, (DD)E, or Fg (Fig. 4). Whereas fibrin stimulates plasminogen formation by t-PA and TNK-t-PA to a similar extent, (DD)E markedly increases the rate of Pg activation by t-PA but not TNK-t-PA. Likewise, Fg has little effect on Pg activation by TNK-t-PA. Because the soluble cofactors, (DD)E and Fg, stimulate Pg activation by TNK-t-PA to a lesser extent than t-PA, whereas fibrin potentiates both activators to a similar extent, these data explain why TNK-t-PA is more fibrin-specific than t-PA. The importance of (DD)E in defining the fibrin specificity of plasminogen activators is highlighted by the observation that (DD)E is as potent as fibrin as a stimulator of t-PA and TNK-t-PA.

As illustrated in Fig. 4A, the relative rate of t-PA- and TNK-t-PA-mediated plasmin formation in the presence of fibrin depends on the Pg concentration. The rate of t-PA-mediated plasminogen activation is higher at low Pg concentrations (0–2 μM), whereas TNK-t-PA appears more active at higher Pg concentrations. This observation may explain some of the discrepancies in the literature. Although the rate of TNK-t-PA-mediated Pg activation in the presence of fibrin has not been reported, Paoni et al. (32) noted that the turnover number for TK-t-PA-mediated activation of Pg in the presence of fibrin is 3-fold higher than that for t-PA. In contrast, Keyt et al. (26) reported that TNK-t-PA has 1.2-fold lower activity in the presence of a plasma clot than t-PA. It is unlikely that this difference reflects the absence or presence of the N mutation, because we and others have found that removal of this glycosylation site has little effect on the kinetics of Pg activation (discussed below, in Tables I and II, and in Ref. 26). The higher relative rate of plasminogen formation determined by Paoni et al. (32) for the TK-t-PA variant may reflect the fact that these investigators used Pg concentrations ranging from 0.9 to 19 μM to measure the maximum rate of Pg activation. In contrast, Keyt and colleagues (26) used 1 μM Pg to compare plasminogen activation by t-PA and TNK-t-PA.

Although the $k_{cat}$ for Pg activation is higher for TNK-t-PA than for t-PA in the presence of fibrin (Table I), so is the $K_{M}$, suggesting that the stability of the ternary activator-substrate cofactor complex is decreased. However, it is important to note...
that at 2 μM Pg, the concentration of Pg found in plasma, the rate of plasmin formation in the presence of fibrin is equivalent for TNK-t-PA and t-PA (Fig. 4A). These data may explain why the two activators produce equivalent thrombolysis in vitro (Fig. 2) and in patients (19, 20).

To identify the mutations that influence the fibrin specificity of TNK-t-PA, we compared TNK-t-PA-induced Bβ1–42 generation in plasma in the absence and presence of a plasma clot with that obtained with T-, K-, TK-, or NK-t-PA (Figs. 1 and 5). Although all of these activators produce equivalent plasma clot lysis, the variants induce less fibrinogenolysis than t-PA (Fig. 5). TK- and TNK-t-PA are the most fibrin-specific, generating the least Bβ1–42. T-t-PA and K-t-PA produce Bβ1–42 levels intermediate to those produced by t-PA and TNK-t-PA, suggesting that the T- and K- mutations contribute to the fibrin specificity of TNK-t-PA in an additive fashion.

Detailed kinetic analysis reveals that in the presence of fibrin, the kcat and KM for K-containing variants are 2- and 3-fold higher, respectively, than those for t-PA, resulting in a catalytic efficiency approximately 1.5-fold lower than that of t-PA (Table I). Our results are consistent with those of others who demonstrated that both the kcat and KM are higher for K-t-PA than for t-PA in the presence of fibrin (32, 49). These data support the concept that although the K mutation increases the rate of TNK-t-PA-mediated Pg activation in the presence of fibrin, it decreases the stability of the ternary cofactor-enzyme-substrate complex.

The soluble cofactor, (DD)E, distinguishes the TNK variants from t-PA. In the presence of (DD)E, the catalytic efficiencies of all the mutants are lower than that of t-PA. Furthermore, the catalytic efficiencies in the presence of (DD)E parallel the extent of Bβ1–42 generation produced by the various activators in the presence of clots. These data suggest that the extent of stimulation by (DD)E has a major impact on Fg degradation.

