

Canine Bone Marrow Stromal Cells Promote Functional Recovery in Mice with Spinal Cord Injury

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ABSTRACT. Regenerative therapy has begun to be clinically applied in humans and dogs to treat neurological disorders, such as spinal cord injury (SCI). Here, we show the therapeutic potential of transplantation of cultured canine bone marrow stromal cells (BMSCs) into mice with SCI. Canine BMSC transplantation therapy was performed, immediately after the spinal cord was injured. Canine BMSC therapy enhanced functional recovery of the hind limbs in mice with SCI. Nestin-positive cells were observed only in the lesion of mice with SCI that received BMSCs. These results suggest that canine BMSCs promote functional recovery in mice with SCI and that migration of nestin-positive cells may contribute to the efficacy of the BMSC treatment.

KEY WORDS: bone marrow stromal cell (BMSCs), canine, mouse, spinal cord injury, transplantation.

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Despite progress in the treatment of spinal cord injury (SCI), recovery from severe paralysis remains difficult. Several cell types, including embryonic spinal cord stem cells [10], Schwann cells [12], olfactory ensheathing glia [11] and bone marrow-derived cells [14], have been used in transplants aimed at spinal cord regeneration. Bone marrow stromal cells (BMSCs) are adherent, non-hematopoietic cells obtained from culturing bone marrow aspirates [14]. Canine BMSCs are technically easy to isolate and expand [7]. The most significant practical advantages of using BMSCs are the capability of autologous transplantation, low cost of culturing and very low risk of teratoma formation [14]. Recently, spinal cord regenerative therapy using bone marrow-derived cells has begun to be clinically applied, leading to promising results in human and veterinary medicine [1, 13, 16]. However, the mechanism of the effects of these cell sources and the cells that are the most effective remain unknown. In the present study, we employed a SCI model to investigate the efficacy of canine BMSC treatment for improving loco-

motor function in immunosuppressed mice. Bone marrow cells were collected from the humeri and femora of a clinically healthy dog (2-year-old male Beagle) under anesthesia. The animal received humane care in compliance with the guidelines for treatment of experimental animals at Yamaguchi University. The bone marrow cells were seeded onto Nunclon™Δsurface (NUNC, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and cultured in Dulbecco's modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin B in a 5% CO₂ incubator at 37°C. After 2 days, non-adherent cells were washed away with medium. The adherent cells were used as canine BMSCs. The culture medium was changed twice a week, and cells in the second or third passage were used. During subculture, flow cytometry analyses were performed utilizing Gallios equipment (Beckman Coulter, Inc., Brea, CA, U.S.A.). Propidium iodide (Sigma-Aldrich, Steinheim, Germany) was used to exclude dead cells from analyses. Data were analyzed using Kaluza software (Beckman Coulter). Monoclonal antibodies against CD11b (AbD serotec, Oxford, U.K.), CD29-PE (Abcam, Cambridge, U.K.), CD44-PE-Cy7 (Biolegend, San Diego, CA, U.S.A.), CD45-e-fluor (eBioscience, Inc., San Diego, CA, U.S.A.) and CD90-APC (eBioscience) were used in this study. Secondary detection of the CD11b antibody was performed using goat polyclonal secondary antibody to mouse IgG-H&L (DyLight® 488) (Abcam). Isotype-identical antibodies (IgGs) were used as

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negative controls.

The surface of cells was positive for CD29, CD44 and CD90 and negative for CD11b and CD45 (Fig. 1A). To confirm the differentiation potential, canine BMSCs were grown in osteogenic and adipogenic differentiation media (Cyagen Biosciences, Inc., Santa Clara, CA, U.S.A.) according to the manufacturer's instructions. After 2 weeks, deposition of bone mineral was observed following alizarin red staining (pH 4.3; AppliChem, Chicago, IL, U.S.A.) (Fig. 1B). Cells containing lipid droplets were observed following Oil Red O staining (Sigma–Aldrich) (Fig. 1B).

The SCI model was performed using female severe combined immunodeficiency mice (n=24). Mice were anesthetized with pentobarbital (50 mg/kg, i.p.), a dorsal laminectomy was performed at the T10 level, and the exposed spinal cord was completely transected with a surgical knife. The animals were divided into 2 groups that were treated with BMSCs or DMEM (control). Cell transplantation was performed immediately after SCI by infusing 1×10^5 cells/ μ l in 12 μ l DMEM using a Hamilton syringe (Hamilton Co., Reno, NV, U.S.A.) into six points rostral and six points caudal to the injury site (1 μ l per location for a total of 12 μ l per animal). Before transplantation, canine BMSCs were labeled using a carboxyfluorescein diacetate-succinimidyl ester cell tracer kit (Invitrogen) according to the manufacturer's instructions. The labeled cells were washed three times and resuspended in DMEM. Cell labeling was confirmed by fluorescent microscope observation.

Motor functional evaluation was performed for each hind limb at 1–4 weeks post-SCI using the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale [2]. Significant increases in BBB scores in the BMSC group compared to the control were observed at 1–4 weeks post-SCI.

