Stem Cell Therapy

Bone marrow-derived mononuclear cells ameliorate neurological function in chronic cerebral infarction model mice via improvement of cerebral blood flow

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ABSTRACT

Background aims: Stroke is a frequently observed neurological disorder that might lead to permanent and severe disability. Recently, various regenerative therapies have been developed, some of which have already been applied clinically. However, their outcomes have not been fully satisfactory. In particular, the development of regenerative therapies for chronic ischemic stroke is greatly needed. Herein intracerebral administration of bone marrow-derived mononuclear cells (BM-MNCs) was assessed as a potential treatment for chronic ischemic stroke using a severe combined immunodeficiency mouse model characterized by minimal vascular variation unrelated to immunodeficiency.

Methods: A reproducible model of permanent middle cerebral artery occlusion was prepared, and intracerebral BM-MNC transplantation was performed 14 days after stroke induction in the infarcted brain.

Results: Sensorimotor behavioral function and cerebral blood flow were significantly improved upon treatment with BM-MNCs compared to control medium injection. The transplanted cells exhibited characteristics of the vascular endothelium and microglia/macrophages. Significant angiogenesis and suppression of astrogliosis and microgliosis were observed in the affected brain. Messenger RNA expression analysis showed significant increases in anti-inflammatory cytokines, A2 astrocyte/anti-inflammatory microglia markers and vascular endothelial markers such as vascular endothelial growth factor and significant decreases in pro-inflammatory cytokines and A1 astrocyte/pro-inflammatory microglia markers following BM-MNC transplantation.

Conclusions: These results suggest that intracerebral administration of BM-MNCs should be considered an effective cell therapy for chronic stroke.

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Introduction

Stroke is one of the major causes of motor impairment, and its incidence has been on the rise with the aging population [1]. Among different types of stroke, cerebral infarction is the most prevalent, and despite current treatment methods, many patients with cerebral infarction end up bedridden, which reduces both quality of life and activities of daily living [1–4]. Currently, as a treatment for cerebral infarction, administration of thrombolytic drugs and thrombectomy can be performed if specific conditions are met in the hyperacute phase; if a favorable therapeutic effect is obtained, complete recovery may be attained without any sequelae [5–7]. However, these treatments can be applied only in limited cases; in many other instances, the sequelae remain [1].

Various regenerative therapies are being developed, some of which have been shown to be effective; however, they are not entirely satisfactory, and more effective methods are needed to address the complete recovery of paralytic symptoms and sensory function [8,9]. Large numbers of mononuclear cells (MNCs) can be obtained from the bone marrow without amplification and prepared immediately from stroke patients via bone marrow
angiogenesis by vascular endothelial progenitor cells and hematopoietic stem cells is the main focus of regenerative medicine and is considered advantageous, as these cells are included in MNCs [12–14]. Therefore, separating only the specific fractions containing vascular endothelial progenitor cells or hematopoietic stem cells may promote angiogenesis efficiently. However, the preparation of those cells is time-consuming and costly. Conversely, MNCs are considered suitable for stroke treatment because of their ease of preparation and high potential for angiogenesis, making them favorable for clinical application [15,16]. Despite several reports on the development of regenerative therapies for stroke using MNCs, the mechanisms underlying their therapeutic effects and their application in the chronic state have not been fully elucidated [17,18]. This is attributed to the difficulty in establishing a stable chronic phase cerebral infarction model in mice, and detailed analysis of such a model has not been sufficiently performed [19,20]. Therefore, in this study, a cerebral infarction mouse model was established using a severe combined immunodeficiency (SCID) mouse known as a strain with reduced cerebral artery variation [19]. Transplantation of bone marrow-derived MNCs (BM-MNCs) was attempted in the SCID mice as a therapeutic method for chronic phase cerebral infarction, and the mechanism underlying their effects was investigated.

Methods

Experimental design

All animal experimental protocols were approved by the Shiga University of Medical Science Institutional Animal Care and Use Committee. All animal procedures were performed in accordance with the guidelines of the Shiga University of Medical Science Research Center for Animal Life Science and the National Research Council’s Guide for the Care and Use of Laboratory Animals. The design of the animal experiments is shown in Figure 1.

Animals

For this study, male 8-week-old SCID mice (CB-17/lcr-scid/scidJcl) were purchased from CLEA Japan, Inc (Tokyo, Japan). C57BL/6-Tg(ubiquitin–green fluorescent protein [GFP])30FlucJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and used for cell transplantation. Mice were housed under a 12-h light/dark cycle and allowed free access to food and tap water.

Animal model of brain infarction

The brain infarction model was created using a previously described method [19]. SCID mice were anesthetized via the intraperitoneal administration of medetomidine (0.15 mg/kg) and butorphanol (0.2 mg/kg). After careful confirmation of deep anesthesia, the skin was disinfected with 70% ethanol, and an incision was made vertically between the left ear canal and left orbit. The connective tissue under the skin was peeled and the surface of the skull was exposed. The left middle cerebral artery (MCA) was confirmed through the skull, and a 2-mm-diameter hole was made in the skull around the MCA. Subsequently, the MCA was exposed, electrocoagulated and disconnected. Thereafter, it was confirmed that the distal portion of the MCA no longer exhibited its red color, and the connective tissue and skin were sutured. After recovery from anesthesia, the behavior of the mice was carefully observed, confirming that they were eating and drinking enough without losing activity due to pain. Among the mice that underwent MCA occlusion (MCAO), those that scored less than 7 on the Basso Mouse Scale (BMS) from day 1 to day 14 after the procedure and survived until day 14 were included in the transplantation study. Mice that died by day 14 or lost more than 20% of their body weight during the study period were excluded. Of the initial 130 mice that underwent MCAO, 20 failed to meet the criteria and 110 advanced to the transplantation study. No mice were dropped from the study.

