Disruption of tissue plasminogen activator gene reduces macrophage migration

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Abstract

Tissue plasminogen activator (tPA) is an essential component of the proteolytic cascade that lyses blood clots. Various studies also suggest that tPA plays important roles in peripheral nerve regeneration. Here we show that disruption of tPA gene reduces macrophage migration after sciatic nerve injury in mice. Moreover, lack of tPA activity attenuates migrating ability of macrophages and affects MMP-9 expression and activity in macrophages in vitro. Addition of ethylenediaminetetraacetic acid (EDTA), which inhibits MMPs, abolished the differences of migration ability of macrophages between tPA$^{+/+}$ and tPA$^{-/-}$ mice. Axonal regeneration is correlated with the increase of macrophage migration, suggesting that tPA may help create a beneficial environment for axonal regeneration through promoting macrophage infiltration. This study shows that tPA may play a role in nerve regeneration through regulating the migration ability of macrophages. This function of tPA may depend on, at least in part, upregulating MMP-9 expression and activity in macrophages.
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Tissue plasminogen activator (tPA), a component of the fibrinolytic system, is a serine protease that converts the zymogen plasminogen (Plg) into the active protease plasmin [1]. tPA is predominantly found in the blood, but is also present and plays various roles in nerve systems [2,3]. In peripheral nerve system, tPA is rapidly induced in sensory neurons and Schwann cells after nerve injury [4,5]. Mice lacking the tPA or Plg gene showed increased axonal degeneration and demyelination and delayed functional recovery after sciatic nerve injury [4,6]. These studies suggest that the tPA/Plg system may play an important role in peripheral nerve degeneration and regeneration after injury. Although tPA deficiency delays nerve recovery after injury, the mechanism of its action in this process is not clear.

After axonal injury, circulating blood monocytes rapidly invade into the site of injury and differentiate into activated macrophages [7]. The importance of macrophages in Wallerian degeneration and nerve regeneration is well documented. During Wallerian degeneration, macrophages play a key role in myelin debris removal in the later phases of repair. In addition to removing degenerated axonal and myelin debris [8], they also clear extracellular matrix (ECM) and induce Schwann cells to proliferate by secreting IL-1 [9]. They also produce neurotrophic factors such as nerve growth factor (NGF) [10] and physically recraft the undamaged ECM of the distal segment. Although macrophages can express tPA [11], which is also correlated with cell migration [12,13], it is not known how tPA affects macrophage migration after nerve injury.

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In the present work, we demonstrate that tPA deficiency decreases the migration ability of macrophages in vivo and in vitro. We also show that lacking tPA gene inhibits the expression of MMP-9 in macrophages. Inhibiting MMPs activity by EDTA can abolish the difference of migration ability between tPA$^{+/+}$ and tPA$^{-/-}$ macrophages. These results indicate that the positive effect of tPA on macrophage migration may be through inducing MMP-9 expression in macrophages and support the potential utility of tPA in the modulation of macrophage inflammatory responses.

Materials and methods

Animals. Colonies of homozygous wild-type (tPA$^{+/+}$) and tPA knockout (tPA$^{-/-}$) C57BL/6J mice were housed with free access to food and water. Animals were treated humanely in accordance with NIH guidelines and the approved procedures of the Institutional Animal Use and Care Committee at Fudan University. Genotype was confirmed by using PCR amplification of genomic DNA from the tail.

Surgery. For sciatic nerve crush injury experiments, adult C57BL/6J male mice (8- to 10-week-old) were used. Mice were anesthetized deeply with pentobarbital (50 mg/kg) by intraperitoneal injection. All surgical protocols were approved by the Institutional Animal Care and Use Committee of Fudan University. For the crush injury, the left sciatic nerve was crushed at midthigh three times (20s each) with watchmaker's forceps. The crush site was marked with India ink. Skin incisions were closed with sutures. For sham controls, the sciatic nerve of the right hindlimb was surgically exposed, but no crush was made.