In the presence of (DD)E, the kcat for Glu-Pg activation by activators containing the K mutation is approximately 6-fold lower than that for t-PA (Table I). In support of this concept, Eastman et al. (49) reported that K-t-PA has a 5-fold lower catalytic efficiency than t-PA in the presence of Pg that was partially degraded by plasmin, reflecting a reduction in kcat. In contrast, we demonstrate that T-containing mutants have a 2.5–3-fold increase in KM compared with t-PA in the presence of (DD)E (Table I). These data suggest that although both the K and T mutations decrease the catalytic efficiency of Pg activation in the presence of (DD)E, they do so via different kinetic mechanisms. Thus, it is the combination of a lower kcat (reflecting the K mutation) and higher KM (reflecting the T mutation) that results in the marked reduction in catalytic efficiencies of TK- and TNK-t-PA relative to t-PA in the presence of (DD)E.

Examination of the ratios of catalytic efficiency in the presence of fibrin, (DD)E or Fg for t-PA, TNK-t-PA or its variants (Table II) reveals that TK- and TNK-t-PA are the most fibrin-specific since their fibrin/(DD)E and fibrin/Fg ratios are the highest, whereas t-PA is the least fibrin-specific because it has the lowest ratios. The N mutation has no effect on fibrin specificity since K- and NK-t-PA have similar kinetic ratios. In contrast, both T- and K-t-PA have higher catalytic efficiencies in the presence of fibrin than in the presence of (DD)E or Fg. Again, these data demonstrate that the T and K mutations combine to increase the fibrin specificity of TNK-t-PA. These results are consistent with the hierarchy of fibrin specificity determined by Bβ1–42 generation, and suggest that the fibrin specificity of the activator is determined by the catalytic efficiency of the activator in the presence of fibrin relative to that in the presence of the soluble cofactors, (DD)E and Fg.

To determine whether differences in the catalytic efficiencies of Pg activation reflect changes in the affinity of the activators for the cofactor, we measured the binding of t-PA, TNK-t-PA, or its variants to fibrin, (DD)E, or Fg. All activators bind to fibrin via two distinct classes of sites (Fig. 6 and Table III). Previously, we and others demonstrated that high affinity binding of t-PA to fibrin is mediated by its finger domain, whereas low affinity binding is mediated by its second kringle domain (14, 16). The results reported here indicate that the K and N mutations do not alter the affinity of the activator for fibrin because K- and NK-t-PA bind to fibrin with affinities similar to that of t-PA. In contrast, the T mutation produces a 3-fold reduction in high affinity binding and a 5-fold decrease in low affinity binding. Although Keyt et al. (26) demonstrated similar reductions in binding to fibrin for T-containing mutants, these investigators reported apparent KM values based on half-maximal binding of activator to various amounts of fibrin, values that do not differentiate between high and low affinity binding. Our findings indicate that the T mutation influences the low affinity, kringle-dependent binding of the activator to fibrin to a greater extent than the high affinity, finger-dependent interaction.

Despite the fact that T-t-PA has reduced affinity for fibrin relative to t-PA, both activators have similar catalytic efficiencies in the presence of fibrin. TK- and K-t-PA also have similar efficiencies in the presence of fibrin, despite differences in low affinity binding of the activators to fibrin. These data suggest that moderate reductions in affinity of these activators for fibrin, caused by additional glycosylation at amino acid 103 (T), do not significantly affect the kinetics of Pg activation in the presence of fibrin.

Like the results with fibrin, T-containing mutants exhibit reduced affinity for (DD)E (Fig. 7A). However, the T mutation produces a greater reduction in the affinity for (DD)E than for fibrin (Table III). In contrast, K- and NK-t-PA bind to (DD)E with affinities similar to that of t-PA. Reduced affinity for (DD)E explains why T-t-PA has a lower catalytic efficiency than t-PA and why both TK- and TNK-t-PA have lower catalytic efficiencies than K-t-PA in the presence of (DD)E. Thus, reduced affinity of the activator for (DD)E and, in turn, reduced catalytic efficiency, contributes to the increased fibrin specificity of TNK-t-PA.

Affinities of the activators for Fg parallel those for (DD)E (Fig. 7B and Table III). However, the catalytic efficiency of plasminogen activation in the presence of (DD)E is an order of magnitude higher than in the presence of Fg. Furthermore, the fold reduction in affinity of the T-containing activators for (DD)E is higher than that for Fg. Taken together, these findings suggest that alterations in Fg affinity have little influence on TNK-t-PA-mediated Pg activation in the presence of a clot.

