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Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice

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Intravenous tissue plasminogen activator (tPA) is used to treat acute stroke because of its thrombolytic activity and its ability to restore circulation to the brain[1,2]. However, this protease also promotes neurodegeneration after intracerebral injection of excitotoxins such as glutamate, and neuronal damage after a cerebral infarct is thought to be mediated by excitotoxins[3–8]. To investigate the effects of tPA on cerebral viability during ischemia/reperfusion, we occluded the middle cerebral artery in wild-type and tPA-deficient mice with an intravascular filament. This procedure allowed us to examine the role of tPA in ischemia, independent of its effect as a thrombolytic agent. tPA-deficient mice exhibited ~50% smaller cerebral infarcts than wild-type mice. Intravenous injection of tPA into tPA$^{-/-}$ or wild-type mice produced larger infarcts, indicating that tPA can increase stroke-induced injury. Since tPA promotes desirable (thrombolytic) as well as undesirable (neurotoxic) outcomes during stroke, future therapies should be aimed at countering the excitotoxic damage of tPA to afford even better neuroprotection after an acute cerebral infarct.

Intravenous injection of the serine protease tissue plasminogen activator (tPA) has been approved by the US Food and Drug Administration as the first agent that combats focal cerebral infarction or stroke. tPA is administered as a thrombolytic agent within 3 hours of the insult with the hope of dissolving the blood clot responsible for initiating cerebral damage[1,2]. However, our work suggests that tPA might also have negative effects on neuronal viability that should be considered relative to its use in stroke patients. It is thought that neuronal injury during focal ischemia in the brain occurs primarily because of accumulation of "excitotoxins" such as the neurotransmitter, glutamate[3–5]. Using direct intracerebral injection of excitotoxins to induce cell death, it has been shown that mice lacking tPA (tPA-deficient mice) are resistant to neuronal degeneration[6,7]. Therefore, tPA, which is produced by both neurons and microglia[8], might play a direct role in mediating excitotoxic neuronal cell injury. In view of the potentially widespread use of tPA in stroke patients, we investigated whether tPA was involved in neuronal death after cerebral ischemia.

Under isoflurane anesthesia, a 6.0 nylon filament coated with silicone was threaded into the lumen of the middle cerebral artery of mice (C57BL/6 and SV129 backgrounds) to produce transient ischemia. After 2 or 3 hours of occlusion, the filament was gently withdrawn to allow tissue reperfusion[9–20]. Body temperature was maintained at 36.5 ± 1.0 °C. Systemic arterial blood pressure was monitored via a femoral arterial line until 30 minutes after all surgical interventions and did not vary among the various groups. Other physiological parameters including arterial blood gases and glucose also did not vary significantly among the groups. Animals were killed 24 hours after the onset of the insult as detailed previously[13,20]. Analyzed by 2,3,5-triphenyltetrazolium (TTC) staining[21], after accounting for cerebral edema[20,22–25], infarct volume decreased from 116 ± 8 mm^3 in control wild-type mice to 69 ± 7 mm^3 in tPA-deficient mice (mean ± s.e.m., n = 19 mice) (Fig. 1*a*). Under these conditions, there is no thrombosis at the occluded site, so the absence of tPA would not contribute to damage via an enhanced clotting cascade. Hence, this technique allowed us to distinguish effects of tPA as a thrombolytic from its neurotoxic effects.

In parallel to the ischemic damage occurring unilaterally on the side of the vascular occlusion in the wild-type mice, we observed a significant increase in tPA activity, most likely due to the accumulation of microglial cells at the site of the injury (Fig. 1, *b* and *c*; cf. normal mice[26]). Activated microglia are known to secrete tPA (ref. 8). Following ischemic injury/reperfusion, the status of microglial cells in the tPA-deficient mice was evaluated using immunohistochemistry against the mature macrophage/microglial cell surface antigen F4/80. Microglia were activated in the wild-type mice but activation was attenuated in tPA$^{-/-}$ mice, as we had observed previously after unilateral intrahippocampal excitotoxic injection in such mice[6].

