Chapter 3

BDNF-overexpression augments bone marrow stromal cell-mediated neuroprotection in the contused rat spinal cord

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Submitted

ABSTRACT

The ability of bone marrow stromal cell (BMSC) transplants to elicit spinal cord repair is thought to result from paracrine effects by secreted trophic factors including brain-derived neurotrophic factor (BDNF). Here we used gene therapy to increase or silence BDNF production in BMSCs and investigated the involvement of BDNF in BMSC-mediated neuroprotection. In a spinal cord organotypic culture, conditioned medium of BMSCs with normal, increased, or decreased BDNF enhanced spinal motoneuron survival averagely 64 % compared with culture medium only. Only with conditioned medium of BMSCs with increased BDNF this neuroprotective effect was sustained. In a rat model of spinal cord contusion, at four weeks post-injury, tissue sparing was 68% improved with a sub-acute transplant of BMSCs with increased or normal BDNF compared with BMSCs with decreased BDNF and without transplant. Motoneuron survival in the contusion with BMSCs with increased BDNF was 33 % higher compared with any other group. Blood vessel density in the contusion with BMSCs with increased BDNF was 35 % higher compared with BMSCs with normal BDNF and 6-fold higher compared with no transplant. BMSCs with decreased BDNF resulted in a 76 % decrease in blood vessel density compared with BMSCs with normal BDNF. Our data show that increased BDNF augments BMSC-mediated neuroprotection in the contused adult rat spinal cord.

INTRODUCTION

Transplantation of bone marrow stromal cells (BMSCs)[1,2] in the injured spinal improves functional recovery[3-9]. BMSCs elicit neuroprotection[3-6] in the damaged spinal cord which is correlated with functional recovery[3]. How BMSCs elicit neuroprotection is poorly understood. It is thought that BMSCs secrete trophic factors exerting paracrine effects[9-16], but which and how trophic factors are involved in BMSC-mediated neuroprotection is unknown.

Protection of neurons in the damaged spinal cord nervous tissue may result from neurotrophic factors[17,18]. BMSCs secrete neurotrophic factors including brain-derived neurotrophic factor (BDNF)[14-16]. BDNF exerts trophic effects on neurons *in vitro* and *in vivo*[19-21]. Administration of BDNF to the injured spinal cord leads to neurotrophic effects including neuronal sparing[22-25]. Transplantation of BMSCs genetically modified to overexpress BDNF improves neuroprotection and repair[26-28]. BDNF is also known to be involved in vascular events[25,29-33], including protecting blood vessels from injury-induced loss, which could also support neuroprotection[34-36]. Together these data validate the candidacy of BDNF as a determinant in BMSC-mediated neuroprotection.

Here, we hypothesized that BDNF secreted by BMSCs is associated with neuronal and vascular sparing in the contused adult rat spinal cord. Gene therapy was used to modulate the secretion of BDNF by BMSCs. To assess the effect of the gene modulations we used a spinal cord organotypic culture to investigate motoneuron survival, and an adult rat model of spinal cord contusion to investigate motoneuron survival, blood vessel density, and nervous tissue sparing.

METHODS

BMSC culture and lentiviral transduction

BMSCs were harvested from femurs from adult female Sprague Dawley rats (200-250 g; Charles River Laboratories, Wilmington, MA) and cultured as previously described[3,37]. After one passage, BMSCs were transduced with lentiviral vectors (LV) encoding for BDNF and green fluorescent protein (GFP; LV-BDNF/GFP) to upregulate BDNF, LV-

BDNFshRNA/GFP to knock down BDNF (five different constructs; clones were purchased from OpenBiosystems; cat. #.: RMM4532-NM-012513), or LV-GFP for controls. LVs were produced by the Viral Vector Core Facility of the Miami Project to Cure Paralysis (University of Miami Miller School of Medicine, Miami, FL) and used at a multiplicity of infection of 100. Transduced BMSCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % Fetal Bovine Serum and 1 % Penicillin/Streptomycin and passaged every three days. Passage 4 (P 4) BMSCs were used for transplantation.

In vitro analysis of BMSCs

Lentiviral transduction of BMSCs in culture was confirmed by verifying stable GFP expression for seven passages. BMSCs in culture were characterized using immunostaining for CD90, CD105, CD45, TrkB and P75 (see below). Culture medium from confluent P 4 BMSC cultures (2 x 10^6 cells) was collected after 24 hours to quantify the amount of secreted BDNF using enzyme-linked immunosorbent assay (ELISA; Promega, Madison, Wi, USA) according to the manufacturer's guidelines. Unconditioned culture medium was used as a negative control. Three biological replicates were quantified per condition and each biological replicate was the average of two technical replicates.