At 4 weeks post-SCI, genomic DNA was prepared from the spinal cord homogenate of 2 mice per groups using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The presence or absence of the sex determination region on the male Y chromosome (*SRY*) in recipient female mice was assessed with PCR. Primer sequences for *SRY* were obtained from published sequences (AF107021; forward primer, 5'-CAAGATGGCTCTAGAGAATCCC-3'; reverse primer, 5'-AGCTGTCCGTGTAGGTGA-3') and amplified a product of 284 bp. The PCR conditions were as follows: incubation at 94°C for 2 min; 40 cycles of incubation at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. PCR products were separated using 2% agarose gel electrophoresis, and gels were stained with ethidium bromide.

Four weeks post-SCI, the remaining mice in each group were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused transcardially with Zamboni solution. The lesion region, including adjacent intact areas of the spinal cord, was excised, immersed in Zamboni solution overnight and cryoprotected by immersion in a series of sucrose solutions (10%, 15% and 20% sucrose in 0.1 M PBS) at 4°C. The tissues were then frozen, embedded in OCT compound (Sakura Finetek Co., Ltd., Tokyo, Japan), sectioned longitudinally at 8 μ m thickness using a cryostat and mounted on (3-aminopropyl) trimethoxysilane (APS)-coated slides for

use in immunohistochemical analysis. Primary antibodies for microtubule-associated protein-2 (MAP-2; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) (1:100) and nestin (Santa Cruz Biotechnology) (1:100) were used in this study.

Canine BMSCs used in this study were CD29⁺, CD44⁺, CD90⁺, CD11b⁻ and CD45⁻ (Fig. 1A) as reported in previous studies [7, 15]. The canine BMSCs showed adipogenic and osteogenic multi-differentiation potential (Fig. 1B). We [13] and other authors [6] previously reported that canine BMSCs differentiate into neuronal cells. Thus, canine BMSCs were confirmed to be capable of differentiation into not only mesoblast-derived cells but also into ectoblast-derived cells, as reported for other species in previous studies.

Although this study was not a double-blind design, canine BMSC transplantation enhanced the functional recovery of the hind limbs in mice with SCI (Fig. 2). Similar functional recoveries were observed in previous reports with other species using different contusion SCI model [4, 8] which were most common type of SCI. The role of transplanted BMSCs remains to be elucidated. Several studies have reported that BMSCs have indirect neuroprotective effects due to secretion of neurotrophic or growth factors, including basic fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and insulin-like growth factor 1 [5, 9]. Transplanted BMSCs integrate into the host spinal cord and contribute to rebuilding of axons and axonal function [5]. Moreover, about 30% of the BMSCs acquire a neuronal phenotype without evidence of cell fusion when co-cultured with neurons [9]. BMSCs can also acquire electrophysiological functions similar to neurons *in vitro* and express neuron-specific receptors [20]. The engrafted BMSCs degrade the extracellular matrix in the glial scar by secreting several proteases, such as matrix metalloproteases, to promote neurite outgrowth from spinal cord neurons [17]. Thus, transplanted BMSCs may play an indirect neuroprotective role through multiple mechanisms. Transplanted cells in this study were detected in SCI lesion site (T10) and lumbar cord in mice at genomic level (Fig. 3A) and histologically near the glial scar around the lesion site at least 4 weeks after treatment (Fig. 3B). PCR amplification was more sensitive than fluorescence staining.

Nestin-positive cells were morphologically fibroblastic, differed from the transplanted cells, and were not observed close to the fluorescently labeled transplanted BMSCs in this study (Fig. 4). Thus, transplanted BMSCs may not differentiate into cells with neuronal phenotypes in mice with SCI. Nestin is an intermediate filament protein and a widely employed marker of multipotent neural stem cells [18, 19]. On the other hand, reactive astrocytes are rather beneficial in promoting neuronal survival by releasing many trophic factors and expressing stem cell markers, such as nestin [3]. Thus, the nestin-positive cells might be derived from reactive astrocytes. Nestin-positive cells in the scar tissue may be associated with the functional recovery of the hind limbs in SCI mice, although the *in vivo* physiological function of nestin remains unknown.

The xenotransplantation model used in this study may not necessarily reflect SCI in canines; however, this model may

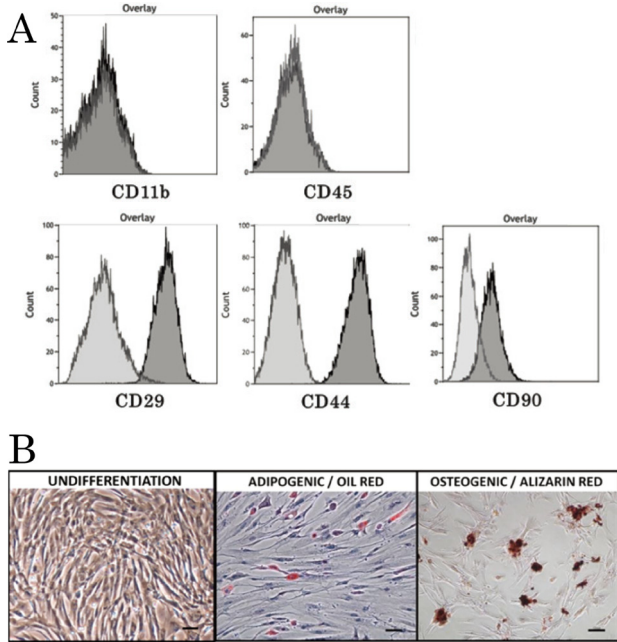


Fig. 1. Characterization of canine BMSCs. (A) Flow cytometry analysis. Canine BMSCs were negative for CD11b and CD45 and positive for CD29, CD44 and CD90. (B) Differentiation potential of canine BMSCs. Canine BMSCs showed adipogenic and osteogenic potential in the presence of appropriate differentiation media.

