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Evaluation of the survival of bone marrow-derived mononuclear cells and the growth factors produced upon intramedullary transplantation in rat models of acute spinal cord injury

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ABSTRACT

Intramedullary bone marrow-derived mononuclear cell (BM-MNC) transplantation has demonstrated neuroprotective effects in the chronic stage of spinal cord injury (SCI). However, no previous study has evaluated its effects in the acute stage, even though cell death occurs mainly within 1 week after injury in all neuronal cells. Moreover, the mechanism underlying these effects remains unclear. We aimed to investigate the survival of intramedullary transplanted allogeneic BM-MNCs and the production of growth factors after transplantation to clarify the therapeutic potential of intramedullary transplanted BM-MNCs and their protective effects in acute SCI. Sprague-Dawley rats were subjected to traumatic SCI and received intramedullary transplantation of $EGFP^{+}$ BM-MNCs ($n = 6$), BM-MNCs ($n = 10$), or solvent ($n = 10$) immediately after injury. To evaluate the transplanted BM-MNCs and their therapeutic effects, immunohistochemical evaluations were performed at 3 and 7 days post-injury (DPI). BM-MNCs were observed at the injected site at both 3 (683 ± 83 cells/mm^2) and 7 DPI (395 ± 64 cells/mm^2). The expression of hepatocyte growth factor was observed in approximately 20% transplanted BM-MNCs. Some BM-MNCs also expressed monocyte chemotactic protein-1 or vascular endothelial growth factor. The demyelinated area and number of cleaved caspase-3-positive cells were significantly smaller in the BM-MNC-transplanted group at 3 DPI. Hindlimb locomotor function was significantly improved in the BM-MNC-transplanted group at 7 DPI. These results suggest that intramedullary transplantation of BM-MNCs is an efficient method for introducing a large number of growth factor-producing cells that can induce neuroprotective effects in the acute stage of SCI.

1. Introduction

Several recent studies have investigated regenerative therapy for spinal cord injury (SCI) (Pego et al., 2012), including transplantation of bone marrow-derived mononuclear cells (BM-MNCs), which contain various cell types such as mature and immature blood cells, immune cells, and stromal cells (Yoshihara et al., 2007; Guo et al., 2012). BM-MNCs are readily available after isolation and do not require culture. In addition, various transplantation methods, including intravenous, intramedullary, and intraventricular methods, have been reported for delivering BM-MNCs to the injured spinal cord (Akiyama et al., 2002; Inoue et al., 2003; Yoshihara et al., 2007; Samdani et al., 2009; Guo et al., 2012). A previous study reported that intramedullary transplantation is superior to intravenous transplantation with regard to the supply of bone marrow-derived mesenchymal stem cells (BMSCs) to the injured spinal cord of rats (Kim et al., 2013). Therefore, intramedullary transplantation may be the best method for providing these cells and their therapeutic effects. However, detailed studies are essential for applying this transplantation method to clinical cases, because needle puncture can aggravate SCI. To date, two studies have tested intramedullary BM-MNC transplantation therapy in the acute stage of SCI and evaluated its effects in the chronic stage. Inoue et al. transplanted BM-MNCs in rat models via the intramedullary route 3 days post-injury (DPI) and demonstrated enhanced remyelination at 3 weeks after SCI (Inoue et al., 2003). Using rat models, Guo et al. performed the transplantation at 1 DPI and demonstrated the suppression of apoptosis at 2 weeks after SCI (Guo et al., 2012). However, as mentioned above, no study has evaluated the therapeutic effects of intramedullary BM-MNC transplantation in the acute stage of SCI. Neuronal cell death reportedly occurs primarily within 1 week in rat SCI (Liu et al., 1997; Casella et al., 2006). Therefore, evaluation in the acute stage of SCI may clarify the protective effects of intramedullary BM-MNC transplantation more accurately.

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Thus far, the mechanisms underlying the therapeutic effects achieved by BM-MNC transplantation remain unclear. Previous studies have attributed these beneficial effects to growth factors secreted by the transplanted cells in pigs and humans (Kamihata et al., 2001; Smiler et al., 2010). A study using mouse multiple sclerosis model reported that the therapeutic effects of BMSCs depend on the secretion of hepatocyte growth factor (HGF) (Bai et al., 2012). HGF has been reported to provide therapeutic effects in central nerve injury, such as the suppression of demyelination, apoptosis, and blood–brain barrier disruption, through the c-Met receptors that are upregulated after injury in rat neurons, oligodendrocytes, and astrocytes (Date et al., 2004; Kitamura et al., 2007). However, as opposed to the effects of BMSCs, the relationship between neuroprotective cytokines and BM-MNC transplantation therapy, particularly in SCI, is not well understood. A recent study in a rat SCI model showed that BM-MNC transplantation into the fourth ventricle induced an increase in HGF levels in the cerebrospinal fluid at 3 days after transplantation (Yoshihara et al., 2007). However, it remains unclear whether the BM-MNCs were the source of HGF. Moreover, one previous study reported that bone marrow cells have the ability to migrate to injured tissue and express several growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and monocyte chemotactic protein-1 (MCP-1) in mouse (Ziegelhoeffer et al., 2004), an angiogenic and neuroprotective chemokine (Ma et al., 2007; Madrigal et al., 2009). We hypothesized that BM-MNCs produce similar growth factors when transplanted into the rat injured spinal cord. Moreover, such growth factors may suppress any secondary damage such as apoptosis in the acute stage. In the present study, we performed intramedullary BM-MNC transplantation immediately after SCI in rat models and investigated the survival of transplanted BM-MNCs and their ability to produce HGF, MCP-1, and VEGF in the injured spinal cord to clarify the therapeutic potential of BM-MNCs. In addition, we investigated the therapeutic effects using immunohistochemistry at 3 and 7 DPI to determine the feasibility and safety of intramedullary BM-MNC transplantation for acute SCI.

