Bone Marrow Stromal Cells Elicit Tissue Sparing after Acute but Not Delayed Transplantation into the Contused Adult Rat Thoracic Spinal Cord

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Abstract

Bone marrow stromal cells (BMSC) transplanted into the contused spinal cord may support repair by improving tissue sparing. We injected allogeneic BMSC into the moderately contused adult rat thoracic spinal cord at 15 min (acute) and at 3, 7, and 21 days (delayed) post-injury and quantified tissue sparing and BMSC survival up to 4 weeks post-transplantation. BMSC survival within the contusion at 7 days post-transplantation was significantly higher with an acute injection (32%) and 3-day delayed injection (52%) than with a 7- or 21-day delayed injection (9% both; \( p < 0.01 \)). BMSC survival at 28 days post-transplantation was close to 0 in all paradigms, indicating rejection. In contused rats without a BMSC transplant (controls), the volume of spared tissue gradually decreased until 46% \( (p < 0.001) \) of the volume of a comparable uninjured spinal cord segment at 49 days post-injury. In rats with BMSC, injected at 15 min, 3, or 7 days post-injury, spared tissue volume was significantly higher in grafted rats than in control rats at the respective endpoints (i.e., 28, 31, and 35 days post-injury). Acute and 3-day delayed but not 7- and 21-day delayed injection of BMSC significantly improved tissue sparing, which was strongly correlated \( (r = 0.79–1.0) \) to BMSC survival in the first week after injection into the contusion. Our data showed that neuroprotective effects of BMSC transplanted into a moderate rat spinal cord contusion depend strongly on their survival during the first week post-injection. Acutely injected BMSC elicit more tissue sparing than delayed injected BMSC.

Key words: BMSC; cell survival; chronic; CNS injury; inflammation; neuroprotection

Introduction

A contusion of the adult spinal cord causes acute death of neural cells at the lesion epicenter and sets off a series of events resulting in additional tissue loss and the formation of cystic cavities (Hagg and Oudega, 2006). Transplantation of growth-promoting cells is considered a promising approach for repair of the contused spinal cord (Oudega and Xu, 2006; Barnett and Riddell, 2007).

Bone marrow stromal cells (BMSC) are among the candidates for cell-based spinal cord repair strategies (Nandoe Tewarie et al., 2006). These mesenchymal cells secrete growth factors that could support repair (Garcia et al., 2004; Mahmood et al., 2004; Song et al., 2004; Chen et al., 2005). Their potential was confirmed by the finding that transplantation of BMSC into the contused adult rat spinal cord resulted in functional recovery (Hofstetter et al., 2002; Wu et al., 2003; Ohta et al., 2004; Zurita and Vaquero, 2004, 2006). The mechanisms underlying these functional improvements observed after BMSC transplantation are incompletely understood.

A BMSC graft could contribute to repair by sparing spinal cord nervous tissue at the site of transplantation. It is known that BMSCs secrete brain-derived neurotrophic factor (BDNF)
(Mahmood et al., 2004), which has been implicated in limiting spinal cord tissue loss after injury (Ruitenberg et al., 2003). At present, the evidence for BMSC grafts to elicit tissue sparing in the injured spinal cord is still ambiguous (Wu et al., 2003; Ohta et al., 2004; Yoshihara et al., 2006).

Following an insult to the spinal cord, a cytotoxic environment develops at the injury epicenter to diminish in strength over time (Hagg and Oudega, 2006). Most likely these circumstances decrease the survival of cells transplanted into the lesion and consequently their effects on spinal cord repair. At present, quantitative evidence of the survival and neuroprotective effects of BMSCs transplanted into a spinal cord contusion is sparse, which obscures their spinal cord repair potential. We transplanted BMSCs into a moderately contused adult rat spinal cord at 15 min (acutely) and at 3, 7, and 21 days (delayed) post-injury and determined tissue sparing and BMSC survival up to 4 weeks post-transplantation.

Methods

Timeline of the experiment

Rats received a moderate contusion of the T9 spinal cord. Some of these rats received a BMSC injection into the injury epicenter at 15 min, 3, 7, or 21 days post-contusion. These BMSC-transplanted rats were euthanized at 15 min, 3, 7, and 28 days post-injection. Other contused rats did not receive the BMSC injection (control rats) and, to match survival times of the BMSC-transplanted rats, were euthanized at 15 min, and 3, 7, 10, 14, 21, 24, 28, 31, 35, and 49 days post-injury. Their spinal cords were removed and prepared for histology to enable analysis of tissue sparing and BMSC numbers. Details on the used techniques are described below.

