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INTRAVENOUS INFUSION OF MESENCHYMAL STEM CELLS PROMOTES FUNCTIONAL RECOVERY IN A MODEL OF CHRONIC SPINAL CORD INJURY

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Abstract—Intravenous infusion of mesenchymal stem cells (MSCs) derived from adult bone marrow improves behavioral function in rat models of spinal cord injury (SCI). However, most studies have focused on the acute or subacute phase of SCI. In the present study, MSCs derived from bone marrow of rats were intravenously infused 10 weeks after the induction of a severe contusive SCI. Open field locomotor function was assessed weekly until 20 weeks post-SCI. Motor recovery was greater in the MSC-treated group with rapid improvement beginning in earlier post-infusion times than in the vehicle-treated group. Blood spinal cord barrier (BSCB) integrity was assessed by the intravenous infusion of Evans Blue (EvB) with spectrophotometric quantitation of its leakage into the parenchyma. In MSC-treated rats, BSCB leakage was reduced. Immunohistochemical staining for RECA-1 and PDGFR-β showed increased microvascula ture/repair-neovascularization in MSC-treated rats. There was extensive remyelination around the lesion center and increased sprouting of the corticospinal tract and serotonergic fibers after MSC infusion. These results indicate that the systemic infusion of MSCs results in functional improvement that is associated with structural changes in the chronically injured spinal cord including stabilization of the BSCB, axonal sprouting/regeneration and remyelination.

Key words: transplantation, mesenchymal stem cell, spinal cord injury.

INTRODUCTION

There are approximately 5.3 million people living with the consequences of spinal cord injury (SCI) worldwide (Wyndaele and Wyndaele, 2006; van den Berg et al., 2010; Piltti et al., 2013). Local and segment-limited primary damage to the spinal cord is characterized by the rupture or contusion of axons and the subsequent development of hemorrhage, ischemia, and edema. The damaged area expands considerably during the first weeks due to secondary damage to neuronal and glial cells. The combination of primary and secondary damage results in necrosis with tissue loss and chronic paralysis with sensorimotor disturbances (Schwab et al., 2006).

A promising cell-based therapy in the treatment of SCI using mesenchymal stem cells (MSCs) is currently being investigated. However, most studies have focused on the acute phase of SCI (Tetzlaff et al., 2011). In an acute model of rodent SCI, direct transplantation of MSCs enhances functional recovery, promotes axonal regeneration, reduces lesion size and protects the corticospinal tract (CST) (Sasaki et al., 2009). Intravenous infusion of MSCs also improves functional outcome after acute (Quertainmont et al., 2012; Matsushita et al., 2015) and acute/subacute (Osaka et al., 2010) contusive SCI. However, there are a limited number of studies evaluating treatment efficacy during the chronic phase of SCI (Tetzlaff et al., 2011). It is an important clinical and research issue to develop approaches to provide therapeutic efficacy in chronic SCI.

In this study MSCs derived from bone marrow were intravenously delivered at 10 weeks after SCI to investigate whether systemic injections improve functional outcome after contusive chronic SCI. Evaluation of the behavioral outcome, histological changes, blood spinal cord barrier (BSCB) disruption, remyelination and sprouting of descending axon tracts were performed to study the structural and functional changes after MSC infusion in a model of chronic SCI in rats.

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Abbreviations: BBB, Basso, Beattie, and Bresnahan; BSCB, blood spinal cord barrier; CST, corticospinal tract; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EvB, Evans Blue; MSCs, mesenchymal stem cells; NDS, normal donkey serum; PBS, phosphate-buffered saline; SCI, spinal cord injury; TCA, trichloroacetic acid.

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EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and the institutional guidelines in Sapporo Medical University. The use of animals in this study was approved by the Animal Care and Use Committee of Sapporo Medical University. All methods and data were reported in accordance with guidelines provided by *Animals in Research: Reporting in Vivo Experiments (ARRIVE) and Minimum Information about a Spinal Cord Injury Experiment (MIASCI)* (Kilkenny et al., 2010; Lemmon et al., 2014).

