Original Article

Bone marrow mesenchymal stem cell transplantation downregulates plasma level and the microglia expression of transforming growth factor β1 in the acute phase of cerebral cortex ischemia

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Abstract

Background: Both bone marrow mesenchymal stem cell (BM-MSC) and transforming growth factor-β1 (TGF-β1) have a strong anti-inflammatory capacity in stroke. But their relationship has not been well addressed. In this study, we investigated how intravenous BM-MSC transplantation in rats effected the expression of TGF-β1 48 h post cerebral ischemia, and we analyzed the main cells that produce TGF-β1.

Methods: We used a distal middle cerebral artery occlusion (dMCAO) model in twenty Sprague–Dawley (SD) rats. The rats were randomly divided into two groups: the ischemic control group and the postischemic BM-MSC transplantation group. One hour after the dMCAO model was established, the rats were injected in the tail vein with either 1 ml saline or 1×10^6 BM-MSCs suspended in 1 ml saline. ELISAs were used to detect TGF-β1 content in the brain infarct core area, striatum and the plasma at 48 h after cerebral infarction. Immunofluorescent staining of brain tissue sections for TGF-β1, Iba-1, CD68 and NeuN was performed to determine the number and the proportion of double stained cells and to detect possible TGF-β1 producing cells in the brain tissue.

Results: Forty-eight hours after ischemia, the TGF-β1 content in the infarcted area of the BM-MSC transplantation group (23.94 ± 4.48 pg/ml) was significantly lower than it was in the ischemic control group (34.18 ± 4.32 pg/ml) (F = 13.534, $P = 0.006$). The TGF-β1 content in the rat plasma in the BM-MSC transplantation group (75.91 ± 12.53 pg/ml) was significantly lower than it was in the ischemic control group (131.18 ± 16.07 pg/ml) (F = 36.779, $P = 0.0002$), suggesting that after transplantation of BM-MSCs, TGF-β1 levels in the plasma decreased, but there was no significant change in the striatum area.

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Immunofluorescence staining showed that the total number of nucleated cells (1037.67 ± 222.16 cells/mm^2) in the infarcted area after transplantation was significantly higher than that in the ischemic control group (391.67 ± 69.50 cells/mm^2) (F = 92.421, $P < 0.01$); the number of TGF-β1$^+$ cells after transplantation (35.00 ± 13.66 cells/mm^2) was significantly reduced in comparison to that in the ischemic control group (72.33 ± 32.08 cells/mm^2) (F = 37.680, $P < 0.01$). The number of TGF-β1$^+$/Iba-1$^+$ microglia cells in the transplantation group (3.67 ± 3.17 cells/mm^2) was significantly reduced in comparison to that of the ischemic control group (13.67 ± 5.52 cells/mm^2) (F = 29.641, $P < 0.01$). The proportion of TGF-β1$^+$/Iba-1$^+$ microglia cells out of all Iba-1$^+$ microglia cells after transplantation ($4.38 \pm 3.18\%$) was significantly decreased compared with that in the ischemic control group ($12.81 \pm 4.86\%$) (F = 28.125, $P < 0.01$).

Conclusions: Iba-1$^+$ microglia is one of the main cell types that express TGF-β1. Intravenous transplantation of BM-MSCs does not cooperate with TGF-β1$^+$ cells in immune-regulation, but reduces the TGF-β1 content in the infarcted area and in the plasma at 48 h after cerebral infarction.

Introduction

Acute ischemic stroke (AIS) is a clinical syndrome that is characterized by an inadequate blood supply to the brain, causing ischemic necrosis of local brain tissues, and leading to neurological deficits. AIS is the most common type of stroke, and it is responsible for high disability and mortality rates. AIS accounts for 69.6%−70.8% of all strokes,[1,2] and it is one of the main causes of death among Chinese people. Mesenchymal stem cells (MSCs) are pluripotent stem cells with self-renewal and pluripotent differentiation potential. For ischemic stroke, intravenous transplantation of allogeneic bone marrow mesenchymal stem cells (BM-MSCs) can exert a protective effect on the brain through various mechanisms, such as immune-regulation, promotion of angiogenesis, and secretion of neurotrophic factors.[3,4]

TGF-β1 is a multifunctional growth factor that is both an immunosuppressive factor and an effective neuroprotective agent. TGF-β1 expression increases in acute and chronic brain injuries, such as stroke, brain trauma, epilepsy, multiple sclerosis and Alzheimer's disease.[5] In ischemic stroke, TGF-β1 can exert a neuroprotective effect through mechanisms such as promoting angiogenesis, immune regulation, inhibiting apoptosis, and inhibiting neurotoxicity.[6−8] TGF-β1 can also reduce acute ischemic blood brain barrier (BBB) disruption and hemorrhagic outcome after intravenous thrombolysis in stroke patients.[9] Applying TGF-β1 to the rat middle cerebral artery occlusion (MCAO) model significantly reduces the volume of cerebral infarction compared to that of the control group.[10] Antagonizing TGF-β1 significantly aggravates brain tissue damage.[11] These findings indicate that TGF-β1 provides a neuroprotective effect in cerebral ischemic injury.

