Intravenous infusion of mesenchymal stem cells inhibits intracranial hemorrhage after recombinant tissue plasminogen activator therapy for transient middle cerebral artery occlusion in rats

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OBJECTIVE Reperfusion therapy with intravenous recombinant tissue plasminogen activator (rtPA) is the standard of care for acute ischemic stroke. However, hemorrhagic complications can result. Intravenous infusion of mesenchymal stem cells (MSCs) reduces stroke volume and improves behavioral function in experimental stroke models. One suggested therapeutic mechanism is inhibition of vascular endothelial dysfunction. The objective of this study was to determine whether MSCs suppress hemorrhagic events after rtPA therapy in the acute phase of transient middle cerebral artery occlusion (tMCAO) in rats.

METHODS After induction of tMCAO, 4 groups were studied: 1) normal saline (NS)+vehicle, 2) rtPA+vehicle, 3) NS+MSCs, and 4) rtPA+MSCs. The incidence rate of intracerebral hemorrhage, both hemorrhagic and ischemic volume, and behavioral performance were examined. Matrix metalloproteinase–9 (MMP-9) levels in the brain were assessed with zymography. Quantitative analysis of regional cerebral blood flow (rCBF) was performed to assess hemodynamic change in the ischemic lesion.

RESULTS The MSC-treated groups (Groups 3 and 4) experienced a greater reduction in the incidence rate of intracerebral hemorrhage and hemorrhagic volume 1 day after tMCAO even if rtPA was received. The application of rtPA enhanced activation of MMP-9, but MSCs inhibited MMP-9 activation. Behavioral testing indicated that both MSC-infused groups had greater improvement than non-MSC groups had, but rtPA+MSCs provided greater improvement than MSCs alone. The rCBF ratio of rtPA groups (Groups 2 and 4) was similar at 2 hours after reperfusion of tMCAO, but both were greater than that in non-rtPA groups.

CONCLUSIONS Infused MSCs may inhibit endothelial dysfunction to suppress hemorrhagic events and facilitate functional outcome. Combined therapy of infused MSCs after rtPA therapy facilitated early behavioral recovery.

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KEY WORDS transplantation; mesenchymal stem cell; stroke; intracranial hemorrhage; recombinant tissue plasminogen activator; rodent; middle cerebral artery occlusion

Reperfusion therapy with intravenous recombinant tissue plasminogen activator (rtPA) administration is the international standard for acute ischemic stroke; however, hemorrhagic complications after rtPA therapy are a major problem that may result in unfavorable clinical outcomes.35 Only a small percentage of stroke patients can receive rtPA therapy because of the narrow therapeutic time window due to elevated risks of symptomatic intracranial hemorrhage. Thus, to suppress the incidence of hemorrhagic events following intravenous rtPA therapy

ABBREVIATIONS BBB = blood-brain barrier; DWI = diffusion-weighted imaging; EvB = Evans Blue; GFP = green fluorescent protein; IL = interleukin; MCA = middle cerebral artery; MMP = matrix metalloproteinase; MSC = mesenchymal stem cell; NF-κB = nuclear factor kappa light-chain enhancer of activated B cells; NS = normal saline; PBS = phosphate-buffered saline; PWI = perfusion-weighted imaging; rCBF = regional cerebral blood flow; ROI = region of interest; rtPA = recombinant tissue plasminogen activator; tMCAO = transient MCA occlusion; TNF = tumor necrosis factor.

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and to extend the therapeutic time window, development of an adjunctive therapy to inhibit endothelial dysfunction would be desirable.

Systemic administration of mesenchymal stem cells (MSCs) after cerebral infarction can reduce stroke volume and improve behavioral function in experimental stroke models.\(^3,6,9,15,19\) Suggested therapeutic mechanisms of MSCs in various models of CNS diseases include secretion of neurotrophic factors that promote neuroprotection, neurogenesis,\(^8\) induction of axonal sprouting, neurovascularization, and immunomodulation.\(^28,33\) In addition to these mechanisms, recent studies have reported that MSCs have the potential to maintain blood-brain barrier (BBB) integrity and reduce BBB leakage that could contribute to their therapeutic efficacy.\(^17\) Several clinical studies using intravenous infusion of MSCs in stroke patients have been performed or are ongoing.\(^7,8\)

In the present study, we tested the hypothesis that intravenous infusion of MSCs inhibits vascular endothelial dysfunction decreasing both the incidence rate of intracerebral hemorrhage and hemorrhagic volume after rtPA therapy using a transient (90-minute) middle cerebral artery occlusion (tMCAO) model in rats. Quantitative analysis of regional cerebral blood flow (rCBF) was performed to assess hemodynamic changes in the ischemic lesion. Additionally, ischemic volume and behavioral performance were recorded during the study period.

