Endogenous Tissue Plasminogen Activator Mediates Bone Marrow Stromal Cell-Induced Neurite Remodeling After Stroke in Mice

Li Hong Shen, MD, PhD*; Hongqi Xin, PhD*; Yi Li, MD; Rui Lan Zhang, MD; Yisheng Cui, MD; Li Zhang, MD; Mei Lu, PhD; Zheng Gang Zhang, MD; Michael Chopp, PhD

Background and Purpose—Bone marrow stromal cells (BMSC) decrease neurological deficits in rodents after stroke and concomitantly induce extensive neurite remodeling in the brain, which highly correlates with the improvement of neurological function. We investigated the effects of endogenous tissue plasminogen activator (tPA) on neurite remodeling after BMSC treatment.

Methods—Adult C57BL/6 wild-type (WT) mice and tPA knockout (tPA−/−) mice were subjected to middle cerebral artery occlusion, followed by an injection of 1 × 10^6 BMSC (n = 18) or phosphate-buffered saline (n = 18) into the tail vein 24 hours later. Behavioral tests were performed at 3, 7, and 14 days after middle cerebral artery occlusion. Animals were euthanized at 14 days after stroke.

Results—The effects of BMSC on functional recovery depended on presence or absence of tPA, even after adjusting for imbalanced stroke severity. BMSC significantly improve functional recovery in WT mice compared to WT controls but show no beneficial effect in the tPA−/− mice compared to tPA−/− controls. Axonal density and synaptophysin-positive areas along the ischemic boundary zone of the cortex and striatum in WT mice are significantly higher than in the tPA−/− mice. BMSC treatment significantly increases tPA protein level and activity only in WT mice.

Conclusions—Our results suggest that endogenous tPA promotes BMSC-induced neurite outgrowth and may contribute to functional recovery after stroke. (Stroke. 2011;42:459-464.)

Key Words: bone marrow stromal cells ■ functional recovery ■ neurite remodeling ■ stroke ■ tissue plasminogen activator

Tissue plasminogen activator (tPA), a member of the fibrinolytic system, is a serine protease that converts the zymogen plasminogen into the active protease plasmin1 and thus cleaves fibrin and dissolves newly formed clots.2 In addition to its role in the circulation, tPA is expressed in the parenchyma of the rodent central nervous system, where it shapes the function of both neurons and glia.3–5 TPA also participates in tissue remodeling through enhancement of cell migration and differentiation, facilitates axonal growth and path-finding,6 and enhances the late phase of long-term potentiation.7,8 Moreover, various studies have also documented the involvement of tPA in peripheral nerve regeneration9,10: tPA activity is localized to neuron growth cones in culture11; tPA mRNA and enzymatic activities are induced in murine embryonic dorsal root ganglia during the period of axonal outgrowth toward their peripheral targets12 and after peripheral nerve injury13; mice lacking tPA show delayed functional recovery after sciatic nerve crush.14 These experimental findings highlight a role for tPA during neural network reconstitution and synaptic plasticity in the nervous system.

Bone marrow stromal cells (BMSC) are a heterogeneous subpopulation of bone marrow cells that include mesenchymal stem and progenitor cells. BMSC transplantation decreases the thickness of glial scar wall,14,15 accelerates axonal sprouting and regeneration,16 and enhances intercortical and intracortical axonal connections,17 which are directly correlated to the recovery of neurological function.18 How BMSC exert these effects, however, is still an open question. Recent in vitro and in vivo data support a critical role of tPA in facilitating neurite outgrowth.5 BMSC modulate endogenous tPA level and activity in the ischemic boundary zone (IBZ).5 Collectively, these experimental data suggest that endogenous tPA is a mediator of BMSC-induced neurite outgrowth. In the present study, C57Bl6 wild-type (WT) and tPA knock-out (tPA−/−) mice were subjected to focal brain ischemia,
BMSC were transplanted, and neurological function and neurite status were evaluated to dissect the role of tPA as well as the interaction between endogenous tPA and BMSC in neurite remodeling after stroke in mice.

