Role of Tissue-Derived Plasminogen Activator (t-PA) in an Excitotoxic Mouse Model of Neonatal White Matter Lesions

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Abstract. White matter (WM) lesions in preterm newborns may lead to cerebral palsy. To study WM lesions in a mouse model, we used intrapallial stereotactic injections of ibotenic acid, an N-methyl-D-aspartate receptor agonist. Previous studies support a contribution of tissue-type plasminogen activator (t-PA) to the brain lesions seen in various adult excitotoxic models. Therefore, we studied both 5-day-old (P5) wild-type mice and t-PA knock-out (t-PA−/−) mice. The ibotenic acid doses required to induce WM cysts were lower in the wild-type mice (EC50 < 0.01 µg/animal) than in the t-PA−/− mice (EC50 = 2.5 µg/animal) (p < 0.01), indicating the existence of t-PA−/−dependent and t-PA−/−-independent mechanisms. Dose-dependent prolonged cyst growth occurred in the wild-type mice only. Early microglial activation and astrogliosis were similar in the wild-type and t-PA−/− mice. In adult mice (P45), demyelination occurred at the injection site in both groups but the astroglial scar was denser in the wild-type than in the t-PA−/− mice. These data support involvement of t-PA at several stages of WM lesion formation. Inactivation of t-PA might confer protection by prolonged hemostasis. The role of t-PA in cyst expansion suggests a new approach to the development of neuroprotective strategies in infants with developing WM lesions.

Key Words: Astrocytes; Glial fibrillary acidic protein; Hemosiderin; Ibotenic acid; Microglia; Myelin basic protein; Neurofilament.

INTRODUCTION

Brain injury in preterm neonates can produce a range of lesions encompassing diffuse or focal periventricular leukomalacia (PVL), germinal matrix-intraventricular hemorrhage, posthemorrhage hydrocephalus, and various patterns of neuronal injury. White matter (WM) damage, of which a common pattern is cystic necrotic PVL, is widely accepted as the best predictor of spastic motor deficits and of cognitive or behavioral disorders that compromise academic performance (1). PVL occurs at a well-defined developmental stage, namely, 24 to 32 weeks after conception. Ultrasonography may show either persistent hyperechoic periventricular lesions or anechoic cystic lesions, both patterns being associated with neurodevelopmental disorders (2). Brain damage is significantly associated with inflammatory or hemodynamic insults to which the WM is highly susceptible at this stage of brain development (3, 4). The pathogenesis of brain damage involves microglial cell recruitment, cytokine and free radical production, and excess glutamate. In the WM, these factors cause astrogliosis and oligodendrogial cell death and axon retraction (4–6).

Animal models of the WM lesions common in human preterm infants have been developed in several species, usually with hypoxia-ischemia and/or inflammatory agents as the insult (7–10). The glutamate excess that occurs after hypoxia-ischemia leads to amino-acid toxicity, a common deleterious mechanism in various brain diseases (11). Intracerebral injection of excitotoxins to immature animals produces lesion patterns that resemble those seen in human patients. AMPA or NMDA gluta-

mater receptor agonists preferentially affect the oligodendrocyte progenitors or astrocyte cells, respectively (12–14). Intracerebral injection of ibotenic acid, an NMDA glutamate receptor agonist, induces cyst formation in mouse pups between 2 and 10 days of postnatal age (P2–P10) (13), which corresponds to the developmental stage at which PVL develops in humans (4). In this in vivo animal model, ibotenic acid induces rapid microglial activation followed by astrocyte death (14). Pro-inflammatory cytokines participate in the excitotoxic cascade (15), while corticosteroids are neuroprotective (16). Subcortical WM lesions eventually develop into a glial scar with an area of defective myelination in adults (13, 14).