Motoneuron survival in spinal cord organotypic cultures

The effect of culture medium conditioned by the differently transduced BMSCs on motoneuron survival was determined using spinal cord organotypic slices prepared from neonatal (postnatal days 1-4) B6.Cg-Tg(Thy1-YFP)16Jrs/J transgenic mice (Jackson Laboratories, Maine, MA) in which spinal motoneurons express (Thy1-driven) yellow fluorescence protein (YFP). The spinal cord was dissected and transferred into dissection medium containing Hank's balanced salt solution (Gibco, Carlsbad, CA) with 4.3 mM NaHCO3, 10 mM Hepes, 33 mM glucose, 0.03 % BSA, 0.15 % MgSO4-7H2O, 100 U/ml penicillin, and 100 U/ml streptomycin. After removal of dura and meninges, the spinal cord was transversely sliced at 350 µm on a McIllwain tissue chopper (Stoelting Co, Wood Dale, IL). Then, 5 slices were kept in inserts with collagen-coated transwell membranes (3.0 µm pore size; Corning, Lowell, MA). Inserts were placed in 6-well tissue culture plates

and incubated with BMSC conditioned culture medium or spinal cord slice culture medium containing 50 % MEM (Gibco), 25 % Hank's balanced salt solution (Gibco), 25 % Heat Inactivated Horse Serum (Hyclone), 25 mM Hepes (J.T. Baker), 200 mM L-glutamine (Gibco), 3.5 g/L d-glucose, 100 U/ml penicillin, and 100 U/ml streptomycin. Slices were kept for five days in vitro (DIV) in a humidified chamber with 5 % CO_2 at 37 °C. Medium was replaced every second day. After 5 DIV, cultures were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for two hours at room temperature. Cultures were then washed three times for 10 min with PBS and stored at 4 ˚C until imaging. Motoneuron survival in the slices was determined under the fluorescent microscope by quantifying YFP-positive motoneurons at 3 and 5 DIV. The numbers of slices per group were averaged.

Spinal cord contusion and BMSC transplantation

A total of 45 adult female Sprague Dawley rats (200-250 g; Charles River laboratories) were anaesthetized with an intraperitoneal injection of 60 mg/kg Ketamine (Butlerschein, Dublin, OH) and 0.5 mg/kg Dexdomitor (Pfizer, New York, NY)[3,38]. The backs were shaved and Lacrilube ophthalmic ointment was applied to the eyes to prevent drying during and after surgery. The skin and underlying muscles were incised and a laminectomy of the ninth thoracic (T9) vertebra was performed. The underlying T8 spinal cord was contused using the Infinite Horizon Impactor at an impact force of 200 kDyne. Subsequently, the muscles were sutured separately and the skin was closed with metal Michel wound clips (Butlerschein). Rats were given 1.5 mg/kg of Antisedan (Pfizer) subcutaneously to reverse Dexdomitor's sedative effect, 6 mg/ml of Gentamicin (VWR, Radnor, PA) intramuscularly and 10 ml of Ringer's solution subcutaneously (Butlerschein). Rats received daily injections of 5 ml Ringer's subcutaneously, 6 mg/kg Gentamicin intramuscularly and 5 mg/kg of Rimadyl subcutaneously (Pfizer) until BMSC transplantation[3,38]. Bladders were manually emptied twice daily until reflex voiding recovered (± 2 weeks).

Three days after the contusion, rats were anaesthetized as described above and the contused spinal cord was re-exposed. Next, 5 x 10^5 BMSCs in DMEM or DMEM alone (volume = 5 µl; n = 10/group) was injected into the contusion epicenter using a glass

pulled needle on a Hamilton syringe held within a micromanipulator[3,38]. After injection, the needle was kept in place for 3 min and then retracted slowly over a 2 min period to prevent back leakage of the injectate. The muscles and skin were closed and the rats treated postoperatively as described above. Daily injections of Ringer's and Rimadyl for three days and gentamicin for four days were given as described above[3,38]. There were 4 experimental groups: BMSC, BMSC-BDNF, BMSC-BDNFshRNA, and DMEM.

Histological and immunocytochemical procedures

One week (n = 3 per group) or 4 weeks (n = 7 per group) after injection, rats were deeply anaesthetized with intraperitoneal injection of 90 mg/kg Ketamine (Butlerschein) and 0.75 mg/kg of Dexdomitor and transcardially perfused with 250 ml of 0.1 M PBS followed by 400 ml of 4% paraformaldehyde[3]. Spinal cords were dissected out, postfixed overnight in the same fixative, and then cryoprotected for 24 h in phosphate-buffered 30 % sucrose for 24 h. Twelve mm long segments centered on the contusion epicenter were sectioned (20 µm) on a Leica CM 1950 cryostat. Every twelfth section was stained with cresyl violet to reveal the cytoarchitecture.

