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Tissue Plasminogen Activator–mediated Fibrinolysis Protects Against Axonal Degeneration and Demyelination after Sciatic Nerve Injury

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Abstract. Tissue plasminogen activator (tPA) is a serine protease that converts plasminogen to plasmin and can trigger the degradation of extracellular matrix proteins. In the nervous system, under noninflammatory conditions, tPA contributes to excitotoxic neuronal death, probably through degradation of laminin. To evaluate the contribution of extracellular proteolysis in inflammatory neuronal degeneration, we performed sciatic nerve injury in mice. Proteolytic activity was increased in the nerve after injury, and this activity was primarily because of Schwann cell–produced tPA. To identify whether tPA release after nerve damage played a beneficial or deleterious role, we crushed the sciatic nerve of mice deficient for tPA. Axonal demyelination was exacerbated in the absence of tPA or plasminogen, indicating that tPA has a protective role in nerve injury, and that this protective effect is due to its proteolytic action on plasminogen. Axonal damage was correlated with increased fibrin(ogen) deposition, suggesting that this protein might play a role in neuronal injury. Consistent with this idea, the increased axonal degeneration phenotype in tPA- or plasminogen-deficient mice was ameliorated by genetic or pharmacological depletion of fibrinogen, identifying fibrin as the plasmin substrate in the nervous system under inflammatory axonal damage. This study shows that fibrin deposition exacerbates axonal injury, and that induction of an extracellular proteolytic cascade is a beneficial response of the tissue to remove fibrin. tPA/plasmin-mediated fibrinolysis may be a widespread protective mechanism in neuroinflammatory pathologies.

Key words: coagulation • extracellular matrix • ancrod • Schwann cells • proteolysis

Introduction

The most common reaction of the nervous system after disconnection of axons from the neuronal body is axon lysis and myelin sheath fragmentation. These processes contribute to functional impairment of the nerve. Axonal and myelin damage are observed in a variety of nervous system diseases with diverse etiologies, such as stroke (Sawlani et al., 1997), spongiform encephalopathies (Liberski and Gajdusek, 1997), Guillan-Barre syndrome (Trojaborg, 1998), insulin-dependent diabetic neuropathy (Said et al., 1992), and multiple sclerosis (Trapp et al., 1998; Arnold, 1999). In these diseases, axonal degeneration is considered to be responsible for permanent disability. It has been proposed that proteases such as plasminogen activators (Reich, 1978; Tsirka et al., 1995; Seeds et al., 1997) and matrix metalloproteinases (LaFleur et al., 1996) may play a role in nervous system damage.

Plasminogen activators (PAs)[1] are serine proteases, which convert plasminogen to plasmin. Plasmin is a potent serine protease that can degrade a variety of proteins, but its primary substrate in vivo is fibrin, the proteinaceous component of blood clots (Bugge et al., 1996). There are two PAs identified in mammals: tissue-type and urokinase-type. The PA/plasmin system can have deleterious effects in pathological settings via proteolysis of tissue proteins. For example, plasmin-catalyzed degradation of laminin promotes excitotoxic-mediated neurodegeneration in the hippocampus (Chen and Strickland, 1997), and plasmin can also degrade myelin basic protein (MBP) in vitro (Cammer et al., 1978; Norton et al., 1990). Conversely, the PA/plasmin system can be beneficial via its fibrinolytic activity, since PA/plasmin-mediated proteolysis

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[1]*Abbreviations used in this paper:* ECM, extracellular matrix; fib, fibrin(ogen); MBP, myelin basic protein; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor-1; plg, plasminogen; PNS, peripheral nervous system; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

plays a critical role in fibrin(ogen) clearance (Bugge et al., 1996).

In experimental nerve injury (Bignami et al., 1982; Sallés et al., 1990), as well as in human neuroinflammatory diseases such as multiple sclerosis, PAs and their inhibitors can be upregulated (Akenami et al., 1997, 1999; Cuzner and Opdenakker, 1999). However, it is not clear if this altered expression is deleterious or a beneficial response of the tissue to the disease. In the current study, we sought to investigate how PAs affect degeneration severity in the peripheral nervous system (PNS) in vivo. To this end, we performed sciatic nerve injury experiments in mice genetically deficient for tPA (Carmeliet et al., 1994), plasminogen (Bugge et al., 1995; Ploplis et al., 1995), and fibrinogen (Suh et al., 1995). Nerve crush in the PNS is a reliable experimental model that allows the study of axonal degeneration (Bridge et al., 1994). Our analysis revealed induction of tPA expression after nerve injury. Axonal and myelin loss were exacerbated in tPA- and plasminogen-deficient mice, whereas fibrinogen depletion was protective. Fibrin deposition was also correlated with nerve damage. These results demonstrate that tPA/plasmin-catalyzed fibrin degradation can protect neurons against axonal injury at least in the PNS.

Materials and Methods

Animals

tPA-deficient mice (tPA$^{-/-}$) (Carmeliet et al., 1994) have been backcrossed to C57Bl/6J for nine generations. Urokinase plasminogen activator (uPA)-deficient mice (uPA$^{-/-}$) (Carmeliet et al., 1994) also have been backcrossed to the C57Bl/6J background. C57Bl/6 mice were used as wild-type controls. Plg-deficient mice (plg$^{-/-}$) (Bugge et al., 1995), which are heterozygous for fib deficiency, fibrinogen-deficient mice (fib$^{-/-}$) (Suh et al., 1995), which are heterozygous for plg deficiency; and combined plg-, fibrinogen-deficient mice (plg$^{-/-}$fib$^{-/-}$) were of mixed genetic background. Heterozygous plg$^{+/-}$fib$^{+/-}$ littermates were employed as controls in all studies. No differences were observed between C57Bl/6J mice and heterozygous plg$^{+/-}$fib$^{+/-}$ mice in the crush injury model. All mice were 8–15-wk old at the start of the experiment. The genotype of all mice was confirmed at the end of the experiments by PCR analysis of genomic DNA extracted from mice tails. All experiments were performed in compliance with institutional guidelines.

Sciatic Nerve Crush Injury

Adult mice were anesthetized by intraperitoneal injection of 2.5% avertin (0.18 ml/10 g body weight; Aldrich Chemical Co.). For the crush injury, sciatic nerves were crushed midthigh three times (20 s each) with watchmaker's forceps, the tips of which had been previously cooled in liquid nitrogen (Funakoshi et al., 1998). The crush site was marked with India ink (LaFleur et al., 1996). Skin incisions were closed with sutures.