Animals

Adult female Sprague-Dawley rats (n = 121, 160–180 g; Harlan, Indianapolis, IN) were used in these experiments. All animals were housed according to the guidelines of the National Institutes of Health and the United States Department of Agriculture. The institutional Animal Care and Use Committee of the University of Miami and Johns Hopkins University approved all surgical procedures.

Culture and lentiviral transduction of BMSCs

BMSCs were obtained from the marrow of femurs and tibia of adult female Sprague-Dawley rats (n = 8), according to a previously published protocol (Azizi et al., 1998). Passage 0 cells were infected with lentivectoral vectors (LV) encoding for green fluorescent protein (GFP) at an MOI of 150. The vectors were generated using the ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA) (Blits et al., 2005). Expression was under control of the human cytomegalovirus promoter and the Woodstuck hepatitis virus post-transcriptional regulatory element (Naldini et al., 1996). The titer of the lentiviral stocks varied from 1–3 × 10⁶TU/mL. Only passage 3 cultures with a transduction rate of about 95% were used for transplantation.

With enzyme-linked immune sorbent assays (ELISA) we determined whether the BMSC secreted BDNF and glial cell-derived neurotrophic factor (GDNF). For this, medium of nearly confluent passage 3 cultures (with an average of 2 × 10⁶ cells) was refreshed, removed 24 h later, and used for BDNF ELISA (BDNF Emax ImmunoAssay System, Promega, Madison, WI) and GDNF ELISA (GDNF Emax ImmunoAssay System, Promega) according to the manufacturer’s instructions. Standard curves of the ELISA kits were linear between 7.8 and 500 pg/mL for BDNF and 15.6 and 1000 pg/mL for GDNF. ELISAs were analyzed with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). The cells in culture secreted 14.1 pg/mL/24 h of BDNF. The amount of GDNF was below the reliability level of the ELISA.

Contusion injury

The rats (n = 108) were anesthetized with 1–2% isoflurane in oxygen and their backs were shaved and cleaned with Betadine and 70% alcohol. Lacrilube ophthalmic ointment (Allergen Pharmaceuticals, Irvine, CA) was applied to the eyes and gentamicin (1.2 mg in 0.03 mL; Abbott Laboratories, North Chicago, IL) was injected intramuscularly. During surgery, rats were kept on a heating pad to maintain their body temperature at 37 ± 0.5°C. A laminectomy was performed at the eighth thoracic vertebra to expose the ninth thoracic spinal cord segment, which was subsequently moderately contused (10 g, 12.5 mm NYU impactor) (Gruner, 1992). To ensure consistency among contused rats, the contusion impact velocity and compression were monitored and all rats with >5% error in these measurements were removed from the study. Rats that remained in the study displayed hindlimb paralysis and scored <3 at 1 day and <7 at 3 days post-injury on the Basso-Beattie-Bresnahan (BBB) scale (Basso et al., 1995). Implementation of these criteria ensured appropriateness of the contusion injury and consistency among the experimental animals. All laminectomies and injuries were performed by the same investigator. After injury, overlying muscles were sutured in layers and the skin was closed with metal wound clips. The rats received 10 mL Ringer’s solution subcutaneously and 1.2 mg gentamicin intramuscularly. The rats were kept in a small animal incubator at 37°C until full recovery and then returned to their cages with ad libitum access to water and food. During the first week post-injury, 1.2 mg gentamicin was administered intramuscularly on a daily basis. The analgesic buprenorphine (0.006 mg; Buprenex, Reckitt Benckiser, Richmond, VA) was administered subcutaneously immediately after surgery and once for the next 3 days. The bladders were expressed manually twice a day until reflex bladder function returned. Urinary tract infection did not occur in any of the rats.