BM-MSCs can inhibit the immune-inflammatory response and promote the production of trophic factors. To analyze their neurotrophic effects, our group has transplanted BM-MSCs intravenously in a rat middle cerebral artery occlusion (dMCAO) model. We found that MSCs not only inhibit microglia in the peripheral area of cerebral infarction but also activate and promote the production of neurotrophic factors 48 h post-transplantation. The neurotrophic factors are mainly IGF-1 and BDNF.[12] Additionally, we found that an early allograft of BM-MSCs in rats with a cerebral infarction can migrate into the brain and express brain derived neurotrophic factor (BDNF) in the cortical infarct area. In addition, BM-MSC transplantation can increase the number and proportion of Iba-1$^+$ microglia that secrete BDNF, which may be one of the mechanisms for its efficacy.[13]

In our previous experiments, we noticed that after BM-MSC transplantation, there was an improvement in rat behavior and a reduction in the infarct volume. We are interested in the anti-inflammation effects of MSC in the secondary injury of stroke, so we selected 48 h as the acute phase of post-stroke pathological process. Because most of the inflammation related cells in the brain peak at this time. It is speculated that the mechanism causing this effect is more likely related to the increase in neurotrophic factors in the rat brain.[12] To identify the related mechanisms of TGF-β1 involvement, we measured the TGF-β1 content in the cerebral infarction area, striatum area, and rat plasma 48 h post cerebral ischemia by ELISA in dMCAO model. Additionally, TGF-β1, Iba-1, CD68 and NeuN colabeled staining and quantitative analysis were used to observe the numbers and the proportion of positive and negative TGF-β1 staining cells in the cerebral infarction to investigate the changes of TGF-β1

expression. Our main goal is to clarify the effects of BM-MSC transplantation on the expression of TGF-β1 after cerebral infarction.

Methods

Animals and groups

All of the animals were provided by Beijing Vital River Laboratory Animal Technologies Co. Ltd. Five specific pathogen free-grade three-week-old male Sprague–Dawley (SD) rats, weighing approximately 100 g were selected for isolation and culture of BM-MSCs. Twenty eight-week-old SD male rats weighing approximately 260–280 g were randomly divided into two groups: ischemic control group, which was intravenously injected with 1 ml of saline through the tail vein 1 h after inducing ischemia; BM-MSC transplantation group, which was intravenously injected with 1 ml of saline containing 1×10^6 BM-MSCs cells through the tail vein 1 h after inducing ischemia. Rats were sacrificed after 48 h. From each group, five rats were used for the extraction of brain tissue proteins, and the remaining five rats were used for immunofluorescence histochemical staining of brain tissue sections. The welfare and ethical treatment of laboratory animals was implemented in accordance with the Regulations on the Administration of Laboratory and Laboratory Animals, which was issued by the Capital Medical University.

Extraction of rat BM-MSCs

The femurs of three-week-old male SD rats weighing approximately 100 g were isolated under sterile conditions, and BM-MSCs were cultured according to a method for whole bone marrow culture.[14] The bone marrow was washed with MEMα (Life Technology, USA) in a petri dish, and then was cultured in complete medium (MEMα + 10% FBS) at a cell concentration of 1×10^6 cells/ml of medium. After incubation at 37 °C and 5% CO_2 for 48 h, the petri dish was gently shaken to remove non-adherent cells and then washed with DPBS twice; then complete medium was added. The medium was changed 12 h later, and the cells were passaged when the cells reached 80% confluency. Sub-culturing was performed by incubating the cells with 0.05% trypsin–EDTA (Life Technology, USA) at 37 °C for 5 min. When BM-MSCs reached passage 3–5, the cells grew well and could be used for transplantation experiments.

Rat dMCAO animal model

The rat dMCAO model was prepared according to the references.[15,16] Male SD rats weighing 260–280 g were selected and anesthetized by compressed nitrous oxide and oxygen gas mixture (70%: 30%) (Surgivet, USA). The rat was placed in the supine position. We made a midline neck incision, approximately 3 cm in length under a microscope (Zeiss, Germany), and exposed the common carotid artery (CCA) on both sides. Then, the animal was placed in the left lateral position, and a 2 cm in length incision was made parallel to the head between the right ear and the right eye approximately. The temporal muscles were separated. The bone window on the skull was opened at approximately 4 mm × 4 mm with a bite forceps (Yuyan Instruments, China) and a high-speed drill (Yuyan Instruments, China). The meninges was gently opened by a micro-forceps (Yuyan Instruments, China).

A microvascular clip was used to clamp the bilateral CCA, and the distal MCA vessel was clamped and was subjected to 25 mA of electrocoagulation. If the electrocoagulation was good and there was no bleeding, micro-scissors (Mailang Instruments, China) were used to cut the blood vessel at the electrocoagulation site. This was to ensure that the blood flow would be completely blocked. Then, 45 min after clamping, the bilateral CCA vascular clamp was removed. The whole operation was performed on an electric blanket to maintain the body temperature of the rats.

Determination of the success of the rat dMCAO model establishment

The model success was confirmed by the hematoxylin and eosin (H&E) staining after microtome sectioning. The neuron morphology in the infarct area was obviously different from those in the normal surrounding area.[17,18]

Cell transplantation

The dMCAO model rats were randomly divided into an ischemic control group and a BM-MSC transplantation group. Cell transplantation was performed as previously reported,[14] in the ischemic control group, 1 ml of saline was injected through the tail vein 1 h after the model was generated. In the BM-MSC transplantation group, 1 ml of saline containing a suspension of 1×10^6 BM-MSCs were injected through the tail vein 1 h after the model was generated.

Extraction of the brain tissue protein and determination of TGF-β1 content in rat infarcted area

After the rat cerebral infarction model was established, we collected venous blood from rats 48 h after transplanting BM-MSCs through the tail vein, and the plasma was collected after centrifugation.