PA In Situ Zymography

For in situ zymographies (Sappino et al., 1993), 10-μm unfixed, cryostat sciatic nerve sections were prepared. Sections were covered by a casein overlay containing 1% low melting point agarose and 25 μg/ml plasminogen, and covered with a coverslip. Plasminogen was prepared from human plasma (Deutsch and Mertz, 1970). Control experiments were performed with overlay mixtures lacking plasminogen. To distinguish uPA from tPA activity, amiloride (Sigma Chemical Co.), a specific inhibitor of uPA enzymatic activity (Vassalli and Belin, 1987), was added in the mixture at 1 mM final concentration. To discriminate between tPA and other fibrinolytic enzymes, tPA-STOP (2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one dihydrochloride salt; American Diagnostica) at a concentration of 50 μg/ml was used. Sections were incubated at room temperature in a humidified chamber. Conversion of plasminogen into plasmin, which in turn lysed the insoluble casein, resulted in the appearance of lytic zones within 6 h. Zones of plasmin-dependent caseinolysis appeared as black areas when photographed under darkfield illumination.

Immunohistochemistry and Immunoblot Analysis

Dissected nerves were embedded in Tissue-Tek OCT, then immediately frozen on dry ice and stored at −70°C until use. Sections were cut longitudinally on a motor-driven Leica cryostat with a retraction microtome and a steel knife at a cabinet temperature of −20°C.

Immunohistochemical staining was performed on cryostat sections (Akassoglou et al., 1997, 1998). Primary antibodies were as follows: rabbit anti-human tPA (1:1,200; Waller and Schleuning, 1985), goat anti–human fibrin(ogen) (1:500; Chemicon International, Inc.), rabbit anti–cow GFAP (1:200; Dako), rabbit anti–mouse laminin (1:1,000; Sigma Chemical Co.), rabbit anti–mouse fibronectin (1:200; Chemicon International, Inc.), rat anti–mouse VCAM-1 (Chemicon International, Inc., 1:50), rabbit anti–human MBP (1:200; DAKO), sheep anti–mouse PA inhibitor 1 (PAI-1) (1:200; American Diagnostica), and rabbit antineuroserpin (1:2,000; provided by D. Lawrence, Department of Vascular Biology, American Red Cross Holland Laboratory, Rockville, MD). Bound antibodies were visualized using either the avidin–biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) and 3-amino-9-ethylcarbazole (Sigma Chemical Co.) as a chromogen, or the avidin–biotin-alkaline phosphatase complex (Vectastain Elite ABC kit; Vector Laboratories), using nitroblue tetrazolium/brom-chlor-indolyl phosphate (Vector Laboratories). Staining specificity for the tPA and fibrin(ogen) antibodies was confirmed using tissue from tPA$^{-/-}$ and fib$^{-/-}$ mice, respectively. Staining specificity for GFAP antibody was confirmed using rabbit IgG. Incubation without the primary antibody served as a negative control. Histochemical Oil Red O staining, which stains lipids and reveals demyelinated areas (Akassoglou et al., 1997), was performed on cryostat sections. Immunoblot for neuroserpin was performed on sciatic nerve extracts as described in Hastings et al., 1997.

Quantification of Axonal Degeneration

8 d after injury, animals were intracardially perfused under deep anesthesia with ice-cold 2% paraformaldehyde, and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 min followed by ice-cold 3% glutaraldehyde in 0.1 M phosphate buffer for 5 min. The injured sciatic nerve was removed, and the region ∼5 mm below the injury site (crush site marked with India ink) was isolated. The noninjured sciatic nerve (contralateral) served as a control. Nerves were immersion fixed for 24 h in phosphate-buffered 3% glutaraldehyde, postfixed in 2% osmium tetroxide solution, and embedded in resin. Semi-thin sections were cut on an Ultra-cut microtome and stained with toluidine blue. A morphometric grid (100 mm^2) was adapted to the microscope, and a minimum of three grids per sample of myelinated axons was counted.

Systemic Defibrinogenation

Mice were depleted of fibrinogen as described in Busso et al. (1998). In brief, mice were anesthetized, their backs shaved, and 14-d mini-osmotic pumps (model 2002; Alza Corp.) filled with a solution of 250 U ancrod per ml of 0.1 M NaCl and 0.1 M Tris-HCl, pH 7.5 (Sigma Chemical Co.) were implanted subcutaneously into their backs (one mini-pump per animal). The insertion sites were closed by sutures. The pumps deliver 0.5 μl/h, so the mice received 3 U ancrod/d. In control animals, buffer-filled mini-pumps were implanted. On day 3 of ancrod infusion, sciatic nerve crush or transection was performed. 8 d after the crush, mice were killed, and tissues were prepared for cryostat or semi-thin sections.

Quantification of Muscle Atrophy

8 d after injury, animals were killed, the skeletal muscles from the injured (ipsilateral) and the control (contralateral) site were isolated, and their net weight was calculated. The percentage of atrophy was determined for each animal by subtracting the weight of the muscle from the injured site from the weight of the muscle of the control site. The difference was multiplied by 100 and divided by the muscle weight of the control site.

Results

tPA Is the Major PA Induced after Sciatic Nerve Injury

Plasminogen activator activity (Bignami et al., 1982) and tPA mRNA (Yuguchi et al., 1997) and protein (Neuberger and Cornbrooks, 1989) expression are increased in the PNS after injury. To address the major type of PA induced after injury, we performed in situ zymography on sciatic nerves of wild-type, tPA$^{-/-}$, and uPA$^{-/-}$ mice. When compared with control uninjured nerve (Fig. 1 A), wild-type sciatic nerve 8 d after crush showed an induction of proteolytic activity (Fig. 1 B, dark zone around nerve). Wild-type control or injured sciatic nerves incubated with an overlay that lacked plasminogen did not show any proteolytic activity (data not shown), indicating that the activity was plasminogen-dependent. tPA$^{-/-}$-injured sciatic nerve did not show appreciable proteolytic activity (Fig. 1 C), whereas uPA$^{-/-}$-injured sciatic nerve showed an induction of proteolytic activity (Fig. 1 D) similar to the injured wild-type control (Fig. 1 B). Addition of tPA-STOP, a specific tPA inhibitor, blocked the activity (Fig. 1 E), whereas addition of amiloride, a specific inhibitor of uPA proteolytic activity, had no effect (Fig. 1 F). After 24 h of assay, a lytic zone started to appear in wild-type sciatic nerves in the presence of tPA-STOP as well as in the tPA$^{-/-}$ crushed sciatic nerves (data not shown), suggesting the presence of low uPA activity. These results show that the proteolytic activity induced after sciatic nerve injury is mainly due to tPA.