**Methods**

**Animals**

The use of animals in this study was approved by the animal care and use committee of Sapporo Medical University, and all procedures were carried out in accordance with institutional guidelines. All MRI evaluations, zymography, behavioral testing, and statistical analyses were performed by persons who were naive with respect to treatment condition.

**Preparation of MSCs From Rat Bone Marrow**

The methodology of MSC culture was based on our previous studies.\(^11,32\) Briefly, bone marrow was obtained from femoral bones in adult Sprague-Dawley rats or green fluorescent protein (GFP)-expressing Sprague-Dawley rats (W-Tg [CAG-GFP]184Ys) diluted to 15 ml with DMEM (Sigma) supplemented with 10% heat-inactivated fetal bovine serum; Thermo Fisher Scientific Inc.), 2 mM l-glutamine (Sigma), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Thermo Fisher Scientific Inc.) and incubated for 3 days (5% CO\(_2\), 37°C). When cultures almost reached confluence, the adherent cells were detached with trypsin–ethylenediaminetetraacetic acid solution (Sigma) and subcultured at 1 × 10\(^4\) cells/ml. In the present study, we used MSCs after 3 passages.

**Cerebral Ischemic Model**

We induced tMCAO for 90 minutes by using a previously described method of intraluminal vascular occlusion.\(^6,9,27\) Adult male Sprague-Dawley rats weighing 280–330 g (n = 222) were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg).

A 20.0- to 22.0-mm length of 3–0 surgical Monosof suture (Covidien), with the tip rounded by heating it near a flame, was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA for 90 minutes with initial diffusion-weighted imaging (DWI)-MRI performed at the 60-minute occlusion period. The initial stroke volume evaluated with DWI was standardized at 220–370 mm\(^3\) and excluded animals outside this range to assess the efficacy of MSCs following rtPA for a large ischemic injury.\(^30\) We used a suture occlusion/reperfusion model in this study to investigate the effects of rtPA and MSCs following complete reperfusion. Physiological parameters (rectal temperature, blood pH, partial pressure of oxygen, partial pressure of carbon dioxide, and blood pressure) were maintained within normal ranges during surgery and transplantation procedures for all animals and did not significantly differ among the experimental groups.\(^9\)

**Experimental Protocols**

The experimental protocol is shown in Fig. 1. Sixty minutes after establishing tMCAO, DWI was obtained to evaluate initial stroke volume. Thirty minutes after DWI, the inserted surgical Monosof suture was removed for reperfusion. Then, the rats were randomized into 4 experimental groups. In Group 1 (1 ml normal saline [NS] + 1 ml vehicle [DMEM]; n = 55), rats were injected intravenously with 1 ml NS immediately after reperfusion, then injected with vehicle after 30 minutes. In Group 2 (rtPA+vehicle; n = 68), rats were injected intravenously with rtPA (10 mg/kg; 1 ml) immediately after reperfusion, then injected with vehicle after 30 minutes. In Group 3 (NS+MSCs; n = 51), rats were injected intravenously with NS (1 ml) immediately after reperfusion, then were injected with MSCs (1.0 × 10\(^6\) cells each) in 1 ml DMEM after 30 minutes. In Group 4 (rtPA+MSCs; n = 48), rats were injected intravenously with rtPA (10 mg/kg; 1 ml) immediately after reperfusion, then injected with MSCs (1.0 × 10\(^6\) cells each) in 1 ml DMEM after 30 minutes. All rats were injected daily with cyclosporine A (10 mg/kg, intraperitoneally).\(^1,3,5,6,10,20,22,28,37\) All intravenous infusions were delivered through the left femoral vein.

