

Lumbar Puncture Delivery of Bone Marrow Stromal Cells in Spinal Cord Contusion: A Novel Method for Minimally Invasive Cell Transplantation

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ABSTRACT

Cell transplantation as a treatment for spinal cord injury is a promising therapeutic strategy whose effective clinical application would be facilitated by non-invasive delivery protocols. Cells derived from the bone marrow are particularly attractive because they can be obtained easily, expanded to large numbers and potentially used for autologous as well as allogeneic transplantation. In this study we tested the feasibility of a novel minimally invasive method—lumbar puncture (LP)—for transplanting bone marrow stromal stem cells (MSC) into a clinically relevant spinal cord contusion model. We further sought to determine optimal protocols for performing such minimally invasive cell transplantation. Sprague-Dawley rats received a moderate contusion injury at the midthoracic level followed by LP transplantation of MSC derived from transgenic rats that express the human placental alkaline phosphatase (AP) reporter gene. The recipients were analyzed histologically to evaluate the extent of cell delivery and survival at the injury site. We found that MSC delivered by LP reached the contused spinal cord tissues and exerted a significant beneficial effect by reducing cyst and injury size. Transplantation within 14 days of injury provided significantly greater grafting efficiency than more delayed delivery, and increasing MSC dosage improved cell engraftment. The techniques described here can easily be translated to patients, thus accelerating clinical application of stem cell therapies.

Key words: cyst; spinal cord injury; stem cells, adult; surgical procedures, minimally invasive; translational

INTRODUCTION

SEVERAL NEURAL and non-neural cell types, including Olfactory ensheathing glia and Schwann cells (Bunge and Pearce, 2003), genetically modified fibroblasts (Murray, 2004) and marrow stromal cells (MSC; Chopp and

Li, 2002), have been shown to improve functional outcomes following transplantation into the injured spinal cord. MSC are clinically attractive because they can be easily obtained from bone marrow, expanded to large numbers and potentially used for autologous transplantation without the need for immune suppression. Recent

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studies of the properties and potential efficacy of MSC have demonstrated that these cells promote axonal growth and produce significant behavioral improvement after direct transplantation into rat spinal cord injury (SCI) models (Chopp et al., 2000; Akiyama et al., 2002; Hofstetter et al., 2002; Ankeny et al., 2004; Ohta et al., 2004; Neuhuber et al., 2005). The mechanism of their action remains controversial. While some studies have suggested that MSC may differentiate into neural phenotypes and potentially be used for cell replacement (Sanchez-Ramos, 2002), others have shown that the changes in morphology and gene expression observed with various differentiation protocols may be misleading or artifacts caused by reagents that disrupt the actin cytoskeleton (Neuhuber et al., 2004; Lu and Tuszynski, 2005). The predominant opinion is therefore that MSC exert their beneficial effects by the secretion of therapeutic factors (Chopp and Li, 2002; Chen et al., 2005) that protect and improve the injury site and promote axonal growth.

Currently, most of the transplantation protocols in SCI utilize direct delivery of cells into the injured parenchyma (Hofstetter et al., 2002; Liu et al., 2002; Bunge and Pearse, 2003). This technique has major disadvantages including the additional trauma resulting from the transplantation surgery which can further compromise the injured tissues, and the vulnerability of cells which are transplanted into the hostile environment of the injured spinal cord, thus leading to reduced survival of the grafted cells. Most importantly, this protocol is difficult to translate to human patients because direct parenchymal transplantation into the spinal cord will require a major neurosurgical procedure under anesthesia with considerable risk of complications in already compromised patients. Therefore, we have been investigating alternative, less invasive strategies of MSC delivery. We have recently shown that cells delivered into the cerebrospinal fluid (CSF) by lumbar puncture (LP) migrate towards a subtotal hemisection injury in the spinal cord (Bakshi et al., 2004; Lepore et al., 2005). However, since most human spinal cord injuries are contusions, we sought to determine the possibility of delivering MSC to the contused spinal cord via LP. Furthermore, we tested different protocols for optimizing the LP delivery of MSC into the contused spinal cord and for maximizing the beneficial effects of the graft.

MATERIALS AND METHODS

MSC Isolation and Labeling

MSC used in these experiments were obtained from Fisher 344 rats that have been transgenically modified to express the human placental alkaline phosphatase (AP)

gene. We have established in earlier experiments (Mujtaba et al., 2002; Han et al., 2004) that the AP gene product is a reliable marker of transplanted cells. AP is expressed in the cell membrane and cytoplasm of all MSC. The expression is stable and, unlike viral vectors that have been used in the past, the expression is not down-regulated following CNS transplantation (Mujtaba et al., 2002). MSC were isolated from the bone marrow of adult AP-transgenic Fisher 344 rats obtained from the femur and tibia as previously described (Lennon et al., 1995; Neuhuber et al., 2004). Briefly, after removing the bones, they were rinsed in saline, epiphyseal plates resected and bone marrow flushed out with a syringe using Hank's buffered saline solution, pooled, and counted. Following centrifugation ($600 \times g$, 10 min), cells were plated at 120×10^6 cells/cm², in 10% fetal bovine serum (HyClone, Logan, UT), 45% Hams' F-12 (Invitrogen, Carlsbad, CA), 45% α -MEM (Invitrogen), supplemented with antibiotics (100 U/mL penicillin G and 100 μ g/mL streptomycin sulfate; Invitrogen). Flasks were incubated in a humidified atmosphere with 5% CO₂ at 37°C. After 8 days, non-adherent cells were removed and remaining cells detached with 0.05% trypsin/0.53 mM EDTA (Invitrogen), and replated at 2000 cells/cm². Subsequently, cultures were passaged at 4-day intervals. The resulting stromal cell fraction represented a population of multipotential stem cells that have been well characterized in their ability to proliferate and differentiate into a variety of mesenchymal phenotypes (Pittenger et al., 1999; Neuhuber et al., 2004). Two animals were adequate to generate enough MSC for the entire study. All experiments were performed with cells passaged less than five times in order to maintain their phenotypic and growth properties.