tPA Is Expressed by Schwann Cells after Sciatic Nerve Injury

Tissue plasminogen activator has been shown to be produced by Schwann cells and the growth cones of peripheral neurons in culture (Krystosek and Seeds, 1984; Clark et al., 1991). To identify the cellular localization of tPA protein in vivo, sciatic nerves before and after crush were stained with an antibody against tPA. When compared with the uninjured sciatic nerve (Fig. 2 A), immunoreactivity specific for tPA was increased as early as 2 d after the crush (Fig. 2 B). The lack of staining in the tPA$^{-/-}$ sciatic nerve with the anti-tPA antibody (Fig. 2 C) showed that the antibody was specific. Staining of parallel sections showed that the tPA immunoreactive cells (Fig. 2 D) had similar morphology to cells positive for GFAP (Fig. 2 E), a Schwann cell marker. In contrast, immunostaining with an antibody against Mac-1, a macrophage-specific marker, showed a different staining pattern than that for tPA (data not shown). These results suggest Schwann cells as a major source of tPA in the sciatic nerve after injury.

To examine the possible role of endogenous inhibitors of tPA, we analyzed the expression of PAI-1 and neuroserpin in the sciatic nerve before and after injury. Immunostaining for PAI-1 revealed that there was no PAI-1 expression before or after injury (data not shown). Neuroserpin, a brain-associated inhibitor of tPA (Osterwalder et al., 1996; Hastings et al., 1997), is constitutively expressed in the sciatic nerve (Lawrence, D., personal communication). Western blot analysis and immunohistochemistry on wild-type and tPA$^{-/-}$ sciatic nerve extracts did not reveal any significant increase of neuroserpin after injury (data not shown). Overall, these data suggest that elevated tPA activity is due primarily to increased expression of the protein, and not to downregulation of protease inhibitors.

Exacerbation of Axonal Degeneration in tPA$^{-/-}$ and plg$^{-/-}$ Mice

Since tPA is the major PA produced after sciatic nerve injury, we compared lesion formation after sciatic nerve crush in wild-type and tPA$^{-/-}$ mice. Toluidine blue staining for myelin in semi-thin cross-sections of crushed

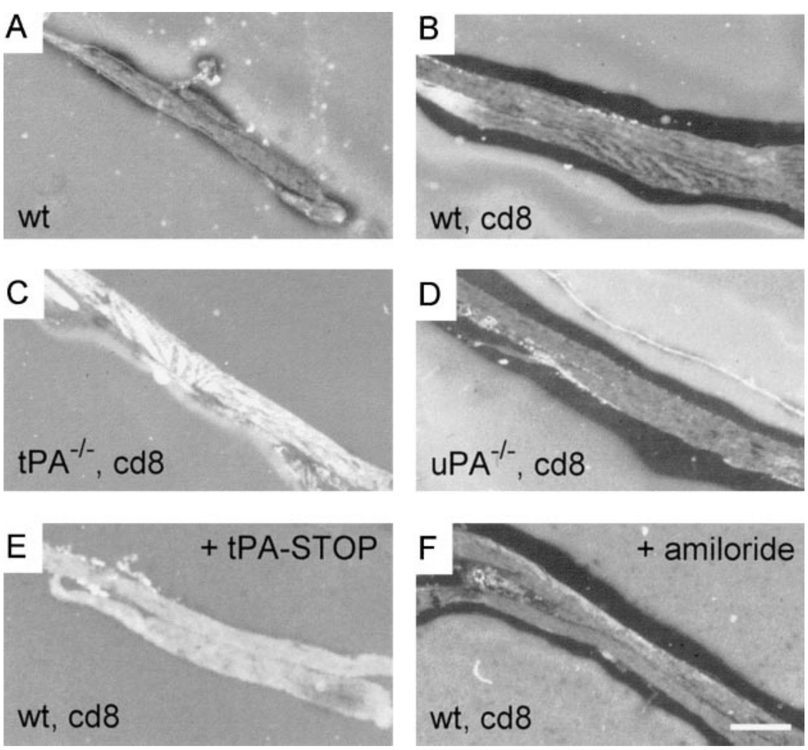

Figure 1. tPA is the major PA induced after sciatic nerve injury. In situ zymography to detect proteolytic activity 8 d after crush (cd8) showed a small proteolytic zone on wild-type uninjured sciatic nerve (A) and an increase of activity after injury (B, dark zone around nerve). C–E show nerves after injury. tPA$^{-/-}$ sciatic nerve (C) did not show any activity, whereas uPA$^{-/-}$ nerve (D) showed proteolytic activity similar to the wild type (B). Proteolytic activity of wild-type sciatic nerve was inhibited by tPA-STOP (E), a tPA inhibitor, but was unaffected by amiloride (F), a uPA inhibitor. Assay time was 6 h. Bar, 1 mm.