BMSC transplantation

Contused rats were anesthetized with an intramuscular injection of 25.7 mg/kg ketamine, 5.14 mg/kg xylazine, and 0.85 mg/kg acepromazine, and their contused spinal cord was exposed. A total of 1 × 10⁶ BMSC in 5 μL DMEM was injected into the contusion epicenter using techniques described previously (Hurtado et al., 2006). BMSC injections were made at 15 min, 3, 7, and 21 days post-contusion. In the 15 min group, the rats remained sedated with the contused spinal cord covered with a saline-moistened gauze until BMSC injection. All transplantations were performed by the same investigator. After injection, muscles were sutured in layers and the skin closed with metal wound clips. Post-surgery care was as described above.
Assessment of BMSC viability during the injection procedures

The effects of the injection procedures on the number of BMSCs were determined in vitro using the same tools and methods as those used for actual transplantation. First, we assessed the viability of $1 \times 10^6$ BMSC in 5 μL DMEM in an Eppendorf tube after 5 h on ice (the typical length of a transplantation session). A sample of these cells was stained with Trypan Blue (1:1; Sigma, St. Louis, MO) and the percentage of living (unstained) BMSCs was calculated using a hemocytometer. Second, we assessed BMSC viability after injection. BMSCs in DMEM were kept on ice for 5 h and then injected into an Eppendorf tube ($1 \times 10^6$ BMSC in 5 μL DMEM per tube) using the same pulled glass injection needle attached to a Hamilton syringe as used for transplantation into the rat spinal cord. These cells were then kept at 37 °C/5% CO₂ for 1, 3, and 5 h ($n=3$ for each time point). BMSCs that were not passed through the glass needle and kept in the incubator for the same time periods served as controls ($n=3$ for each time point). At the selected times, and immediately after passing through the glass needle (time point 0), cells were stained with Trypan Blue (1:1) and the percentage of living (unstained) BMSCs was calculated using a hemocytometer. The effect of passing through the glass needle was determined by the difference in the percentages of living BMSC between control and time point 0 cells. Whether the incubation conditions had affected BMSC death in time was calculated from the difference in viability between control cells and cells that were passed through the glass needle at 1, 3, and 5 h. Third, we determined the difference in the percentages of living BMSC between control cells and cells that were passed through the glass needle at 1, 3, and 5 h ($n=3$ for each time point). At the selected times, and immediately after passing through the glass needle (time point 0), cells were stained with Trypan Blue (1:1) and the percentage of living (unstained) BMSCs was calculated using a hemocytometer. The effect of passing through the glass needle was determined by the difference in the percentages of living BMSC between control and time point 0 cells. Whether the incubation conditions had affected BMSC death in time was calculated from the difference in viability between control cells and cells that were passed through the glass needle at 1, 3, and 5 h. Third, we determined how many cells passed through the needle. A sample of $1 \times 10^6$ BMSC in 5 μL DMEM that was injected into an Eppendorf tube using a pulled glass needle attached to a Hamilton syringe was stained with trypan blue to calculate the total number of living (unstained) BMSCs using a hemocytometer.

Histological procedures

At 15 min, 3, 7, and 28 days after BMSC injection, rats were anesthetized as above (note that the 15-min group remained anesthetized until fixation). Control rats that were contused but did not receive a BMSC transplant were anesthetized 15 min, and 3, 7, 10, 14, 21, 24, 28, 31, 35, and 49 days post-injury. These time points correspond with the survival times of the transplanted animals. All measurements were taken by the same investigator. From each rat transplanted with BMSCs, every tenth cryostat section (200 μm intervals) was used to determine the volume of a 5 mm long segment centered at the contusion/transplantation epicenter and that of spared tissue within this segment. Tissue was considered spared if it lacked cavities, areas with high density of infiltrating small cells, resembling neutrophils and lymphocytes, and neurons with darkly stained cytoplasmic Nissl bodies (Takami et al., 2002). The volume of spared tissue was expressed as a percentage of the average volume of a comparable uninjured spinal cord segment.

Measurement of BMSC number within the contused spinal cord

From each rat transplanted with BMSCs, every tenth cryostat section was covered with a glass slip with Vectashield with DAPI (4'-6-diamidino-2-phenylindole, Vector Laboratories, Burlington, CA) and used to determine the number of GFP-positive BMSCs in the contusion. In these sections, in a blinded fashion, the contusion area with GFP-positive cells was outlined under a 2.5x objective. Then, under a 63x objective (oil immersion), grids of 250 by 250 μm (counting frame area, 625 μm²) were placed over the outlined area and each GFP-positive cell with a recognizable nucleus was counted using the optical fractionator function of Stereo Investigator (MBF Bioscience, Williston, VT). The sampling interval was: $x=250 \mu m$, $y=250 \mu m$. All measurements were taken by the same investigator. The resulting numbers were used to calculate the total number of GFP-positive BMSC within the contusion. These total numbers were then expressed as a percentage of the total number found at 15 min post-injection.

