Development/Plasticity/Repair

Functional Recovery in Traumatic Spinal Cord Injury after Transplantation of Multineurotrophin-Expressing Glial-Restricted Precursor Cells

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Demyelination contributes to the physiological and behavioral deficits after contusive spinal cord injury (SCI). Therefore, remyelination may be an important strategy to facilitate repair after SCI. We show here that rat embryonic day 14 spinal cord-derived glial-restricted precursor cells (GRPs), which differentiate into both oligodendrocytes and astrocytes, formed normal-appearing central myelin around axons of cultured DRG neurons and had enhanced proliferation and survival in the presence of neurotrophin 3 (NT3) and brain-derived neurotrophin factor (BDNF). We infected GRPs with retroviruses expressing the multineurotrophin D15A (with both BDNF and NT3 activities) and then transplanted them into the contused adult thoracic spinal cord at 9 d after injury. Expression of D15A in the injured spinal cord is five times higher in animals receiving D15A–GRP grafts than ones receiving enhanced green fluorescent protein (EGFP)–GRP or DMEM grafts. Six weeks after transplantation, the grafted GRPs differentiated into mature oligodendrocytes expressing both myelin basic protein (MBP) and adenomatus polyposis coli (APC). Ultrastructural analysis showed that the grafted GRPs formed morphologically normal-appearing myelin sheaths around the axons in the ventrolateral funiculus (VLF) of spinal cord. Expression of D15A significantly increased the percentage of APC⁺ oligodendrocytes of grafted GRPs (15–30%). Most importantly, 8 of 12 rats receiving grafts of D15A–GRPs recovered transcranial magnetic motor-evoked potential responses, indicating that conduction through the demyelinated VLF axons was restored. Such electrophysiological recovery was not observed in rats receiving grafts of EGFP–GRPs, D15A–NIH3T3 cells, or an injection of an adenovirus expressing D15A. Recovery of hindlimb locomotor function was also significantly enhanced only in the D15A–GRP-grafted animals at 4 and 5 weeks after transplantation. Therefore, combined treatment with neurotrophins and GRP grafts can facilitate functional recovery after traumatic SCI and may prove to be a useful therapeutic strategy to repair the injured spinal cord.

Key words: spinal cord injury; remyelination; glial-restricted precursor cells; transplantation; rat; myelin repair

Introduction

Demyelination contributes to the dysfunction of the traumatically injured spinal cord in both humans and experimental ani-

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erwise degenerate. Thus, the relationship between remyelination and functional recovery after traumatic SCI remains unresolved.

Both neurotrophin 3 (NT3) and brain-derived neurotrophic factor (BDNF) regulate neuronal development and axonal regeneration (Xu et al., 1995). They are also important mediators of myelination. Mice lacking functional trkC or NT3 are deficient in both mature oligodendrocytes and OPCs (Kumar et al., 1998). NT3 enhances the survival and proliferation of OPCs in vitro (Barres and Raff, 1994; Kumar et al., 1998; Yan and Wood, 2000; Franklin et al., 2001) and in vivo (Barres et al., 1994). Myelination by oligodendrocytes is also enhanced by NT3 in both cultures of neurons and the injured CNS (McTigue et al., 1998; Yan and Wood, 2000; Jean et al., 2003). BDNF is important for myelin formation in peripheral nerve during development, because inactivation of BDNF signaling by deleting trkB receptors causes myelin deficits both in vivo and in vitro (Cosgaya et al., 2002).

In this study, we grafted glial-restricted precursor cells (GRPs) genetically modified to express D15A, a multilineurotroph that binds trkB and trkC and has both BDNF and NT3 activities, genetically modified to express D15A, a multilineurotroph that binds trkB and trkC and has both BDNF and NT3 activities. The injury severity was adjusted to cause enough loss of myelin and/or axons to result in electrophysiological and hindlimb locomotor deficits but also sufficient sparing of demyelinated axons to enable remyelination (Cao et al., 2005). We postulated that D15A would enhance remyelination by engrafted GRPs and thus increase functional recovery. Transcranial magnetic motor-evoked potential (tCMMEP) responses were used to objectively measure the conduction of demyelinated axons after SCI (Cao et al., 2005; Loy et al., 2002). The relationship between increased remyelination and electrophysiological and behavioral functional recoveries was carefully analyzed. We report here that only the combined treatment with D15A administration and GRP transplantation led to remyelination of demyelinated axons, restoration of axonal conduction, and improvement in hindlimb locomotor recovery.

Materials and Methods

All animal care and surgical interventions were undertaken in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996), and with the approval of the University of Louisville Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Preparation of GRPs. GRPs were immunopanned from embryonic day 14 Fischer 344 rat spinal cord using an A2B5 antibody and a protocol modified from previous studies (Mayer-Proschel et al., 1997; Mujtaba et al., 1999). Growth medium, which consisted of DMEM/Ham's F12 (In-vitrogen, Carlsbad, CA), N2 and 2× B27 supplements (each at 1×; Invitrogen), fibroblast growth factor 2 (FGF2) (20 ng/ml), and platelet-derived growth factor aa (PDGFaa) (10 ng/ml), was changed every other day. After 5 d, the cells were passaged. For fluorescence-activated cell sorting (FACS) analysis of embryonic rat GRPs, the cells were stained live with self-conjugated antibodies against A2B5–phycoerythrin and NG2–FITC and analyzed on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ).