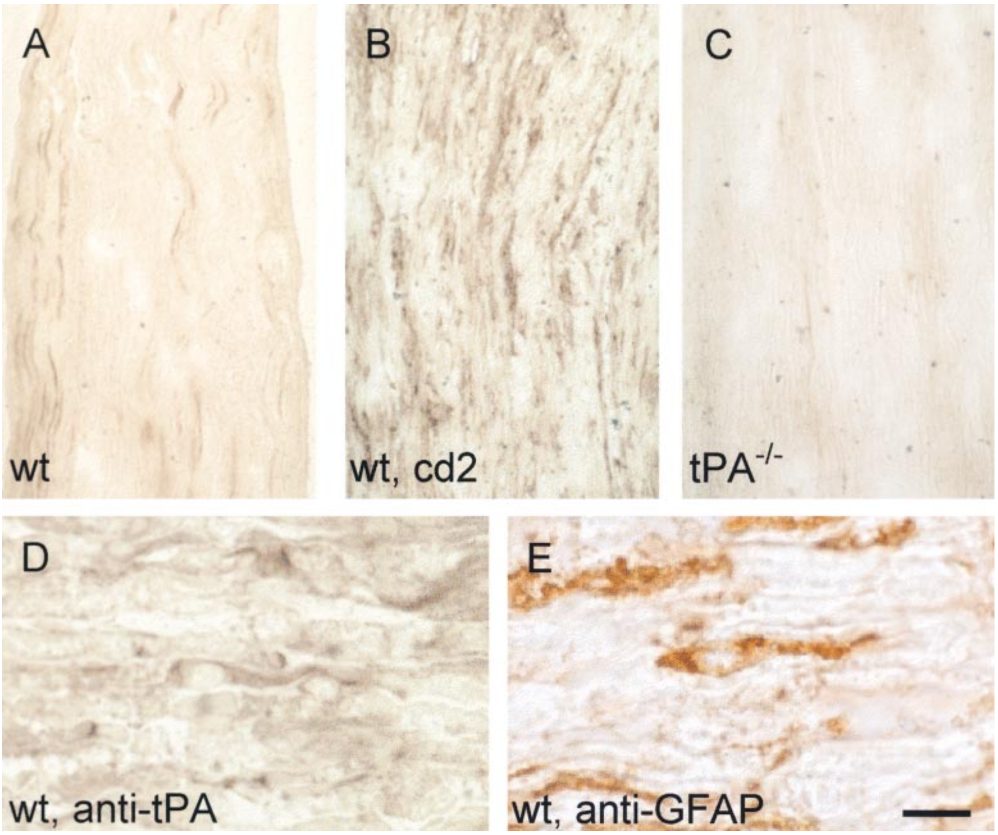

Figure 2. tPA is produced by Schwann cells after sciatic nerve crush. Immunocytochemistry with an antibody against tPA on longitudinal cryostat sections of wild-type, uninjured sciatic nerve revealed tPA staining only of the sciatic nerve vasculature (A). As early as 2 d after crush (cd2) tPA immunoreactivity was increased in the endoneurium (B). Sciatic nerve from a tPA$^{-/-}$ mouse showed no immunoreactivity with the tPA antibody (C). Immunostaining of parallel sections of a wild-type sciatic nerve 2 d after crush showed similar morphology between tPA immunoreactive cells (D) and GFAP-positive Schwann cells (E). The number of GFAP-positive cells was equivalent in wild-type and tPA$^{-/-}$ nerves before and after crush, suggesting that the number of Schwann cells is not significantly different in the two genotypes. Bar: (A–C) 93 μm; (D–E) 18 μm.

nerves after 8 d showed a dramatic decrease in myelinated axons in tPA$^{-/-}$ mice (Fig. 3 D), when compared with wild-type mice (Fig. 3 C). Similarly, light microscopy analysis of the lesion at 8 d with Oil Red O, which stains the lipids associated with the myelin sheath, revealed increased myelin debris in tPA$^{-/-}$ mice (Fig. 3 F, aggregates of lipid), when compared with wild-type controls (Fig. 3 E). To examine whether the difference in demyelinating damage persisted at later times, we analyzed sciatic nerves 22 d after crush. Since at later times the myelin and axonal debris has been removed, Oil Red O staining is not informative. Therefore, tissue morphology was examined with an antibody against MBP, which stains myelinated axons. In accordance with our observation 8 d after the crush, tPA$^{-/-}$ mice had decreased immunoreactivity for MBP (Fig. 3 H), when compared with wild-type controls (Fig. 3 G). This result shows that, at later times after crush injury, there are fewer myelinated axons in tPA$^{-/-}$ mice than in wild-type mice.

To quantitate the effect of tPA on axonal demyelination, we counted myelinated axons in semi-thin sections. This quantification showed (myelinated axons/0.1 mm^2) the following: 10.3 ± 2.4 in control, and 2.1 ± 0.3 in tPA$^{-/-}$ (Fig. 4 D). The decrease in the number of myelinated axons in tPA$^{-/-}$ mice compared with control mice was statistically significant ($P < 0.01$).

To assess whether the increase in axonal degeneration in the absence of tPA was due to its proteolytic function, we performed crush injury in mice genetically deficient for plasminogen, which is the primary substrate for tPA. Plg$^{-/-}$ mice (Fig. 4 A) showed a similar reduction in myelinated axons as tPA$^{-/-}$ mice (Fig. 3 F). Quantification of myelinated axons/0.1 mm^2 showed 2.8 ± 0.7 in plg$^{-/-}$ (Fig. 4 D). The decrease in myelinated axons in plg$^{-/-}$ mice compared with control mice was statistically significant ($P < 0.02$), and there was no significant difference between tPA and plg$^{-/-}$ mice. These results indicate that tPA reduces axonal loss and demyelination in the PNS primarily through its proteolytic effect on plasminogen. These results do not exclude a subtle nonproteolytic effect of tPA, as has been observed in other systems (Kim et al., 1999; Rogove et al., 1999).

Loss of Fibrinogen Rescues Exacerbation of Axonal Degeneration Observed in plg$^{-/-}$ Mice

Removal of fibrinogen rescues mice from most of the effects of plasminogen deficiency (Bugge et al., 1996). However, the resistance of plasminogen-deficient mice to excitotoxic neuronal degeneration in the hippocampus is not affected by the removal of fibrinogen (Tsirka et al., 1997a). To determine if fibrin(ogen) was playing a role in inflammatory neuronal degeneration, we performed nerve crush in mice with fibrinogen deficiency (fib$^{-/-}$) or a double deficiency for plasminogen and fibrinogen. Fib$^{-/-}$ mice were similar to wild-type mice in myelinated axons (Fig. 4, B and D). The decrease in myelinated axons observed in plg$^{-/-}$ mice (Fig. 4 A) was alleviated by genetically superimposing fibrinogen deficiency (plg$^{-/-}$fib$^{-/-}$; Fig. 4, C and D). These results indicate that tPA/plasmin-mediated deg-

