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CXCR4$^+$CD45$^-$ BMMNC subpopulation is superior to unfractionated BMMNCs for protection after ischemic stroke in mice

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ABSTRACT

Cell-based therapy is considered to be a promising therapeutic strategy for stroke treatment. Although unfractionated bone marrow mononuclear cells (BMMNCs) have been tried in both preclinical and clinical trials, the effective subpopulations need to be identified. In this study, we used fluorescence-activated cell sorting to harvest the CXCR4$^+$CD45$^+$ and CXCR4$^+$CD45$^-$ BMMNC subpopulations from transgenic mice that express enhanced green fluorescent protein. We then allogeneically grafted unfractionated BMMNCs or a subpopulation into mice subjected to transient middle cerebral artery occlusion (tMCAO) and compared the effects on stroke outcomes. We found that CXCR4$^+$CD45$^-$ BMMNCs, but not CXCR4$^+$CD45$^+$ BMMNCs, more effectively reduced infarction volume and neurologic deficits than did unfractionated BMMNCs. Brain tissue from the ischemic hemisphere of mice treated with CXCR4$^+$CD45$^-$ BMMNCs had higher levels of vascular endothelial growth factor and lower levels of TNF-α than did tissue from mice treated with unfractionated BMMNCs. In contrast, CXCR4$^+$CD45$^+$ BMMNCs showed an increase in TNF-α. Additionally, CXCR4$^+$CD45$^+$ and CXCR4$^+$CD45$^-$ populations exhibited more robust migration into the lesion areas and were better able to express cell-specific markers of different linages than were the unfractionated BMMNCs. Endothelial and astrocyte cell markers did not colocalize with eGFP$^+$ cells in the brains of tMCAO mice that received CXCR4$^+$CD45$^+$ BMMNCs. *In vitro*, the CXCR4$^+$CD45$^-$ BMMNCs expressed significantly more *Oct-4* and *Nanog* mRNA than did the unfractionated BMMNCs. However, we did not detect gene expression of these two pluripotent markers in CXCR4$^+$CD45$^+$ BMMNCs. Taken together, our study shows for the first time that the CXCR4$^+$CD45$^-$ BMMNC subpopulation is superior to unfractionated BMMNCs in ameliorating cerebral damage in a mouse model of tMCAO and could represent a new therapeutic approach for stroke treatment.

1. Introduction

Cell transplantation-based regenerative therapy provides us with a promising approach for stroke treatment (Bliss et al., 2010; Burns and Steinberg, 2011; Liu et al., 2014a; Misra et al., 2012). Compared with other cell sources, bone marrow mononuclear cells (BMMNCs) have attracted the interest of many researchers because their use avoids ethical concerns, and they are easy to obtain and purify. They can be harvested allogeneically or autologously from bone marrow within hours, no cell culture procedures are needed, and they can be administered immediately into the recipient through various routes. Over the past decade, evidence from preclinical studies has shown that grafting BMMNCs after cerebral ischemia provides substantial therapeutic effects (Boltze et al., 2011; Fujita et al., 2010; Hess and Hill, 2011; Mendez-Otero et al., 2007; Prasad et al., 2012). Despite progress in this field, the detailed mechanism through which BMMNCs exert their protective effects in cerebral ischemia remains elusive.

BMMNCs harbor a heterogeneous population that contains mature and immature cells in the myeloid and lymphoid lineages, such as mesenchymal stem cells (MSCs), hematopoietic stem cells

Abbreviations: BMMNC, bone marrow mononuclear cell; tMCAO, transient middle cerebral artery occlusion; FACS, fluorescence-activated cell sorting; HSC, hematopoietic stem cell; EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; TCSC, tissue-committed stem cell; CXCR4, C–X–C chemokine receptor type 4; SDF-1, stromal cell-derived factor 1; eGFP, enhanced green fluorescent protein.

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(HSCs), and endothelial progenitor cells (EPCs) (Arnous et al., 2012; Civin and Gore, 1993; Crosby et al., 2000; Dominici et al., 2006; Savitz, 2013). In reality, BMMNCs harvested from bone marrow by density centrifugation contain very few stem cells (~2% to 4% HSCs/EPCs and ~0.01% MSCs) (Malliaras and Marban, 2011). Bone marrow-derived stromal cells, or MSCs, are currently a promising cell source in stroke therapy. MSCs are capable of self-renewal and can differentiate into various cell linages, including cartilage, bone, adipose, hepatocytes, and neurons (Duenas et al., 2014; Pittenger et al., 1999; Prockop, 1997). It has been reported that human MSCs can migrate into the rat brain and acquire a neuronal phenotype *in vivo* (Azizi et al., 1998). More importantly, MSCs function as a "cytokine and trophic factors factory" that supports other cell types (Caplan and Dennis, 2006). Despite the advantages of MSCs, obtaining sufficient quantities requires cell culture. Therefore, autologous MSCs cannot be obtained in the acute stage after stroke, limiting their application.