Materials and Methods

Animal Middle Cerebral Artery Occlusion Model and Cell Transplantation

Adult male WT C57BL/6J mice (n = 18, Charles River, Wilmington, MA) and tPA−/− mice with C57BL/6J background (n = 18, Jackson Laboratory, Bar Harbor, ME) weighing 22 to 25 grams were used in this study. All experiments were conducted in accordance with the standards of the Institutional Animal Care and Use Committee of Henry Ford Hospital. Mice were subjected to permanent monofila-

ment middle cerebral artery occlusion (MCAO). At 24 hours after ischemia, randomly selected mice received BMSC (derived from C57BL/6J mice) or vehicle administration. Approximately 1 × 10⁶ BMSC in 0.5 mL phosphate-buffered saline or phosphate-buffered saline alone was slowly injected via the tail vein over a 5-minute period into each mouse. Immunosuppressant was not used in any animal in this study. Animal mortality for the WT and tPA−/− animals was similar, ~40%. All mice were euthanized at 14 days after MCAO, among which 12 were used for protein extraction (n = 3/group), and the remaining 24 brains (n = 6/group) were embed-

ded in paraffin.

Behavioral Tests

A modified neurological severity score (mNSS) and Foot-fault tests were performed by a blinded investigator before MCAO and at 1, 3, 7, and 14 days after MCAO, as previously described.19

Histological and Immunohistochemical Assessment

Mouse brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and then brain blocks were embedded in paraffin. One coronal paraffin slide (6-μm-thick) from each of the 7 brain blocks (1-mm-thick) of each mouse was stained with hematoxylin and eosin. The 7 coronal brain sections were traced using a Global Laboratory Image analysis system (Data Translation) for lesion volume evaluation. The infarct volume is presented as a percentage of total contralateral hemisphere volume. A standard paraffin-embedded block (within the center of the lesion of MCAO) corresponding to coronal coordinates bregma 0 to 1.0 mm was obtained, from which a series of 6-μm-thick sections were analyzed using light and fluorescent microscopy (Olympus BH-2). After deparaffinizing, brain sections were pro-

cessed for either Bielschowsky silver–Luxol fast blue staining or standard immunostaining. Primary antibodies against tPA (polyclonal antibody, 1:50; Santa Cruz Biotech) or synaptophysin (monoclonal antibody, 1:1000; Chemicon) were used.

Direct Casein Zymography Measuring tPA Activity in the Brain

Brain tissues from mice (n = 3/group) along the IBZ ipsilateral to the injury were extracted and homogenized. Protein was isolated with Trizol (Invitrogen) following a standard protocol. tPA activity was measured as previously described.5

Quantification

Immunoreactive signals were analyzed with National Institutes of Health Image software (Image J) based on evaluation of an average of 3 histology slides (6-μm-thick, 54-μm interval, every 10 slides) from the standard block of each animal. For measurements of synaptophysin and tPA immunostaining areas, 8 fields of view were digitized under a ×40 objective and the MCID computer imaging analysis system. The positive area was measured in each field of view. For measurements of axon density, 5 fields of view along the IBZ within the ipsilateral striatum were digitized and evaluated.

Statistical Analysis

The behavioral scores were evaluated for normality and data transformation was considered if data were not normal. As a result, ranked data were used for the analysis because modified neurological severity score data were not normal. Two-way ANOVA with factors of BMSC and tPA was used to test factor effects on stroke severity at baseline and functional recovery after treatment. The baseline functional score was included in the analysis if there was imbalanced stroke severity among groups. Analysis using PROC MIXED in SAS23 started testing for BMSC and tPA interaction, followed by a subgroup analysis, if the P value of the F-test with degree of freedom (1, (N1−1)*(N2−1)) was significant at the level of 0.05, where N1 and N2 are numbers of mice in WT and tPA−/− groups, respectively. The subgroup analysis was conducted through CONSTRCT statement under PROC MIXED with F-test with degree of freedom (1, N1/(N2−1)). All histological and immunohistochemical measurements were analyzed using standard parametric testing procedures.