**MRI Studies and Measurement of Infarct Volume**

Rats were anesthetized with ketamine (75 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). Each rat was placed in an animal holder/MRI probe apparatus and positioned inside the magnet. The animal’s head was held in place inside the imaging coil. All MRI measurements were performed using a 7-T, 18-cm bore superconducting magnet (Oxford Magnet Technologies) interfaced to a UNITY INOVA console (Oxford Instruments and Varian Inc.) as described previously.\(^6,12\)

Briefly, DW images were obtained from a 1.0-mm-thick coronal section with a 0.5-mm gap using a 30 mm × 30 mm field of view (TR 3000 msec, TE 37 msec, b value 1000 sec/mm\(^2\)) and reconstructed using a 128 × 128 image matrix. The T2-weighted images were obtained from a 1.0-mm-thick coronal section with a 0.5-mm gap using a 30 mm × 30 mm field of view (TR 3000 msec, TE 30
Combination therapy of MSCs and rtPA for transient MCAO in rats

msec) and reconstructed using a 256 × 256 image matrix. Accurate positioning of the brain was performed to center the image slice 5 mm posterior to the rhinal fissure with the head of the rat held in a flat skull position.

DW images were obtained 60 minutes after tMCAO, and T2-weighted images were obtained at 1, 4, 7, 14, 21, and 28 days after tMCAO. The ischemic lesion area was calculated from MR images using imaging software (Scion Image, beta version 4.0.2, Scion Corporation) based on the previously described method.18 Lesion volume (mm³) was determined by analysis of high-intensity areas on 9 serial corona images collected through the cerebrum. For each slice, the higher-intensity lesions on DWI and T2-weighted imaging, where the signal intensity was 1.25 times higher than the counterpart in the contralateral brain lesion, were marked as the ischemic lesion area, and infarct volume was calculated taking slice thickness (1 mm/slice) into account. The presence of intracerebral hemorrhage was counted when there was a low-intensity signal on T2-weighted imaging, where the signal intensity was 1.25 times higher than the counterpart in the noninfarcted hemisphere.

Histological Processing for GFP

For immunohistological analysis, 4 rats in each group were used. Rats were deeply anesthetized with ketamine and xylazine (75/10 mg/kg, intraperitoneally), perfused with saline and 0.1 M phosphate buffer, and processed for standard frozen sectioning. Cryosections were cut using a cryostat and mounted on glass slides. Sections were washed in phosphate-buffered saline (PBS) and 0.1% Tween 20 three times, blocked in 5% normal donkey serum and 0.3% Triton X-100 for 30 minutes, and then incubated overnight in primary antibodies diluted with 5% normal donkey serum, 0.3% Triton X-100, and PBS at 4°C. The cryosections were processed for immunolabeling for chicken polyclonal anti-antibody (20 μm thick, 1:1000; Abcam, ab13970) to detect GFP. After being washed in PBS-Tween 20 four times, sections were incubated in secondary antibody AF 488-conjugated goat anti–chicken immunoglobulin Y for GFP (1:1000; Abcam, 150173), counterstained with DAPI, and coverslipped with VECTASHIELD (Vector Laboratories). The frozen sections were examined using confocal microscopy with Ex/Em (405 nm/410–502 nm for DAPI; 488 nm/499–553 nm for GFP; LSM780 ELYRA S.1 system, Zeiss).

Gelatin Zymography

Gelatin zymography was performed using brain tissue removed from rats on Day 1 after tMCAO, as previously described.13 Four rats in each group were used. Briefly, brain samples were homogenized immediately after perfusion in x10 volume lysis buffer (150 mM NaCl, 1% sodium dodecyl sulfate, 0.1% deoxycholic acid, and 50 mM Tris-HCl, pH 7.5) containing protease inhibitors on ice. After centrifugation at 9000g for 15 minutes at 4°C, the supernatant was collected and stored at –80°C until use. Total protein concentration of each supernatant was determined by Thermo BCA protein assay (Pierce). The activity of matrix metalloproteinase–9 (MMP-9) in each sample was measured using a gelatin-zymography kit (Primary Cell), according to the manufacturer’s instructions. In brief, each sample containing 20 μg of protein was diluted with the homogenizing buffer in the kit,
mixed with an equal volume of sample buffer, and loaded for electrophoresis for 2 hours. The gels were washed and incubated for 48 hours in incubation buffer at 37°C, then stained with Coomassie blue and scanned. To measure band intensities, densitometric analysis was performed using TotalLab Quant.