Sections were immuno-blocked and permeabilized in 5 % NGS and 0.03 % Triton for 30 min at room temperature. Subsequently, sections were incubated with primary antibodies for 2 h at room temperature and then overnight at 4 ˚C. Used primary antibodies were rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200; DAKO) to stain reactive astrocytes, mouse monoclonal rat endothelial cell antibody (RECA-1; 1:100; Abd Serotec MCA 970 GA) to stain blood vessels. After washing twice in PBS, sections were incubated with secondary antibody Alexa Fluor 594 or 488 at a concentration of 1:200 for two h at room temperature. Sections were stained with DAPI (2 µl/ml) for 3 min to stain nuclei, covered with glass slips in fluorescent mounting medium (Dako North America, Inc. Carpinteria, CA, USA), and stored at 4 °C.

Quantification of motoneurons and blood vessels

The number of motoneurons (all cells > 25 µm) was determined in cresyl violet-stained sections. Motoneuron number was expressed as a percentage of the number of motoneurons in a comparable uninjured spinal cord segment.

Blood vessels were quantified in RECA-1-stained sections using MATLAB® Software (MathWorks)[39]. In the three middle sections, at 20 X magnification, a sample (320 µm x 420 µm) was taken in the gray matter about 600 µm laterocaudally to the injury epicenter and all RECA-1 positive structures were outlined[3]. The area fraction of the outlined structures was determined in each sample and averaged per rat. Averages for all rats per group were averaged.

Assessment of GFP-positive BMSC number and spared tissue volume

The number of GFP-positive BMSCs in the contusion was determined in DAPI-only stained sections using StereoInvestigator (MicroBrightField Inc., Colchester, Virginia, USA). In each section with GFP-positive cells, the transplanted area was outlined manually at 10 x magnification and covered with a 250 × 250 µm grid. At 60 X magnification with oil immersion, GFP-positive cells were marked using the optical fractionator with a 60 × 60 µm counting frame[38]. Results were averaged per group and expressed as a percentage of the number of transplanted cells.

The volume of spared nervous tissue was determined in cresyl violet-stained sections using the Cavalieri estimator function in StereoInvestigator® Software (MicroBrightField Inc). In each twelfth section the volume of spared tissue in a 3.4 mm long segment centered at the contusion epicenter was determined. Beyond the rostral and caudal borders of this segment cavities were absent. Within the analyzed segment, tissue was considered spared if it lacked cavities, areas with densely packed small cells resembling infiltrated neutrophils and lymphocytes, and neurons with darkly stained cytoplasmic Nissl bodies[38]. The Gundersen Coefficient of Error was < 0.05 for all measurements. The volume of spared tissue was expressed as a percentage of the average volume of a comparable uninjured spinal cord segment and displayed as mean ± SEM.

Statistical analysis

For all results, one-way ANOVA with Tukey's post-hoc analysis was used to compare differences among the experimental groups. Differences were accepted as statistical significant with $p < 0.05$.

Ethics and surgical approval

Rats used in this study were housed according to the National Institutes of Health and the United States Department of Agriculture guidelines. Rats were kept within a double-barrier facility, air in the cages was continuously refreshed and water and food were available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

RESULTS

In vitro characterization of modified BMSCs

We characterized the transduced BMSCs in vitro and they were found to have stable GFP expression for at least seven passages. Transduction of BMSCs with LV-BDNF/GFP or LV-BDNFshRNA/GFP did not change the presence of typical BMSC surface markers CD90 (Fig. 1A, 1F, 1K, respectively) and CD105 (Fig. 1B, 1G, 1L, respectively) or the absence of the hematopoietic marker CD45 (Fig. 1E, 1J, 1O, respectively) compared to BMSCs transduced with LV-GFP[3,38]. BMSCs transduced with LV-BDNF exhibited an increase in staining for the BDNF receptors, TrkB (Fig. 1H) and P75 (Fig. 1I), while BMSCs transduced with LV-BDNFshRNA exhibited a decrease in staining for these receptors (Fig. 1M, 1N) compared to BMSCs transduced with LV-GFP. ELISA was used to determine the effectiveness of the used LVs in P4 BMSCs. BMSCs transduced with LV-BDNF/GFP secreted 60.4 ± 8.3 pg/ml BDNF in 24 h, which was over six-fold higher than that secreted by BSMCs transduced with LV-GFP (9.4 ± 0.3 pg/ml/24 h) (Fig. 1P; $p < 0.05$). BMSCs transduced with LV-BDNFshRNA secreted an amount of BDNF below the linear range of the ELISA (7.8 pg/ml; ig. 1P). DMEM does not contain BDNF.

