Molecular requirements for safer generation of thrombolytics by bioengineering the tissue-type plasminogen activator A chain

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Summary. Background: Thrombolysis with tissue-type plasminogen activator (t-PA) is the only treatment approved for acute ischemic stroke. Although t-PA is an efficient clot lysis enzyme, it also causes damage to the neurovascular unit, including hemorrhagic transformations and neurotoxicity. Objectives: On the basis of the mechanism of action of t-PA on neurotoxicity, we aimed at studying the molecular requirements to generate safer thrombolytics. Methods: We produced original t-PA-related mutants, including a non-cleavable single-chain form with restored zymogenicity (sc*-t-PA) and a t-PA modified in the kringle 2 lysine-binding site (K2*-t-PA). Both sc*-t-PA and K2*-t-PA showed fibrinolytic activities similar to that of wild-type t-PA on both euglobulin-containing and plasma-containing clots. In contrast to wild-type t-PA, the two mutants did not promote N-methyl-D-aspartate receptor-mediated neurotoxicity. Conclusions: We designed t-PA mutants with molecular properties that, in contrast to t-PA, do not induce neurotoxicity. Keywords: neurotoxicity, thrombolytic, tissue-type plasminogen activator.

Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease of the neurovascular unit released by endothelial cells [1], neurons [2], and glial cells [3]. t-PA is an unusually active zymogen, with full intrinsic activity and low zymogenicity [4]. t-PA promotes fibrinolysis via the conversion of the inactive fibrin-bound zymogen plasminogen into plasmin. In the brain, t-PA shows critical functions and dysfunctions through either plasminogen-dependent or plasminogen-independent mechanisms [5]. Intravenous t-PA can also cross the disrupted blood–brain barrier [6], and thus can induce a neurotoxic effect [5–7].

Since the approval of recombinant t-PA (alteplase, Boehringer-Ingelheim, Paris, France) for the acute treatment of ischemic stroke, experimental data have favored the idea that, beyond its beneficial vascular effects, t-PA may have damaging properties in the cerebral parenchyma [7]. Indeed, t-PA promotes hemorrhagic transformations [8–11]. Moreover, in the brain parenchyma, its interaction with the N-methyl-D-aspartate receptor (NMDAR) in neurons activates signaling processes that result in neuronal death [8]. Such an interaction of t-PA with the N-terminal domain of GluN1 and subsequent neuronal death is reported to be dependent on its kringle 2 domain [12].

Current experimental strategies to improve safer thrombolysis include the use of a plasminogen activator inhibitor-1-derived hexapeptide to inactivate the pro-neurotoxicity of t-PA [13], and a soluble annexin A2 to improve the efficacy of t-PA-induced fibrinolysis [14]. Desmodus rotundus plasminogen activator (DSPA), a serine protease secreted by the D. rotundus salivary glands, shares 66% identity with human t-PA, but lacks a kringle domain as compared with t-PA. DSPA [15] was developed as a third-generation thrombolytic devoid of the above mentioned side-effects of t-PA. Indeed, a number of studies have emphasized the absence of excitotoxicity of this exclusively single-chain serine protease [16–18]. Nonetheless, although DSPA was proposed as a safer thrombolytic for stroke, the last phase III clinical trial (DIAS-2) was unsuccessful [19]. Concerns about the conclusions of the trial could be raised, as the patient inclusion criteria may have been heterogeneous in this study.
On the basis of the foregoing, we generated and characterized two new mutants of t-PA. These mutants include point mutations that yield, for one mutant, a non-cleavable single-chain form of t-PA (sc*-t-PA), and for the second one, a form modified in the kringle 2 lysine-binding site (LBS) that is unable to interact with NMDARs (K2*-t-PA).

Materials and methods

Amino acids are numbered from the N-terminal serine of the mature Rattus norvegicus t-PA sequence (UniProtKB: P19637).