BM-MNC transplantation therapy in a mouse model of cerebral infarction

Whole bone marrow cells were obtained from the bilateral humerus, femur, tibia and ilium of the GFP transgenic mice. BM-MNCs were isolated from whole bone marrow cells by the specific centrifugal method using Ficoll-Paque (GE Healthcare, Chicago, IL, USA). Whole bone marrow cells were suspended in 4 mL phosphate-buffered saline (PBS) and carefully overlaid on 3 mL of Ficoll-Paque medium. The Ficoll gradients were centrifuged at 400 × g for 30 min without a brake. A BM-MNC layer was collected using a sterile pipette, washed in PBS, counted using a hemocytometer and suspended in Dulbecco’s Modified Eagle’s Medium and

Figure 1. MCAO and schematic experimental design of BM-MNC transplantation therapy in mouse model of chronic cerebral infarction. (A) Left panel shows the main trunk of the left MCA after left temporal craniotomy in SCID mouse. Black dotted lines show the left MCA margin. The two pictures on the right show the MCA after electrocoagulation and cutting. (B) Optical image of mouse brain tissue with cerebral infarction under stereomicroscope. The vertical white dotted line shows the median line of the whole brain. The horizontal white dotted line indicates a line drawn across the bregma. The center red spot shows the bregma. The lower left dot indicates the point of BM-MNC administration, which is 3 mm left of and 2 mm caudal to the bregma. (C) Timeline of the experimental procedure and evaluation protocol. Orange arrows show the time points of left MCAO procedure (day 0) and BM-MNC administration (day 14). On day 14, mice were divided into three groups: sham (burr hole only), medium CTL (burr hole + medium injection) and BM-MNCs (burr hole + BM-MNC injection). The small black squares on the gray lines indicate when each behavioral or histological experiment (left) was performed. The black arrows on the gray lines indicate that all evaluations were performed on day 28. CTL, control.
Ham F-12 (#048-2978S; Wako, Osaka, Japan) with 5% fetal bovine serum (biosera, Cholet, France). The concentration was adjusted to 1 × 10^7 MNCs/3 μL for transplantation.

Two weeks after cerebral infarction, the SCID mice were randomly divided into three groups: sham treatment, medium control, and BM-MNCs. Allocation concealment procedures were applied, and all experiments were performed in a blinded manner. Mice were anesthetized by intraperitoneal administration of drugs as described earlier and fixed to a stereotactic apparatus (SR-6; Narishige Scientific Instrument Lab, Tokyo, Japan). The skin was disinfected with 70% ethanol, and a midline incision was made. A small burr hole was created on the left side. In the sham treatment group, a burr hole was created without injection. In the BM-MNC group, BM-MNCs (1 × 10^7 MNCs/3 μL) were injected slowly for 5 min using a Hamilton syringe (GL Sciences, Tokyo, Japan) with a 30-gauge (G) needle 3 mm to the left, 2 mm dorsal to the bregma and 2.5 mm vertical from the dura. In the medium control group, 3 μL of Dulbecco’s Modified Eagle’s Medium and Ham F-12 was injected at the same location without BM-MNCs. The skin was closed following the procedure.

Behavioral tests

The motor and sensory functions of mice with brain infarction were assessed before (pre-) and on day 1, day 7, day 14, day 15, day 21 and day 28 after stroke using the grid walk test, cylinder test, BMS and von Frey test. On the day of MCAO or the day before cerebral infarction, after several rounds of training in the same methods, basal control function was evaluated as a pre-stroke condition. On day 14, these data were evaluated before the treatment intervention. In the grid walk test, mice were placed on a wire grid (20 × 20 × 20 cm) with many squares measuring 1.0 × 1.0 cm each, and a video was recorded for 5 min. The video recordings were manually observed in slow motion. The number of missed forelimb steps on the affected side was counted for the first 100 steps. The deficit score was calculated as the percentage of missed steps per 100 steps [21]. In the cylinder test, mice were placed in a cylinder (diameter 11.5 cm and height 20 cm), and their movements were recorded using a video camera. In this test, the frequency of the use of the forelimbs was separately counted on both the ipsilateral and contralateral sides to assess sensorimotor function. The recordings were made until the mice reared at least 20 times in contact with the wall of the cylinder or until 10 min had elapsed. The video recordings were later manually observed in slow motion. Two points were evaluated: fine touch and paw dragging on the impaired side. For fine touch, the frequency of contact with the wall by the right, left or both forelimbs was counted and the ratio of impaired side use was calculated using the following formula: (number of ipsilateral contacts + [bilateral contacts]) / (number of ipsilateral + contralateral + bilateral contacts) [22]. The ratio of paw dragging was calculated using the following formula: (number of paw drags) / (total number of paw touches) [23,24].

For BMS, each mouse was placed individually in a 50 × 50 × 30-cm open field (LimeLight; Neuroscience, Tokyo, Japan). The locomotion of each mouse was observed for 5 min, and movement was evaluated using a BMS score of 0–9 points [25,26]. In the von Frey test, mice were placed individually in a clear cage on a wire mesh floor to measure the withdrawal response time of the right hind paw to a von Frey filament using the up–down method [27]. The 0.4-G, 0.6-G, 1-G, 1.4-G, 2-G, 4-G and 6-G von Frey filaments (Tactile Test [Aesthesiology] Semmes–Weinstein von Frey Aesthesiometer; Muromachi Kikai Co, Ltd, Tokyo, Japan) were applied to the mid-plantar surface of the right hind paw until it bent. The test started with a 1-G filament, and the up or down filament was applied according to the Dixon table [27]. Next, the tabular value was determined using the filament response patterns [28]. Subsequently, a 50% withdrawal threshold was calculated using the tabular values [27].

2,3,5-triphenyltetrazolium chloride staining

Mice were exsanguinated and perfused with PBS on ice under deep anesthesia (midazolam [4 mg/kg] + medetomidine [0.15 mg/kg] + butorphanol [0.2 mg/kg]). The whole brain was carefully removed, cut into 2-mm coronal sections and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St Louis, MO, USA) for 20 min at room temperature [19,29]. After TTC staining, the non-infarcted area (red) was measured using ImageJ 1.53e (National Institutes of Health, Bethesda, MD, USA), and the ratio of the pre-served area to the whole brain or cortex was calculated using the following formula: (ipsilateral non-infarct area) / (contralateral non-infarct area).