Most investigators who have studied the use of cell transplantation for cerebral ischemia have used mixed BMMNCs. However, the migration and beneficial effects of BMMNCs require the cell surface expression of CXCR4. Many studies have documented that BMMNCs expressing this marker undergo rapid mobilization during cerebral ischemia in response to the chemokine gradient formed by stromal cell-derived factor-1 (SDF-1), which is secreted in the ischemic penumbra, especially by astrocytes and endothelial cells (Hill et al., 2004; Wang et al., 2012). Compared with $CXCR4^-$ BMMNCs, $CXCR4^+$ BMMNCs exhibit greater migratory capacity and are more effective at improving neovascularization, releasing trophic factors, and facilitating tissue repair after acute ischemia (Seeger et al., 2009). In addition, the tissue-committed stem cell (TCSC), a population of non-adherent $CXCR4^+$ cells, express mRNA for various markers of progenitor cells and can circulate into peripheral tissues, where they contribute to regeneration after tissue damage (Kucia et al., 2005, 2007; Ratajczak et al., 2004, 2007). It has been reported that hypoxia upregulates the expression of CXCR4 in ischemic regions (Tang et al., 2009). In addition, CXCR4 knockout donor cells have significantly less survival potential than do wild-type donor cells in the recipient brain (Shichinohe et al., 2007). These findings suggest that the optimum cells for stroke therapy should be $CXCR4^+$.

The vast majority of BMMNC populations contain committed HSCs, which maintain all blood lineages, including erythrocytes, platelets, monocytes, granulocytes, and lymphocytes (Civin and Gore, 1993). HSCs have been shown to mobilize from bone marrow to peripheral blood circulation during stroke, and the concentration of HSCs in blood correlates with neurofunctional improvements in patients after stroke (Taguchi et al., 2009). It has been reported that allogeneic grafting of HSCs reduced post-ischemic inflammation and improved outcome in a mouse stroke model (Schwarting et al., 2008). Furthermore, HSCs were shown to transdifferentiate across tissue-lineage boundaries into various terminal cell types, including non-HSC (Jang et al., 2004; Krause et al., 2001; Orlic et al., 2003), microglia, and macroglia cells (Eglitis and Mezey, 1997). However, the transdifferentiation of HSCs has been debated vigorously (Fukuda and Fujita, 2005; Murry et al., 2004; Wagers et al., 2002). Possible explanations, such as cell fusion (Terada et al., 2002; Ying et al., 2002) and epigenetic changes in recipient tissues (Hochedlinger and Jaenisch, 2003; Jaenisch, 2002), are not fully able to explain the mechanisms of HSC transdifferentiation. It has been reported that the CXCR4 receptor is widely expressed on both HSCs and TCSCs. CD45, a cell surface marker uniquely expressed on HSCs (Thomas, 1989), can be used to separate $CXCR4^+$ BMMNCs into a $CXCR4^+CD45^+$ subpopulation enriched in HSCs and a $CXCR4^+CD45^-$ subpopulation highly enriched in non-hematopoietic TCSCs (Kucia et al., 2005). To the best of our knowledge, no report has described the effects of $CXCR4^+CD45^+$ and $CXCR4^+CD45^-$ BMMNCs on outcome of ischemic stroke.

In this study, we examined whether one subpopulation of BMMNCs provides better protection after ischemic stroke than unfractionated BMMNCs. We found that $CXCR4^+CD45^-$ BMMNCs are superior to both $CXCR4^+CD45^+$ BMMNCs and unfractionated BMMNCs for improving stroke outcomes.

2. Materials and methods

2.1. Transient middle cerebral artery occlusion (tMCAO) and experimental groups

All studies were carried out in accordance with the guidelines for animal research and approved by the Institutional Animal Care and Use Committee at Zhengzhou University. All efforts were made to minimize animal suffering and reduce the number of animals used. Adult male C57BL/6J mice (stock number, J000664; weight, 25–30 g; 10–12 weeks old; Animal Center of Nanjing University School of Medicine, Nanjing, China) were housed at room temperature with a 12-h light/dark cycle in a pathogen-free environment and were given free access to food and water throughout the study. One technician performed all surgical procedures and was blinded to animal group assignment. Cerebral infarction was induced by tMCAO as previously described with slight modifications (Longa et al., 1989). Briefly, anesthesia was maintained with 1.5% halothane in air and was delivered via a snout mask. Body temperature was maintained at 37 °C throughout the surgical procedure with a heating pad. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a ventral midline incision. A round-tip, silicone-coated 6-0 nylon filament was gently inserted into the lumen of the ICA to the opening of the middle cerebral artery (MCA). The length of the filament was approximately 12 ± 2 mm and determined according to the weight of each mouse. Successful MCAO was defined as a decrease in cerebral blood flow (CBF) of more than 80% compared with that of the contralateral hemisphere, as measured by laser-Doppler flowmetry (Moor Instruments, Devon, UK). For the sham operation, mice underwent the same procedure, with the only difference being that the filaments did not occlude the MCA and were withdrawn from the ICA immediately. After 90 min of MCAO, the filaments were withdrawn into the stump of the ECA to initiate blood flow reperfusion. After surgery, an investigator blinded to group assignment allocated the tMCAO animals into one of four treatment groups: vehicle, unfractionated BMMNCs, $CXCR4^+CD45^+$ BMMNCs, and $CXCR4^+CD45^-$ BMMNCs. The sham-operated animals received the same allocation and treatments.

2.2. Donor BMMNC isolation

Pathogen-free eGFP transgenic mice (C57BL/6J-Tg (CAG-eGFP); 5–6 weeks old, purchased from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China) were used as cell donors. This mouse strain carries an eGFP-expressing gene controlled by chicken β-actin promoter in all tissues. The mice were euthanized by CO_2 inhalation, and bone marrow was flushed from medullary cavities of the humeri, femora, and tibiae. After the bone marrow was subjected to 1.084 g/mL Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences, Uppsala, Sweden) density centrifugation, the buffy coat layer was aspirated and resuspended in 100 mM phosphate-buffered saline (PBS, pH7.4). The viability of the isolated BMMNCs was assessed by trypan blue (Sigma–Aldrich, St. Louis, MO, USA) resistance staining. Approximately 1.2×10^8

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