To determine the effect of intravenous injection of tPA into tPA-deficient or wild-type mice (C57BL/6 or SV129), we administered intravenous tPA at 0.9–1.0 mg/kg (similar to the human dosage in the North American and European clinical trials)[1,2]. We found that tPA dramatically increased the size of cerebral infarction when occlusion was due to the intraluminal filament, causing a near doubling

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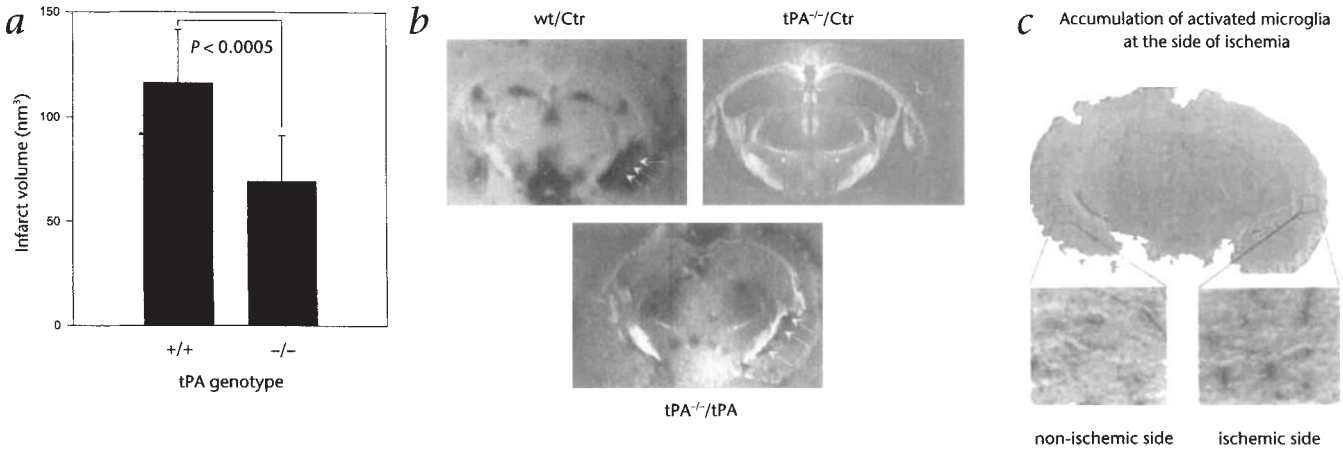

Fig. 1 Tissue plasminogen activator-deficient mice manifest smaller cerebral infarcts than wild-type mice, which display increased tPA activity at the site of ischemia. ***a***, Wild-type C57BL/6 (+/+) mice ($n = 9$) had larger infarction volumes than tPA-deficient (−/−) mice ($n = 10$; $^*P < 0.0005$). Mice underwent 3 h of ischemia/21 h of reperfusion via the intraluminal suture technique. Coronal sections were stained for viability with TTC and infarct volumes digitally quantified. Values are mean + s.e.m. Significant differences were observed between the two groups by a Student's *t*-test. ***b***, Local increase in tPA activity in the murine brain in response to focal ischemia/reperfusion or exogenous tPA injection. Histological zymography of coronal sections was used to reveal tPA activity, as described under Methods. tPA activity assay of wild-type mice (*top left*), but not tPA-deficient mice (*top right*), revealed a marked increase in tPA levels in the infarcted left hemisphere (indicated by arrows). In a tPA-deficient animal receiving intravenous tPA (1 mg/kg via the femoral vein), tPA activity increased most notably at the site of the infarction (*bottom*; arrows point to the black zones of lysis due to tPA activity). ***c***, Accumulation of activated microglia at the site of ischemia/reperfusion. The antibody F4/80 was used as a marker of microglial activation, as described under Methods. The insets show higher magnification (×400) of selected cortical areas. Note the extensive arborization and thicker cell bodies of the F4/80$^+$ microglial cells on the ischemic side.

of the damage in tPA-deficient mice and approximately a 33% increase in wild-type mice (Fig. 2). No hemorrhage or other bleeding diathesis occurred in any of the animals, as analyzed at postmortem examination. In wild-type, but not tPA$^{-/-}$ mice, ischemia induced an increase in tPA activity, as assessed by histological zymography (Fig. 1*b*, *top*). The tPA-deficient mice were also useful in demonstrating that intravenous administration of exogenous tPA resulted in the apparent entry of tPA activity into the brain (Fig. 1*b*, *bottom*). Taken together, these data reinforce our supposition that toxic actions of tPA may, under these conditions, adversely affect cerebral damage. Of note, however, in the face of large cerebral infarcts in wild-type mice (for example, induced by 3 hours rather than 2 hours of vascular occlusion), injection of tPA did not enhance the size of the stroke over that in control animals not receiving tPA (data not shown), suggesting a ceiling effect. This result is consistent with our finding that large infarcts activate a high level of endogenous tPA activity in the area of the insult (as in Fig. 1*b*, *top left*).