Surgery

All experiments were performed with adult female Sprague-Dawley rats with pre-operative weights of 250–300 g. The Drexel University Institutional Animal Care and Use Committee approved all experimental protocols. Moderate contusion injuries were performed at midthoracic level using the MASCIS Impactor device that drops a 10-g weight on the exposed spinal cord from a height of 25 mm. This model of spinal cord contusion injury is standard and has been widely used in the field (Young, 2002). All rats were immune-suppressed with Cyclosporine A (CsA) at 10 mg/kg/day beginning 3 days before transplantation and continuing until sacrifice.

Lumbar Puncture

Lumbar puncture was performed under inhalation (isoflurane) anesthesia using techniques described earlier

LUMBAR PUNCTURE DELIVERY OF MSC FOR SPINAL CONTUSION

(Bakshi et al., 2004). Animals were anesthetized and placed on an operating surface that flexes the spine. A small (1-cm) longitudinal incision was made in the skin over the L3–5 spinous processes and the skin retracted. A human neonatal lumbar puncture needle (25-gauge \times 1, ref. NR 405073 from Becton Dickinson, NJ) was advanced into the spinal canal at L3–4 or L4–5 level. Proper placement of the needle in the lumbar subdural space was indicated by three signs: a feeling of give at the time of entry (soft sign), a tail flick (more definitive sign), and presence of CSF in the needle hub (most definitive sign). Once proper needle placement was confirmed, the CSF present in the needle hub was aspirated using a micropipette and an appropriate dose of MSC (uniformly diluted at 50,000 cells/ μ L of basic culture medium) was injected into the CSF over 30 sec. An additional 10 μ L of saline was administered to flush the needle, and the LP needle was withdrawn after replacing the stylet. The skin was stapled and the animal was returned to its cage. The entire procedure took between 3–5 min.

Experiment 1: Cell survival and accumulation. Eighteen rats received a moderate contusion injury. Eleven rats received 2×10^6 MSC in 40 μ l of medium by LP 1 week after the injury. Rats were sacrificed at 1 week ($n = 3$), 2 weeks ($n = 2$), or 6 weeks ($n = 6$) after transplantation. Six control animals received an injury but no transplant, and were sacrificed 6 weeks after injury.

Experiment 2: Window of opportunity. Twenty-four rats received a moderate contusion injury. Five groups ($n = 5$ each, except for 20 days where $n = 4$) received cell transplantation by LP (2×10^6 MSC per animal) at 4, 9, 13, 20, or 27 days after injury. Animals were sacrificed 2 weeks following transplantation and injured cord tissues were analyzed histologically. Because differences between individual time points were small, data from 4, 9, and 13 days were pooled to represent the early time point (<14 days) and compared to data from 20 and 27 days, pooled to represent the later time point (>14 days), using one-way ANOVA with significance set at 0.05.

Experiment 3: Dose-response. Fifteen rats were given moderate contusion injuries, followed 9 days later by the transplantation of vehicle only ($n = 4$), 1×10^6 ($n = 4$), 2×10^6 ($n = 4$), or 4×10^6 ($n = 3$) MSC by LP. Animals were sacrificed and analyzed histologically 2 weeks after transplantation.

Experiment 4: Multiple transplantations. Twelve rats were subjected to a moderate spinal cord contusion injury. One group of control animals ($n = 6$) received a

single dose of 2×10^6 MSC by LP 9 days following injury. The experimental group ($n = 6$) received three doses of 2×10^6 MSC each at weekly intervals beginning 9 days following injury. All animals were sacrificed and analyzed histologically 30 days after injury.

Histology

Animals were perfused with 4% paraformaldehyde, the spinal cords dissected out, cryoprotected in 30% sucrose, and frozen in TissueTek. Axial sections of 20 μ m in thickness were obtained from the injured segments using a cryostat. Histological analysis was performed on all spinal cords and the presence of transplanted cells was detected using alkaline phosphatase (AP) histochemistry and immunofluorescence methods.

AP histochemistry. Sections were washed three times in X-Gal PBS, heat-treated at 60°C in X-Gal PBS for 1 h to inactivate endogenous alkaline phosphatase, briefly washed in AP buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5), and incubated at room temperature in the dark with AP buffer containing 1.0 mg/mL NBT, 0.1 mg/mL BCIP, and 5 mM levamisole (Sigma) in AP buffer for 2 h. Slides were coverslipped in aqueous mounting media and visualized using a Leica light microscope.

Immunofluorescence. Selected slides were ringed with rubber cement and hydrated with PBS. Non-specific immunoreactivity was blocked with 10% goat serum for 1 h and then primary antibody (monoclonal, anti-human AP; 1:200, Serotec) diluted in PBS-0.2% Triton was applied for overnight incubation at room temperature. The next morning, primary antibody was removed by three rinses with PBS, and secondary antibodies (FITC-conjugated goat anti-mouse; Jackson ImmunoResearch) were applied at a dilution of 1:200 for 2 h in the dark. The secondary antibody was then rinsed and slides coverslipped with Vectashield-Hardset containing DAPI for nuclear staining. Images were acquired using the Leica DMRB microscope with fluorescent and DIC optics, fitted with a high-resolution Photometrics Sensys cooled CCD digital camera connected to a PowerMac computer running Signal Analytics IP Labs Spectrum software for acquisition and processing of 12-bit images.

