Tissue Plasminogen Activator (tPA) Deficiency Exacerbates Cerebrovascular Fibrin Deposition and Brain Injury in a Murine Stroke Model

Studies in tPA-Deficient Mice and Wild-Type Mice on a Matched Genetic Background

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Abstract—Although the serine protease, tissue plasminogen activator (tPA), is approved by the US Food and Drug Administration for therapy to combat focal cerebral infarction, the basic concept of thrombolytic tPA therapy for stroke was challenged by recent studies that used genetically manipulated tPA-deficient (tPA−/−) mice, which suggested that tPA mediates ischemic neuronal damage. However, those studies were potentially flawed because the genotypes of tPA−/− and wild-type control mice were not entirely clear, and ischemic neuronal injury was evaluated in isolation of tPA effects on brain thrombosis. Using mice with appropriate genetic backgrounds and a middle cerebral artery occlusion stroke model with nonsiliconized thread, which does lead to microvascular thrombus formation, in the present study we determined the risk for cerebrovascular thrombosis and neuronal injury in tPA−/− and genetically matched tPA+/+ mice subjected to transient focal ischemia. Cerebrovascular fibrin deposition and the infarction volume were increased by 8.2- and 6.7-fold in tPA−/− versus tPA+/+ mice, respectively, and these variables were correlated with reduced cerebral blood flow up to 58% (P<0.05) and impaired motor neurological score by 70% (P<0.05). Our findings indicate that tPA deficiency exacerbates ischemia-induced cerebrovascular thrombosis and that endogenous tPA protects the brain from an ischemic insult, presumably through its thrombolytic action. In addition, our study emphasizes the importance of appropriate genetic controls in murine stroke research. (Arterioscler Thromb Vasc Biol. 1999;19:2801-2806.)

Key Words: tissue plasminogen activator ▪ cerebrovascular events ▪ fibrin ▪ neuroprotection ▪ ischemia

The majority of acute ischemic strokes in humans are due to thrombotic or thromboembolic occlusions.1–3 This forms the rationale for use of thrombolytic agents in patients with acute ischemic stroke. Intravenous injection of the serine protease, tissue plasminogen activator (tPA), has been approved by the US Food and Drug Administration to combat focal cerebral infarction or ischemic stroke, and modest but significant results have been reported in stroke victims if tPA is administered within 3 to 6 hours of the insult.4–6

The neuroprotective effects of tPA were demonstrated in initial experimental stroke studies in rodents7–9 but were not confirmed in later studies in mice lacking tPA (tPA−/−) due to genetic engineering.10 Studies in tPA−/− versus wild-type mice raised the possibility that endogenous tPA mediates excitotoxin-induced neuronal degeneration11 and is responsible for ischemic neuronal damage.10 It has been suggested that these neurotoxic effects of tPA are independent of its desirable thrombolytic action.10 These findings, although in contradiction with previously suggested physiological roles of tPA in brain development,12 learning, memory,13 and long-term potentiation,14,15 have challenged the concept of tPA lytic therapy for stroke. Whether tPA treatment for ischemic stroke may be neurotoxic has become a controversial issue in the treatment of stroke patients.

The work arguing for an ischemia-induced neurotoxic role of tPA has, however, compared ischemic brain injury in tPA−/− mice versus wild-type mice that were on different genetic backgrounds. In addition, ischemic neuronal injury was evaluated in isolation of tPA’s beneficial effect on brain thrombosis.10 Because genetic backgrounds16–20 and throm-
Results and Discussion
The validity of the tPA deficiency stroke model was assessed by the ability of tPA to thrombolymically lyse intravascular clot in the MCA. In the control group, MCA lumi- nal thrombi were present in 83±1% of animals (n=10) and approximately 60% of the midline was infarcted. With a concentration of 1.0 U/kg/min of tPA, the rate of clot dissolution was 89±1% (n=6) compared with 0% in the vehicle group (n=9). With a concentration of 0.1 U/kg/min, the rate of clot dissolution was 44±8% (n=6) compared with 0% in the vehicle group (n=9). These results are consistent with previous reports showing a dose-dependent lysis of thrombi with tPA.

Methods

Animals
Mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed at the Medical Research Institute of New York, where they were back-crossed every 8 to 10 generations to generate heterozygotes, thus excluding major genetic drift between both lines. The tPA−/− animals were routinely screened by tail bleeds to verify their tPA deficiency. The tPA+/− C57BL/6 wild-type mice were used to control for strain-related differences in susceptibility to stroke. Male mice weighing between 22 and 27 g were subjected to a modified middle cerebral artery (MCA) occlusion technique as described below.