To differentiate GRPs in vitro, the cells were plated in polymethine/fibronectin-coated six-well plates. The following day, the FGF2 was removed, and 30 ng/ml thyroid hormone [tri-iodothyronine (T3)] was added to the growth medium. Thus, differentiation medium consisted of DMEM/Ham's F12, 1× N2 and 2× B27 supplements, PDGFaa (10 ng/ml), and T3 (30 ng/ml), and medium was changed every other day. After 8 d, the cells were fixed and immunohistochemically analyzed for their differentiated phenotypes.

Before transplantation, GRPs were labeled by infection with the LZRS retrovirus encoding either enhanced green fluorescence protein (EGFP) or D15A–EGFP (see below). In some experiments, double labeling with both bromodeoxyuridine (BrDU) and EGFP was used. For BrDU labeling, undifferentiated passage 2 GRPs were incubated in 10 μM BrDU overnight. This resulted in >90% labeling efficiency, with no loss of cell viability. For retroviral labeling, GRPs were treated with 1 μg/ml polybrene for 1 h, followed by incubation in growth medium containing LZRS–EGFP or LZRS–D15A–EGFP retrovirus for 4 h. Routinely, ~60% cells were infected. Two hours before transplantation, the labeled cells were detached from the dishes using a cell lifter, collected by centrifugation at 1000 × g for 4 min, and resuspended in 1 ml of culture medium. After cell count and viability assessment with trypan blue in a hemacytometer, the cell suspension was centrifuged a second time and resuspended in a smaller volume to give a density of 5 × 10⁴ viable cells per microliter. NIH3T3 cells were cultured in DMEM/Ham's F12 containing 10% FBS and subsequently infected with LZRS–D15A–EGFP, as described above.

Construction and characterization of the D15A–LZRS retrovirus. EGFP and D15A (Urfer et al., 1994) cDNAs were cloned into the LZRS retroviral vector (Kinsella and Nolan, 1996). To generate high titer virus, FöX cells (provided by Dr. Gary Nolan, Stanford University, Stanford, CA) were transfected using GenePORTER 2 (Gene Therapy Systems, San Diego, CA), which routinely gave transfection efficiencies of 50–65%. Selection with 2 μg/ml puromycin begins 48 h later. To harvest virus supernatant, media was changed to the appropriate serum-free media without mitogens overnight and harvested the next day. We routinely obtain titers of 5 × 10⁵ to 5 × 10⁶ pfu/ml.

In vitro myelination by GRPs. GRPs were cocultured with embryonic day 15 Sprague Dawley dorsal root ganglion (DRG) neurons, prepared as described previously (Plant et al., 2002). Approximately 5000–7000 neurons were plated per culture. For myelination experiments, cocultures were prepared by adding 50,000 GRPs to purified DRG neuron cultures. Two groups of DRG neuron cultures were maintained without GRPs. The DRG neuron cultures and the cocultures were fed every 2–3 d with Neurobasal medium containing B27 supplement and l-glutamine for 2 weeks, after which they were divided into four groups, with addition of 10 ng/ml NT3, BDNF, both NT3 and BDNF, or no addition, respectively, and were cultured for an additional 2 weeks. The total period of coculture was thus 4 weeks. Subsequently, cultures were processed for either myelin basic protein (MBP) immunohistochemistry or ultrastructural analysis.

Construction of D15A adenovirus. The cDNA encoding D15A was cloned into the E1-deleted Ad5Sc1 and replication-deficient Ad5-D15A adenovirus was isolated as described previously (Gao et al., 1998). The titer was 1.1 × 10¹¹ pfu/ml.

Secretion and bioactivity of D15A–GRPs in vitro. Secretion and bioactivity of secreted neurotrophin in D15A–GRPs were analyzed in vitro on trkC–PC12 cells (Tsoulfas et al., 1993). The trkC–PC12 cells were grown in DMEM containing 10% horse serum and 5% fetal bovine serum. They were induced to differentiate by adding NT3 for 3 d into the growth medium, and a standard curve was developed using increasing concentrations of recombinant human NT3 (250 and 500 pg/ml, 1, 2, 4, 6, and 10 ng/ml). The differentiated trkC–PC12 cells whose processes were three times longer than their cell body were counted, and the percentage of differentiated/total PC12 cells was calculated. To collect conditioned medium from D15A– or EGFP–GRPs, GRPs were infected with D15A or EGFP retroviruses and grown in DMEM for 2 d. Conditioned medium was collected, and 10% horse serum and 5% fetal bovine serum were added. The trkC–PC12 cells were differentiated for 3 d. The percentage of differentiated trkC–PC12 cells in each conditioned medium were counted as described above and were used to determine the bioactivity of the respective conditional mediums using the standard curve established as above. The concentration of NT3 in DMEM, D15A–, or EGFP–GRPs were also assayed by commercially available sandwich technique ELISA kit (R & D Systems, Minneapolis, MN) following the recommended protocol of the manufacturer.