Chemicals

N-methyl-D-aspartate (NMDA) was from Tocris (Bristol, UK); Spectrofluor 444FL and Spectrozyme were from American Diagnostica (ADF Biomedical, Neuville-sur-Oise, France); and 6-aminocaproic acid (ε-ACA), Dulbecco’s modified Eagle’s medium (DMEM), poly(L-lysine), cytosine β-D-arabinoside, extravidin and hygromycin B were from Sigma-Aldrich (L’Isle d’Abeau, France). The QuickChange XL kit was from Stratagene (La Jolla, CA, USA). Plasminogen was from Calbiochem (Nottingham, UK). Lipofectamine 2000, fetal bovine and horse sera and laminin were from Invitrogen (Cergy Pontoise, France). t-PA (alteplase) was from Boehringer-Ingleheim (Paris, France).

Construction of wild-type t-PA (wt-t-PA) and ΔK2-t-PA mutants in the pcDNA5/FRT vector

The rat t-PA cDNA sequence was amplified by PCR with the following primers: 5′-CCGGGATCCTCCTACAGAGCGACC-3′ and 5′-GGCAAGCTTTTGCTTCATGTTGCTTTGAATCCAGTT-3′ (with a His6 tag at the N-terminal position). PCR products were inserted into a pcDNA5/FRT vector (Invitrogen). Fusion PCR was performed to obtain ΔK2-t-PA from wt-t-PA, by use of the same protocol, with the following primers: 5′-CAGGCCACGTGGAGTCCTGAGTTGGTCCCTTAGG-3′ and 5′-TCCACCTGCGGCCTG-3′. Final constructs were automatically sequenced.

Site-directed mutagenesis

Mutagenesis of wt-t-PA (t-PA W254R) was performed with the QuikChange XL Kit and the following primers: 5′-GGACCGAAAGCTGACCGGAATATTGCGACATGTCC-3′ and 5′-GGCATGTCGCAATATTCCCGTGTCCTGAGTCTGAGTTGGTCCCTTAGG-3′ and 5′-TCCACCTGCGGCCTG-3′. Final constructs were automatically sequenced.

Human embryonic kidney (HEK)-293 cell cultures and stable transfection

Stable HEK-293 cells transfected with the pFRT/lacZeo vector (HEK-FlpIn; Invitrogen) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were transfected with Lipofectamine 2000. Positive clones were isolated by hygromycin B selection. The quality of the transfection was assessed by quantitative RT-PCR.

Bioreactor production of the t-PA-related mutants

To produce high yields of mutants, stable transfected HEK-293 cells were grown in a laboratory-scale bioreactor (CELLine AD 100; Dominique Dutscher SAS, Brumath, France).

Purification of His6 mutants

Purification was performed with a nickel–nitrioltriacetic acid metal-affinity chromatography matrix (Qiagen, Courtabouef, France). t-PA variants were then conditioned in an NH4HCO3 0.5 mM buffer and stored.

SDS-PAGE plasminogen–casein zymography

A zymography assay was performed by addition of plasminogen (4.5 μg/mL) and casein (1%) in 10% SDS–polyacrylamide gels. Electrophoresis was performed at 4 °C. Gels were washed with Triton X-100 (2.5%) and incubated for 2 h at 37 °C. Caseinolytic bands were visualized after Coomassie staining.

Amidolytic activity assay

t-PA variants were incubated in the presence of a fluorogenic substrate (5 μM) (Spectrofluor FL444). The reaction was carried out at 37 °C in 50 mM Tris (pH 8.0) containing 150 mM NaCl in a total volume of 100 μL. The amidolytic activity was measured as the change in fluorescence emission at 440 nm (excitation at 360 nm). With Spectrozyme, an amidolytic substrate (Spectrozyme t-PA [Spt-Pa]), wt-t-PA and sc*-t-PA (0.3 nM) were incubated with increasing concentrations of Spt-Pa (0–1 mM, 12 doses).
in a microplate (200 μL per well), and OD405 nm was recorded every minute with a microplate spectrophotometer (ELx 808; BioTek, Colmar, France). Then, the maximal velocity (Vmax) of the reaction was calculated, and the data were plotted as follows:

\[ V = \frac{V_{\text{max}} [\text{SptPA}]}{K_M + [\text{SptPA}]} \]

