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Genetics

Neuronal death in the central nervous system demonstrates a non-fibrin substrate for plasmin

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ABSTRACT **Mice deficient for plasminogen exhibit a variety of pathologies, all of which examined to date are reversed when the animals are also made fibrin(ogen) deficient. These results suggested that the predominant, and perhaps exclusive, physiological role of plasminogen is clearance of fibrin. Plasminogen-deficient mice also display resistance to excitotoxin-induced neurodegeneration, in contrast with wild-type mice, which are sensitive. Based on the genetic interaction between plasminogen and fibrinogen, we investigated whether resistance to neuronal cell death in the plasminogen-deficient mice is dependent on fibrin(ogen). Unexpectedly, mice lacking both plasminogen and fibrinogen are resistant to neurodegeneration to levels comparable to plasminogen-deficient mice. Therefore, plasmin acts on substrates other than fibrin during experimental neuronal degeneration, and may function similarly in other pathological settings in the central nervous system.**

Deficiency for plasmin(ogen) (plg$^{-/-}$) in mice leads to high mortality, rectal prolapse, ulcerations of the gastrointestinal tract and the skin, and impaired wound healing (1). Plasmin can degrade fibrin efficiently, and its role in fibrinolysis is well established. Because all of the plg$^{-/-}$ pathologies are also associated with deposits of fibrin(ogen), either in the vasculature or extravascularly (1, 2) in the affected organs and tissues, it was possible that the abnormalities were due to impaired fibrinolysis. To determine the extent to which fibrin deposition was critical for the pathologies observed in plg$^{-/-}$ mice, animals with a combined plg–fibrinogen (fib) deficiency (plg$^{-/-}$; fib$^{-/-}$ mice) were produced (3, 4). These double deficient mice had a normal life-span, nearly lesion-free organs, and normal wound healing, indicating that all the plg$^{-/-}$ pathologies were corrected. These results suggested that the primary role of plasmin(ogen) is fibrinolysis (4).

In this report, we show that in the brain plasmin functions in a fibrin–cleavage-independent mechanism. This is evident using a model of excitotoxin-induced hippocampal neuronal death involving the plg activator (PA)/plasmin(ogen) proteolytic cascade, since plg$^{-/-}$;fib$^{-/-}$ mice are as resistant to neurodegeneration as plg$^{-/-}$ mice. Therefore, we suggest that in the central nervous system (CNS) plasmin has a substrate that is different from fibrin, revealing a non-fibrinolytic role for plasmin.

MATERIALS AND METHODS

Intrahippocampal Injection of Kainic Acid. Adult male mice, weighing approximately 25 g, were injected intraperitoneally (i.p.) with atropine (0.6 μg per g of body weight) and then were deeply anesthetized with an i.p. injection of 2.5% avertin (0.02 ml/g of body weight). They were placed in a stereotaxic apparatus and injected unilaterally with 1.5 nmol of kainic acid in 0.3 μl of PBS into the hippocampus (5, 6). The coordinates of the injection were: bregma −2.5 mm, medial-lateral 1.7 mm, and dorsoventral 1.6 mm. The excitotoxin was delivered over 30 sec. After kainic acid was delivered, the injection needle remained at the above coordinates for another 2 min to prevent reflux of fluid. Five days after the injection, the sections were mounted onto slides, dehydrated through increasing ethanol gradients, and stained with cresyl violet (5, 6) to assess neuronal survival. Normal morphology of the neuronal cells, as well as rRNA staining as revealed by the staining for cresyl violet, indicate the presence of viable neurons. Six mice were injected for each of the four genotypes studied.

Quantification of Neuronal Loss over CA1–CA3 Hippocampal Subfields. Wild-type, plg$^{-/-}$, fib$^{-/-}$, and plg$^{-/-}$; fib$^{-/-}$ mice were injected and the tissue was processed as above. Serial sections of 30 μm were prepared and stained with cresyl violet. Five consecutive sections from the dorsal hippocampus of two mice from each genotype and treatment were matched. The hippocampal subfields (CA1–CA3) on these sections were traced from camera lucida drawings of the hippocampus. The length of each subfield was measured by comparison to 1 mm standards traced under the same magnification. Each subfield was then further subdivided as containing intact (completely spared/normal morphology, no dead neurons present in the field), partially lost (few intact neurons present/disruption of the cellular morphology, yet maintenance of some cresyl violet-stained cellular "ghosts"), or totally lost (completely eliminated/no cells present, no cresyl violet staining) pyramidal neurons. The length for intact, partially lost, and totally lost neurons over each hippocampal subfield was averaged across sections, and the standard deviations were determined.