Evans Blue Analysis

Four rats in each group were used for Evans Blue (EvB) analysis. Rats were anesthetized with ketamine and xylazine (75/10 mg/kg, intraperitoneally). The 4% EvB, which was dissolved in 0.9% saline, was injected as a single bolus dose of 1 ml/kg via the right femoral vein after infusion of MSCs or vehicle. At Day 1, rats were deeply anesthetized with ketamine and xylazine (75/10 mg/kg, intraperitoneally) and transcardially perfused with ice-cold PBS to remove the intravascular dye. The whole brains were removed, and the macroscopic images were acquired using a Nikon AZ100 microscope with a ×0.5 objective. The whole brains were dissected into coronal 1-mm sections using a vibratome. The coronal slices were examined with a Nikon AZ100 microscope with a ×0.5 objective.

For extraction to quantify the total EvB content in the tissue, after taking the macroscopic images, the brains were placed in 1:2 weight (mg):volume (ml) ratios of 0.9% saline and homogenized for 1 minute using a sonifier (Branson Ultrasonic Corporation). Samples were incubated in 37°C for 60 minutes, then ×2 weight of 50% was added in each sample and homogenized for 1 minute with a beam homogenizer. Homogenized samples were centrifuged at 10,000g for 20 minutes to remove tissue debris, and the supernatants were added to a 96-well plate (30 μl per well, each plate supplemented with 90 μl of 95% ethanol and thoroughly mixed) for fluorescence spectroscopy (620 nm/680 nm).

Treadmill Stress Test

Rats were trained 20 min/day for 2 days a week to run on a motor-driven treadmill (Muromachi Inc.) at a speed of 20 m/min with a slope of 20° before tMCAO. The maximum speed at which the rats could run on a motor-driven treadmill was recorded on Days 1, 4, 7, 14, 21, and 28 after tMCAO.

Statistical Analysis

All statistical analyses were performed using JMP 11.1 for Windows (SAS Institute Inc.). Categorical data in contingency tables were analyzed using the chi-square test, and, if appropriate, the Bonferroni test for post hoc analysis to determine any significant differences between each pair of groups. Continuous data were assessed for normality using the Shapiro-Wilk test; normally distributed continuous data were analyzed by 1-way ANOVA, and the Tukey-Kramer test was used to compare the subgroups if significance was found. Continuous data that did not pass this normality test were compared using the Kruskal-Wallis test, and the Steel-Dwass test was used to compare the subgroups if significance was found.

Results

Hemorrhagic Events After Intravenous rtPA

Representative images are shown in Fig. 2A for NS+vehicle (Group 1), rtPA+vehicle (Group 2), NS+MSCs (Group 3), and rtPA+MSCs (Group 4). Low-intensity areas suggesting hemorrhage were detected in the T2-weighted imaging in all groups. Quantitative analysis demonstrated that acute hemorrhagic events in Group 2 were higher than those in the other 3 experimental groups in terms of incidence rate (Group 1: 9.5%; Group 2: 50%; Group 3: 3.6%; Group 4: 10%, p < 0.01, chi-square test; Fig. 2B) and hemorrhagic volume (Group 1: 0.14 ± 0.01 mm³; Group 2: 3.20 ± 1.54 mm³; Group 3: 0.01 ± 0.01 mm³; Group 4: 0.48 ± 0.39 mm³, p < 0.01, Kruskal-Wallis test; Fig. 2C). It should be noted that hemorrhagic events after rtPA therapy in Group 2 were reduced by MSC infusion in Group 4 (40% reduction, p < 0.01, chi-square test; Bonferroni test for post hoc: volume 2.72 mm³ reduction, p < 0.05, Steel-Dwass test) suggesting that intravenous infusion of MSCs has the potential to reduce the incidence and volume of intracerebral hemorrhage induced by rtPA therapy.

