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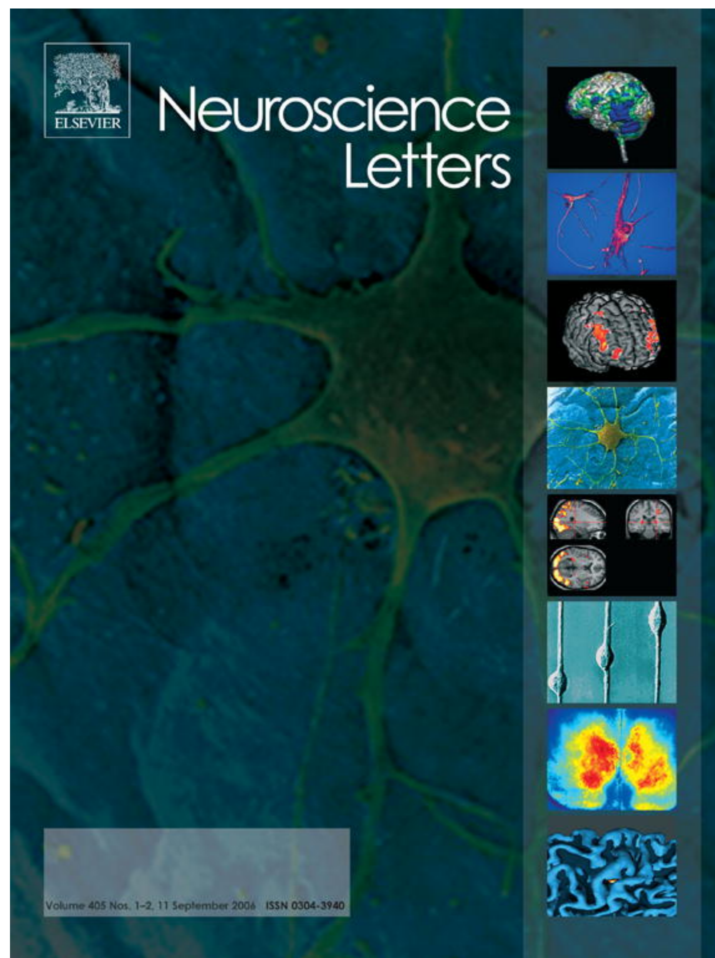

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Selective migration and engraftment of bone marrow mesenchymal stem cells in rat lumbar dorsal root ganglia after sciatic nerve constriction

María Florencia Coronel [1], Patricia Leonor Musolino [1], Marcelo José Villar *

School of Biomedical Sciences, Austral University, Av. Pte. Perón 1500, B1629AHJ Pilar, Buenos Aires, Argentina

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Abstract

Bone marrow mesenchymal stem cells (MSCs) preferentially migrate to the injured hemisphere when administered intravenously to rats with traumatic or ischemic brain injuries. In this study, we have investigated the localization of MSCs injected into the lumbar-4 dorsal root ganglion (L4-DRG) of rats with a sciatic nerve single ligature nerve constriction (SLNC). MSCs were isolated by their adherence to plastic, cultured until confluence and labelled with Hoechst. Animals with a unilateral injection of MSCs were subjected to an ipsilateral, bilateral or contralateral SLNC. After 9 days, they were perfused and the lumbar DRGs were dissected out, cut in a cryostat and observed with a fluorescence microscope. Large numbers of Hoechst-positive cells were observed in the injected L4-DRG, distributed around primary afferent neurons, resembling the anatomical localization of glial cells. In animals with an ipsilateral SLNC, some cells were detected in the ipsilateral L3, L5 or L6-DRGs but not in the contralateral ganglia. In animals with a bilateral lesion, MSCs migrated to both the ipsilateral and contralateral DRGs whereas in animals with a contralateral ligature, MSCs migrated to the contralateral DRGs. These results suggest that MSCs preferentially engraft in DRGs hosting primary sensory neurons affected by a lesion of their peripheral branches. Further studies should be carried out in order to elucidate the molecular mechanisms involved in this migration and homing, in order to evaluate the possible use of MSCs as a new therapeutic strategy for the treatment of peripheral nerve neuropathies.
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Keywords: Stem cell trafficking; Primary afferent neurons; Peripheral nerve lesion; Peripheral neuropathies