Table. Bone Marrow Stromal Cell Effects in Wild-Type and Tissue Plasminogen Activator Knockout Mice*

<table>
<thead>
<tr>
<th></th>
<th>WT PBS</th>
<th>WT BMSC</th>
<th>tPA−/− PBS</th>
<th>tPA−/− BMSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranked mNSS at day 1 (baseline)†</td>
<td>25.4</td>
<td>13.39</td>
<td>25.3</td>
<td>12.37</td>
</tr>
<tr>
<td>Ranked mNSS at day 3‡</td>
<td>25.8</td>
<td>13.31</td>
<td>26.0</td>
<td>11.87</td>
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<tr>
<td>Ranked mNSS at day 7†</td>
<td>30.9</td>
<td>14.58</td>
<td>21.0§</td>
<td>10.65</td>
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<tr>
<td>Ranked mNSS at day 14‡</td>
<td>28.0</td>
<td>11.75</td>
<td>15.9§</td>
<td>6.08</td>
</tr>
<tr>
<td>% Foot-fault test at day 1 (baseline)</td>
<td>25.4</td>
<td>4.79</td>
<td>25.9</td>
<td>5.41</td>
</tr>
<tr>
<td>% Foot-fault test at day 3†</td>
<td>21.2</td>
<td>3.43</td>
<td>18.3§</td>
<td>3.38</td>
</tr>
<tr>
<td>% Foot-fault test at day 7†</td>
<td>18.0</td>
<td>4.08</td>
<td>12.0§</td>
<td>2.89</td>
</tr>
<tr>
<td>% Foot-fault test at day 14†</td>
<td>14.6</td>
<td>3.37</td>
<td>8.5§</td>
<td>2.73</td>
</tr>
</tbody>
</table>

BMSC indicates bone marrow stromal cells; mNSS, modified Neurological Severity Score; PBS, phosphate-buffered saline; tPA, tissue plasminogen activator; WT, wild-type.

*The stroke severity was significantly different between WT and tPA−/− at baseline (before the BMSC treatment) but was balanced between assigned treated and control groups within each mouse type. Therefore, all the analyses of BMSC effects were adjusted for the baseline stroke severity.

†P < 0.05 for testing tPA by BMSC interactions.

‡Ranked within each time interval.

§P < 0.05 compared to PBS within each mouse type.
were performed by observers blinded to the treatments and analyzed using the similar analysis approach, as described.

**Results**

**Neurological Outcome and Lesion Volume**

Stroke severity measured by Foot-fault and modified neurological severity score was significantly reduced in tPA−/− mice, compared to WT mice, but stroke severity was balanced between the assigned treatment groups for each mouse type. BMSC by tPA interactions were observed (P<0.05) on days 7 and 14 in modified neurological severity score test, and on days 3, 7, and 14 in Foot-fault test adjusting for unbalanced stroke severity at the baseline, which indicated that effects of BMSC on the functional recovery depended on tPA presence/absence. In WT mice, BMSC significantly improved functional recovery at days 7 and 14 (P<0.05), respectively. In contrast, in the tPA−/− mice, no BMSC effect was observed on functional recovery at any time points (Table). In addition, after treatment with BMSC, the more severely injured WT mice showed significantly superior recovery in percentage of left Foot-fault, compared to the less injured tPA−/− mice (P<0.05), after adjusting for baseline unbalanced stroke severity. Therefore, tPA is required for the functional benefit of BMSC.

Ischemic lesion volumes 14 days after the onset of stroke for the WT mice were 17.7%±3.45% (MCAO alone) and 15.9%±2.78% (MCAO+BMSC); for the tPA−/− mice, volumes were 11.5%±1.33% (MCAO alone) and 10.9%±2.08% (MCAO+BMSC). No BMSC and tPA interaction was observed, and there was no BMSC effect in either WT or tPA−/− mice in terms of lesion volume.