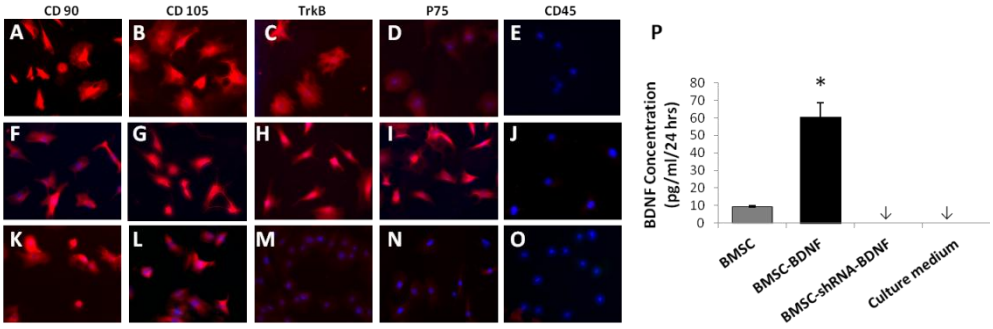

Fig. 1. Characterization of lentivirally modified BMSCs. Compared to control BMSCs (BMSC; A-E), lentiviral transduction of BMSCs with BDNF (BMSC-BDNF; F-J) or shRNA-BDNF (BMSC-shRNA-BDNF; K-O) does not change the expression of BMSC surface markers CD90 (A, F, K) and CD105 (B, G, L) or the absence of hematopoietic markers CD45 (E, J, O) but changes the expression of TrkB (C, H, M) and P75 (D, I, N). BMSC-BDNF exhibited increased staining for TrkB (H vs. C) and P75 (I vs. D) and BMSC-shRNA-BDNF exhibited decreased staining for TrkB (M vs. C) and P75 (N vs. D). Amounts of BDNF in conditioned medium of all types of modified BMSCs and DMEM (culture medium) was determined using ELISA (P). BDNF-hypersecreting BMSCs (BMSC-BDNF) secreted more than 6 times more BDNF than control BMSCs (BMSC). BDNF-hyposecreting BMSCs (shRNA-BDNF-BMSC) secreted amounts of BDNF undetectable for the ELISA. DMEM (Culture medium) also contained no detectable amounts of BDNF. Arrows indicates concentrations below the linear range of the ELISA (<7.8 pg/ml). Error bars display standard error of the mean (SEM). *$p < 0.05$.

Increased BDNF sustains BMSC-mediated neuroprotection

We used a spinal cord organotypic culture system to examine the neuroprotection potential of the modified BMSCs. Incubation with conditioned medium from cultured BMSCs transduced with LV-GFP, LV-BDNF, or LV-BDNFshRNA for 3 days resulted in a 58 % (M = 62.6 ± 6.4), 77 % (M = 70.1 ± 6.2), and 57 % (M = 62.1 ± 3.6) increase in YFP-positive motoneurons compared to incubation with spinal cord organotypic culture medium (Fig. 2A; $p < 0.05$). This result showed that the BMSCs, regardless of their respective genetic modification, secreted trophic factors leading to a similar degree of motoneuron sparing and that BDNF is not a necessary factor for motoneuron protection *in vitro*. Interestingly, at 5 days *in vitro* the protective effect on motoneurons was sustained with conditioned medium from BDNF-hypersecreting BMSCs (M = 66.8 ± 5.6; 87 % increased over incubation with spinal cord organotypic culture medium) but not with conditioned medium from BDNF-hyposecreting BMSCs (M = 35.6 ± 4.4) or from control BMSCs (M =

49.8 ± 3.6) (Fig. 2B). Photographic examples are provided of spinal cord slices and YFP-positive ventral motoneurons in conditioned medium from normal BMSCs (C and G, respectively), BDNF-hypersecreting BMSCs (D and H), BDNF-hyposecreting BMSCs (E and I), and with incubation in the defined spinal cord organotypic culture medium (Culture medium) (F and J). The data demonstrated that increased BDNF levels sustain spinal motoneuron sparing *in vitro*.