After being subjected to deep anesthesia, the animals were decapitated. The cerebral cortex of the infarcted areas and the striatum below the infarcted cortex tissues were isolated (Supplementary Figure 1), washed with precooled PBS (4 °C), and put into the protein extraction liquid, which containing protease inhibitors. The brain tissue was thoroughly homogenized and centrifuged at $12000 \times g$ for 1 h at 4 °C, the supernatant containing the protein was then removed. The protein was quantified by a BCA protein assay kit (Thermo Fisher, USA). The TGF-β1 content in certain amount of rat brain tissue extract (equal to 40 μg total protein) was measured according to the instructions of an ELISA kit (Abnova Biotechnology, USA).

Immunohistochemistry and cell counting

The damage of dMCAO infarct model used in this experiment focuses in the parietal and temporal cortex. Rats were anesthetized and transcranial perfusion was performed using 0.9% saline at room temperature (RT), followed by cold 4% formaldehyde (PFA). The brains were then removed, postfixed in 4% PFA for 24 h, and stored in 30% sucrose/PBS at 4 °C. All brains were sectioned on a vibrating microtome (Leica, Germany) at 40 μm thickness, and every 12 sections was taken for staining counting analysis.[19]

In brief, immunofluorescence staining was performed according to previous reports,[16,20,21] floating brain sections were incubated in 0.3% Triton-100/PBS for 30 min and blocked with 2% donkey serum in PBS for 30 min at RT. Sections were incubated overnight with a biotin-conjugated anti-TGF-β1 primary antibody at a dilution of 1:500 (Abnova Biotechnology, USA). On the following day, sections were rinsedin TBS 3 times for 5 min and then they were incubated for 2 h at RT with Cy3-conjugated secondary antibodies at a dilution of 1:300 (Immune-Jackson Inc., CA, USA). The sections were re-blocked with 2% donkey serum in PBS for 30 min, and then incubated with anti-Iba-1 1:300 (Cell Signaling, USA), anti-CD68 1:300 (Merck, Germany), and anti-NeuN 1:300 (Abcam, USA) respectively.

After being washed with PBS for three times, FITC-conjugated secondary antibodies (1:300, Immune-Jackson Inc., CA, USA) was added and incubated with the sections for 2 h at RT, which was followed by DAPI treatment for 20 min. Then, the sections were mounted onto slides. Positive cells were counted using a TCS SP5 II confocal laser scanning microscope (Leica, Wetzlar, Germany) at 200× magnification. The confocal settings, such as gain and offset, were designed to ensure that all pixels of all the selected sections were within the photomultiplier detection range. The settings were maintained to ensure that all images were collected with the same parameters.

For the statistical analysis, we counted the cells in the cortical infarct areas. The counting region is a 1.6 mm × 0.8 mm rectangle (Supplementary Figure 1), which is located in the infarct cortex area and 2.5 mm away from the demarcation line between the infarct and the surrounding area. ImageJ software (National Institutes of Health, USA) was used to mark the 800 μm × 800 μm cortex in the infarcted area.

The cells that were positive for single staining of TGF-β1, and double staining of TGF-β1 with Iba-1, CD68 or NeuN, within the detection range were counted based on the number of cells per square millimeter and corrected for section thickness.

Statistical analyses

Data were acquired in a blinded and unbiased fashion. Data are expressed as the mean ± standard error of the mean (SEM). The comparisons were analyzed by one-way analysis of variance (one-way ANOVA) and Bonferroni Dunnett corrections using SPSS 19.0 (IBM Corp., USA). The level of significance of all comparisons was set at $P < 0.05$.

Results

Comparison of the TGF-β1 content in the cortex infarct area, ipsilateral striatum and plasma of rats

Forty-eight hours after establishing the cerebral infarction in rats, the TGF-β1 content in the infarcted area in the BM-MSC transplantation group (23.94 ± 4.48 pg/ml) was significantly lower than it was in the ischemic control group (34.18 ± 4.32 pg/ml) (F = 13.534, $P = 0.006$). This suggests that TGF-β1 content decreased after transplantation of BM-MSCs. In the injured striatum, the TGF-β1 content in the BM-MSC transplantation group (13.13 ± 7.95 pg/ml) was not significantly different from that in the ischemic control (10.71 ± 6.99 pg/ml) (F = 0.262, $P = 0.623$). Forty-eight hours after ischemia induction, the TGF-β1

content in the rat plasma in the BM-MSC transplantation group (75.91 ± 12.53 pg/ml) was significantly lower than it was in the ischemic control group (131.18 ± 16.07 pg/ml) (F = 36.779, $P = 0.0002$), suggesting that after transplantation of BM-MSCs, the TGF-β1 content in plasma decreased.

The total number of cortical nucleated cells increases, but the TGF-β1 expressing cells decreases in rats with cerebral infarction

Statistical data of immunofluorescence staining showed that the total number of nucleated cells in the infarcted area of the ischemic control group (391.67 ± 69.50 cells/mm^2) was significantly different from that of the transplanted group (1037.67 ± 222.16 cells/mm^2) (F = 92.421, $P < 0.01$).

The number of TGF-β1$^+$ cells in the ischemic control group cells was 72.33 ± 32.08 cells/mm^2, while BM-MSC treatment reduced the number of TGF-β1$^+$ cells in the same area to 35.00 ± 13.66 cells/mm^2 (F = 37.680, $P < 0.01$). The percentage of TGF-β1$^+$ cells out of the total number of nucleated cells in the transplantation group ($3.37 \pm 0.63\%$) was lower than it was in the ischemic control group ($18.47 \pm 5.24\%$), and the difference was statistically significant (F = 106.180, $P < 0.01$).