Assessment of BMSC migration

We found that transplanted BMSC had migrated away from the injection site. Their number, location, and distance from the contusion epicenter were determined in one series of cryostat sections of each of the transplanted rats. Because it is possible that BMSC had migrated beyond 8 mm, which was the approximate distance from the injury epicenter to the edge of the section in rostral and caudal direction, we also sectioned and examined the contiguous 10 mm long rostral and caudal spinal cord segment. All GFP-positive BMSC outside of the contusion were counted manually under a 40x objective.

Statistical analysis

We used SigmaStat (Systat Software, San Jose, CA) for the statistical analyses. The t test was used to determine differences between groups, which were accepted at $p < 0.05$. The
relationship between tissue sparing and BMSC number was
determined by the Pearson correlation coefficient (r).

Results

BMSC transplants in the contused spinal cord

With all transplantation paradigms, GFP-positive BMSCs
were found within the contusion at 15 min after the injection
(Fig. 1A–C). The number of BMSCs within the contusion was
noticeably decreased at 7 days (Fig. 1D–F) and 28 days (Fig.
1G–I) after injection.

Spared tissue in the contused adult rat spinal cord

We qualitatively assessed the contused spinal cord segment
of adult rats without (controls) and with a BMSC transplant
using toluidine blue-stained transverse semi-thin plastic sec-
tions of the contusion epicenter and cresyl violet-stained
horizontal cryostat sections of the contused spinal cord seg-
ment (Fig. 2). In transverse plastic sections, a loosely orga-
nized transplant that filled up the contusion was found at 3
days after an acute injection (Fig. 2A). At 28 days after a BMSC
injection into the 21-day-old contusion, the spinal cord was
not only decreased in size but also contained one or more

FIG. 1. BMSC transplants within a moderate contusion in the adult rat thoracic spinal cord decrease in size during 28 days
post-injection. The transplant is shown at 15 min (A–C), 7 days (D–F), and 28 days (G–I) after an injection at 15 min (acute),
7 days, and 21 days, respectively, post-injury. All microphotographs are from horizontal cryostat sections. (A) Scale bar,
600 μm in A–I.

Spared tissue in the contused adult rat spinal cord

FIG. 2. Spared tissue in the contused adult rat spinal cord after BMSC transplantation. (A) Loosely organized tissue in
contusion epicenter at 3 days after an acute BMSC injection. (B) Cavities present in the contusion epicenter at 28 days after a
21-day delayed transplantation of BMSCs. Note that the spinal cord has also decreased in size. (C) At 3 days after acute BMSC
injection, damaged tissue but not cavities was observed. (D) At 28 days after acute injection, large cavities were found in the
contused segment. (E) With a 7-day delayed BMSC injection, damaged tissue and a few small cavities were found at 3 days
post-transplantation. (F) At 28 days after BMSC injection into the 7-day-old contusion, large cavities were found in the contused
segment. Images are from toluidine blue-stained transverse semithin plastic sections (A and B). Images are from cresyl violet-
stained horizontal cryostat sections (rostral to the left) (C–F). (A) Scale bar, 150 μm in A and B; (C) scale bar, 600 μm in C–F.
large cavities (Fig. 2B). On horizontal sections, after an acute injection, cavities were not present at 3 days (Fig. 2C) but could be found at 28 days (Fig. 2D). With a 7-day delayed injection, small cavities were found at 3 days (Fig. 2E) and much larger ones at 28 days (Fig. 2F) post-injection.

Using Stereo Investigator software, we determined that the volume of spared tissue in the contused spinal cord segment gradually decreased to 10.1 ± 1.7 mm³ at 49 days post-injury in control rats and to 11.5 ± 0.9 mm³ at 49 days post-injury in BMSC-transplanted rats (Table 1). Because narrowing of the contused segment occurs, we determined the volume of spared tissue relative to that of a comparable segment from uninjured and untreated spinal cord (21.9 ± 3.4 mm³; SD, n = 5; Fig. 3A). We found that with BMSCs injected at 15 min, 3 days, or 7 days after injury the volume of spared tissue was significantly higher in transplanted rats than in control rats at the respective endpoints (i.e., 28, 31, and 35 days post-injury).

We then determined the relative change in spared tissue volume in transplanted and control rats between the same time periods after contusion (Fig. 3B). Importantly, in BMSC-transplanted rats the volume of spared tissue at 15 min after injection was similar to that found in control rats matching survival times. The results show that tissue sparing during the 28 days that BMSCs were present was increased with acute and 3-day delayed but not 7- and 21-day delayed BMSC transplantation compared to the loss that occurs in control rats within the same time periods.