Detection of GFP-MSCs In Vivo

Histological analysis at 1 day after GFP-MSC (green) infusion indicated that the infused cells survived and were distributed to the infarcted hemisphere (Fig. 2D). There were few GFP-MSCs in the contralateral hemisphere. To determine whether autofluorescence of the MSCs was present at the wavelengths used to study GFP fluorescence, we examined these sections of animals infused with MSCs derived from wild-type Sprague-Dawley rats. No green cells were observed in these lesions (data not shown).

Gelatin Zymography

MMP-9 contributes to hemorrhagic transformation after reperfusion. Gelatin zymography indicated that on the ipsilateral (lesion) side of the brain, MMP-9 activity was high in Groups 1 and 2 (NS+vehicle and rtPA+vehicle, respectively). However, when MSCs were infused with (Group 4) and without rtPA (Group 3), MMP-9 activity was low (Fig. 3). It should be noted that the rtPA+MSCs group (Group 4) had significantly lower MMP-9 activity than the rtPA+vehicle group (Group 2; p < 0.01, Tukey’s post hoc test) had, and MMP-9 activity in the NS+MSCs group (Group 3) was significantly lower than the NS+vehicle group (Group 1 [NS+vehicle] = 5.01 ± 0.30; p < 0.01, Tukey’s post hoc). MMP-9 activity was not statistically significant among the groups on the contralateral (nonlesion) side of the brain (Fig. 3). These data suggest that MSC infusion might decrease the activity of MMP-9 and suppress hemorrhagic events.

Evans Blue Analysis

To study the mechanistic insight beyond the MMP-9 activation, the status of endothelial function 1 day after treatments was evaluated to analyze the EvB dye extravasation (Fig. 4). The pattern of BBB leakage evaluated with EvB in the 4 groups is similar to that of MMP-9 activity. Macroscopic images demonstrated intense blue EvB extravasa-
Combination therapy of MSCs and rtPA for transient MCAO in rats

J Neurosurg Volume 127 • October 2017

In the infarcted lesion on the ipsilateral side 1 day after tMCAO with treatments compared with the contralateral side (Fig. 4A–H). Intense blue in the infarcted hemisphere was observed in the following order: rtPA+vehicle (Group 2) > NS+vehicle (Group 1) > rtPA+MSCs (Group 4) > NS+MSCs (Group 3; Fig. 4A–H). Quantitative analysis of total EvB content was high in Groups 1 and 2 (NS+vehicle and rtPA+vehicle, respectively). However, when MSCs were infused with (Group 4) and without rtPA (Group 3), EvB extravasation was low (Fig. 4I). It should be noted that the rtPA+MSCs group (Group 4) had lower EvB content than the rtPA+vehicle group had (Group 2; p < 0.05, Tukey’s post hoc test), and EvB content in the NS+MSCs group (Group 3) was lower than that in the NS+vehicle group (Group 1 [NS+vehicle], p < 0.05, Tukey’s post hoc test). EvB content was not statistically significant among the groups on the contralateral (nonlesion) side of the brain (p < 0.01; Fig. 4I). Collectively with the data for MMP-9 activity, these data suggest that MSC infusion might suppress hemorrhagic events via inhibition of endothelial dysfunction.

Ischemic Lesion Volume by MRI Analysis

The ischemic lesion volume was estimated for the 4 experimental groups using in vivo MRI (see Methods). Sample images for the 4 experimental groups at 7 time points are shown in Fig. 5A. Quantification of high-intensity volume is shown in Fig. 5B. DW images were obtained at 60 minutes [row “Pre (DWI)” in Fig. 5A] and confirmed no difference in the initial stroke volume among the groups (Group 1 [NS+vehicle] = 274 ± 12 mm$^3$, n = 9; Group 2 [rtPA+vehicle] = 279 ± 11 mm$^3$, n = 12; Group 3...
[NS+MSCs] = 267 ± 8 mm$^3$, n = 11; Group 4 [rtPA+MSCs] = 276 ± 14 mm$^3$, n = 9; $F[3,37] = 0.12$, ANOVA, $p = 0.95$). T2-weighted images were obtained from the 4 groups on Days 1, 4, 7, 14, 21, and 28 after tMCAO induction. In all groups, estimated lesion volume gradually decreased over the time course of 28 days after tMCAO (Fig. 5B). The MSC-treated groups displayed greater volume reduction compared with vehicle-infused groups at Days 14, 21, and 28 ($p < 0.01$, Tukey's post hoc test; Fig. 4B). The lesion volume of the rtPA+MSCs group (Group 4) was 22% (Day 14), 21% (Day 21), and 25% (Day 28) of the rtPA-vehicle group (Group 2), respectively. Although there was no statistical significance, there were trends that the reduction in lesion volume was greatest for the rtPA+MSCs group (Group 4) on Days 4, 7, 14, 21, and 28, and the ischemic lesion volume was highest for the rtPA-vehicle group (Group 2) over the time course until Day 28 (Fig. 5B). Collectively, these results suggest that intravenous infusion of MSCs had greater therapeutic efficacy for reducing the ischemic lesion volume when rtPA therapy was performed.