Quantitative Analysis

The pattern of expression of the hPAP transgene does not allow discrimination of individual cells, making cell counting technically difficult. Therefore, volumes occupied by transplanted cells were used to approximate the number of cells grafted into the injured tissue. Similarly,

volumes of cyst, injury and spared white matter were used to estimate changes in these parameters. To calculate volumes, we used Cavalieri's volume estimator technique (Rosen and Harry, 1990). This commonly used stereological tool allows estimation of volumes by analyzing the area of interest in random samples (Ankeny et al., 2001; Ma et al., 2001; Ankeny et al., 2004). Random sampling was performed by acquiring images from every twentieth section stained using AP histochemistry starting in the healthy tissue rostral from the injury continuing through the injury site and ending in the healthy tissue caudal to the injury. The injury site was defined as an obvious discontinuity in the density of small cells compared to the neighboring healthy tissues as described earlier (Takami et al., 2002). Because of variability among animals, which was most likely due to efficiency of perfusion, all volumes were normalized to spinal cord volume within the same range of sections and displayed as volume fractions (Howard and Reed, 1998). Spinal cord volume was determined by tracing the whole spinal cord section in every 20th section stained with Cresyl violet and performing numerical integration with Cavalieri's correction to calculate the volume.

Graft volume determination. To determine graft volume, AP-positive grafts in each of the twenty sections were carefully outlined. The non-specifically labeled dura lateral to the graft was excluded as was a band of average dural thickness dorsal to the graft. In some cases, AP-positive cells appeared to be adhered to the dura lateral to the injury; these cells were not included in the measurements. AP-positive structures ventral to the injury site most likely were non-specifically labeled blood vessels that were also excluded from the measurements.

Cyst and injury size determination. Specific measurements were obtained by tracing cyst (empty areas within a section) and injury site (area of disrupted white and gray matter including cysts) as shown in Figure 2A,B.

Determination of spared white matter. To determine white matter sparing, random samples were obtained as described above and stained with cyanine R to detect myelin. Spared white matter was defined as the remaining uninterrupted tissue positive for myelin (Fig. 2C).

All volumes (graft, cyst, injury and spared white matter) were calculated using numerical integration with Cavalieri's correction. All data are displayed as mean percentages or respective volumes normalized to cord volume \pm standard error of the mean (SEM).

Correlation analysis was performed between volume fraction of transplanted cells and cyst or injury volume.

Statistical Analysis

Data from all experiments were analyzed by one-way ANOVA with post-hoc analysis using the Bonferroni and Mann-Whitney *U* tests where appropriate. Significance was set at $p < 0.05$ for all comparisons. Correlations were performed using simple linear regression followed by ANOVA with significance set at $p < 0.05$.

RESULTS

MSC Survival and Accumulation

This experiment was designed to determine cell survival and accumulation following MSC transplantation by LP. One week after a moderate contusion at midthoracic level 2×10^6 MSC were delivered by LP. Transplanted MSC, identified by AP histology, were congregated along the dorsal surface of the injured cord, at the point of maximal impact (Fig. 1A). Transplanted cells formed a dense network and the specificity of AP histochemistry was confirmed by immunofluorescence staining with antibodies against human AP (Fig. 1A, inset).

Similar to our previously published results (Han et al., 2002, 2004; Bakshi et al., 2004), co-immunostaining with human AP antibodies and DAPI also established that grafted MSC were intact and the observed AP staining was not due to uptake of dead AP-positive cells by macrophages. To further confirm that macrophages did not take up AP-positive cells, we co-immunostained sections containing MSC grafts with antibodies against human AP and ED1, a macrophage marker. We did not find co-localization of ED1-positive macrophages and immunostaining for AP (data not shown). Sections of injured tissue from rats that had not received MSC transplants served as controls for the specificity of AP histochemistry (Fig. 1B). The extent of cell survival as determined by graft volume observed at 6 weeks was not significantly reduced relative to week 1 (Fig. 1C) suggesting that once MSC cells have entered the injured spinal cord after LP delivery, they survive well and show little intraspinal migration.

To determine if accumulation of MSC at the injury site was random or selective, we stained sections rostral and caudal of the injury site using AP histochemistry (Fig. 1D). We did not observe MSC in any segments of the spinal cord other than at the injury site. A possible reason for the lack of cells in segments of uninjured cord may be that cells do not attach in the subdural space and subsequently are lost during tissue processing. To more specifically determine if accumulation of MSC is selective or not, we have performed experiments using fluorescent beads instead of cellular transplants (data not shown). We found that fluorescent beads accumulate