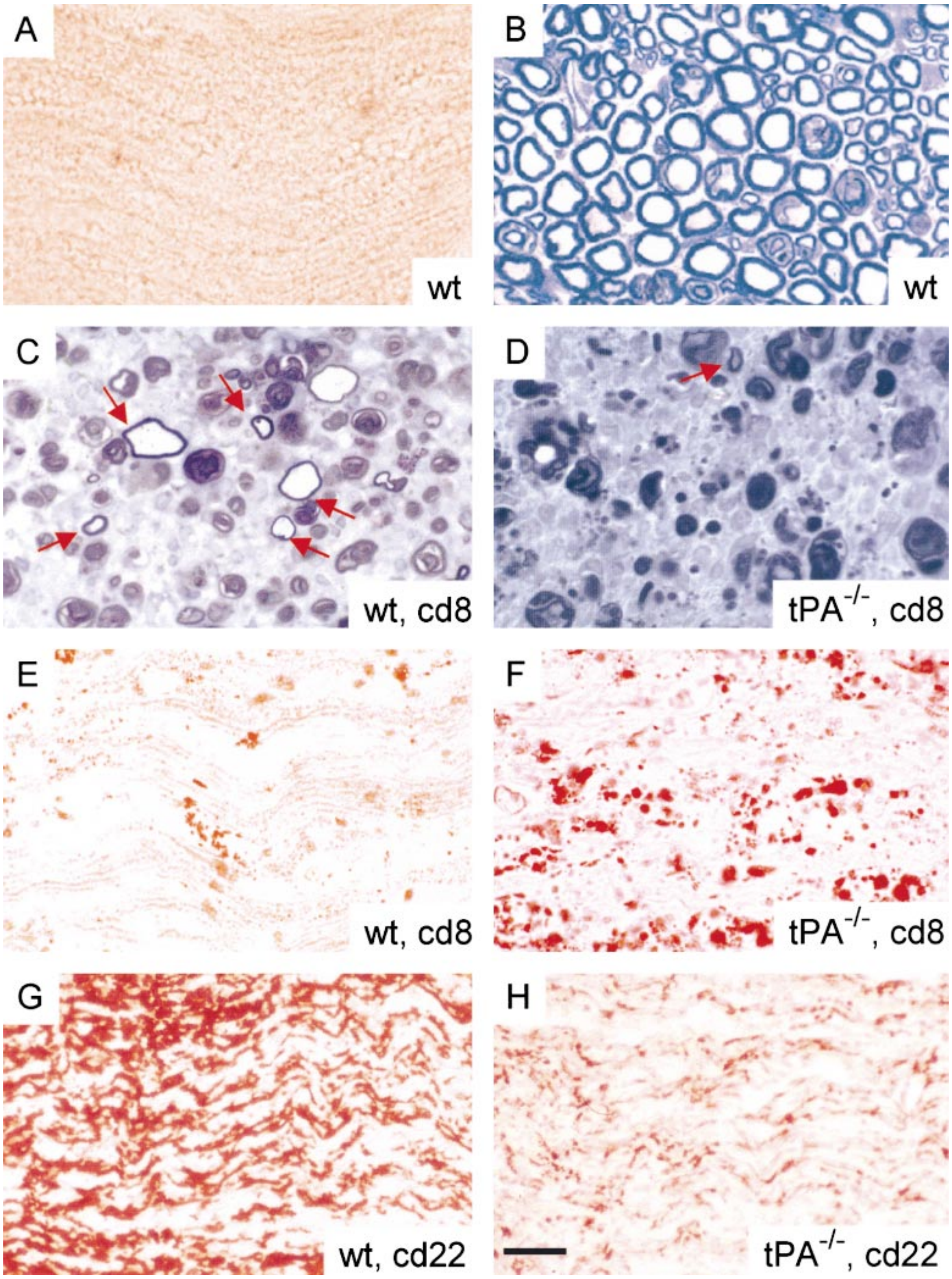

Figure 3. Axonal degeneration and demyelination are exacerbated in tPA-deficient mice after sciatic nerve crush. Oil Red O staining of cryostat sections of uninjured wild-type sciatic nerve (A) revealed normal myelin distribution, and toluidine blue staining of sciatic nerve semi-thin cross-sections showed normal axon morphology (B). 8 d after crush (cd8), toluidine blue staining of semi-thin cross-sections of wild-type mice (C) demonstrated more myelinated axons (arrows) than $tPA^{-/-}$ mice (D). Oil Red O staining showed increased accumulation of myelin and lipid debris in the $tPA^{-/-}$ (F) compared with the wild-type (E) sciatic nerve. 22 d after crush (cd22), staining for myelin basic protein revealed fewer myelinated axons in $tPA^{-/-}$ (H) than in wild-type (G) nerves. Bar, 18 μm.

radation of fibrin(ogen) protects axons from degeneration and demyelination. Quantification of myelinated axons/ 0.1 mm^2 showed the following: 12.0 ± 2.8 in $fib^{-/-}$, and 15.2 ± 4.0 in $plg^{-/-}fib^{-/-}$ (Fig. 4 D). The increase in the number of myelinated axons in the $plg^{-/-}fib^{-/-}$ mice compared with the $plg^{-/-}$ mice was statistically significant ($P < 0.03$).

Fibrin(ogen) Deposition Increases after Nerve Injury and Correlates with Axonal Degeneration

To address the involvement of fibrin(ogen) in axonal degeneration and myelin loss, we performed immunocytochemistry with an antibody against fibrin(ogen). A partial nerve crush at the sciatic nerve revealed that the crushed part of the nerve, which underwent degeneration (Fig. 5 A), had extensive deposition of fibrin(ogen), whereas the immediately adjacent, uninjured region was free of fibrin(ogen) (Fig. 5 B). Absence of staining of a $fib^{-/-}$ sciatic nerve documented the specificity of the antibody against fibrin(ogen) (not shown). This staining indicates that fibrin(ogen) deposition is spatially correlated with degeneration.

We also examined other extracellular matrix (ECM) proteins for their involvement in sciatic nerve injury. In the mouse hippocampus, tPA-mediated degradation of lami-

