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Survival, Integration, and Axon Growth Support of Glia Transplanted Into the Chronically Contused Spinal Cord

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Due to an ever-growing population of individuals with chronic spinal cord injury, there is a need for experimental models to translate efficacious regenerative and reparative acute therapies to chronic injury application. The present study assessed the ability of fluid grafts of either Schwann cells (SCs) or olfactory ensheathing glia (OEG) to facilitate the growth of supraspinal and afferent axons and promote restitution of hind limb function after transplantation into a 2-month-old, moderate, thoracic (T8) contusion in the rat. The use of cultured glial cells, transduced with lentiviral vectors encoding enhanced green fluorescent protein (EGFP), permitted long-term tracking of the cells following spinal cord transplantation to examine their survival, migration, and axonal association. At 3 months following grafting of 2 million SCs or OEG in 6 µl of DMEM/F12 medium into the injury site, stereological quantification of the three-dimensional reconstructed spinal cords revealed that an average of 17.1 ± 6.8% of the SCs and 2.3 ± 1.4% of the OEG survived from the number transplanted. In the OEG grafted spinal cord, a limited number of glia were unable to prevent central cavitation and were found in patches around the cavity rim. The transplanted SCs, however, formed a substantive graft within the injury site capable of supporting the ingrowth of numerous, densely packed neurofilament-positive axons. The SC grafts were able to support growth of both ascending calcitonin gene-related peptide (CGRP)-positive and supraspinal serotonergic axons and, although no biotinylated dextran amine (BDA)-traced corticospinal axons were present within the center of the grafts, the SC transplants significantly increased corticospinal axon numbers immediately rostral to the injury–graft site compared with injury-only controls. Moreover, SC grafted animals demonstrated modest, though significant, improvements in open field locomotion and exhibited less foot position errors (base of support and foot rotation). Whereas these results demonstrate that SC grafts survive, support axon growth, and can improve functional outcome after chronic contusive spinal cord injury, further development of OEG grafting procedures in this model and putative combination strategies with SC grafts need to be further explored to produce substantial improvements in axon growth and function.

Key words: Schwann cells; Olfactory ensheathing glia; Regeneration; Sensory; Spinal cord injury; Chronic; Contusion

INTRODUCTION

Injury to the spinal cord results in a loss of cellular and axonal integrity, producing deficits in sensory and motor function that are not amenable to restoration in the clinical setting. The absence of a therapy to substantially ameliorate the detrimental effects of acute spinal cord injury (SCI) has led to more than 200,000 chronically paralyzed individuals in the US alone (28). Whereas recent developments in the spinal cord injury (SCI) research field have led to the investigation of a number of molecular and cellular repair strategies in experimental models of chronic SCI (2,16,18,19,31,39, 40,47–50,84,95,103,104), testing of therapeutic interventions at a chronic stage is still substantially understudied compared with acute and subacute SCI (48).

The implementation of a promising strategy for chronic SCI repair faces a number of physiological obstacles, such as a well-developed growth-inhibitory glial scar (25,96,99), a reduced intrinsic ability of neurons to regrow at longer periods postinjury due to downregulation of growth-associated molecules (6,101,102), and the potential of causing neuronal cell death by reinjuring axons through an invasive therapy (46,85).

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Of the many treatment strategies employed for SCI repair, cellular grafting to bridge the injury site where significant tissue has been lost provides a substrate for axonal regrowth. This has been a foundation for many of the successful acute and subacute therapies (11,27, 67,82). Some of the more frequently used cellular interventions have employed fibroblasts (40,49,59,95,104), embryonic stem cells (60,63,100), bone marrow stromal cells (13,45,61,70), fetal tissue (3), peripheral nerve grafts (18,19,82), macrophages (34,77,94), Schwann cells (10,11,72,73,97), and olfactory ensheathing glia (9,32,78,79,89), often in combination with molecular or pharmacological strategies aimed at increasing neurotrophin levels (40,59,87,105,108), masking or degrading inhibitory molecules (12,29), or activating or inhibiting intracellular regulators of growth (61,69,73). Cells presently eligible for transplantation therapy in the clinic, however, are limited due to a lack of necessary qualities required for autologous harvesting and use. Those cells that can be harvested from a donor must also be able to divide to produce significant numbers in vitro and be easily purified of other contaminating cell types using methods and materials that are readily approvable by the Federal Drug Administration (88).

Schwann cells (SCs), the axonal ensheathing and myelinating glia of the peripheral nervous system (PNS), which are critical for the regenerative potential of the injured PNS, are excellent candidates for transplantation. SCs produce a trophic-rich environment by secreting multiple growth factors and extracellular matrix molecules (76) that support the elongation of axons (11). Furthermore, SCs are capable of integration into host central nervous system (CNS) tissue, support the growth of several types of CNS axons in a variety of injury models (10,11,72,73,97), and myelinate fibers in cord grafts (23,97,109). Importantly, SCs could be obtained from a biopsy of peripheral nerve from a spinal cord-injured individual, amplified to significant numbers through tissue culturing procedures, and then autologously transplanted into the injured spinal cord without the potential of immune rejection. A number of studies have shown that combination strategies employing SCs are capable of promoting supraspinal axon growth and functional improvements in acute SCI models (10,11,73).

Olfactory ensheathing glia (OEG) are also promising glial cells for use in transplantation to repair the injured spinal cord. Native to the olfactory mucosa and olfactory bulb, OEG are proposed to be important in the guidance of olfactory nerves extending into the CNS from continually renewing sensory neurons in the mucosa. OEG exhibit numerous characteristics that make them an ideal cell for transplantation into the injured spinal cord. They are able to: surround growing axons, preventing their contact with inhibitory molecules and CNS glia; express growth-promoting neurotrophic factors, adhesion molecules, and extracellular matrix proteins (8,89); reportedly migrate significantly after transplantation from the injured spinal cord milieu into the contiguous host cord (79,81); and myelinate CNS axons (33,51,58). Furthermore, grafting of OEG into the injured spinal cord has been demonstrated to facilitate long-tract regeneration and behavioral recovery in a number of SCI paradigms (9,29,56,78,79,89). Recent advances in tissue culturing procedures have shown the potential of OEG to be harvested from either the olfactory bulb (89) or nasal mucosa for autologous transplantation into a spinal cord-injured individual.

To date, however, neither SCs nor OEG have been investigated for their potential to repair the chronically contused spinal cord, to examine whether they are able to survive and integrate following transplantation, support chronically injured axon growth, or improve functional outcome. The current study was designed to address these basic questions. We employed enhanced green fluorescent protein (EGFP)-encoding lentiviral vector infection of SCs and OEG to allow long-term tracking (3 months) of the cells following transplantation into the moderately contused spinal cord at 2 months postinjury, a time in which open field locomotion has reached a plateau according to the BBB score and when the development of a significant spinal cord fluid-filled cyst has occurred. This time point has also been chosen in other studies of chronic SCI therapies (31,40). We report that, although grafted SCs and OEG were identified at 3 months posttransplantation, OEG survival was very poor. SCs, however, were found in modest numbers, supported growth of supraspinal and ascending axons, and improved functional outcome after chronic injury.