In vitro astrocyte cell death can be induced by conditioned media from enriched microglial cell cultures exposed to ibotenic acid (14). Similarly, activated macrophages can induce cavitation in confluent astrocyte cell cultures (17). Microglial cells release a variety of inflammatory agents (18), including the serine protease tissue plasminogen activator (t-PA) (19). In vivo models of ischemia or stroke in the adult rodent brain show that microglial cells are responsible for neuronal death in the hippocampus after exposure to kainate or transient ischemia-reperfusion. These effects are caused, at least in part,
by t-PA, which may act at several levels (20, 21). Activation of plasminogen produces plasmin, which degrades extracellular matrix proteins and can lead to neuronal death (22, 23). Microglial activation by t-PA can occur via nonproteolytic mechanisms (24, 25). Finally, t-PA may interfere with NMDA excitotoxic mechanisms, since it cleaves the NR1 subunit of the NMDA receptor, thereby increasing agonist-induced calcium influx (26).

Recombinant human t-PA (hrt-PA) is commonly used in adults to achieve thrombolysis in the heart or kidney or, less often, in the brain. It has been suggested that administering hrt-PA to preterm babies with intraventricular hemorrhage may dissolve intraventricular blood clots and prevent hydrocephalus (27). However, the potential neurotoxicity of t-PA in adult animals indicates a need for further investigations of the risk/benefit ratio of hrt-PA in newborns. PVL in preterm neonates is of major concern as a source of permanent neurological and cognitive disability. Therefore, we designed the present study to investigate the potential role of endogenous t-PA in a neonatal mouse model of WM damage. To this end, we compared ibotenic acid-induced lesions on P5 in wild-type mice and in t-PA knock-out (t-PA<sup>−/−</sup>) mice (28).

**MATERIAL AND METHODS**

**Animals**

The study was performed in t-PA<sup>−/−</sup> mice and wild-type mice with the same genetic background (C57-B16/129; 75/25). The animals were permanently housed by pairs in a room with a controlled temperature (21°C ± 2°C) and a 12-hour light/12-hour dark cycle. They were fed rodent laboratory chow and given water ad libitum. The day of birth was P1. Randomization was achieved by using 1 or 2 pups as controls in each litter, while the remaining pups received a single injection. Brains from 2 to 4 litters were combined in each experimental group. All protocols and procedures complied with the guidelines established by the Institut National de la Santé et de la Recherche Médicale (INSERM) for laboratory animals and were performed under the supervision of a licensed expert (PL, license number: 76.A.16).

**Excitotoxic Lesion Model**

Ibotenic acid (Sigma, St. Quentin Fallavier, France), an agonist of both NMDA and metabotropic glutamate receptors, was used to mimic hypoxic-ischemia in P5 mice (13). In this model, ibotenic acid injection induced developmental abnormalities in the cortex and WM that were inhibited by the NMDA antagonists MK-801, kynurenic acid, and magnesium (14, 29, 30) but not by the metabotropic glutamate receptor antagonist L-AP3 (13). P5 pups each received a single injection of ibotenic acid, 0.01 to 20 µg in 2 µl of sterile phosphate buffered saline (PBS, pH 7.4) as previously described (13). The injection was given in the frontoparietal cortex, the corresponding WM being the corpus callosum radiation in mice. The needle was inserted perpendicular to the cortex surface. Five days after the injection, the brains were collected, fixed in 4% formalin, embedded in paraffin, and cut into 10-µm slices in the coronal plane. The lesions were identified by light microscopy examination of sections stained with cresyl violet (Sigma). We focused on the WM, where ibotenic acid induced cyst-like lesions with well-defined cellular borders on P10 (called “cysts” hereafter). The frequency and size of cysts and noncystic lesions were recorded in each experimental group. Lesion size was estimated based on the rostrocaudal dimension calculated as the number of consecutive 10-µm-thick slices in the lesion visible by microscopy (13). All microscope examinations were performed by an expert who was unaware of the group assignment of the animals. The same lesions have been observed in different genetic backgrounds (Swiss, NMRI) (14, 16).

The time-course of ibotenic acid (5 µg)-induced lesions was evaluated by examining animals killed 1 h to 5 days after ibotenic acid injection. In addition, the long-term outcome of the lesions produced by 0.1 or 5 µg of ibotenic acid injected on P5 was evaluated in wild-type and t-PA<sup>−/−</sup> mice killed 40 days after the injection (i.e. on P45).

