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Transplantation of Bone Marrow Stem Cells as well as Mobilization by Granulocyte-Colony Stimulating Factor Promotes Recovery after Spinal Cord Injury in Rats

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ABSTRACT

Emerging clinical studies of treating brain and spinal cord injury (SCI) with autologous adult stem cells led us to compare the effect of an intravenous injection of mesenchymal stem cells (MSCs), an injection of a freshly prepared mononuclear fraction of bone marrow cells (BMCs) or bone marrow cell mobilization induced by granulocyte colony stimulating factor (G-CSF) in rats with a balloon-induced spinal cord compression lesion. MSCs were isolated from rat bone marrow by their adherence to plastic, labeled with iron-oxide nanoparticles and expanded *in vitro*. Seven days after injury, rats received an intravenous injection of MSCs or BMCs or a subcutaneous injection of G-CSF (from day 7 to 11 post-injury). Functional status was assessed weekly for 5 weeks after SCI, using the Basso-Beattie-Bresnehan (BBB) locomotor rating score and the plantar test. Animals with SCI treated with MSCs, BMCs, or G-CSF had higher BBB scores and better recovery of hind limb sensitivity than controls injected with saline. Morphometric measurements showed an increase in the spared white matter. MR images of the spinal cords were taken *ex vivo* 5 weeks after SCI using a Bruker 4.7-T spectrometer. The lesions populated by grafted MSCs appeared as dark hypointense areas. Histology confirmed a large number of iron-containing and PKH 26-positive cells in the lesion site. We conclude that treatment with three different bone marrow cell populations had a positive effect on behavioral outcome and histopathological assessment after SCI, which was most pronounced after MSC injection.

Key words: G-CSF; lesion; marrow cells; mesenchymal stem cells; MRI; nanoparticles; repair

INTRODUCTION

TRANSPLANTED STEM CELLS can either replace missing populations of cells or rescue cells in the injured brain or spinal cord by the production of cytokines (interleukins) and/or neurotrophic factors that facilitate regeneration. Autologous adult stem cells, such as bone marrow cells (BMCs), may have ethical and legislative advantages over fetal or embryonic stem cells. Adult bone marrow provides a source of circulating blood progeni-

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tor cells derived from hematopoietic stem cells, but bone marrow is also a source of nonhematopoietic cells that can differentiate into a variety of cell types including bone (Rickard et al., 1994), cartilage (Ashton et al., 1980), muscle (Ferrari et al., 1998), glia, and neurons (Azizi et al., 1998; Woodbury et al., 2000). Their ability to secrete substances such as cytokines (interleukins) and trophic factors (Eaves et al., 1991; Majunder et al., 1998; Bjorklund et al., 2002) may, in addition to the replacement of lost cells, facilitate regeneration and rescue partially damaged cells, thus leading to functional recovery after spinal cord injury (SCI). Also, the transplantation of acutely isolated BMCs leads to extensive remyelination (Sasaki et al., 2001; Akiyama et al., 2002; Inoue et al., 2003). A further advantage of acutely isolated BMCs is that they can be transplanted into patients within a few hours after harvesting and without expansion in cell culture. In addition, the transplantation of mesenchymal stem cells (MSCs) has also been shown to improve functional recovery after CNS injury (Chopp et al., 2000; Hofstetter et al., 2002; Syková and Jendelová, 2005).

The administration of granulocyte colony stimulating factor (G-CSF) is known to mobilize hematopoietic stem cells from the bone marrow into the peripheral blood (Demetri and Griffin, 1991). G-CSF has been used extensively for bone marrow reconstitution and stem cell mobilization (Weaver et al., 1993). The subcutaneous administration of G-CSF enhanced the availability of circulating hematopoietic stem cells to the brain and their capacity for neurogenesis and angiogenesis in rats with cerebral ischemia (Shyu et al., 2004). In addition, G-CSF itself may also have anti-apoptotic and anti-inflammatory effects as well as an effect on neovascularization (Murphy et al., 2004). To date, no one has compared the effect of different bone marrow cell populations on morphological and functional recovery after SCI. We therefore compared the effect of (1) pluripotent cells containing nonhematopoietic stem cells that can differentiate along multiple mesenchymal cell lineages—MSCs (Prockop, 1997); (2) the freshly isolated mononuclear fraction of bone marrow containing stromal cells, hematopoietic and nonhematopoietic stem and precursor cells and lymphocytes—BMCs (Sasaki et al., 2001; Akiyama et al., 2002); or (3) G-CSF-mobilized endogenous BMCs containing mainly hematopoietic stem cells, but also progenitor cells and lymphocytes.

METHODS

Experimental Design

In this study, 60 male Wistar rats (Velaz, Prague, Czech Republic) were used. Their body weights ranged between 300 and 330 g to ensure uniform anatomical measurements inside the spinal channel. A balloon-induced spinal cord compression lesion was performed to mimic human SCI (Vanicky et al., 2001). Seven days after SCI, the rats were randomly assigned into four groups. The first group ($n = 15$) received an intravenous injection of MSCs, the second group ($n = 15$) received an intravenous injection of BMCs, and the third group ($n = 15$) received a subcutaneous injection of G-CSF for 5 days starting at 7 days post-injury. The control group ($n = 15$) received a single intravenous injection of saline 7 days after SCI.

This study was performed in accordance with the European Communities Council Directive of 24th of November 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic.

Spinal Cord Surgery

The rats were anesthetized by passive inhalation of 1.5–2.0% isoflorane (Forane; Abbott Laboratories Ltd., London, UK) in air. An adequate level of anesthesia was determined by monitoring the corneal reflex and withdrawal to painful stimuli applied to the hind limb. The SCI model has been described in detail previously (Vanicky et al., 2001). Briefly, under aseptic conditions, the animal's back was shaved, and a 2-cm midline incision was made to expose the T10-L1 spinous processes. Under a surgical microscope, a small laminectomy (1.5 mm diameter) was performed in the vertebral arch of T10 using a drill. The periosteal membrane was opened, and a 2-French Fogarty catheter (Baxter Healthcare Corp., Irvine, CA), filled with saline and connected to a gastight 50-μL Hamilton syringe, was inserted into the epidural space. The center of the balloon rested at the T8-T9 level of the spinal cord. After these surgical manipulations, we stabilized the body temperature at 37°C and also the concentration of the anesthetic at 1.75% isoflurane for 5 min. The balloon was rapidly inflated with 15 μL of saline for 5 min. Subsequently, the catheter was deflated and removed; the muscles and skin were sutured in anatomical layers.

