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Title: Mesenchymal stem cell transplantation reduces glial cyst and improves functional outcome following spinal cord compression.

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ABSTRACT

BACKGROUND: Mesenchymal stem cells (MSCs) are multipotent stem cells that have a supportive role in regenerative therapies, especially in the central nervous system, where spontaneous regeneration is limited. MSCs can exert a paracrine activity and modulate the inflammatory response after a central nervous system injury. Spinal cord injury (SCI) leads to permanent neurologic deficits below the injury site, owing to neuronal and axonal damage. Among experimental treatments after SCI, cell transplantation has emerged as a promising approach.

METHODS: Using a compression injury model in the mouse spinal cord, MSCs were acutely transplanted into the lesion cavity; injured mice without the graft served as controls. After 26 days, the survival of MSCs was investigated, and their effect on the formation of glial cyst and on injury-related inflammation was evaluated.

RESULTS: Grafted MSCs remained permanently undifferentiated. The lesion volume was reduced by 31.6% compared with control mice despite the fact that astroglial and microglial activation was not altered by the graft. Sensory and motor tests showed that MSC cell therapy results in improvement on a battery of behavioral tests compared with control mice: MSC-treated mice versus control mice scored 0.00 versus 0.50 in the posture test, 0.00 versus 1.50 in the hindlimb flexion test, 3.00 versus 2.25 in the sensory test, and 7.50 mistakes versus 15.83 mistakes in the foot-fault test.

CONCLUSIONS: These results underscore the therapeutic potential of MSCs, making them promising treatments for central nervous system pathologies.

INTRODUCTION

Mesenchymal stem cells (MSCs) are self-renewing, multipotent stem cells that can differentiate into several types of mesenchymal tissues (35). MSCs can also play an important supportive role in regenerative therapies, particularly in the CNS (where spontaneous regeneration is very limited), supporting axonal growth and the maintenance of synaptic connections, preventing neuronal death by reducing apoptosis and limiting free radical generation (17). MSCs also secrete cytokines and growth factors, thus exerting a paracrine influence on damaged tissues (11, 5). And finally, MSCs can interact with the host immune system, modulating the inflammatory response to insult (31, 15).

Spinal cord injury (SCI) approximately concerns 6 million people worldwide, and profoundly affects the quality and expectancy of life of the victims, most of whom are relatively young (25, 39). Traumatic SCI leads to hemorrhage, ischemia, edema, a strong inflammatory response, reactive gliosis, the formation of glial scars and cystic cavities, and loss of neurons and glia; it is often accompanied by severe neurological dysfunction and disability (8, 25).

Stem cells (SCs) hold great promise for treating SCI. In an earlier paper, we have demonstrated the benefits of using MSCs in a lesion model that involves spinal cord hemisection (3). Here we explore the potential benefits of using MSCs in a murine compression SCI model, focusing our analysis on how MSCs alter the inflammatory response and the formation of the glial cyst, and on how these changes influence functional recovery. To our knowledge, this is the first report to employ undifferentiated MSCs in an acute SCI model, and to document lesion volume reduction and behavioral improvement.

MATERIALS AND METHODS

Experimental animals

Two month-old C57BL/6J male mice (Harlan-Italy, San Pietro al Natisone, Italy) were used to produce the SCI model. Animals had free access to food and water. All experimental procedures on live animals were performed according to the European Communities' Council Directive of 24

November 1986 (86/609/EEC) and University of Torino's institutional guidelines for animal welfare (DL 116/92); efforts were made to minimize the number of animals used and their suffering.

BCF1 mice, which express Enhanced Green Fluorescent Protein (EGFP) under the beta-actin promoter, were kindly provided by Dr. M. Okabe (Osaka University, Suita, Japan, 32), and were bred in our animal facility: EGFP mice were sacrificed and MSCs were harvested from bone marrow (see below).

Isolation and culture of MSCs

Murine MSCs were isolated and expanded in vitro for transplantation, as previously described (3). 15 seven-to-nine week-old EGFP mice were anesthetized and killed by cervical dislocation; their tibias and femurs were cleared of muscle and connective tissue, and bone marrow cells were aspirated using a 22-gauge needle; cells were washed twice (5 minutes each) by centrifugation at 1000 rpm in Eagle's alpha minimum essential medium (a-MEM; Sigma) containing 2 mM L-glutamine (Invitrogen-Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco).