The animals were killed under deep anesthesia on 2, 8, and 14 days after injury. Immediately after respiratory arrest, the mouse's thoracic cavity was opened, and the body tissues were perfused with ice-cold PBS (40 ml) through a needle inserted into the left ventricle of the heart. A 5 mm piece of sciatic nerve including the crush site was prepared for next experiments. Each experimental group consisted of at least five animals.

Preparation of macrophages. Normal tPA$^{+/+}$ and tPA$^{-/-}$ mice were sacrificed. Five milliliters of ice-cold PBS containing heparin (10 U/ml) was injected into the peritoneal cavity and aspirated out. Peritoneal lavages were centrifuged at 800g for 10 min and the pellet was resuspended in RPMI1640-10% fetal calf serum (FCS). Cells were counted using hemocytometer and plated at density of 0.5×10^6 cells per well in 24-well plates. The non-adherent cells were washed out after 2 h with warm PBS, fresh medium was added, and the adherent population was characterized as macrophages after staining with F4/80 antibody. Cells produced by this procedure were >95% positive for macrophages.

Immunofluorescence. Dissected sciatic nerves were embedded in Tissue-Tek OCT (Tissue-Tek, Torrance, CA) and immediately frozen in dry ice. Eight micron longitudinal sections were cut on a cryostat and mounted onto slides.

Immunofluorescence staining was performed on cryostat sections. For immunofluorescence, the following primary antibodies were used: rabbit anti-matrix metalloproteinase-9 (MMP-9) (1:2000, Chemicon, Temecula, CA), rat anti-F4/80 (1:50, Serotec, Oxford, UK). The sections were blocked in 5% normal goat serum in PBS for 1 h at room temperature and then incubated with primary antibody in 3% normal goat serum in PBS overnight at 4 °C. After rinsing, the sections were incubated in appropriate fluorescent-conjugated secondary antibodies and examined under Olympus BX61 microscope. Images were acquired with a SPOT digital camera and figures were prepared with Adobe Photoshop 7.0 and Microsoft Power Point program. Sections incubated without the primary antibody served as negative controls (data not shown).

Western blot analysis. Total protein was isolated from peritoneal macrophages and sciatic nerve, respectively. Protein concentration was determined by using a BCA protein kit (Pierce, Rockford, IL). Protein abundance was calculated by multiplying the volume of each sample by its concentration.

Aliquots of 20 µg were separated by 10% polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dried milk in PBS at room temperature for 1 h. Membranes were incubated with primary antibody for MMP-9 followed by incubation with anti-rabbit IgG-HRP. To ensure equal loading of protein, membranes were incubated with anti-β-actin antibody (1:1000; Sigma, St. Louis, MO). The protein abundance in each lane was normalized to the β-actin abundance. Detection of HRP was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Gelatin zymography. Gelatin zymography was adapted from the procedure described by Muir [14]. Total protein was isolated from peritoneal macrophages. Aliquots of 20 µg were separated by 10% polyacrylamide gel containing gelatin (2 mg/ml, Sigma) at 4 °C. After electrophoresis, the SDS was extracted from the gel by washing with 2.5% Triton X-100 for 30 min. The gel was incubated for 24 h at 37 °C in 100 ml of 1× LSCB buffer (0.05 M Tris, pH 7.6, 0.2 M NaCl, 5×10^{-3} M CaCl$_2$, and 0.02% Brij-35). Staining with 0.125% Coomassie blue in 50% MeOH/10% acetic acid, followed by destaining with the same solvent, revealed transparent zones of lysis against the dark background at M_r 92 kDa, corresponding to MMP-9.

Oil red O staining. Saturated oil red O (Sigma, St. Louis, MO) was diluted to 0.7% and filtered immediately before use. Sections for oil red O staining were fixed in 60% isopropanol and stained with oil red O in a closed container for 10–15 min. The background was cleared by rinsing in 60% isopropanol, and then the samples were washed in water. Sections were examined under Olympus BX61 microscope.

Quantification of axonal regeneration, MMP-9 and F4/80 positive cells. For regeneration analysis after sciatic nerve crush, the nerves were collected 2 mm distal to the crush site. The samples were fixed in 3% glutaraldehyde in phosphate buffer for 24 h, postfixed in 2% osmium tetroxide solution, and embedded in resin. Consecutive semi-thin sections were cut on an Ultra-cut microtome and stained with toluidine blue. A morphometric grid (0.1 mm^2) was adapted to the microscope, axons with clear myelination were counted in at least three randomly selected grids per sample.