After surgery animals were housed in pairs, to reduce the stress from isolation, on a 12-h light/dark cycle with a standard rat diet and water *ad libitum*. Gentamicine® (Lek, Ljubljana, Slovenia), at a dose of 1 mg per animal i.m., was administered for 3 days after SCI, the first dose 15 min before surgical manipulation. Depo-Medrol® (Pharmacia, Puurs, Belgium), at a dose of 2 mg per animal i.m., was administered to all rats weekly to prevent cell transplant rejection.

After SCI, manual bladder expression was performed twice a day during the early post-operative period; with the improvement of the animals' condition, it was performed once a day until the end of the second week, by which time a reflex bladder was established. The rats were monitored for evidence of urinary tract infection or any other sign of systemic disease. Body weight was measured once a week to monitor overall recovery from the SCI.

Preparation of MSCs, BMCs, and G-CSF Treatment

Rat MSCs were isolated and labeled with iron-oxide nanoparticles according to a protocol described previously (Jendelová et al., 2003). Briefly, bone marrow was collected from 1-month-old Wistar rats by flushing femurs and tibias under sterile conditions with complete medium composed of DMEM (PAA-Laboratories GmbH, Pasching, Austria), 10% fetal bovine serum (PAA-Laboratories GmbH, Pasching, Austria) and penicillin/streptomycin (100 U/mL; Gibco, Invitrogen Corp., Paisley, Scotland, UK). Cells were then plated in 80-cm^2 culture flasks. After 3 days, nonadherent cells were removed along with the culture medium, and only adherent cells were further cultured in complete medium for four to six passages. An Endorem® (Guerbet, Roissy, France) suspension (200 μL per 20 mL of culture medium, i.e., 2.2 mg of iron) was added to the rat MSC cultures 5 days prior to transplantation. After 72 h, the contrast agent was washed out. The cells from several flasks were pooled, and the number of cells was estimated by counting in a Burke chamber. The final concentration was adjusted to about 2×10^6 cells/0.5 mL. Prior to transplantation the cells were labeled using the lipophilic membrane dye PKH 26 (Sigma, St. Louis, MO), a fluorescent cell marker, according to the manufacturer's instructions.

To prepare BMCs, a bone marrow cell mass obtained from the femurs and tibias of 1-month-old rats was diluted with Hanks balanced salt solution (Gibco, Invitrogen Corp.) to obtain a 4-mL cell suspension. The bone marrow cell suspension was then centrifuged (400*g*, 40 min) with 3 mL of Ficoll solution (Ficoll-Paque TM Plus, Amersham Pharmacia-Biotech AB, Uppsala, Sweden). The mononuclear fraction was collected and resuspended in phosphate-buffered saline (PBS) at a concentration of approximately 2×10^6 cells/0.5 mL.

MSCs and BMCs were transplanted into the periphery via a single femoral vein injection of approximately 2×10^6 cells in 0.5 mL of PBS, flushed with 0.5 mL of sterile saline, 7 days after SCI. During transplantation, the rats were anesthetized as previously described for spinal cord surgery. Cell viability was measured in the remaining cells after transplantation using trypan blue staining (Sigma, St. Louis, MO). MSCs remaining after transplantation were re-cultured, and cell viability was assessed by the fluoresceindiacetate-propidium iodide protocol of Jones and Senft (1985).

For bone marrow mobilization after SCI, we administered G-CSF (Neupogen, Roche, Basiley, Switzerland) intravenously into the femoral vein at a dose of 5 μg per 100 g of body weight for 5 consecutive days according to the manufacturer's protocol. Treatment was performed from day 7 to 11 after SCI.

To check the uptake of nanoparticles from dead cells, we injected the contrast agent Endorem (2.5 μL in 0.5 mL of PBS) intravenously into rats with SCI. This concentration corresponds to the amount of iron in 2 million labeled cells.

Evaluation of Neurological Outcome

All rats were behaviorally tested before SCI and 1, 7, 14, 21, 28, and 35 days after SCI. Hind limb performance was evaluated by two independent observers, using the Basso-Beattie-Bresnahan (BBB) open field locomotor test developed by Basso et al. (1995). Movements of the hind limbs during locomotion were quantified using a scale ranging from 0 to 21, where 0 reflected no locomotor activity, and 21 reflected a normal performance. In this scoring system, the evaluation of leg movements is based strictly on objective criteria.

The response to radiant heat was determined using a plantar test apparatus (Ugo Basile, Comerio, Italy). Rats were placed in a transparent plastic chamber upon a glass floor and allowed to acclimate for 10 min. The radiant heat source was placed under the glass floor directly beneath the hind paw. Withdrawal latency was defined as the time between the activation of the heat source and hind paw withdrawal, which resulted in the termination of the heat source. A limit time of 35 sec was used in order to prevent tissue damage, and a 5-min interval between consecutive stimulations of the same hind paw was employed. Testing was performed three times on each side, the latencies for each side were averaged, and the average values were used for statistical analysis.