To culture MSCs, marrow cells were plated at a density of 700,000 cells/cm^2 in 19.5 cm^2 polystyrene dishes (BD Biosciences) that were pre-treated with a coating of fetal bovine serum (FBS; Sigma): they were grown in a-MEM supplemented with 10% FBS, in a humidified atmosphere of 95% air with 5% CO$_2$ at 37°C. Medium was replaced on day 4 to remove free floating cells, and then replenished every 2-3 days.

At 10 days *in vitro*, adherent cells were retrieved by trypsinisation (Trypsin, Invitrogen-Gibco) and immunodepleted of CD11b-positive granulocytic cells by magnetic cell sorting: cells were incubated with MicroBeads conjugated to monoclonal rat anti-mouse/human CD11b antibody (MiltenyiBiotec GmbH, BergischGladbach, Germany) and loaded onto a MACS column (MiltenyiBiotec). Cd11b-negative cells were harvested, washed and re-plated onto dishes as described above.

When the cells were near confluence, they were incubated with trypsin for 5 min at 37°C. The cell suspension was diluted 1:2 at each passage. Before transplantation, MSCs were counted on a Burker chamber and re-suspended in saline solution at a final concentration of 50×10^3 cells/µl.

Surgery and Cell Injection Procedures

Mice underwent surgical procedures to produce a spinal cord compression. Animals were divided into the following groups: (i) spinal cord injury (SCI) + saline solution group (vehicle treated – VT mice) **n** = 19; (ii) SCI + transplantation of mesenchymal stem cells (MSC mice) **n** = 14. Vehicle treated mice were the same as in (2).

Spinal cord compression was induced according to Farooque (9). Briefly, adult C57BL/6J mice were deeply anaesthetized with 3% isoflurane vaporized in O_2/N_2O (50:50). A laminectomy was performed at the level of L2 neuromer. Compression was applied onto the spinal cord for 5 min, gently laying a round plate (diameter 1.5 mm), oriented longitudinally over the dorsal aspect of spinal cord, and weighing 10 g, in order to produce moderate injury. The plate was removed after 5 min. Immediately after injury, the suspension of MSCs (10^5 cells in 2 µl saline) was slowly (over about 60 seconds) injected at depth 1 mm via a glass micropipette (outer tip diameter 50 µm) assembled on a stereotaxic apparatus into the spinal cord, into the SCI lesion cavity. VT animals received vehicle alone.

Behavioural tests

Injured mice (VT, **n** = 12; MSC, **n** = 8) underwent a battery of behavioural tests prior to surgery to establish a baseline for comparison with post-surgical/transplantation values; all were then studied at 4, 7, 12, 19 and 26 days post injury (dpi), using the following tests: posture, foot-fault test, hindlimb flexion, and sensory test.

Posture test. The mouse was placed on a smooth flat surface and its general posture was observed, from the back. The following scores were applied: 0 = not different from normal, 1 = balance shifted to the lesioned side (adapted from 38).

Foot-fault test. The mouse was placed on an elevated wire grid. Each time that its injured paw slipped between the wire, as it moved along the grid with a weight-bearing step, one foot-fault was recorded. The total number of steps taken (N = 30), and the number of foot-faults for the injured hindlimb, were counted (34, 45).

Hindlimb flexion test. The animal's head was covered with a hand and the hindlimbs were gently pulled toward the tail and turned over the sole. If retractive power was the same for both hindlimbs, the score would be 0; if the hindlimb retractive power was weaker than normal, the score was 1; if the lesioned hindlimbs were extended abnormally, but were retractable when the sole was touched with a finger, the score was 2; if the hindlimbs were extended abnormally and were not retractable when the sole was touched with a finger, the score was 3 (38).

Sensory test. The mouse was touched with a blunt stick on each side of the body and the reaction to the stimulus was observed and scored as follows: Scores indicate the following: 3 = mouse reacted by turning head and was equally startled by the stimulus on both sides; 2 = mouse reacted slowly to stimulus on the impaired side; and 1 = mouse did not respond to the stimulus (12).

Histological examination

Twelve, nineteen and twenty-six days after compression/transplantation, animals were deeply anaesthetized by intraperitoneal injection of chloral hydrate and perfused transcardiacally with 4% buffered paraformaldehyde (PFA, pH 7.4). The spinal cord was removed, cut between T8 and L2 vertebral segments and post-fixed in PFA for 2 h at 4°C.