For MMP-9 or F4/80 positive cell quantifications, immunostained longitudinal nerve sections were used. The center of the crush site was adapted to the microscope and positive cells were counted in 0.2 mm^2 area. Five animals in each group were analyzed.

Matrigel invasion analysis. Macrophage invasion was evaluated by using a nitrocellulose filter of 8 µm pore size (BD, Bioscience, San Jose, CA) coated with 100 µl Matrix-gel (Sigma, St. Louis, MO). RPMI-1640 (100 µl) containing 5×10^6 cells/ml was added to the upper compartment of the Boyden chamber. To induce invasion, 400 µl RPMI-1640 containing 10^{-8} M formyl-Met-Leu-Phe (FMLP) peptide (Sigma, St. Louis, MO) was put into the lower compartment. After 20 h of incubation at 37 °C containing 5% CO$_2$, membrane filter was removed and the cells were stained with crystal violet. Data were expressed as the average of five random fields.

RNA isolation and quantitative real time PCR. Total RNA was isolated from normal peritoneal macrophages, using Trizol reagent (TaKaRa, Japan), according to the manufacturer's instructions. Two micrograms of RNA was reverse-transcript using TaKaRa RT kit and the cDNAs were analyzed by 7300 real time PCR system (Applied Biosystems, Foster City, CA), participated with primers for β-actin, MMP-9.

Statistical analysis. All data, expressed as means ± SEM, were analyzed with the Student's t test. Differences were considered statistically significant at $P < 0.05$.

Results

tPA deficiency inhibits macrophage migration after nerve injury

Mammalian peripheral nerve injury is always accompanied by Wallerian degeneration that is marked by

the invasion of a large number of macrophages [15]. In order to analyze the effect of tPA deficiency on macrophage migration after sciatic nerve injury, we performed immunofluorescent staining with F4/80 antibody to detect the change of macrophage number in nerve (Fig. 1). Staining with an anti-F4/80 antibody revealed few macrophage infiltration in unoperated nerve of tPA$^{+/+}$ and tPA$^{-/-}$ mice. In tPA$^{+/+}$ mice, there was a significant increase in migrated macrophages by two days after crush. However, there were still few migrated macrophages in tPA$^{-/-}$ mice at this time point. Compared with wild-type controls, tPA$^{-/-}$ mice still showed a significant reduction in macrophage infiltration on 8 days after injury. Although macrophage influx in crushed nerve on 14 days was much less than 8 days after injury, tPA$^{-/-}$ mice also showed significantly less F4/80 staining in comparison with tPA$^{+/+}$ mice. These findings suggest that tPA may be involved in recruitment of inflammatory macrophages to the sciatic nerve after injury.

Fig. 2. tPA deficiency decreases MMP-9 protein level after nerve injury. MMP-9 protein level was analyzed by Western blots. Loss of tPA gene lowered MMP-9 protein expression after nerve injury.

Double immunofluorescence staining with MMP-9 showed that most of migrated macrophages in sciatic nerve expressed MMP-9 on 8 days and 14 days after injury. On two days after injury, however, MMP-9 is not mainly expressed by the macrophages migrated into crushed nerve. To further investigate the protein level of MMP-9 in nerve, we performed Western blots. As shown in Fig. 2, MMP-9 protein levels were lower in tPA$^{-/-}$ mice compared with tPA$^{+/+}$ mice, consistent with the result of immunofluorescence staining.

Exogenous tPA promotes macrophage migration

In order to determine the effect of exogenous tPA on macrophage migration after sciatic nerve injury, tPA$^{+/+}$ mice were used. Both sides of sciatic nerve were crushed. The left sciatic nerve was wrapped with a piece of gelfoam (Nanjing Jinling Pharmaceutical Co., Ltd) soaked with tPA (100 μg/ml) in PBS solution on seven days after crush. The right sciatic nerve was wrapped with a piece of gelfoam soaked in PBS as a control. Seven days after implantation of the gelfoam, the mice were sacrificed and the nerve sections were stained with F4/80 antibody. The result is shown in Fig. 3. After treatment with tPA, the number of macrophages ($39.25 \pm 9.18/0.2$ mm^2) as revealed by F4/80 staining was significantly increased compared to the one of PBS control ($19.17 \pm 4.54/0.2$ mm^2). This finding reveals that exogenous tPA promotes macrophage migration after nerve injury.