**Loss of BMSCs transplanted into the moderately contused rat spinal cord**

We found that 95% of BMSCs survived a period of 5 h on ice and 92% survived when passed through a pulled glass needle. Thus, our methods could have resulted in a maximum of 13% death among BMSCs injected into the contusion. From the intended 1 million cells, 6.7 × 10⁵ BMSC passed through the needle alive. Taking the greatest possible percentage of deaths due to transplantation into account, this implies that 8 × 10⁵ BMSCs were injected into the contusion of which 1.3 × 10⁵ cells (13%) were dead. To correct for variability due to our procedures, BMSC numbers were expressed as a percentage of the calculated average of 6.7 × 10⁵ BMSC passing through the needle alive.

### Table 1. Comparison of Volumes of Spared (Intact) Tissue in Contused Spinal Cord Segments in Rats With and Without BMSC Transplant

<table>
<thead>
<tr>
<th>Time of Tr</th>
<th>Time after Tr</th>
<th>BMSC transplant</th>
<th>Controls (no BMSC)</th>
<th>Time after con</th>
<th>% of control</th>
</tr>
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<tbody>
<tr>
<td>15 min</td>
<td>15 min</td>
<td>20.6 ± 3.6</td>
<td>22.3 ± 1.9</td>
<td>15 mo</td>
<td>−7.6</td>
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<tr>
<td>3 d</td>
<td>19.3 ± 1.4</td>
<td>18.7 ± 1.1</td>
<td>3 d</td>
<td>3.2</td>
<td></td>
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<tr>
<td>7 d</td>
<td>16.6 ± 1.8</td>
<td>13.2 ± 0.8</td>
<td>7 d</td>
<td>25.8**</td>
<td></td>
</tr>
<tr>
<td>28 d</td>
<td>15.7 ± 0.8</td>
<td>11 ± 1.6</td>
<td>28 d</td>
<td>42.7***</td>
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</tr>
<tr>
<td>3 d</td>
<td>15 min</td>
<td>18.4 ± 1.2</td>
<td>18.7 ± 1.1</td>
<td>3 d</td>
<td>−1.6</td>
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<tr>
<td>7 d</td>
<td>16.5 ± 0.9</td>
<td>13.2 ± 1.4</td>
<td>7 d</td>
<td>27.3**</td>
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<tr>
<td>28 d</td>
<td>15.8 ± 1.8</td>
<td>11.8 ± 2.2</td>
<td>31 d</td>
<td>33.9**</td>
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<tr>
<td>7 d</td>
<td>15 min</td>
<td>14.1 ± 1.2</td>
<td>13.2 ± 0.8</td>
<td>7 d</td>
<td>6.8</td>
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<tr>
<td>3 d</td>
<td>14.4 ± 0.9</td>
<td>13.9 ± 1.2</td>
<td>10 d</td>
<td>3.6</td>
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<tr>
<td>7 d</td>
<td>13.2 ± 0.6</td>
<td>12.2 ± 1.2</td>
<td>14 d</td>
<td>8.2</td>
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<tr>
<td>28 d</td>
<td>12.7 ± 1.1</td>
<td>11.8 ± 1.7</td>
<td>35 d</td>
<td>7.6</td>
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<tr>
<td>21 d</td>
<td>15 min</td>
<td>13.2 ± 1.2</td>
<td>12.2 ± 1.7</td>
<td>21 d</td>
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<tr>
<td>3 d</td>
<td>13.1 ± 1.4</td>
<td>11.9 ± 1.3</td>
<td>24 d</td>
<td>10.1</td>
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<tr>
<td>7 d</td>
<td>12.8 ± 0.8</td>
<td>11 ± 1.6</td>
<td>28 d</td>
<td>16.4</td>
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<tr>
<td>28 d</td>
<td>11.5 ± 0.9</td>
<td>10.1 ± 1.7</td>
<td>49 d</td>
<td>13.9</td>
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Rats were contused and received a BMSC transplant (Tr) at 15 min, 3, 7, or 21 days post-injury (column 1). The transplanted rats were euthanized at 15 min, 3, 7, or 28 days after BMSC injection (column 2). The spinal cords were removed and histologically prepared to determine the volumes of spared (intact) tissue (column 3, ± SD). These were then compared to those in control rats, which were similarly contused but did not receive BMSCs (column 4, ± SD). Survival times of control rats after contusion (con; column 5) were matched with the total survival times (i.e., pre-injection time plus post-injection time) of BMSC-injected rats. The difference (as percentage of the volume in controls) was calculated and tested for significance (column 6). A positive value indicates more spared (intact) tissue in the BMSC-transplanted spinal cord. No asterisks, not significant; *p < 0.05; **p < 0.01; ***p < 0.01.
Discussion