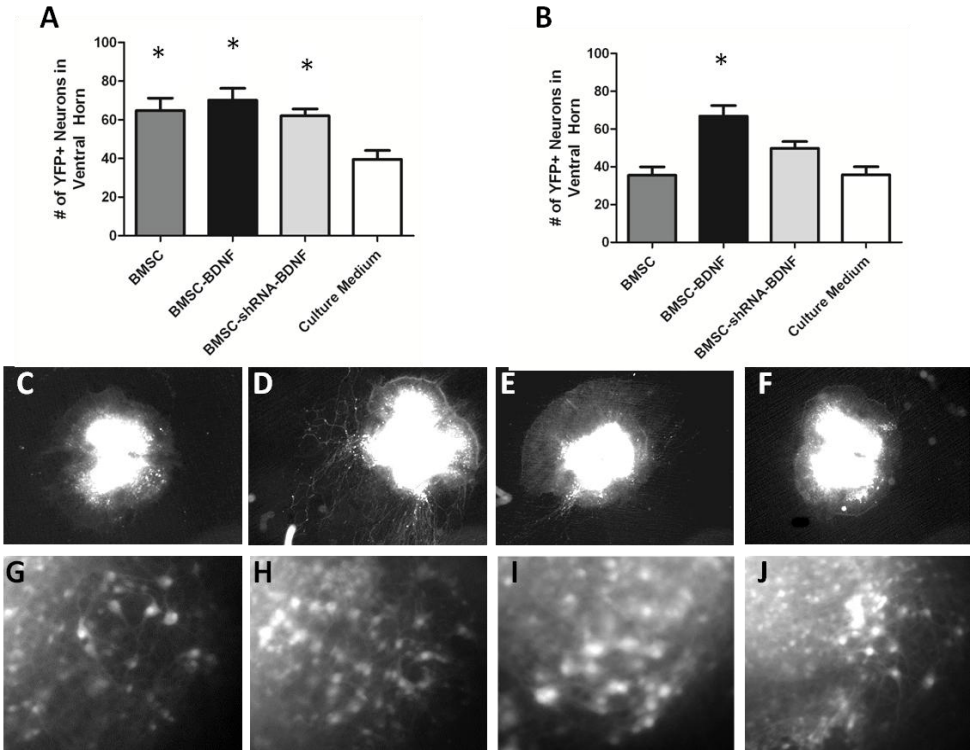

Fig. 2. Sustained in vitro protection of ventral horn motor neurons by BMSC-BDNF. (A) At day three in vitro all types of BMSC conditioned medium resulted in more live YFP-positive neurons in the ventral horn of organotypic spinal slices than unconditioned medium. At five days in vitro, conditioned medium from BDNF-hypersecreting BMSCs (BMSC-BDNF), but not that from the other BMSC types, sustained motoneuron protection. (C-F) shows an example of a spinal cord slice and (G-J) shows YFP-positive ventral motoneurons in conditioned medium from normal BMSCs (C and G, respectively), BDNF-hypersecreting BMSCs (D and H), BDNF-hyposecreting BMSCs (E and I), and with incubation in the defined spinal cord organotypic culture medium (Culture medium) (F and J). Error bars display standard error of the mean (SEM). *$p < 0.05$.

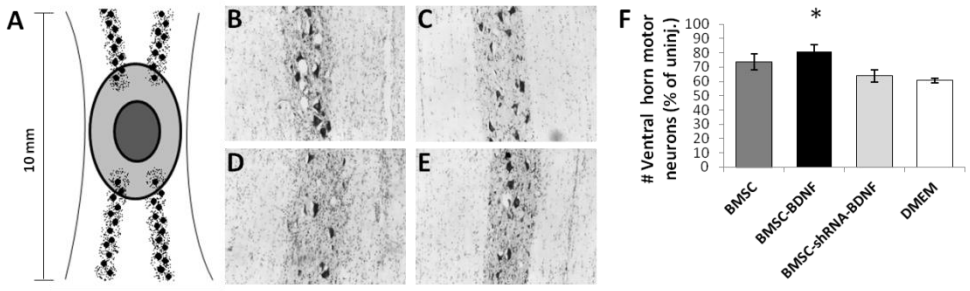

Fig. 3. BDNF-hypersecretion enhances motoneuron survival in a contused spinal cord. (A) Schematic representation of the quantification of ventral motoneurons in cresyl violet-stained sections of a 10-mm long spinal cord segment centered on the contusion epicenter. Motoneurons were found in the ventral spinal cord of rats injected with normal BMSCs (B), BDNF-hypersecreting BMSCs (C), BDNF-hyposecreting BMSCs (D), and DMEM (E). (F) Bar graph showing that 33% more motoneurons were spared in the group of rats that received BDNF-hypersecreting BMSCs compared to the knockdown and DMEM groups. Error bars display standard error of the mean (SEM). *$p < 0.05$.

BDNF-hypersecretion enhanced motoneuron survival in a contused spinal cord

Our in vitro data and previous studies showed that BDNF has protective effects on spinal motoneurons. We examined whether manipulation of BDNF secretion in a BMSC transplant would affect motoneuron survival in a 10-mm long spinal cord segment centered on the contusion epicenter (Fig. 3A). In rats with transplants of BMSCs transduced with LV-BDNF (Fig. 3B), 80.6 % ± 5.3 motoneurons relative to the number in a comparable uninjured spinal cord segment were present compared to 60.7 % ± 1.6 in rats with DMEM injections (Fig. 3E), which represents a 33 % increase in motoneuron number ($p<0.05$; Fig. 3F). Transplantation of BMSCs transduced with LV-GFP (Fig. 3C) or LV-BDNFshRNA (Fig. 3D) did not result in a statistically significant increase in the number of motoneurons (M = 73.9 ± 5.6, M = 63.9 ± 4.3, respectively; Fig. 3F).