Surgical procedures. Surgical procedures were as described previously (Cao et al., 2001, 2002, 2005). Briefly, after anesthetization with Nembutal (50 mg/kg, i.p.), adult female Fischer 344 rats received a dorsal laminectomy at the ninth thoracic vertebral level (T9) to expose the spinal cord, and then a 150 kdyn contusive SCI using the Infinite Horizons (IH; Lexington, KY) impactor. At 9 d after injury, rats were randomly assigned to five groups, as shown in Table 1, which received DMEM, D15A–GRPs, EGFP–GRPs, D15A–NIH3T3 cells, or D15A adenovirus, respectively. Animals were reanesthetized as above, and the laminectomy site was re-exposed. Four injections were made at 1 mm cranial to, caudal to, and left and right of the lesion at a depth of 1.3 mm and 0.6 mm laterally from midline. At each site, 2 μl of cell suspension or vehicle was injected through a glass micropipette with an outer diameter of 50–70 μm and the tip sharp-beveled to 30–50° at a rate of 0.5 μl/min, as described previously (Cao et al., 2001, 2002). Thus, a total of 400,000 cells were grafted into each injured spinal cord. For the adeno-viral experiment, a total of 10^9 pfu of Ad5–D15A in 4 μl of DMEM was injected into four sites of the injured spinal cord, as described above. The animals were allowed to survive for 5 weeks after transplantation.

ELISA assessment of in vivo neurotrophin expression in the injured spinal cord. A total of 12 animals received 150 kd IH contusion SCI as described above. Eight days later, they were then randomly divided into three groups, which received 8 μl of D15A–GRPs, EGFP–GRPs, or DMEM, respectively, at four sites as described above. One week after transplantation, animals were killed with an overdose of Nembutal, and 8 mm of spinal cord (4 mm from the injured epicenter cranially and caudally, respectively) was used for protein isolation. Spinal cords were homogenized with buffer consisting of 137 mM NaCl, 20 mM Tris-HCl, 1% NP40, and 1X protease inhibitor cocktail set III (Calbiochem, La Jolla, CA). Protein concentrations were calculated with the BCA protein assay (Pierce, Rockford, IL). Expression of NT3 was examined by commercially available sandwich technique ELISA kit (R & D Systems) following the recommended protocol of the manufacturer. Oxidized horseradish peroxidase enzymatic products in the microplate wells were read at 450 nm absorbance using a SpectraMax Plus384 plate reader (Molecular Devices, Sunnyvale, CA). Human recombinant NT3 (R & D Systems) was used to generate a standard curve. NT3 levels were then normalized against total protein. Differences of NT3 expression among three groups was compared with repeated-measures ANOVA followed by Student–Newman–Keuls post hoc test. The level of statistical significance was set at p < 0.05.

Behavioral assessment. Open-field locomotor testing using the Basso–Beattie–Bresnahan (BBB) locomotor rating scale (Basso et al., 1995) was performed at day 7 after injury and once weekly thereafter for 5 weeks after grafting. All of the animals were coded, and behavioral assessments were performed by two investigators blinded with respect to the treatment groups. The mean BBB scores were tallied by injured groups and plotted as a function of time after injury. Changes in BBB scores over time for the four groups were analyzed using repeated-measures ANOVA with the between-groups factor. The degree of freedom was corrected for unequal variance using the Greenhouse–Geisser correction. Differences among the groups and each group over the 6 postinjury test weeks were performed using Tukey’s honestly significant difference (HSD) post hoc t tests.

Table 1. Description of experimental groups

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<tr>
<td>Total</td>
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<td>44</td>
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Five animals died unexpectedly during the course of the experiment; one from group 1, one from group 3, two from group 4, and one from group 5. Early data from these animals were omitted from the analysis. Four animals from each group, except for group 5, were initially tested for tcMMEP responses and survival/differentiation of grafted GRPs in the contused spinal cord. After we observed a robust survival of grafted GRPs and the return of tcMMEP responses in the D15A–GRP–grafted animals, an additional eight rats were included in each group, except for groups 1 and 6, for evaluation of BBB score, tcMMEP responses, and histology. NA, Not applicable; n (BBB), number of animals for which BBB scores were obtained; n (tcMMEP), number of animals for which tcMMEP responses were recorded; n (total), total number of animals at the start of study.

Figure 1. Demyelination after contusive SCI. A, B, In the VLF of normal adult spinal cord, darkly stained compact myelin, the thickness of which is proportional to axon diameter, surrounded each axon. C, D, In the VLF at 1 week after SCI, many myelin sheaths had degenerated and numerous intramyelinic vacuoles were seen (arrows). D, A macrophage was observed in close relation to degenerating myelin (open arrowhead). C, D, The axons surrounded by degenerating myelin appeared morphologically normal (asterisks). C, These axons could be distinguished from those that were undergoing degeneration (curved arrows). E, F, Some demyelinated axons survived for at least 1 month after injury (arrows). E, F, Healthy-appearing demyelinated axons were observed in the VLF at the injury epicenter (E) and also a few millimeters away from the epicenter (F, arrow). These axons are adjacent to astrocytic processes (arrowheads). Scale bars, 2.5 μm.