Histological Evaluation and Assessment of Spinal Tissue Sparing

At 35 days post-injury, the animals were deeply anesthetized with chloral hydrate (400 mg/kg, Fluka, Riedel-de Haen, Seelze, Germany) and transcardially perfused with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The entire spinal cord was left in the spinal channel overnight, then removed

and post-fixed in the same fixative and kept at 4°C until further processing. Three randomly selected spinal cords from the MSC group were left in the bone and stored in a plastic tube for *ex vivo* MRI, then cut into 8-μm-thick sagittal frozen sections. For comparing the size of the spinal cord lesions, we randomly selected three spinal cords from each of the four groups and cut them into serial sagittal frozen sections. Sections were stained for Prussian blue (visualization of iron) and counterstained with nuclear red. Unstained sections were mounted with a medium designed to prevent photobleaching in order to observe PKH 26–positive cells. A double staining method for colocalizing iron oxide nanoparticles in macrophages was performed. Samples were incubated with a mouse monoclonal antibody (ED1, diluted 1:100) directed against the rat homologue of human CD68, a marker of monocytes and macrophages (Serotec, Oxford, UK). Antigen-antibody complexes were visualized using a biotin-streptavidin detection system (LSAB2 System, HRP, DakoCytomation,) with 3,3′-diaminobenzidine as the chromogen (Fluka Chemie, GmbH). Iron was detected by Prussian blue staining as described above.

For morphological analysis, a 2-cm-long segment of the spinal cord containing the lesioned site was dissected and embedded in paraffin. The whole segment was serially cut, and a series of 20 sections (thickness 5 μm) were collected (1 mm distance between individual sections). The sections were mounted onto gelatin-coated glass slides, and then stained with Luxol Fast Blue and Cresyl Violet. We used this staining to facilitate discriminating between gray and white matter at low magnification.

Every section was imaged using a digital camera (Axiocam, Zeiss, Oberkochen, Germany); high-resolution images were used to delineate the spared white and gray matter, and their areas were measured using image analysis software (SigmaScan Pro 5, Aspire Software International, Leesburg, VA). For statistical analysis, 11 lesion-centered sections were used from each spinal cord. The volume of the spared tissue in the 11-mm-long segment was calculated as the sum of cross-sectional areas multiplied by the distance between them.

Magnetic Resonance Imaging and Detection of Nanoparticles

Ex vivo magnetic resonance (MR) imaging was used to confirm the presence of nanoparticle-labeled MSCs, transplanted intravenously, in the spinal cord lesion (Syková and Jendelová, 2005). MR images were obtained with a 4.7-T Bruker spectrometer using a whole-body resonator. The spinal segments, fixed *ex vivo* in a fixative solution, were placed in a 50-mL polypropylene test tube and then centered within the magnet. A three-dimensional (3D)–gradient echo sequence was used for data acquisition. Sequence parameters were TR = 25 msec and TE = 5.1 msec, and number of acquisitions = 128. 3D images were obtained with the dimensions: FOV = 6 × 3 × 2.4 cm, and matrix = 256 × 128 × 96.

Statistical Analysis

In individual animals, Basso, Beattie, and Bresnahan (BBB) scores and plantar scores were averaged across hind limbs, and inter-group differences were analyzed by using the non-parametric Kruskall-Wallis and Mann-Whitney *U* tests. Morphometric measurements were used to construct plots of consecutive cross-sectional areas of the spared tissue at individual levels of the spinal cord, rostral and caudal to the lesion center. The differences at each level were analyzed using the Kruskall-Wallis and Mann-Whitney *U* tests. Body weights at individual survival time points and the calculated volumes of the spared tissue within the 11-mm-long segments were compared by the unpaired Student's *t*-test. All values are expressed as means ± standard error of the mean (SEM). Differences between groups were considered statistically significant if $p < 0.05$.

RESULTS

Animal Care and Recovery after Spinal Cord Injury

The average body weights of all animals with SCI did not significantly differ at 35 days survival time. As a rule, hematuria was observed within the first days after SCI. The retention of urine was encountered in paraplegic animals, persisting for 2–3 weeks after spinal cord injury, so manual bladder expression was required. Seven days after SCI, MSCs or BMCs were injected intravenously or the first injection of G-CSF was administered. Rats from the control group received an intravenous injection of saline. No acute reaction to cell implantation or G-SCF treatment was observed. The cell viability of the BMCs and MSCs was confirmed after transplantation by negative staining for trypan blue. The cell viability of re-cultured MSCs was also assessed by the fluoresceindiacetate-propidium iodide protocol of Jones and Senft (1985). The viability of both types of cells was >90%.

Behavioral Testing

The BBB locomotor score was determined in animals before SCI and at days 1, 7, 14, 21, 28, and 35 after (Fig. 1A). All rats achieved the maximum score of 21 before SCI. One day after spinal cord compression, all rats were completely paraplegic; they had a BBB score of 0. The control, saline-treated rats gradually recovered motor per-

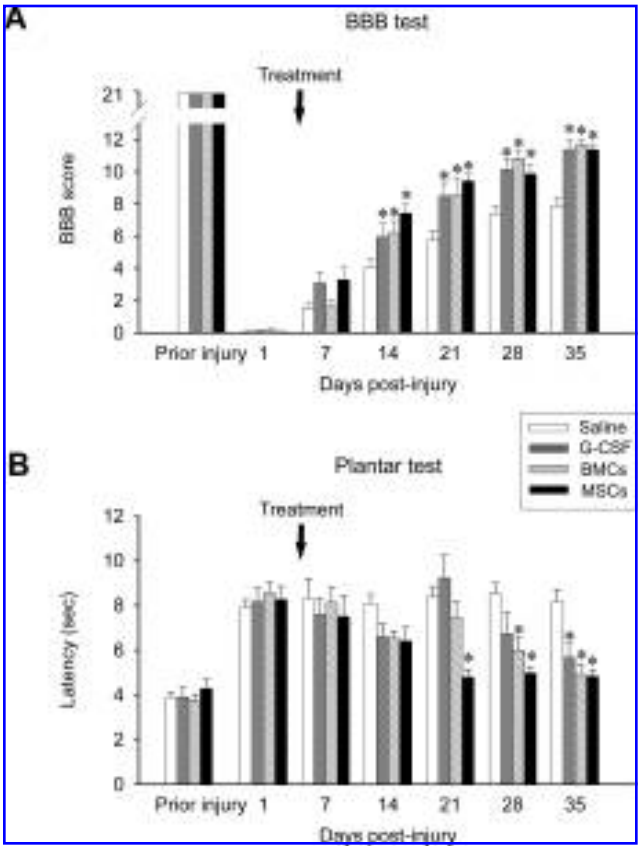

FIG. 1. Behavioral outcome after treatment with MSCs, BMCs, or G-CSF. (**A**) Behavioral open-field BBB motor scores of MSC, BMC, and G-CSF treated rats were significantly higher than those of the saline-injected (control) animals, 14, 21, 28, and 35 days after SCI ($p < 0.05$). The scores of the MSC, BMC, and G-CSF treated animals were not significantly different from one another at any time point. (**B**) Time course of the animals' response to radiant heat measured with the plantar test in the treated and saline-injected rats. In all treated rats, the latency times decreased as their recovery progressed. The most pronounced effect was seen in MSC treated rats. The latency time in the saline-injected (control) rats did not change during the 35-day survival period. Data are averaged between right and left hind limbs and expressed as mean ± SEM. *$p < 0.05$ compared to control group. For abbreviations, see text.