The effect of tPA deficiency on circulating levels of peripheral blood leucocytes

After nerve injury, circulating blood monocytes rapidly invade into the site of injury and differentiate into activated macrophages. Therefore, the circulating levels of peripheral blood leucocytes may be involved in the number of macrophage migration at the site of injury. To determine whether tPA deficiency has an effect on circulating levels of peripheral blood leucocytes, we used Giemsa-staining to count the number of total leucocytes, monocytes, neutrophils, and lymphocytes (Fig. 4). The results showed that there was no significant difference on the number of these cells between tPA$^{+/+}$ and tPA$^{-/-}$ mice. This result suggests that tPA deficiency has no effect on circulating levels of peripheral blood leucocytes.
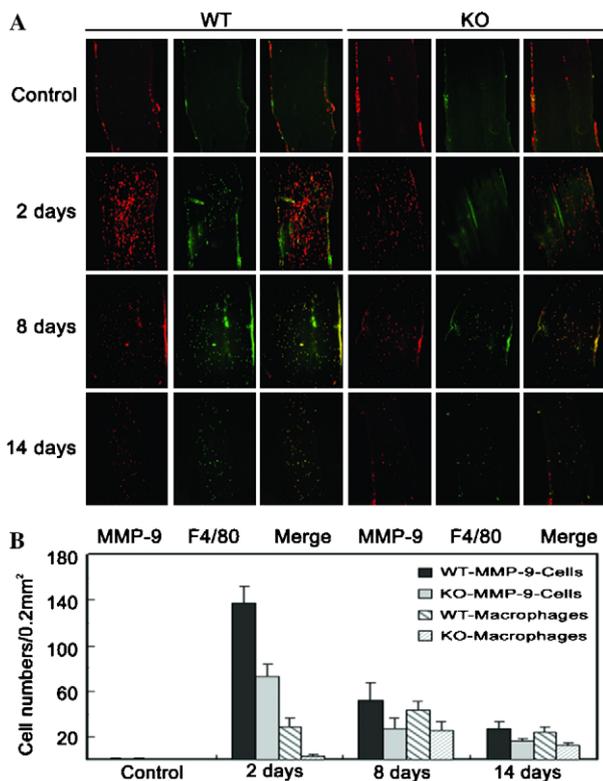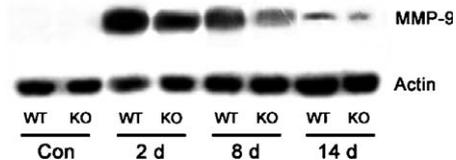