**Fibrin agarose zymography**

Proteins (10 μg) and reference proteins (10 μL of t-PA 6 IU mL⁻¹, urokinase plasminogen activator [u-PA] 2.5 IU mL⁻¹, and plasmin 200 nM) were electrophoresed in a 8% polyacrylamide gel under non-denaturing conditions. SDS was then exchanged with 2.5% Triton X-100 before the gel was carefully overlaid on a 1% agarose gel containing 1 mg mL⁻¹ bovine fibrinogen, 100 nM plasminogen, and 0.2 NIH unit mL⁻¹ bovine thrombin. Zymograms were allowed to develop at 37 °C for 12 h. Active proteins in cell lysates were identified by reference to the migration of known markers (u-PA, t-PA, and plasmin).

**Clot lysis time**

Human plasma was obtained from citrated blood, and the euglobulin fractions, containing β-globulins and γ-globulins, were separated by isoelectric precipitation. The euglobulin solution (100 μL), or the plasma, was supplemented with 15 mM calcium chloride and each of the t-PA mutants at 25, 30, 35 and 40 IU, or 900, 1000, 1100 and 1200 IU, respectively. The time to clot lysis was recorded by OD measurements at 405 nm and 37 °C by reference to the International Reference Preparation (IRP 98/714). Tests were performed in duplicate (from three independent experiments). Results are expressed as the time needed to obtain 50% clot lysis.

**Neuronal cell culture**

Neuronal cultures were prepared from fetal mice cortices (embryonic day 15–16), as previously described [8]. Various treatments were performed after 14 days in vitro.

**Excitotoxic neuronal death**

Excitotoxic neuronal death was induced by exposure of cortical neurons to NMDA alone or in the presence of one of the t-PA mutants for 1 h, as previously described [20]. NMDA was incubated at 50 μM final concentration in serum-free DMEM supplemented with 10 μM glycine. t-PA variants were used at 0.3 μM amidolytic activity final concentration (relative to alteplase), unless otherwise mentioned. Neuronal death was quantified 24 h later by measuring the activity of lactate dehydrogenase released from damaged cells into the bathing medium with a cyto-

toxicity detection kit (Roche Diagnostics, Mannheim, Germany).

**Statistical analyses**

All statistical analyses were performed with the two-tailed Kruskall–Wallis test, followed by post hoc comparisons with the two-tailed Mann–Whitney test. Results are expressed as mean ± standard deviation. A P-value of < 0.05 was considered to indicate statistical significance.

**Results**

**Generation of new thrombolytics originating from t-PA**

Structural differences between human t-PA (UniProtKB: P00750), rat t-PA (UniProtKB: P19637) and bat DSPA (hereafter termed DSPA; UniProtKB: P98119) were studied by the use of multiple alignments. DSPA contains a single kringle domain with a similar amino acid sequence to t-PA’s kringle 1 domain (Fig. 1A) and the absence of a constitutive LBS (Fig. 1A, black boxes). t-PA’s kringle 2 domain contains a constitutive LBS formed by Asp237, Asp239, and Trp254. Analysis of the primary sequence of DSPA revealed a lack of the cleavage site Arg276–Iso277, which is present in t-PA (Fig. 1B).