Bone marrow mesenchymal stem cells (MSCs) are multipotent stem cells that have the capacity to give rise to several types of cells: osteoblasts, chondrocytes, adipocytes and miocytes [20,21]. Under certain culture conditions, MSCs can be induced to differentiate to cells with a Schwann cell morphology which harbour characteristic markers such as S-100, the nerve growth factor low affinity receptor p75, the glial fibrillary acidic protein (GFAP) and the sulfatide glycolipid O4 [7,28]. They can also differentiate *in vitro* into neuron-like cells that express neural markers such as nestin and NeuN [1,22]. Furthermore, MSCs have the capacity to differentiate into myelin-forming cells *in vivo* [23,28] and *in vitro* [7,18], and following peripheral nerve or spinal cord lesions, they were shown to repair demyelinated axons [7,18,23,28]. MSCs administered intravenously to rats subjected to traumatic [14,15] or ischemic [4] brain injuries preferentially migrate to the injured hemisphere [14,15], where they increase the expression of growth factors [15] and improve the functional recovery of these animals [4,14,15]. Furthermore, human and rat MSCs have been shown to migrate and engraft in the brains of albino rats: the cells migrated from the injection site along known pathways for migration of neural stem cells to successive layers of the brain [2]. Moreover, it has been shown that intravenously administered MSCs have the ability to migrate to the spinal cord and dorsal root ganglia (DRGs) of control mice and differentiate into neuroectodermal and microglial cells [6]. In the present study, we have investigated the fate and localization of MSCs injected into the lumbar-4 DRG (L4-DRG) of rats with an ipsilateral, bilateral or contralateral sciatic nerve single ligature nerve constriction (SLNC).

Sprague Dawley male rats (200–300 g) were sacrificed using an overdose of chloral hydrate (1.5 g/kg, i.p.) and their tibiae and femurs were dissected out from attached muscle and connective tissues. The epiphysis of the bones was removed and the marrow was extracted with 3 ml of DMEM (GIBCO/BRL) using a 15 G

* Corresponding author. Tel.: +54 2322 482948; fax: +54 2322 482204.
E-mail address: mvillar@cas.austral.edu.ar (M.J. Villar).
[1] These authors equally contributed to this work and therefore should be regarded as joint first authors.

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needle and a 3 ml syringe. Red cells were lysed with 0.15 M buffered ammonium chloride solution and the remaining cells washed twice with phosphate-buffered saline (PBS). The cells were then centrifuged through a density gradient (Ficoll-Paque Plus, 1.077 g/ml, Pharmacia) for 30 min at $400 \times g$. The interface containing mononuclear cells was washed with PBS and centrifuged for 10 min at $250 \times g$. The cells were then suspended at a concentration of 10×10^6 cells/ml in DMEM, 10% fetal bovine serum, 50 μg/ml gentamicine, 2.5 μg/ml anfotericine B and 50×10^6 cells were plated in 25 cm^2 cell culture flasks. After 3 days, the non-adherent cells (afterwards referred to as bone marrow non-adherent mononuclear cells, BNMCs) were removed by replacing the culture medium. Medium was changed every 4–5 days until confluence was reached. Prior to transplantation, MSCs were marked after incubation for 24 h with the fluorescence nuclear marker bis-benzamide (Hoechst 33258, Sigma, 1 μg/ml) [12]. They were harvested by incubation with 0.25% trypsin–1 mM EDTA, washed with PBS and suspended at a concentration of 50×10^6 cells/ml in PBS.

Three groups of lesioned animals were evaluated. All of them received a unilateral intraganglionic (L4-DRG) injection of MSCs and were subjected to either an ipsilateral ($n = 6$), bilateral ($n = 6$) or contralateral ($n = 6$) "medium" SLNC (mSLNC) of the sciatic nerve. All animals were sacrificed after 9 days of survival. Two control groups were also evaluated, one consisted of animals with a unilateral injection of MSCs and an ipsilateral mSLNC that were sacrificed immediately after ($n = 4$); the other one included animals with a unilateral injection of BNMCs and an ipsilateral mSLNC with 9 days of survival ($n = 6$).

Adult Sprague Dawley rats (200–300 g, Fucal, Buenos Aires, Argentina) were anesthetized with chloral hydrate (350 mg/kg, i.p.) and their right L4-DRG was exposed using a micro bone rongeurs after dissection of the aponeurotic and the paraspinal muscle group. For MSC transplantation, a suspension of cells (2×10^5 MSCs in 4 μl PBS) was injected into the ganglion *via* a drawn glass micropipete (70–100 μm tip diameter) using a micropump syringe injector, over a 60 s period of time. Sham animals were injected with BNMCs (2×10^5 BNMCs in 4 μl PBS) previously labelled with Hoechst. After the muscular-aponeurotic and skin individual suture, the sciatic nerve constriction was performed as described below.