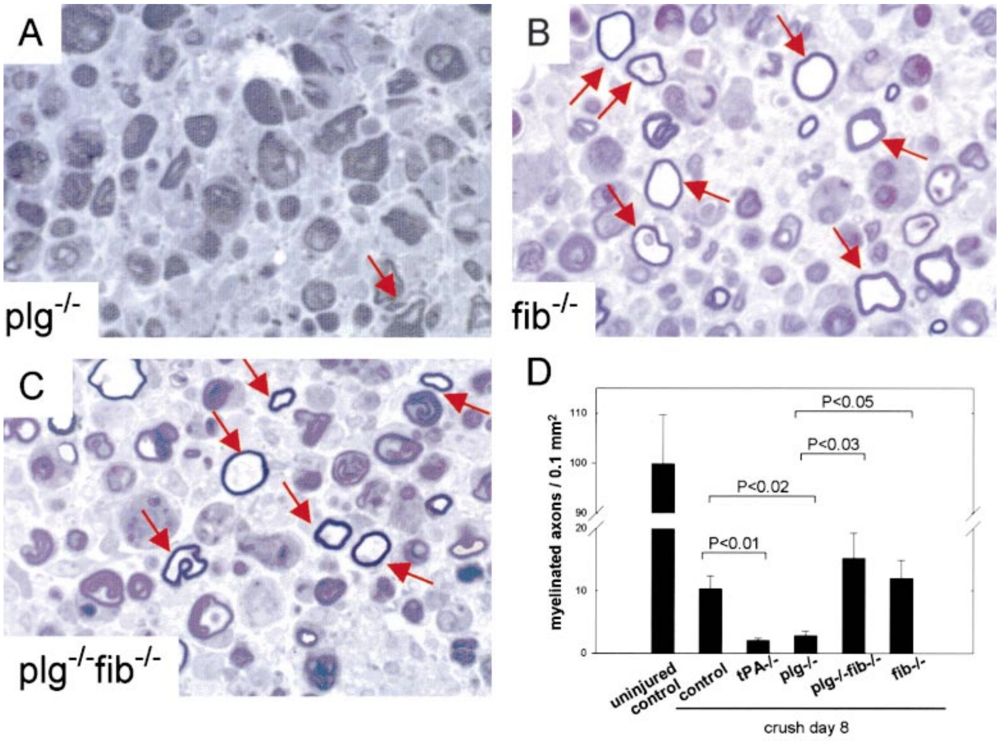

Figure 4. tPA protects from axonal degeneration through a proteolytic mechanism. Toluidine blue staining of sciatic nerve semi-thin cross-sections of plg$^{-/-}$ mice (A) reveals exacerbated axonal damage. Fib$^{-/-}$ mice (B) and plg$^{-/-}$fib$^{-/-}$ (C) mice showed myelinated axons similar in number to wild-type mice (C). (D) Quantification of myelinated axons. First column shows uninjured sciatic nerve (n = 4). After crush, tPA$^{-/-}$ (n = 6) and plg$^{-/-}$ (n = 4) nerves showed significantly fewer myelinated axons compared with control injured nerve (n = 5) (P < 0.01 and P < 0.04, respectively). Crushed fib$^{-/-}$ (n = 7) and plg$^{-/-}$fib$^{-/-}$ (n = 4) nerves showed the same number of axons as control crushed nerves. Plg$^{-/-}$fib$^{-/-}$ nerves showed more myelinated axons compared with tPA$^{-/-}$ nerves (P < 0.04). Uninjured sciatic nerves from all genotypes had similar number of myelinated axons and similar morphology. Data are expressed as means ± SEM. Statistical comparisons between medians were made with the *t* test. Scale as in Fig. 3.

nin after injury is implicated in neurodegeneration (Chen and Strickland, 1997). To examine the expression pattern of laminin in sciatic nerve injury, we stained sciatic nerves before and after crush with an antibody against laminin. Laminin was expressed in the normal and the tPA$^{-/-}$ sciatic nerve, and this expression was not significantly altered after injury (data not shown). Another ECM protein, fibronectin, was equally expressed at low levels in wild-type and tPA$^{-/-}$ sciatic nerves, and not altered by injury. To examine if differences in adhesion molecules could contribute to the exacerbated phenotype observed in tPA$^{-/-}$ mice, we stained wild-type and tPA$^{-/-}$ mice for VCAM-1, which is upregulated after injury (Castano et al., 1996). Wild-type and tPA$^{-/-}$ mice showed similar upregulation of VCAM-1 after crush. These results suggest that laminin, fibronectin, and VCAM-1 do not contribute to the altered peripheral nerve phenotype of tPA$^{-/-}$ mice.

Pharmacological Depletion of Fibrin(ogen) Protects Mice from Axonal Degeneration

Administration of ancrod, a Malayan pit viper (*Calloselasma rhodostoma*) venom protein, leads to consumption of systemic fibrinogen and drastically reduces plasma fibrinogen levels (Bell et al., 1978). Therefore, under these conditions, excessive fibrin deposition should be diminished, which might result in less nerve damage. Ancrod (3 U/d) was administered to tPA$^{-/-}$ mice for 3 d before crush, and then throughout the experimental period, without any effects on survival. tPA$^{-/-}$ mice treated with ancrod showed dramatically reduced fibrin(ogen) immunoreactivity (Fig. 6 B) when compared with untreated tPA mice (Fig. 6 A).

Histopathological analysis revealed that depletion of fibrin(ogen) rescued tPA$^{-/-}$ mice from exacerbated axonal degeneration. tPA$^{-/-}$ mice treated with ancrod showed more myelinated axons (Fig. 6 D) when compared with untreated tPA$^{-/-}$ mice (Fig. 6 C). Quantification of myelinated axons/0.1 mm^2 showed the following: 2.1 ± 0.3 in tPA$^{-/-}$, and 5.9 ± 0.6 in tPA$^{-/-}$ treated with ancrod (Fig. 6 E). The increase in myelinated axons in the tPA$^{-/-}$ mice treated with ancrod compared with the untreated tPA$^{-/-}$ mice was statistically significant ($P < 0.01$).

Although we detected no morphological differences in the uninjured sciatic nerves of the various mouse genotypes, the possibility existed that subtle anatomical changes due to gene disruption might be playing a role in the phenotype. The ancrod experiment is important in eliminating this possibility. Since the tPA$^{-/-}$ mice used were highly inbred and, therefore, genetically identical, the reversal of the phenotype by fibrinogen depletion indicates that acute fibrin deposition is responsible for exacerbating the nerve damage in the tPA$^{-/-}$ mice.