tcMMEP responses. All tcMMEP responses were obtained from stimulation of awake, nonanesthetized, restrained rats, as described previously (Magnuson et al., 1999; Loy et al., 2002; Cao et al., 2005). Briefly, rats were placed in a prone position on a wooden board and wrapped in a cloth stockingette tacked to the board surface. The hindlimbs were pulled through the cloth and left exposed to enable the insertion of needle electrodes into the gastrocnemius muscle of each hindlimb, with the active electrode placed into the gastrocnemius muscle belly and the reference electrode placed near the distal tendon. The ground electrode was placed subcutaneously between the coil and recording electrodes. tcMMEP responses were elicited by activation of subcortical structures with an electromagnetic coil placed over the cranium. A single magnetic pulse with a stimulus intensity of 60 mV and a gain of 500 or 5000 was used. Data from four responses measured 1 min apart were averaged. The onset latency to the initiation of response and amplitude were recorded.
Figure 2. Differentiation of and myelination by GRPs in vitro. A, B, Proliferating GRPs expressed specific GRP markers, A2B5 (A) and NG2 (B). C, As determined by FACS analysis, ~97% coexpress both markers. After differentiation for 8 d, most GRPs became mature oligodendrocytes and expressed MBP in culture (D). In coculture with DRG neurons, GRPs differentiated into mature oligodendrocytes and myelinated DRG axons as shown by MBP immunohistochemistry (E). Ultrastructural analysis showed that these GRPs formed central myelin on DRG axons (F). The arrowheads point to typical outer cytoplasmic loops. The arrow indicates an astrocyte process in close apposition to the oligodendrocyte myelin. G, After differentiation for 8 d in the presence of NT3, BDNF, or both, the percentage of galactocerebroside-positive (Galc^*) oligodendrocytes increased significantly compared with the control (ANOVA single factor between groups; \( F = 5.509; \) df = 3; \( p < 0.01 \)). H, NT3 or/and BDNF also increased the total numbers of surviving cells and Brdu^+ cells after 8 d differentiation. \( p < 0.001 \). Error bars represent SEM. Scale bars: A, B, D, E, 50 \( \mu \)m; F, 2 \( \mu \)m.

All animals were evaluated once per week for 6 weeks. The latencies and amplitudes of tcMMEP responses over different survival times were analyzed using repeated-measures ANOVA with the between-groups factor, followed by Tukey’s HSD post hoc test.

Immunohistochemistry. For the histological analysis of the grafted cells, rats were anesthetized with 80 mg/kg Nembutal and perfused transcardially with 0.01 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord segments that received the grafts were removed, cryoprotected in 30% sucrose buffer overnight at 4°C, and embedded in OCT compound. Some cords were transversely sectioned at 16 \( \mu \)m, and others were cut longitudinally. In the presence of 10 ng/ml NT3 (B) or undiluted D15A–GRPs, or/and BDNF also increased the total numbers of surviving cells and Brdu^+ cells after 8 d of differentiation. \( p < 0.001 \).

Histological analyses. The total volume of spared white matter in the injured epicenter was determined as described previously (Cao et al., 2005). Briefly, after the final behavioral and physiological assessments, rats were anesthetized with an overdose of Nembutal (80 mg/kg) and perfused transcardially with 0.01 M PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord segments that received the grafts were removed, cryoprotected in 30% sucrose buffer overnight at 4°C, and embedded in OCT compound. Some cords were transversely sectioned at 16 \( \mu \)m on a cryostat, and others were cut longitudinally.

Figure 3. Secretion of NT3 by D15A–GRPs in vitro and in vivo. The bioactivity of D15A–GRPs was examined on trkC–PC12 cells. A, Undifferentiated trkC–PC12 cells had round cell bodies without processes. B, C, In the presence of 10 ng/ml NT3 (B) or undiluted D15A–GRPs conditioned medium (C), trkC–PC12 cells differentiated into multipolar neurons with several long processes. Expression of NT3 in the injured spinal cord was also examined by ELISA at 1 week after transplantation (2 weeks after injury). As shown in D, expression of NT3 in rats that received grafts of D15A–GRPs is significantly higher than those that received grafts of EGFP–GRPs or DMEM medium (\( F = 159.9; \) df = 2, 9; \( * p < 0.001 \)). Data are the mean ± SD (\( n = 4 \)).
Electrophysiological recovery after engrafment of D15A–GRPs. In the normal adult rat, the latency of the monosynaptic tcMMEP response was 6.2 ± 0.3 ms in the amplitudes ranging from 9 to 12 mV (Fig. 3A, A', F, G). B, B', The tcMMEP responses disappeared after moderate (150 kdyn) contusion injury. C, G. At 1 week after transplantation, tcMMEP responses were partially restored, although the amplitudes were still small. D, E, G. Over the 4 week survival time, the amplitude of tcMMEP responses continued to increase. Note the 10-fold difference in gain between the recordings in A and A' and those in B–E'. B–E', In control animals, the restoration of tcMMEP responses was never observed. F, Latencies of the recovered tcMMEP responses shortened after D15A–GRP engrafment. Repeated ANOVA measures showed that latencies were significantly different over survival time (F = 7.0; df = 4, 24; p < 0.05). Tukey’s HSD post hoc t tests showed that latencies of recovered tcMMEP responses decreased significantly by weeks 3 (*p < 0.05), 4, and 5 (**p < 0.01), compared with 1 week after graft. Latencies on weeks 4 and 5 were also significantly shorter than those in week 2 (p < 0.05). All latencies in D15A–GRP-grafted animals were significantly longer than those in normal uninjured animals. G, Repeated-measures ANOVA showed that the amplitudes of recovered tcMMEP responses (G) changed significantly over the survival time (F = 5.9; df = 4, 24; p < 0.005). Turkey’s HSD post hoc t test showed that amplitudes in week 5 after graft were significantly higher than those in all other weeks (week 5 vs weeks 1 and 3, p < 0.01; week 5 vs weeks 2 and 4, p < 0.05). Error bars represent SEM.