Samples were transferred overnight into 30% sucrose in PB (phosphate buffer) 0.1 M at 4°C for cryoprotection, embedded in cryostat medium (Killik; Bio-Optica, Milan, Italy) and cut on the cryostat (Microm HM 550) in serial transverse 50 μm-thick sections, and either stored free-floating

in PBS at 4°C, or mounted onto gelatin-coated slides, and processed for immunostaining. Before performing any reactions, however all sections were mounted on slides with PBS, coverslipped, and examined on a Nikon Eclipse E800 epifluorescence microscope under a FITC-filter to count surviving transplanted cells.

Immunofluorescence and immunohistochemistry

EGFP[+]-MSCs were counted in all sections: in order to avoid double counting in consecutive sections, only cells contained entirely within each section were counted - partial cells, cut at the edges of the section, were not included in the counts.

For immunofluorescence (VT, **n** = 8; MSC, **n** = 5), serial sections (one every 600 μm) were immunostained as follows: non-specific binding sites were blocked by immersing tissue for 30 min at room temperature in 10% normal donkey serum (Sigma) in PBS, then left overnight at 4°C in primary antibody made up in the same solution. The following antibodies were used: 1:500 polyclonal rabbit anti-glial fibrillary acidic protein (GFAP; DakoCytomation, Denmark), 1:200 monoclonal mouse anti-microtubule associated protein-2 (MAP-2; Chemicon, Temecula, CA, USA), 1:200 monoclonal mouse anti-nestin (Chemicon), 1:200 polyclonal rabbit anti-NG2 chondroitin sulfate proteoglycan (Chemicon), 1:500 monoclonal anti-neuronal nuclei (NeuN) (made in mouse; 1:10; Chemicon), 1:200 monoclonal mouse anti-vimentin (DakoCytomation), 1:400 polyclonal rabbit anti-Ki67 (Novocastra Laboratories Ltd., Newcastle, United Kingdom), 1:150 monoclonal mouse anti-synaptophysin (Immunological Sciences, Rome, Italy). The next day, sections were washed in PBS, and incubated in 1:200 cyanine 3-conjugated anti-rabbit or anti-mouse secondary antibodies (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as appropriate.

For counterstaining, cells were incubated for 30 min with 0.001 g/ml Bisbenzimide in PB 0.1M, and rinsed with PBS. Finally, coverslips were mounted with a drop of PB 0.1M.

Sections were examined with a Nikon Eclipse E800 light and epifluorescence microscope and photographed with a Nikon Coolpix 995 digital camera. Photomicrographs were manipulated and mounted in plates with the Photoshop CS2 software, with autocontrast enhancement. In order to check for double staining and for 3D reconstructions, some preparations were examined also with an Olympus Fluoview 300 confocal laser scanning microscope (CLSM).

For immunohistochemistry, sections were incubated in a humidified chamber for 30 min at RT in PBS containing 0.3% Triton X-100; endogenous peroxidase activity was neutralized by incubating the sections for 30 min in presence of 0.3% H_2O_2. Non-specific binding sites were blocked with 0.3% Triton X-100 and 10% normal goat serum (NGS; Sigma-Aldrich) in PBS for 1 h at RT, and then sections were incubated at 4°C overnight with polyclonal rat anti-mouse CD11b antibody (1:100; Serotec, Oxford, United Kingdom) in the same solution. On the following day sections were incubated with secondary goat anti-rat antibody (1:50; Serotec) in 2% NGS, followed by avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min. Immunoreactivity was visualized with the Vector SG kit (Vector Laboratories). Finally, sections were dehydrated in ascending alcohols and cover-slipped with Eukitt mounting medium (Bio-Optica).

To evaluate microglial and astroglial activation, GFAP and CD11b immunoreactivities, respectively, were analyzed (VT, **n** = 4; MSC, **n** = 4). Two blind observers quantified the density of immunopositive profiles with the use of Scion Image (Scion Corporation, Frederick, MD, USA) by. For semiquantitative analysis, we considered three sections, one at the lesion site, one 600 μm rostral to it and another one 600 μm caudal to it. In particular, we quantified immunoreactivity in the laminae I, II, III, IV, V (represented as the average of GFAP- or CD11b-immunoreactive percentages of spinal area in the whole dorsal horn for a thickness of 1200 μm) and IX. These areas were photographed using a Nikon Coolpix 995 digital camera at 40x. The percentage of the overall GFAP- or CD11b-positive area was quantified using the Scion Image software for Windows (freeware version of NIH image, Scion Corporation, Frederick, MD, USA), accordingly to 2, 3, 40.