2. Materials and methods

2.1. Experimental design

Contusive SCI was induced in the spinal cord of healthy female Sprague–Dawley (SD) rats (Tokyo Laboratory Animal Science, Tokyo, Japan) aged 10 weeks (n = 26). Their body weights ranged between 215 and 250 g. Immediately after the injury, rats (n = 6) were injected with BM-MNCs harvested from three SD-Tg (CAG-enhanced green fluorescent protein; EGFP) male rats (380–400 g) aged 10 weeks (Japan SLC, Shizuoka, Japan) to evaluate transplanted BM-MNCs. As a great number of tissue sections were used for evaluation of transplanted BM-MNCs, another BM-MNC-transplanted group was prepared for the evaluation of therapeutic effects. Rats (n = 10) were injected with BM-MNCs derived from five male SD rats (385–390 g) aged 10 weeks. Another group of rats received an injection of solvent without cells; these served as a control group (n = 10). Then, 50% rats from each group were euthanized for immunohistochemistry at 3 or 7 DPI. BM-MNCs prepared from one rat were injected into two rats for evaluation at the two time points. All rats were housed in a clean room under a 12-h light–dark cycle and a temperature of 26 °C. The study was approved by the Animal Care and Use Committee of Nippon Veterinary and Life Science University (Approval code: 26S-7). All experimental animals were treated in accordance with the guidelines issued by the Animal Care and Use Committee of Nippon Veterinary and Life Science University.

2.2. Preparation of BM-MNCs

To prepare BM-MNCs, bone marrow cells were collected under sterile conditions as described in a previous study, with some minor changes (Wu et al., 2003). Briefly, the donor rats were euthanized in a transparent box using isoflurane (Pfizer, Pearl River, NY, USA) saturated in an absorbent material (Kimwipe; Nippon paper crecia, Tokyo, Japan). Rats were carefully observed and respiratory and cardiac arrest confirmed. The epiphyses were removed from the femurs and tibiae and the marrows were slowly flushed out with 10 ml saline to obtain a cell suspension. The bone marrow cells suspension was filtered through a 100-μm mesh cell strainer (Corning; Corning, NY, USA).

BM-MNCs were isolated as previously described, with some minor changes (Ishida et al., 2004). Briefly, the collected bone marrow cell suspension was carefully loaded on density gradient media (Lymphoprep: AXIS-SHIELD, Dundee, UK) and centrifuged at 800 ×*g* for 20 min. The mononuclear cell layer above the gradient media layer was collected and resuspended in serum-free Dulbecco's modified eagle medium (DMEM, Sigma, St Louis, MO, USA). All cells derived from SD-Tg (CAG-EGFP) rats express EGFP and can be identified as EGFP$^+$ cells in the recipient animals through fluorescent microscopy (Wu et al., 2003).

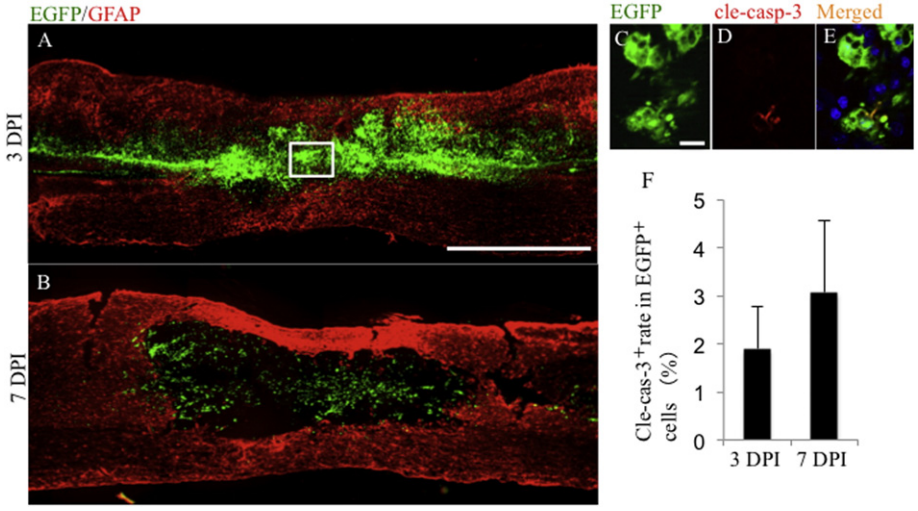

Fig. 1. Immunohistochemical evaluation of transplanted BM-MNCs in a rat model of acute spinal cord injury. (A): Transplanted EGFP$^+$ cells (green) are observed at the epicenter of the injury site, with a high cell density spread in the cranial and caudal directions over the injury area. (B): The number of cells has decreased at 7 DPI. GFAP (red) is merged to clarify the lesion site in A and B. Scale bar = 1 mm. (C): A few EGFP$^+$ cells immunoreact with cle-casp-3 (red; D). Scale bar = 50 μm. (E): A merged image showing colocalization of EGFP and cle-casp-3 at 3 DPI EGFP$^+$ cells were selected from the center of the lesion area, shown as a white square in A. (F): Cle-casp-3 immunoreactivity in EGFP$^+$ cells can be observed at the two time points.