Preparation of MSCs from rat bone marrow

MSC culture preparation was based on our previous studies (Kim et al., 2006; Ukai et al., 2007; Suzuki et al., 2013; Takayanagi et al., 2015). Briefly, bone marrow was obtained from the femoral bones of eight adult wild type and two GFP-expressing Sprague–Dawley rats (W-Tg (CAG-GFP)184Ys), diluted to 25 ml with Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM L-glutamine (SIGMA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Thermo Fisher Scientific Inc.) and incubated for 3 days (5% CO_2, 37 °C). MSCs generated from a single donor were infused into two recipients. When cultures almost reached confluence, the adherent cells were detached with a trypsin–EDTA solution (SIGMA) and subcultured on 150 mm^2 Tissue Culture Dish (1030-150: IWAKI, Tokyo, Japan; surface area: 148 cm^2) at 5×10^5 cells/ml with 14 ml culture medium, thus plating density is about $3.4 \times 10^3/cm^2$. The phenotype analysis of surface antigen on MSCs were $CD45^-$, $CD73^+$, $CD90^+$ and $CD106^-$ (Kim et al., 2006). The cultured MSCs were used for transplantation after three passages.

SCI model

Contusive SCI was performed as described previously (Matsushita et al., 2015). Briefly, adult male Sprague–Dawley rats (250-300 g) were anesthetized with an intraperitoneal (IP) injection of ketamine and xylazine (90/4 mg/kg). After incision, the T9 vertebra was stabilized, a laminectomy performed at the T9-10 level spinal cord, and a 200-kdyn contusion delivered using the Infinite Horizons impactor (Precision Systems and Instrumentation, LLC, Lexington, KY, USA). Appropriate post-operative care, including twice-daily manual bladder expression for up to 14 days, was provided for all animals. Rats were housed in an atmosphere of 50% humidity at a temperature of 24 ± 2 °C.

Thirty-two rats received contusive SCI in this study. Sixteen rats were used for Basso, Beattie, and Bresnahan (BBB) behavioral testing (n = 8/MSC group, n = 8/Vehicle group). These 16 animals were used for immunofluorescence studies (PKC-γ and 5-HT; n = 4/MSC group, n = 4/Vehicle group) and plastic/EM studies (n = 4/MSC group, n = 4/Vehicle group). Another eight rats were used for GFP study (n = 4/MSC group, n = 4/Vehicle group) and the other eight rats were used for Evans Blue (EvB) study (n = 4/MSC group, n = 4/Vehicle group).

Behavioral testing

Open field locomotor function was assessed using the BBB locomotor rating scale (Basso et al., 1995) by an evaluator blinded to treatment condition. Rats were scored 2 days prior to SCI induction, 1 week post-contusion, and at weekly intervals thereafter until sacrifice at 20 weeks post-SCI (n = 8/MSC group, n = 8/Vehicle group).

Transplantation procedure

Rats were randomized and received intravenous infusion of MSCs (1.0×10^6) in 1.0 ml total fluid volume (DMEM) or vehicle (1.0 ml fresh DMEM alone) via femoral vein 10 weeks after SCI. All rats were injected daily with cyclosporine A (10 mg/kg, IP) (Liu et al., 2006; Osaka et al., 2010; Zheng et al., 2010; Alexanian et al., 2011; Davies et al., 2011). The inclusion criterion for this study was a BBB score of less than eight at 10 weeks after SCI induction.

Assessment of BSCB integrity with EvB

Assessment of BSCB integrity with EvB was performed as described with minor modifications (Wang and Lai, 2014; Matsushita et al., 2015). Briefly, six hours prior to sacrifice, a 4% solution of EvB (4 ml/kg) was infused into the femoral veins of each rat (n = 4/MSC group, n = 4/Vehicle group) at 11 weeks post-contusion (i.e., 1 week after MSC or vehicle infusion). After perfusion with phosphate-buffered saline (PBS), each spinal cord was removed and stripped of its meninges. To quantify EvB dye extravasation, three 1-cm segments of the spinal cord were used: one centered on the impact site and one each rostral and caudal to the lesion. The sections were removed, weighed, homogenized in 7.5% (w/v) trichloroacetic acid (TCA), and centrifuged for 15 min at 10,000 rpm at 4 °C. Absorbance of the supernatant was measured at 620 nm using a microplate fluorescence reader (Infinite M1000 Pro, Tecan Group Ltd., Kawasaki, Japan) and EvB concentrations were calculated using standard curves obtained by serial dilution of EvB in 50% TCA followed by the subtraction of background values obtained for cords processed without EvB infusion. Values were expressed as μg of EvB per g of spinal cord tissue.

Histological processing

For immunohistological analysis, rats were deeply anesthetized with ketamine and xylazine (90/4 mg/kg), perfused with saline and 4% paraformaldehyde in 0.1 M phosphate buffer, and processed for standard frozen sectioning. Cryosections were cut using a cryostat and mounted on glass slides. Sections were washed in PBS and 0.1% Tween 20 (PBS-T) three times, blocked in 5% normal donkey serum (NDS) and 0.3% Triton X-100 in