Analysis of the lesion volumes

In order to study the effects of stem cells on the lesion, in a specific set of experiments we analysed some spinal cords (VT, **n** = 9; MSC, **n** = 7) with the Neurolucida software program (Microbrightfield Inc., VT, USA), as performed in (2). Spinal cords were dissected at 26 dpi, frozen, and serially cut on the cryostat, as previously described. One of every two 50 μm-thick sections (total spinal cord segment was 1700 μm-thick) was drawn at the computer using the Neurolucida software program and the volume obtained was analysed with the Neurolucida Explorer program (Microbrightfield Inc.). The volume of the lesion was then expressed as a percentage of the total volume of the segment analyzed.

Statistics

Data are shown as mean ± SEM (standard error of the mean) and inter-group differences were statistically compared with paired Student's t-test, two tails. Differences were considered significant when $P \leq 0.05$.

RESULTS

We report on the results of the effects of MSCs that are acutely grafted in a compression injury model of spinal cord damage. Neuronal survival, inflammation and, lesion size were analysed and compared between transplanted and control groups.

Survival and distribution of grafted MSCs

MSCs were injected into the site of lesion, immediately after compression injury. At 26 dpi, few MSCs were detected at the lesion site (<1%) (Figure 1a-d); moreover MSCs did not migrate far from the site of injection, but were able to invade the damaged area and penetrate the lesion site (Figure 1). The surviving cells have a fibroblast-like morphology, mainly rounded: they are rarely

emitting a short process (Figure 1b-d). Furthermore, no transplanted MSCs were positive for neuronal or glial markers, suggesting their ability to remain undifferentiated.

Quantification of astrogliosis and microgliosis

To study the neuroinflammatory response of the tissue to the injury, we quantified the density of GFAP- and CD11b-immunopositive profiles (Figure 2a-d) at three rostrocaudal levels, focusing on laminae I, II, III, IV, V and IX; counts were made at 26 dpi.

The densities of GFAP- and CD11b-immunopositive profiles occupied 13.79% ± 3.15% and 16.00% ± 2.72% of the area considered to be GFAP+ in saline-treated controls and MSC-treated mice, respectively, and 14.97% ± 1.53% and 12.79% ± 1.20% of the area considered to be CD11b+ in the control and MSC-treated animals, respectively. Therefore, stem cell transplantation did not alter astrogliosis or microglial activation.

Analysis of the lesion volume

The lesion size was reconstructed with use of the Neurolucida software program. At 26 dpi, the glial cyst could be clearly outlined due to the increased natural colour of the lesion area. A 1700 μm-long spinal cord segment was serially reconstructed on the computer (Figure 2e-f). The lesion volume, expressed as a percentage of the total volume of the segment analysed, was 9.5% ± 2.19 in VT mice and 6.5% in MSC ones. These data show a reduction in the lesion volume of 31.6% ± 1.72 in the MSC-grafted mice vs the VT ones.

Behavioural tests

To examine whether MSC grafts led to functional improvement after SCI, we used a battery of tests to evaluate the behaviour of injured mice: posture, foot-fault test, hindlimb flexion and sensory response. Animals were tested at 4, 7, 12, 19 and 26 dpi. Day 0 corresponds to baseline, pre-

surgical performance. In every test, the transplanted animals displayed a more rapid recovery of motor and sensory functions (Figure 3).

Posture. Even though the initial (at 4 dpi) performance of the grafted MSC mice was highly compromised, by 12 dpi they exhibited a better recovery than the VT animals (0.13 vs 0.33); this level of performance gradually improved until, by 26 dpi, they showed a normal posture (score 0.00 ± 0.00), compared to 0.50 ± 0.33 in the VT mice.

Foot-fault test. The performance of MSC mice strongly improved, particularly between the 4th and 12th dpi, after which the recovery was slower, until the 26th dpi, when MSC mice showed 7.50 ± 0.59 of mistakes.

Hindlimb flexion. MSC mice displayed a remarkable recovery starting from 12 dpi, when the score was 0.50 ± 0.34 compared to a score of 0.83 ± 0.40 for the VT animals. By 26 dpi, the transplanted mice exhibited total recovery at 26 dpi (score 0.00 ± 0.00), while VT mice still showed a score similar to 4 dpi.