Iba-1$^+$ cell is one of the major contributors to TGF-β1 expression

We have shown that in the ipsilateral ischemia core cortex, Iba-1$^+$ cells are one of the main sources of BDNF production. We performed double immunohistochemical staining for TGF-β1 and Iba-1 (Fig. 1) at 48 h after ischemia, and found that in the infarct areas, a number of TGF-β1 staining overlapped with Iba-1. Iba-1$^+$ microglia cells in the transplantation group (83.67 ± 31.88 cells/mm^2) were significantly reduced in comparison to that of the ischemic control group (106.67 ± 20.21 cells/mm^2) (F = 4.455, $P = 0.046$). TGF-β1$^+$/Iba-1$^+$ microglia cells in the transplantation group (3.67 ± 3.17 cells/mm^2) were significantly reduced in comparison to that of the ischemic control group (13.67 ± 5.52 cells/mm^2) (F = 29.641, $P < 0.01$). The proportion of double-positive microglia cells (TGF-β1$^+$/Iba-1$^+$) out of all nucleated cells in the transplantation group ($0.35\% \pm 0.28\%$) was significantly lower than it was in the ischemic control group ($3.49\% \pm 1.02\%$) (F = 102.462, $P < 0.01$). Additionally the TGF-β1$^+$/Iba-1$^+$ proportion out of the total number of Iba-1$^+$ microglia cells in the transplantation group

($4.38\% \pm 3.18\%$) was lower than it was in the ischemic control group ($12.81\% \pm 4.86\%$) (F = 28.125, $P < 0.01$), while the proportion of TGF-β1$^+$/Iba-1$^+$ microglia cells out of the total number of TGF-β1$^+$ cells in the transplantation group ($10.48\% \pm 8.12\%$) decreased dramatically when compared to that of the ischemic control group ($18.89\% \pm 7.24\%$) (F = 9.720, $P = 0.005$) (Figs. 1 and 4).

These results suggest that cerebral infarction in rats significantly reduces the number of TGF-β1$^+$/Iba-1$^+$ microglia in the infarcted area after BM-MSC transplantation (Figs. 1 and 4).

CD68$^+$ microglia and NeuN$^+$ neurons are partly responsible for TGF-β1 expression after the induction of ischemia

CD68$^+$ microglia and TGF-β1 expression
After ischemia, CD68$^+$ microglia cells were quantified (72.33 ± 21.87 cells/mm^2), and the BM-MSC treatment dramatically increased the cell number to 122.67 ± 47.80 cells/mm^2 (F = 11.003, $P = 0.003$). The proportion of CD68$^+$ microglia out of all nucleated cells in the transplantation group ($11.82\% \pm 4.74\%$) was significantly lower than it was in the ischemic control group ($18.47\% \pm 3.59\%$) (F = 13.055, $P = 0.003$). TGF-β1$^+$/CD68$^+$ double-positive cells were quantified (7.67 ± 7.71 cells/mm^2) after ischemia induction, and the BM-MSC treatment did not change the cell number significantly (9.00 ± 6.41 cells/mm^2) (F = 0.212, $P = 0.650$).

The proportion of TGF-β1$^+$/CD68$^+$ microglia cells out of total nucleated cells showed no difference between the transplantation group ($0.87\% \pm 0.53\%$) and the ischemic control group ($1.96\% \pm 1.78\%$) (F = 3.579, $P = 0.072$). Additionally, the proportion of TGF-β1$^+$/CD68$^+$ microglia cells out of all CD68$^+$ microglia cells and TGF-β1$^+$ cells between the transplantation group and ischemic control group was not different (F = 0.521, $P = 0.478$) (Figs. 2 and 4).

NeuN$^+$ neurons and TGF-β1 expression
BM-MSC treatment after ischemia induction caused the number of NeuN$^+$ neurons (302.33 ± 40.88 cells/mm^2) to increase almost three-fold over that of the control (115.33 ± 35.40 cells/mm^2) (F = 143.488, $P < 0.01$). The proportion of NeuN$^+$ neurons out of all nucleated cells between the transplantation group and the ischemic control group showed no difference (F = 0.047, $P = 0.830$).

After ischemia, the number of TGF-β1$^+$/NeuN$^+$ cells was 8.00 ± 5.66 cells/mm^2 field and BM-MSC

treatment did not cause any difference in the cell number (6.33 ± 3.17 cells/mm^2) (F = 0.793, $P = 0.383$). The proportion of TGF-β1$^+$/NeuN$^+$ cells out of total nucleated cells in the transplantation group ($0.61\% \pm 0.26\%$) was significantly lower than it was in the ischemic control group ($2.04\% \pm 1.44\%$) (F = 10.981, $P = 0.003$).

The proportion of TGF-β1$^+$/NeuN$^+$ double-positive cells out of the total number of NeuN$^+$ neurons was $6.94 \pm 6.06\%$ after ischemia, and BM-MSC treatment reduced the proportion to $2.09 \pm 0.94\%$ (F = 8.943, $P = 0.007$). However, the proportion of TGF-β1$^+$/ NeuN$^+$ double-positive cells out of all TGF-β1$^+$ cells between the transplantation group and ischemic control group showed no difference (F = 2.487, $P = 0.129$) (Figs. 3 and 4).