Figure 4.
Results

Demyelination in contusion SCI

We first determined whether demyelinated axons would survive in the contused VL, where tcMMEP responses are conducted (Loy et al., 2002; Cao et al., 2005). In the normal spinal cord, axons in the VL appeared as round or slightly oval profiles with varying diameter. A darkly stained rim of compact myelin, whose thickness was proportional to the axon diameter, surrounded each axon (Fig. 1A,B). One week after injury, the most evident pathological change in the VL myelin was the presence of vacuoles (Fig. 1C,D, arrows) as the myelin layers separated. Macrophages and/or microglia observed close to the degenerating myelin contained phagocytosed material (Fig. 1D, arrowhead). Importantly, many demyelinated axons appeared morphologically normal (Fig. 1C,D, asterisks), although some showed axoplasmic organelle condensation, suggesting axonal degeneration (Fig. 1C, curved arrowhead). One month after injury, the number of demyelinated axons in the VL decreased compared with 1 week after injury. However, many healthy-appearing demyelinated axons were still evident (Fig. 1E,F, arrows), and they were in close apposition to astrocytic processes (Fig. 1E,F, arrowheads). Some axons were wrapped by thin myelin relative to their axonal diameters (Fig. 1E, open arrowhead), suggesting remyelination. Thus, ultrastructural analysis demonstrated that demyelination occurred in the VL after contusive SCI, and some of these demyelinated axons could survive for at least 1 month after injury.

Differentiation of and myelination by GRPs in vitro

Proliferating, passage 2 GRPs stained with antibodies directed against A2B5 and the GRP-specific chondroitin sulfate proteoglycan NG2 (Fig. 2A,B); 97% coexpressed both markers (Fig. 2C), ~6% of these cells expressed the bipotential oligodendrocyte/astrocyte precursor marker O4 (data not shown), and none expressed the more mature oligodendrocyte markers O1, RIP (receptor-interacting protein), or MBP, or the astrocyte-specific proteins GFAP or vimentin (data not shown). After 8 d in culture, in the absence of FGF2 and PDGFaa, but in the presence of T3, 80% of the cells matured to express MBP (Fig. 2D). When cultured in medium containing 10% FBS, >50% of the cells developed into GFAP+ astrocytes (data not shown). No neurons were observed under any differentiating conditions. Thus, the purified GRPs were bipotential and could differentiate into both oligodendrocytes and astrocytes.

To test the capacity of GRPs to myelinate axons, we cocultured GRPs with purified embryonic DRG neurons (Plant et al., 2002). After coculture for 4 weeks, numerous GRPs were observed to myelinate the DRG axons as assessed by MBP expression (Fig. 2E). Ultrastructural analysis of the myelin formed by these GRPs showed it to be CNS myelin (Fig. 2F). GRP-derived astrocyte processes were in close apposition to these myelinated axons (Fig. 2F, arrow). These in vitro studies demonstrated that the isolated GRPs differentiated into mature astrocytes and oligodendrocytes, the latter forming CNS-type myelin.

To study the effect of NT3 and BDNF on GRP maturation, we differentiated the GRPs in vitro in the absence of FGF2 and PDGFaa but in the presence of NT3, BDNF, or both. After differentiation for 8 d, the percentage of galactocerebroside-positive cells was significantly increased in the presence of NT3, BDNF, or both (Fig. 2G), indicating that the differentiation into oligodendrocytes was enhanced by NT3 and BDNF. NT3 had been shown previously to be important for the survival and proliferation of oligodendrocytes in vivo (Barres et al., 1994). We then determined whether NT3 and/or BDNF affected the number of surviving and proliferating GRPs during their differentiation. After differentiation for 8 d, the total number of cells was significantly increased in the presence of either NT3, BDNF, or both (Fig. 2H). To determine whether the increase was attributable to enhanced proliferation, dividing cells were labeled with BrdU for 24 h on day 8 after differentiation in the presence or absence of neurotrophins. The number of BrdU+ cells was significantly increased in the presence of NT3, BDNF, or both (Fig. 2H). Thus, NT3 and BDNF enhanced the survival, proliferation, and differentiation of GRPs into oligodendrocytes in vitro.