Sensory test. As for the hindlimb flexion test, at 26 dpi the transplanted group showed a complete recovery (score 3.00) compared with VT (2.25 ± 0.55).

DISCUSSION

Having already obtained positive results with the use of MSC transplants in a hemisection model, we have now evaluated MSC transplantation as a possible therapeutic approach in the spinal cord compression model.

Methodological issues

SCI induces both acute and chronic changes in spinal cord. Here we choose to transplant MSCs in the acute phase of SCI, suggesting that early intervention with cells may influence various outcomes of damage or repair such as the evolution of the occurring glial scar, 27).

Moreover, we choose the compression model to mimic tissue destruction observed in human patients. However, in this model respiratory movements allow a difficult control of the position of the applied weight (43), thus affecting reproducibility of the SC damage. This partially justifies the more significant results obtained with the previous model, the hemisection injury.

On the other hand, the compression model applied on the mouse is easy to perform and specifically affects the major murine corticospinal bundle, being the lesion mainly located posteriorly. This in contrast to the human, where it is located in the anterolateral bundle.

Several different stem cell types have been tested in experimental models of SCI (5), demonstrating the safety and practicability of such procedures. As reviewed by Gögel *et al.* (13), researchers have used embryonic stem cells (ESCs) derived from the blastocyst, induced pluripotent stem cells reprogrammed by fibroblasts and hematopoietic or mesenchymal stem cells. In particular, ESCs have the ability to differentiate and promote functional recovery (18, 1), but their use has been associated with allodynia (23); their derivation from living embryos also raises some ethical controversy. In contrast, MSCs have been used now in two models of SCI, they do not cause allodynia, confer neuroprotection, stimulate neurorepair/neuroregeneration and their use does not involve use of live embryos (3, 1).

Survival and cell fate

We have transplanted MSCs during the acute therapeutic time window: one month after engraftment, less than 1% of 10^5 grafted cells survived. Despite this low rate of cell survival, the numbers are likely an underestimate, due to the section thickness, the presence of cell clusters in which individual profiles are difficult to resolve, and the long post-transplantation time interval.

With regard to the fate of MSCs, the grafted cells were negative for neuronal as well as glial markers. Our results are similar to those obtained by Gu and coll. (14): 300,000 MSCs were injected after spinal contusion and, 8 weeks after injury, did not express neuronal, astrocytic or oligodendrocytic markers, though assuring lesion volume reduction and axonal regrowth in the

transplanted rats. Therefore MSC differentiation is not required to observe histological effects/functional improvement. Even though under specific *in vitro* conditions MSCs may display neuronal morphology and express neuron-specific markers (37, 45), this could be an artifact of the culture conditions (6); taken together with the present results, this suggests the need for further investigations of MSC properties.

However, our results indicate that, even though MSCs remain undifferentiated, they nonetheless contribute to functional recovery, presumably via a neurotrophic role. In fact, as reviewed by Joyce and coworkers (17), the injected MSCs have the ability to home to the site of injury, attracted to areas of hypoxia, apoptosis or inflammation: here the cells may secrete bioactive substances that modulate the local immune system, prevent apoptosis, and promote angiogenesis as well as proliferation. Specifically, MSCs can release molecules with trophic (SCF, LIF, M-CSF, NGF, NT-3), immunomodulatory (PGE-2, TGF-β1, LIF), anti-apoptotic and angiogenetic (VEGF, bFGF, IL-6, IGF-1) properties, which are fundamental for tissue repair at the injury site (30, 24, 27).

Analysis of neuroinflammation and lesion volume

The compression model of SCI allowed us to study the inflammatory response and the formation of the glial cyst.

Astrogliosis and microglial activation are not significantly affected by cell transplantation (as already reported for the transplantation of neural precursor cells under the same experimental conditions as described here (2)). The level of GFAP expression appears, however, to be slightly higher in MSC mice compared to control animals. Although reactive astrocytes have long been considered detrimental to the repair of injured spinal cord, believed to form a physical or chemical barrier to axonal regeneration, recent studies demonstrate that reactive astrocytes can support spinal cord repair, repair the blood-brain barrier and restrict inflammation (36). Faulkner and coworkers (10) have performed a focused experiment, where they produced a moderate crush injury in mice in

combination with the ablation of reactive astrocytes by antiviral agent ganciclovir: in absence of reactive astrocytes, they observed extensive tissue and cell degeneration, failure in wound reduction and persisting motor impairment. Similar results were obtained by Myer and coll. (28) by ablating reactive astrocytes after moderate controlled cortical impact, determining greater 60% loss of cortical tissue.