Muscle Atrophy after Nerve Injury Is Decreased after Fibrin(ogen) Depletion

To assess a possible functional consequence of fibrin(ogen) depletion, we examined skeletal muscle weight decay after nerve crush. The gastrocnemius muscle atrophies after sciatic nerve injury due to muscle denervation and the percentage of atrophy corresponds to the degree of nerve

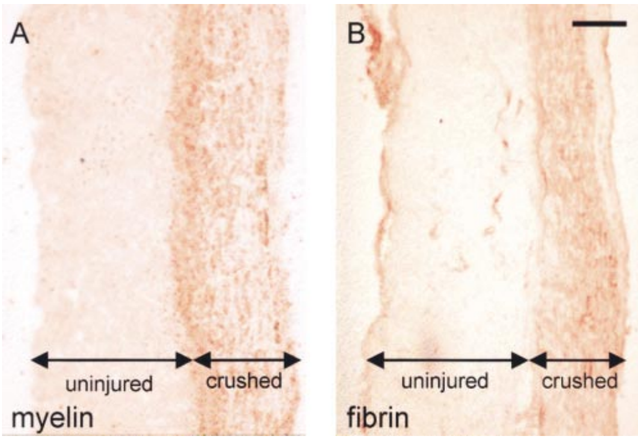

Figure 5. Fibrin(ogen) deposition increases after sciatic nerve injury and correlates with axonal degeneration and demyelination. Staining of parallel sections of a partially crushed wild-type sciatic nerve 8 d after injury with Oil Red O (A), a myelin stain, and antifibrinogen antibody (B) revealed that the crushed part of the nerve, which underwent axonal degeneration (A, Oil Red O stained aggregates represent myelin debris accumulation), also had extensive deposition of fibrin(ogen) (B), whereas the immediately adjacent, uninjured region was free of fibrin(ogen). Double-headed arrows indicate uninjured and crushed regions. Bar, 165 μm.

damage (Funakoshi et al., 1998). Therefore, a direct measure of functional impairment can be obtained by assessing muscle weight (Fig. 7). Muscle mass of wild-type mice dropped 24.5 ± 2.7% 8 d after the sciatic nerve crush compared with the unlesioned, contralateral side. Exacerbation of muscle mass atrophy in the tPA$^{-/-}$ mice 8 d after crush (40.1 ± 3.8%; $P < 0.005$ compared with wild-type mice) indicated increased axonal damage in the absence of tPA. 22 d after crush, muscle atrophy was 81% for a tPA$^{-/-}$ mouse, whereas for a wild-type mouse it was 66%. This preliminary evidence suggests that increased muscle atrophy persists at later time points after injury. After treatment with ancrod, there was less muscle atrophy in tPA$^{-/-}$ mice (16.6 ± 3.4%). The decrease in muscle mass atrophy in the ancrod-treated tPA$^{-/-}$ mice compared with the untreated tPA$^{-/-}$ was statistically significant ($P < 0.001$). This observation agrees with the histopathological observation that depletion of fibrin(ogen) protects tPA$^{-/-}$ mice from exacerbated axonal damage. Overall, these results suggest that tPA-mediated fibrinolysis protects motor axons after nerve injury.

Discussion

The studies reported here demonstrate that upregulation of tPA and plasmin is beneficial in reducing axonal damage; fibrin(ogen) deposition correlates with axonal degeneration; and depletion of fibrin(ogen) rescues mice from exacerbated axonal damage. These results indicate that plasmin-mediated removal of fibrin(ogen) deposits is critical for limiting axonal degeneration and demyelination.

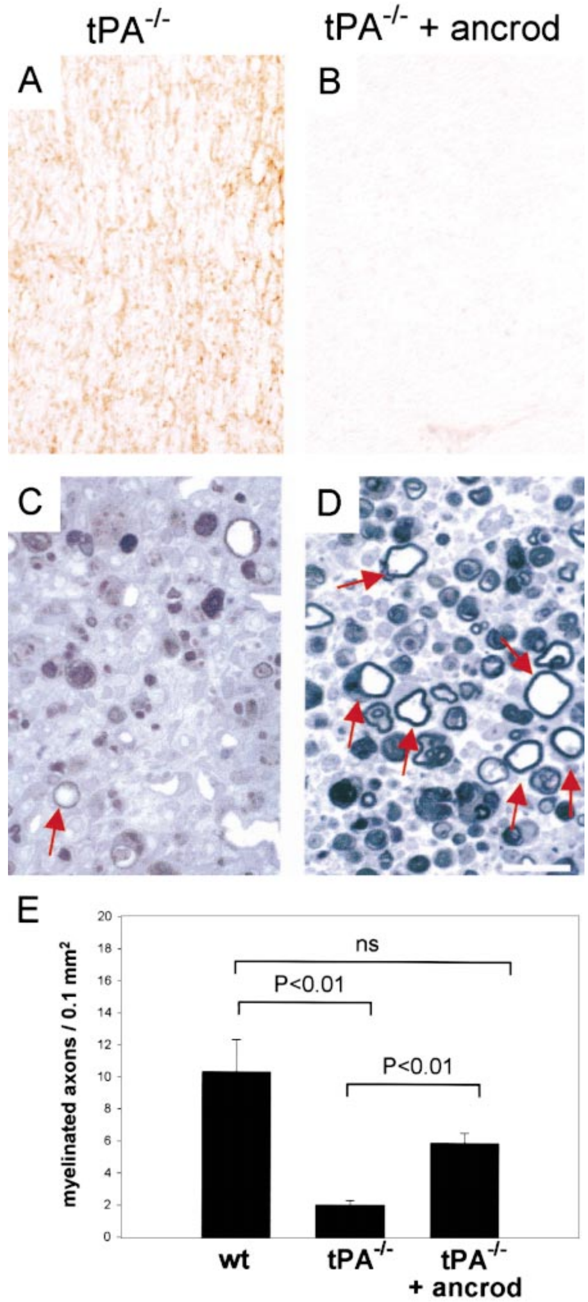

Figure 6. Pharmacological depletion of fibrin(ogen) reduces axonal damage in tPA$^{-/-}$ mice. Immunostaining for fibrin(ogen) revealed deposition of fibrin(ogen) in tPA$^{-/-}$ (A) mice, whereas ancrod-treated tPA$^{-/-}$ (B) mice showed little fibrin(ogen) immunoreactivity. Toluidine blue staining of semi-thin cross-sections of crushed sciatic nerve (8 d after injury) of tPA$^{-/-}$ mice treated with ancrod showed an increase of myelinated axons (D) compared with buffer-treated tPA$^{-/-}$ mice (C). (E) Quantification of myelinated axons 8 d after sciatic nerve crush. tPA$^{-/-}$ mice treated with ancrod ($n = 4$) showed significantly more myelinated axons than buffer-treated tPA$^{-/-}$ mice ($n = 6$, $P < 0.01$). The difference between tPA$^{-/-}$ mice treated with ancrod and wild-type controls ($n = 5$) was not statistically significant (ns). Data are expressed as means ± SEM. Statistical comparisons between medians were made with the t test. Bar: (A and B) 113 μm; (C and D) 22 μm.