Electrophysiological and locomotor functional recovery after engraftment of GRPs

We next asked whether the combination of GRP transplantation and neurotrophin delivery could restore electrophysiological conduction after demyelination and, more importantly, enhance the recovery of hindlimb locomotor function after SCI. Before engraftment, GRPs were infected with a retrovirus encoding EGFP or D15A–EGFP. Additional groups of injured animals were grafted with D15A-infected NIH3T3 cells or injected with 4 × 10 pfu of an adenovirus encoding D15A or DMEM. We then determined whether the increase was attributable to enhanced proliferation, dividing cells were labeled with BrdU for 24 h on day 8 after differentiation in the presence or absence of neurotrophins. The number of BrdU+ cells was significantly increased in the presence of NT3, BDNF, or both (Fig. 2H). Thus, NT3 and BDNF enhanced the survival, proliferation, and differentiation of GRPs into oligodendrocytes in vitro.
NT3 at concentrations of 0.25, 0.5, 1, 2, 4, 6, and 10 ng/ml. The percentage of differentiated trkC–PC12 cells as 26.5% in the undiluted D15A–GRPs conditioned medium, which was equal to the bioactivity of NT3 at 6.43 ± 0.48 ng/ml determined by the recombinant human NT3 standard curve of trkC–PC12 cells. Our ELISA assay showed that D15A–GRP conditioned medium had 6.54 ± 0.05 ng/ml (mean ± SD; n = 3) NT3, which confirmed the secretion of NT3 into D15A–GRP conditioned medium. No NT3 was detected in DMEM or EGFP–GRP-conditioned medium by either bioactivity or ELISA assay. These results showed that D15A–GRPs secreted biologically active D15A into the culture medium. We also used an ELISA assay to examine whether D15A–GRPs could stably secret D15A into the injured spinal cord. As shown in Figure 3D, expression of NT3 in animals that received grafts of D15A–GRPs (53.7 ± 5.8 pg/mg) was significantly higher compared with ones that received grafts of EGFP–GRPs (11.6 ± 3.6 pg/mg) or DMEM (8.8 ± 1.2 pg/mg) at 1 week after transplantation (2 weeks after injury). These results demonstrate that D15A–GRPs secreted D15A into the injured spinal cord.

Because tcMMEP responses provide a reproducible, noninvasive, and objective assessment of axonal conduction and functional integrity of descending VLF motor pathways in both normal and injured spinal cord (Fehlings et al., 1987; Loy et al., 2002), they were used to monitor the electrophysiological function of VLF axons after grafting. In the normal adult rat, the latency of the monosynaptic tcMMEP response was 6.2 ± 0.3 ms and the amplitudes of this normal response ranged from 9.0 to 21.0 mV, with an average of 16.0 ± 3.8 mV (Loy et al., 2002; Cao et al., 2005) (Fig. 4A, A'). After a 150 kdyn contusive SCI, tcMMEP responses disappeared (Fig. 4B, B'). One week after transplantation of D15A–GRPs, tcMMEP responses were partially restored in 8 of 12 rats; however, the latencies (8.41 ± 0.46 ms) were significantly longer (p < 0.001), and the amplitudes (0.7 ± 0.6 mV) were significantly smaller (p < 0.001) than in normal animals (Fig. 4C, F, G). Note the 10-fold difference in gain between the recordings in Figure 4, A and A'. The latencies of tcMMEP responses in D15A–GRP transplanted animals continued to become shorter over time (Fig. 4D–F). The latencies at 2 and 3 weeks after graft were 8.14 ± 0.48 and 7.81 ± 0.22 ms, respectively. The latencies at week 3 after graft were significantly shorter compared with week 1 after graft (p < 0.01) but were still significantly longer than the latencies in normal animals. At weeks 4 and 5 after grafting, the latencies dropped to 7.52 ± 0.56 and 7.41 ± 0.36 ms, which were significantly shorter than at weeks 1 and 2 after grafting (all p < 0.05) but were still significantly longer than in normal animals (p < 0.01). Although there was a strong trend for mean amplitudes of the returned tcMMEP to increase from week 1 to week 4 after grafting (0.67 ± 0.59 mV in week 1, 1.13 ± 0.71 mV in week 2, 0.95 ± 0.68 mV in
week 3, and 1.33 ± 1.02 mV in week 4, respectively), they were not significantly different from each other (Fig. 4G). However, the amplitudes in week 5 postgraft (2.57 ± 1.56 mV) were significantly higher than in weeks 1–4 after graft (p < 0.05) (Fig. 4G). In the animals that received EGFP–GRPs (Fig. 4C–E′), D15A–NIH3T3 cells, D15A adenosin, or DMEM injection (data not shown), the return of tcMMEP responses was never observed. The D15A adenosin was used as a control for D15A expression in the absence of an exogenous cellular graft. Thus, the conduction of tcMMEP responses through the initially demyelinated axons in the VLF was partially restored only by the transplantation of D15A–GRPs.

We further asked whether the electrophysiological restoration of tcMMEP responses correlated with improvement of hindlimb locomotor function. Consistent with our (Cao et al., 2005) and other (Scheff et al., 2003) published reports, in 150 kdyt-injured animals, BBB scores were improved over time in each group (Fig. 5). BBB scores were significantly higher at week 5 and 6 (p < 0.01) than at week 1 after injury in all four groups. More importantly, BBB scores in D15A–GRP-grafted animals were 15.6 and 15.2 at 4 and 5 weeks after graft, respectively, which were significantly higher than those for the D15A–NIH3T3 cell-grafted group (12.2 and 12.6), or the DMEM-injected animals (13.1 and 12.7), respectively (all p < 0.05) (Fig. 5). Although there was a trend for greater improvement of BBK scores in EGFP–GRP-grafted animals at 4 and 5 weeks after transplantation, respectively, this improvement over D15A–NIH3T3 cell-grafted and DMEM-injected animals did not reach statistical significance. BBB scores among the four groups were not significantly different from week 1 to week 4 after injury. Therefore, our behavioral results were consistent with the electrophysiological data; significant enhancement of hindlimb function was only observed in the D15A–GRP-grafted animals starting at 5 weeks after injury (4 weeks after transplantation).