formance, reaching scores of 7–9 points by day 35. Such scores are indicative of the so-called intermediate phase of recovery (7–10 points), defined as no weight support with plantar placement of the paws. This intermediate phase of recovery was reached in rats treated with MSCs as early as 14 days after injury and in rats treated with BMCs or with G-CSF as early as 21 days after injury. By day 35 all treated rats achieved significantly higher BBB scores (10–12 points) than did control rats at the same time point. They could support their weight on plantar steps and had begun to coordinate the movement of their hind limbs and fore limbs. In all treatment groups, the BBB scores were significantly higher at days 14, 21, 28, and 35 than those seen in control animals ($p < 0.05$; Fig. 1A). BBB values showed no significant differences ($p < 0.05$) among the MSC, BMC, and G-CSF treated groups at any time point.

To test sensitivity changes, we used the plantar test. Immediately after SCI, the rats showed a longer latency of hind limb withdrawal (Fig. 1B). In control rats the reaction time did not significantly shorten during the evaluation period. The rats from all treatment groups displayed shorter reaction times to the heating stimulus than did control animals as their recovery progressed. In MSC-treated rats latency times decreased, indicating the recovery of sensitivity in their hind limbs, already at day 21. Rats that received BMCs or G-CSF recovered more slowly than those that received MSCs. Statistical analysis (treatment versus control groups) showed that hind limb sensitivity was significantly improved in the MSC treated group beginning 21 days post-injury, in the BMC treated group 28 days after injury, and in the G-CSF treated group 35 days after spinal cord injury (Fig. 1B).

Morphological Evaluation of Lesions and MR Imaging of MSCs Labeled with Iron-Oxide Nanoparticles

Figure 2 shows typical examples of lesions in control rats and MSC, BMC, or G-CSF treated animals. The lesions were considerably smaller in all three treatment groups than in the control animals, suggesting a possible positive effect of bone marrow cells on lesion repair (Fig. 2A–D). Staining for iron (Prussian blue) revealed many cells containing nanoparticles in the lesion site (Fig. 2D, E). On serial sections, Prussian blue–positive cells corresponded to cells labeled with PKH 26, as seen in Figure 2E,F. To exclude the possibility that free nanoparticles were taken up by macrophages, we performed immunohistochemical and Prussian blue staining to colocalize iron in activated microglia/macrophages. Although we found strong positive staining using ED1 antibody in the lesions, Prussian blue staining did not, for the most part, colocalize with the ED1 staining (Fig. 2G).

The fate of transplanted MSCs labeled with iron-oxide nanoparticles was followed by *ex vivo* MRI. MR images of longitudinal spinal cord sections from lesioned nongrafted animals showed the lesion cavity as inhomogeneous tissue with a strong hyperintensive signal (Fig. 3A). Lesions in grafted animals were seen as dark hypointense areas (Fig. 3C), while the lesions of animals injected with nanoparticles suspended in PBS were visible as weak hypointense signals (Fig. 3E). Histological

evaluation confirmed only a few iron-containing cells in lesioned control animals (Fig. 3B), strong positivity for iron in grafted animals (Fig. 3D) and a few weakly stained Prussian blue-positive cells in animals injected intravenously with a PBS-nanoparticle suspension (Fig. 3F).

Morphological measurements of the cross-sectional areas of the white matter showed a statistically significant increase in all groups treated with MSCs, BMCs, or G-SCF, compared to controls, cranially to the lesion center (Fig. 4A). Caudally to the lesion center, the cross-sectional areas of the white matter were significantly greater only in the MSC and BMC groups. In consecutive sections of the gray matter, a statistically significant increase in the area of spared gray matter was seen only in the MSC treated group, caudally to the lesion center (Fig. 4B).

The volume of the spared tissue in 11-mm-long segments of the spinal cord was determined as the sum of cross-sectional areas multiplied by the distance between them. A statistically significant increase in the volume of spared white matter was observed in the MSC and BMC treated groups, compared to controls. The spared white matter volume in the G-CSF treated group was also increased, but the increase did not reach statistical significance (Fig. 5A). No statistically significant differences were observed among the groups treated with MSCs, BMCs or G-CSF. The volume of the spared gray matter in 11-mm-long segments with the SC lesion at their center was not significantly different among any of the four groups (Fig. 5B).

DISCUSSION

In our study, we demonstrated that the intravenous injection of MSCs or BMCs as well as the mobilization of BM with G-CSF significantly improved the recovery of hind limb motor function in rats with a spinal cord compression lesion. We found improved behavioral parameters after SCI in comparison with the control saline treated group. The recovery of hind limb sensitivity was faster and more apparent after an injection of MSCs, and similar results were seen histologically. The spared cross-sectional area of white matter was significantly greater in all treated animals; the volume of spared white matter was significantly greater only in MSC and BMC treated animals. The spared cross-sectional area of gray matter was significantly greater in MSC treated animals. The volume of spared gray matter was not significantly influenced by any of the treatments.

