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Research report

Comparison of the therapeutic effects of bone marrow mononuclear cells and microglia for permanent cerebral ischemia

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HIGHLIGHTS

- We treated rats with bone marrow mononuclear cells (BM-MNCs) and microglia after stroke.
- The distribution or morphology of transplanted BM-MNCs and microglia was observed *in vivo*.
- We compared the therapeutic effects of BM-MNCs and microglia for stroke.
- BM-MNCs improved post-stroke functional outcome and reduced brain water content and lesion volume.

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ABSTRACT

In this study we transplanted bone marrow mononuclear cells (BM-MNCs) or microglia into rats that had undergone permanent cerebral ischemia and observed the distribution or morphology of transplanted cells *in vivo*. In addition, we compared the effects of BM-MNCs and microglia on infarct volume, brain water content, and functional outcome after permanent cerebral ischemia. BM-MNCs and microglia were obtained from femur and brain, respectively, of newborn rats. Adult rats were injected with vehicle or 3 million BM-MNCs or microglia via the tail vein 24 h after permanent middle cerebral artery occlusion (pMCAO). The distribution or morphologic characteristics of transplanted BM-MNCs (double stained with BrdU/Cd34 or BrdU/CD45) and microglia (double stained with BrdU/Iba-1) were detected with immunofluorescent staining at 3 or 7 and 14 days after pMCAO. Functional deficits were assessed by the modified neurologic severity score at 1, 3, 7 and 14 days after pMCAO. Brain water content was assessed at 3 days, and infarct volume was determined at 14 days. We observed more BrdU/CD45 and BrdU/Iba-1 double-stained cells than BrdU/CD34 double-stained cells around the infarcted area. Some infused microglia showed the morphology of innate microglia at 7 days after pMCAO, and the number increased at 14 days. BM-MNC-treated rats showed significantly reduced infarct volume and brain water content compared to vehicle- and microglia-treated rats. In addition, BM-MNC treatment reduced neurologic deficit scores compared to those in the other groups. The results provide evidence that infusion of BM-MNCs, but not microglia, is neuroprotective after permanent cerebral ischemia.

1. Introduction

Currently, use of tissue plasminogen activator is the only strategy available for protecting the brain after ischemic stroke. However, recent research has shown cell therapy to be a promising new avenue for the treatment of stroke [1,2]. Transplanting different cell types, such as neural stem cells, progenitor cells, cord blood cells, and bone marrow stromal cells (BMSCs) could minimize neural injury and potentially restore function of damaged tissue [3,4].

One source of cells shown to improve functional outcome in animal models of ischemic stroke is bone marrow. Although studies have demonstrated the high therapeutic value of BMSCs for ischemic stroke in animals [5–8], BMSCs are generally considered more difficult to culture and purify than bone marrow mononuclear cells (BM-MNCs). BM-MNCs comprise mesenchymal and hematopoietic stem cells. Clinical research into their use

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in heart disease therapy has drawn attention to their potential therapeutic value [9]. Indeed, BM-MNCs have been shown to differentiate into microglia-like cells and may provide a therapeutic effect for Alzheimer's disease in animal models [10,11]. Several laboratories have reported that BM-MNCs migrate to the boundaries of infarcts and promote stroke recovery after transient cerebral ischemia in rats [12–14]. Furthermore, in multiple small clinical trials, intravascular injection of BM-MNCs appears to be safe and feasible in stroke patients [15,16].

Microglia are resident cells in the brain that have a monocyte lineage. After stroke they can be activated to have the characteristics of monocytes or macrophages. However, published results are conflicting in regard to the role of microglia in the pathophysiology of cerebral infarction. In a recent report, Narantuya et al. [17] found that transplantation of human microglia into a rat transient cerebral ischemia model provides neuroprotection and improves functional recovery. However most investigators have concluded that microglia are detrimental when activated in the acute phase of stroke [18,19].

To the best of our knowledge, no study has examined the therapeutic effects of BM-MNCs and primary microglia on stroke outcomes in a permanent cerebral ischemia model. The aims of our study were to observe the morphologic characteristics of transplanted microglia and the distribution of exogenous BM-MNCs and microglia *in vivo* and to evaluate whether transplantation of each cell type affects infarct volume, brain water content, and functional recovery after permanent cerebral ischemia in rats.

2. Materials and methods

2.1. Animals

All studies were performed in accordance with the NIH and institutional guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee at Zhengzhou University, Zhengzhou, China. Adult, male Sprague-Dawley rats (260–300 g, 3 months old) were purchased from the Animal Experimental Center of Zhengzhou University. They were housed under standard conditions with a 12-h light/dark cycle and given free access to food and water throughout the study. All procedures were designed to minimize animal suffering and the number of animals used.

2.2. Preparation of BM-MNCs and microglia

BM-MNCs were collected from femurs of 1- or 2-day-old Sprague-Dawley rats ($n = 48$) and were purified by density gradient centrifugation as previously described [20]. Flow cytometry analysis (FACSCalibur, BD Biosciences, San Jose, CA) with monoclonal anti-CD34 (a marker of hematopoietic stem/progenitor cells, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD45 (a marker of monocytes and a negative marker of mesenchymal stem cells, BD Biosciences) antibodies showed that $11.3 \pm 1.28\%$ of the collected BM-MNCs were positive for CD34 and $86.1 \pm 2.12\%$ were positive for CD45.