**Immunohistological Stainings**

A polyclonal rabbit antibody to bovine glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark) was used to assess astroglial reactivity at various times between 1 h and 5 days after ibotenic acid injection, as well as at P45. A monoclonal mouse anti-myelin basic protein (MBP) (Chemicon, Euromedx, Mundolsheim, France) and a mouse monoclonal anti-human neurofilament (200 KD clone RT97, Chemicon) were used to study WM myelination and axonal density, respectively, 40 days after the ibotenic acid injection. The 10-µm brain sections were exposed to the appropriate nonimmune serum in PBS (blocking buffer) then incubated overnight at room temperature with the anti-GFAP, anti-MBP, or anti-neurofilament antibody (1:250, 1:500, or 1:1,000, respectively, in blocking buffer), incubated for 45 min with appropriate biotinylated anti-IgG (1:100 in blocking buffer), and finally subjected to indirect immunoperoxidase staining using the avidin-biotin complex technique (Vector, Tebu, Les Ulis, France) and DAB urea-peroxide (Sigma). Controls run omitting primary antibodies were consistently negative.

The density of astrocytes highly positive for GFAP was evaluated in wild-type and t-PA<sup>−/−</sup> mice during the 5 days following a 5-µg ibotenic acid injection. To this end, 2 independent observers unaware of treatment group assignment used an arbitrary 4-point scale, where 0 indicated very few or no positive cells, 1 low density, 2 medium density, and 3 high density. The optical density (OD) of glial scar labeled by GFAP antibodies was measured in P45 mice on digitalized pictures, using the background outside the section as the reference (Microvision Inst., Evry, France). OD was measured in the core of the glial scar over an average surface area of 1,000 µm², in 2 sections per animal, by an investigator unaware of treatment group assignment and of genotype.

**Histological Stainings**

Activated microglial cells were stained with biotinylated Griffonia simplicifolia I lectin B4 (IB4) (Vector). Luxol fast blue (VWR, Fontenay sous Bois, France) was used to stain myelin in the P45 mice. Hematoxylin and eosin (H&E) (VWR) served to identify inflammatory cells 48 h and 5 days after
T-PA AND EXCITOTOXICITY IN IMMATURE MOUSE CORTEX

Fig. 1. Effect of increasing doses of ibotenic acid (mg/pups) on the white matter of wild-type (open bars) and t-PA−/− (shaded bars) mice on postnatal day 5 (P5). A: Rate of occurrence of cysts (%). B: Rostro-caudal cyst size (μm). The Fisher exact test was used to compare rates and the Newman-Keuls test to compare sizes. Asterisks indicate significant differences between wild-type and t-PA−/− mice: *p<0.05; **p<0.01; and ***p<0.001.

Statistical Analysis

Mean lesion sizes (± standard error of the mean, SEM) were calculated in each group. Ibotenic acid dose-response relations and cyst size changes according to the time since ibotenic acid injection were studied using two-way ANOVA and the post-hoc Newman-Keuls test. The number of brains analyzed in each condition was 9 to 19 (usually 12). Rates of occurrence were evaluated using the Fisher exact test. The median ODs (interquartile range) of GFAP labeling in P45 mice were compared using the nonparametric Kruskal-Wallis test (2 to 8 brains per group).

RESULTS

Effect of Graded Doses of Ibotenic Acid on White Matter in t-PA−/− and Wild-Type Mice