RESULTS AND DISCUSSION

To determine if plasmin might have substrates different than fibrin(ogen) in contexts other than the reported plg$^{-/-}$ phenotypes (1), we investigated the fibrin-dependence of a neuronal degeneration model that involves tissue plg activator (tPA) and plasmin(ogen). In this model, mice are injected unilaterally into the hippocampus with the glutamate analog kainate (KA), which leads to loss of pyramidal neurons on the injected side (5, 6). Using this assay, mice deficient for tPA or plg are resistant to neuronal cell death, implicating the tPA/plasmin extracellular proteolytic system in neurodegeneration. However, mice deficient for fib are susceptible to neuronal death (7). This experimentally induced pathological situation is analogous to the physiological situations explored by Bugge *et al.* (4): plg$^{-/-}$ mice show a different phenotype than wild-type or fib$^{-/-}$ mice. Thus, using the same genetic logic of

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Abbreviations: tPA, tissue plasminogen activator; plg, plasminogen; fib, fibrinogen; KA, kainate; CNS, central nervous system.
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Bugge *et al.* (4), if the resistance (abnormal phenotype) of plg-deficient mice was dependent on fib, then the $plg^{-/-}$ mice would again be rendered sensitive (wild-type phenotype) to neuronal degeneration in the absence of fib.

Wild-type mice, $plg^{-/-}$, $fib^{-/-}$, or doubly deficient mice ($plg^{-/-}$;$fib^{-/-}$) were subjected to the neuronal degeneration experimental paradigm. The results of unilateral, intrahippocampal KA injection demonstrate that mice with a combined deficiency in plg and fib are resistant to neuronal degeneration (Fig. 1). The degree of neuronal survival was quantitated in the four genotypes: mice deficient in both plg and fib were resistant to neuronal cell death to a level comparable to plg-deficient mice (Fig. 2). In particular, $\approx$100.0 $\pm$ 0.0% of the neurons in both plg-deficient and doubly deficient mice were maintained intact in the CA1 region after KA injection, 100.0 $\pm$ 0.0% in the CA2, and 90.1 $\pm$ 1.3% in plg deficient (vs. 93.4 $\pm$ 1.8% in plg; fib deficient) in the CA3 subfield. These numbers should be compared with the equivalent numbers for wild-type (0.0 $\pm$ 0.0% in the CA1, 37.5 $\pm$ 11.9% in the CA2, and 4.6 $\pm$ 1.3% in the CA3) and the fib-deficient mice (0.0 $\pm$ 0.0% in the CA1, 0.0 $\pm$ 0.0% in the CA2, and 0.0 $\pm$ 0.0% in the CA3). The effect is specific for neuronal cells, since microglial activation was comparable to wild type in all the four genotypes tested (ref. 7 and data not shown).

Therefore, there appears to be a substrate for plasmin other than fibrin(ogen) that influences neuronal degeneration. In addition, immunohistochemical experiments using an anti-fibrin(ogen) antibody show no fibrin(ogen) in the mouse

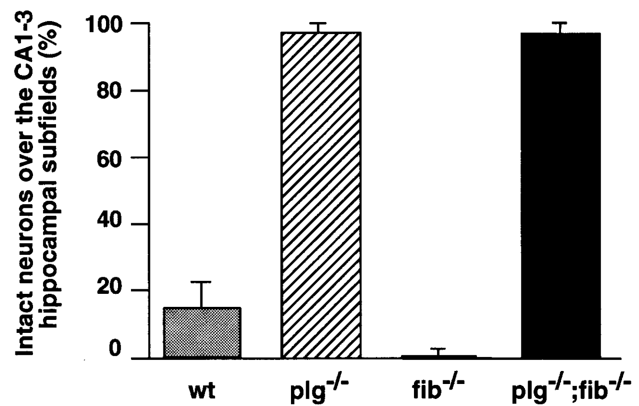

FIG. 2. Quantification of neuronal survival over CA1–CA3 hippocampal subfields in wild-type, plg-, fib-, and plg/fib-deficient animals after KA injection. Mice from the four different genotypes were subjected to intrahippocampal injection of the excitotoxin KA. The degree of neuronal survival was quantitated, as described in the *Materials and Methods*. The percentage of spared neurons over the CA1–CA3 hippocampal subfields in each genotype was averaged and plotted.

hippocampus (data not shown). Sections from fibrin(ogen)-deficient mice were used as negative controls, whereas the efficiency of the antibody was tested on liver sections of wild-type mice (3). Fibrin deposits in the brain have been reported only in pathological situations that include extensive