MATERIALS AND METHODS

Cultures

Schwann Cells. SCs were obtained from sciatic nerves of adult female Fischer rats (Harlan Co., Indianapolis, IN) as described previously (66). Briefly, the nerves were minced and placed in culture dishes in DMEM/10% heat-inactivated fetal bovine serum (HI-FBS) without mitogens. Two weeks later, after outward migration of fibroblasts, the explants were transferred to new dishes where they were enzymatically dissociated and then replated in DMEM/10% FBS supplemented with three mitogens: bovine pituitary extract (2 mg/ml, Invitrogen Corporation, Carlsbad, CA), forskolin (0.8 μg/ml), and heregulin (2.5 nM, Genentech, San Francisco, CA) (62). Cells were grown to confluency and passaged to new dishes three times. Their purity for grafting, tested by a method described previously (97), was found to be 95–98% with minimal fibroblast contamination (2–5%).

Olfactory Ensheathing Glia. Highly purified cultures of OEG were prepared from the nerve fiber layer of the olfactory bulb of adult female Fischer rats (Harlan Co.) using a procedure modified from one described previously (80). Care was taken to minimize the inclusion of nonnerve fiber layer bulb tissue, and the pia was removed. OEG were dissociated and, unlike the Ramón-Cueto and colleagues' (80) method, were exposed for 5 days to forskolin (0.8 μg/ml) and pituitary extract (2 mg/ml) in DMEM/F12/10% FBS before purification by p75 immunopanning as described by Takami and colleagues (97). A second period in mitogens preceded harvesting at confluency for transplantation. Checking the purity was similar to SCs except that p75 rather than S100 was used with Hoechst nuclear staining. Purity was found to be 94–98%. Other cells present in these cultures appeared to be fibroblasts and SCs.

Lentiviral Vectors

Lentiviral Vector Preparation. The gene encoding EGFP was subcloned into a lentiviral vector plasmid. This plasmid contained the cytomegalovirus (CMV) promoter to drive transgene expression and the Woodchuck posttranscriptional regulatory element (WPRE) to enhance mRNA transport. The plasmid was purified using an Endofree Maxiprep Kit (Qiagen, Valencia, CA) and dissolved at 1 μg/μl in a buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (TE). The transfection and viral harvesting process took place over a 5-day period. To maximize confluency, 293T cells were cultured in T75 culture flasks. Each flask was passaged to four flasks and grown to maximum confluency. These flasks were then passaged to four 10-cm dishes each in Iscoves modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% HI-FBS and gentamicin (basal iscove medium; IMDM/10%). This resulted in approximately 5×10^6 cells per dish. Day 2 of the preparation incorporated transfection. Two hours prior to transfection IMDM/10% was refreshed. A calcium phosphate-mediated transfection was performed, introducing the VSV-G, p874 packaging, and lentiviral vector plasmids. The 293T cells were then incubated overnight at 37°C, 5% CO_2. On day 3, the medium was removed and IMDM with 2% HI-FBS was added. On day 4, the first harvest of virus (24 h) was performed. The medium was removed from the 10-cm dish and collected in two 50-ml tubes. On day 5, 8 ml of IMDM/2% was replaced for a second harvest. The medium was spun down at 1500 rpm for 5 min and the supernatant filtered through a 0.2-μm filter to remove cell debris. Virus was then concentrated by ultracentrifugation at $20{,}000 \times g$ and resuspended in phosphate-buffered saline (PBS). After this, the vectors were titered for transducing units on 293T cells or using an enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Promega, Madison, WI) for quantifying p24 core protein concentrations.

Lentiviral Vector Transduction of Glia to Express EGFP. Virus concentrated in PBS was used to infect SCs and OEG. Equal volumes of virus containing medium and Dulbecco's modified Eagle medium with 10% HI-FBS (DMEM/10%), 2 μM/ml forskolin, 20 mg/ml pituitary extract, and 2.5 nm/ml heregulin (triple mitogens) were added to each culture of SCs to yield a final volume of 8 ml. In the case of OEG, the cultures were infected by the addition of a predetermined volume of concentrated virus. A multiplicity of infection (MOI) of 50 was used in all cultures to ensure high transduction efficiency.

Animals

Adult female Fischer rats (Harlan Co., $n = 51$; 180–200 g) were housed according to NIH and the *Guide for the Care and Use of Animals*. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. Prior to surgery, rats were anesthetized (45 mg/kg ketamine, 5 mg/kg xylazine) by IP injection. An adequate level of anesthesia was determined by monitoring the corneal reflex and withdrawal to painful stimuli for the hind limbs. The back region was shaved and aseptically prepared with chlorhexidine (Phoenix Pharmaceutical Inc., St Joseph, MO). Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA) was applied to the eyes to prevent drying. During surgery, the rats were kept on a homeothermic blanket system (Harvard Apparatus Ltd., Kent, England) to maintain the body temperature at 37 ± 0.5°C as measured by rectal probe.

Moderate Thoracic Contusion Injury. Contusion injury was induced by the weight drop device developed at New York University (42). Following anesthesia, a vertical incision was made along the thoracic vertebra and the superficial muscle and skin retracted. A laminectomy performed at thoracic vertebra T8 exposed the dorsal surface of the spinal cord underneath (T9) without disrupting the dura mater. Stabilization clamps were placed around the vertebrae at T6 and T12 to support the column during impact. The exposed spinal cord was moderately injured by dropping a 10.0-g rod from a height of 25.0 mm. The contusion impact height, velocity, and compression were monitored. Animals ($n = 5$) were excluded immediately when height or velocity errors exceeded 6% or if the compression distance was not within the range of 1.75–2.25 mm. After injury, the muscles were sutured in layers and the skin closed with metal wound clips. The rats were allowed to recover in a warmed cage with water and food easily accessible.

Gentamicin (5 mg/kg, intramuscular; Abbott Laboratories, North Chicago, IL) was administered immediately postsurgery and then daily for 7 days. The analgesic, Buprenex (0.01 mg/kg of 0.3 mg/ml, subcutaneous; Reckitt Benckiser, Richmond, VA) was delivered postsurgery and daily for 2 days. The rats were maintained for 8 weeks after injury with weekly behavioral testing. At 8 weeks, animals were randomly placed into three experimental groups for transplantation studies: (i) injured, no transplantation ($n = 12$), (ii) injured, SC transplantation ($n = 12$), and (iii) injured, OEG transplantation ($n = 12$). Ten uninjured animals served as controls. From each group, six rats were used for anterograde tracing with biotinylated dextran amine (BDA) and immunochemical detection of calcitonin gene-related protein (CGRP)-positive fibers, and six for anterograde tracing from the sciatic nerve with cholera toxin beta subunit (CTB) and immunochemical detection of 5-HT-positive fibers.

Transplantation. Prior to implantation, EGFP transduced SCs and OEG were harvested from culture via trypsinization, then centrifuged, resuspended, and counted. SCs and OEG were then resuspended as aliquots of 2×10^6 cells in DMEM/F12 medium and were kept on ice for no more than 20 min prior to surgery. Eight weeks after injury, rats were reanesthetized with 2% halothane and the injury site was exposed. Either (i) 2×10^6 SCs in 6 μl of DMEM/F12 medium or (ii) 2×10^6 OEG in 6 μl of DMEM/F12 medium were injected into the epicenter of the contused area. Cells were injected at a depth of 1 mm using a 10-μl Hamilton syringe with a pulled, beveled glass pipette tip (200 μm diameter), backfilled with mineral oil, and held in a micromanipulator with a microinjector capable of delivery of a specific volume over time (World Precision Instruments Ltd., Sarasota, FL). Injections were administered over a 3-min period, and the injection pipette was kept in place for an additional 3-min to minimize leakage upon withdrawal. After the injections, the muscle layers and the skin were closed separately and animals received postoperative care as described above.