Discussion

Due to the obvious aging trend of the Chinese population, the incidence of stroke is on the rise, resulting in a huge disease burden of the society. Despite the improvement in the clinical diagnosis and treatment technology of cerebrovascular disease, the
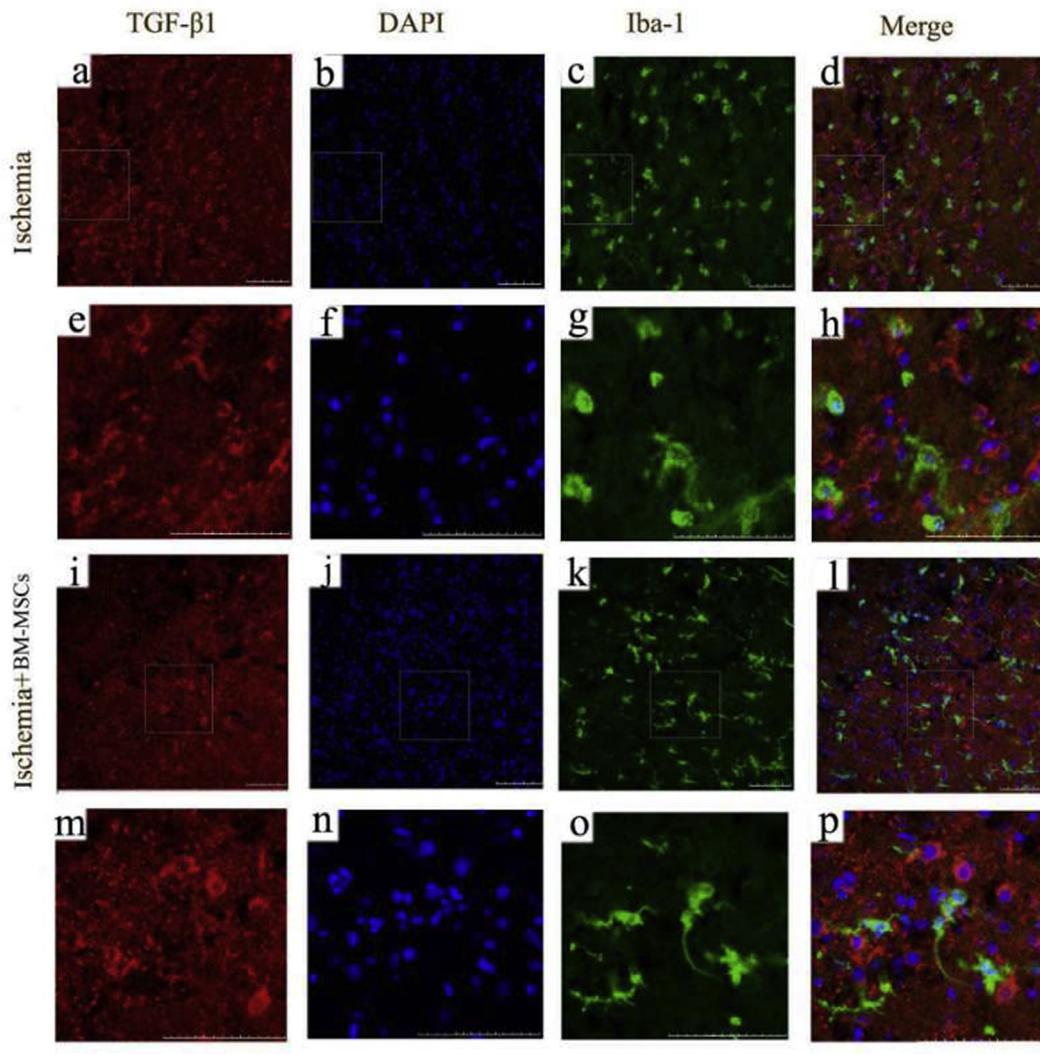