The transplantation of autologous stem cells from adult bone marrow might therefore be a promising strategy for the treatment of CNS injuries or neurodegenerative diseases. The use of cells originating in bone marrow has several advantages over other cell sources, such as the immediate availability of healthy donor tissue, and there has been no reported tumorogenesity. Bone marrow as a source of cells has been already approved in human medicine for the treatment of hematopoietic diseases. MSCs (Chopp et al., 2000; Akiyama et al., 2002; Hofstetter et al., 2002) have been successfully used in the treatment of different experimental models of spinal cord injury, but the mechanisms or factors that promote reduced deficit are still unknown. One possible effect of cell therapy is that the grafted cells integrate into the host tissue and replace damaged or lost cells. MSCs can differentiate into neuron-like cells and glia (Jacobs et al., 1987; Prockop, 1997; Azizi et al., 1998; Eglitis et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Woodbury et al., 2000; Jendelová et al., 2003), but we have no clear evidence that MSCs functioned in this way in the present study. In our previous experiments (Jendelová et al., 2003), we injected MSCs into rats with a cortical photochemical lesion and studied the differentiation of the grafted cells. We found that only a few (<3%) BrdU-labeled MSCs expressed the neuronal marker NeuN and expressed the astrocytic marker GFAP. In a subsequent study (Jendelová et al., 2004; Syková and Jendelová, 2005), in which we injected nanoparticle-labeled MSCs into rats with a spinal cord injury, we found that nanoparticle-labeled MCSs populated the lesion site and the rats exhibited functional improvement; however, we did not observe any neurons stained for iron.

The use of iron-oxide nanoparticles for labeling cells and their MRI detection has been reported previously (Jendelová et al., 2003, 2004; Lee et al, 2004; Bulte et al. 2002, Hoehn et al., 2002). The size of the labeling nanoparticles is within the nanometer range, with only a minimal effect on cell viability and function. Several *in vitro* studies performed to monitor the effect of iron particles on cell growth and metabolism have shown no negative effects (Bulte et al., 2001; Lewin, et al., 2000; Jendelová et al., 2003, 2004). Histochemical staining for iron (Prussian blue staining) demonstrated the presence of Prussian blue-positive cells in tissue sections in areas that corresponded well to the dark regions in MRI images. A few iron-containing Prussian blue-positive cells were occasionally found in an experimental group injected with saline only. The iron most likely originated in hemorrhages and iron degradation products released from iron-containing proteins (such as hemoglobin, ferritin and hemosiderin). The hemorrhage degradation products were mainly localized in microglia/macrophages, because macrophages constitute the major cellular pathway for regulating the content and distribution of iron in mammals (Brock, 1989). However, the iron released from