Anterograde Axonal and Retrograde Neuronal Tracing. For anterograde tracing, at 16 weeks postinjury (8 weeks posttransplantation) rats were reanesthetized with 2% halothane and either (i) 10% biotinylated dextran amine (BDA; Molecular Probes, Eugene, OR) was injected stereotaxically and bilaterally into the hind limb area of the motor cortex (2×0.5 μl) or (ii) 1.5% cholera toxin B subunit (CTB; List Biological Laboratories Inc., Campbell, CA) was injected into the right sciatic nerve (1×1.5 μl). Brains from anterogradely traced animals were sectioned and examined to ensure accurate tracer injection into the hind limb area of the motor cortex in each animal.

Histology

At 19 weeks after injury (11 weeks posttransplantation), rats were deeply anesthetized (70 mg/kg ketamine, 10 mg/kg xylazine), 0.1 ml heparin was injected into the spleen, and the rats were transcardially perfused first with 200 ml of physiological saline and then with 500 ml of ice-cold phosphate-buffered 4% paraformaldehyde (0.1 M, pH 7.4). The brain and spinal cord were removed and postfixed overnight in the same fixative at 4°C, and then transferred to phosphate-buffered 30% sucrose for 48 h at 4°C for tissue cryoprotection. The T7–T11 thoracic spinal cords (20-mm-long piece), which contained the lesion and graft site, were dissected and embedded in phosphate-buffered 10% gelatin solution (Difco Laboratories, Detroit, MI) to prevent further damage during histological procedures. Initially the cords were placed in liquid gelatin for 4 h at 37°C, followed by their embedding in the same gelatin solution for 1 h at 4°C to solidify the gelatin. The embedded spinal cord blocks were then fixed overnight in 4% paraformaldehyde in phosphate buffer (0.1 M, ph 7.4) and then left for 48 h in phosphate-buffered 30% sucrose. Gelatin-embedded cord segments were cut into 30-μm-thick sagittal sections using a freezing microtome. Every sixth section was collected (for 6 series) in PBS (0.1 M, pH 7.4) and stored at 4°C until further processing.

Immunochemistry. Every other sixth sagittal section was immunochemically stained for fluorescent microscopy. Sections were initially transferred into 1× Tris-buffered saline (TBS), washed, and then blocked with 5% heat-inactivated normal goat serum at room temperature for 1 h. Following removal of the blocking solution, sections were incubated overnight at room temperature in the primary antibodies, a mix of the monoclonal antibody anti-glial fibrillary acidic protein (GFAP, 1:1000; Sternberger Monoclonals Inc., Lutherville, MA) and one of the following polyclonal antibodies: anti-serotonin (5-HT, 1:5000; Incstar Corp., Hudson, WI), anti-CGRP (1:1000; Peninsula Laboratories Inc., San Carlos, CA), or goat anti-CTB (1:20,000; List Biological Laboratories Inc.). After the primary antibodies, sections were washed three times with 1× TBS and then incubated for 1 h at room temperature with the fluorescent secondary antibodies, a mix of Alexa 660-conjugated donkey anti-mouse (1:200; Molecular Probes) and Alexa 594-conjugated goat anti-rabbit or rabbit anti-goat (1:200; Molecular Probes) in 1× TBS buffer containing 1% heat-inactivated normal goat serum. Following three washes with 1× TBS, sections were mounted onto Snowcoat X-tra slides (Surgipath, Winnipeg, Manitoba)

and coverslipped with Vectashield mounting medium (Vector Laboratories, Inc., Birmingham, CA) containing the nuclear dye Hoechst 33342 and kept at 4°C.

For fluorescent visualization of BDA-traced corticospinal tract axons, every other sixth section was initially transferred into 1× TBS, washed, and then blocked with 5% heat-inactivated normal goat serum at room temperature for 1 h. Following removal of blocking, sections were incubated for 1 h with horseradish peroxidase steptavidin conjugated to rhodamine (Vector Laboratories Inc.). Following three washes with 1× TBS, sections were mounted onto Snowcoat X-tra slides and coverslipped with Vectashield mounting medium (Vector Laboratories Inc.) containing the nuclear dye Hoechst 33342 and kept at 4°C.

Stereological Quantification of EGFP Cell Survival. In one series of mounted sagittal sections immunostained with GFAP, EGFP-positive cells were counted using an unbiased method employing computer-assisted microscopy and StereoInvestigator software (MicroBright Field) throughout the 3D structure of the spinal cord (n = 6–7/transplant group). In each section the boundary of the EGFP graft was first traced with a 20× objective and logged into the StereoInvestigator software using serial section manager, which tracked the positions of each section (~10 sections per spinal cord at 180-μm intervals) within the *Z*-axis of the 3D reconstruction of the spinal cord. Then, using optical fractionator, the traced contour of each section was divided into 50 × 50-μm counting frames; these frames typically encompassed two to four cells. Next the grid size was automatically determined by the software to permit counting of 20 sampling sites within each traced contour of each section. In each sampling site the number of EGFP-positive cells with Hoechst-stained nuclei was then marked with a dissector probe under a 63× objective. Algorithms for estimating the *Z*-stack total counts of EGFP-positive cells within the 3D structure of each spinal cord were performed according to StereoInvestigator 3.0 (Micro BrightField). The coefficient of error (CE) (Gundersen) for each estimated count was less than 10%.

Quantitative Assessment of Traced Fibers. BDA- or CTB-labeled axons were examined in every sixth section of the T8–T10 cord segments (i.e., every 180-μm interval spanning the width of the spinal cord). Labeled axons were visualized using fluorescence microscopy. The number of BDA-labeled axons was determined by counting all labeled axons crossing arbitrary lines placed perpendicular to the rostral–caudal cord axis at 2000 and 500 μm rostral to the graft–host or injury–host interface (identified by the GFAP–EGFP or $GFAP^+$–GFAP border) and at the epicenter of the graft/injury site under oil immersion with a 63× objective. The number of CTB-labeled axons was similarly analyzed, but at 2000 and 500 μm caudal to the graft–host or injury–host interface and at the epicenter of the graft/injury site. The number of fibers from each section, approximately 10 sections per spinal cord, was then summed for each rat. To estimate the number of fibers per section, the totals were then divided by the number of sections examined. Tracing efficiency was also examined across animals by examining dye intensity in the dorsal columns of five transverse sections from either the C2 or L5 spinal cord for BDA and CTB tracing, respectively, from each animal. Images taken selectively of only the dorsal columns in each of the sections were analyzed using intensity-measuring software (van der Lest et al., University of Njimegen, The Netherlands). The software automatically measured, and gave, in arbitrary units, the total pixel intensity of the dye staining in each image. These values were then averaged over the five sections to give a mean BDA or CTB intensity for each animal as a measure of tracing efficiency.

Determination of 5-HT and CGRP Fiber Growth. The numbers of 5-HT-labeled axons were determined by counting all labeled axons crossing arbitrary lines placed perpendicular to the rostral–caudal axis of the spinal cord at 2000 and 500 μm rostral to the graft–host or injury–host interface (identified by the GFAP–EGFP or $GFAP^+$–GFAP border) and at the epicenter of the graft/injury site under oil immersion with a 63× objective. The number of CGRP-labeled axons was similarly analyzed, but at 2000 and 500 μm caudal to the graft–host or injury–host interface and at the epicenter of the graft/injury site. The number of fibers from each section, approximately 10 sections per spinal cord, was then summed for each rat. To estimate the number of fibers per section, the totals were then divided by the number of sections examined.