**Neurite Remodeling in the IBZ**

Synaptophysin, a presynaptic vesicle protein, is an indicator of synaptic plasticity and synaptogenesis.24 As shown in Figure 1, WT mice (Figure 1A) showed higher synaptophysin expression in the striatum of the IBZ compared to the less severely injured WT mice showed significantly superior recovery in percentage of left Foot-fault, compared to the less injured tPA−/− mice (P<0.05), after adjusting for baseline unbalanced stroke severity. Therefore, tPA is required for the functional benefit of BMSC.

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**Figure 1.** Synaptophysin staining shows axonal remodeling is increased after bone marrow stromal cell (BMSC) treatment in wild-type (WT) mice, but not in tissue plasminogen knockout (tPA−/−) mice. A–C, Compared to WT control animals, WT mice with BMSC treatment show significantly higher expression of synaptophysin immunopositive signals, whereas tPA−/− mice with and without BMSC transplantation have significantly lower signals in the ischemic boundary zone. *P<0.05 vs WT control. Scale bars: 25 μm.

**Figure 2.** Bielshowsky silver and Luxol fast blue double-staining shows axonal remodeling is increased after bone marrow stromal cell (BMSC) treatment in wild-type (WT) mice, but not in tissue plasminogen knockout (tPA−/−) mice. A and B, Axonal density in the ischemic boundary zone (IBZ) in WT mice is significantly higher than that of the tPA−/− mice; corresponding higher magnification pictures in the IBZ are also shown. C, Quantitative data of axon density demonstrate that BMSC treatment significantly increased axonal density in WT mice, whereas no effect shows in tPA−/− animals in the IBZ. *P<0.05 vs WT control. Scale bars: 50 μm.

**Figure 3.** tPA protein level in the IBZ in mice subjected to MCAO. A, B: C: there is no tPA signal in tPA−/− mice; D–a: tPA immunostaining shows that tPA protein is significantly increased in WT mice treated with BMSC; D–b: Direct casein zymography demonstrates tPA activity in WT mice, and 0 tPA activity in tPA−/− mice. *P<0.05 versus MCAO alone. Scale bars: 50 μm.
tPA Level and Activity in the IBZ

tPA is the primary source of plasminogen activator in the brain. Immunostaining with tPA antibody showed positive signals in WT mice (Figure 3A, B, D-a) that were absent in tPA−/− mice (Figure 3C). This indicates the lack of tPA protein in knockout mice. To identify the specific cell types that express tPA in the IBZ, double-staining immunohistochemistry of brain sections were performed. tPA labeling was colocalized with neuronal marker MAP2 (Figure 4A), astrocytic marker glial fibrillary acidic protein (Figure 4B), microglial marker IB4 (Figure 4C), or endothelial marker von Willebrand factor (Figure 4D). Consistent with these findings, direct casein zymography detected a lytic tPA band in WT mice only (Figure 3D-b). No tPA activity is detectable in tPA−/− mice with or without BMSC treatment.

Discussion

Our data demonstrate that BMSC infusion at 24 hours after MCAO significantly improves neurological recovery in WT mice, consistent with previous results. Concurrent with the amelioration of functional deficits, WT mice receiving BMSC treatment have significantly higher synaptophysin level, higher axonal density, and more robust increase in tPA protein and activity in the IBZ compared to WT controls. In tPA−/− mice, however, BMSC treatment induces no functional improvement and no increase in synaptophysin expression and axonal density. In addition, compared with WT control mice, tPA−/− mice with and without BMSC treatment show significantly less neurite remodeling in the IBZ. Collectively, these data strongly suggest that endogenous tPA is required for BMSC to stimulate brain plasticity and to improve neurological deficits in mice after focal brain ischemia.