Indeed some authors have directly injected glial-restricted precursor-derived astrocytes in an attempt to promote axon regeneration and functional recovery in the acute phase of SCI (7).

Similarly to our results, Yang (41) observed that MSC administration following middle cerebral artery occlusion can determine an increase of activated glia, associated to a good brain integrity and significant motor recovery. Therefore it is possible that activated glia play some role in the regenerative response.

Also, reactive microglia could limit neurodegeneration, in particular by releasing neuroprotective factors such as bFGF (basic fibroblast growth factor) and NGF (4), and anti-apoptotic molecules, such as IGF-1 (21).

Moreover activated microglial cells (identified by their expression of CD11b) provide protection from inappropriate stimuli. When activated, they retract their processes, become motile, and move to the injury site: here microglia can adopt an amoeboid morphology and assume a phagocytic role that is essential for the elimination of apoptotic cell debris (22). We have observed both of these transitional states in our samples.

In addition, our results show that MSCs remain undifferentiated following injury, and are able to invade the damaged area and penetrate the glial cyst. Morphometric analysis revels 31.6% reduction in lesion volume in treated animals relative to controls, in agreement with Gu's data (14) and demonstrating contribution of the MSCs to limit glial cyst size. Also Keimpema and coworkers (19) administered a suspension of bone marrow stem cells (BMSCs) in a model of cerebral ischemia, reporting a significant lesion size reduction; they suggest the involvement of activated

microglia, arguing that implanted BMSCs might achieve this by instructing activated microglia to provide a stronger anti-apoptotic response.

Finally, as mentioned above, MSCs can secrete active agents that promote angiogenesis, immunomodulation and neurogenesis. Some authors have also proposed that MSCs produce substances such as fibronectin, which can counteract the inhibitory effects of the glial scar (20).

Functional outcome

The behavioural tests of general posture, resistance force, sensory functions and coordination, show improvements in all aspects. Our histological results correlate with this behavioural improvement: transplanted mice show significantly better performance in terms of somatic sensation and locomotor activity compared to the VT mice. Moreover, our sensory tests show no allodynia-like hypersensitivity, reported by some investigators after grafts (16).

We thus propose that MSCs exert a supportive function on damaged spinal cord tissue, with several factors cooperating to achieve this effect: as reviewed by Parr and co-workers (33), MSCs promote neuroprotection by reducing apoptosis and demyelination, by decreasing the size of glial scar and by creating cellular bridges; they secrete trophic factors which induce sprouting and axonal regeneration, induce vascular effects (such as oedema reduction) and repair of the blood-brain barrier In summary MSCs can establish a dynamic relationship with the host SCI environment, by supporting axonal growth, guiding regenerating axons and bridging the lesion (44). We hypothesize that MSCs, penetrating into the glial cyst and reducing its volume, can enhance axonal regeneration: besides being able to degrade nerve-inhibitory molecules at the injury site, MSCs can secrete nerve-permissive matrix components (such as laminin, fibronectin, and collagen) that can contribute to the decrease in cavitation size (44). Consequently the reduction of the cystic cavity can clearly explain the behavioural improvement observed after SCI.

Concluding remarks

Our study shows that early transplantation of MSCs into the compressed spinal cord reduces the glial cyst, resulting in neuroprotection and consequently supporting functional recovery after injury. These findings support our positive results with use of MSC grafts in a mouse spinal injury model, where they promote the sprouting of raphespinal axons caudal to the lesion (3). Such preclinical results identify MSCs as strong candidates for cell transplantation therapy following SCI, in order to limit the histological and functional consequences of the damage. Further studies are needed to identify the pattern of molecular expression of MSCs under normal and experimental conditions, to elucidate and to further enhance their mechanisms of action.

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FIGURE LEGENDS

Figure 1 - Survival and distribution of MSCs

(a-d) MSCs have a fibroblast-like shape, rarely emitting a few short processes. After grafting, they preferentially invade the damaged area, penetrating the glial cyst (yellowish).

(e, f) The lesion area (outlined) is well evident both by natural colour **(e)**, and by GFAP-immunoreaction **(f)**.