Behavioral Testing

Gross hind limb performance was evaluated weekly using the open field locomotor test (BBB) (5). Animals were initially tested for 8 weeks postinjury prior to transplantation, after which they were randomly assigned to each of the three experimental groups. During the week of transplantation and the subsequent week (9 and 10 weeks postinjury) animals were not tested. Thereafter, animals were scored weekly for 6 weeks and then testing was stopped and the animals were anterogradely traced. Footprint analysis was performed prior to anterograde tracing at 16 weeks postinjury (8 weeks posttransplantation) using a procedure modified from de Medinaceli and colleagues (20). The animal's hind paws were inked to record footprints on paper that covered a runway 1 m long and 7 cm wide. A series of at least three sequential steps was used to determine the mean values for each

measurement of stride length, limb rotation, and base of support, as described previously (73).

Statistical Analysis

One-way ANOVA followed by the Bonferroni post hoc test was used for comparing results of anterograde tracing and axon counts after immunohistochemical detection of specific fiber types. A mixed factorial (repeated measures) ANOVA followed by the Tukey-Kramer test was used for comparison of weekly functional recovery patterns after injury (14,90). Differences were accepted to be statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, compared with injury-only controls. All errors are given as standard deviations.

RESULTS

In Vitro Preparation of EGFP-Labeled Glia by Lentiviral Vector Infection

Within 48 h of EGFP lentiviral vector infection at a MOI of 50, 98–99% of both SCs and OEG expressed the transgene. A MOI of 50 was optimal with lesser MOIs resulting in reduced transduction efficiency (data not shown). Observation of transduced cells for several weeks after EGFP infection compared with nontransduced cell controls demonstrated that introduction of the transgene did not affect either proliferation or morphology of the two cell types (data not shown).

Graft Survival and Integration

The transplantation of 2 million SCs into the injury epicenter at 2 months postinjury led to a $17.1 \pm 6.8\%$ cell survival as measured 3 months later through stereological counting of EGFP-positive cells. In contrast, only $2.3 \pm 1.4\%$ of the 2 million transplanted OEG were present at this time (Fig. 1). These results are similar to those obtained when the two types of glia are transplanted subacutely (1 week) postinjury (74,75) and agree with earlier reports that OEG appear to survive well only when grafted into healthy tissue near large injury sites (79,81). In OEG-grafted animals, EGFP OEG were observed as patches of cells within the injury site, although these patches rarely contained more than 10 cells. A rare example of a large patch of transplanted OEG is shown in Figure 2A. In SC grafted animals, EGFP SCs were found as a relatively continuous grafted mass within the injury epicenter, often interspersed with regions of debris or immune cells and surrounded by an astroglial boundary (Fig. 2B). Significant migration of EGFP cells into healthy host spinal cord was not observed with either cell type.

The majority of EGFP SCs were spindle shaped with processes extending sometimes up to 60 μm from their oval-shaped cell bodies. The surviving OEG exhibited a wide range of shapes, spindle shaped, tri- or multipolar, flattened, or rounded (Fig. 2A). In contrast to transplantation of SCs at 1 week postinjury, when most cells align parallel to the rostral–caudal axis of the spinal cord (74,75), cells introduced into the 2-month-injured spinal cord displayed not only parallel formations, but also both swirling patterns and arrangements that were perpendicular to the rostral–caudal axis of the spinal cord (Fig. 2B). At 1 week postinjury, the spinal cord tissue is relatively intact at the injury site and is filled with infiltrating immune cells (44); at this time there is little cavitation. At 2 months postinjury, however, there is significant cavitation and the formation of a fluid-filled cyst at the injury epicenter (44,93).

Axon Growth Responses in the Chronically Injured Spinal Cord

Neurofilament-immunoreactive (NF-IR) axons displayed robust penetration of SC grafts (Fig. 3A). NF-IR axons were found entering SC grafts from the host cord and from the dorsal roots. Although grafted OEG did not form a substantive graft, small groups of surviving OEG were also observed to be closely associated with numerous NF-IR axons.

Phenotypic Identification of Responsive Axons Present Within the Grafts

Growth of ascending fibers, CTB traced or CGRP positive, was observed predominantly around, but also within, SC grafts (Fig. 3B). However, there was no significant difference in the number of CTB-labeled, long-tract ascending axons traced from the sciatic nerve that were present either caudal to, or within, injury trabeculae and SC or OEG grafts (Fig. 4A). On the other hand, significantly greater numbers of CGRP-positive axons were seen in SC (32 ± 12 fibers/section, $p < 0.01$) than in OEG grafts (9 ± 2 fibers/section), or in injury trabeculae (15 ± 6 fibers/section) (Fig. 4B). The significantly larger number of CGRP-positive axons found caudal to and within SC grafts than CTB-labeled ascending axons may imply that these axons originated from nearby dorsal roots. 5-HT-positive serotonergic axons also were able to enter the SC grafts in limited numbers (15 ± 4 fibers/section) (Figs. 3D, 4C). Furthermore, more serotonergic axons were found within 500 μm of the rostral host cord–graft/injury interface after SC transplantation (117 ± 21 fibers/section, $p < 0.01$) compared with injury-only controls (75 ± 13 fibers/section) (Fig. 4C).

Determination of Nonresponding Supraspinal Axons in the Chronically Contused Spinal Cord

BDA-labeled corticospinal axons were examined at three locations relative to the grafts, 2000 and 500 μm rostral to and within the grafts. Corticospinal axons were

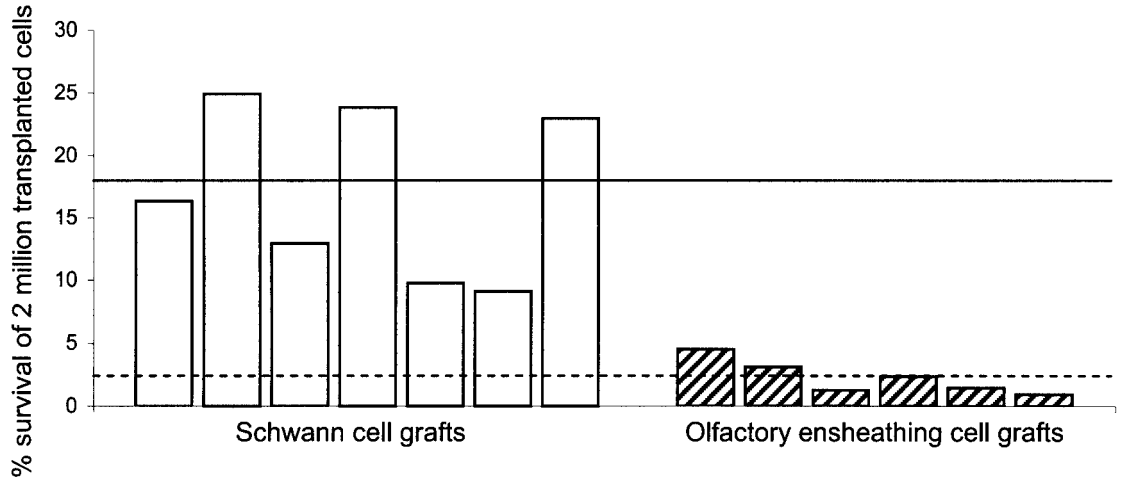

Figure 1. EGFP-labeled Schwann cells (SCs) exhibit greater survival than olfactory ensheathing glia (OEG) when transplanted into the chronic contusion injury site. Stereological quantification of EGFP-positive cells in the 3D structure of the injured spinal cord demonstrates that there is a significant loss of both cell types at 11 weeks posttransplantation. The graph shows the number of EGFP SCs or OEG counted per rat expressed as a percentage of the original number of cells transplanted. Solid line: average SC graft survival; dotted line: average OEG graft survival.

present immediately rostral (within 500 μm) to the graft–host interface (Figs. 3E, 4D). The majority of these fibers displayed axonal swellings and retraction bulbs in the area just above SC grafts (Figs. 3F). Although corticospinal axons were found in regions containing low concentrations of grafted SCs (Fig. 3F), these axons were not seen within the concentrated cellular area at the center of the SC grafts where fiber numbers were counted (Fig. 4D). Similar results have been observed following acute SC transplantation (74,75,97). Although the SC grafts did not support the growth of chronically injured corticospinal axons, greater numbers of these axons were found immediately rostral to the injury/graft site after SC grafting; 2.4-fold more axons (34 ± 9 fibers/section, $p < 0.001$) were found within 500 μm of the rostral host cord–graft interface than in injury-only controls (14 ± 5 fibers/section) (Fig. 4D).