The sciatic nerve was exposed and dissected free from the surrounding tissue at a mid-thigh level. It was then wrapped with a thin strip (5 mm long) of polyethylene and constricted to a mSLNC with a reduction of 40–80% of its original diameter [3]. The degree of constriction of each nerve was confirmed after perfusion of the animals and dissection under a surgery microscope using a 10 mm ruler, and also by microscopical observation of 16 μm sections. After 9 days of survival, the animals were deeply anesthetized using an overdose of chloral hydrate (1.5 g/kg, i.p.), and perfused through the heart with 60 ml of warm (37 °C) Tyrode's buffer (pH 7.4), followed by 60 ml of fixative (4% paraformaldehide and 0.2% picric acid in 0.16 M phosphate buffer, pH 7) [27] at 37 °C and 300 ml of the same fixative at 4 °C. The ipsilateral and contralateral L3–L6-DRGs and thoracic 10 (T10)-DRGs were removed and post fixed in the same fixative for 90 min at 4 °C. The tissues were then rinsed in 20% sucrose in phosphate buffer (pH 7.2) containing 0.1% sodium azide and stored at 4 °C.

Tissues were embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, USA) and cut longitudinally at 14 μm thickness in a cryostat (Microm, Heildeberg, Germany). Sections were mounted onto chrome alume-gelatin coated slides, allowed to dry for at least 1 h and examined with a Nikon Eclipse E-800 photomicroscope under fluorescence illumination using proper filters. Hoechst-positive MSCs present in each DRG section were counted. The total number of engrafted cells was calculated including all the DRGs affected by the sciatic nerve ligature. The results were expressed both as the mean value ± S.E.M. and as a percentage of the amount of injected cells. Photographs were taken using a Nikon DXM 1200 digital camera (Tokyo, Japan). Resolution, brightness and contrast of the digital images were optimized using the Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

Intraganglionic injection of MSCs and ipsilateral mSLNC: Hoechst-positive cells were observed in the injected L4-DRG and also in the other lumbar ganglia affected by the nerve lesion (ipsilateral L3, L5 and L6) (Fig. 1a–c). MSCs migrated to the ipsilateral L5-DRG in all the animals studied, whereas 83% of them showed engraftment in the ipsilateral L3 and/or L6-DRGs. The number of engrafted MSCs (389.00 ± 40.34) represented 0.19% of the total amount of cells injected. No MSC could be detected in either the contralateral lumbar DRGs, or the ipsilateral thoracic ganglia. In L4-DRG, large numbers of Hoechst-positive cells could be observed distributed along the intraganglionic nerve fibers and surrounding primary afferent neurons, with characteristic glial localization (Fig. 1a and b). A similar distribution was observed in the ipsilateral L3, L5 (Fig. 1c) and L6-DRGs.

Intraganglionic injection of MSCs and bilateral mSLNC: When bilateral lesions were made, migration and engraftment of MSCs occurred in both the ipsilateral (L3, L5 and L6) and contralateral (L3–L6) ganglia (Fig. 1d). Migration to the ipsilateral L5 and L6-DRGs and to the contralateral L5-DRG occurred in 100% of the animals studied. In 66% of them migration to the ipsilateral and/or contralateral L3-DRGs was observed. Engraftment occurred in the contralateral L4 and/or L6-DRGs in 83% of the animals studied. The total number of engrafted cells (565.83 ± 23.12), including the 7 ganglia in which homing occurred, represented 0.28% of the injected MSCs. No Hoechst-positive cells could be detected in T10-DRGs. MSC distribution in the injected L4-DRG, as well as in the ganglia where engraftment occurred, was exactly the same as that described above.