BDNF-hypersecreting BMSCs augment BMSC-mediated blood vessel protection

Previously, we have demonstrated that BMSC transplants results in increased blood vessel numbers in a contused spinal cord[3]. Here, we examined the role of BDNF in this protective effect by determining the density of blood vessels near the contusion (Fig. 4A). Blood vessels were present in rats with BMSCs transduced with LV-GFP (Fig. 4B), LV-BDNF (Fig. 4C), LV-BDNFshRNA (Fig. 4D), and in rats with DMEM injections (Fig. 4E). Quantification

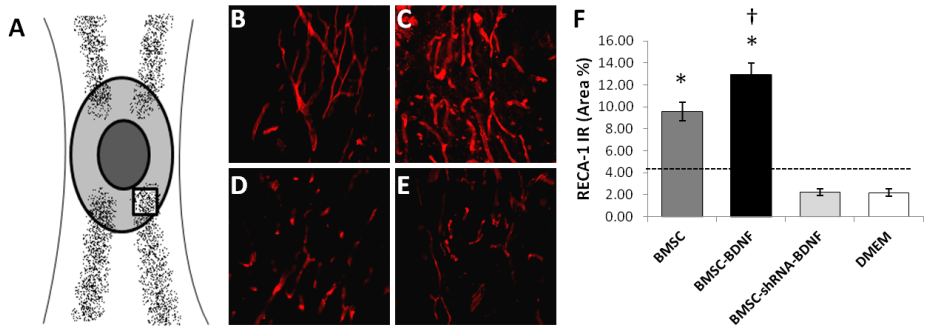

Fig. 4. BDNF-hypersecretion augment BMSC-mediated blood vessel sparing in a contused spinal cord. (A) Schematic of the contusion injury with gray matter and location where blood vessel density was determined. RECA-1-positive blood vessels were found in gray matter near the contusion in rats with normal BMSCs (B), BDNF-hypersecreting BMSCs (C), BDNF-hyposecreting BMSCs (D), and DMEM (E). (F) Bar graph showing that rats with BDNF-hypersecreting BMSCs had a 6-fold increase and rats with an injection of normal BMSCs had a 4-fold increase in blood vessel density compared to DMEM. Rats that received BDNF-hyposecreting BMSCs had a lower density of blood vessels compared to rats with normal BMSCs. Dotted line in F indicates mean blood vessel density in uninjured controls. Error bars display standard error of the mean (SEM). *$p < 0.05$ compared to DMEM and $^{\dagger}p < 0.05$ compared to BMSC.

revealed a significant six-fold increase ($p < 0.05$) in blood vessel density in rats with BDNF-hypersecreting BMSCs compared to rats with DMEM injections (Fig. 4F). Rats with BMSCs transduced with LV-GFP had a significant four-fold increase in blood vessel density compared with rats with DMEM injections (Fig. 4F). Overexpressing BDNF in a BMSC transplant resulted in a 35 % increase and silencing BDNF expression in a 76 % decrease in blood vessel density compared to normal BMSCs (Fig. 4F).

Tissue sparing 4 weeks after BMSC transplantation

Previous research showed that intraspinal BMSC transplants result in larger volumes of spared nervous tissue[3,38]. Here, we examined the role of BDNF in BMSC-mediated neuroprotection in the contused spinal cord. At four weeks post-injection, the volume of spared tissue in rats that received BMSCs transduced with LV-GFP or LV-BDNF was 68 % larger ($p < 0.05$) in both groups compared to that in rats with DMEM injections (Fig. 5). Spared tissue volume in rats with BDNF-hyposecreting BMSCs was similar as in rats with DMEM injection (Fig. 5). These results show that BDNF is necessary for BMSC-mediated tissue sparing but overexpressing BDNF does not lead to larger volumes of spared nervous tissue.