Immunohistochemistry

Mice were deeply anesthetized via intraperitoneal administration of midazolam (4 mg/kg) + medetomidine (0.15 mg/kg) + butorphanol (0.2 mg/kg) and perfused transcardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer for fixation. After perfusion fixation, whole mouse heads were kept with 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. The fixative was replaced with 15% sucrose in 0.1 M phosphate buffer the next day. Subsequently, the sucrose buffer was replaced several times with fresh buffer. The fixed brains were isolated, embedded in an optimal cutting temperature compound (Tissue-Tek; Sakura Finetek Japan Co, Ltd, Tokyo, Japan) and frozen in liquid nitrogen. The frozen brains were cut into 10-μm sections using a cryostat (CM3050 S; Leica). For immunostaining, the sections were incubated with 5% normal goat serum in 0.3% Triton X-100 in PBS at room temperature for 30 min to block non-specific immunoreactions. After blocking, the sections were incubated at 4°C overnight with the following primary antibodies: sheep anti-von Willebrand factor (vWF) (1:50) (Abcam, Cambridge, UK), rabbit anti-microtubule-associated protein 2 (MAP2) (1:100) (Cell Signaling Technology, Inc, Danvers, MA, USA), rabbit anti-β-gal fibrillary acidic protein (GFAP) (1:100) (EnCor Biotechnology Inc, Gainesville, FL, USA) and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:100) (Wako). The next day, the sections were incubated at 4°C overnight with a secondary antibody–donkey anti-sheep Alexa Fluor 555 (1:500) (Thermo Fisher Scientific, Waltham, MA, USA) or donkey anti-rabbit Alexa Fluor 555 (1:1000) (Thermo Fisher Scientific)—against the corresponding species. The sections were then mounted using VECTASHIELD containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc, Burlingame, CA, USA). A Leica TCS SP8 X confocal laser scanning microscope was used to observe the fluorescence images using Leica Application Suite X software.

To investigate the effect of the treatment on the surrounding tissue, immunohistochemistry was conducted on more than four sections per mouse (with an interval of greater than 20 μm) starting from the implantation site and extending 0.5 mm caudally. The sections were stained for vWF, MAP2, GFAP and Iba1. For vWF staining, the number of positively stained ring-shaped structures was counted in a 0.5 × 0.5-mm area of the ipsilateral cortex and compared between the control and treatment groups. For MAP2 staining, the fluorescence intensity was measured in a 0.5 × 0.5-mm area using ImageJ 1.53e, and the ratio against the contralateral side was calculated and evaluated for treatment effects. For GFAP and Iba1 staining, the number of positively stained cells was counted per 4,6-diamidino-2-phenylindole-
positive number in a 1 × 1-mm area of the ipsilateral thalamus and compared between the control and treatment groups.

Messenger RNA gene expression analysis

On the 14th day after treatment (28 days after cerebral infarction), brains were removed under deep anesthesia, cut into 2-mm widths 1 mm caudal to the implant site to avoid inflammation by needle injury, divided into ipsilateral and contralateral sides and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen brains using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer’s protocol. Reverse transcription was performed from 1 μg total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Kusatsu, Japan) according to the manufacturer’s protocol. Quantitative reverse transcription polymerase chain reaction was performed using Luna Universal qPCR Master Mix (M3003L; New England Biolabs, Ipswich, MA, USA) on a LightCycler 480 System II (Roche Diagnostics, Manheim, Germany) according to the manufacturer’s protocol.

We used the following primer pairs: interleukin (IL)-4 forward 5′- TCAACCCCGACTGGTTGTC-3′ and IL-4 reverse 5′- TGTTCTCGTGGTGCTGGAGG-3′; IL-10 forward 5′- CCAAGCTTGGCCGAGGAG-3′ and IL-10 reverse 5′- TTTTCAAGGCGGAATTGCTG-3′; transforming growth factor beta (TGF-β) forward 5′- CAGAGCTGCGCTTGCAGAG-3′ and TGF-β reverse 5′- GTTACGACCCGGTGTACCAAG-3′; tumor necrosis factor alpha (TNF-α) forward 5′- CAGCCCGTTGAGCAAG-3′ and TNF-α reverse 5′- GATACAAATCCGTCGAGGTGTTG-3′; IL-6 forward 5′- ACGGCTTCCTGCTCATACA-3′ and IL-6 reverse 5′- CATTCCACAGATTTCCCAAG-3′; IL-1β forward 5′- CAACGCAAAGTTGATTCTCTCCATG-3′ and IL-1β reverse 5′- GATCCACACTCTCTCCAGCTG-3′; insulin-like growth factor 1 (IGF-1) forward 5′- TGGCGAGAAGGCTGGTTTATA-3′ and IGF-1 reverse 5′- CTGCTGTAACGATGAAGCCCTG-3′; CD86 forward 5′- CACACCTTGGAGCAAGAA-3′ and CD86 reverse 5′- TTAGTTTCGGTGGACCTTG-3′; inducible nitric oxide synthase (iNOS) forward 5′- TGGGACGAGTTGTTGAGG-3′ and iNOS reverse 5′- GTAGGTGAGGGCTTGGCTGA-3′; transglutaminase 1 (TGM-1) forward 5′- CCAAGCCTTATCGGACCACT-3′ and TGM-1 reverse 5′- GTAGGTGAGGGCTTGGCTGA-3′; transglutaminase 2 (TGM-2) forward 5′- CACGTCGTAGCAAACCACCAAGTGG-3′ and TGM-2 reverse 5′- GTAGGTGAGGGCTTGGCTGA-3′; and arginase 1 (Arg1) forward 5′- CACGTCGTAGCAAACCACCAAGTGG-3′ and Arg1 reverse 5′- TGTGGAGAGTGTGTGTCG-3′. GAPDH was used as a reference gene to determine the relative expression levels of the target genes using the comparative cycle threshold method.