Fig. 1. tPA deficiency reduced macrophage invasion after sciatic nerve injury. Nerve sections were double immunostained for F4/80 (green) and MMP-9 (red) and the images were merged (A). Loss of tPA gene decreased macrophage number (F4/80+) in nerve on 2, 8, and 14 days after injury. Macrophages are the major cellular source of MMP-9 on 8 days and 14 days after injury. Macrophages or MMP-9 positive cell numbers were quantified in the sciatic nerves of tPA$^{+/+}$ and tPA$^{-/-}$ mice (B). tPA deficiency significantly decreased either macrophages and MMP-9 positive cell numbers at different time point after injury. WT, tPA$^{+/+}$ mice; KO, tPA$^{-/-}$ mice; scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
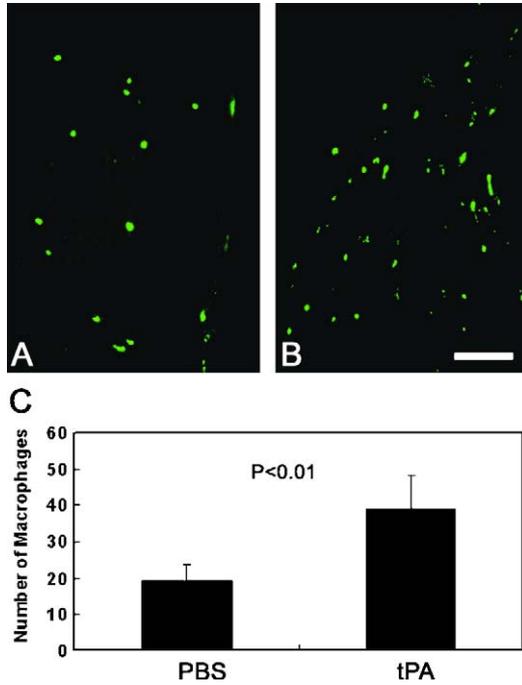
Fig. 3. Exogenous tPA promotes macrophage migration after nerve injury. Seven days after tPA treatment, nerve sections were stained with anti-F4/80 antibody. tPA treatment (B) increased macrophage number compared to PBS (A) treated nerves. (C) Quantification of macrophage number in nerves of different treated animals. tPA treatment significantly increases macrophage number ($P < 0.01$). Scale bars, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

tPA deficiency represses migration ability of peritoneal cavity macrophages

In addition to its proteolytic lysis of fibrin, tPA activity is correlated with neuronal migration [13]. PC 12 cells over-expressing tPA migrated faster than control cells [16]. These studies suggested that tPA plays a role in cell migration. To study the influence of tPA on macrophage motility, we analyzed the effect of tPA deficiency on the migration ability of macrophages in Boyden chamber assay. As shown in Fig. 5, tPA deficiency (B: 30.17 ± 13.83/fields) resulted in significantly reduced recruitment of macrophages when compared with wild-type control (A: 63.67 ± 13.18/fields). In addition, administration of EDTA (10 μM), an inhibitor of MMPs, into

Fig. 5. tPA deficiency represses peritoneal cavity macrophage migration. Macrophages migrated through Matrigel-coated membrane were stained with crystal violet. The migration was quantified by blind counting of the migrated cells on the lower surface of the membrane. The difference in migration levels between tPA$^{+/+}$ (A) and tPA$^{-/-}$ (B) macrophages was statistically significant ($P < 0.01$). Adding 10 μM EDTA to the upper compartment of the Boyden chamber can significantly reduce migrated macrophage number and remove the difference of migration levels between tPA$^{+/+}$ (C) and tPA$^{-/-}$ (D) macrophages ($P > 0.05$). The quantified result of the experiment is represented on the histogram (E). WT, tPA$^{+/+}$ macrophages; KO, tPA$^{-/-}$ macrophages; scale bars, 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
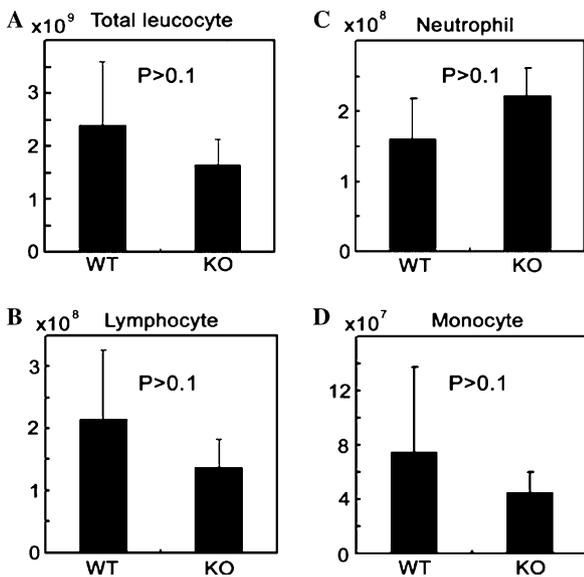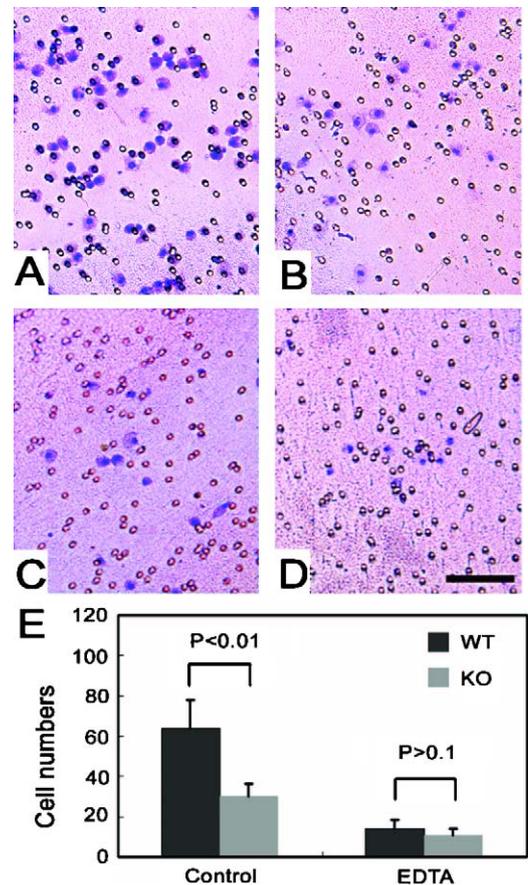