Survival of BMSCs transplanted acutely (15 min post-injury) or delayed (3, 7, and 21 days post-injury) into a moderately contused adult rat spinal cord is low with few or none of the cells left at 28 days post-injection. BMSC loss at the 7-day post-injection time point was 68% with acute and 48% with 3-day delayed injections, which was significantly lower than with 7- and 21-day (91% both) delayed injections. This indicates that the contusion environment is more deleterious to transplanted BMSCs during the second and fourth weeks after impact than during the first week after impact. In control rats, the amount of spared tissue gradually decreased in time until 46% of that in a comparable uninjured spinal cord segment at 49 days post-injury. The presence of BMSCs resulted in increased amounts of spared tissue compared to controls. Acute and 3-day delayed, but not 7- and 21-day delayed, injection of BMSCs significantly improved tissue sparing. There was a large positive correlation between spared tissue volume and BMSC number with injections during the first week post-contusion.

Effects of BMSCs on tissue sparing were assessed by determining the volume of spared (intact) tissue (see above for criteria; also see Takami et al., 2002) within the contused spinal cord segment. This revealed an increased volume of spared tissue at 7 and 28 days post-injury with an acute BMSC injection and at 6, 10, and 31 days post-injury with a 3-day delayed BMSC injection. This particular approach of measuring spared tissue is employed regularly but it does not take into account narrowing of the spinal cord, which typically occurs after a contusion. Therefore, we also determine the volume of spared tissue relative to that of a comparable segment from uninjured and untreated spinal cord (Takami et al., 2002). This revealed an increased volume of spared tissue at the above-mentioned time points but also at 35 days post-injury with a 7-day delayed BMSC injection.

Our results demonstrated that acutely transplanted BMSCs contributed to sparing of nervous tissue following a moderate contusion of the thoracic rat spinal cord better than delayed transplanted BMSCs did. The reduced loss of the acutely transplanted BMSCs during the first week after injection correlated closely with the increased volumes of tissue sparing. Previously, it was reported that acutely transplanted BMSCs reduced the size of cavities at 3 weeks after an injection into the contusion (Wu et al., 2003) and at 5 weeks after an injection into the fourth ventricle (Ohta et al., 2004). In the present study, we have assessed spared tissue volume relative to that of a comparable uninjured spinal cord segment, which typically occurs after a contusion. Therefore, we also determine the volume of spared tissue relative to that of a comparable segment from uninjured and untreated spinal cord (Takami et al., 2002). This revealed an increased volume of spared tissue at the above-mentioned time points but also at 35 days post-injury with a 7-day delayed BMSC injection.

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BMSC migration into adjacent spinal nervous tissue

The number of BMSCs in the transplant site could decrease as BMSC migrate away from the injection site. We found that a few days after injection BMSCs started to migrate away mainly from the rostral and caudal aspects of the transplant (Fig. 5A). One week after injection, BMSCs were found in the dorsal and ventral columns, some as far as 7 mm away from the contusion epicenter. Most of these cells were spindle-shaped with a small nucleus and several extensions (Fig. 5B). With a 3-day post-injury injection, a total of 2100 ± 849 BMSCs at 7 days and 820 ± 99 at 28 days post-transplantation had migrated away from the transplant site (Fig. 5C). Significantly more BMSCs (p < 0.001) were found rostral (80 ± 14%) than caudal (20 ± 9%) to the transplantation site. The total number of migrated BMSCs represented 1.2% of the number of BMSCs in the contusion at 15 min post-injection and 2.4% of the number at 7 days post-injection. At 28 days post-injection, almost twice as many BMSCs were found outside of the contusion compared to within. Thus, BMSCs that had migrated away from the injection site into the adjacent spinal cord tissue appeared to be protected compared to those that remained within the contusion.