Measurement of cerebral blood flow

Cortical surface cerebral blood flow (CBF) in mice was measured using a laser speckle flowmetry imaging system (Omezogene; Ome-gawave Inc, Tokyo, Japan) as previously reported [30]. Under anesthesia with 2.0% isofluorane, mice were placed in the prone position, and a linear skin incision was made to expose the surface of the skull. The skull surface was exposed to a 780-nm near-infrared laser light. The scattered light was detected through a hybrid filter using a charge-coupled device camera placed above the head of the mouse. A hybrid filter was used to remove all light other than measured laser light (wavelength 780 nm) and surface-reflected light. The speckle contrast, which indicates the number and velocity of moving erythrocytes, was measured from the raw speckle images obtained in high-resolution mode (638 × 480 pixels, two images/s) using the software (laser speckle blood flow imager) installed in Omezogene. Red indicated high CBF and blue indicated low CBF. Ten consecutive raw speckle images were simultaneously acquired, and the images were averaged five times. The images were analyzed using the LIA software installed in Omezogene. A line drawn from bregma to lambda was used as a guide for setting the size and position of the regions of interest. A square was drawn with the bregma—lambda line on one side and divided into four equal parts parallel to the line, with the outer three quarters defined as the MCA region and the caudal half of the outer quarter defined as the core region. CBF was obtained in arbitrary units using Omezogene, and the corresponding area in the contralateral hemisphere was used to measure CBF and calculate the ipsilateral to contralateral ratio. After measurement of CBF, the skin was closed. No mice were died by these procedures.

Statistical analysis

Data were presented as mean ± standard deviation. The Kruskal–Wallis test was used to calculate statistical significance for comparison of multiple datasets. The Friedman test was used for behavioral tests at each time point. The Mann–Whitney U test was used for two group comparison. Statistical significance was set at P < 0.05. SPSS Statistics 25 (International Business Machines Corporation, Armonk, NY, USA) was used for the analysis.

Results

Experimental design of BM-MNC cell therapy for MCAO-induced chronic cerebral infarction mouse model

SCID mice were selected for the cerebral infarction model because these mice have less arterial variation than other strains (Figure 1). The left MCA was coagulated and cut to generate the cerebral infarction model (Figure 1A). BM-MNCs were implanted 2 mm caudal to and 3 mm left of the bregma at a depth of 2.5 mm (Figure 1B). After MCAO at 8 weeks of age, hemisilateral motor and sensory dysfunction was confirmed using behavioral tests. Fourteen days after MCAO, BM-MNCs were administered (Figure 1C). For evaluation of the therapeutic effects of BM-MNCs, a sham group (trepanation only) and a medium control group were prepared, and behavioral tests were compared pre-MCAO and day 1, day 7, day 14, day 15, day 21 and day 28 post-MCAO. The histological examination, including TTC staining, was performed on day 14 and day 28, and immunohistochemistry for vWF, MAP2, GFAP and Iba1 was performed on day 28 after MCAO in the BM-MNC group. In addition, messenger RNA (mRNA) expression was analyzed on day 28. Moreover, CBF was measured pre-MCAO and 14 days and 28 days post-MCAO.

Therapeutic effects of BM-MNCs on neurological behavior in chronic MCAO

The grid walk and cylinder tests were performed to evaluate the therapeutic effects on sensorimotor functional recovery after cerebral infarction with or without BM-MNC treatment (Figure 2A,B). The frequency of step failure of the affected forelimb on the grid was calculated pre-MCAO and day 1, day 7, day 14, day 15, day 21 and day 28 post-MCAO using the grid walk test (Figure 2A). The frequency increased to approximately 15% on day 1 after MCAO and decreased to 10% from day 7 to day 14. However, the failure frequency decreased significantly in the BM-MNC group after treatment, whereas no change was observed
from day 15 to day 28 in the medium group. Cylinder tests were performed on the affected forelimb pre-MCAO and day 1, day 7, day 14, day 15, day 21 and day 28 post-MCAO (Figure 2B,C). The number of times the affected forelimb was used was counted while standing on the hindlimbs as the fine touch (Figure 2B). The ratio of failures in wall contact with the affected forelimb was evaluated using the cylinder test as the forelimb dragged (Figure 2C). The ratio of use of the affected forelimb decreased similarly in both the medium control and BM-MNC groups after MCAO until day 14, whereas the ratio of use tended to gradually improve in the BM-MNC group from day 14 onward. The ratio in the BM-MNC group improved significantly on day 28 compared with that observed in the medium group (Figure 2B). The ratio of wall contact failure with the affected forelimb also increased similarly in the control and BM-MNC groups after cerebral infarction; however, the failure ratio improved significantly in the BM-MNC group compared with the medium group on day 15 (Figure 2C).

Figure 2. Motor and sensory behavioral analysis in the mouse model of chronic cerebral infarction after BM-MNC transplantation therapy. (A) The percentage of step failure on the affected forelimb in all steps in the grid walk test is shown Pre and day 1, day 7, day 14, day 15, day 21 and day 28 post-infarction in both medium control (n = 14) and BM-MNC (n = 9) groups. (B,C) Ratio of utilization of affected forelimb in the cylinder test. (B) Ratio of fine touch of the affected forelimb (ipsilateral side) in contact with the cylinder wall and (C) ratio of drag on contact with the cylinder wall with the affected forelimb (ipsilateral side) are shown Pre and day 1, day 7, day 14, day 15, day 21 and day 28 post-infarction in both medium control (n = 17) and BM-MNC (n = 17) groups. (D) Basso Mouse Scale is shown Pre and day 1, day 7, day 14, day 15, day 21 and day 28 post-infarction in both medium control (n = 16) and BM-MNC (n = 15) groups. (E) The 50% response threshold of the affected hindlimb in the von Frey test is shown Pre and day 1, day 7, day 14, day 15, day 21 and day 28 post-infarction in both medium control (n = 12) and BM-MNC (n = 9) groups. Black squares show the medium control group and black circles show the BM-MNC group. Error bars show mean ± SD. *P < 0.05. Pre, pre-infarction; SD, standard deviation.
For BMS, another motor function test, mouse was observed in an open field and scored on a 9-point scale (Figure 2D). Both groups demonstrated a decrease in scores after cerebral infarction, and there was no significant difference in the subsequent course between the two groups. A von Frey test was also performed, and sensory function was evaluated by measuring the 50% withdrawal sensory threshold of the affected hindlimb. In both groups, superficial sensation was impaired on day 1 after cerebral infarction and gradually improved during the course of the study, with no significant difference observed between the two groups (Figure 2E).