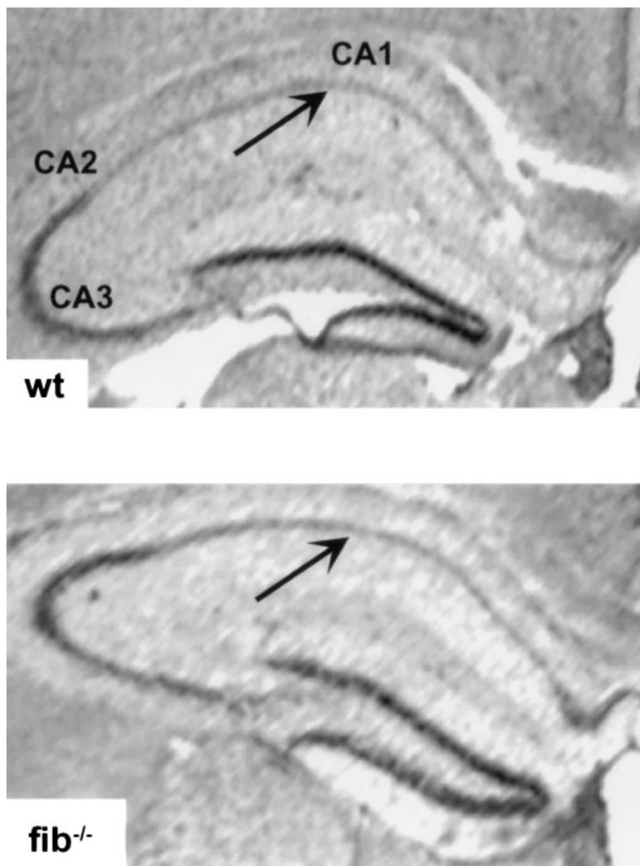

FIG. 1. Plasmin functions in neuronal cell death independently of fibrin clearance. Cresyl violet-stained coronal sections through the hippocampus reveal the neuronal degeneration generated 5 days after KA injection. (*Upper Left*) Hippocampus from heterozygous $plg^{+/-}$;$fib^{+/-}$ mouse (wt) showing substantial degeneration on the injected side (ipsilateral). CA1, CA2, and CA3 denote the hippocampal subfields. DG, dentate gyrus. (*Upper Right*) Hippocampus from $plg^{-/-}$ mouse showing minimal degeneration on the injected side. (*Lower Left*) Hippocampus from $fib^{-/-}$ mouse showing extensive degeneration on the injected side. (*Lower Right*) Hippocampus from $plg^{-/-}$;$fib^{-/-}$ mouse showing minimal degeneration on the injected side. In each case, the uninjected side (contralateral) showed no detectable degeneration. Arrows show the site of injection. The genotypes were coded until completion of the experiments and scoring of the results.

disruption of the blood-brain barrier, infiltration of cells, and exposure of the brain parenchyma to blood-derived proteins (8, 9).

These findings represent the first definitive demonstration of a phenotype in plg$^{-/-}$ mice that is not affected by fibrin-(ogen) removal. To date, the exact function of tPA/plasmin(ogen) in the hippocampus is unknown. Although it is possible that plasmin(ogen) may function in a nonproteolytic way, this possibility is rather unlikely for two reasons:

(*i*) tPA-deficient mice are also resistant to excitotoxic neuronal death (6), and these mice have normal levels of CNS plg (S.E.T., unpublished observations). Because there is no detectable urokinase plg activator in the hippocampus (7, 10), no plasmin is being generated in the CNS of the tPA-deficient mice. All these factors taken together indicate that plg needs to be activated to mediate cell death.

(*ii*) Wild-type mice are protected against neurodegeneration by α_2-antiplasmin (7), suggesting that the activity of plasmin is required for neuronal death.

The results presented here derive from an experimental protocol that subjects the hippocampus to a challenge and generates a pathology. However, tPA protein and activity and plg protein are present in the unperturbed mouse brain (10–12), suggesting that plasmin may have a normal function in the CNS that does not involve fibrinolysis. It is also possible that the overexpression of tPA activity, associated with overstimulation of hippocampal neuronal activity (6, 13), represents a fibrin-independent role for plasmin in pathological settings that produce excess proteolysis. If this latter case pertains, non-fibrin substrates of plasmin may be involved in other pathological settings. As such substrates, one could envision matrix metalloproteinases, growth factors (e.g., transforming growth factor-β) that are known to be activated by plasmin cleavage, or other extracellular matrix proteins (14). Some of the matrix metalloproteinases (15), as well as tissue inhibitor of metalloproteinases-1 (16), synthesized by neurons, have been shown to display neuroprotective properties in cases of excitotoxicity, elicited either by deposits of amyloid peptide, or KA lesions, respectively. Furthermore, transforming growth factor-β1 conferred protection from cell death to hippocampal neurons that had been subjected to transient global ischemia (17), whereas transforming growth factor-β2 was able to potentiate excitotoxicity elicited by *N*- methyl-D]aspartate in rat cortical neurons (18). These findings further suggest that the balance of proteolytic activity may be critical for neuronal survival in pathological events and may be an important contributor to disease pathogenesis.

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