Fig. 4. The effect of tPA deficiency on circulating levels of leucocytes in peripheral blood. Peripheral blood sections were stained with Giemsa solution. Total leucocytes, monocytes, neutrophils, and lymphocytes were quantified in blood sections. There was no significant difference between tPA$^{+/+}$ and tPA$^{-/-}$ mice. WT, tPA$^{+/+}$ mice; KO, tPA$^{-/-}$ mice.

upper well of Boyden chamber significantly inhibited macrophage migration. And there was no significant difference between tPA-positive (C, 13.89 ± 4.4/fields) and tPA-negative macrophages (D, 6.46 ± 3.91/fields) after treated with EDTA. This finding reveals that tPA may affect macrophage migration through MMPs activity.

tPA deficiency downregulates MMP-9 mRNA level in macrophage

After sciatic nerve crush, mice lacking tPA gene showed attenuated MMP-9 activity [17]. In human cerebral microvascular endothelial cells, MMP-9 was upregulated when recombinant tPA was added [18]. These findings indicated that tPA plays a role in MMP-9 expression and activation. To examine whether tPA has an effect on MMP-9 expression and activation in macrophages, we isolated mature macrophages from peritoneal cavity. Assessed by Western blot (Fig. 6A), MMP-9 protein levels in macrophages from tPA$^{+/+}$ mice were significantly higher than that from tPA$^{-/-}$ mice. Gelatin zymography showed that MMP-9 activity of tPA$^{+/+}$ macrophages was also significantly higher than tPA$^{-/-}$ macrophages (Fig. 6B). Moreover, there was a significantly higher MMP-9 mRNA level in tPA$^{+/+}$ macrophages compared with tPA$^{-/-}$ macrophages. The result of quantitative real time PCR reveals that tPA has an effect on the expression of MMP-9 mRNA (Fig. 7).

tPA deficiency delays axonal regeneration and myelin debris clearance

Removing the deposited myelin debris is the main function of macrophages migrated into the crushed nerve. It can help create a beneficial environment for axonal regeneration. Toluidine blue staining showed a dramatic decrease in myelinated axons in tPA$^{-/-}$ mice compared
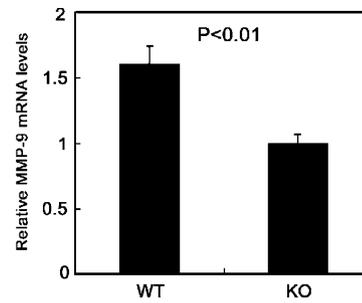

Fig. 7. tPA deficiency decreases MMP-9 mRNA level in macrophages. Quantitative real time PCR analysis demonstrated that tPA deficiency decreased MMP-9 mRNA level in peritoneal cavity macrophages. Real time PCR amplification of housekeeping gene β-actin was performed in an identical manner to serve as control.