**Figure 3.** TTC staining in brain sections in the mouse model of chronic cerebral infarction after BM-MNC transplantation therapy. (A) The left panel shows a top view of the mouse brain with cerebral infarction under stereomicroscope. The center red spot shows the bregma, the lower left red spot indicates the point of puncture and the white line indicates a horizontal line 1 mm posterior to the injection site along which the brain was sectioned. The middle and right panels show TTC staining of the section. The green line outlines the ipsilateral non-infarcted area and the blue line outlines the contralateral non-infarcted area, showing the hemisphere in the middle panel and the cortex in the right panel. Below is the formula for area ratio, which calculated the ipsilateral to contralateral ratio of the non-infarcted area in each hemisphere and cortex. (B) Representative TTC-stained pictures and schematic illustrations of the mouse brain are shown for day 14 and day 28 in the sham, medium control and BM-MNC groups after cerebral infarction. In the schematic illustrations, the cerebral infarction and defect lesion are shown as gray areas. (CD) Ipsilateral to contralateral area ratio of the non-infarcted area is shown for day 14 (n = 5) and day 28 in the sham (n = 5), medium control (n = 7) and BM-MNC (n = 5) groups in (C) hemisphere or (D) cortex. Each circle shows an individual value as a dot plot. Error bars show mean ± SD. SD, standard deviation.
Evaluation of residual brain volume by TTC staining after BM-MNC treatment

The stroke area was evaluated by TTC staining following treatment with BM-MNCs after cerebral infarction (Figure 3). Coronal brain sections were prepared from the BM-MNC and medium control (day 14), sham operation, medium control and BM-MNC (Figure 3A,B). Staining revealed a lack of cortical area on the ipsilateral side, and the patterns were the same in all four groups (Figure 3B). Next, the area ratio of the residual area on the ipsilateral side was calculated against that on the contralateral side (Figure 3A) and the area ratio among the three groups (Figure 3C). This ratio did not differ significantly among the three groups. Additionally, the ratio of the residual cortical area on the ipsilateral side was calculated and compared among the three groups (Figure 3A,D), and no significant difference was observed (Figure 3D). These results demonstrated that treatment had no effect on the infarction volume on day 28 compared with that observed on day 14.

Characterization of transplanted cells in infarcted brain after BM-MNC cell therapy

Next, immunohistochemistry was performed to investigate the mechanisms underlying the beneficial effects of BM-MNCs on behavioral phenotypes in the BM-MNC group (Figure 4). Coronal brain sections were prepared at the transplant site containing transplanted BM-MNCs from GFP transgenic mice (Figure 4A). To examine the properties of the transplanted cells, sections containing GFP-positive cells were selected and stained with anti-vWF antibody as a marker of the vascular endothelium, anti-MAP2 antibody as a neuronal marker, anti-GFAP antibody for astrocytes and anti-Iba1 antibody for microglia/macrophages. With regard to vWF staining, GFP-positive cells were observed along the blood vessel wall in the thalamus, and the rims of GFP-positive cells were stained with the vWF antibody (Figure 4B). At the infarction area in the cortex of brain sections, MAP2, GFAP and Iba1 staining was assessed along with GFP-positive cells (Figure 4C). Several GFP-positive cells accumulated in the infarction area, and most were merged with Iba1 staining. In the thalamic region of the ipsilateral side, after BM-MNC injection, several GFP-positive cells were observed and merged with Iba1 without MAP2 or GFAP staining (Figure 4D). To analyze the angiogenic effects of the transplantation therapy on the vasculature, vWF immunohistochemistry was performed on day 28 at the cortical and thalamic areas of infarcted brains of the medium and BM-MNC groups (Figure 4E–H). The number of vWF-positive cells was significantly higher on the ipsilateral side of the cortex (Figure 4E,F) and thalamus (Figure 4G,H) in the BM-MNC group compared with the medium group.

Evaluation of neuronal cell loss, astrogliosis and microgliosis with BM-MNC therapy

To analyze the therapeutic effects on pathological conditions in the infarct area, immunohistochemistry for MAP2, GFAP and Iba1 was performed in the medium and BM-MNC groups (Figure 5). In coronal brain sections containing the hippocampal region, MAP2 staining was observed at day 28. Cortical lesions on the ipsilateral side showed atrophy and loss of MAP2 staining, whereas those on the contralateral side were preserved (Figure 5A). The ratio of MAP2-positive intensity in a certain region of the cortical area on the ipsilateral side to the mirror image area on the non-ipsilateral side was calculated and compared between the medium control and BM-MNC groups (Figure 5A,B). No difference was observed in the intensity ratio between the two groups (Figure 5B). This result was consistent with the TTC staining results (Figure 3), which suggested that the injection did not affect the infarction volume. Next, the number of GFAP-positive cells was compared in brain sections with GFAP staining between the medium control and BM-MNC treatment groups (Figure 5C). Many GFAP cells accumulated in the cortical lesions on the ipsilateral infarction side. In addition, as a notable observation in the control medium group, GFAP-positive cells accumulated in the thalamic area of the ipsilateral side as well as in the infarction area against the contralateral side (Figure 5C; also see supplementary Figure 1A). By contrast, GFAP staining was strongly suppressed in the thalamic region of brain sections from the BM-MNC group, and the number of GFAP-positive cells was significantly decreased in the ipsilateral thalamic area of the BM-MNC group compared with that observed in the medium control group (Figure 5C,D). From these results, it was clear that BM-MNC treatment suppressed gliosis in the thalamus after stroke.