Scale bar = 500 μm in **a** and **e-f**, 100 μm in **b**, 50 μm in **c-d.**
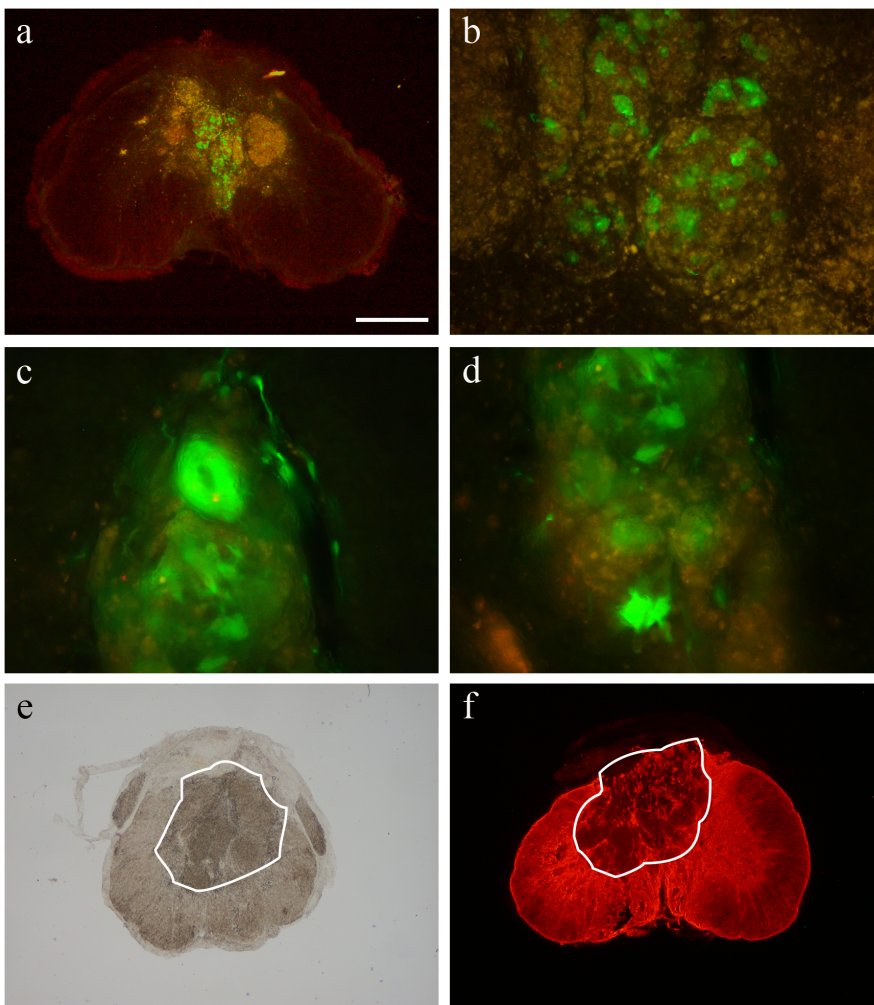
Figure 2 - Analysis of astrogliosis, microgliosis and lesion volume

(a, b) GFAP-immunoreactivity: hypertrophic astrocytes are present in the vicinity of the lesion site. Astrogliosis is comparable in VT **(a)** and MSC animals **(b)**.

(c, d) CD11b-immunoreactivity: the lesion site is filled with a large number of ramified and ameboid microglial cells; however microgliosis is comparable in the two experimental groups, VT **(c)** and MSC mice **(d)**.