We generated three mutants derived from rat t-PA (R. norvegicus wt-t-PA): (i) a rat t-PA genetically engineered with complete deletion of its kringle 2 domain (from Cys181 to Cys262), hereafter termed ΔK2-t-PA; (ii) a rat t-PA containing a tryptophan to arginine point mutation at position 254 (W254R), hereafter termed K2*-t-PA; and (iii) an exclusively single-chain rat t-PA obtained by an arginine to serine point mutation at position 276 (R276S), hereafter termed sc*-t-PA (Table 1). When subjected to SDS-PAGE and immunoblotting, wt-t-PA, sc*-t-PA and K2*-t-PA showed similar
molecular masses, whereas ΔK2-t-PA showed a molecular mass reduced by 15 kDa (Fig. 2A). sc*-t-PA was present in its exclusively single-chain form, whereas wt-t-PA, K2*-t-PA and ΔK2-t-PA were present in both their single-chain and two-chain forms (at 35 kDa and 25 kDa for ΔK2-t-PA). As a control, the purified sc*-t-PA was incubated with 5 nM plasmin at 37 °C for 24 h prior to SDS-PAGE immunoblotting. Plasmin did not cleave sc*-t-PA as it did wt-t-PA (Fig. 2B). Thus, the R276S point mutation (sc*-t-PA) leads to the generation of a non-cleavable form of t-PA.

t-PA is known to bind and cleave several substrates in addition to plasminogen (such as the GluN1 subunit), with no identified allosteric regulator. Therefore, we evaluated the intrinsic proteolytic activity of each of the t-PA variants. For this purpose, amidolytic activity assays with a fluorogenic substrate (Spectrofluor) (Fig. 2C) and plasminogen-containing zymography assays (Fig. 2D) were performed for the different t-PA-related mutants. The data revealed that, although kringle 2-related mutants (DK2-t-PA and K2*-t-PA) showed amidolytic activity similar to that observed for wt-t-PA, sc*-t-PA did not.

Table 1 Biochemical characteristics of the tissue-type plasminogen activator (t-PA) variants: (i) sequence provided in the Supplementary Data; (ii) fibrinolytic activities obtained from euglobulin clot lysis time assay by reference to the International Reference Preparation (IRP 98/714), using the time to obtain 50% clot lysis

| Name          | Sequence/mutation | Two-chain form | Amidolytic activity | Apparent fibrinolytic activity (IU mg⁻²) |
|---------------|-------------------|----------------|---------------------|----------------------------------------|
| Human t-PA    | UniProtKB: P00750 | Yes            | Yes                 | –                                      |
| DSPAαz1       | UniProtKB: P98119 | No             | Limited             | –                                      |
| Rat wt-t-PA   | UniProtKB: P19637†| Yes            | Yes                 | 203 605                                |
| Rat kMPA      | Arg276→Ser        | No             | Limited             | 184 136                                |
| Rat K2MPA     | Tryp254→Arg       | Yes            | Yes                 | 155 559                                |
| Rat AK2-t-PA  | Cys181 to Cys262 deletion | Yes | Yes | 319 903 |

DK2, Desmodus rotundus plasminogen activator.

![Fig. 2.](image) Biochemical characterization of the tissue-type plasminogen activator (t-PA) variants. (A) Equal amounts (100 ng) of wt-t-PA, sc*-t-PA, K2*-t-PA and ΔK2-t-PA mutants were subjected to immunoblotting. (B) Time-course of plasmin-dependent cleavage (5 nm) of wt-t-PA and sc*-t-PA mutants. Although wt-t-PA was converted by plasmin, sc*-t-PA remained uncleaved. (C, D) Activity of the t-PA-related mutants measured either on a fluorogenic substrate (C) or by plasminogen–casein zymography assays (D); ***P < 0.01. (E, F) Michaelis–Menten plots for wt-t-PA and sc*-t-PA activity with Spectrozyme t-PA (SptPA) and the corresponding $V_{max}$ and $K_M$. AU: arbitrary unit; OD: optical density; tc-t-PA: two-chain t-PA.
To further investigate the behavior of the sc-t-PA variants as compared with wt-t-PA, we determined the \( K_M \) values of these two plasminogen activators by using the amidolytic Spectrozyme as the substrate. The data showed that the point mutation within the cleavage site of t-PA led to a four-fold increase in the \( K_M \) as compared with wt-t-PA (430 \( \mu \)m and 110 \( \mu \)m, respectively; see Fig. 2E,F). Hereafter, concentrations of the t-PA mutants are normalized to their intrinsic amidolytic activity, unless otherwise mentioned.