The occurrence rate and size of subcortical WM cysts were measured in t-PA−/− and wild-type mice 5 days after injection of graded doses (0.01 μg–20 μg) of ibotenic acid (Fig. 1). Whatever the dose and genotype, lesions occurred consistently. In wild-type animals, cysts were more common, whereas the proportion of noncystic lesions was larger in the t-PA−/− mice. Higher ibotenic acid doses were associated with a larger proportion of cystic lesions. Among the wild-type mice, cysts were extremely common (72%–93%), even with the lower doses (0.01 μg–2.5 μg), and cysts were consistently found with the higher doses (Fig. 1A). Cyst size increased along the 0.01-μg to 5-μg dose range (p<0.001; two-way ANOVA) but did not increase further from 5 μg to 20 μg (542 μm–561 μm) (Fig. 1B). In the t-PA−/− mice, only 50% of the pups injected with doses no greater than 5 μg had cysts, but this proportion increased with the dose, reaching 80% in the pups given 20 μg. Cysts occurred at significantly lower rates in the t-PA−/− mice than in the wild-type mice over the 1-μg to 5-μg dose range (Fig. 1A). Interestingly, whereas the cyst occurrence rate increased with the ibotenic acid dose, cyst size in the t-PA−/− mice showed no significant variation (168 μm–300 μm) across the dose range used in the study (Fig. 1B). With high ibotenic acid doses, cyst size in the t-PA−/− mice was significantly smaller than in the wild-type mice (p<0.01; Newman-Keuls test) (Fig. 1B).

Noncystic lesions in wild-type mice were seen only in a few pups injected with low doses (0.01 μg–2.5 μg) of ibotenic acid, precluding a statistical evaluation. However, noncystic lesion size did not seem influenced by the dose of ibotenic acid (data not shown). In the t-PA−/− mice, ibotenic acid (0.01 μg–5 μg) produced noncystic lesions in nearly 50% of the animals. These noncystic lesions were always smaller than the cystic lesions and did not increase in size with the dose of ibotenic acid (170 ± 10 μm (n = 6), 270 ± 40 μm (n = 6), 240 ± 61 μm (n = 7), 135 ± 29 μm (n = 7), 145 ± 46 μm (n = 6), 93 ± 18 μm (n = 3), and 175 ± 25 μm (n = 2) in the groups injected with 0.01, 0.1, 1, 2.5, 5, 10, and 20 μg, respectively). In the t-PA−/− mice, the cystic or noncystic lesions were never larger than 289 μm, even with high doses of ibotenic acid, and were similar in size to those seen in the wild-type mice with low doses (Fig. 1).

Cyst formation within 5 days after ibotenic acid administration occurred even with low doses in the wild-type mice (EC50 < 0.01 μg), whereas higher doses (>2.5 μg) were needed in the t-PA−/− mice to obtain a 50% cyst occurrence rate. Conversely, high doses were required (EC50 – 2.5 μg) to induce the development of the large cystic lesions observed 5 days after ibotenic acid injection in the wild-type mice. Large cystic lesions did not occur in the t-PA−/− mice.

Histology of Lesions

Light microscopy of cresyl violet-stained brain sections of P10 mice injected with ibotenic acid (0.01–20
µg) on P5 showed both cortical and WM lesions (Fig. 2). The disruption in tissue architecture was consistently more severe in the cortex than in the WM. In the wild-type and t-PA−/− mice, the needle insertion site was seen only as a slender track confined to the cortex, indicating that the lesions were induced by the ibotenic acid rather than by vehicle injection (Fig. 2A, B). The nature of the lesions varied with the dose of ibotenic acid and the genotype of the animals (Fig. 2C–F). Noncystic lesions were seen mainly in the t-PA−/− mice and contained compact nests of cells heavily stained by cresyl violet (Fig. 2D). Noncystic lesions were found in all the ibotenic acid-injected animals that did not have cysts. Cysts surrounded by cell infiltrates were seen in a few wild-type and t-PA−/− brains.