To assess the possibility that an adverse effect of tPA during cerebral ischemia influenced neuronal viability, neuronal loss was quantified in the hippocampus, as we have previously described for direct excitotoxic insults (see Methods). We observed a dramatic difference in the number of surviving neurons in the CA1–CA3 subfields following 3 hours of ischemia and 21 hours of reperfusion in the wild-type versus tPA-deficient brains. Neuronal loss was much more marked in wild-type compared with tPA$^{-/-}$ mice (Fig. 3; 74 ± 9% neuronal loss in wild-type compared with 10 ± 3% in tPA-deficient mice; mean ± s.d., $n = 6$ brains; $P < 0.001$). Similarly, more extensive neuronal cell death was also evident in the cerebral cortex of wild-type compared with tPA$^{-/-}$ mice, but we chose to quantify the hippocampus where the direct comparison of samples is more obvious and quantifiable in this model (Fig. 3).

We also monitored regional cerebral blood flow by the laser Doppler flowmetry technique, as previously described[13,20,27–29], to ensure that there were no differences between control and tPA-deficient mice, either before, during or after the induction of ischemia. We found that with reperfusion the cerebral blood flow was partially restored to levels that were not significantly different between the wild-type, tPA-deficient or tPA-injected mice for each group of experiments (data not shown).

By using an intraluminal filament rather than thrombosis to produce cerebral ischemia, our experiments allowed us to dissociate vascular from excitotoxic events in tPA-deficient and wild-type mice. Our findings suggest that tPA may not only act to enhance the dissolution of thromboses, as previously shown, but may also contribute to excitotoxic damage of neurons. Augmentation of neuronal damage by tPA during focal cerebral ischemia may occur at least under some circumstances and especially after relatively small infarcts. Thus, therapeutic intervention with tPA in the nervous system may represent a two-edged sword. Since the administration of tPA is known to diminish overall cerebral infarct size in humans, we speculate that even better protection could be attained if the neurotoxic effects of tPA could be ameliorated while leaving its thrombolytic activity intact. Future therapies with CNS-specific serine protease inhibitors could prove useful in this regard.

Methods

Animals and model of focal cerebral ischemia. Wild-type and tPA-deficient male mice (on C57BL/6 and SV129 backgrounds) weighing 23–27 g were housed in a 12-h light/dark cycle and permitted food and water intake *ad libitum*[6]. Transient ischemia/reperfusion was performed using the intravascular filament model to occlude the middle cerebral artery unilaterally for 2 or 3 h followed by reperfusion for the remainder of the 24-h period[9,20]. Infarct volumes were digitally quantified on 1.5-mm-thick coronal sections after correction for edema by measuring TTC staining, as described previously[20–25]. In a group of animals, after anesthesia and perfusion with 10 ml normal saline, the cerebral arteries were revealed by stain-