Fig. 1. TGF-β1 and Iba-1 immunofluorescence staining in the infarcted area for the ischemic control group and the BM-MSC transplantation group at 48 h. a–d: TGF-β1, DAPI, and Iba-1 immunostaining images and combined image (original magnification 200×) 48 h after ischemia induction in the ischemic control group; e–h: Enlarged area of the squares in a–d (original magnification 600×); i–l: The BM-MSC transplantation group, TGF-β1, DAPI, and Iba-1 immunostaining (original magnification 200×) 48 h after ischemia induction; m–p: Enlarged area of the squares in i–l (original magnification 600×). Scale bar, 50 μm. TGF-β1: transforming growth factor-β1; BM-MSC: bone marrow mesenchymal stem cell.
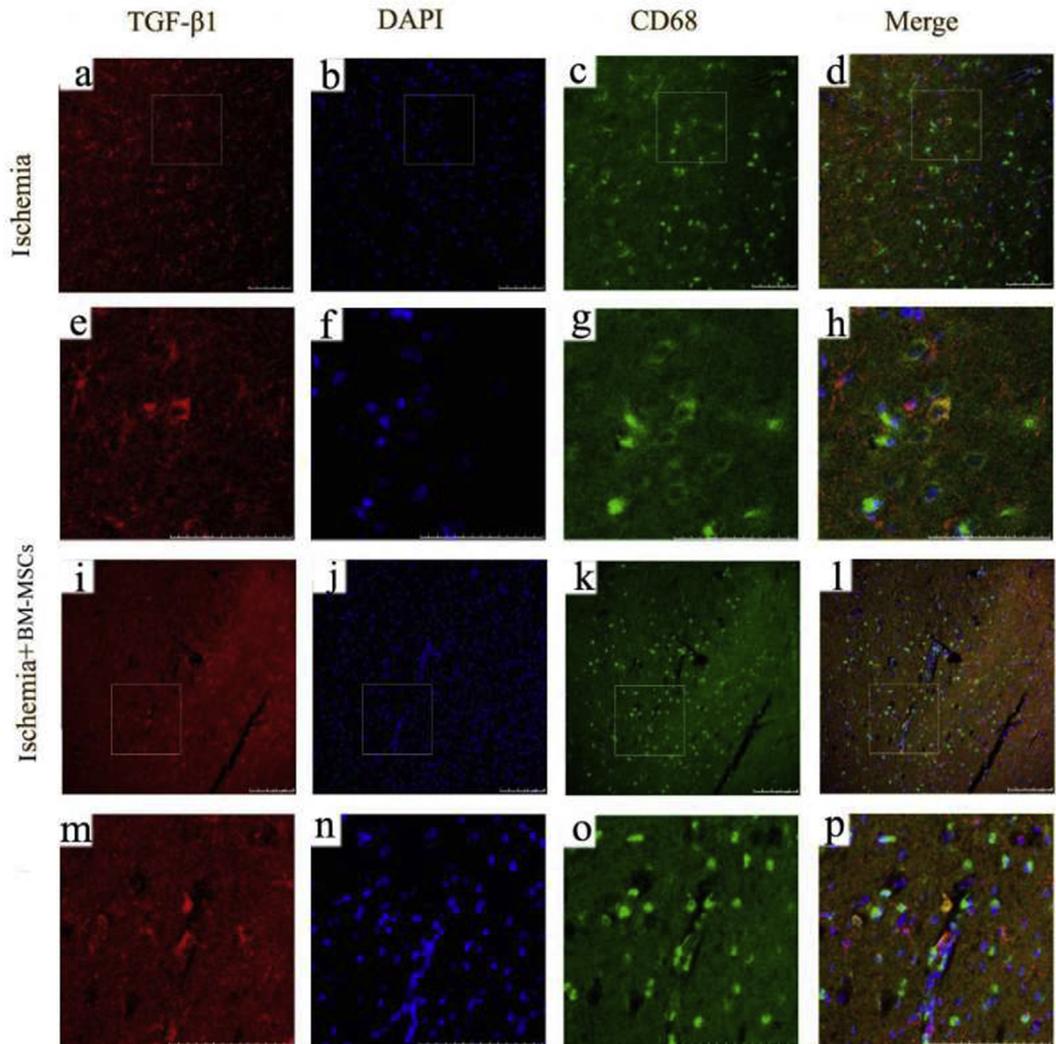
Fig. 2. TGF-β1 and CD68 immunofluorescence staining in the infarcted cortex area for the ischemic control group and the BM-MSC transplantation group at 48 h. a−d: TGF-β1, DAPI, and CD68 immunostaining images and merged images (original magnification 200×) 48 h after ischemia induction in the ischemic control group; e−h: Enlarged area of the squares in a−d (original magnification 600×); i−l: The BM-MSC transplantation group, TGF-β1, DAPI, and CD68 immunostaining (original magnification 200×) 48 h after ischemia induction; m−p: Enlarged area of the squares in i−l (original magnification 600×). Scale bar, 50 μm. TGF-β1: transforming growth factor-β1; BM-MSC: bone marrow mesenchymal stem cell.

survivors still usually exhibit different degrees of neurological deficits. The deficits may affect future ability to work or even to live. Stem cell research and tissue repair/regeneration have emerged as promising solutions for such problems. Up to now, there have been more than 50 clinical trials using neural stem cells or adult stem cells to treat cerebral infarction on the website clinicaltrials.gov.[22−27]

Both TGF-β1 and BM-MSC are important immunosuppressive factors in the pathophysiology process of cerebral ischemia. In our study, we wanted to address the following question: Does intravenously transplanted BM-MSCs have a synergistic or antagonistic effect with TGF-β1 in acute phase?