General Preparation
Mice were initially anesthetized with metofane and maintained with 50 mg/kg IP pentobarbital. Atripline methyl nitrate (0.18 mg/kg IP) was given as premedication to prevent airway obstruction by mucus secretion. Animals were anesthetized in a 37°C heating pad. The right femoral artery was cannulated with a siliconized, uncoated, 6-0 8-mm-long prolene suture was used in animal care guidelines at the University of Southern California and approved by the National Institutes of Health.

Stroke Model
A modified intravascular MCA occlusion technique with a non-siliconized, uncoated, 6-0 8-mm-long prolene suture was used in mice weighing 22 to 27 g versus the commonly used 10- to 12-mm-long nylon filament coated with silicone that does not have thrombogenic properties.

Blood Flow and Head Temperature Measurements
Cortical cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (LDF) and a tissue perfusion monitor (Transonic BLF21). Animals were placed in a stereotactic head frame, and readings were obtained 2 mm posterior to the bregma, both 3 and 6 mm to each side of the midline, by using a stereotactic micromanipulator and keeping the angle of the probe perpendicular to the cortical surface. LDF probes (0.8-mm diameter) positioned on the cortical surface were connected to a tissue perfusion monitor (Transonic BLF21). The procedure was considered to be technically successful when a ≥50% reduction in relative CBF was observed immediately after the placement of the occluding suture.

Neurological Deficits
Neurological examinations were performed at 24 hours after reperfusion. The neurological findings were scored on a 5-point modified scale: no neurological deficit (0); failure to extend left forepaw fully; (1) turning to left; (2) circling to left; (3) unable to walk spontaneously; and (4) stroke-related death (5).

Blood Analysis
Arterial blood gases (pH, PaO2, PaCO2) were measured before and during MCA occlusion using aABL 30 acid-base analyzer (Radiometer).

Measurement of Volume of Injury
The area of injury was delineated by incubation of unfixed 1-mm coronal brain slices in 2% TTC in phosphate buffer (pH 7.4). Serial coronal sections were displayed on a digitizing video screen equipped with an imaging system (Jandel Scientific). The volume of injury was calculated by summing the affected areas from each coronal section and multiplying by the thickness of each section. Brain infarction and edema were calculated using the Swanson correction.

Histopathology
Detection of fibrin by immunostaining was performed on brain tissue sections previously treated with TTC. Tissue fixed in 10% buffer formalin was processed, and 7-μm-thick, paraffin coronal sections from each block were cut and stained. Fibrin was localized using a grading scale: 1, fibrin deposition limited to intravascular space; 2, fibrin deposition in the intravascular lumen and the perivascular space; and 3, fibrin lattices in the extravascular or parenchymal tissue only. For immunostaining, anti-mouse fibrin II antibody (NYB-T2G1, Accurate Chemical Science Corp) was used. Routine control sections included deletion of primary antibody, deletion of secondary antibody, and the use of an irrelevant primary antibody. All analyses assessed visually were performed by 1 observer blinded to the specimen source or timing.

Detection of Fibrin in Brain Tissue Sections by Quantitative Western Blot
In brief, after being stained with TTC, a 1-mm section of brain tissue was divided into contralateral and ipsilateral hemispheres. Other organs (heart, lung, liver, etc) were also collected. Tissue was homogenized in 10 mmol/L sodium phosphate buffer, pH 7.5, 0.1 mol/L 1-aminocaproic acid, 5 mmol/L trisodium EDTA, 10 U aprotinin/mL, 10 U heparin/mL, and 2 mmol/L PMSF. The homogenate was agitated for 14 hours at 4°C, and the particulate material was sedimented by centrifugation at 10 000g for 10 minutes, resuspended in extraction buffer without PMSF, sedimented again, and finally dispersed in 3 mol/L urea. The suspension was agitated for 2 hours at 37°C, vigorously vortexed, and centrifuged at 14 000g for 15 minutes. The supernatant was aspirated and the sediment dissolved at 65°C in reducing SDS buffer, subjected to SDS–polyacrylamide gel electrophoresis (8%), and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp) by electrophoretic transfer. Fibrin was visualized with anti-mouse fibrin II antibody (given above) and an enhanced chemiluminescence system (Amersham Corp). Fibrin standards were prepared by clotting a known amount of murine fibrinogen (Sigma Chemical Co) with an excess of thrombin in the absence of calcium. The films were scanned with a Hoefer GS 300 scanning densitometer interfaced to an IBM personal computer with a DT 2805 analog and digital system (Data Translation), and data were converted into micrograms of fibrin per 0.1 gram of tissue.

