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Effect of repeated allogeneic bone marrow mononuclear cell transplantation on brain injury following transient focal cerebral ischemia in rats

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ABSTRACT

Aims: Transplantation of bone marrow mononuclear cells (BMMCs) exerts neuroprotection against cerebral ischemia. We examined the therapeutic timepoint of allogeneic BMMC transplantation in a rat model of focal cerebral ischemia, and determined the effects of repeated transplantation outside the therapeutic window.
Main methods: Male Sprague–Dawley rats were subjected to 90 minute focal cerebral ischemia, followed by intravenous administration of 1×10^7 allogeneic BMMCs or vehicle at 0, 3 or 6 h after reperfusion or 2×10^7 BMMCs 6 h after reperfusion. Other rats administered 1×10^7 BMMCs at 6 h after reperfusion received additional BMMC transplantation or vehicle 9 h after reperfusion. Infarct volumes, neurological deficit scores and immunohistochemistry were evaluated 24 or 72 h after reperfusion.
Key findings: Infarct volumes at 24 h were significantly decreased in transplantation rats at 0 and 3 h, but not at 6 h, after reperfusion, compared to vehicle-treatment. Even high dose BMMC transplantation at 6 h after reperfusion was ineffective. Repeated BMMC transplantation at 6 and 9 h after reperfusion reduced infarct volumes and significantly improved neurological deficit scores at 24 and 72 h. Immunohistochemistry showed repeated BMMC transplantation reduced ionized calcium-binding adapter molecule 1, 4-hydroxy-2-nonenal and 8-hydroxydeoxyguanosine expression at 24 and 72 h after reperfusion.
Significance: Intravenous allogeneic BMMCs were neuroprotective following transient focal cerebral ischemia, and the therapeutic time window of BMMC transplantation was >3 h and <6 h after reperfusion in this model. Repeated transplantation at 6 and 9 h after reperfusion suppressed inflammation and oxidative stress in ischemic brains, resulting in improved neuroprotection.

Introduction

Transplantation of bone marrow cells reportedly exerts neuroprotection against cerebral ischemia (Chen et al., 2001; Bliss et al., 2007). We previously showed that either transplantation of autologous bone marrow mononuclear cells (BMMCs) or bone marrow stromal cells (BMSCs) reduced infarct volume following transient focal cerebral ischemia in rats (Kamiya et al., 2008; Suda et al., 2011). However, it is difficult to prepare sufficient numbers of autologous BMMCs for transplantation at the acute phase of stroke, because collection of BMMCs requires surgical procedures. In addition, BMSCs also require enough time to culture cells, thus adding to the difficulty of preparing BMSCs at the acute phase of stroke. Moreover, we confirmed "allogeneic" and "autologous" BMMC transplantations were equally neuroprotective in a rat stroke model (Kamiya et al., 2011). Therefore, allogeneic BMMCs may be a therapeutic option for immediate transplantation or repeated transplantation. The benefit of cell transfusion therapy for the acute phase of cerebral ischemia includes attenuation of inflammation and inhibition of antioxidant processes (Bliss et al., 2007; Yang et al., 2011).

The present study was designed to determine the therapeutic time window of allogeneic BMMC transplantation in a rat model of transient focal cerebral ischemia, and to examine whether repeated BMMC transplantations outside the therapeutic time window achieved further neuroprotection. In addition, the mechanisms of neuroprotection were investigated in relation to inflammation and oxidative stress.

Materials and methods

Ischemia model

All experimental procedures were approved by the institutional guidelines for animal use and care. A total of 134 male Sprague–Dawley rats, weighing 250 to 300 g, were used in this study. Anesthesia was

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performed with 5% halothane and maintained with 0.5 to 1.0% halothane in 70% N_2O and 30% O_2 mixture during all surgical procedures. The rats were subjected to transient focal cerebral ischemia for 90 min using an intraluminal suture technique (Ueda et al., 2013; Nito et al., 2011; Suda et al., 2011). Briefly, the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) on the right side were carefully exposed via a midline cervical incision. The ECA and the CCA were double-ligated using a silk suture. A silicon rubber-coated round-tip nylon surgical thread was inserted into the ICA (approximately 18 mm from the bifurcation) via a small puncture in the CCA to occlude the origin of the middle cerebral artery (MCA). A silk suture around the CCA was tightened to prevent bleeding from the puncture site. After 90 min of MCA occlusion, reperfusion was performed by withdrawing the suture. A PE-50 catheter was inserted into the tail artery for blood sampling and arterial blood pressure monitoring to confirm that physiological variables were within normal limits. Rectal temperature was maintained at 37.0 ± 0.5 °C during the procedure.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) analyses were performed using Unity-INOVA-300 system with a 7 T/18 cm horizontal magnet (Agilent Technologies., CA, USA), as described previously (Ueda et al., 2013; Nito et al., 2011). The continuous arterial spin labeling method (Williams et al., 1992), modified with a centrally encoded variable-tip-angle gradient echo technique (Ewing et al., 2003), was performed to obtain coronal images of cerebral blood flow (CBF) at the level of the bregma, to confirm successful MCA occlusion and CBF reduction.