In this study, we established a rat dMCAO model and used ELISA to measure the TGF-β1 content in the cerebral infarction area, striatum area, and plasma at 48 h after cerebral infarction. To find what type of cells are the sources of TGF-β1 expression, TGF-β1 was used in combination with staining for the markers of microglia cells and neurons, such as Iba-1, CD68 and NeuN. Quantitative analysis was applied to observe the number
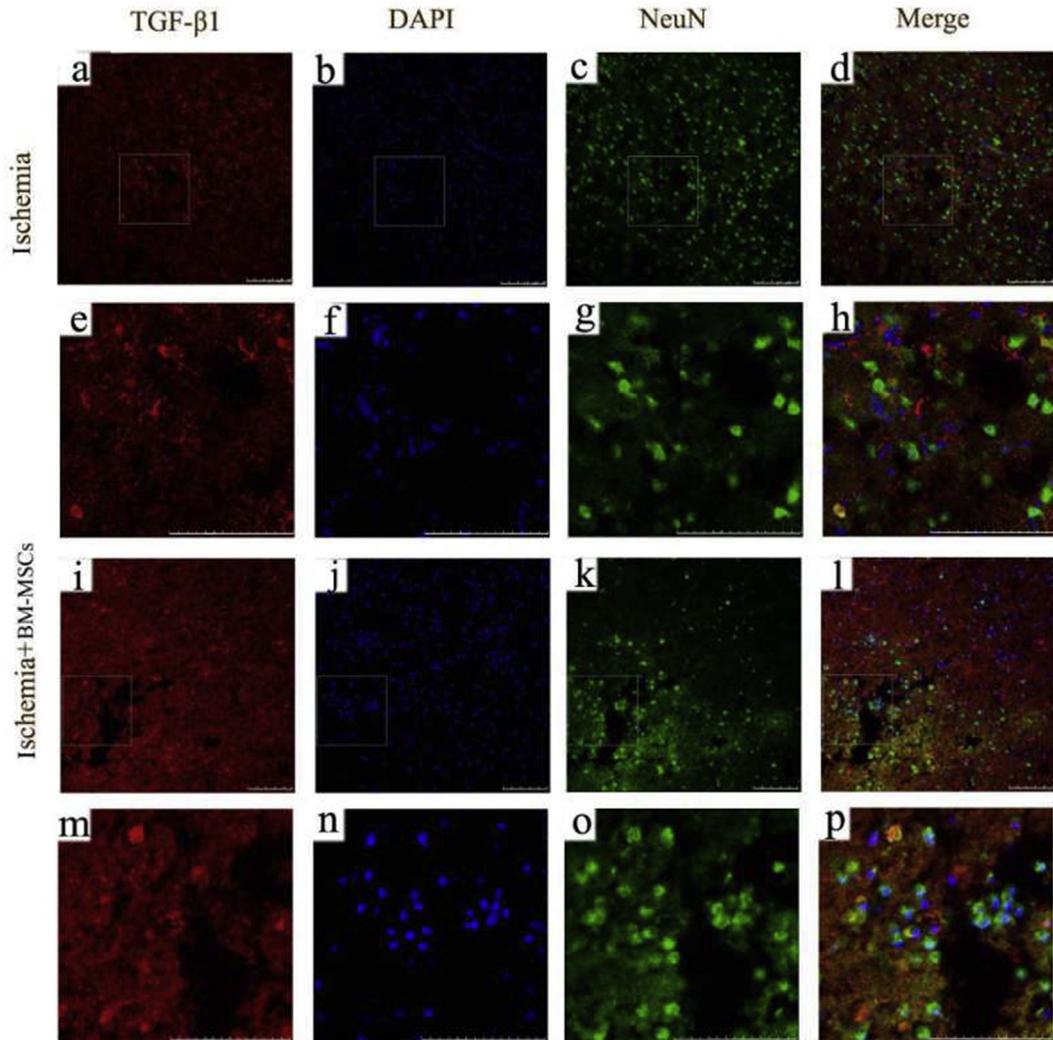
Fig. 3. TGF-β1 and NeuN immunofluorescence staining in the infarcted cortex area for the ischemic control group and the BM-MSC transplantation group at 48 h. a—d: TGF-β1, DAPI, and NeuN immunostaining map and combined graph (original magnification 200×) 48 h after ischemia induction in the ischemic control group (original magnification 600×); e—h: Enlarged area of the squares in a—d; i—l: The BM-MSC transplantation group, TGF-β1, DAPI, and NeuN immunostaining (original magnification 200×) 48 h after ischemia induction; m—p: Enlarged area of the squares in i—l (original magnification 600×). Scale bar, 50 μm. TGF-β1: transforming growth factor-β1; BM-MSC: bone marrow mesenchymal stem cell.

and the ratio of single and double TGF-β1 positive cells in the cerebral infarction area. Additionally, the impacts of BM-MSC transplantation on the number and proportion of TGF-β1+ cells were analyzed too.

We explored the potential effects of plasma TGF-β1 on the brain TGF-β1 level. In the physiological condition, the TGF-β1 in periphery circulation can not go through BBB freely.[28] In the acute phase of cerebral ischemia, the TGF-β1 in blood may go into the brain easier due to the breakdown of BBB. In our experiment, BM-MSC transplantation significantly reduced the plasma TGF-β1 concentration in the periphery circulation, therefore may reduce the amount of TGF-β1 go into the brain too. Our results suggest the reduction of TGF-β1 in blood may contribute to the decreasing of TGF-β1 in the infarct area after BM-MSC transplantation.

Microglia and astrocytes are widely distribute in the entire brain. They not only maintain the normal function of neurons,[29] but also constitute the immune defense system of the central nervous system together.[30] TGF-β1 is a multifunctional pleiotropic cytokine that
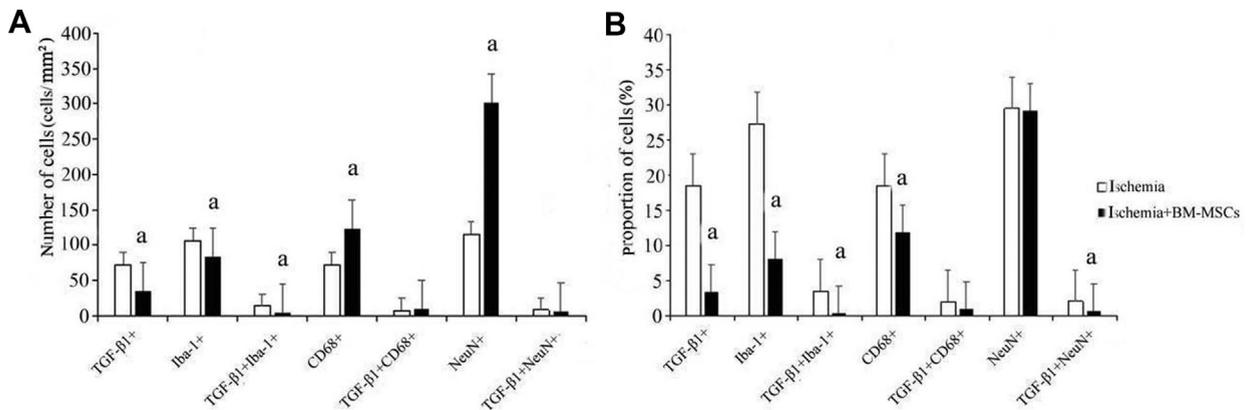
Fig. 4. The summary of the number and proportion of TGF-β1^{+} single and double positive cells in the infarct cortical area. A: The number of TGF-β1^{+}, Iba-1^{+}, CD68^{+}, NeuN^{+} single positive cells and TGF-β1^{+}/Iba-1^{+}, TGF-β1^{+}/CD68^{+}, TGF-β1^{+}/NeuN^{+} double positive cells at 48 h after ischemia induction; B: The proportion of these cells out of all nucleated cells at 48 h after ischemia induction. [a] $P < 0.05$, BM-MSC transplantation group vs ischemic control group. TGF-β1: transforming growth factor-β1; BM-MSC: bone marrow mesenchymal stem cell.