**Differentiation of engrafted GRPs in the injured spinal cord**

We next addressed whether the functional recovery in the D15A–GRP-grafted animals was potentially mediated by remyelination. Six weeks after grafting, robust survival of transplanted GRPs was observed in D15A–GRP-grafted animals (Fig. 6A–C) and also in EGFP–GRP-grafted animals (data not shown) with cells distributed around the cavities throughout the injury site. Many more grafted cells were localized in the ventral than dorsal spinal cord, likely because of the increased spared white matter in the ventral spinal cord (Fig. 6A) and the VLF graft location. The grafted cells migrated widely, up to 4 mm rostrocaudally in the white matter. In longitudinal sections, D15A–GRP processes could be traced by EGFP immunohistochemistry several millimeters along the axons (Fig. 6C). Many engrafted D15A–GRPs showed typical oligodendrocyte morphology with multiple processes closely apposing NFM \(^+\) axons (Fig. 6D).

To determine the maturation state of the grafted D15A–GRPs, cells were double stained with EGFP and mature oligodendrocyte markers MBP and APC. As shown in Figure 6D, grafted GRPs express MBP \(^+\) myelin with the long EGFP \(^+\)/MBP \(^+\) processes apposing the longitudinal axons in the white matter, suggesting that some grafted D15A–GRP formed mature myelin. Many grafted GRPs expressed APC (Fig. 6E). Although APC has been reported previously to label Schwann cells in addition to oligodendrocytes after SCI (McTigue et al., 2001), we did not observe colabeling with APC and the Schwann cell marker S-100β (data not shown). The light microscopic results were confirmed by EGFP-immunohistochemical EM that showed that the grafted D15A–GRPs were in the areas of new central myelin sheaths in the VLF (Fig. 6G,H). To examine whether D15A expression enhanced GRP differentiation in vivo, the percentages of EGFP \(^+\) cells expressing APC were counted in the D15A–GRP and EGFP–GRP-grafted groups. The percentage of APC \(^+\)-expressed GRPs was more than twofold greater in the D15A–GRP-grafted animals (Fig. 7A). These data were consistent with the in vitro results and suggested that BDNF and NT3 enhanced the survival, differentiation, and myelination of grafted GRP-derived oligodendrocytes in vivo. Many grafted GRPs still expressed NG2, an oligodendrocyte precursor marker, suggesting that many of the grafted cells remain undifferentiated (data not shown). Some of the grafted EGFP \(^+\) GRPs became GFAP \(^+\) astrocytes (Fig. 5F) with morphologies distinct from the oligodendrocytes just described above. These cells usually had small cell bodies with fewer processes.

We finally asked whether the functional recovery observed in the D15A–GRP-grafted animals might also be attributable, in part, to increased sparing of white matter as a result of the presence of D15A. The total volume of spared white matter in 4 mm of the spinal cord rostral and caudal to the injury epicenter was 8.92 ± 1.05, 7.96 ± 0.9, 8.06 ± 0.66, and 7.38 ± 0.29 mm\(^3\) in animals receiving D15A–GRPs, EGFP–GRPs, D15A–NIH3T3 cells, or DMEM (mean ± SEM; ANOVA; F = 3.099; df = 3; p = 0.064).

**Discussion**

In this study, we showed that many demyelinated but otherwise intact axons exist in the VLF after contusive SCI and that the demyelinated axons survived for at least 1 month after the injury. These results are consistent with previous histological studies showing that there is chronic demyelination of axons after traumatic SCI in both humans (Bunge et al., 1993; Guest et al., 2005) and experimental animals (Blight, 1983, 1993; Cao et al., 2005; Totoiu and Keirset, 2005). Electrophysiological analyses showed that the normal tcMMEP responses are absent after SCI, and such conduction deficits persist chronically, also consistent with findings in the injured human (Alexeeva et al., 1997, 1998) and rodent (Blight, 1993) spinal cord. Mitotically competent OPCs are harbored in the adult spinal cord (Horner et al., 2000) and proliferate after SCI (Ishii et al., 2001; McTigue et al., 2001; Yamamoto et al., 2001). These cells have the potential to differentiate into oligodendrocytes in vivo (Engel and Wolswijk, 1996).
and in vivo (Gensert and Goldman, 1997). However, the persisting conduction deficits after traumatic SCI indicate that endogenous oligodendrocytes and/or OPCs fail to fully remyelinate the demyelinated axons and restore electrophysiological conduction. Because massive oligodendrocyte death attributable to apoptosis occurs acutely after SCI, with some oligodendrocyte death observed a few segments away from the injury epicenter (Crowe et al., 1997; Liu et al., 1997; Springer et al., 1999), it is likely that endogenous OPCs are unable to completely restore lost myelin in the injured spinal cord. Endogenous remyelination in the chemically demyelinated adult rat VLF is predominantly by invading Schwann cells, but is incapable of chronically restoring tcMMEP responses (Talbott et al., 2005). Increasing the number of cells with the ability to differentiate into oligodendrocytes by transplantation may be one very important means for replacing lost myelin. However, transplantation of EGFP–GRPs did not restore tcMMEP responses in the contused spinal cord, suggesting that increasing the number of GRPs alone is not enough to restore the function of demyelinated axons and that additional factors are required.