Statistics
Physiological variables, infarction, and edema volumes were compared between groups by Student’s t test and ANOVA. Nonparametric data (neurological outcome scores) were subjected to the Kruskal-Wallis test. A value of P<0.05 was considered statistically significant.
Results

First, we examined the anatomy of the circle of Willis in mixed tPA−/− and tPA+/+ 129/Sv and C57BL/6 strains and C57BL/6 mice, as the cerebral vascular pattern may influence the susceptibility of mice to cerebral ischemia.18–20 Figure 1A illustrates the complete circle of Willis in a mixed 129/Sv and C57BL/6 tPA−/− mouse after intracardiac Evans blue perfusion; the same vascular pattern was found in a mixed tPA+/+ control mouse (not shown). The C57BL/6 mouse strain has a well-developed forebrain circulation including the MCA, and the anterior circulation is similar to that of the mixed 129/Sv and C57BL/6 strain.18–20 However, in contrast to the mixed strain, the C57BL/6 mouse has a dysgenetic posterior cerebral communicating artery, and contributions of the posterior circulation to the forebrain anterior and middle cerebral circulations are negligible in this strain.18–20

We used 8-mm-long, 6-0, uncoated, nonsiliconized prolene sutures that may have different occluding and thrombogenic properties in comparison with the more-often-used 10- to 12-mm-long nylon filament coated with silicone, which does not lead to thrombus formation.10 The position of thread in the MCA is shown in Figure 1B. In all animals, rectal temperature was maintained at 37±1°C. The blood pressure, gases, and the pH before and after 3 hours of MCA occlusion were within the physiological range in all studied groups. After 3 hours of occlusion, animals were allowed a 24-hour reperfusion period before they were killed for analysis. Brain injury at 24 hours in tPA+/+ mice was significant and involved the lateral, ventromedial, and dorsolateral cortex and the lateral and medial striatum, in contrast to limited injury in the tPA+/+ control strain (Figure 1C). On the other hand, the injury in C57BL/6 mice was significantly greater than in both tPA−/− and tPA+/+ mice on the 129/Sv and C57BL/6 backgrounds, reflecting significant genetic differences between the 2 strains (Figure 1C). Figure 2A illustrates that the volumes of brain infarction (upper panel) and edema (Swanson correction, lower panel) are shown. Values are mean±SE. n=6. P<0.05 for (a) tPA+/+ mice compared with tPA−/− mice, both on the 129/Sv and C57BL/6 mixed background; (b) C57BL/6 mice compared with 129/Sv and C57BL/6 mixed wild-type mice; (c) C57BL/6 mice compared with tPA−/− mice; and (ns) nonsignificant for brain edema in C57BL/6 mice compared with 129/Sv and C57BL/6 mixed wild-type mice. B. Changes in CBF during MCA occlusion and reperfusion in a tPA+/+ mouse (upper panel) and a tPA−/− mouse (lower panel), both on the mixed 129/Sv and C57BL/6 background, as determined by LDF. OCC. and REP. denote the onset of occlusion and the reperfusion phase, respectively. Mean±SD, from 4 or 5 measurements. *P<0.05 by ANOVA.
Figure 3 shows fibrin deposits in microvessels in the ischemic lesioned hemisphere in tPA<sup>-/-</sup> mice (A through E) and modest occasional immunostaining for fibrin in tPA<sup>+/+</sup> mice on 129Sv and C57BL/6 background (G and H) after 3 hours of MCA occlusion followed by 24 hours of reperfusion. Tissue was fixed in formalin and paraffin embedded. Seven-micron sections from matched regions in the ischemic and contralateral nonischemic hemisphere were reacted with the anti-mouse fibrin antibody. The dark brown reaction product shows fibrin in the ischemic lesioned hemisphere. I, tPA<sup>-/-</sup> mice: A, Two large venules (V, arrows) with intravascular fibrin, blood cells, and perivascular fibrin deposits; numerous fibrin-rich, small microvessels, ie, arterioles, venules, and capillaries (arrows); B, Several capillaries with intraluminal fibrin deposits (arrows). C, No significant staining in the contralateral hemisphere. D, Large venule on the cortical surface (V, arrow), and several capillaries positive for fibrin. E, Fibrin deposits and leakage from a microvessel. F, Intraluminal fibrin deposits in brain capillaries. II, tPA<sup>++/+</sup> mice: G, Absence of intraluminal or extraluminal fibrin deposits in brain capillaries (arrows). H, Moderate intraluminal fibrin deposits in the outer part of a large cortical venule (large arrow) are seen occasionally in the tPA<sup>++/+</sup> mouse; negative staining for fibrin in brain capillaries (small arrows).