can be produced both by microglia cells and astrocytes in response to brain injury.[31,32]

Astrocytes are one of the main sources secreting trophic factors, cytokines and growth factors in the central nervous system. It was found that astrocyte is the main cell type that increase TGF-β1 after stroke in the MCAO model.[33] Although these experiments were performed on MCAO model at 14 days after the ischemia injury, obviously different from that we observed TGF-β1 expression in dMCAO model at 48 h after ischemia, the results about astrocyte are not especially novel, so we did not focus on astrocytes in this article.

In this study, at 48 h after cerebral ischemia induction in rats, one of the main TGF-β1-producing cells is activated Iba-1^{+} microglia/macrophages in the cortical infarct core. The transplantation of BM-MSCs reduced the number of TGF-β1[+]/Iba-1^{+} microglia cells. The proportion of TGF-β1+/Iba-1^{+} cells in the total Iba-1^{+} microglia cells was eventually reduced too, indicating that after BM-MSCs transplantation, fewer microglia cells are capable of expressing TGF-β1 due to unknown mechanisms.

As an anti-inflammatory cytokine, TGF-β1 can transform microglia from an activated state to a resting state in the central nervous system. Our data lead to a confirmation that BM-MSCs exert a neuroprotective mechanism in the dMCAO model after transplantation may not occur through immunosuppression.

At 48 h after dMCAO induction, the function of BM-MSCs is not immune-suppressive. The reasons for the failure of BM-MSCs to promote immunosuppression and inflammatory regulation may be as follows: BM-MSCs require a certain amount of time to exert anti-inflammatory effects, and in vitro experiments usually require 4 d.[34] The observation point of this experiment is 48 h; BM-MSCs need activation by higher levels of inflammatory factors in the body, such as TNF-α, IFN-γ, and IL-1β, which can play a role in inhibiting lymphocyte proliferation and in inhibiting microglial cells.[35] The dMCAO model in this experiment had relatively minor damage, and there might not have been a sufficiently high concentration of inflammatory factors in the cerebral infarct area at 48 h after transplantation.

The BM-MSCs which was in the in-activated status were capable of suppressing the expression of TGF-β1 in microglia cells. The mechanisms are worth being studied in the future.

Our previous studies showed that the behavioral improvement and the reduction of infarct volume in rats after transplantation of BM-MSCs was likely to be related to the increase of neurotrophic factors in the rat brain.[12] TGF-β1 is considered as a neurotrophic factor in some research articles. The expression of TGF-β1 in microglia/macrophages regulates the expression of plasminogen activator inhibitor 1 (PAI-1) in astrocytes in a paracrine manner, thereby promoting the activity of endogenous tissue-type plasminogen activator (tPA) and inducing neuronal recovery processes.[36]

Our results showed that after 48 h, BM-MSC transplantation did not promote the expression of the immunosuppressive factor TGF-β1. That means TGF-β1 is not the neurotrophic factor by which BM-MSC exert its neuroprotective effects in dMCAO model.

Finally, it is accepted that the ability of BM-MSCs to exert immune-suppression and anti-inflammation effects may be time related. In the acute phase, BM-

MSC transplantation did not promote the expression of TGF-β1 at 48 h, but mainly promote the production of neurotrophic factors, which are produced by transplanted BM-MSCs and via the induction of host cell production. The function of intravenous grafting of BM-MSCs, no matter neurotrophic effects or immune and inflammation inhibitory effects, may vary at different time points after the cerebral ischemia.[12]

Therefore, we will further study and explore the mechanisms that play a role in BM-MSC effects, for example the crosstalk of BM-MSCs and TGF-β1 at other time points.

In conclusion, intravenous transplantation of BM-MSCs reduced the plasma TGF-β1 levels at 48 h after ischemia and transplantation. Iba-1$^+$ microglia is the main cell type producing TGF-β1. Transplantation of BM-MSCs reduces both the total number of TGF-β1$^+$ cells and the TGF-β1 expressing proportion of Iba-1$^+$ microglia cells in the cerebral infarction area. In the acute phase of dMCAO rat model, intravenously transplanted BM-MSCs have an antagonistic effect with TGF-β1 with the respect to immune-regulation.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdtm.2020.05.005.

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