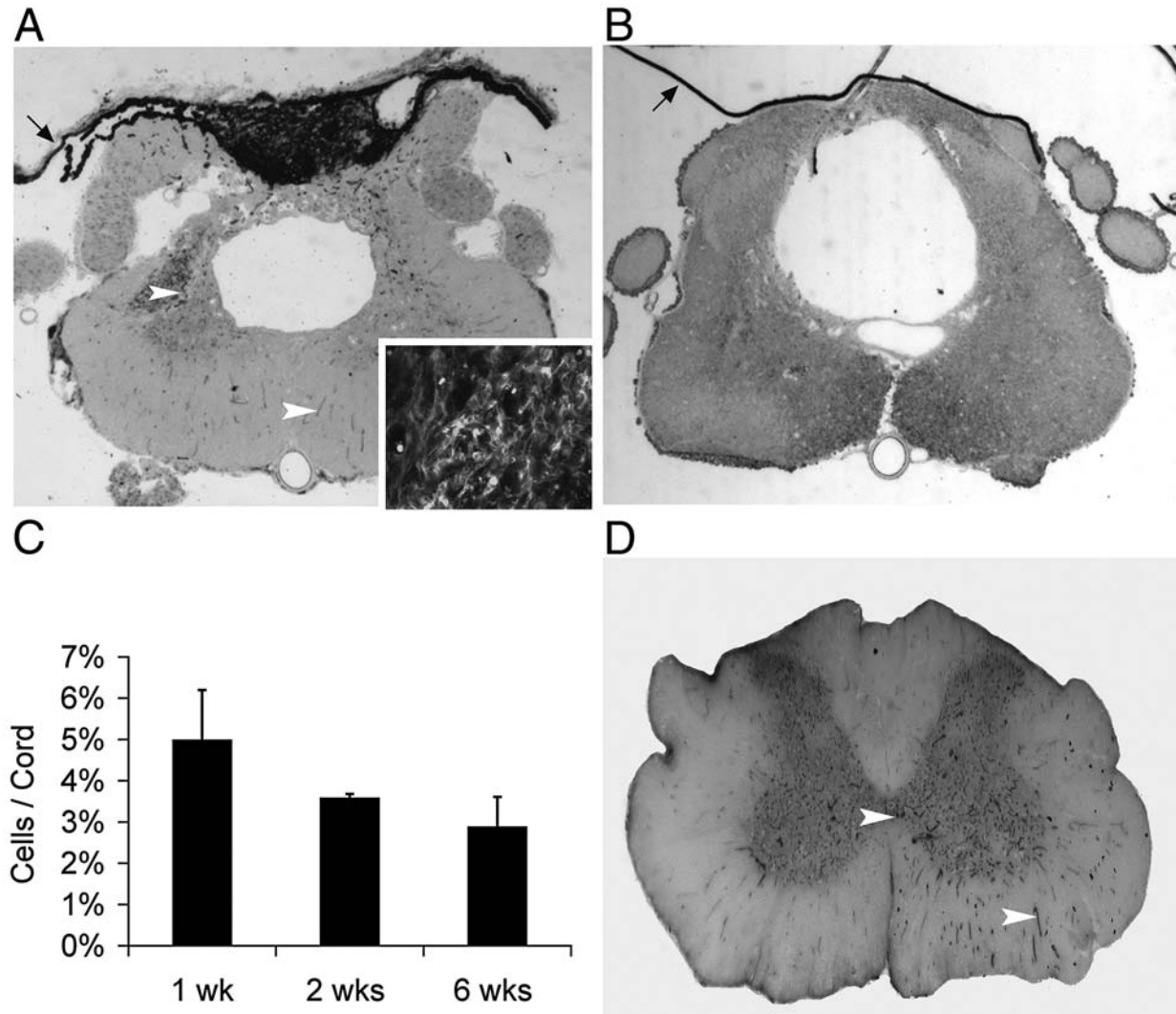
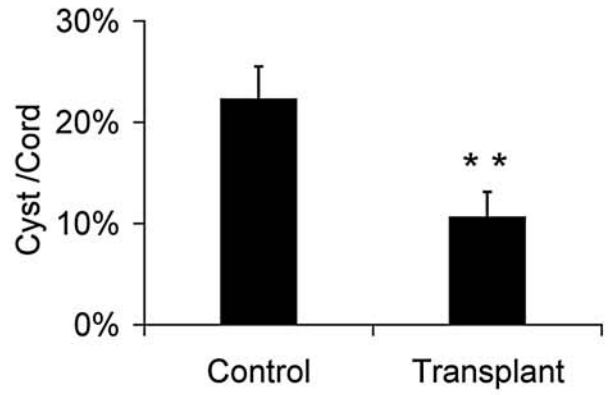
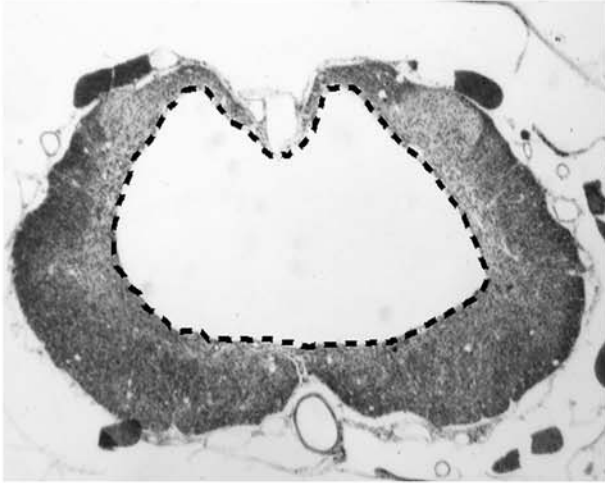


FIG. 1. Bone marrow stromal cells (MSC) delivered by lumbar puncture (LP) accumulate at the injury site. Axial spinal cord sections were stained by AP histochemistry (black staining). **(A)** Two weeks after transplantation of MSC via LP into moderately contused animals, transplanted MSC accumulated at the injury site. Immunostaining of MSC grafts with antibodies against human AP further confirmed the specificity of AP histochemistry (inset). **(B)** To control for non-specific AP staining, injured controls that had not received MSC transplants were stained using AP histochemistry. No AP staining was observed at the injury site. Non-specific AP staining was found in the dura (arrow). **(C)** Graft volumes were determined 1, 2, and 6 weeks after LP delivery and are shown as the mean percentages (\pm SEM) of AP-positive graft volumes normalized to cord volume. Cells were still present at the injury site after 6 weeks, even though graft volume decreased over time. **(D)** To confirm that MSC selectively accumulated at the site of injury, AP histochemistry was performed on spinal cord sections distal of the injury site. Except for non-specific staining of blood vessels, which can be found in most sections (see arrowheads in a,d), no AP-positive staining was observed in cervical (rostral of the injury site) or lumbar (caudal of the injury site; data not shown) sections of the cord suggesting that MSC do not accumulate in uninjured tissue.