**Time-Course of Development of Lesions Induced by 5 µg Ibotenic Acid in Wild-Type and t-PA−/− Mice**

The time-course of lesion development was studied in both genotypes after injection of 5 µg of ibotenic acid, since this was the lowest dose that produced the maximum effect in the wild-type mice. Lesion size was measured from 1 h to 5 days after the injection. After a short latency period (1–24 h), bleeding was noted at the ibotenic acid or vehicle injection site in both genotypes. The blood was eliminated by the tissue processing and section staining procedures and mechanical tissue disruption was then visible (Fig. 2H). The histological characteristics of the lesions changed as the time since injection increased. During the first 2 days following ibotenic acid injection, no differences were observed between wild-type and t-PA−/− mice regarding the occurrence rate of a cavity at the injection site produced by injection-induced physical damage (Fig. 3A). From 48 h onward, cysts rimmed by a smooth border were seen (Fig. 2G). From 3 days after ibotenic acid injection onward, the cyst rate was significantly lower in the t-PA−/− mice than in the wild-type mice (Fig. 3A). Cyst size changes with time since ibotenic acid injection differed significantly between the wild-type and the t-PA−/− mice (p < 0.02; two-way ANOVA) (Fig. 3B). In the wild-type mice, cyst size increased significantly from one day to the next during the first 4 days after ibotenic acid injection (p < 0.001; two-way ANOVA) but showed no change between days 4 and 5 (Fig. 3B). Conversely, in the t-PA−/− mice, cyst size tended to decrease from 2 days onward, although not significantly (Fig. 3B). However, the effects of ibotenic acid injection on lesion development over 5 days were significantly different between the 2 genotypes (p < 0.001; two-way ANOVA). The Newman-Keuls analysis showed significant cyst size differences 3, 4, and 5 days postinjection (p < 0.05; p < 0.001, and p < 0.001, respectively) (Fig. 3B). Progression toward noncystic lesions occurred only in the t-PA−/− mice, in a few animals as early as 24 h postinjection and in a significant number from day 3 onward. In these mice, lesion size did not increase between day 3 and day 5 postinjection (100 µm–200 µm, data not shown).

**Histological Characterization of Damaged Area at Various Postinjection Times**

Astrocytosis was evaluated semiquantitatively over the 5-day period following the ibotenic acid injection on P5. In noninjected P5 controls, strongly GFAP-positive astrocytes were found in low densities in both the wild-type and the t-PA−/− mice (Fig. 4A). These GFAP-positive cells were not visible 1 to 6 h after 5 µg of ibotenic acid in the wild-type or t-PA−/− mice (Fig. 4A) but were detected again from 1 day postinjection onward. Astrocytosis seemed more marked in the t-PA−/− than the wild-type mice but, in both groups, peaked 2 days postinjection and remained similarly prominent thereafter (Fig. 4A). In noninjected P10 mice, a uniformly distributed population of GFAP-positive astrocytes was seen throughout the WM and overlying cortex (not shown). Conversely, in both genotypes, the density of GFAP-positive astrocytes was high around the noncystic (Fig. 4B) or cystic lesions (Fig. 4C, D) 5 days after the ibotenic acid injection (on P10).

Histochemical labeling by IB4 performed 2 days post-injection showed reactive microglia (Fig. 4E) in both the wild-type and the t-PA−/− mice injected with either 0.1 or 5 µg of ibotenic acid. Conversely, 5 days postinjection, no IB4-labeled reactive microglia was seen. The vascular endothelium was consistently labeled by IB4. H&E staining also showed microglial cells in the WM on P7 in both genotypes (Fig. 4F).

In noncystic lesions 5 days after ibotenic acid injection, H&E staining showed red blood cells admixed with fibrin deposits and surrounded by mononuclear inflammatory infiltrates. These cells contained vesicular nuclei with 1 or 2 nucleoli and eosinophilic or clear cytoplasm, identifying them as macrophages (Fig. 4G). The absence of foamy cells in these lesions was coherent with the very immature myelination at this stage, as evidenced by Luxol fast blue staining (not shown). At the periphery of the lesion, astrocytes were identified based on their morphological appearance (Fig. 4G). Perls staining showed residual changes from bleeding in the core and at the periphery of noncystic lesions (Fig. 4H). Blue staining at the periphery of cysts was also observed.