In subsequent analyses, Iba1 staining was performed on brain sections from the medium control and BM-MNC treatment groups (Figure 5E,F). On the ipsilateral side in the medium control group, several Iba1-positive cells accumulated in the thalamic lesion against the contralateral side, similar to that in the cortical lesion (Figure 5E; also see supplementary Figure 1B). However, this accumulation of Iba1-positive cells was significantly suppressed in the BM-MNC group, as observed by GFAP staining (Figure 5E,F). These results suggested that BM-MNC treatment has therapeutic effects on microgliosis in the thalamus after stroke. To rule out the influence of the needle injection on the location of sections, including the hippocampus, the number of MAP2, GFAP and Iba1-positive cells was compared in brain sections between the sham and medium control groups, and no significant difference was observed, confirming that the injection procedure itself did not significantly affect the number of these cells (see supplementary Figure 2A–C).

Therapeutic effect of BM-MNCs on CBF in cerebral infarction model mice

To analyze the therapeutic effects of BM-MNC transplantation on CBF, laser speckle flowmetry was used to measure cortical surface CBF, and the CBF ipsilateral to contralateral ratio was calculated in the MCA and core regions (Figure 6). Before comparing the treatment and non-treatment groups, the natural course of the CBF ratio was evaluated in the MCA and core regions pre and post day 14 and day 28 after stroke (sham) (Figure 6A). In both regions of the MCA and the core of the MCA, a significant decrease in CBF ratio was observed on day 14 and day 28 after cerebral infarction compared with that at pre-cerebral infarction. However, no significant difference was observed between the levels on day 14 and day 28 post-cerebral infarction. Next, the CBF ratio was measured in both the medium control and BM-MNC groups, and the effects of treatment were evaluated on day 28 after stroke (Figure 6B). The CBF ratio was significantly higher in the BM-MNC group than the medium group in both the MCA and core regions. Therefore, injection of BM-MNCs was suggested to improve the reduction in blood flow in the MCA area caused by cerebral infarction.

mRNA gene expression analysis in brain tissues of stroke after BM-MNC therapy

To analyze the detailed pathological changes and therapeutic effects in BM-MNC-treated mice, mRNA expression was investigated in brain tissues (Figure 7). On the 14 days after BM-MNC treatment (28 days after cerebral infarction), mRNA was extracted from each of the bilateral hemispheres with a 2-mm thickness 1 mm posterior to the transplantation site (Figure 7A). Several types of mRNA expression were analyzed using quantitative reverse transcription polymerase chain reaction for cytokines, pro-inflammatory/anti-inflammatory microglia markers, A1/A2 astrocyte markers, growth factors and endothelial cell markers (Figure 7; also see supplementary Figure 2D). The expression of these markers was significantly decreased in the BM-MNC-treated group compared with the medium control group (Figure 7B). These results suggested that BM-MNC treatment suppressed inflammation and pro-inflammatory cytokine expression in the brain tissue of stroke mice.
Figure 4. Immunohistochemistry analysis of brain sections in the mouse model of chronic cerebral infarction after BM-MNC transplantation therapy. (A) Top view of a mouse brain with cerebral infarction under stereomicroscope. The center red spot shows the bregma, the lower left red spot indicates the point of puncture and the white line indicates a horizontal line across the injection site along which the brain was sectioned. (B) The panels show the two series of GFP signal (green), vWF immunohistochemistry staining (red) and DAPI (blue) nuclear stain in the thalamic area of the brain section 14 days after transplantation of BM-MNCs. Scale bar = 20 μm. (C,D) Immunohistochemistry staining of MAP2 (red), GFAP (red) and Iba1 (red) with DAPI (blue) nuclear stain in the (C) cortical or (D) thalamic area of the brain section 14 days after transplantation of BM-MNCs (green). Each area of white square in the middle panel is enlarged to the each right side. Arrowheads indicate cells expressing Iba1 with GFP-expressing BM-MNCs. Scale bar = 50 μm. (E) Left side shows schematic illustration of mouse brain with cerebral infarction area (gray). Right side shows the immunostaining pictures with vWF antibody in the red square area of brain sections in the medium control and BM-MNC groups. Scale bar = 50 μm. (F) The number of vWF-positive vessels of ring-shaped structures in the ipsilateral cortex is shown for the medium control (n = 6) and BM-MNC (n = 6) groups. Each circle shows an individual value as a dot plot. Error bars show mean ± SD. (G) Left side shows schematic illustrations of mouse brain with cerebral infarction area (gray). Right side showed the immunostaining pictures with vWF antibody in the red square area of brain sections in the medium control and BM-MNC groups. Scale bar = 50 μm. (H) The number of vWF-positive vessels of ring-shaped structures in the ipsilateral thalamus is shown for the medium control (n = 5) and BM-MNC (n = 5) groups. Each circle shows an individual value as a dot plot. Error bars show mean ± SD. *P < 0.05. DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation.
Figure 5. Histological analysis of infarction area, astrogliosis and microgliosis of brain sections in the mouse model of chronic cerebral infarction after BM-MNC transplantation therapy. (A) Immunohistochemistry staining of MAP2 (red) in brain sections 28 days after cerebral infarction. The upper and lower panels show the medium control and BM-MNC groups, the left and right panels show the contralateral and ipsilateral sides of the cortex and the middle panels show the whole brain section. The white squares (500 $\times$ 500 $\mu$m) in the middle panels are magnified on right or left side. (B) The ratio of intensity of MAP2 staining in the ipsilateral to contralateral cortex (500 $\times$ 500 $\mu$m) is shown for the medium control ($n = 5$) and BM-MNC ($n = 5$) groups. Each circle shows an individual value as a dot plot. Error bars show mean $\pm$ SD. (C) Immunohistochemistry staining of GFAP (red) and DAPI (blue) in brain sections 28 days after cerebral infarction. The upper and lower panels show the medium control and BM-MNC groups, the left and right panels show the contralateral and ipsilateral sides of the thalamus and the middle panels show the whole brain section. The white squares (250 $\times$ 250 $\mu$m) in the middle panels are magnified on right or left side. The length of one side in the enlarged right and left pictures is 250 $\mu$m. The white square in the lower left corner of the ipsilateral side panel of the medium control group shows magnified GFAP-positive cells. (D) The number of GFAP-positive cells in the ipsilateral thalamus is shown for the medium control ($n = 5$) and BM-MNC ($n = 5$) groups. Each circle shows an individual value as a dot plot. Error bars show mean $\pm$ SD. *P < 0.05. DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation.
Expression levels were normalized by GAPDH mRNA expression, ipsilateral to contralateral ratio was calculated for each level of mRNA expression and all ratios were compared between the medium and BM-MNC groups. Among the anti-inflammatory cytokines, IL-4 mRNA expression was significantly higher in the BM-MNC group than the medium control group (Figure 7B); however, no significant difference was observed in IL-10 between the two groups (Figure 7C; also see supplementary Figure 3). Among the inflammatory cytokines, mRNA expression of TNF-α, IL-6 and IL-1β was significantly suppressed in the BM-MNC group (Figure 7D–F). Among the pro-inflammatory microglia markers, CD86 expression was significantly suppressed in the BM-MNC group (Figure 7G); however, no significant difference was observed in iNOS expression between the two groups (see supplementary Figure 3A). Among the anti-inflammatory microglia markers, CD206 expression was significantly higher in the BM-MNC group than the medium control group (Figure 7H); however, Arg1 expression levels demonstrated no significant difference between the two groups (see supplementary Figure 3B).