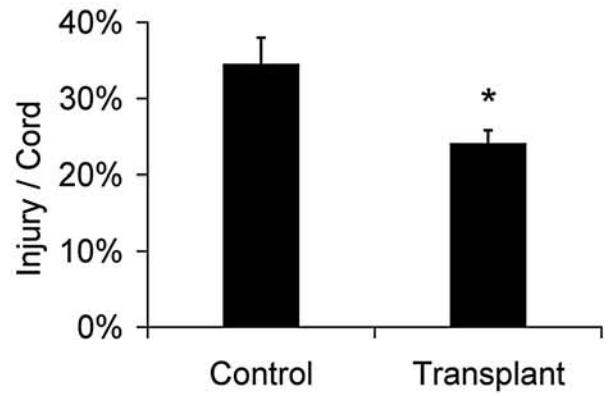
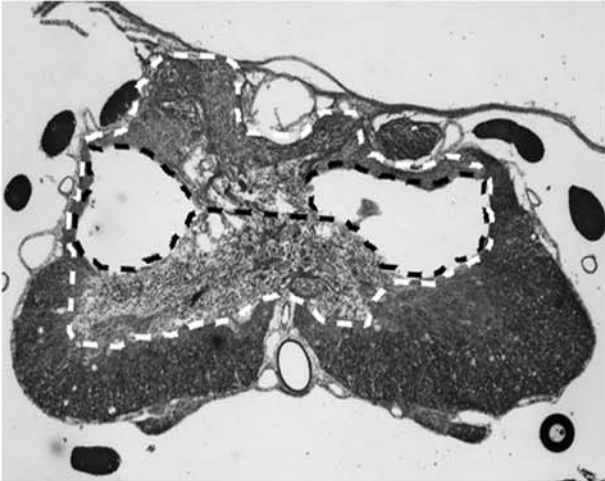
along the length of the spinal cord but do not selectively enter the contusion site. In studies done on animals with partial hemisections, we got similar results after LP delivery of fluorescent beads (Lepore et al., 2005). Very few beads accumulated at the site of hemisection com-

pared to the number of accumulated cells delivered by LP suggesting that while easy accessibility at the injury site may be one reason for cell accumulation, chemotactic signaling and presence of specific adhesion molecules are likely to play a role as well.

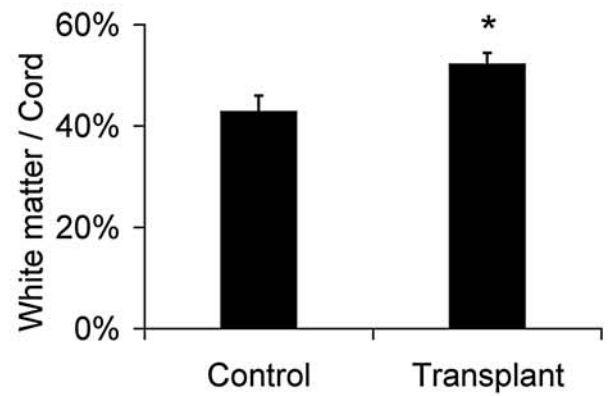
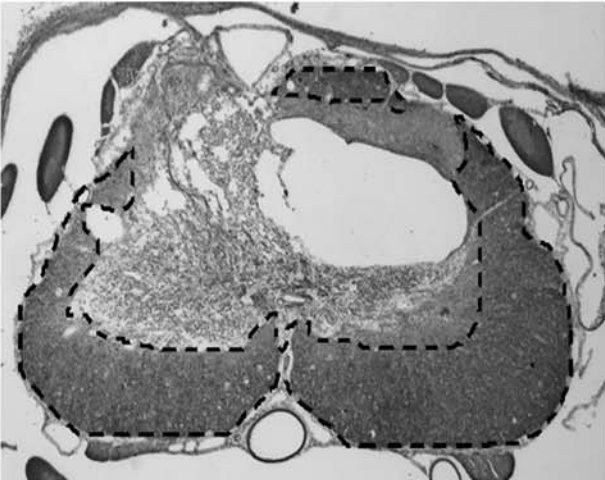
A Cyst size



B Injury size



C White matter sparing



Histological evidence of protection. To determine the ability of transplanted MSC to provide tissue protection, we evaluated the effects of the grafted cells on the host spinal cord by calculating the volumes of cysts (Fig. 2A), size of injury (Fig. 2B), and spared white matter (Fig. 2C) at 6 weeks after transplantation in comparison with controls. To correct for possible variability in the cord sizes, we expressed all volumes as a percentage of cord volumes. We observed that cyst volumes were significantly reduced in the transplanted group (11% of the corresponding cord) in comparison to the control group (22% of corresponding cord; $p < 0.01$; Fig. 2A). We also determined that the transplanted group of animals had significantly smaller injury sizes compared to controls (24% in transplanted group versus 35% in control group; $p < 0.01$; Fig. 2B). Analysis of spared white matter volumes revealed that the transplanted group of animals had significantly greater myelin sparing when compared with the injury-only animals (52% versus 43%; $p < 0.05$; Fig. 2C).

Reduced cyst and injury volumes correlate with grafting efficiency. We noted a negative correlation between the extent of MSC engrafted at the injury site and the size of cyst (Fig. 3A; $R = -0.68$; $p < 0.05$) or injury (Fig. 3B; $R = -0.56$; $p < 0.08$). These results suggest a relationship between MSC transplantation and reduction of cyst size and injury volumes in transplanted animals.