**Kringle 2-related mutants (\( \Delta K2 \)-t-PA and \( K2^* \)-t-PA) are fibrinolytic and do not promote NMDAR-mediated neurotoxicity**

Kringle 2-related mutants were characterized for their ability to initiate fibrinolysis on fibrin-agarose plates. \( \Delta K2 \)-t-PA and \( K2^* \)-t-PA activated plasminogen into plasmin in the presence of fibrin, as did wt-t-PA (Fig. 3A). To quantify the fibrinolytic activity of the kringle 2-related mutants, an in vitro clot lysis assay, performed on euglobulin fractions from platelet-poor human plasma clots as the substrate, revealed that \( \Delta K2 \)-t-PA had enhanced fibrinolytic activity as compared with wt-t-PA (\( +57\% \), \( n = 3 \); Fig. 3B). \( K2^* \)-t-PA showed decreased fibrinolytic activity as compared with wt-t-PA (\( -24\% \), \( n = 3 \); Fig. 3B). As \( \alpha 2 \)-antiplasmin may influence t-PA-induced fibrinolysis, the fibrinolytic activity of each of the t-PA mutants generated was tested against clots derived from whole plasma. \( \Delta K2 \)-t-PA showed increased fibrinolytic activity (\( +131\% \), \( P < 0.02 \); Fig. 3C) and \( K2^* \)-t-PA showed decreased fibrinolytic activity (\( -65\% \), \( n = 3 \), \( P < 0.02 \); Fig. 3C) as compared with wt-t-PA.

To estimate the effect of kringle-related t-PA mutants on NMDAR-mediated neurotoxicity, pure cultures of cortical neurons were subjected to NMDA exposure either alone or in combination with either purified \( \Delta K2 \)-t-PA or \( K2^* \)-t-PA (0.3 \( \mu \)m) prior to measurement of neuronal death 24 h later. wt-t-PA caused a 39% potentiation of NMDAR-mediated excitotoxicity (71% of the cells died, as compared with 51% with NMDA alone), an effect similar to what was observed for human t-PA-containing alteplase (Fig. 4A; \( n = 3 \), \( P < 0.01 \)), whereas \( \Delta K2 \)-t-PA and \( K2^* \)-t-PA (Fig. 4B; \( n = 4 \), \( P < 0.01 \)) showed no potentiation of NMDA-mediated neurotoxicity. Thus, substitution of Trp254, a constitutive amino acid of the LBS of kringle 2 of t-PA, confirms that kringle 2 is critical in mediating the pro-neurotoxicity of t-PA. Accordingly, similar experiments performed in the presence of \( \epsilon \)-ACA, a lysine analog that is known to compete with the LBS of t-PA, showed that blockage of LBS function prevented wt-t-PA-induced potentiation of NMDAR-mediated neurotoxicity (Fig. 4C; \( n = 5 \), \( P < 0.01 \)).

A zymogenic t-PA (sc-t-PA) is also a fibrinolytic that does not promote NMDAR-mediated neurotoxicity

We tested both the fibrinolytic activity and the neurotoxicity of sc-t-PA. In contrast to its reduced intrinsic amidolytic activity (Fig. 2C,D), sc-t-PA remained fibrinolytic in the presence of fibrin (\( -10\% \) on the euglobulin clot lysis assays, \( n = 3 \) [Fig. 5A,B] and \( +27\% \) on the plasma clot lysis assays, \( n = 3 \) [Fig. 5C]). This mutant was then tested for its ability to influence NMDAR-mediated neurotoxicity in cortical neurons. sc-t-PA did not potentiate NMDAR-dependent excitotoxicity as compared with wt-t-PA (\( n = 3 \), \( P < 0.01 \); Fig. 6).