Figure 3 shows fibrin deposits in microvessels in the ischemic lesioned hemisphere in tPA<sup>-/-</sup> mice. The immunostaining for fibrin was performed after TTC staining. Several small arterioles, venules, and capillaries contained intraluminal fibrin deposits (Figure 3A) of grade 1 on the scale for fibrin localization in an MCA model. Extravascular deposition of fibrin grade 2 was also found, suggesting breakdown of the blood-brain barrier (Figure 3A). Figure 3B shows several capillaries in the ischemic hemisphere positive for fibrin, in contrast to insignificant fibrin staining in a matched region of the contralateral hemisphere (Figure 3C). Figures 3D, 3E, and 3F illustrate fibrin deposits in a large pial venule and parenchymal capillaries, intraluminal and perivascular deposits around a small microvessel, and fibrin deposits within capillaries, respectively. Several vessels contained entrapped blood cells (eg, see venules in Figure 3A or the small microvessel in Figure 3E), possibly reflecting polymorphonuclear response (indicated by positive staining for polymorphonuclear markers; not shown) as reported previously.

Fibrin deposits and fibrin-rich thrombi were found occasionally after 24 hours of reperfusion in tPA<sup>++/+</sup> wild-type control mixed-strain mice (Figure 4C). The relative increase in the amount of fibrin in brain section IV (the level of the optic chiasm) was ~8.2-fold based on Western blot analysis and in sections III and V, between 5- and 6-fold. In the absence of ischemic challenge, there were no fibrin deposits in brain under basal conditions in any strain of either tPA<sup>-/-</sup> or tPA<sup>++/+</sup> mice. Fibrin deposition in the ischemic hemisphere in tPA<sup>++/+</sup> C57BL/6 mice subjected to ischemia was barely detectable (not shown) and was similar to that in tPA<sup>-/-</sup> 129Sv and C57BL/6 mixed-strain control mice, indicating that the fibrin level was not related to the difference in injury volumes between the 2 strains. No genotypic differences in endothelial lining, such as denudation, that could precipitate fibrin deposition were observed, and the immunoblot signal for fibrin deposition in control nonischemic brain was undetectable in all groups of mice (data not shown).

**Discussion**

This study in tPA<sup>-/-</sup> and wild-type tPA<sup>++/+</sup> mice on a matched genetic 129Sv and C57BL/6 mixed background indicates that tPA deficiency precipitates and markedly augments ischemia-induced cerebrovascular fibrin deposition and microvascular thrombotic obstructions. This in turn correlates with enhanced brain infarction and edema volumes, reduced CBF during MCA occlusion, and the reperfusion phase after the ischemic insult, and impaired motor neurological score 24 hours after focal cerebral ischemia in...
tPA−/− mice. The study strongly suggests that tPA protects the brain from an ischemic insult through its desirable thrombolytic action.

Cerebral protection from tPA has been shown in earlier stroke studies7–9 that led to clinical trials with tPA. Unwanted side effects of tPA treatment for stroke were also noted, including a higher rate of intracerebral hemorrhage,28 downstream displacement of thrombolysed material, breakdown of the blood-brain barrier, and reperfusion injury.29 However, direct neurotoxic effects from tPA have not been reported until recently. Recent studies in tPA−/− 129/Sv and C57BL/6 mixed-strain mice compared with C57BL/6 mice that served as a wild-type tPA+/+ control suggested that tPA mediates neuronal damage after cerebral ischemia.10 These studies raised a question whether tPA should be discontinued as an antistroke agent because of its neurotoxicity,10 or alternatively, whether clinical tPA trials should better define the inclusion/exclusion criteria for tPA treatment in the stroke population if tPA does not harm neurons.1–3

The present study compared 2 wild-type tPA+/+ mouse strains, the C57BL/6 strain and the 129/Sv and C57BL/6 mixed strain, and tPA−/− versus tPA+/+ mice on matched 129/Sv and C57BL/6 mixed genetic background. Because the volume of ischemic brain injury with the vascular occlusion method is inversely proportional to the length of thread inserted into the MCA,17 we used shorter threads to increase the sensitivity of measurements. In contrast to commonly used siliconized nylon sutures, which do not lead to thrombus formation,10 we used nonsiliconized thread that might have different thrombogenic properties30 and does lead to microvascular thrombus formation. Our data confirmed that the wild-type C57BL/6 strain is significantly more susceptible to cerebral ischemia than other strains for reasons that are not well understood.17–20 For example, infarcts in C57BL/6 mice are 7-fold larger than in 129J mice17 and 9.6-fold larger than in mixed 129/Sv and C57BL/6 mice as shown in the present study, and there is also significantly higher susceptibility to ischemia in C57BL/6 mice compared with 129/Sv mice.18–20