Optimization of Protocols

Window of opportunity. This experiment was performed to determine the optimal therapeutic window following injury during which MSC transplantation by LP can be performed effectively (Fig. 4A). Early grafting of 2×10^6 MSC (i.e., grafting within 2 weeks of injury; $n = 15$) lead to significantly better engraftment ($p < 0.05$) compared to late cell grafting (performed between 2 and 4 weeks after injury; $n = 9$). However, we still observed engraftment of MSC after delivery at late time points suggesting that while cell delivery during the 2-week window after injury is more efficient, cells can still be delivered up to 4 weeks post injury.

Dose-response relationships. This experiment was designed to derive a dose-response curve for MSC transplantation by LP, where response is defined as histological cell grafting efficiency. Four groups of rats were given moderate contusion injuries followed 9 days later by transplantation of vehicle only ($n = 4$), 1×10^6 ($n = 4$), 2×10^6 ($n = 4$), or 4×10^6 ($n = 3$) MSC by LP. Animals were analyzed histologically 2 weeks after transplantation. We did not find a strong positive correlation ($R = 0.45$) between the cell dosage and the volume of MSC grafts observed at the injury site suggesting that only a limited number of MSC delivered at a given time point can efficiently engraft at the site of injury (Fig. 4B).

Multiple transplantations. One of the unique advantages of the LP delivery technique is the possibility of performing multiple transplantations that might lead to improved grafting efficiency. This experiment was designed to test if multiple LP transplantations lead to improved cell grafting compared to a single dose of MSC delivered by LP. We tested if delivering three doses of 2×10^6 MSC at weekly intervals improves the cell grafting efficiency compared to a single dose. Analysis of the results revealed that animals that received three doses of MSC had 2.3 times better cell grafting than those that received single dose (Fig. 4C) suggesting that supplemental doses of MSC have an additive effect and result in improved cell grafting efficiency.

DISCUSSION

Cells Delivered by LP Reach the Site of Injury

We have previously demonstrated that MSC as well as lineage restricted neural precursor cells reach a cervical spinal hemisection injury after delivery by LP (Bakshi et al., 2004; Lepore et al., 2005) but are not seen in the surrounding uninjured cord. Because most human spinal cord injury is in the form of a contusion, in this study we sought to determine if MSC delivered by LP would also accumulate at the site of a contusion injury. After

FIG. 2. Cyst and injury size are reduced in animals that received bone marrow stromal cell (MSC) grafts via lumbar puncture (LP), while amounts of spared white matter are increased. Typical axial spinal cord sections from control group (A) or transplanted group (B,C) were stained with Nissl myelin (cyst and injury size) or cyanine R (white matter sparing) at 6 weeks after transplantation. Volumes of cysts, injury size and spared white matter were determined using Cavalieri's volume estimator technique and normalized to total cord volume. Data are displayed as mean percentages \pm SEM of cyst, injury or white matter volumes normalized to cord volume. (A) Cyst size was quantified by measuring the area of the cyst in every twentieth section (black dashed line in A,B) and calculating the volume. Transplanted animals had significantly smaller cysts (graph; $**p < 0.01$) than controls. (B) Injury size was quantified by measuring the area of the injury (disrupted tissue) in every twentieth section (white dashed line in B) and calculating the volume. Transplanted animals had significantly smaller injuries (graph; $*p < 0.05$) than controls. (C) White matter sparing was calculated by tracing the spared (non-disrupted) white matter in every twentieth section (black dashed line in C) and calculating the volume. Transplanted animals had significantly more spared white matter (graph; $*p < 0.05$) than controls.

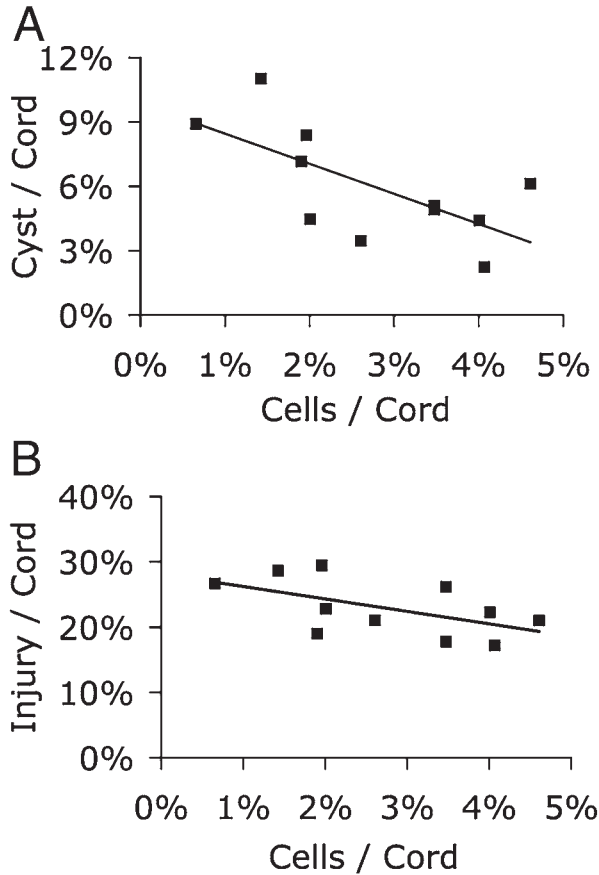


FIG. 3. Graft volumes negatively correlate to cyst and injury size volumes. Correlation analysis was performed on volumes occupied by grafted cells versus cyst or injury volumes in a group of animals from experiment 3. Negative correlations were observed between cell grafting efficiency and cyst volume ($R = -0.68$; $p < 0.05$) (A) and between cell grafting efficiency and injury volume ($R = -0.56$; $p < 0.08$) (B).

demonstrating successful cell grafting, we established the anatomical benefits achieved by MSC grafting, i.e. reduced lesion size indicating attenuation of the secondary injury. Finally, we optimized the protocols for performing MSC delivery by LP for SCI.