with tPA$^{+/+}$ mice (Fig. 8A and B). Similarly, oil red O staining exhibited there was an increase in myelin debris deposition at the injury site of sciatic nerve of tPA$^{-/-}$ mice
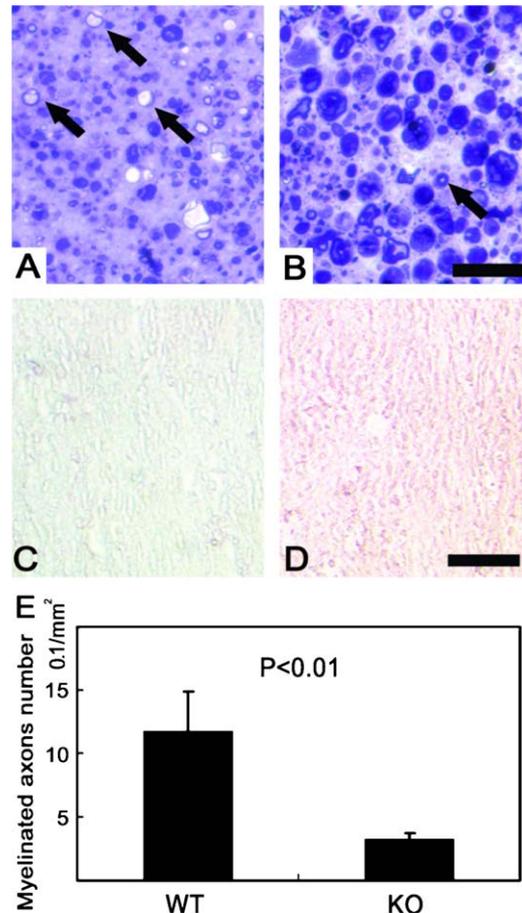

Fig. 8. tPA deficiency delays axonal regeneration and myelin debris clearance after sciatic nerve crush. Toluidine blue staining for myelin in semi-thin cross-sections of crushed nerves after 8 days showed a decrease in myelinated axons (arrows) in tPA$^{-/-}$ mice (B), when compared with tPA$^{+/+}$ mice (A) Scale bars (B) 20 μm. Oil red O staining showed an increased accumulation of myelin and lipid debris in tPA$^{-/-}$ mice (D) compared with tPA$^{+/+}$ mice (C) Scale bars (D) 50 μm. Quantification of myelinated axons 8 days after crush (E) showed significantly fewer myelinated axons in tPA$^{-/-}$ nerves compared with controls ($P < 0.01$). WT, tPA$^{+/+}$ mice; KO, tPA$^{-/-}$ mice.
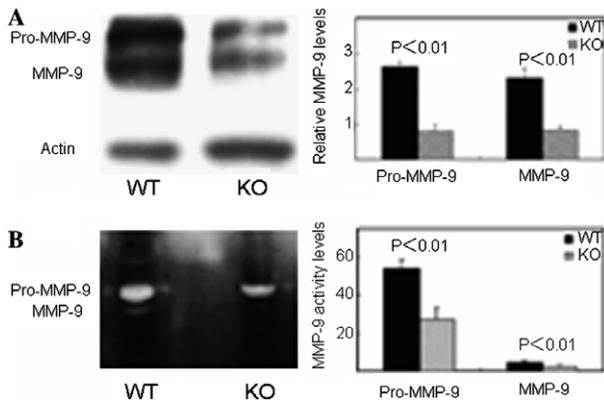

Fig. 6. tPA deficiency reduces MMP-9 protein and activity levels in macrophages. Western blot (A) and Zymographic analysis (B) showed an induction of MMP-9 secretion and activation by tPA. Although tPA$^{-/-}$ mice also showed MMP-9 expression in macrophages, the protein and activity levels remained below those observed in tPA$^{+/+}$ mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

compared to that of tPA$^{+/+}$ mice (Fig. 8C and D). To quantitate the effect of tPA on axonal regeneration, we counted the myelinated axons in semi-thin sections. This quantification showed that the number of myelinated axons in tPA$^{+/+}$ mice ($11.8 \pm 3.1/0.1 \text{ mm}^2$) was more than that of tPA$^{-/-}$ mice ($3.2 \pm 0.5/0.1 \text{ mm}^2$) (Fig. 8E). These results indicate that tPA promotes axonal regeneration and myelin debris clearance after sciatic nerve injury.