**FIG. 3.** Gelatin zymography. A: Activation of MMP-9 in the contralateral (nonlesion side) was low but high in the ipsilateral (lesion side) brain for NS+vehicle and rtPA+vehicle groups (1 and 2). Activation of MMP-9 was greatly reduced in the MSC-treated groups (3 and 4) on the lesion side. B: Densitometric analysis by zymography. **p < 0.01 versus contralateral hemisphere; ††p < 0.01 versus ipsilateral Group 1, §§p < 0.01 versus ipsilateral Group 2, ANOVA, $F[3,12] = 15.4$, Tukey’s post hoc test.

Dynamic Susceptibility Contrast-Enhanced Perfusion-Weighted Image

To assess rCBF, the PWI-derived CBF maps allowed further quantitative analysis of the hemodynamic changes in the defined lesion in the ischemic brain (see Methods). The rCBF ratio between rtPA therapy groups (Groups 2 and 4) were similar, and the rCBF ratio between non-rtPA therapy groups (Groups 1 and 3) was also similar at 2 hours after reperfusion of tMCAO, although the rCBF ratio in the rtPA therapy groups were about 23% higher than in the non–rtPA therapy groups (Fig. 5C). These results suggest that the acute rCBF ratio could be upregulated to 23% by the rtPA therapy with and without receiving intravenous infusion of MSCs ($F[3,10] = 8.14$, $p < 0.01$, ANOVA).

Behavioral Function

The maximum velocity at which the rats could run on a motor-driven treadmill was recorded. Before tMCAO, all rats reached a velocity of 70 m/min. Twenty-four hours after tMCAO, maximum velocity on the treadmill test was at
its maximum deficit. Both the MSC-infused groups (Group 3 [NS+MSCs], n = 11 rats, and Group 4 [rtPA+MSCs], n = 9 rats) had greater maximum velocity from 4 to 28 days than non-MSC-treated controls had (Group 1 [NS+vehicle], n = 9 rats, and Group 2 [rtPA+vehicle], n = 12 rats); moreover, Group 4 attained a higher velocity than Group 3 did from Day 7 to Day 28. These results indicate that intravenous administration of MSCs improved functional outcome and that the combination of infused MSCs with rtPA therapy could facilitate quicker recovery compared with MSC delivery alone (Group 3 [NS+MSCs]). The rtPA therapy alone (Group 2) did not provide functional recovery compared with the vehicle control (Group 1) during the study period even though CBF was increased in Group 2. These data are summarized in Fig. 6.

Discussion

In the present study, we examined both the incidence rate of intracerebral hemorrhage and hemorrhagic volume after rtPA therapy with intravenous infusion of MSCs in the acute phase of tMCAO and demonstrated that infused

![EvB analysis](image-url)
MSCs reduce endothelial dysfunction. Although the rtPA therapy with vehicle infusion (without MSCs) had the highest incidence rate of intracerebral hemorrhage and greatest stroke volume, intravenous infusion of MSCs with rtPA therapy after tMCAO resulted in a greater reduction in both the incidence rate of intracerebral hemorrhage and hemorrhagic volume.