FIG. 3. BMSC transplantation elicits tissue sparing in the contused adult rat spinal cord. (A) Bar graph showing the volumes of spared tissue in the contused spinal cord segment in rats without (control, open bars) and with (closed bars) BMSC transplants. Spared tissue volume is expressed as a percentage of the volume of a comparable uninjured spinal cord segment. Time points of the x-axis refer to control rats. The survival times of BMSC-transplanted rats that matched these time points were 15 m/15 m, 15 m/3 d, 15 m/7 d, 3 d/7 d, 7 d/7 d, 21 d/15 m, 21 d/3 d, 15 m/28 d, 3 d/28 d, 7 d/28 d, and 21 d/28 d (see also Table 1). Asterisks indicate a significant difference control and BMSC transplanted rats (*p < 0.05; **p < 0.03; ***p < 0.001). (B) Decrease in spared tissue volume occurring during the 28-day time period. The volume at the endpoint is expressed as a percentage of the volume at the starting point. The time periods refer to control rats. The matching time points of transplanted rats are listed above.

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FIG. 3. BMSC transplantation elicits tissue sparing in the contused adult rat spinal cord. (A) Bar graph showing the volumes of spared tissue in the contused spinal cord segment in rats without (control, open bars) and with (closed bars) BMSC transplants. Spared tissue volume is expressed as a percentage of the volume of a comparable uninjured spinal cord segment. Time points of the x-axis refer to control rats. The survival times of BMSC-transplanted rats that matched these time points were 15 m/15 m, 15 m/3 d, 15 m/7 d, 3 d/7 d, 7 d/7 d, 21 d/15 m, 21 d/3 d, 15 m/28 d, 3 d/28 d, 7 d/28 d, and 21 d/28 d (see also Table 1). Asterisks indicate a significant difference control and BMSC transplanted rats (*p < 0.05; **p < 0.03; ***p < 0.01). (B) Decrease in spared tissue volume occurring during the 28-day time period. The volume at the endpoint is expressed as a percentage of the volume at the starting point. The time periods refer to control rats. The matching time points of transplanted rats are listed above.

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to that of a comparable segment from an uninjured and untreated spinal cord to account for the normally occurring shrinkage. Despite this difference in approach, our results are in agreement as they indicated that acutely transplanted BMSC exert neuroprotective actions on the nervous tissue within the contused segment. The effects of these neuroprotective actions remain visible until 28 days post-transplantation. In 2006, Yoshihara and collaborators demonstrated that BMSC transplantation into a 9-day-old moderate contusion did not result in neuroprotection within the contused segment, which is in concurrence with our findings that delayed transplanted BMSCs fail to exert neuroprotective actions. One potential mechanism by which transplanted BMSCs result in neuroprotection may be by secreting BDNF (Ruitenbergh et al., 2003; Patist et al., 2004). We used ELISA to confirm that the BMSCs used for transplantation in our study produced and secreted BDNF. Future studies in which the production of BDNF by BMSCs or the actions of BDNF are blocked will help to elucidate the role of BMSC-derived BDNF on neuroprotection.

Our data showed that 0.02% of acutely transplanted BMSCs had survived up to 28 days in a moderate spinal cord contusion. In a previous study, it was shown that 0.17% of BMSC acutely injected into and near a moderate-severe contusion (25 mm, NYU impactor) in the rat spinal cord had survived at 5 weeks post-injury/injection (Hofstetter et al., 2002). When BMSCs were grafted into a 7-day-old contusion, six times more cells (0.99%) had survived at 5 weeks post-injury (i.e., 4 weeks post-injection). In our study we used a less severe contusion injury but found a lower BMSC survival at time points that were close (acute injection, 28 days survival here vs. 35 days survival observed by Hofstetter et al. [2002]) or exactly matching (7-day delayed injection, 28 days survival here vs. 35 days survival observed by Hofstetter et al. [2002]) to that of a comparable segment from an uninjured and untreated spinal cord to account for the normally occurring shrinkage. Despite this difference in approach, our results are in agreement as they indicated that acutely transplanted BMSC exert neuroprotective actions on the nervous tissue within the contused segment. The effects of these neuroprotective actions remain visible until 28 days post-transplantation. In 2006, Yoshihara and collaborators demonstrated that BMSC transplantation into a 9-day-old moderate contusion did not result in neuroprotection within the contused segment, which is in concurrence with our findings that delayed transplanted BMSCs fail to exert neuroprotective actions. One potential mechanism by which transplanted BMSCs result in neuroprotection may be by secreting BDNF (Ruitenbergh et al., 2003; Patist et al., 2004). We used ELISA to confirm that the BMSCs used for transplantation in our study produced and secreted BDNF. Future studies in which the production of BDNF by BMSCs or the actions of BDNF are blocked will help to elucidate the role of BMSC-derived BDNF on neuroprotection.