Hindlimb Function After Glia Transplantation Into the Chronically Injured Spinal Cord

The gross locomotion of all animals was evaluated during the first 8 weeks postinjury prior to their random allocation into the three experimental groups. Statistical comparison of the three groups of animals demonstrated that there was no significant difference in BBB scores at week 8 preceding transplantation (injury only, 8.7 ± 1.2; SC graft, 8.3 ± 0.9; OEG graft, 8.4 ± 0.9) (Fig. 5). At this stage, most of the animals exhibited sweeping hindlimb movements and occasional plantar placement, with and without weight support, while in stance only. Following a 2-week recovery period, in which no behavioral analysis was conducted, there was no significant improvement in BBB scores over the subsequent 6 weeks in the injury-only and OEG-transplanted groups (BBB scores at 16 weeks endpoint: 8.7 ± 0.7 and 8.8 ± 0.9). In the SC-grafted group, however, there was a modest, but significant, improvement in BBB scores at 7 and 8 weeks posttransplantation, with a group average BBB score at 8 weeks of 10.2 ± 0.8 ($p < 0.05$) (Fig. 5). These animals were able to support their body weight and perform occasional weight-supported plantar stepping but exhibited no forelimb–hindlimb coordination.

Footprint analysis of the hind limbs was performed at 16 weeks postinjury (8 weeks posttransplantation). The base of support, determined as the distance between the left and right hind paw central pads, was determined to be 2.1 ± 0.2 cm in uninjured animals; after injury the base of support increased to 4.6 ± 0.4 cm. Following injury, rats exhibited a decrease in trunk stability, necessitating greater interlimb spread (base of support) to offset this deficit and support their body weight during locomotion. The animals performed predominantly sweeping movements of the hind limbs during locomotion. In SC-grafted animals, which exhibited better weight support during locomotion, their base of support was significantly improved to 3.8 ± 0.2 cm ($p < 0.01$) (Fig. 6A). OEG transplantation did not improve the base of support. The degree of hind paw rotation, the angle formed by the intersection of a line through the print of the third digit and a line through the central pad parallel to the walking direction, was found to be $11.7 \pm 0.80°$ in uninjured animals; after injury this parameter increased to a

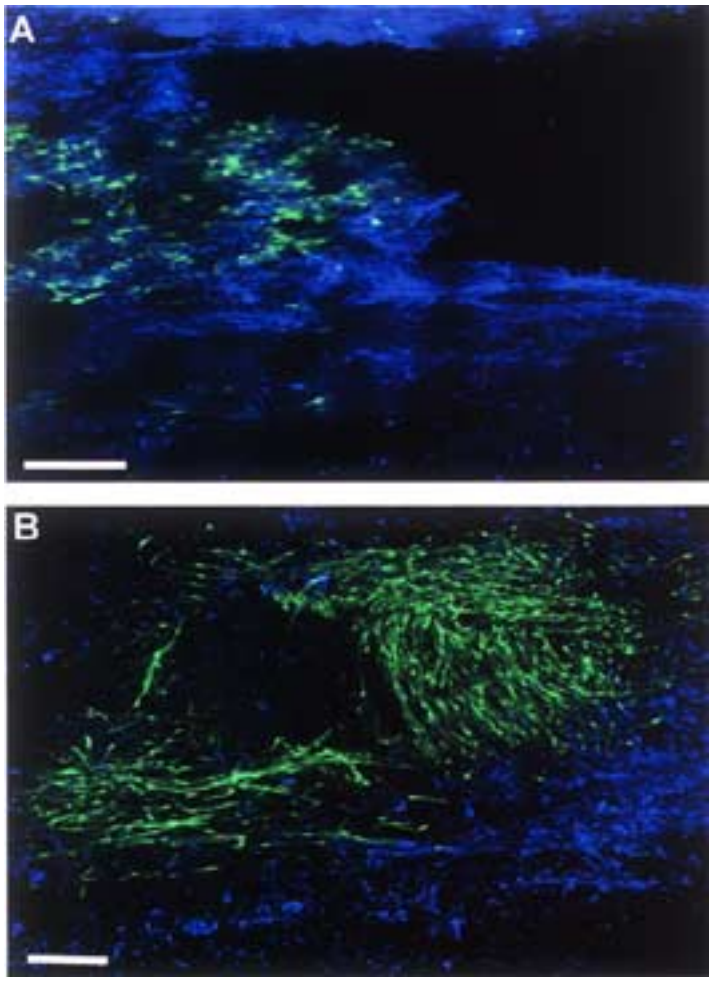

Figure 2. EGFP-labeled Schwann cells (SCs) and olfactory ensheathing glia (OEG) survive for up to 11 weeks following transplantation into the chronically contused spinal cord. (A) This section contains many more grafted EGFP OEG than is normally seen; it is shown to demonstrate their characteristic shapes. EGFP OEG (green), usually found in the center of the cavity, are surrounded by host astroglial cells stained with GFAP (blue). The OEG are more varied in shape than SCs, displaying more rounded cell bodies and shorter, more numerous processes. (B) Numerous grafted EGFP SCs (green) are found within the injured spinal cord surrounded by host astroglial cells stained with GFAP (blue). The SCs exhibit oval-shaped cell bodies with long bipolar processes and are positioned in various orientations relative to the rostral–caudal axis of the spinal cord. Scale bar: 200 μm.

mean of $36.2 \pm 1.0°$ as the hind paws were abnormally angled during sweeping of the hind limbs throughout locomotion. In animals that received SCs, foot exorotation significantly improved ($34.1 \pm 0.70°$, $p < 0.001$); the animals were able to bring their hind limbs closer together with weight supported steps during locomotion (Fig. 6B). There was no improvement in animals that received OEG transplantation. The final parameter examined, stride length, was reduced after injury (uninjured, 10.3 ± 0.8; injured, 7.9 ± 0.6, $p < 0.001$); neither type of cell transplant was able to improve this injury-induced deficit (Fig. 6C).