The molecular mechanisms that underlie hemorrhagic events after rtPA therapy remain unclear; however, previous studies suggested the deleterious role of MMP-9 in the development of vascular damage by disrupting vascular integrity during reperfusion in acute stroke. Ischemic insult causes an activation of MMP-9 mediated through elevation of cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. However, it has been suggested that transplanted MSCs may protect damaged tissue by blocking TNF-α and IL-1β. Exogenous rtPA also upregulates nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) via the protease-activated receptor 1 pathway. NF-κB triggers activation of MMP-9 expression. Recent studies have suggested that MSCs secrete TNF-inducible gene 6 protein, which decreased Toll-like receptor 2/NF-κB signaling through direct interaction with CD44 (for review see Prockop and Oh). Thus, the reduction of NF-κB might inhibit activation of MMP-9. Taken together, the infused MSCs might protect the disruption of vasculature by nondegradation of basal lamina and/or extracellular matrix with the reduced expression of MMP-9. Quantitative analysis of the EvB leakage demonstrated that dysfunction of the BBB might support the findings of MMP-9. Thus, it is conceivable that infused MSCs inhibit the endothelial dysfunction and may reduce BBB disruption to decrease both the incidence rate of intracerebral hemorrhage and hemorrhagic volume even though rtPA was infused in the acute phase of cerebral ischemia.

The rtPA-infused groups (Groups 2 and 4) showed increased rCBF in the lesion area 2 hours after reperfusion, and MSC-infused groups had therapeutic efficacy, which

FIG. 5. Characterization of ischemic lesion size by MRI analysis. A: Axial DW images were obtained 60 minutes after tMCAO induction (Pre [DWI]). T2-weighted images were obtained from the 4 groups at Day 1, Day 4, Day 7, Day 14, Day 21, and Day 28 after tMCAO induction from the 4 groups (NS+vehicle, rtPA+vehicle, NS+MSCs, rtPA+MSCs). Bar = 3 mm. B: A summary of lesion volume evaluated with DWI (pre) and T2-weighted imaging were obtained 1, 4, 7, 14, 21, and 28 days after reperfusion in 4 experimental groups. C: Summary of rCBF evaluated with PWI. *p < 0.05, **p < 0.01, Tukey’s post hoc tests.
Combination therapy of MSCs and rtPA for transient MCAO in rats

MSCs may contribute to vascular stabilization and remodelling of the BBB after injury, thereby protecting intact tissue and reducing edema. MSCs may also induce angiogenesis, such as brain-derived neurotrophic factor at early post-infarct approach for acute CNS insult is that beneficial effects of MSC infusion in experimental cerebral ischemia models. Interestingly, there was a synergistic effect of rtPA therapy and MSC infusion on ischemic stroke, indicated by greater functional improvement and reduced stroke volume over the study period, although the rtPA therapy alone (Group 2) did not provide therapeutic efficacy. MSC may enhance the therapeutic efficacy contributed in part by improvement in microcirculation by rtPA. Yet, improvement in microcirculation by rtPA alone did not provide functional improvement. Thus, rtPA may improve microcirculation, allowing for greater therapeutic potential of the MSCs, and the MSCs are initially important because they reduce the potential hemorrhagic complications of rtPA treatment.

In the present study, we showed that there is accumulation of intravenously administered MSCs in the injured region in CNS diseases, including stroke, which is consistent with previous studies. It could be possible that infused MSCs migrated into the injured CNS tissue and provide beneficial effects. A hypothetical sequence of potential therapeutic mechanisms in a cell-based therapeutic approach for acute CNS insult is that beneficial effects may be provided from neuromodulators by MSC release, such as brain-derived neurotrophic factor at early post-MSC infusion times (days). MSCs could also support trophic effects for vulnerable neurons and antiinflammatory responses and the reduction of edema, thus leading to enhanced tissue sparing following trauma. Over time, MSCs may contribute to vascular stabilization and remodeling of the BBB after injury, thereby protecting intact tissue and reducing edema. MSCs may also induce angiogenesis, remyelination, and axonal sprouting.

Cell-based therapy is considered appropriate for several neurological diseases, including stroke. The use of rtPA is now an established stroke treatment within several hours of ischemic stroke onset. It is conceivable that patients who receive acute rtPA therapy could be infused with MSCs in a clinical setting in the future. This study might encourage this protocol, which could facilitate the therapeutic effects of MSCs in stroke.

Conclusions

Systemically administered MSCs reduced intracranial hemorrhage by rtPA after tMCAO. The rtPA therapy also might enhance the therapeutic efficacy of MSCs.

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**Disclosures**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**


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