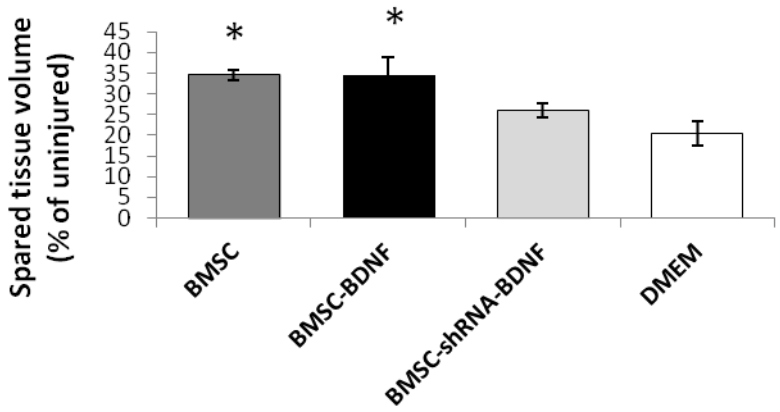

Fig. 5. Tissue sparing 4 weeks after BMSC transplantation. Four weeks after transplantation, a protective effect of BMSCs on nervous tissue is present. The bar graph shows that transplantation of normal BMSCs (BMSC) and BDNF-hypersecreting BMSCs (BMSC-BDNF) resulted in increased volumes of spared tissue compared with rats with DMEM or BDNF-hyposecreting BMSCs. The latter group was not significantly different from the DMEM group. Error bars display standard error of the mean (SEM). *$p < 0.05$.

BDNF-hyposecretion affects BMSC survival in a spinal cord contusion

Survival of a BMSC transplant in damaged nervous tissue is limited[38]. We examined whether increased or decreased BDNF secretion would affect BMSC survival in a contusion environment. One week after transplantation into a three-day old spinal cord contusion, transplanted BMSCs transduced with LV-GFP (Fig. 6A) or LV-BDNF (Fig. 6B) appeared to be mostly spindle-shaped, which was in agreement with our previous study[38]. Stereology-based quantification showed that 13.7 ± 2.2 % of the transplanted BMSCs transduced with LV-GFP and 17.2 ± 4.9 % of BMSCs transduced with LV-GFP were present in the contusion (Fig. 6D). Transplanted BMSCs transduced with LV-BDNFshRNA were not found in the contusion (Fig. 6C). The data show that BDNF is necessary for BMSC survival in a spinal cord contusion but overexpressing BDNF does not lead to improved survival.

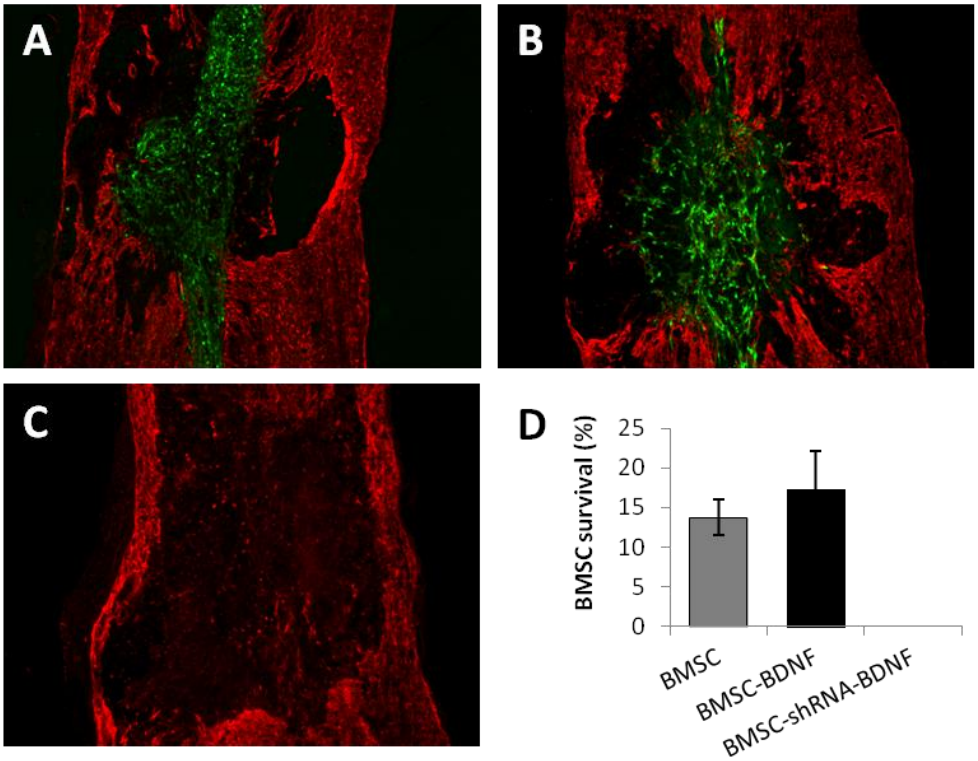

Fig. 6. BDNF-hyposecretion affects BMSC survival in a contusion. One week after transplantation into the contused spinal cord, BMSCs appeared mostly as spindle-shaped cells in transplants of normal BMSCs (BMSC) and of BDNF-hypersecreting BMSCs (BMSC-BDNF) (A,B). BDNF-hyposecreting BMSCs were not detected in the contusion (C). Quantification revealed similar numbers in transplants of normal and BDNF-hypersecreting BMSCs (D). Error bars display standard error of the mean (SEM).