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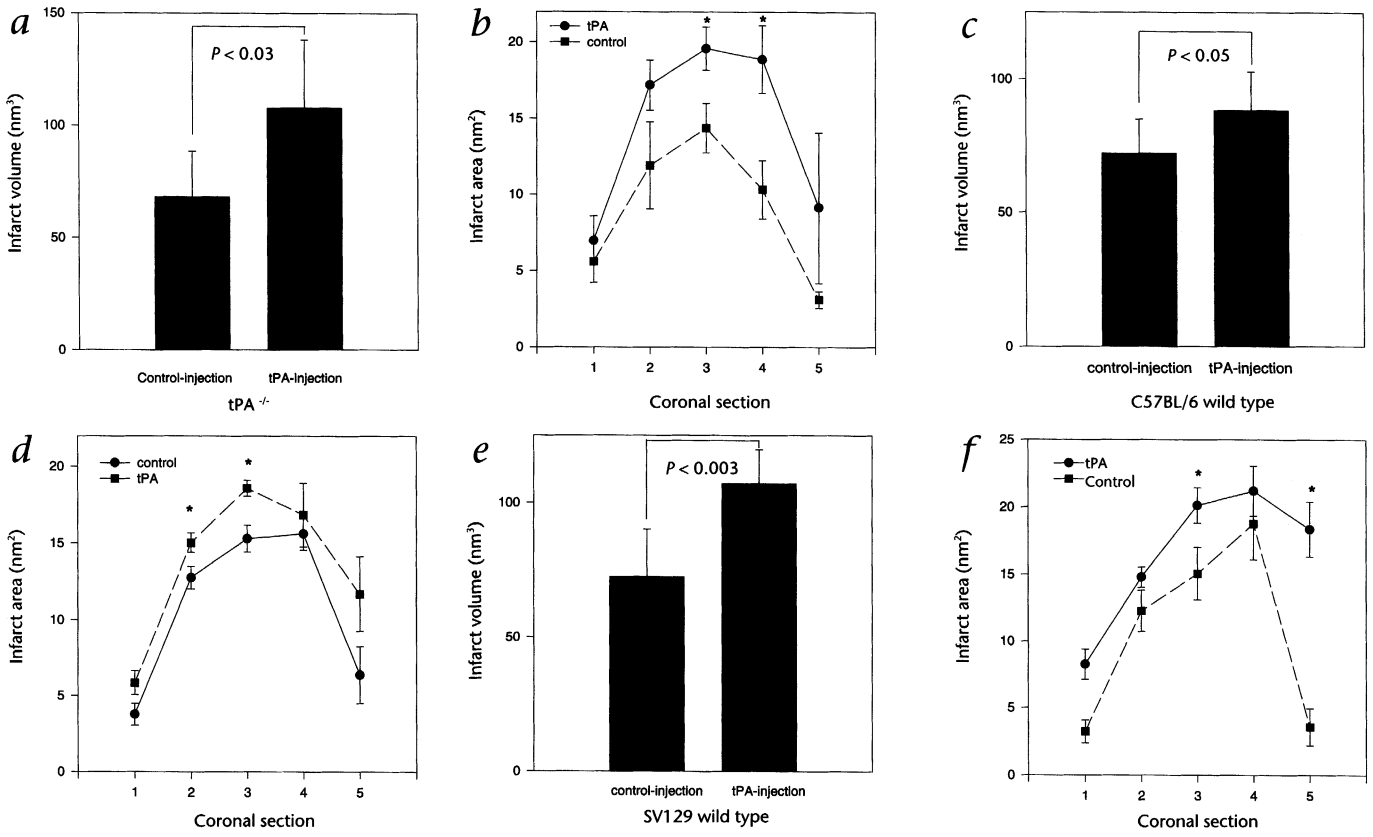

Fig. 2 Injection of tPA via the femoral vein 2 h after vascular occlusion with an intraluminal filament increases infarct size in tPA-deficient or wild-type mice. ***a***, Infarct volumes of saline-injected (*left bar*, $n = 5$) and tPA-injected (1 mg/kg) tPA-deficient mice (*right bar*, $n = 6$) undergoing a 3-h occlusion of the middle cerebral artery (MCA) followed by 21 h of reperfusion (means + s.e.m.; $P < 0.03$). ***b***, Infarct area for each of the five coronal sections of the same brains as in *a* (means ± s.e.m.; $P < 0.03$). Each coronal slice was 1.5 mm thick. ***c***, Infarct volumes of equimolar control peptide (BSA)-injected (*left bar*, $n = 8$) or tPA-injected (0.9 mg/kg) wild-type (C57BL/6) mice (*right bar*, $n = 9$) undergoing a 2-h vascular occlusion followed by 22 h of reperfusion (means + s.e.m.; $P < 0.05$). ***d***, Infarct area for each of the five coronal sections of the same brains as in *c* (means ± s.e.m.; $P < 0.05$). ***e***, Infarct volumes of wild-type (SV129) mice ($n = 6$ in each group) injected with control solution (Genentech tPA diluent) or tPA (0.9 mg/kg) and undergoing a 2-h vascular occlusion followed by 22 h of reperfusion (means + s.e.m.; $P < 0.003$). ***f***, Infarct area for each of the five coronal sections of the same brains as in *e* (means ± s.e.m.; $P < 0.003$).

ing and demonstrated to have developed normally in tPA-deficient mice via further perfusion with 5 ml of a 1% suspension of carbon black particles in India ink[13,20]. When tPA was injected, it was administered intravenously, 10% initially by bolus, followed by constant infusion over 20 to 30 min. The formula for the carrier used to dilute tPA was obtained from the manufacturer (Genentech, South San Francisco, CA) and was used as one of the control injections; the diluent consisted of 0.02 M arginine phosphate at pH 7.3 with 0.001% Tween. All experiments were performed in a randomized fashion with the experimenters masked to the treatment.