Gene expression of the A1 astrocyte marker Amigo2 was significantly lower in the BM-MNC group than the medium group (Figure 7I). By contrast, A2 astrocyte marker TGM-1 was significantly higher in the BM-MNC group than the medium group (Figure 7J). Among the growth factors, the angiogenesis-related molecule VEGF was significantly higher in the BM-MNC group, whereas no significant difference was observed in TGF-β or IGF-1 (Figure 7K; also see
Among the vascular endothelial markers, vWF gene expression was significantly higher in the BM-MNC group than the control group (Figure 7L); however, no significant difference was observed in CD31 expression (see supplementary Figure 3E). Pro-inflammatory microglia- and A1 astrocyte-related genes were downregulated, whereas anti-inflammatory microglia- and A2 astrocyte-related genes were upregulated. These results suggested that BM-MNC treatment suppresses astrogliosis and
microgliosis and promotes angiogenesis, which is consistent with the results observed in histological studies.

Discussion

In this study, intracerebral administration of BM-MNCs improved motor function in a mouse model of chronic cerebral infarction through recovery of CBF. Histological analysis revealed that BM-MNCs colonized the cortex and thalamus 2 weeks after administration and showed features of vascular endothelium or microglia/macrophages. Additionally, astrogliosis and microgliosis were significantly suppressed in the ipsilateral thalamus of the BM-MNC group, whereas angiogenesis was significantly increased in the ipsilateral cortex. In the mRNA gene expression analysis, the genes related to anti-inflammatory cytokines, A2 astrocytes and anti-inflammatory microglia markers were down-regulated. In addition, the expression of vWF as a vascular endothelial marker and VEGF was strongly elevated in the BM-MNC group.

Regulated. In addition, the expression of vWF as a vascular endothelial marker and VEGF was strongly elevated in the BM-MNC group. Therefore, the administration of BM-MNCs suppressed inflammation and neoangiogenesis in stroke lesions, leading to improvement in the stroke phenotype.

Here, therapeutic effects were stably observed through BM-MNC transplantation for chronic stroke in our experiments, which is the first report to clearly show the mechanism of its therapeutic effect in mice. One important reason for these results was the choice of the mouse infarction model. As a cerebral infarction model, SCID mice were used to induce focal cerebral infarction by transcranial MCAO. Cerebral infarction models are mainly divided into two types: global and focal ischemia [31]. The focal ischemia model was selected because it is more relevant to human cerebral infarction [32]. In humans, approximately 70% of cerebral infarctions occur in the MCA region; therefore, this region was selected for model creation [33]. Of several methods for producing focal ischemia models, transcranial MCAO was selected because of its visual confirmation of peripheral blood flow blockage, high reproducibility of infarct size and neurological dysfunction and low mortality [31,34–36]. Disadvantages of transcranial MCAO due to the surgical procedure include the following: partial destruction of skin, muscle, bone, dura mater, brain surface and blood vessels; cerebrospinal fluid leakage and intracranial infection; and change in intracranial pressure, brain temperature and blood–brain barrier (BBB) permeability [31,36–38]. This was addressed by establishing a control group for treatment. SCID mice were selected as the cerebral infarction model owing to their high reproducibility of cerebral infarction and long-term survival [19]. As previously reported, SCID mice have less inter-individual variability in vasculature than the commonly used C57/BL6 mice, resulting in a more reproducible cerebral infarction model [19,20].

To evaluate the therapeutic effect of BM-MNC transplantation on behavioral responses, the grid walk, cylinder and von Frey tests as well as BMS were performed and compared between the medium control and BM-MNC groups. Behavioral tests showed improvement in the grid walk and cylinder tests in the BM-MNC group compared with the medium group, which indicated an improvement in sensorimotor function because both of these tests are thought to reflect that function [39,40]. However, the von Frey test, as a sensory function test, and the BMS, as a motor function test, did not reveal any differences between the two groups. Regarding motor function, the grid walk and cylinder tests are capable of detecting finer differences than the BMS [41,42], and motor function was improved; however, we did not observe any improvement in sensory function. To the best of our knowledge, BM-MNC transplantation alone has resulted in functional recovery in the acute and subacute phases in many studies [10,17,43,44]. However, there are no reports of functional recovery in the chronic phase following BM-MNC transplantation [17]. There is only one report showing a therapeutic effect in combination with training [45]. In this study, direct intracerebral administration of 1 × 10⁶ cells per mouse was performed, which is a relatively large amount in the local brain area compared with that reported in other studies, and intraventricular or intravenous administration was used [18]. This difference may have led to functional recovery.