DISCUSSION

Identification of therapies capable of repairing the chronically injured spinal cord is a challenging and essential direction for the amenability of SCI research to the majority of persons with spinal cord injury. The present report investigated the survival of SC and OEG grafts and their promotion of axon growth and functional restitution in the chronically contused rat spinal cord. Both cell types have demonstrated efficacy in acute contusive SCI paradigms (73,97). We demonstrate using EGFP-labeled cells that, although both SCs and

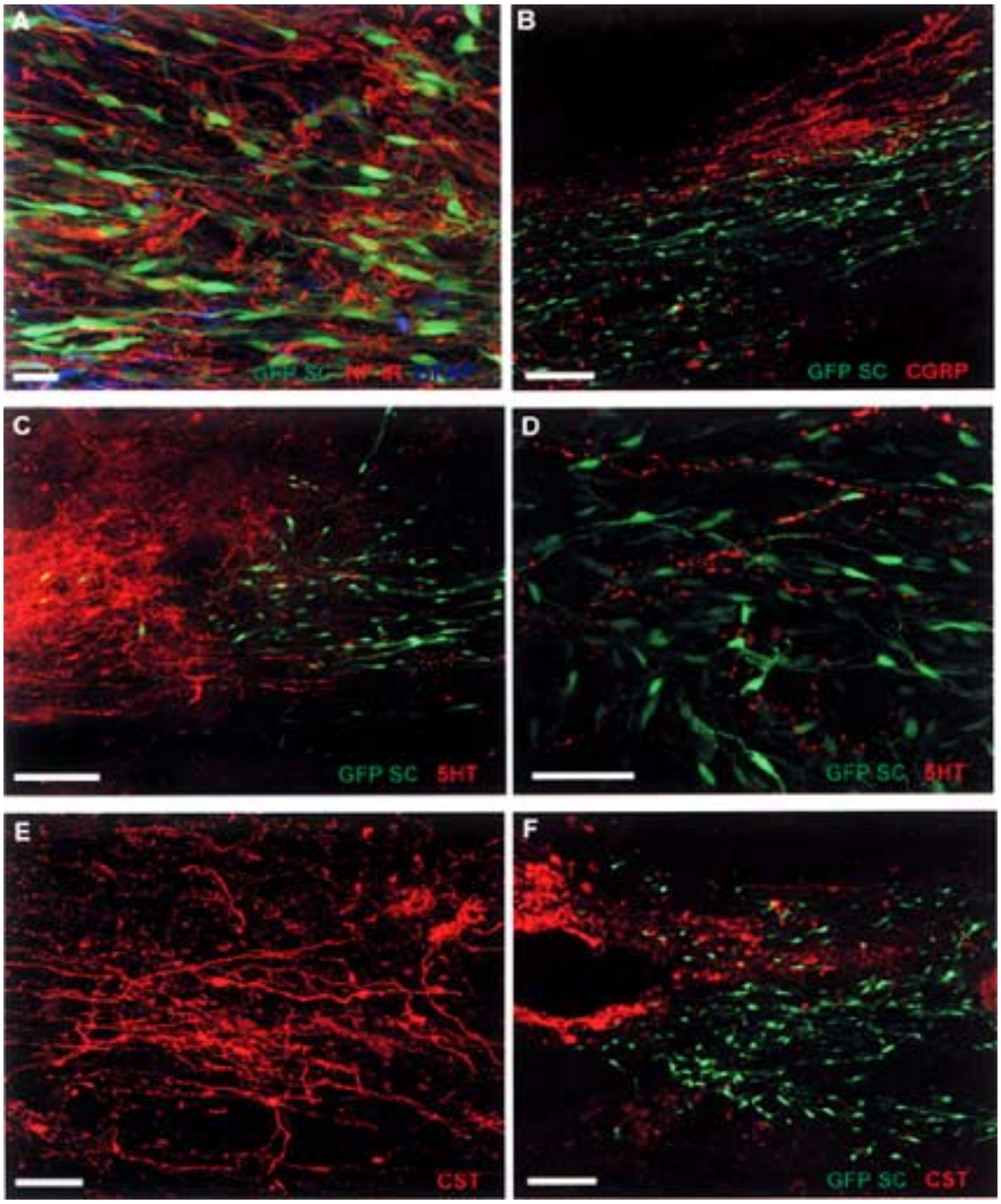

Figure 3. Schwann cell grafts support serotonergic and CGRP, but not corticospinal, axon growth in the chronically contused spinal cord. (A) Grafted SCs (green) support the growth of numerous neurofilament-positive axons (red) into the graft/injury site from the host spinal cord and the dorsal roots. Association of transplanted SCs with neurofilament-positive fibers is evident to a limited extent. Processes from nearby host astrocytes (blue) can be seen invading the SC graft. Scale bar: 20 μm. Projection image thickness: 30 μm. (B) CGRP-positive sensory axons (red), both from nearby dorsal roots and ascending from the caudal spinal cord, are seen growing around and through the transplanted SCs (green). Scale bar: 100 μm. (C) Numerous 5-HT-positive serotonergic axons (red) are present immediately rostral to the SC graft (green)/injury site. Scale bar: 100 μm. (D) Some of these serotonergic axons are able to also grow into the transplanted SC grafts for significant distances but are rarely seen past the graft–caudal cord interface. Scale bar: 50 μm. Projection image thickness: 10 μm. (E) BDA-traced corticospinal tract axons (red) in host spinal cord 2000 μm rostral to a SC graft. Many of the fibers exhibit characteristic axon swelling following axotomy. Scale bar: 50 μm. (F) BDA-traced corticospinal tract axons at the rostral host cord–SC graft interface. The corticospinal tract axons are unable to grow across this interface and many axons exhibit retraction bulbs and axon swellings. Scale bar: 100 μm.