BMMC extraction

The femur bone was extracted from donor animals (n = 68) and bone marrow was obtained as described previously (Kamiya et al., 2008, 2013) and then heparinized. BMMCs were isolated by density-gradient centrifugation with Ficoll-Paque (Iihosi et al., 2004; Kamiya et al., 2008, 2013). The fraction containing BMMCs was stained using Turk's solution and trypan blue, and cells were microscopically counted using a Burker-Turk counting chamber. BMMCs (1×10^7) were then diluted in 1 ml phosphate-buffered saline (PBS) and stored on ice until transplantation. Because 1×10^7 of the BMMCs were sufficient for neuroprotection (Chen et al., 2001; Yang et al., 2011), this dose was prepared for all transplantation experiments in this study.

BMMC transplantation

A PE-50 catheter was inserted into the left femoral vein of each recipient rat. In experiment-1, allogeneic BMMCs in PBS (1×10^7 cells in 1 ml of PBS) or vehicle (1 ml of PBS) were intravenously transplanted through the catheter at 0, 3 or 6 h after reperfusion (each n = 8). In addition, a high dose of allogeneic BMMCs (2×10^7 cells in 1 ml of PBS) were also transplanted at 6 h after reperfusion to confirm dose-dependent effects (n = 8). In experiment-2, another set of animals, administered 1×10^7 BMMCs or vehicle at 6 h after reperfusion, received additional BMMC transplantation of the same dose or vehicle at 9 h after reperfusion (each n = 6). Vehicle-treated animals in experiment-2 were administered 1 ml of PBS instead of BMMCs. A preliminary study showed the effects of transplantation on neuroprotection were equal between autologous and allogeneic BMMCs (Kamiya et al., 2011), thus allogeneic transplantation was used in the present study because the repeated transplantation in experiment-2 needs more BMMCs and too much autologous BMMC extraction is so invasive to the recipient rats.

Measurement of infarct volume

To measure infarct volume, animals in experiment-1 were deeply anesthetized using 5% halothane, and were decapitated at 24 h after reperfusion (n = 8). The brains were carefully removed and cut into 2 mm-thick coronal sections. The slices were stained with 2,3,5-triphenyltetra-zoliumchloride (TTC). The animals in experiment-2 were deeply anesthetized using 5% halothane and were perfusion-fixed with 4% paraformaldehyde followed by a brief flush using heparinized saline at 24 or 72 h after reperfusion (n = 6). The brains were carefully removed and stored in 4% paraformaldehyde overnight, and 20-μm-thick coronal frozen sections were cut at 2 mm intervals on a cryostat. Each section was mounted on a poly-L-lysine coated slide glass, and stained with hematoxylin and eosin (HE). Infarct volumes were calculated by adding the total infarct areas on TTC or HE-stained sections using Image J software version 1.42q (NIH, Maryland, USA).

Evaluation of motor function

To evaluate motor function, neurological deficit scores were evaluated at 24 or 72 h after reperfusion using a five-point scale as previously described (Ren et al., 2011): 0: no neurological deficit, 1: failure to fully extend the right forepaw, 2: circling to the right, 3: falling to the right, and 4: unable to walk spontaneously.

Immunohistochemistry

Immunohistochemistry was performed using coronal frozen brain sections from experiment-2. Briefly, the sample sections were incubated with 10% goat serum in PBS to block nonspecific reactions, followed by incubation with rabbit polyclonal antibodies against ionized calcium-binding adapter molecule 1 (Iba-1) as a marker of activated microglia (1:500, Wako Pure Chemical Industries, Ltd., Osaka, Japan), mouse monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE) as a marker of lipid peroxidation (1:50, Japan Institute for the Control of Aging, Shizuoka, Japan) or mouse monoclonal antibody against 8-hydroxydeoxyguanosine (8-OHdG) as a marker of oxidative DNA damage (1:50, Japan Institute for the Control of Aging, Shizuoka, Japan) overnight at 4 °C. Then, the sections were processed with biotinylated goat anti-rabbit IgG or anti-mouse IgG (Vector Laboratories, CA, USA) at room temperature for 1 h, followed by avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min. The labeled secondary antibodies were visualized using diaminobenzidine. Each process was followed by several brief washes with PBS. The Iba-1 positive cells in the cortical ischemic boundary zone were counted at three randomly chosen square fields (0.4 mm^2).

Statistical analysis

Statistical analysis was performed using Statview version 5.0 software (SAS Institute, CA, USA). An analysis of variance (ANOVA) followed by Scheffe's post-hoc test was performed for comparisons of infarct volumes and immunohistochemical cell counts, and data were presented as mean ± standard deviations (SDs). A Mann–Whitney *U*-test was used to compare neurological deficit scores, and data were expressed as median and interquartile range. Statistical significance was set at $p < 0.05$.

Results

Therapeutic time window of allogeneic BMMC transplantation

Infarct volumes assessed by TTC-stained sections at 24 h after reperfusion in the vehicle and single transplantation groups at 0, 3 and 6 h after reperfusion were 262 ± 81, 49.0 ± 7.7, 107 ± 39, and 195 ± 96 mm^3, respectively. Infarct volumes were significantly decreased in the single