**Long-Term Outcome of the Ibotenic Acid-Induced Lesions in Wild-Type and t-PA−/− Mice**

Persistent lesions in P45 (adult) mice were dense GFAP-positive glial scars with WM atrophy and/or disruption (corpus callosum radiation). No such lesions were visible in the wild-type or t-PA−/− mice injected with PBS. Glial scars were consistently present in wild-type and t-PA−/− mice given 0.1 or 5 µg of ibotenic acid (Fig.
Fig. 2. Typical characteristics of brain lesions induced by intracortical injections of PBS or ibotenic acid in cresyl violet-stained brain sections on P10 (A–G) or 3 h postinjection (H). A, B: Effect of PBS injection into a wild-type pup (A) and a t-PA<sup>−/−</sup> pup (B). C, D: Effect of 0.1 µg ibotenic acid in a wild-type pup (C) and a t-PA<sup>−/−</sup> pup (D). E, F: Large cyst and small cyst induced by 5 µg ibotenic acid in a wild-type pup (E) and a t-PA<sup>−/−</sup> pup (F), respectively; G: Typical cyst surrounded by heavily stained cells induced 5 days after injection on P5 of ibotenic acid 5 µg to a wild-type pup; H: Cavity and tissue disruption 3 h after the injection of ibotenic acid 5 µg into a wild-type pup. The arrows show the injection site and the arrowhead a noncystic lesion. Abbreviations: C, cyst; Ca, postinjection cavity; V, lateral ventricle; WM, white matter. Scale bar =100 µm.
Fig. 3. Time-course study of white matter lesion during the 5 days following an intracortical injection of ibotenic acid 5 μg into wild-type mice (open bars) and t-PA−/− mice (shaded bars). Rate of occurrence of lesions (A) and size of lesions (μm) (B) at 1, 3, and 6 hours (h) and 1 to 5 days (d) postinjection. The Fisher exact test was used to compare rates and the Newman-Keuls test to compare sizes. Asterisks indicate significant differences between wild-type and t-PA−/− mice after ibotenic acid injections: *p < 0.05; **p < 0.01; ***p < 0.001. # Indicates a significant increase in lesion size versus the value 1 h postinjection in the wild-type mice, as shown by a Newman-Keuls test: **p < 0.001.

Atrophy of the corpus callosum was visible on the cresyl violet-stained sections from the vast majority of animals of both genotypes, even with the low dose (0.1 μg) ibotenic acid (Table). Further investigation with specific myelin markers (e.g., Luxol fast blue, MBP, or neurofilament antibodies) showed atrophy or disruption of the corpus callosum radiation. Disruption was not observed after PBS injection to the wild-type or t-PA−/− mice (Fig. 5E, G, I) but was present in 50% of mice after 0.1 μg or 5 μg ibotenic acid injection in each genotype group (Fig. 5F, H, J, Table). In the P45 mice, lesion size as measured on cresyl violet- and Luxol-stained sections showed no significant differences between the wild-type and t-PA−/− mice.

DISCUSSION

Role of t-PA in Neonatal White Matter Lesions Induced in Mice by Ibotenic Acid Injections

The present study provides evidence that t-PA gene inactivation conferred neuroprotection against the excitotoxic effects of ibotenic acid in neonatal mice. The time-course study showed that the lesions observed 5 days postinjection in the wild-type animals developed slowly over several days. Nevertheless, the lesions are determined very early after ibotenic acid injection, since the NMDA receptor antagonist MK-801 fully prevented neurotoxicity when administered immediately after ibotenic acid but had no effect when given after a 4-hour lag. These data are consistent with participation of t-PA in at least 2 distinct mechanisms involved in WM damage: early potentiation of the cyst-inducing effects of ibotenic acid and subsequent stimulation of cyst growth. The evidence of a cyst growth phase provided by this study is a major finding as it suggests the existence of a window for neuroprotective therapy.

Involvement of t-PA-Dependent and t-PA-Independent Pathways at Various Stages of Ibotenic Acid-Induced White Matter Injury