It is possible that the use of the highly susceptible C57BL/6 mouse to serve as a control for the effects of ischemia in mismatched tPA-deficient mouse strains, eg, the mixed 129/Sv and C57BL/6 strain in a previous study,10 could in addition to the differences in the ischemia models, eg, without thrombosis10 versus with thrombosis and cerebrovascular fibrin deposition (present study), account for differences in the results and data interpretation between the 2 studies.

Mice with combined tPA and urokinase deficiencies spontaneously deposit fibrin in several organs associated with ischemic necrosis.31,32 The singly deficient mice, either urokinase PA−/− or tPA−/−, do not develop spontaneous fibrin deposits under basal conditions, but they are significantly more susceptible to development of venous or arterial thrombosis if exposed to provocative stimuli (eg, endotoxin) or subjected to injury of the peripheral arteries.31,32 Our present data concur with these studies by demonstrating increased brain fibrin deposition, enhanced brain injury, and reduced CBF in tPA-deficient mice versus genetically matched tPA+/+ mice challenged by an ischemic insult. It is possible that small differences in the CBF within the critical range of 0% to 30% of baseline during the 3 hours of MCA occlusion may have a major influence on neuropathological outcome in tPA−/− versus genetically matched tPA+/+ mice. On the other hand, under basal conditions, there was no detectable fibrin deposits in brains of either tPA−/− or tPA+/+ mice. Because the anatomy of the cerebral vascular pattern in tPA−/− versus genetically matched tPA+/+ mice was the same and genotypic differences in endothelial lining, such as denudation, that could precipitate fibrin deposition were not observed, it is most likely that enhanced cerebrovascular fibrin deposition and brain injury in tPA−/− mice are related to the lack of fibrinolytic tPA action. However, one cannot rule out that other potentially important hemostatic differences in brain endothelial cells may exist between tPA−/− and tPA+/+ mice that can also contribute to alterations in the coagulation profile within the brain microcirculation in tPA−/− versus tPA+/+ mice.

The 2 tPA+/+ wild-type strains, namely, mixed 129/Sv and C57BL/6 mice and C57BL/6 mice, did not develop significant cerebrovascular fibrin deposits when subjected to focal ischemic stroke, and only occasionally did some venules and/or capillaries display moderate positive intraluminal fibrin staining. This may indicate that endogenous tPA in brain capillaries and/or in the circulation of these mice is sufficient to maintain significant thrombolytic activity. The 2 control tPA−/− strains did not exhibit significant differences in either CBF during occlusion and the reperfusion phase or in the edema volume or pattern of forebrain cerebral circulation. Thus, it is most likely that as-yet-unidentified genotypic factors and possibly poor communication between posterior and forebrain circulations in C57BL/6 mice17–20 compared with 129/Sv and C57BL/6 mixed mice that exhibit a normal circulatory pattern may be responsible for the significantly larger infarction volumes in the gray matter and
higher neurological scores in C57BL/6 mice versus the 129/Sv and C57BL/6 mixed strain or other mouse strains. Fibrin deposition in ischemic brain can be precipitated by procoagulant transformation of brain microvascular endothelium, formation of a platelet plug, or leukocyte-endothelial interactions during reperfusion. Previous studies have established a link between ischemic brain injury and reduced antithrombotic mechanisms in brain microcirculation in the presence of major stroke risk factors. In humans, the antithrombotic factor protein C is protective in ischemic stroke. The present study confirms that a deficiency of tPA favors fibrin deposition in brains challenged by a thrombotic ischemic insult, which in turn enhances ischemic brain injury. These findings provide direct evidence for a causal relationship between reduced fibrinolytic activity and ischemic neuronal damage, suggesting that endogenous tPA promotes desirable thrombolytic and protective effects in cerebral ischemia, and that factors other than tPA must be responsible for ischemic neuronal damage. In addition, the present study emphasizes the importance of appropriate genetic controls in murine stroke research.

Acknowledgment
This work was supported by National Institutes of Health grant HL63290 to B.V.Z.

References
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doi: 10.1161/01.ATV.19.11.2801

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