Discussion

It has been reported that plasminogen activator (PA) system is involved in periphery nerve injury and recovery [5,6]. There are two types of PAs, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Previous studies showed that tPA is the major PA induced after sciatic nerve injury [4]. tPA deficient mice show exacerbated nerve degeneration and impaired remyelination after nerve injury [4,6]. In this study, the effect of tPA on macrophage migration after sciatic nerve injury was investigated. Our data showed that tPA deficiency could attenuate migration ability of macrophages after injury. Moreover, exogenous tPA could facilitate macrophages invading into the injured site after sciatic nerve crush. These results indicate that tPA may enhance macrophage migration after nerve injury.

Infiltrated macrophages form an important part of cellular response to peripheral nerve injury and play a key role in myelin debris removal after nerve injury. Macrophages also produce an array of growth factors and cytokines after nerve injury. In peripheral nerves, regeneration does not occur without an influx of inflammatory macrophages [19]. In the present study, oil red O staining revealed that myelin debris deposition was increased in tPA$^{-/-}$ mice when compared to tPA$^{+/+}$ mice. In addition, myelinated axons were also decreased in tPA$^{-/-}$ mice when compared to tPA$^{+/+}$ mice. These data reveal that tPA may enhance myelin debris clearance and axonal regeneration depending on promoting macrophage recruitment into the injured peripheral nerve.

Earlier studies [13] showed that blocking tPA activity could inhibit mouse granule neuron migration, suggesting that tPA is required for its effect on granule cell migration. Otherwise, PC12 cells overexpressing tPA regenerate neuritis in complex extracellular matrix more extensively than control cells [16]. The potential mechanism responsible for the improved cell migration is that tPA cleaves plasminogen into plasmin, which in turn degrades components in the ECM to enable cell to invade to a greater extent. In support of this hypothesis, plasmin was shown to degrade laminin soon after excitotoxic injury [20]. However, some studies also note that tPA may play a signaling role in migration by acting on a cell-surface receptor independent of its catalytic ability [21,22].

MMP-9, which is a member of MMPs, a family of Zn^{2+}-dependent extracellular proteases, is also implicated in peripheral nerve regeneration [9,14]. It is believed to be a physiologically relevant mediator of degradation of ECM components such as type IV collagen and gelatin [23,24] and promote nerve regeneration by creating a permissive environment for axonal elongation. Our data showed that MMP-9 was mainly expressed by macrophages on 8 and 14 days after nerve injury. However on two days after nerve injury, MMP-9 was not mainly expressed by macrophages. We hypothesize that MMP-9 might be mainly expressed by neutrophils migrated into crushed nerve on two days after nerve injury. Otherwise, MMP-9 is also considered to be an important component in the progression of macrophage inflammatory response. Liver X receptors can impact macrophage inflammatory responses by impressing MMP-9 expression [25]. MMP-9 also promotes neutrophil migration in pancreatitis-associated lung injury [26]. Plasmin, activated by tPA, can activate MMP-9 [27]. Therefore, a deficiency of tPA can affect the activities of MMP-9 after peripheral nerve injury [17]. Recent studies have documented that tPA was involved in regulating MMP-9 protein expression and this response was mediated by the low density lipoprotein receptor related protein (LRP), which avidly binds tPA and possesses signaling properties [18,28]. Our results further showed that tPA participated in upregulating MMP-9 mRNA, protein ,and activity levels in peritoneal cavity macrophages. This finding suggests that the regulation of MMP-9 expression in macrophages may be a mechanism that tPA can impact macrophage inflammatory responses.

Taken together, tPA not only degrades fibrin and other extracellular matrix proteins, but also increases macrophage recruitment to scavenge myelin debris. tPA mediated macrophage migration can promote axonal regeneration and this pathway might be used as a new approach to accelerate nerve regeneration after injury. This approach would be especially valuable if it could be accomplished locally at the site of injury.

Acknowledgments

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