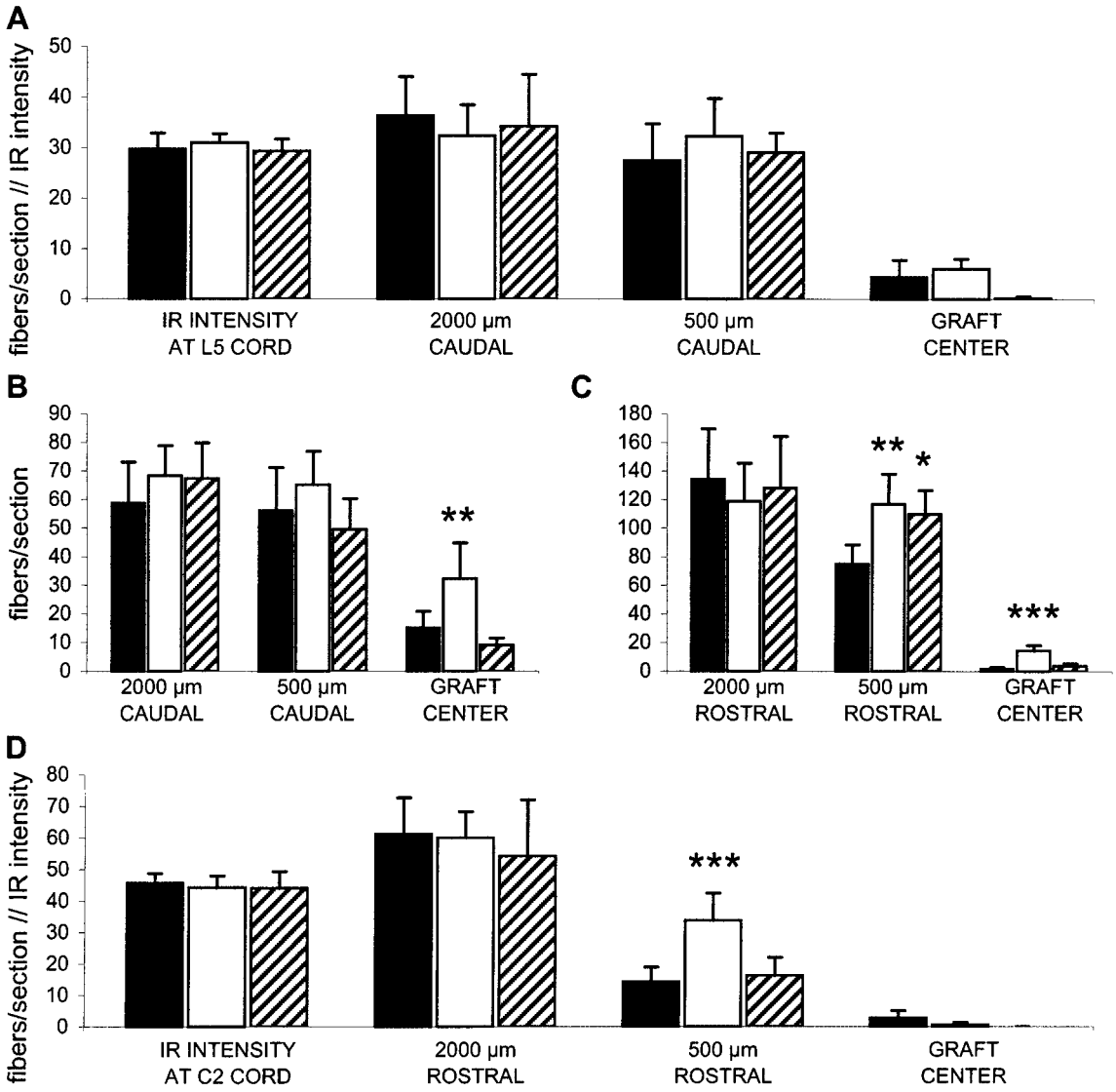

Figure 4. Cell grafts increase axon numbers rostral to injury/graft and are supportive for growth of selected ascending and supraspinal fiber populations. (A) Quantification of CTB-labeled ascending axons from the sciatic nerve shows that provision of a SC or OEG graft into the chronically contused spinal cord did not enhance their growth caudal to, or into, the injury/graft site. No difference in efficacy of CTB tracing, measured within the lumbar 5 cord, was seen among treatment groups. (B) Immunochemical detection of a specific ascending axon type; those that are CGRP positive, however, demonstrated that SC transplantation significantly increased the growth of these fibers into the injury/graft site. (C) Characterization of 5-HT-positive serotonergic fibers from the brain stem demonstrated that both SC and OEG grafts increased their numbers rostral to the injury/graft while SC grafts, in contrast to injury-only controls, supported some growth of these fibers into the injury/graft site. (D) Examination of BDA-labeled corticospinal fibers from the sensorimotor cortex showed that transplantation of SCs into the injured spinal cord increased their numbers immediately rostral to the injury/graft compared to injury-only controls, but these grafts did not support corticospinal axon growth to the graft center. No difference in efficacy of BDA tracing, measured within the cervical 2 cord, was seen among treatment groups. Filled bars: SCI only; open bars: SC graft; striped bars: OEG graft. Note for (A) and (D) that immunoreactive intensity of the CTB or BDA tracing in L5 or C2 transverse spinal cord dorsal columns, respectively, is presented in arbitrary units on the same *x*-axis as fiber numbers/section. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with injury-only controls.

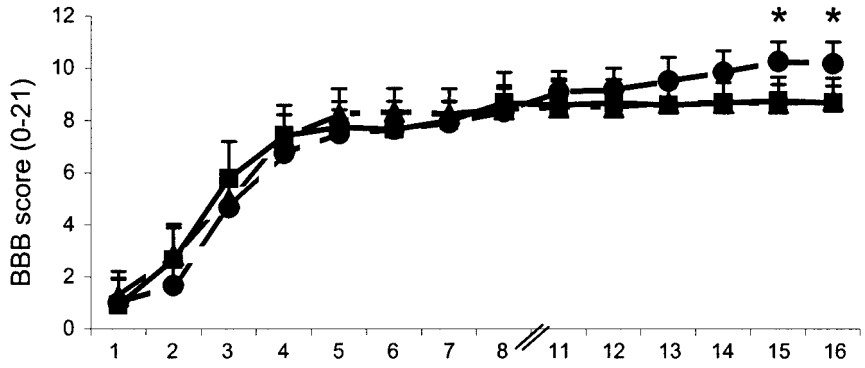

Figure 5. Schwann cell grafts improve gross locomotion after injury. Open field locomotor ability was assessed weekly using the BBB score from 1 to 8 weeks postinjury and then following transplantation for 6 weeks (11–16 weeks postinjury). Animals receiving SC grafts demonstrated a modest, but significant, improvement in open field locomotion compared with injury-only controls. Legend: ◆: SCI only; ●: SC graft; ■:OEG graft. $*p < 0.05$ compared with injury-only controls.

OEG are present at 3 months posttransplantation, only SCs survive in significant numbers to form a graft capable of bridging the injured spinal cord and acting as a substrate for axonal growth of both CGRP- and 5-HT-positive axons. Additionally, SC grafting facilitated modest, though significant, improvements in hind limb function during overground locomotion.

To accomplish the histological aims of the study, we employed lentiviral vectors to introduce EGFP into the glial cells for efficient and comprehensive labeling, allowing visualization of the cells' morphology and long-term tracking in vivo as described elsewhere (24,98). Cell infection of lentiviral EGFP is superior to many of the cell trackers previously used for identification of the survival, spatial extent, axonal growth support, and migration of grafted cells within the injured spinal cord. Other labels used for cell tracking generally suffer from nonuniform or inadequate labeling of the entire cell, short-lived exposure or loss of the signal during cell division, leakage of the label, and transfer of the label to endogenous cells upon grafted cell death, laborious methods required for visualization or noncompatibility of visualization methodology with immunochemistry procedures that preclude them from specific use in vivo (43,55,74,75). Cell labeling with lentiviral vectors encoding EGFP addresses all these shortcomings of other labels (15,74,75), though its use has been very limited in spinal cord transplantation studies to date.

Transplantation of SCs and OEG into the spinal cord injury site resulted in quite disparate abilities of the two cell types to survive, integrate, and form a substantive structure upon which endogenous axons could grow. Whereas SCs exhibited modest survival and formed grafts capable of bridging the injured cord, OEG survival was poor. The successful use of direct OEG grafting in previous reports has been confined largely to small spinal cord lesion models (52,57,58) or to grafting of the cells into healthy tissue near the spinal cord injury site (79,81,87,89). It is not presently known why OEG appear to be more susceptible to cell death than SCs within the hostile milieu of the injury site. Experiments designed to compare responses of SC and OEG to various proinflammatory cytokines, glutamate, or free radicals, or to examine immune cell reactions to the grafted cells early after introduction into the injured spinal cord may shed light on this issue. It has been shown that the use of anti-inflammatory compounds 1 week prior to, or during, OEG transplantation into the acutely injured spinal cord can improve the ability of OEG grafts to promote axon growth (68,72) and functional recovery (72).