Histological zymography of tPA activity. Wild-type or tPA$^{-/-}$ mice were subjected to 2 h of focal ischemia. After 10 min of reperfusion, the animals were killed and their brains frozen as described previously[6]. Then, 12 µm consecutive coronal brain cryostat sections were subjected to histological zymography to visualize tPA activity with or without amiloride [1 mM, a specific urokinase (uPA) inhibitor][6,26,30]. The sections were overlaid with a milk-agarose substrate matrix containing 50 µg/ml of purified human plasminogen and 1 mg/ml amiloride[26]. Photographs were taken under dark-field illumination after a 3-h incubation at 37 °C.

Identification of brain macrophages with F4/80 antibody. Wild-type mice were subjected to 2 h of focal ischemia/10 min reperfusion; the ani-

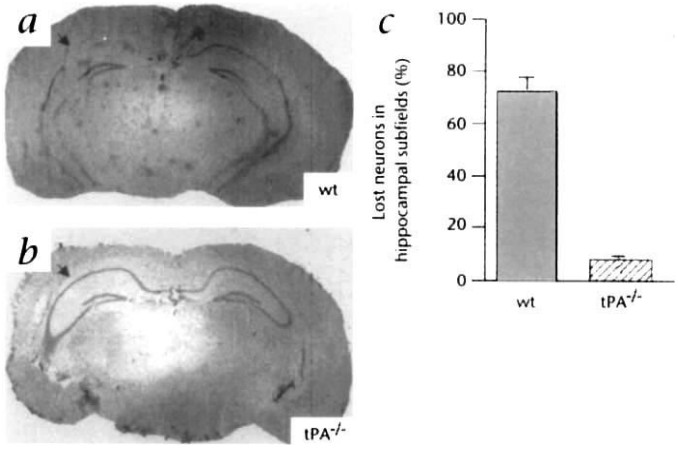

Fig. 3 Quantification of hippocampal neuronal cell loss after 3 h of unilateral cerebral ischemia followed by 21 h of reperfusion with the intraluminal filament model in wild-type (C57BL/6) versus tPA-deficient mice. ***a***, Striking neuronal loss (arrow) in the CA1–CA3 hippocampal subfields of a wild-type (wt) mouse on the side of the infarct. ***b***, Relatively intact neurons (arrow) in the CA1–CA3 hippocampal subfields of a tPA-deficient mouse ipsilateral to the infarct. ***c***, Quantification of hippocampal pyramidal cells (CA1–CA3) reveals significantly more neuronal loss on the side ipsilateral to the cerebral infarct in wild-type compared with tPA$^{-/-}$ mice ($n = 6$; $P < 0.001$ by *t*-test). Values represent means + s.d.

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mals were killed, and their brains were frozen and sectioned (12 μm). Microglia were revealed by immunohistochemistry with an antibody to the mature macrophage/monocyte antigen F4/80 (Serotec, Washington, DC), as described previously[6].

Assessment of neuronal damage. Quantification of neuronal loss in the CA1–CA3 hippocampal subfields was performed as we have previously described[6,8,31]. Briefly, wild-type or tPA$^{-/-}$ mice were subjected to 3 h of ischemia/21 h reperfusion as detailed above. The mice were then killed, and the brains were removed and quickly frozen in cold acetone in a dry ice-ethanol bath. Serial cryostat sections 12 μm in thickness were cut and stained with cresyl violet. Four consecutive sections from the dorsal hippocampus of mice from each genotype were visually matched. The hippocampal subfields (CA1–CA3) in each of these sections were traced from camera lucida. The length of each subfield was then measured by comparison to 1-mm standards traced under the same magnification. In each subfield, we scored the length of tissue with viable pyramidal neurons (having normal morphology) and length of tissue with neuronal loss (no cresyl violet staining and no cells present). The lengths representing intact neurons and neuronal loss for each hippocampal subfield were averaged across all sections and standard deviations calculated.

Measurement of regional cerebral blood flow (rCBF). The initial reading of rCBF was assigned a value of 100%, and subsequent readings were expressed relative to this value. Flow was monitored in the ischemic core as judged by the fact that the rCBF fell to virtually 0% during occlusion of the middle cerebral artery. rCBF was measured by laser Doppler flowmeter (Vasamedics BPM, Minneapolis, MN), using methods that we have previously described[20]. A probe tip was fixed ~6 mm lateral and 2 mm posterior to the bregma in the anesthetized animals. This site corresponds to the distal arterial supply of the middle cerebral artery.

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