One of the most significant findings of this study is that MSC delivered by LP (at the lumbar level) can actually reach the distant contused spinal cord (at the midthoracic level). There seems to be a considerable degree of selectivity to the “homing effect” of grafted MSC because our unpublished results show that fluorescent polystyrene beads that are delivered by the same LP protocols show very little penetration into the contusion site. Other studies have suggested that molecules released or present at the injury site can attract MSC. This “homing” may be mediated by the presence of CXCR4 receptor on hMSC reacting to expression of SDF-1 α in injured tissues (Ji et al., 2004).

We believe that the LP delivery method will have major implications for clinical translation of transplantation therapies in SCI. Most investigators in the past have used direct parenchymal injection into the contused spinal cord with minor variations that included delayed transplantation or injection not only into the injury epicenter, but also caudal and rostral to the injury (Hofstetter et al., 2002; Bunge and Pearse, 2003). While this strategy might be acceptable for studying the potential efficacy of transplants in experimental models, there are serious limitations to translating highly invasive protocols to the clinic. For example, there are considerable risks associated with transplanting cells directly into the spinal cords of patients because the injured neural tissues respond poorly to manipulation. Apart from the possibility of injuring the spared spinal cord, subjecting an acutely injured SCI patient to anesthesia and a major neurosurgical procedure leads to considerable morbidity. Thus, as the experimental models improve and get ready to be clinically tested there is an urgent need to develop more minimally invasive methods for transplanting therapeutic cells.

Several studies attempted to address these concerns by testing intravenous or intraventricular cell transplantation (Yandava et al., 1999; Garbuzova-Davis et al., 2003). Mahmood et al. (2003) injected MSC intravenously following experimental traumatic brain injury but could demonstrate only few cells at the injured site. We confirmed this result in spinal cord injury and demonstrated in our previous studies that intravenous cell delivery was inefficient and had several theoretical and practical limitations (Bakshi et al., 2004). For example, cells in the intravenous compartment are likely to get trapped in extra-CNS organs (liver, lungs) and have a prolonged exposure to the reticulo-endothelial system leading to rapid elimination. Another study has demonstrated the possibility of delivering MSC and neural stem cells into the contused spinal cord by injecting the cells into the 4th ventricle also noting a reduction in cyst volume in transplanted animals (Ohta et al., 2004). Although their results are comparable to ours we believe that because LP is considerably less invasive and easier to perform in human beings, our procedure provides superior safety and is more likely to be translated to the clinics.

LP-Delivered Cells Result in Decreased Injury and Cyst Size

To test the critical issue of whether transplanted cells delivered by LP benefit the host, we performed quantitative analysis that measured the extent of cyst formation, injury size and spared white matter in transplanted animals compared to controls. Our results demonstrate significant reductions in cyst and injury size as well as improved white matter sparing following MSC trans-

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plantation by LP. As total spinal cord volumes of transplanted animals were often smaller than those of controls, we normalized all measured volumes to total spinal cord volume to ensure that measurements reflected genuine changes and not artifacts caused by, for example, tissue shrinkage. Nevertheless, the significant differences in cyst and injury size (which were 100% and 50% larger in injured controls compared to transplanted animals, respec-

tively) were still significant in non-normalized analysis of injury size only. Only the relatively modest improvement in white matter sparing (which was no more than 20% different between controls and transplanted animals and consequently less robust than the reduction in cyst and injury size) was not significant in non-normalized measurements. The relationship between MSC engraftment and reduction in cyst and injury size has a significant negative correlation indicating that introducing more MSC leads to better histological outcomes. These results are consistent with earlier observations showing that MSC produce growth factors and cytokines that can provide tissue protection and promote axonal growth and regeneration (Chopp and Li, 2002; Hofstetter et al., 2002; Ohta et al., 2004; Chen et al., 2005). Based on these observations, we predict that minimally invasive LP transplantation of MSC should produce significant behavioral improvements; however, functional testing of animals will be essential to ultimately determine efficacy of cells delivered using this technique. These longer-term experiments are in progress and their results will be reported separately.

Optimal Protocols for Performing LP Transplantation

In this set of experiments, we studied the critical variables associated with the LP technique to define protocols for performing the most effective delivery of cells. In the first experiment, we determined that early delivery of cells (within 14 days of injury) yields significantly better cell grafting efficiency compared to later cell grafting. The threshold of 14 days for pooling the data was chosen for its likely clinical significance. In most studies so far, cell transplants have been delivered to the injury site after a short delay but within a 2-week window of the initial injury. This 2-week period is considered the window of opportunity for effective cell therapy (Rosen-