Our data from t-PA−/− mice constitute convincing evidence that t-PA contributes to the various steps of the complex process leading to excitotoxin-induced WM cyst formation in neonatal mice. The potential of low-dose ibotenic acid to induce cyst formation was significantly different 5 days postinjection between the wild-type and the t-PA−/− mice. A 250-fold higher dose was needed to induce cysts in the t-PA−/− mice (EC50 ~ 2.5 μg) than in...
PA−/− pups given ibotenic acid 0.1 μg. GFAP-labeling on P10 in wild-type (C) and t-PA−/− (D) pups given ibotenic acid 5 μg. E: IB4 labeling of activated microglia in a P7 wild-type mice given ibotenic acid 0.1 μg. F: High-power view of an H&E-stained section from the brain of a P7 t-PA−/− pup given ibotenic acid 0.1 μg. G: High-power view of an H&E-stained noncystic lesion in a t-PA−/− pup given ibotenic acid 0.1 μg. H: Perls staining of noncystic lesions in a P10 t-PA−/− pup given ibotenic acid 0.1 μg. Hemosiderin is seen as a blue deposit. The small black arrows indicate blood vessels (E) and the large black arrows indicate macrophages (G). The arrowheads show microglial cells (E, F). Abbreviations: C, cyst; Ca, early postinjection cavity; WM, white matter. Scale bar = 50 μm.
Fig. 5. Typical characteristics of brain lesions in P45 mice 40 days after an intracerebral injection of PBS or ibotenic acid. GFAP staining of an astroglial reaction in a wild-type pup (A) and a t-PA<sup>−/−</sup> pup (B) after an injection of ibotenic acid 0.1 µg. GFAP staining of an astroglial reaction in a wild-type pup (C) and a t-PA<sup>−/−</sup> pup (D) after ibotenic acid 5 µg. Luxol fast blue staining of sections from wild-type mice injected with PBS (E) or ibotenic acid 0.1 µg (F). MBP immunostaining of t-PA<sup>−/−</sup> mice injected with PBS (G) or ibotenic acid 0.1 µg (H). Neurofilament immunostaining of t-PA<sup>−/−</sup> mice injected with PBS (I) or ibotenic acid 0.1 µg (J). The arrows point to areas of disruption of the corpus callosum and the arrowheads to areas of reactive astrocytosis. Abbreviations: Cx, cortex; WM, white matter. Scale bar = 100 µm.
the wild-type mice (EC50 < 0.01 μg), indicating that t-PA potentiated excitotoxin-induced cyst formation. However, the ability of ibotenic acid to induce cyst formation in t-PA−/− deficient mice (provided the dose was sufficiently high) supports the concomitant existence of a t-PA− independent mechanism of toxicity. Growth of existing cysts required both high doses of ibotenic acid and the presence of t-PA, indicating that low and high doses operate via distinct mechanisms that both involve t-PA.

Delayed fibrin clot lysis in vivo related to impaired plasmin generation is an established characteristic of t-PA−/− mice (31). Greater clot stability at the injection site in our experiments might have protected the brain tissue from secondary bleeding. Thus, the decreased cyst formation rate in the t-PA−/− mice 3 days after ibotenic acid 5 μg may be ascribable, at least in part, to prolonged hemostasis. This finding is congruent with evidence that an imbalance between clotting factors and thrombolytic factors was a risk factor for neonatal brain lesions and cerebral palsy in humans (32). Studies of the vascular endothelium are needed, as NMDA receptors on endothelial cells may be another potential target for ibotenic acid (33).

In addition to the role for endothelial t-PA in thrombolysis, t-PA produced by the central nervous system (CNS) contributes to physiological responses and to disease processes. Neurons, astrocytes (34), and microglia (20, 34) release t-PA. Moreover, the t-PA inhibitors plasminogen activator inhibitor-1 and neuroserpin are secreted by astrocytes and neurons, respectively (34, 35). Plasminogen mRNA and protein have been detected in the CNS after kainate injection (36, 37). Thus, the CNS contains all the t-PA/plasmin system components needed to regulate proteolysis. In the cascade of events set in motion by ibotenic acid, the site or sites where t-PA operates remained to be determined. Both endothelial and CNS t-PA are probably involved, as exogenous hrt-PA had different effects when given intracerebrally versus intraperitoneally in our model (data not shown). In addition, systemic plasmin inhibitors protected against cyst growth (data not shown).