(e, f) Morphometric analysis of glial cyst: the lesion volume is reduced by 31.6% in MSC mice **(f)** compared with VT **(e)**.
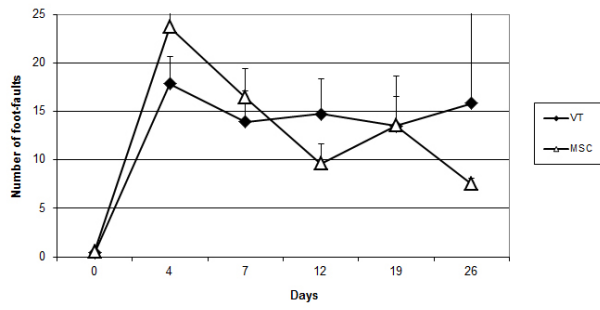
Scale bar = 50 μm in **a-d.**
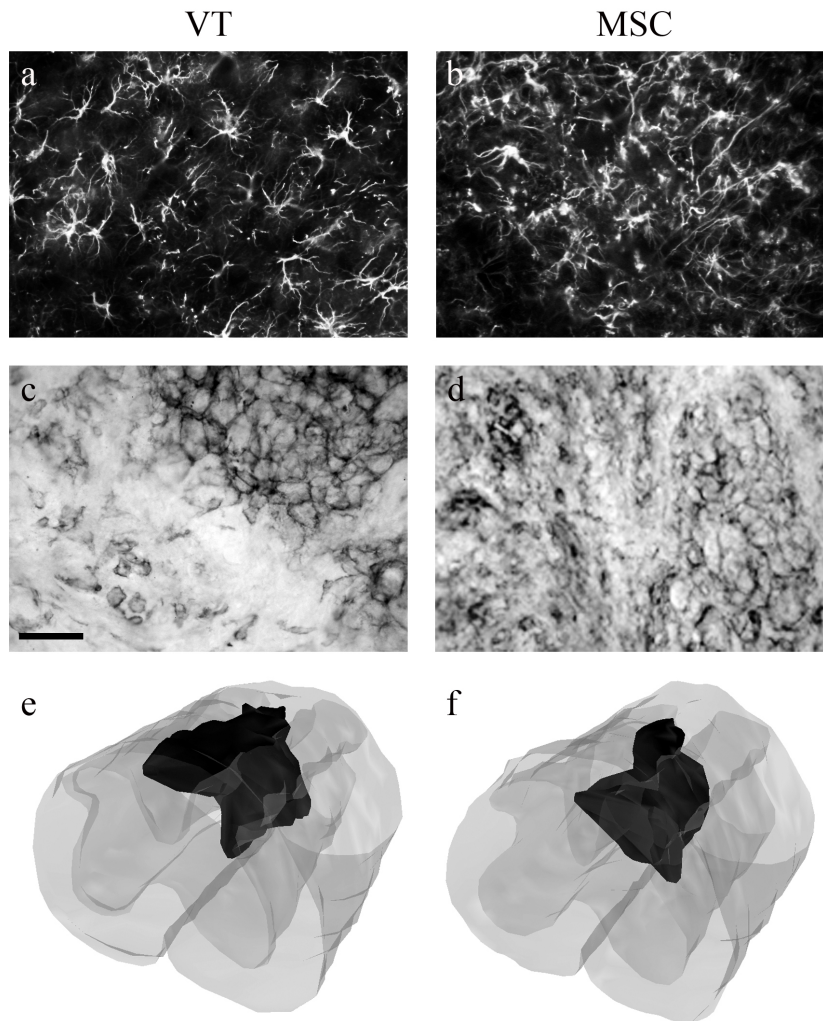
Figure 3 - Behavioural tests

MSC (*empty triangles*) and VT (*filled black diamonds*) mice underwent a battery of behavioural tests (posture, foot-fault test, hindlimb flexion and sensory test), to compare their recovery post injury. The graph shows behavioural scores obtained before injury/transplantation (day 0) and 4, 7, 12, 19 and 26 days post injury. The scores are expressed as mean ± SEM.
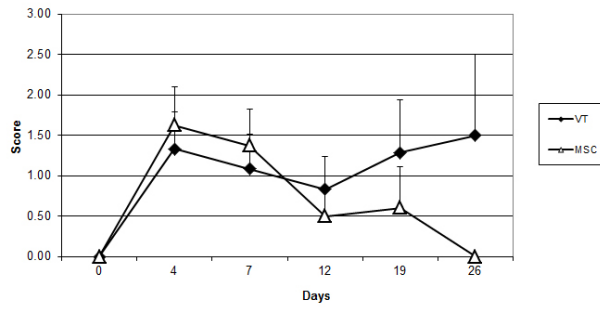
POSTURE
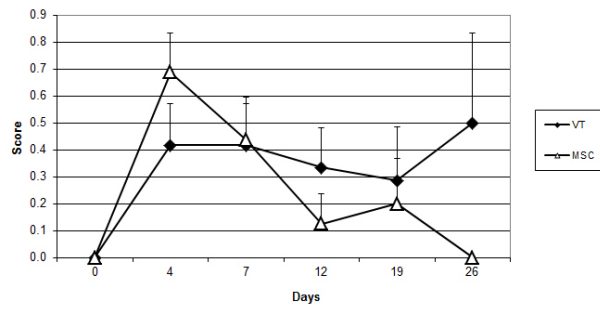
FOOT-FAULT TEST

HINDLIMB FLEXION

SENSORY TEST