DISCUSSION

The repair-supporting effects of BMSC transplants in the injured spinal cord[3-9] are thought to result from paracrine actions by secreted trophic factors[9-16]. We used gene therapy to manipulate BDNF production by BMSCs and assess its role in BMSC-mediated neuroprotection. Our experiments revealed that *in vitro* increased BDNF amounts sustained motoneuron protection. In the contused spinal cord, BDNF overexpression by grafted BMSCs protected motoneurons and blood vessels; spared tissue volume was unaffected. The data suggest that enhancing BDNF levels in BMSC transplants elicit neuronal and vascular effects that may be beneficial for recovery after spinal cord injury.

With an organotypic culture system we showed that motoneuron survival in spinal cord slices was similar in conditioned medium with normal, increased, or decreased amounts of BDNF. This finding indicated that BDNF is not necessary for *in vitro* spinal motoneuron survival and that other trophic factors secreted by BMSCs are sufficient for this neuroprotective effect. After the first days in culture, motoneuron survival was only sustained with increased BDNF amounts. Thus increased BDNF is vital to maintain the motoneuron survival-promoting effects exerted by the trophic molecules secreted by BMSCs. Possibly; BDNF sustains the survival of motoneurons by preventing or delaying apoptotic pathways[21,40].

Transplantation of BDNF-hypersecreting BMSCs enhanced motoneuron survival in the ventral gray matter in the contused adult rat spinal cord. This finding is in agreement with our *in vitro* observation that conditioned medium from BDNF-hypersecreting BMSCs promoted and sustained motoneuron survival in spinal cord organotypic cultures. BDNF is known to exert neurotrophic actions on neurons[9-16] and treatment of injured spinal cord with BDNF[22-25] or with BDNF-hypersecreting cells promotes neuron survival[26-28,41-44]. In our spinal cord organotypic culture, BDNF was found not to be a necessary factor for motoneuron sparing; other BMSC-derived factors were sufficient. We were unable to verify whether this finding would translate to the *in vivo* spinal cord injury model because silencing BDNF production in BMSCs resulted in rapid loss of transplanted BMSCs.

The finding that survival of BMSCs with decreased BDNF was inferior to that of BMSCs with normal or increased BDNF in a contusion environment is intriguing. To our knowledge, this is the first demonstration of transplantation of BMSCs with silenced BDNF production into the injured spinal cord. In culture, we observed no difference in survival of any of the differently modified BMSCs used in this study. Also, BDNF hypersecretion did not enhance BMSC survival *in vitro* or *in vivo*. How can we explain the decrease in BMSC transplant survival in the absence of BDNF? It is possible that grafted BMSCs are protected by BDNF from apoptotic death[21,40] due to injury-related events including oxidative stress, hypoxia, and/or transplant preparation-related events such as detachment which induces

anoikis. Possibly, the observed lower expression of the BDNF receptors, TrkB and P75 in BDNF-hyposecreting BMSCs contributed to the lower survival in the contusion. In culture, injury-related events are absent and cells are attached; thus, BDNF would not be required for their protection. Further research is needed to elucidate the role of BDNF in BMSC transplant survival in a spinal cord contusion.

In the contused spinal cord with a transplant of BDNF-hypersecreting BMSCs the volume of spared nervous tissue was similar as with normal BMSCs. Our finding that a transplant of normal BMSCs resulted in improved tissue sparing corroborated with data from our previous studies[3,38] as well as that from other groups[4-6]. The lack of additional nervous tissue sparing with increased BDNF was also shown with a complete spinal cord transection[25] but not in a partial, dorsal column, transection[44]. Possibly, the severity of the injury is a determinant in BDNF-mediated tissue sparing. As discussed above, early death of BMSCs with decreased BDNF production prevented determining whether BDNF is necessary for BMSC-mediated tissue sparing.

A transplant of BMSCs with increased BDNF production significantly augmented BMSC-mediated increases in blood vessel density near the injury epicenter. Previously, we showed that BMSC transplants resulted in increased vascularization in the contused spinal cord[3]. Here, we extend this observation and show that BMSC-secreted BDNF possibly plays a role to elicit this effect. Other studies have also implicated BDNF in vascular events in the injured nervous tissue[25,29-33], possibly through upregulation of vascular endothelial growth factor[30]. It is possible that the effects of increased BDNF on motoneuron sparing and blood vessel sparing as we demonstrate in our study are related. Enhancing blood vessel number through angiogenic/angioprotective effects may have resulted in neuroprotection[34-36]. Future research will need to elucidate the interplay between BDNF-mediated vascular and neuronal sparing.

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