<table>
<thead>
<tr>
<th>Time of Tr</th>
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<th>No. of BMSC (±SD)</th>
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<tr>
<td>15 min</td>
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<tr>
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<td>137818 ± 13710</td>
<td>59 ± 6</td>
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<tr>
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<td>32 ± 3</td>
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<tr>
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<td>650 ± 1170</td>
<td>0 ± 1</td>
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<tr>
<td>7 d</td>
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<td>87678 ± 13034</td>
<td>52 ± 8</td>
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<td>450 ± 495</td>
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<tr>
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<td>19154 ± 4107</td>
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<tr>
<td>28 d</td>
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<td>4300 ± 3387</td>
<td>2 ± 2</td>
<td>***</td>
<td>n.s.</td>
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</tbody>
</table>

Rats were contused and received BMSC transplants (Tr) at 15 min, 3, 7, or 21 days post-injury (column 1). The rats were euthanized at 15 min, 3, 7, or 28 days after injection (column 2) and the spinal cord was histologically prepared to determine the number of GFP-positive BMSC within the contusion using stereology (column 3). These numbers were expressed as a percentage of the number at 15 min after injection for each group (column 4). Within each group (i.e., injection paradigm; column 5), BMSC numbers at 3, 7, and 28 days post-injection were significantly lower (**p < 0.01) compared to the previous time points (15 min, 3, and 7 days), demonstrating the significant gradual decrease in BMSC number regardless of the injection time. Between groups, at the same time point (column 6), BMSC number at 15 min post-injection was not different (n.s.). BMSC number at 3 days post-injection was significantly higher with acute (&p < 0.05) and 3-day delayed injections (&p < 0.03) compared to 21-day delayed injections. BMSC number at 7 days post-injection was significantly higher with acute (#p < 0.01) and 3 days delayed (##p < 0.005) compared to 7- and 21-day delayed injections. BMSC number at 15 min and 28 days post-injection were not different (n.s.).
the contusion lesion. Possibly, macrophages are directly involved in transplanted BMSC loss, which would explain the greater loss at injection times when more macrophages can be found in the contusion. The relationship between macrophages and transplanted BMSCs within the injured spinal cord is still poorly understood.

We observed that some of the transplanted BMSCs migrated from the contusion into the rostral and caudal white matter. To our knowledge, this is the first evidence that BMSCs transplanted into a contusion site in the adult rat thoracic spinal cord migrate into the adjacent nervous tissue. Neuhuber and colleagues (2005) described that human BMSCs grafted in a hemisection in the adult rat cervical spinal cord migrated into the nearby spinal tissue. Our data suggested that the conditions at and nearby the 3-day-old spinal cord contusion permitted migration of the injected BMSCs. In the injured adult central nervous system, chemotactic factors and cytokines such as macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, and interleukin-8 have been implicated in BMSC migration (Wang et al., 2002).

In sum, we have gathered evidence that BMSCs transplanted immediately and 3 days after a contusion survive better within the lesion environment during the first week after injection compared to BMSC transplanted at 7 and 21 days post-injury. The improved survival is transient but the BMSC transplant elicited neuroprotective effects that resulted in improved tissue sparing up to 28 days after injection.

**FIG. 4.** BMSC survival in a moderate contusion in the adult rat thoracic spinal cord is low. (A) The number of BMSCs transplanted acutely (15 min post-injury) or delayed (3, 7, and 21 days post-injury) into a moderate contusion decreases over 28 days post-injection. The numbers are expressed as a percentage of the numbers found at 15 min post-injection. (B) BMSC death within the moderate contusion at 7 days post-injection is significantly lower after acute and 3-day delayed injections than with 7- and 21-day delayed injections. Percentages are relative to the number at 15 min post-transplantation. Bars represent standard error of the mean. *p < 0.01; **p < 0.005.

**FIG. 5.** BMSC transplanted into the moderately contused adult rat spinal cord contusion migrate into spinal cord white matter. (A) GFP-positive BMSC (arrow) "leaving" the transplant (T) at its rostral edge. (B) Several GFP-positive BMSC that have migrated approximately 5 mm into the rostral lateral white matter (WM). (C) Bar graph showing the number of BMSC that migrated out of a transplant at 7 and 28 days post-injection. BMSC were injected at 3 days post-injury. All microphotograph confocal images are from horizontal 20 µm thick cryostat sections. (A) Scale bar, 15 µm in A and B.
Acknowledgments

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Author Disclosure Statement

No conflicting financial interests exist.

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