Intraganglionic injection of MSCs and contralateral mSLNC: In this group of animals, MSCs migrated from the injected L4-DRG to the contralateral ganglia (L3–L6), affected by the sciatic nerve ligature (Fig. 1e). MSCs migrated to the contralateral L4 and L5-DRGs in all the animals studied, whereas in 66% of them implantation occurred in the contralateral L3 and/or L6-DRGs. The total count of engrafted MSCs (401.60 ± 43.85) represented 0.20% of the total amount of cells injected. No migration was observed either to the ipsilateral L3, L5 or L6-DRGs, or to both the ipsilateral and contralateral T10-DRGs. In the injected L4-

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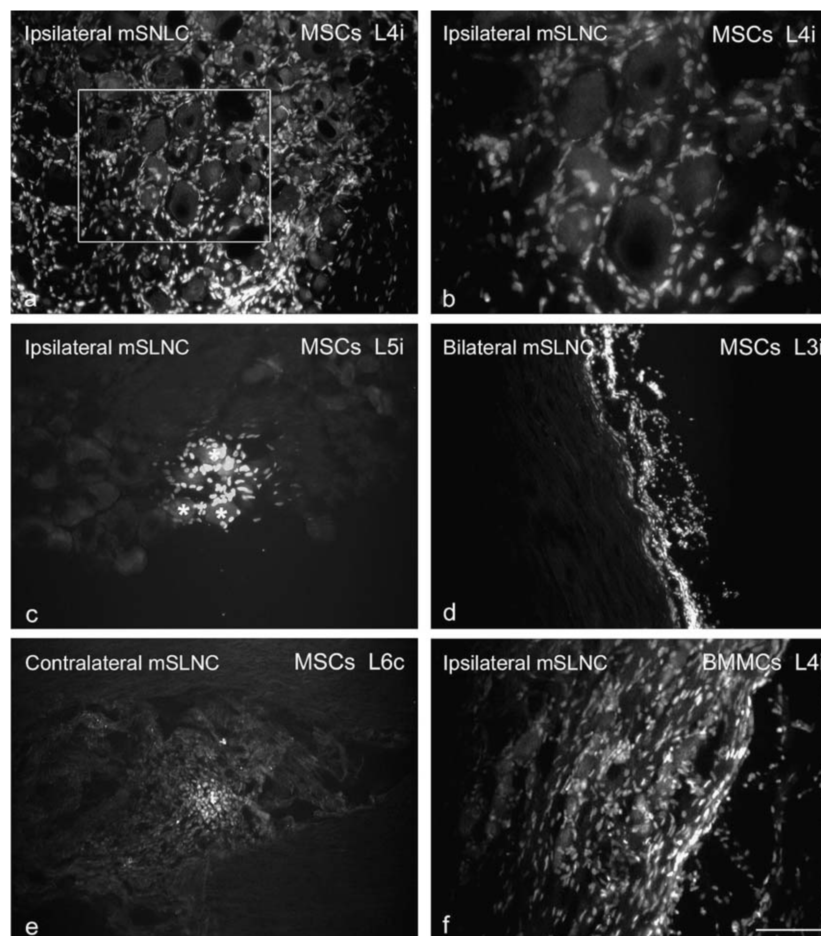

Fig. 1. Immunofluorescence micrographs showing Hoechst-positive MSCs in dorsal root ganglia of rats of different experimental protocols. In (a) and (b) the perineuronal localization of Hoechst-positive MSCs in an injected L4-DRG after 9 days of survival is observed. Boxed area in (a) is shown at a higher magnification in (b). Engraftment of MSCs in the ipsilateral L5-DRG (c) after an ipsilateral lesion, ipsilateral L3-DRG (d) after a bilateral lesion and contralateral L6-DRG (e) after a contralateral lesion, is shown. Asterisks in (c) indicate primary sensory neurons. In (f) the heterogeneous distribution of Hoechst-positive BNMCs in the injected L4-DRG is shown. Calibration bar: 50 μm (b); 100 μm (a, c, d and f); 200 μm (e).

DRG, MSCs distributed in a heterogeneous way without a clear relationship to neurons or fibers.

Control animals: BNMCs injected into the L4-DRG from lesioned animals showed a heterogeneous distribution (Fig. 1f) and did not migrate to any of the other DRGs. When animals were sacrificed immediately after surgery, MSCs localized mostly in the periphery of the injected DRG and no Hoechst-positive cells could be observed in any of the other evaluated ganglia.

In the present study, we show a selective migration and engraftment of bone marrow mesenchymal stem cells in lumbar dorsal root ganglia of animals subjected to a peripheral nerve ligature. Previous studies have shown that intravenously administered MSCs migrate to the DRGs of control mice [6]. However, to our knowledge, there are no reports showing a selective engraftment of MSCs in DRGs affected by a peripheral lesion.