No significant migration of surviving glia, SCs or OEG, from the injury milieu into nearby healthy tissue of the contiguous cord was observed in any of the animals 3 months posttransplantation. Some cells from both transplantation groups were found in dorsal roots immediately adjacent to the injury site or in the central canal. The inability of transplanted SCs to migrate into the undamaged host cord after grafting in various SCI models has been well documented (11,109). SC migration into healthy CNS tissue appears to be limited by the glial scar (110), especially by interactions with astrocytes and the extracellular matrix molecules or cell adhesion molecules produced as recapitulated in coculture studies in vitro (37,38,41,53,107). This restriction of SC migration by host CNS glia may reflect the important function of preventing SC CNS invasion across the dorsal root entry zone in the normal spinal cord (30). In contrast to other reports (79,81), we did not observe migration of the lim-

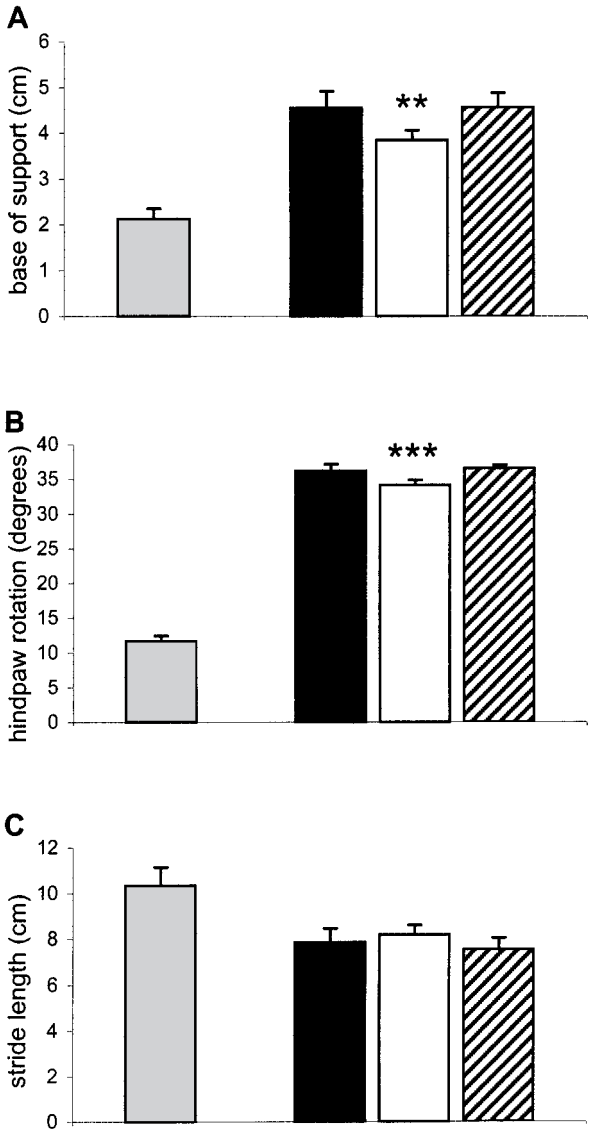

Figure 6. Schwann cell grafts improve base of support and foot rotation after injury. (A) The distance of the base of support between the hind paws. (B) The angle of foot exorotation of the hind paws. (C) The stride length. Light shaded bars: no injury; filled bars: SCI only; open bars: SC graft; striped bars: OEG graft. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with injury-only controls.

ited number of OEG that had survived transplantation in the current study. The failure of OEG to migrate may simply be due to the poor survival of the grafted cells or the placement of the cells into the center of the contusion injury, rather than in nearby healthy tissue (79, 81,87); the injury site may not have been permissive for their migration. It will be important in future work to examine if transplantation of OEG on either side of the contusion injury in healthy tissue can lead to improved survival and promote axon growth. It is uncertain in such a paradigm, however, whether following a large injury if additional grafting of other cells, such as SCs, a matrix, or a biopolymer construct, may also be required to physically bridge the injured cord as used previously (81).

The limited survival of OEG following transplantation precludes interpretation in the present study of the ability of OEG grafts to support axon growth after chronic contusion injury. SC grafts, however, did bridge the chronically injured cord, supported growth of CGRP-positive and serotonergic axons and significantly increased the numbers of both corticospinal and serotonergic axons immediately rostral to the injury/graft. None of these fibers, however, were seen exiting the grafts to enter the contiguous cord. Growth of chronically injured supraspinal and ascending axons into grafts has been observed with other cellular transplants (16,31,49), though pronounced growth from the grafts into the contiguous cord is only observed with additional therapies (16,61, 69,73,104). Limited growth of supraspinal fiber systems is seen after acute spinal cord contusion and SC grafting (72,73,97), so it is surprising that modest growth of these fibers is seen in the chronically injured spinal cord, where the growth potential of axotomized neurons would likely be more limited due to a highly formed glial scar and reduced expression of growth-associated gene programs (6,25,96,101,102).

One explanation for this finding could be a reduction in the presence of inhibitory myelin debris in the chronic injury due to phagocytosis by infiltrating macrophages (7,17,36). A reduction in inhibitory myelin and myelin-associated proteins could have created a more favorable environment for axonal sprouting into the grafts. Myelin and a number of myelin-associated proteins are potent inhibitors of CNS regeneration after spinal cord injury (26,64,92). The ability of SC grafts to reduce axonal dieback after injury has been observed in acute SCI models (72,73,97); however, at the time of grafting in the current study, 2 months postinjury, these axons would have been expected to already have undergone significant dieback (71,97). It is thus unlikely that the SCs are actually reducing axonal dieback. It may be that the production of growth factors by grafted SCs, such as nerve growth factor (4), brain-derived neurotrophic factor (65), ciliary neurotrophic factor (35,83), and glial cell line-derived neurotrophic factor (106), may have diffused into the contiguous host cord and promoted limited sprouting of these fibers into cord regions immediately adjacent to the graft; many of these fibers would then be obstructed from further growth by the glial scar. The presence of numerous retraction bulbs at the graft–host interface would support this scenario of abortive sprouting.

This capacity for axon growth support reported here

was accompanied by moderate gains in stepping and hind paw position at 7–8 weeks posttransplantation in SC-grafted animals that were statistically significant compared with injury-only controls. Because none of the fiber systems examined in the current study showed extensive growth across SC grafts that could account for this restitution, such as the corticospinal or serotonergic systems, which are important in locomotion (21,22,81, 91), it is probable that changes in local spinal networks may have facilitated these modest improvements. SC bridges have been shown previously to support growth of numerous spinal cord proprioceptive axons (97,109), which are able to modulate various aspects of movement (1,54); the growth of these fibers may have been responsible for the observed improvements. Examination of the response of proprioceptive neurons to SC grafts in this chronic SCI model will be an important step in future experiments. The noradrenergic system, also involved in locomotion, was not investigated in the current study and may have contributed to the observed increased function. However, this may be unlikely because SC grafts in the subacute contusion injury paradigm have been shown previously to be unable to support growth of these axons and thus would not be expected to grow after chronic SCI (97).

The current study explored the feasibility and efficacy of SC and OEG transplants in the chronically contused spinal cord using EGFP-labeled cells to allow assessment of survival, migration, and ability to facilitate ascending and supraspinal axon growth. We reiterate, as previously described (74,75,89), the poor ability of highly purified OEG (from adult animals) grafts to survive when directly transplanted into larger spinal cord injuries, despite the less inflammatory environment of the chronically injured cord (36). In contrast, transplanted SCs were able to survive and form a substantive graft for the support of both ascending, CGRP-positive and supraspinal, serotonergic axons. Furthermore, the SC-grafted animals demonstrated a gradual improvement in open field locomotion and hind paw positioning that, though modest, was significantly different from injured controls at endpoint. The present report demonstrates the therapeutic potential of SC transplantation for chronic spinal cord injury repair and paves the way for the examination in chronic SCI of the efficacy of a number of SC combination therapies that have shown promise in acute SCI paradigms.

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