Based on our previous observation that the binding of t-PA to (DD)E is mediated by its second kringle domain (14), we determined whether differences in the affinities of the activators for (DD)E reflect their affinities for l-lysine. Consistent with binding of the activators to (DD)E and Pg, l-lysine binds to T-containing variants with affinities lower than that for t-PA (Table III). These data confirm that the lysine binding properties of the activator are reduced by the addition of glycosylation at amino acid 103 and are consistent with a reduced affinity of T-containing activators for (DD)E and Fg.

Our studies give insight into the biochemical differences between t-PA and TNK-t-PA and help to explain why TNK-t-PA is more fibrin-specific than t-PA. The two agents have equivalent fibrinolytic properties because fibrin stimulates TNK-t-PA and t-PA to a similar extent. In contrast, TNK-t-PA produces less fibrinogenolysis than t-PA because (DD)E produces less stimulation of TNK-t-PA than t-PA. These observa-
tions highlight the importance of (DD)E as a determinant of the fibrin specificity of plasminogen activators. We have demonstrated that both the T and K mutations contribute to the decreased stimulation of TNK-t-PA by (DD)E via two distinct mechanisms. Glycosylation addition within the first kringle (T) decreases the affinity of TNK-t-PA for (DD)E by reducing it mechanisms. Glycosylation addition within the first kringle (T) decreases the affinity of TNK-t-PA for (DD)E by reducing its lysine binding properties, whereas the tetra-alanine substitution in the protease domain (K) decreases the rate of plasmin formation in the presence of (DD)E. Thus, the T and K mutations combine to decrease the catalytic efficiency of Pg activation by TNK-t-PA in the presence of (DD)E, and this in turn increases the fibrin specificity of TNK-t-PA relative to t-PA. Our studies also suggest that the fibrin specificity of TNK-t-PA could be enhanced further by eliminating its lysine binding properties.

Acknowledgments—We thank Dr. Michael Nesheim for many helpful discussions and Alan Stafford for excellent technical advice.

REFERENCES
1. Collen, D., and Lijnen, H. R. (1986) Haemostasis 16, (Suppl. 3) 25–32
2. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) J. Biol. Chem. 257, 2912–2919
3. Horrevoets, A. J. G., Pannekoek, H., and Nesheim, M. E. (1997) J. Biol. Chem. 272, 2183–2191
4. Lijnen, H. R., Van Hoe, B., and Collen, D. (1990) Thromb. Res. 57, (Suppl. X) 45–54
5. de Vries, C., Veerman, H., Koomen, E., and Pannekoek, H. (1990) J. Biol. Chem. 265, 15347–15352
6. Masek, M. W., Siebenlist, K. R., Voskuilen, M., and Nieuwenhuizen, W. (1998) Thromb. Haemostasis 79, 796–801
7. Verheijen, J. H., Nieuwenhuizen, W., and Wijngaards, G. (1982) Thromb. Res. 27, 377–385
8. Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., and Chang, G. T. G. (1983) Biochem. Biophys. Res. Commun. 120, 123–142
9. Collen, D. (1997) Thromb. Haemostasis 78, 742–746
10. Rao, A. K., Pratt, C., Berke, A., Jaffe, A., Ockene, I., Schreiber, T. L., Bell, W. R., Knatterud, G., Robertson, T. L., and Terrin, M. L. (1988) J. Am. Coll. Cardiol. 11, 1–11
11. Olexa, S. A., and Budzynski, A. Z. (1979) J. Biol. Chem. 254, 4925–4932
12. Weitz, J. I., Leslie, B., and Ginsberg, J. (1991) J. Clin. Invest. 87, 1082–1090
13. Stewart, R. J., Fredenburgh, J. C., Lee, A. Y., Rischke, J. A., and Weitz, J. I. (1990) Blood 76, 680–685
14. Nesheim, M. E., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541–21548
15. Klement, G., Klement, P., Smith, S., and Weitz, J. (1995) Thromb. Haemostasis 73, 139 (abstr.)
Identification of the Mechanism Responsible for the Increased Fibrin Specificity of 
TNK-Tissue Plasminogen Activator Relative to Tissue Plasminogen Activator 
Ronald J. Stewart, James C. Fredenburgh, Beverly A. Leslie, Bruce A. Keyt, Janice A. 
Rischke and Jeffrey I. Weitz 

J. Biol. Chem. 2000, 275:10112-10120. 
doi: 10.1074/jbc.275.14.10112

Access the most updated version of this article at http://www.jbc.org/content/275/14/10112

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 15 of which can be accessed free at 
http://www.jbc.org/content/275/14/10112.full.html#ref-list-1