Specificity of the Developmental White Matter Lesions

In the adult rat hippocampus, t-PA is involved in kainate excitotoxicity (20) and hypoxic-ischemic lesions (21). Microglia was shown to release t-PA responsible for neuronal toxicity (19, 38). In these models, plasmin activation is a key factor in inducing neuronal death (19, 22, 23), but t-PA also exerts plasmin-independent effects. In particular, Nicole et al (26) showed that t-PA cleaved the NR1 subunit of the NMDA receptor and enhanced NMDA-evoked Ca2+ currents, suggesting a potential link between t-PA and NMDA toxicity. In addition, t-PA also has nonproteolytic effects, one of the most notable being induction of a microglia autoactivation loop (25). In the adult WM, activated macrophage/microglial cells have deleterious effects (17). In neonatal mice, NR1 expression colocalizes with microglial markers for a short period corresponding to the ontogenic window of WM vulnerability to ibotenic acid (14). However, only indirect in vitro evidence is available to support the presence of NMDA receptors in these cells (39), and the presence of functional NMDA receptors in the microglia has not been established (40). Thus, whether the microglia on P5 contains functional NMDA receptors in vivo remains to be confirmed. Astrocytes are probably an early target of the neurotoxic cascade, since GFAP staining disappears rapidly after ibotenic acid injection (14). This effect is not due to t-PA, as it was similar in the wild-type and t-PA−/− mice in our study. Thus, the differences between genotypes regarding the lesions on P10 are probably not related to an effect on astrocytes during the early stages of lesion genesis.

Intracerebral ibotenic acid injection on P5 induces massive neuronal death in the cortex of wild-type mice and, to a lesser extent, of t-PA−/− mice (not shown). Conceivably, the effect of t-PA deficiency against WM lesions in our model may be ascribable, at least in part, to...
primary neuroprotection of the cortex. However, WM lesions also result from a tissue-specific cascade, as in this model some agents exert targeted neuroprotective effects (e.g., vasoactive intestinal peptide [41] and melatonin [42]) and other deleterious effects (nociceptin [43]) on the WM yet have no influence on the cortical toxicity of ibotenic acid.

Potential Therapeutic Benefits and Risks of Using t-PA in Human Neonates

According to our findings, during a well-defined period of vulnerability in neonates, t-PA may exert toxic effects on the neonatal brain parenchyma, particularly the WM, by enhancing glutamatergic-dependent mechanisms that lead to cyst formation. In addition, further damage may occur from recurrent bleeding due to thrombus dissolution. Consequently, great caution is in order regarding ongoing therapeutic trials of exogenous t-PA given to dissolve intraventricular clots with the goal of preventing hydrocephalus in preterm neonates (27).

Glial scars in the WM are physical barriers for migrating cells such as pre-oligodendrocytes. We found no difference in the histological labeling of activated microglia or in GFAP-staining of astrocytes during the 5 days following ibotenic acid injection to wild-type or t-PA−/− mice. Nevertheless, astrocyte density was reduced in adult t-PA−/− mice given ibotenic acid on P5. As ibotenic acid induced not only a local scar but also damage in a larger cortical and subcortical territory, the reduced astrocytosis in adult t-PA−/− mice represented significant neuroprotection.

This model may produce data relevant to clinical practice. Cysts induced by ibotenic acid in wild-type mice mimic anechoic PVL lesions in humans. The reduced cyst formation and concomitant increase in compact cell nests in the t-PA−/− mice indicate that this model may be useful for investigating echogenic PVL lesions in human neonates (4, 44). Neonatologists have noted that diffuse PVL lesions have become more common and multiple cysts less common in recent years (44). A possible explanation is the more widespread use of perinatal glucocorticoid treatment to prevent neonatal respiratory distress syndrome. In the mouse model used in our study, glucocorticoid treatment reduced the rate of cyst formation but increased the rate of noncystic lesions (16), supporting a role for inflammation in the excitotoxin-induced cascade and a link between this inflammation and t-PA. Finally, our finding that ibotenic acid-induced lesions continued to grow for several days after the injection indicates that their may be a window of opportunity for treatments designed to halt lesion growth. The potential benefit of agents that inhibit the t-PA-plasminogen-metalloprotease cascade is under investigation in this model in our laboratory.

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