**Discussion**

Although thrombolysis with t-PA is clearly beneficial for stroke patients [21] (NINDS, ECASS, and Epithet), the benefits are limited to a low percentage of patients, owing to a 4.5-h therapeutic window and possible deleterious effects of t-PA. Thus, the development of a new class of thrombolytics with higher fibrinolytic efficiency, a lower
risk of hemorrhagic transformations and reduced neurotoxicity is of seminal importance. Several clues in rodent models indicate that the benefit of t-PA-induced thrombolysis is counterbalanced by side-effects such as neurotoxicity [7]. At the mechanistic level,
the t-PA–NMDAR interaction triggers a dramatic increase in NMDAR signaling that leads to increased neuronal death [8,12,20,22]. This mechanism: (i) requires the proteolytic activity of t-PA; (ii) is dependent on the kringle 2 domain of t-PA; and (iii) requires GluN1/GluN2D-containing NMDARs. Furthermore, immunotherapy to prevent the interaction of t-PA with the GluN1 subunit of the NMDAR dramatically reduces ischemic damage and improves neurological scores [23]. Similarly, although intravenous t-PA augments the lesion volume in a rat model of NMDA-induced excitotoxic lesions, DSPA (which lacks the kringle 2 domain) does not [24]. Nevertheless, despite both its increased fibrinolytic activity and its lack of neurotoxicity as compared with t-PA, the last clinical trial performed with DSPA was unsuccessful [19].

The present study indicates that the kringle 2 domain (via the LBS-constitutive tryptophan at position 254) is critically involved in mediating the t-PA-induced potentiation of NMDAR-induced neuronal death. In addition, deletion of t-PA’s kringle 2 domain increases its fibrinolytic activity, a finding that was previously reported for DSPA [18]. Thus, as reported for DSPA, lack of the kringle 2 domain or disruption of its LBS is conducive to a lack of neurotoxicity [12]. Moreover, comparative studies of the primary structures of DSPA and t-PA suggest a potential role of the low zymogenicity of t-PA in its damaging effects. We found that, in the absence of fibrin, the non-cleavable single-chain t-PA (sc*-t-PA, R276S) partly restored its amidolytic zymogenicity. Furthermore, inhibition of its intrinsic activity led to the generation of a form of t-PA that lacked neurotoxicity. Interestingly, the fibrinolytic activity of sc*-t-PA is revealed by fibrin clots. These data are in agreement with a study demonstrating that mutation of the cleavage site of sc-t-PA (R276G) lowers its intrinsic amidolytic activity [25].

We generated and characterized a set of original fibrinolytics derived from t-PA: a fibrinolytic K2*-t-PA characterized by a lack of pro-neurotoxicity, and an sc*-t-PA characterized by both 4.4-fold reduced amidolytic activity and lack of pro-neurotoxicity, but with conserved fibrinolytic activity. The apparent fibrinolytic activity measured for each t-PA mutant, with the euglobulin-containing clot lysis assay, confirmed previous data. As previously found for desmonteplase [18,26], AK2-t-PA showed increased fibrinolytic activity (+ 57%) as compared with wt-t-PA. In contrast, a point mutation in its kringle 2 domain (W254R) led to a decrease in its apparent fibrinolytic activity (− 24%). These data are in agreement with a study demonstrating that mutation of the cleavage site of sc-t-PA lowers its intrinsic amidolytic activity [25].

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t-PA mediates its deleterious effects via an interaction with NMDAR and activation of platelet-derived growth factor (PDGF)-CC to PDGF-C [8,12,20,28], as well as through a mechanism involving its kringle 2 domain. The point mutation W254R in the kringle 2 domain of t-PA may lower its ability to activate PDGF-CC and thus to limit hemorrhagic transformations [9]. These in vitro data provide the basis for further studies to evaluate the efficacy of this new generation of fibrinolytics in experimental models of thrombosis, prior to their possible use in clinical situations.

In conclusion, the present article reports that molecular engineering can produce novel and efficacious molecular variants based on existing therapeutic drugs. We postulate that a combination of the mutations W254R/W253R and R276S/R275S in rat and human sequences, respectively, may provide a relevant and optimized fibrinolytic treatment with future clinical applications.

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary data refer to the Rattus norvegicus pcDNA5/FRT plasmid used to produce t-PA variants through these experiments.

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