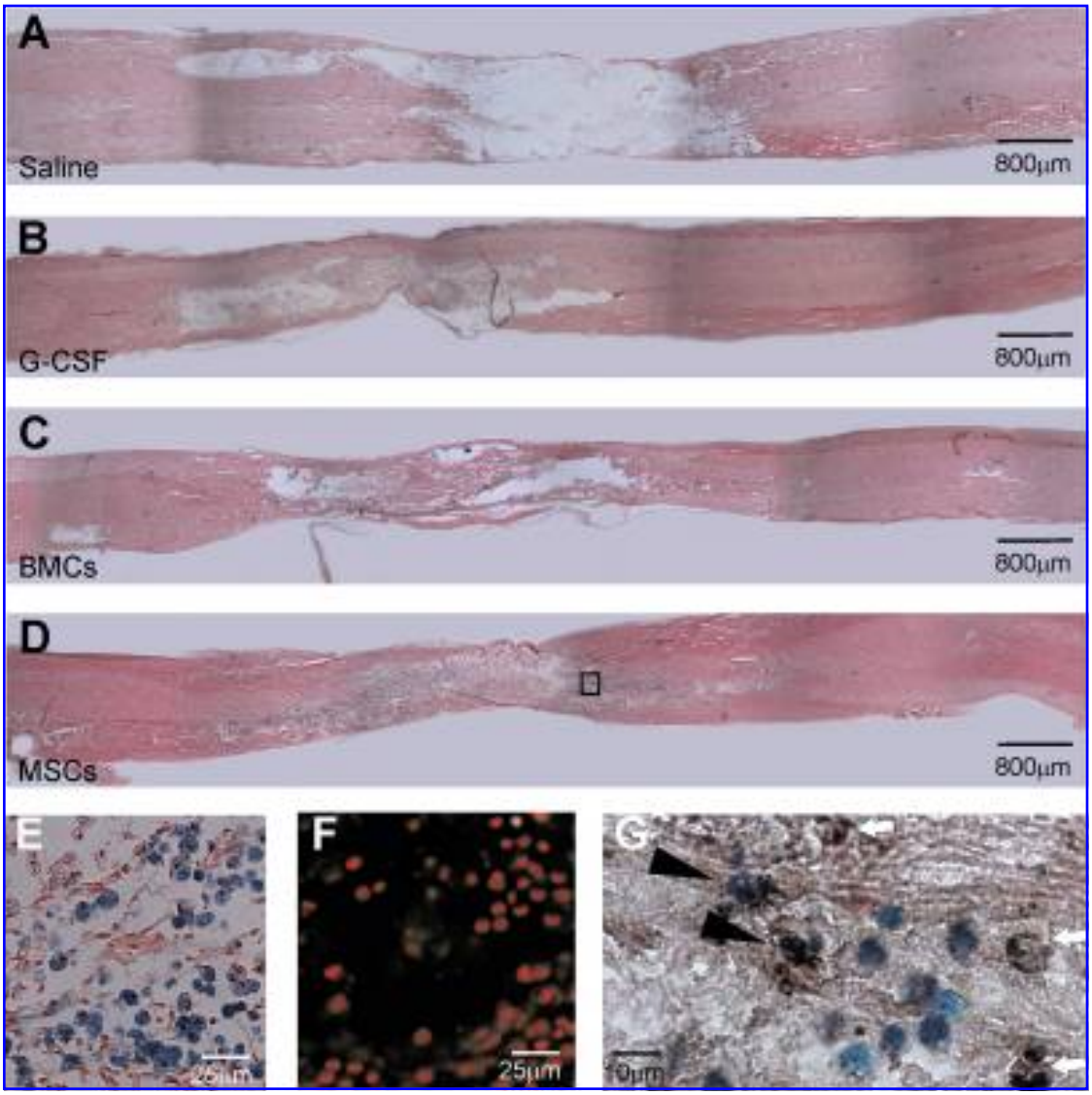

FIG. 2. Comparison of spinal cord lesions 4 weeks after the intravenous injection of MSCs, BMCs, or G-CSF. (**A**) Longitudinal section of a spinal cord lesion after saline injection. There is a large cavity in the center of the lesion. (**B**) Longitudinal section of a spinal cord lesion after the injection of G-CSF. (**C**) Longitudinal section of a spinal cord lesion after the injection of BMCs. The lesion length and cavity formation were smaller in animals treated with G-CSF or BMCs. (**D**) Longitudinal section of a spinal cord lesion after the injection of MSCs. The most pronounced effect was seen in animals treated with MSCs. A spinal cord compression lesion is populated with intravenously injected MSCs labeled with superparamagnetic nanoparticles. (**E**) Prussian blue staining of a spinal cord lesion with intravenously injected nanoparticle-labeled MSCs. The lesion is populated with Prussian blue–positive cells (magnified region from D). (**F**) Intravenously injected MSCs were also detectable in the spinal cord lesion using the membrane florescent dye PKH 26 (serial section to E). (**G**) Immunostaining with ED-1 antibody revealed a number of macrophages in the lesion (white arrows). The majority of Prussian blue–positive cells did not colocalize with ED-1 staining; however, a few macrophages were Prussian blue–positive (black arrowheads). For abbreviations, see text.

iron-containing proteins, if present at all, did not produce effects as large as those produced by superparamagnetic iron-oxide nanoparticles in labeled cells. In order to demonstrate that iron-oxide nanoparticles released from dead or dying cells were not responsible for the hypointense areas observed on MR images, we injected only the contrast agent Endorem into rats with a SCI. No host cells in the lesion took up the nanoparticles except macrophages. However, Prussian blue staining was much weaker than in the case of lesions containing grafted

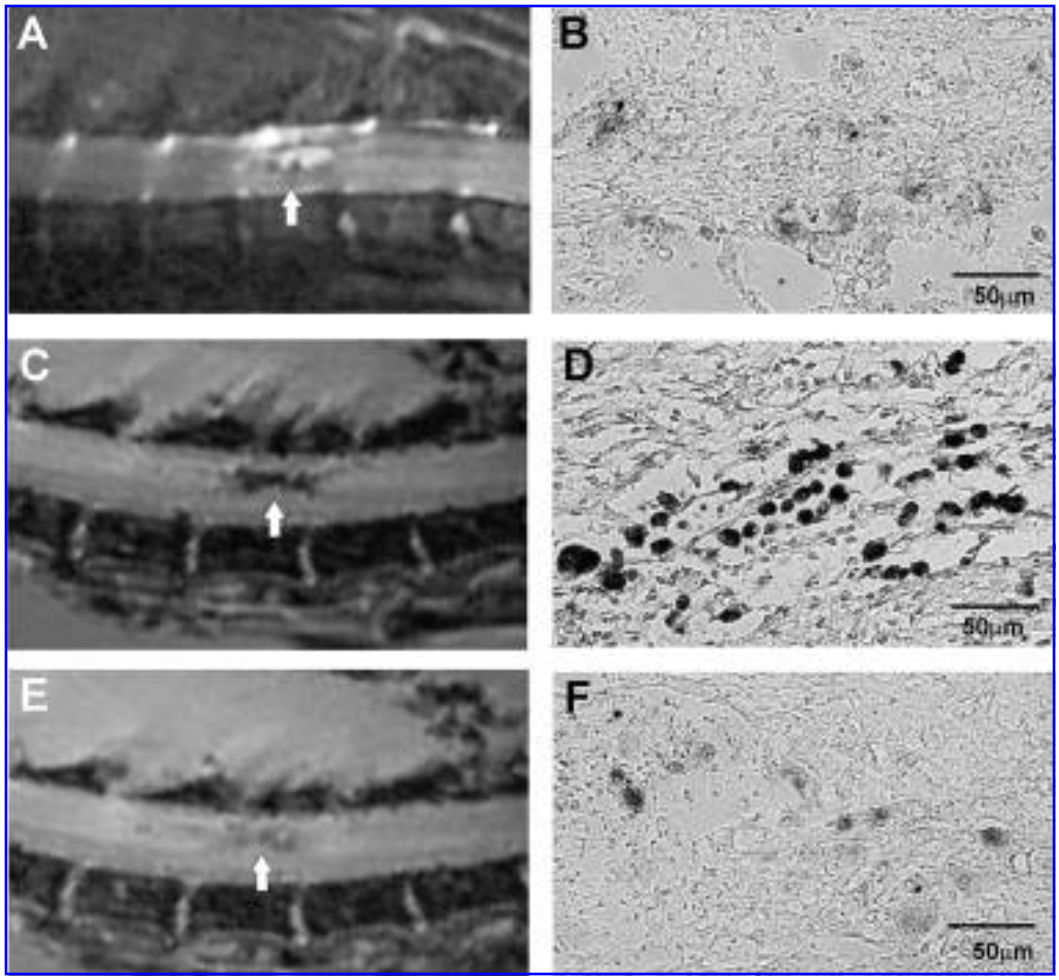

FIG. 3. MR images and Prussian blue staining of a spinal cord compression lesion injected with saline, contrast agent, or nanoparticle-labeled MSCs. (**A**) Longitudinal MR image of a spinal cord lesion 5 weeks after induction. The formation of a lesion cavity is visible as a strong hyperintensive signal. (**B**) A few cells weakly stained for Prussian blue (dark dots) can be seen in a spinal cord lesion in an animal without implanted cells. (**C**) Longitudinal MR image 4 weeks after MSC grafting. The lesion with nanoparticle-labeled cells is visible as a dark hypointensive area (arrow). (**D**) Dense Prussian blue staining of a lesion populated with nanoparticle-labeled MSCs (black dots). (**E**) Longitudinal MR image 4 weeks after the injection of contrast agent. A weak hypointensive signal can be seen in the center of the lesion. (**F**) Weak Prussian blue positivity (dark dots) observed in a spinal cord lesion in an animal injected with contrast agent alone. For abbreviations, see text.

nanoparticle-labeled cells, and MR images did not show the dark hypointensity seen in grafted animals. Our results are in agreement with the findings of Lee et al. (2004), who monitored nanoparticle-labeled olfactory ensheathing glia grafted into injured rat spinal cord. They described a small quantitative mismatch between the distributions of MR contrast and histological markers due to the redistribution of nanoparticles into macrophages.