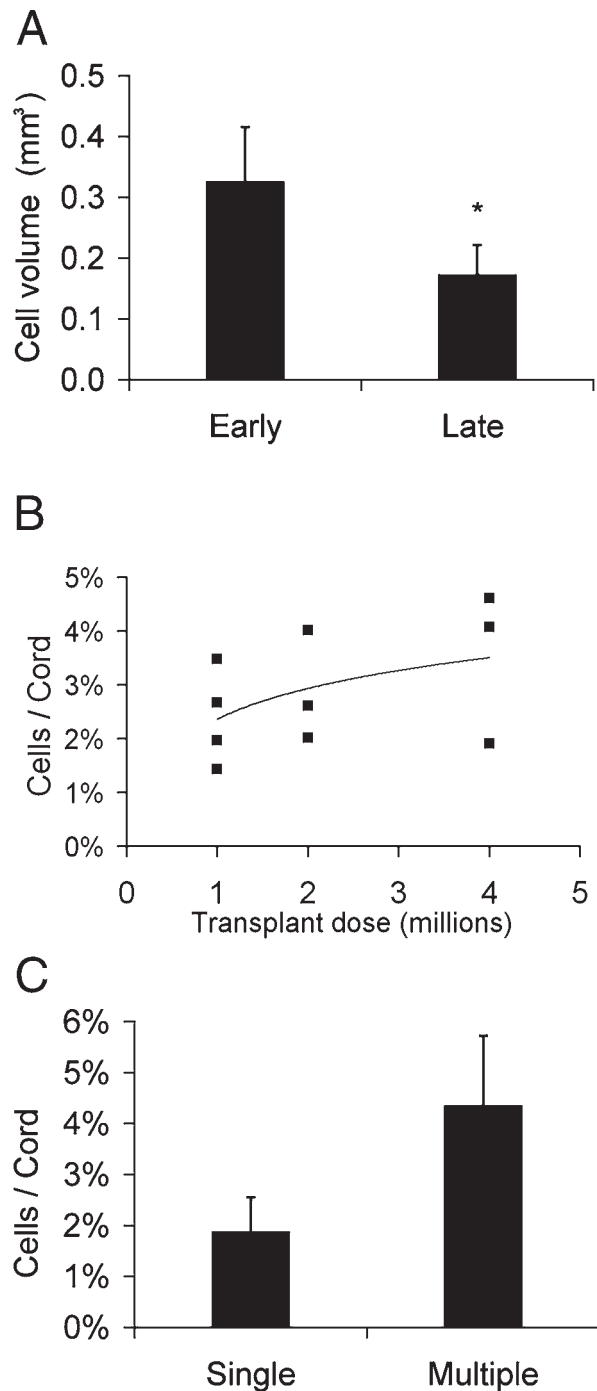


FIG. 4. Effects of timing of bone marrow stromal cell (MSC) delivery, dosage, and multiple lumbar punctures (LPs). **(A)** Window of opportunity: contused animals received 2×10^6 MSC at different times after injury. According to time of transplant, animals were pooled into two groups: early (<14 days) and late (>14 days). Early transplant times resulted in significantly better grafting efficiency ($*p < 0.05$), indicating that the optimal window of opportunity for transplants is within the first 2 weeks after injury. **(B)** Dose-response curve: four groups of rats received LP transplants of vehicle, 1×10^6 , 2×10^6 , or 4×10^6 MSC 9 days after injury. There was no significant correlation between cell dosage and graft volume ($R = 0.45$). **(C)** Multiple LPs: nine days after injury, two groups of contused animals received either a single dose of 2×10^6 MSC or three doses of 2×10^6 MSC at weekly intervals. In animals receiving multiple LP transplantations, a 2.3-fold increase in cell grafting efficiency was observed.

zweig and McDonald, 2004). Survival of grafted cells is limited during the acute phase because of the ongoing inflammatory response; however, it has been shown that cellular transplants delivered within 2 weeks after injury provide considerable tissue protection (Shibayama et al., 1998; Ankeny et al., 2004; Rosenzweig and McDonald, 2004). After the initial 2-week period cell transplants tend to survive better and still provide benefits like trophic support and a permissive matrix for axonal growth (Chen et al., 2005); however, the tissue-protective effect is lost (Shibayama et al., 1998). In addition, it is likely that in the acute phase after injury, the process of chemokine secretion that accompanies first the innate and later the adaptive immune response, contributes to attraction and 'homing' of MSC towards the injured tissues. In the later sub-acute phase expression levels of chemokines change and this may result in decreased 'homing' of transplanted cells. Furthermore, the progressive healing and gliosis around the contused area might gradually prevent MSC from accessing the injured tissues (Rosenzweig and McDonald, 2004).

Next, we sought to determine the optimal dose of MSC needed for effective grafting. However, we did not find significantly increased graft volumes after single injections of higher numbers of MSC suggesting that grafting efficiency is limited by capacity and fluid properties of the CSF rather than the optimal therapeutic dose. We therefore propose that multiple LPs using lower cell doses at each time may be a better option to achieve both efficient engraftment of cells and an optimal therapeutic cell dose.

As noted before, one of the advantages of performing cell transplantation by LP is the possibility of multiple cell deliveries. We tested this possibility in the final optimization experiment. We found that animals that received multiple transplantations had on average 2.3 times more cells than those that received a single dose. Large variations in the extent of cell grafting precluded the possibility of obtaining statistical significance. Furthermore, all animals in this experiment received their first cell dose 9 days after injury. Based on the window of opportunity experiment, it is possible that if LP delivery had been started earlier (within 3 days) and carried on for multiple doses, significant improvement in cell engraftment would have been observed. As a caveat, the dosages delivered in the two groups were different, i.e. the multiple injection group received 6×10^6 cells (2×10^6 per LP over 3 LP) compared to 2×10^6 for the control group. However, considering results from our optimal dose experiment, we presume that a single injection of 6×10^6 cells would not have resulted in efficient engraftment, and therefore, graft volumes obtained with single injection of 2 or 6×10^6 cells would not have significantly differed. Our results suggest that multiple LP transplantations have an additive effect on cell engraftment and

may be a valuable alternative leading to better cell engraftment and thus, more tissue protection.

CONCLUSION

We have demonstrated the feasibility of delivering MSC by LP into a contusion model of SCI. We have also established that MSC transplanted in this minimally invasive method reach the contused spinal cord and exert significant beneficial effects on the host tissue. Finally, we have described optimal protocols for performing such transplantation experiments. We predict that the novel technique described here would be rapidly adopted by the field, and if successful, should be easy to translate into the clinical setting, thus benefiting patients of SCI and other neurological diseases.

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