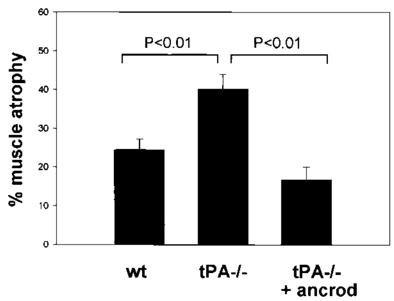

Figure 7. Depletion of fibrin(ogen) reduces muscle atrophy. Muscle atrophy 8 d after sciatic nerve crush was increased in the absence of tPA and decreased after fibrinogen depletion. Muscle mass in wild-type mice (n = 7) dropped 24.5 ± 2.7% compared with the unlesioned, contralateral side, whereas in tPA$^{-/-}$ mice (n = 7), muscle mass dropped 40.1 ± 3.8% ($P < 0.005$ compared with wild-type mice). After depletion of fibrinogen, muscle mass in tPA$^{-/-}$ mice treated with ancrod (n = 5) muscle mass dropped 16.6 ± 3.4% ($P < 0.001$ compared with tPA$^{-/-}$ mice). Data are expressed as means ± SEM. Statistical comparisons between medians were made with the t test.

Plasmin Can Be Beneficial or Pathogenic for Neuronal Degeneration Depending on Its Substrate

tPA$^{-/-}$ (Tsirka et al., 1995) and plg$^{-/-}$ (Tsirka et al., 1997b) mice are resistant to excitotoxin-induced neuronal death in the hippocampus, indicating that a tPA/plasmin proteolytic cascade promotes neuronal degeneration under these conditions. In this model of neuronal death, fib deficiency does not affect the neurodegeneration-resistant phenotype of the plg$^{-/-}$ mice (Tsirka et al., 1997a), demonstrating that fibrin is not the primary substrate for plasmin in the hippocampus. Further experiments indicated that a major plasmin substrate in the hippocampus is laminin (Chen and Strickland, 1997). In contrast, after sciatic nerve crush, exacerbation of axonal degeneration and demyelination observed in tPA$^{-/-}$ and plg$^{-/-}$ mice is rescued in the absence of fibrinogen, showing that fibrin(ogen) is the primary plasmin substrate during axonal degeneration and demyelination under inflammatory conditions.

A difference between excitotoxic neuronal death and inflammatory axonal degeneration is the participation of serum proteins in lesion formation. After excitotoxin injection, blood-brain barrier breakdown does not appear to contribute to excitotoxic neuronal damage (Chen et al., 1999). In contrast, under inflammatory conditions, disruption of the blood–brain barrier and fibrin deposition are early expressions of immune effector activity and precede clinical signs and axonal degeneration (Bush et al., 1993). Neuronal death in the PNS is also often associated with enhanced vascular permeability of the blood–nerve barrier (Koh et al., 1993). A preliminary examination of blood–nerve barrier disruption by IgG immunostaining revealed no difference between wild-type and tPA$^{-/-}$ mice (data not shown).

The present findings, coupled with previous results (Wang et al., 1998; Nagai et al., 1999; Kilic et al., 1999), reveal that a specific proteolytic system (e.g., tPA/plasmin) can be either deleterious or beneficial after different challenges to the nervous system. The significant distinction as to whether plasmin-mediated proteolysis is beneficial or a liability may be the component(s) of the ECM targeted. More specifically, plasmin degradation of fibrin may be advantageous, whereas excessive plasmin-mediated degradation of other matrix components may be associated with pathologic manifestations.

Fibrin Deposition as a Contributor to Axonal Degeneration

How does fibrin(ogen) deposition contribute to axonal damage? Fibrin(ogen) is necessary for various inflammatory responses in vivo (McRitchie et al., 1991; Tang and Eaton, 1993), and deficiency in fibrinolysis can contribute to pathogenic processes (Idell et al., 1989; Bugge et al., 1996; Kitching et al., 1997; Busso et al., 1998; Drew et al., 1998). Fibrin deposition also has been reported in multiple sclerosis, where it has been shown to correlate with demyelinated plaques (Claudio et al., 1995).

Fibrin is deposited after injury in both wild-type and tPA$^{-/-}$ mice. Fibrin depletion improves the degeneration phenotype of tPA$^{-/-}$ or plasminogen$^{-/-}$ mice, but not wild-type mice. Further experiments may reveal subtle quantitative or spatial aspects of fibrin deposition that can explain why tPA$^{-/-}$ mice have increased damage.

Persistent fibrin(ogen) deposition might affect axonal degeneration by influencing inflammation, with consequent aggravation of the injury. Local matrices may drive the inflammatory response by providing a temporary scaffold for inflammatory cell adhesion and migration and by increasing chemotaxis at sites of inflammation (Tang and Eaton, 1993). However, analysis of the inflammatory profile of the sciatic nerve lesions did not reveal any major differences in the macrophage or T cell number between wild-type, tPA$^{-/-}$, and systematically defibrinogenated wild-type mice 8 d after injury (Akassoglou, K., and S. Strickland, unpublished observations).

The plasmin/fibrin axis could also contribute to inflammation by upregulation of proinflammatory cytokines. Cytokines regulate the balance between proteases and their inhibitors, and proteolysis can modulate cytokine activity (Cuzner and Opdenakker, 1999). Interestingly, fibrin upregulates interleukin 1 (Perez and Roman, 1995) and tumor necrosis factor (Formica et al., 1994), two pro-inflammatory cytokines which are involved in inflammatory axonal damage and demyelination in both the peripheral (Creange et al., 1997) and central nervous system (Akassoglou et al., 1998; Wiemann et al., 1998). A more detailed analysis of the inflammatory response and cytokine expression after injury may reveal an immunoregulatory role for fibrin(ogen) in axonal damage and demyelination.

Our results suggest that tPA/plasmin-mediated fibrinolysis reduces axonal damage and, therefore, components of this pathway might be used as a new approach to protect axons from injury. In situations of chronic demyelination, a large percentage of the damage might occur via a fibrin-mediated pathway. In this case, depletion of fibrin(ogen) or prevention of fibrin(ogen) deposition might protect against demyelination. This approach would be especially valuable if it could be accomplished locally at the site of inflammation. Thus, the identification of fibrin(ogen) deposition as a new contributing factor to axonal degeneration may yield additional strategies to prevent damage in the nervous system.

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