When L4-DRGs corresponding to lesioned nerves were injected, MSCs acquired a striking perineuronal localization, resembling glial/satellite cells. This pattern of distribution seemed to be related to at least three different factors: period of time after the lesion, cell type injected and presence of primary afferent neuronal injury. This perineuronal localization was acquired in an active and time dependent fashion as it could not be observed immediately after the injection. It was also characteristic of MSCs, since another cell fraction of bone marrow, the BNMCs, distributed heterogeneously in the ganglia after the same survival time. Interestingly, when MSCs where injected in L4-DRGs from non-lesioned animals, the cells distributed throughout the ganglia in a non-organized manner similar to that of BNMCs. The characteristic localization adopted by MSCs in the affected ganglia suggests an association with a selective role in the injured nervous tissue.

MSCs migrated from the injected L4-DRG specifically to the other ganglia affected by the sciatic nerve injury, showing a selective migratory tropism of these cells for the lesioned ganglia. Engraftment occurred preferentially in the DRGs that host the major number of primary afferent neurons that integrate the sciatic nerve, L4 and L5. Homing occurred in a minor proportion in the other lumbar ganglia, L3 and L6. Migration of MSCs could occur both *via* the cerebrospinal fluid and/or the vascular system and the engraftment is probably mediated by

chemokines, cell surface receptors or/and antigens expressed by primary afferent neurons in response to the lesion. Tissue damage probably creates a favourable microenvironment (cytokine millieu, extracellular matrix) that enables efficient homing of the circulating stem cells [25,26]. In the lumbar DRGs, the expression of certain cell adhesion molecules (CAMs) such as TAG-1 [24] and gicerin [9] is regulated after peripheral nerve damage. The expression of other CAMs such as N-cadherin, NCAM and L1-CAM is regulated by changes in the firing pattern of neural impulses [10], like those associated to sciatic nerve lesions. Moreover, select chemotactic factors and cytokines expressed in the injured brain such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1α) and interleukin-8 (IL-8), have been implicated in both, rat and human MSC migration *in vitro* [25,26]. Also, tissue extracts prepared from rat ischemic brains increase MSC migration in *in vitro* studies using a microchemotaxis chamber [25,26].

MSCs express several adhesion-related antigens, *i.e.* the integrin subunits α4 (CD49d), α5, β1, integrins αvβ3 (CD51/CD61) and αvβ5 (CD51/b5), ICAM-1 (CD54), VCAM-1 (CD106) and CD44H [5]. MSCs also express both the mRNA and protein of the receptors CX3CR1, CXCR4, CCR2 and CCR5, each of them corresponding to the chemokines fractalkine, stromal cell-derived factor 1 (SDF-1), MCP-1 and MIP-1α, respectively [11]. In a model of left hypoglossal nerve avulsion, the chemokines fractalkine and SDF-1, have been demonstrated to partially mediate the trafficking of MSCs transplanted into the lateral ventricle to the impaired nucleus in the brain [11]. On the other hand, SDF-1 and its receptor CXCR4 have been detected in rat glial and neuronal cells *in vitro* [19], in the developing DRG [17], as well as in both, Schwann cells and vascular cells from the adult rat sciatic nerve [8]. Besides, a transient increase in the mRNA levels of one of the isoforms of SDF-1, SDF-1β has been observed in the distal stump of the sciatic nerve following crush injury [8]. For these reasons, SDF-1 may be at least one of the factors mediating MSC homing in the injured DRG. However, mechanisms involving other molecules cannot be excluded. Their identification is of major importance for understanding and designing MSC-mediated cell therapy for trauma and diseases of the nervous system.

MSCs have become one of the most interesting targets for the study of tissue and organ regeneration because of their plasticity. MSCs have many features that make them attractive for cell and gene therapy: they are easy to isolate from aspirates from bone marrow by their adherence to plastic and they can be readily expanded in culture by repeated passage [21]. Furthermore, the use of these adult stem cells obviates the ethical and immunological issues associated with embryonic stem cells. In addition, MSCs produce a wide range of cytokines and growth factors both *in vitro* and *in vivo* [13,15,16] that have been demonstrated to promote endogenous restorative mechanisms of injured tissues [15], inducing functional recovery [14].

Our studies showing a selective engraftment of MSCs in lesioned nervous tissues (DRGs) support their possible application as a cell therapy for peripheral nervous system repair. Further investigations should be carried out in order to evaluate a possible role of MSCs in nervous tissue regeneration.

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