Hofstetter et al. (2002) measured the membrane properties of neuron-like MSCs in a study in which *in vitro* differentiation was induced with 2-mercaptoethanol. The cells in culture did not show typical neuronal properties such as action potentials or voltage-gated Na^+ and K^+ currents. Nevertheless, the potential usefulness of MSCs in cell therapies does not necessarily rest on the direct replacement of lost or damaged neurons. A more likely explanation for the possible beneficial effect of MSCs could be that the grafted cells, when migrating into an environment of injury, express factors beneficial to the nervous tissue or activate compensatory mechanisms and endogenous stem cells within the tissue (Chopp et al., 2000). MSCs secrete cytokines such as CSF, interleukins, stem cell factor (SCF) (Eaves et al., 1991; Majunder et al., 1998), nerve growth factor (NGF), brain-derived neu-

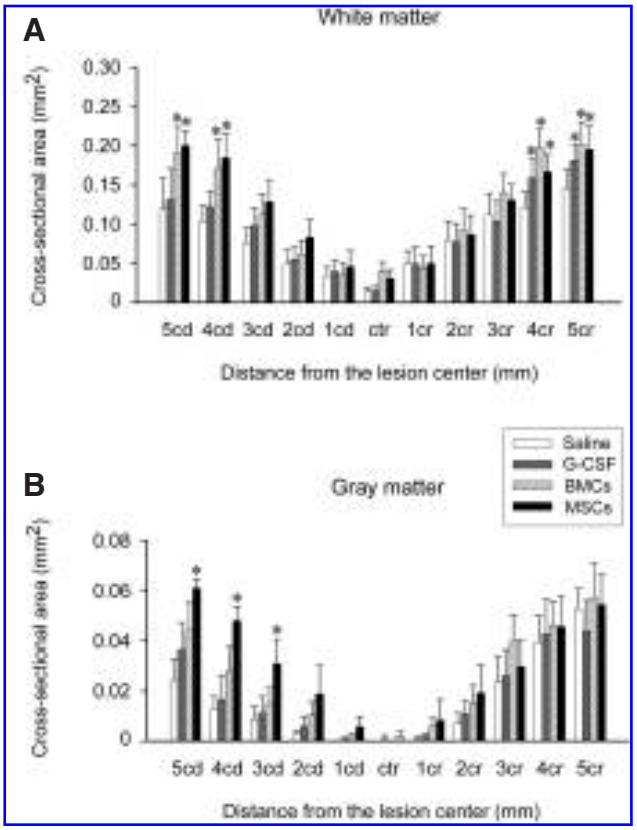

FIG. 4. Craniocaudal distribution of the white and gray matter in the center of the lesion in animals treated with MSCs, BMCs, G-CSF, or saline. (**A**) Graph of consecutive cross-sectional areas of the spared white matter at a defined distance from the lesion center. Asterisks mark statistically significant differences between treated and saline-injected (control) animals at the cranial as well as at the caudal end of the spinal cord segment. (**B**) Graph of consecutive cross-sectional areas shows the craniocaudal distribution of the spared gray matter in the center of the lesion in individual groups. Differences in cross-sectional area reached statistical significance at the caudal end of the lesion between the MSC and saline-injected rats. All data are presented as means ± SEM. $*p < 0.05$ compared to saline-injected (control) rats. cr, cranial; cd, caudal. For other abbreviations, see text.

rotrophic factor (BDNF), hepatocyte growth factor (HGF), and vascular endothelial cell growth factor (VEGF) (Bjorklund et al., 2002). It has also been reported that MSCs stimulate glial cells to produce neurotrophic factors such as NGF and BDNF (Majunder et al., 1998; Mahmood et al., 2002; Wang et al., 2002). The progressive ischemia that accompanies acute injury extends to the surrounding white matter, leading to additional axonal and neuronal death and necrosis (secondary injury); (Kobrine et al., 1975; Fehlings et al., 1989; Tator and Koyanagi, 1997). Microcirculatory impairment, ruptured blood vessels, and damaged cells and axons increase the extracellular concentrations of excitatory amino acids, initiating excitotoxicity. This in turn triggers the secondary loss of neighboring intact nerve fibers, neurons, and oligodendrocytes. Oligodendrocytes may also be lost after SCI through apoptosis (programmed cell death). Days or weeks after the initial trauma, the apoptotic death of oligodendrocytes, located as far as four segments away from the trauma site, amplifies the original injury (Beattie et al., 2000).

BMCs, which include hematopoietic stem cells, macrophages, lymphocytes, as well as marrow stromal cells, have previously been successfully used in the treatment of different experimental models of spinal cord injury (Sasaki et al., 2001; Akiyama et al., 2002; Inoue et al., 2003). Sasaki et al. (2001) reported that the transplantation of BMCs into demyelinated spinal cord leads to extensive remyelination, demonstrating that BMCs can