We observed that many grafted GRPs either remained undifferentiated (NGF−) or differentiated into astrocytes, with only a small number of cells (~12%) differentiating into APC+ oligodendrocytes. These data suggest that full oligodendrocyte differentiation of the grafted GRPs is restricted and is consistent with previous studies that showed that oligodendrocyte differentiation of exogenous GRPs is restricted after traumatic SCI (Han et al., 2004; Hill et al., 2004). We showed recently that, after demyelination of the VLF by injection of the glial toxic ethidium bromide, which kills both oligodendrocytes and astrocytes, the number of endogenous OPCs increases, and these proliferating OPCs express two transcription factors important in oligodendrocyte development, Nkx2.2 and Olig2 (Talbott et al., 2005). However, mature oligodendrocyte differentiation of these proliferating endogenous OPCs is restricted. Previous studies showed that the differentiation of multipotent neural stem cells engrafted into the normal or contusive spinal cord is restricted to an astrocytic lineage, although these neural stem cells have the potential to differentiate into oligodendrocytes, neurons, and astrocytes in vitro (Chow et al., 2000; Shihabuddin et al., 2000; Cao et al., 2001; Hill et al., 2004; Hofstetter et al., 2005). Together, these studies suggest that the microenvironment in the injured spinal cord may be inhibitory for full oligodendrocyte differentiation from endogenous and/or grafted GRPs. The factor(s) responsible for this inhibition remain to be determined. Induction of NSCs or GRPs along the oligodendrocyte lineage before transplantation may be an effective approach for enhancing oligodendrocyte differentiation in the injured spinal cord (Liu et al., 2000; Körstendt et al., 2005). Expression of growth factors that promote oligodendrocyte differentiation and maturation may also increase remyelination in the injured spinal cord (McTigue et al., 1998; Bambakidis and Miller, 2004).

It is possible that a lack of trophic factors necessary for oligodendrocyte maturation in the injured spinal cord may result in the failure of endogenous OPCs and grafted GRPs to fully differentiate into myelinating oligodendrocytes after SCI. Transplantation of fibroblasts, genetically modified to secrete NT3, into the contused spinal cord of rats increases myelination by endogenous OPCs (McTigue et al., 1998). However, present data showed that administration of D15A alone, by either transplantation of D15A–expressing fibroblasts or the injection of an adenovirus encoding D15A into the injured spinal cord, failed to result in the recovery of tcMMEP responses or enhanced hindlimb locomotor function. This result suggests that increasing remyelination by endogenous OPCs by administration of neurotrophins alone is insufficient to remyelinate enough demyelinated axons to facilitate electrophysiological and behavioral recovery. This may reflect the fact that the number of endogenous OPCs in the adult spinal cord is limited (Sim et al., 2002). As discussed above, transplantation of EGFP–GRPs alone is also not enough to facilitate functional recovery after SCI.

In these experiments, only the transplantation of D15A–GRPs into the injured spinal cord restored tcMMEP responses and hindlimb locomotor function. Histological analysis showed that the grafted D15A–GRPs differentiated into MBP+ oligodendrocytes in the injured spinal cord, and EM analysis confirmed that the grafted D15A–GRPs formed central myelin around the axons. Quantitation of white matter sparing at the lesion epicenter, however, showed no difference between the D15A–GRP-grafted group and the other groups, including EGFP–GRP and D15A–NIH3T3 grafts, indicating that with D15A–GRP grafts, functional recovery was likely attributable to the enhanced remyelination by grafted GRPs of spared or regenerated axons. In vivo experiments showed that NT3 and BDNF increased the proliferation and survival of differentiating GRPs supporting similar mechanism(s) in vivo. Consistent with this interpretation, delivery of neutralizing NT3 antibodies to the developing optic nerve significantly reduced the number of oligodendrocytes (Barres et al., 1994). NT3 also enhanced the survival and proliferation of oligodendrocyte lineage cells in culture (Barres and Raff, 1994; Yan and Wood, 2000). Oligodendrocytic differentiation of the grafted D15A–GRPs was twofold greater than that seen with EGFP–GRPs, consistent with in vivo oligodendrocyte differentiation data. Myelination by GRPs of cultured neurons was significantly increased by NT3 (Yan and Wood, 2000; Rubio et al., 2004). D15A most likely promotes remyelination by directly enhancing the differentiation and myelination capacity of endogenous and grafted GRPs.

It is also possible the D15A-expressing GRPs enhance the regeneration of some axons after SCI because BDNF and NT3 can facilitate regeneration of numerous supraspinal pathways involved in locomotion (Onifer et al., 2000). Astrocytic differentiation of the engrafted GRPs could provide a permissive substrate for regeneration (Privat, 2003). Moreover, GRP grafts into the contused spinal cord can reduce the expression of proteoglycans inhibitory for axonal regeneration (Hill et al., 2004). However, any regenerating axons would require remyelination by the engrafted GRPs. The possible contribution of regenerating fibers to the functional recovery observed in the present study remains to be determined. Although the early recovery of tcMMEP responses would be consistent with remyelination rather than regeneration, the significant locomotion recovery at weeks 5 and 6 after injury may also reflect regeneration.

These data support an emerging concept in designing strategies to repair the injured CNS; combination strategies will be necessary for optimal functional return (Lu et al., 2004; Pearse et al., 2004; Sivasankaran et al., 2004). Determining which combination of therapies to use is likely to be both lesion dependent and empirically delineated. Functionally useful remyelination by engrafted stem cells will require at least partial lineage restriction of the cells, appropriate trophic support, and inhibition of the signal cascades that preclude cell-specific differentiation.

References


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