In response to massive neuronal death and denervation after focal ischemia, neurons undergo axonal sprouting and establish new synaptic connections, which may underlie the partial recovery of neurological function over time. Cultured medium from BMSC increases neurite outgrowth in
primary cultured neurons, and BMSC treatment facilitates axonal remodeling in rodents. Although the exact mechanisms are still not clear, we have demonstrated that endogenous tPA is a key mediator of BMSC-induced neurite outgrowth. Therefore, we used loss of function tPA mice in the present study to elucidate BMSC–endogenous tPA interaction and its impact on neurite remodeling after MCAO. In agreement with our hypothesis, BMSC facilitate neurite remodeling and improve neurological deficits after focal brain ischemia in WT mice but show no effects in tPA mice.

Being the major serine protease in the central nervous system, tPA can be produced in the rodent brain by neurons, astrocytes, microglia, and endothelial cells. Regardless of its cellular source, tPA is secreted and functions in the extracellular space. Endogenous tPA level is tightly regulated both intracellularly and extracellularly within the cells. tPA transcript is modulated in an immediate-early manner and, once in the extracellular space, tPA activity is controlled by inhibitors, including neurserpin and plasminogen activator inhibitor-1. This regulation assumes the role of tPA in modulating learning, synaptic plasticity, cell death, and the permeability as well as coupling of the neurovascular unit under physiological conditions. After ischemic brain injury, animal studies show an increase in endogenous tPA activity within the ischemic tissue, and genetic deficiency of tPA is associated with decrease in the volume of ischemic lesion and preservation of the function of the blood–brain barrier. Local tPA level is also significantly increased after peripheral nerve injury, and tPA is crucial for axonal regeneration. Mice show delayed functional recovery after sciatic nerve crush. Taken together, this experimental evidence outlines a complicated and controversial role for tPA after injury. Our data are consistent with previous studies showing that tPA mice have smaller lesion volume but less robust axonal remodeling after permanent MCAO compared with WT animals. The major message of this study, however, is that endogenous tPA mediates the beneficial effects of BMSC treatment of brain ischemia. We therefore propose that BMSC injected at 24 hours after MCAO regulates the endogenous tPA/plasminogen activator inhibitor-1 system in such a way that guarantees a consistently increased tPA level and activity in the IBZ in the subacute phase after ischemic attack, which facilitates neurite remodeling and thus may lead to the recovery of neurological deficits.

Conclusion

tPA has pleiotropic effects that can affect brain remodeling, including neurogenesis and angiogenesis. tPA stimulates the expression of MMP, particularly MMP-9, which enhances progenitor cell migration and contributes to neurogenesis and angiogenesis. Likewise, tPA can cleave pro-trophic factors, such as nerve growth factor and brain-derived neurotrophic factor, into active factors that contribute to many aspects of brain plasticity. Regarding how local tPA facilitates axonal remodeling, there are many possibilities. Previous studies show that tPA indirectly through plasmin, or directly activates the N-methyl-D-aspartate recep-

tor, which can then induce nitric oxide release and foster neurotogenesis. Given the multifaceted effects of tPA on brain tissue, we cannot exclude other possibilities like plasmin-dependent degradation of extracellular matrix and fibrin clearance and plasmin-independent proteolytic cleavage of precursor forms of neurotrophins, such as pro-brain-derived neurotrophic factor to the mature neurotrophin brain-derived neurotrophic factor. These actions can either clear the way for sprouting axons or promote the innate capacity of neurite outgrowth, thus facilitating axonal remodeling after massive neuronal death caused by ischemic attack. Further studies are warranted to clarify the mechanisms by which tPA accelerates axonal regeneration.

Acknowledgments

The authors thank Theradigm for providing primary cultures of BMSC, and Cindi Roberts and Qing-e Lu for technical assistance. tPA mice were generously provided by Dr Costantino Iadecola.

Source of Funding

This work was supported by NINDS grants P01 NS42345, and R01 NS66041.

Disclosure

None.

References

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Stroke. 2011;42:459-464; originally published online January 6, 2011; doi: 10.1161/STROKEAHA.110.593863

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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