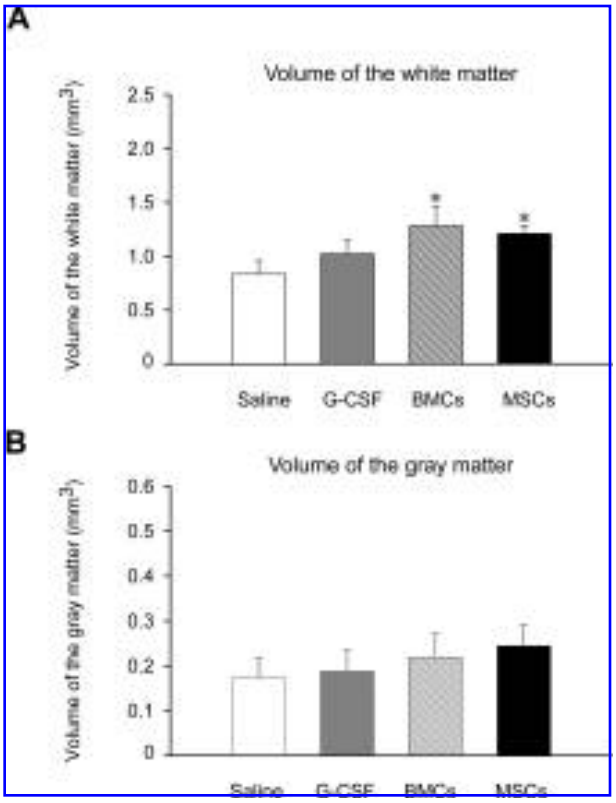

FIG. 5. Analysis of the spared white and gray matter volume in the center of the lesion. (**A**) The total volume of the white matter in 11-mm-long segments of the spinal lesion in the treated and saline-injected animals. (**B**) The total volume of gray matter in the center of the spinal cord lesion. No statistically significant differences were observed between treated and saline-injected animals. All data are presented as means ± SEM. $*p < 0.05$ compared to saline-injected (control) rats.

differentiate into myelinating phenotype cells *in vivo*. However, the concept of grafted BMCs turning into cells of lineages not normally derived from BMCs is still a topic of debate, and as yet we do not have sufficient data to prove the phenotype of grafted BMCs in a spinal cord lesion. Nevertheless, there are several reasons supporting the use of BMCs in SCI therapy. One reason is that the identities of the subpopulations responsible for neuronal differentiation remain unknown. Secondly, the neuronal protective roles of not only MSCs, but also of hematopoietic stem cells, are well known (Chen et al., 2002; Chong et al., 2002). Hematopoietic stem cells secrete many cytokines, including trombopoietin and interleukin 11 (Dame et al., 2003; Mehler et al., 1993). These cytokines are known to be essential factors for the survival and differentiation of neuronal progenitor cells. A recent clinical study was performed by Park et al. (2005) on six patients with SCI. A combination of autologous BMCs implanted as early as 7 days after SCI and subsequent repetitive mobilization of bone marrow cells with granulocyte macrophage-colony stimulating factor (GM-CSF) resulted in five out of six patients showing improved motor and/or sensory function. Our Phase I clinical trial (Motol Hospital in Prague) is currently underway investigating the use of autologous BMC implantation in patients ($n = 20$) with a transversal spinal cord lesion. (Syková et al., in press; Syková et al, 2005). This trial compares intraarterial (via a. vertebralis, $n = 6$) versus intravenous administration of BMCs ($n = 14$) in a group of subacute (10–33 days post-SCI, $n = 8$) and chronic patients (2–18 months, $n = 12$). For patient follow-up, MEP, SEP, MRI and the ASIA score are being used. Partial improvement in the ASIA score and partial recovery of MEP or SEP have been observed in all subacute patients who received cells via a. vertebralis ($n = 4$) and in 1 out of 4 subacute patients who received cells intravenously.

The functional deficit after SCI is mostly due to white matter damage rather than gray matter damage. We observed that MSC or BMC transplantation significantly increases the spared volume of white matter in the lesion center. We therefore propose that the functional benefits observed in both groups may not be due to the integration of the transplanted cells into the spinal cord tissue, but due to the production of beneficial factors that lead to increased protection from secondary damage. Moreover, the influence of MSCs or BMCs is most likely not due to one specific action, but to the synchronized actions of many factors.

G-CSF in humans mobilizes bone marrow cells that are a mixture of hematopoietic progenitor cells (mainly $CD34^+$), similar in characteristics to marrow-derived stem cells (Rice and Reiffers, 1992). In addition to the mobilization of hematopoietic progenitor cells, G-CSF itself might have several mechanisms by which it can affect recovery from neuronal injuries: G-CSF receptors are found on neurons and glia, so G-CSF may exert a specific neuroprotective effect on these cells (Schabitz et al., 2003). Thus, G-CSF may confer neuroprotection after stroke by inhibiting excitotoxicity and inducing the transcription of neuroprotective genes (Schabitz et al., 2003). G-CSF (filgrastim) has been used after neuronal injury to reduce the risk of sepsis in patients with traumatic brain injury (Heard et al., 1998). In our study we found that the effect of G-CSF treatment was less profound than that of MSC or BMC implantation. The improvement of hind limb sensitivity after G-CSF treatment had a slower time course than that following cell treatment, and the histological assessment of spared white and gray matter showed that the increase in spared tissue was not statistically significant. It was shown that the number of white blood cells in the peripheral blood increases after G-CSF treatment (Rice and Reiffers, 1992). Therefore, G-CSF may not only exert a direct beneficial effect on spinal cord tissue, but can also lead to an increase in the number of systemic neutrophils. This neutrophilia can then have an adverse effect on the development of a spinal cord lesion as part of the inflammatory processes in the lesion. This hypothesis is in agreement with Fukumoto's findings, in which he and his colleagues reported that treatment with G-CSF can lead to the aggregation of mobilized inflammatory cells that induce cerebral and myocardial infarctions (Fukumoto et al., 1997). In contrast, Taguchi (Taguchi et al., 2004) found that $CD34^+$ cells play a positive role in neuroregeneration by inducing neovascularization in the ischemic zone of the mouse brain, producing growth factors or cytokines and providing a favorable environment for neurogenesis; in the spinal cord the effect of $CD34^+$ cells mobilized by G-CSF may reflect the same mechanisms. Since we did not inject isolated hematopoietic progenitor cells, we cannot determine whether G-CSF-mobilized hematopoietic progenitor cells are less potent in spinal cord regeneration than nonhematopoietic progenitor cells or if G-CSF had any negative effects on SCI repair.

In conclusion, our results show that treatment with different cell populations obtained from bone marrow (MSCs, BMCs and the endogenous mobilization of bone marrow cells) has a beneficial effect on behavioral and histological outcomes after SCI. It is not clear whether the injection of MSCs or BMCs or G-CSF treatment induces functional and morphological improvement through the same mechanisms of action.

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