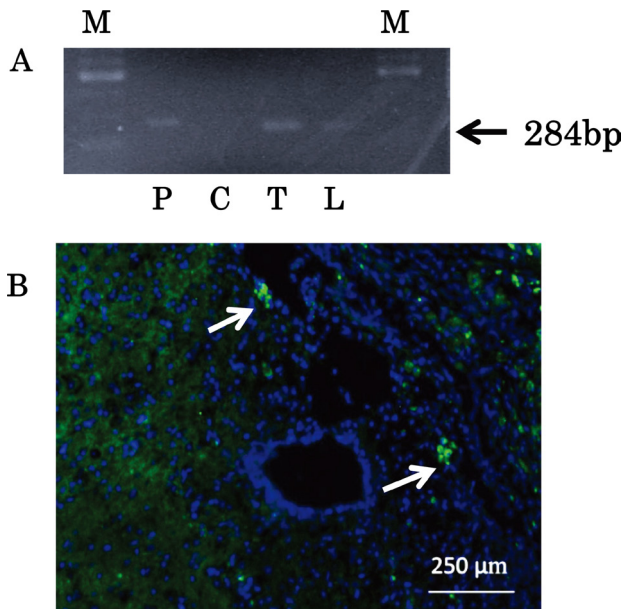


Fig. 3. Detection of donor cells. (A) PCR amplification of canine SRY was observed in the SCI lesion site (T10) and lumbar cord in mice. P, positive control (male dog gDNA); C, cervical cord; T, thoracic cord; L, lumbar cord; M, marker. (B) Localization of fluorescently labeled cells transplanted into the spinal cord. Fluorescent areas (arrows) were detected near the glial scar around the lesion site in the mouse spinal cord. Bar=250 μ m.

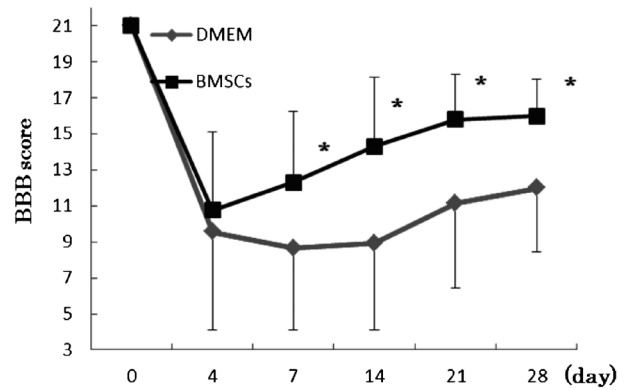


Fig. 2. Comparison of the BBB locomotor rating scores in mice with SCI with or without canine BMSC transplantation therapy. Data are the mean \pm SD. The Mann-Whitney U test was used to compare groups. * P <0.05 compared to control. DMEM, Dulbecco's modified Eagle Medium.

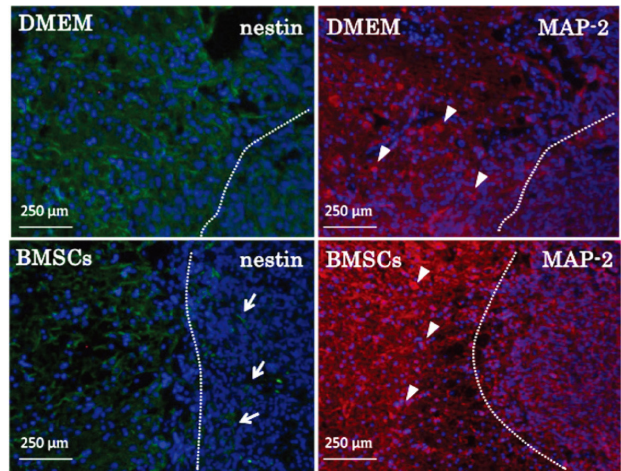


Fig. 4. Immunohistochemical analysis of mouse spinal cord following treatment with canine BMSCs. A small number of nestin-positive cells in the glial scar were observed only in the BMSC group (arrows). A few cells positive for neuronal markers (MAP-2; arrowheads) were observed outside the glial scar, but none were observed in the scar. The dotted line shows the border between the intact spinal cord (left) and the glial scar (right). DMEM, Dulbecco's modified Eagle Medium. Bar=250 μ m.

establish a certain standard to evaluate the most effective cell sources, the number of cells required for treatment, the timing of transplantation and other factors.

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