Rats used for the collection of BM-MNCs were also used for the culture of microglia. Microglia were isolated from mixed glial culture with a slightly modified version of a previously described method [21]. Briefly, the whole brains of rats were stripped of meninges, mechanically dissociated, and filtered through 70-μm nylon mesh to recover dissociated cells. To harvest enough cells, the dissociated cells were centrifuged and resuspended in Dulbecco's Modified Eagle's Medium (DMEM-F12; HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; HyClone) and antibiotics. This preparation of mixed microglia was seeded onto culture dishes coated with poly-L-lysine (Corning, Cambridge, MA) and incubated for 7 days at 37 °C. We then used a previously described method to obtain an enriched culture of microglial cells [22,23]. The mixed glia culture was shaken, and the primary selected microglia were cultured in DMEM that contained 10% FBS (2×10^5 cells/mL). Immunocytochemical analysis was carried out as follows to identify the purity of microglia. Microglia were fixed with 4% paraformaldehyde and incubated with rabbit anti-CD11b/c antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate-buffered saline (PBS) containing 5% goat serum and 3% Triton X-100. Then the cells were rinsed with PBS three times and incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Stained cells were viewed and analyzed under a bright-field microscope. Immunocytochemical analysis indicated that the purity of the cultured microglia was more than 95%.

We also prepared a portion of BM-MNCs and microglia to test their migration capabilities. We labeled the cells with bromodeoxyuridine (BrdU) by incubating them in cell culture medium containing 12 μg/mL BrdU (Sigma, St. Louis, MO, USA) for 24 h. Incorporation of BrdU into the cells was confirmed by immunocytochemistry. The experimental procedure was the same as that used for detection of CD11b/c above except that rabbit anti-BrdU antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used.

2.3. Permanent middle cerebral artery occlusion (pMCAO)

Adult, male rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (400 mg/kg). pMCAO was induced by occluding the left middle cerebral artery (MCA) with a nylon monofilament as described previously [24,25]. Body temperature of the rats was monitored throughout surgery by a rectal probe and maintained at 37 ± 0.5 °C with a heating pad. After surgery, the wound was sutured and rats were returned to their cages, which were maintained at 29 °C. Sham-operated rats ($n = 16$) were subjected to the same surgical procedure, except that the MCA was not occluded. Successful MCAO was defined as more than 80% decrease in cerebral blood flow and was confirmed by Laser-Doppler flowmetry. Rats were excluded from the study if the cerebral blood flow did not decrease by more than 80% after pMCAO. The mortality was recorded during the experimental and recovery periods.

2.4. Injection of cells

Eighty rats that underwent pMCAO were randomly divided into four groups as follows: no treatment (16 rats), vehicle treatment (16 rats), treatment with microglia (24 rats), treatment with BM-MNCs (24 rats). Cell-treated rats were administered 3 million primary microglia or BM-MNCs by tail vein infusion at 24 h after MCA occlusion. Vehicle-treated rats were injected with an equal volume of cell culture medium. Blood pH, arterial blood gases (PaO_2, $PaCO_2$), and body temperature were measured before the pMCAO procedure, 30 min after the procedure, and 30 min after cell injection.

2.5. Assessment of migration capabilities of microglia and BM-MNCs

On day 3 after pMCAO, four rats each from microglia- and BM-MNC-treated groups were anesthetized with pentobarbital sodium solution, and brains were cut into 30-μm sections for detection of BrdU-labeled cells. Briefly, sections were blocked for 2 h with 1% bovine serum albumin (BSA) in PBS-Tween-20 (PBS-T) and then incubated overnight at 4 °C with rabbit anti-BrdU (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-T containing 1% BSA. The sections were then rinsed three times in PBS-T (10 min each) and finally incubated for 2 h at room temperature with CFL555-conjugated secondary antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). The stained cells around the infarcted area were observed under a fluorescence microscope (Olympus CKX41, Olympus, Japan). Images of the areas were captured with a digital camera (Olympus, Japan) by an investigator blinded to experimental group. In addition, we double stained sections for BrdU (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and CD34 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or CD45 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at 3 days after occlusion ($n = 4$). Double staining of BrdU (1:200; Santa Cruz) and ionized calcium-binding adaptor molecule 1 (Iba-1) (1:500; Wako Pure Chemical Industries, Osaka, Japan) was carried out at 3, 7 and 14 days after occlusion ($n = 4$). The sections were incubated overnight at 4 °C with the above antibodies. After being rinsed in PBS-T three times for 10 min each, the sections were incubated for 2 h at room temperature with CFL555- or CFL488-conjugated secondary antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). The sections then were rinsed at 4 °C in PBS-T and PBS three times for 10 min each and coverslipped with VECTASHIELD (Santa Cruz Biotechnology, Santa Cruz, CA). Double-stained cells were observed under a fluorescence microscope (Olympus CKX41, Olympus, Japan).

2.6. Analysis of brain water content

The rats ($n = 6$) were anesthetized and decapitated on day 3 after pMCAO as previously described [26] for determination of water content. Briefly, cerebral tissue was divided with a blade into two hemispheres from the anatomic midline. The left hemisphere was immediately weighed with an electronic analytical balance to obtain the wet weight. Then brain samples were dried at 100 °C in an electric blast drying oven for 24 h and reweighed to obtain the dry weight. Brain water content was calculated as: (wet weight − dry weight)/wet weight × 100% [27].

2.7. Determination of infarct volume

Rats used for the determination of infarct volume were anesthetized with pentobarbital sodium solution and killed on day 14 after pMCAO ($n = 6$). Brains of these rats were removed and cut into five 2-mm-thick coronal sections on a Vibratome (Vibratome, St. Louis, MO). The brain slices were quickly immersed in 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) in 0.1 M PBS (pH 7.4) for 20 min at room temperature and then stored in phosphate-buffered 4% paraformaldehyde overnight before analysis. The area of damaged parenchyma (unstained tissue) was measured on the posterior surface of each slice using Sigma