In this study, BM-MNC treatment improved motor function but not sensory function. The absence of a significant improvement in sensory disturbance could be attributed to gradual spontaneous improvement in both the BM-MNC and medium groups, indicating the difficulty in recognizing a significant difference between the two groups. As sensory impairment includes subjective factors, the evaluation of sensory impairment in mice was more difficult than the evaluation of motor function. Sensory impairment may also exhibit hyperalgesia; therefore, a highly accurate assessment of sensory deficits is required in mice. However, as its therapeutic effects for cerebral infarction have been recognized, it would be desirable to evaluate it in primates and/or in clinical practice.

The features of the transplanted BM-MNCs were included in this study. Various cell types have been used in transplantation therapies for cerebral infarction, including BM-MNCs, adipose-derived mesenchymal stem cells [46], bone marrow-derived mesenchymal stem cells [47], dental pulp cells [48], olfactory epithelial cells [49], embryonic stem cells [50], neural stem cells [51] and induced pluripotent stem cells [52]. Compared with other cell types, BM-MNCs have the following advantages: they can be autologously transplanted, bone marrow cells can be harvested, MNCs can be separated by relatively simple procedures, there is no requirement of culture or induction of differentiation, MNCs can be freshly prepared without cryopreservation and they can be collected and administered at any time [53]. Furthermore, local administration in the brain was selected as the transplantation approach in this study. Unlike intravenous injections, which may result in cell trapping in organs and difficulty in crossing the BBB, local administration by needle insertion minimizes these concerns, as most of the transplanted cells are not trapped in any organ and it is not necessary to consider the BBB during transplantation [54]. BM-MNC transplantation has resulted in improvement in neuronal function by ameliorating age-related decline in cerebral metabolism, suggesting that BM-MNC transplantation is more useful in the treatment of cerebral infarction, which is primarily a disease of older individuals [55,56]. The disadvantages of BM-MNCs include the risk of bleeding and infection from bone marrow aspiration and the lack of potential for direct differentiation into neurons and astrocytes. Indeed, among progenitor cells, neuronal and other stem cell populations have a high potential to differentiate directly into neurons and glial cells [50,52,57]. In this regard, our strategy has several disadvantages. However, our aim was not to increase the number of neurons, but rather to modify the niche around the neurons and the environment in ischemic brain lesions using MNCs. As shown in the mRNA expression study, several genes related to anti-inflammatory and neuroprotective effects were elevated, indicating that brain inflammation was suppressed and that the environment surrounding the neurons was improved under ischemic conditions. This led to an improvement in the perfusion of ischemic brain lesions. These results were the most unique aspect of our strategy, which differed from previous stem cell transplantation aimed at supplying additional neurons.

Another important consideration in the clinical application of regenerative therapy is resolution of the differences between mice and humans. In mice, BM-MNCs at 1 × 10⁶ cells/3 μL were transplanted once into the thalamus on the ischemic side (3 mm lateral, 2 mm caudal and 2.5 mm deep) 14 days after cerebral infarction, but the same procedure cannot be conducted in humans. First, the correspondence between the 14th day in mice and the equivalent period in humans is unclear, and this period is believed to be the duration
during which secondary damage to the thalamus persists, although this remains unclear. Second, we investigated the treatment of cortical infarction in mice caused by MCAO; however, the extent to which this corresponds to human ischemia remains unclear. Furthermore, in humans, the ischemic sites may vary among patients, making it unclear which specific sites are suitable for treatment. Third, ischemic symptoms are milder in mice than in humans. Cerebral infarction in humans is believed to result in severe paralysis, as seen in this study, although the severity of symptoms differs between mice and humans [36]. Moreover, whether the severity of symptoms in cerebral infarction is suitable for treatment remains unknown. Fourth, the transplantation site was the ipsilateral thalamus; however, since the size of the brain differs between mice and humans, the distance between the lesion and transplantation site in the human brain is not clear. Fifth, in this study, the number of cells to be transplanted into mice was set at $1 \times 10^6$ cells/$\mu$L; however, the appropriate number for humans remains unclear. Sixth, BM-MNCs were transplanted into the thalamus of mice as a single injection; however, considering that humans have larger brains than mice, it remains unclear whether increasing the number of injection sites or injections would be beneficial.

As mentioned earlier, to address the gap between animal and human research, many issues remain to be solved. However, potential recommendations for cell therapy in stroke, such as the Stem Cell Therapies as an Emerging Paradigm in Stroke and Stroke Treatment Academic Industry Roundtable, have been published [58,59]. According to these publications, our study meets the recommendations in several respects. A focal infarction model was used in this study, which is recommended because it closely resembles human cerebral infarctions. In addition, none of the mice died after transplantation, indicating the high level of safety of the treatment. Randomization and concealment of allocations were also implemented for unbiased results. Moreover, inclusion and exclusion criteria were established. As mentioned earlier, to address the gap between animal and human research, many issues remain to be solved. However, potential recommendations for cell therapy in stroke, such as the Stem Cell Therapies as an Emerging Paradigm in Stroke and Stroke Treatment Academic Industry Roundtable, have been published [58,59]. According to these publications, our study meets the recommendations in several respects. A focal infarction model was used in this study, which is recommended because it closely resembles human cerebral infarctions. In addition, none of the mice died after transplantation, indicating the high level of safety of the treatment. Randomization and concealment of allocations were also implemented for unbiased results. Moreover, inclusion and exclusion criteria were established.

Among the conclusions of this study is that MNCs hold high potential for cell transplantation therapy.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: TT, MK and NO. Acquisition of data: TK. Analysis and interpretation of data: TK. Drafting or revising the manuscript: TK, TT, KN and AT. All authors have approved the final article.

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Data Availability

The data presented in this study are included in the article and supplementary materials. Further inquiries should be addressed directly to the lead contact upon reasonable request.

Supplementary materials

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