Schwann Cell But Not Olfactory Ensheathing Glia Transplants Improve Hindlimb Locomotor Performance in the Moderately Contused Adult Rat Thoracic Spinal Cord

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Cultured adult rat Schwann cells (SCs) or olfactory ensheathing glia (OEG), or both, were transplanted in the adult Fischer rat thoracic (T9) spinal cord 1 week after a moderate contusion (10 gm, 12.5 mm, NYU impactor). Rats received either a total of 2×10^6 cells suspended in culture medium or culture medium only (controls). At 12 weeks after injury, all grafted animals exhibited diminished cavitation. Although in medium-injected rats 33% of spinal tissue within a 5-mm-long segment of cord centered at the injury site was spared, significantly more tissue was spared in SC (51%), OEG (43%), and SC/OEG (44%) grafted animals. All three types of glial grafts were filled with axons, primarily of spinal origin. SC grafts contained more myelinated axons than SC/OEG and OEG grafts. Both types of SC-containing grafts expressed more intense staining for glial fibrillary acidic protein and chondroitin sulfate proteoglycan compared with OEG-only grafts. Retrograde tracing demonstrated that the number of propriospinal and brainstem axons reaching 5–6 mm beyond the grafted area was significantly higher with SC and SC/OEG grafts but not with OEG-only grafts compared with controls. Corticospinal fibers terminated closer to the lesion epicenter in all grafted animals than in controls. With SC-only grafts, a modest but statistically significant improvement in hindlimb locomotor performance was detected at 8–11 weeks after injury. Thus, in addition to this functional improvement, our results show that an SC graft is more effective in promoting axonal sparing/regeneration than an SC/OEG or OEG graft in the moderately contused adult rat thoracic spinal cord.

Key words: spinal cord injury; transplantation; contusion injury; axonal sparing; axonal regeneration; propriospinal axons; corticospinal tract; brainstem axons; neuroprotection

Injury to the adult mammalian spinal cord results in progressive tissue damage, which causes permanent functional deficits (for review, see Amar and Levy, 1999). Acute treatment with anti-inflammatory agents such as methylprednisolone (for review, see Oudega et al., 1999; Takami et al., 2002) or interleukin-10 (Bethea et al., 1999; Takami et al., 2002) limits injury-induced tissue damage. However, despite application of neuroprotective treatments, severe functional deficits are still observed, and in fact, at present, spinal cord injury (SCI) has very poor clinical prospects.

Experimental strategies aimed at promoting axonal sparing/regeneration and restoring functional deficits suggest that there may be a window of opportunity for repair after SCI (for review, see Bunge, 2001; Jones et al., 2001). Several cellular grafting strategies result in some behavioral improvements of experimentally induced paralysis (Iwashita et al., 1994; Cheng et al., 1996; Grill et al., 1997; Li et al., 1997; Liu et al., 1999; McDonald et al., 1999; Ramón-Cueto et al., 2000; Coumans et al., 2001).

An amply researched and reputable cell type for spinal cord repair is the Schwann cell (SC), the myelin-forming glial cell of the peripheral nervous system (for review, see Bunge, 1994; Bunge and Kleitman, 1999). An SC graft bridging a transected adult rat thoracic spinal cord promotes regeneration and myelination of propriospinal axons (Xu et al., 1995a, 1997, 1999). The combination of an SC graft with methylprednisolone (Chen et al., 1996) or with neurotrophic factors (Xu et al., 1995b; Menei et al., 1998) promotes axonal regeneration from brainstem neurons as well.

Another glial type that has shown promise in SCI repair is olfactory ensheathing glia (OEG) (for review, see Franklin and Barnett, 2000; Kleitman and Bunge, 2000; Ramón-Cueto, 2000; Plant et al., 2001; Raisman, 2001). OEG grafts promote axonal growth and functional improvements in different SCI models (Li et al., 1997, 1998; Ramón-Cueto et al., 1998, 2000). Moreover, combining an SC graft with OEG placed into the transected cord stumps promotes regeneration of descending and ascending axons into the caudal and rostral cord, respectively (Ramón-Cueto et al., 1998).

The repair abilities of SCs and OEG in the adult cord have been studied in complete or partial transection models (for review, see Kleitman and Bunge, 2000; Ramón-Cueto, 2000; Bunge, 2001; Plant et al., 2001) and in demyelination models (for review, see Blakemore et al., 2000) (see also Imaizumi et al., 1998, 2000). Although a common type of damage to the human spinal cord is a contusive injury (Bunge et al., 1997; Kakulas, 1999), studies on

Received Feb. 15, 2002; revised April 23, 2002; accepted May 9, 2002.

This work was funded by the Christopher Reeve Paralysis Foundation, National Institute of Health Grants NS09923 and PO1NS38665, and The Miami Project. M.O. is a Werner Heumann Memorial International Scholar. We are grateful to Andrew B. Weber and Pratik P. Desai for histology; Annemarie N. Ali for help with image analysis; Roland Garcia-Rojas, Paulo Diaz, and Dr. Alexander E. Marcillo for inducing the contusion injury; and Yelena Pressman for cell culture. Lyudmila Rusakova, Rosemary Abril, and Kim Loor are thanked for their help with animal care and behavioral testing, and Robert Camarena for assistance in photography. The gift of the 192 hybridoma cell line from Dr. Eric Shooter (Stanford University) is gratefully acknowledged.

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 0270-6474/02/226670-12$15.00/0

the repair capacity of SCs (Martin et al., 1991, 1996) and OEG in clinically relevant contusion models are sparse. In the present study, the effects of purified adult rat SCs or OEG, or both, on spinal tissue sparing, axonal sparing/regeneration, and behavioral improvement were assessed in moderately contused adult Fischer rat thoracic spinal cord. We used the term "sparing/regeneration" in this paper because spared and regenerated axons could not be distinguished. To our knowledge, this study is the first to compare the reparative capacity of SCs and OEG in a clinically relevant contusive SCI model.

MATERIALS AND METHODS

Schwann cell purification. Highly purified SC cultures were obtained from sciatic nerve of adult female Fischer rats (Charles River Laboratories, Wilmington, MA) as described previously (Morrissey et al., 1991). To determine the purity of the SCs used for grafting, samples of the harvested SCs were plated onto culture dishes, cultured for 3 hr, stained for S100, and then coverslipped with Citifluor (UKC Chemical Laboratory, Canterbury, England) containing 100 μM Hoechst nuclear dye (Sigma, St. Louis, MO) to compare numbers of S100-positive cells with Hoechst-labeled cells. The purity of the SCs used for implantation was 95–98%.

Olfactory ensheathing glia purification. Highly purified cultures of OEG were obtained from the nerve fiber layer of the olfactory bulb of adult female Fischer rats as described previously (Ramón-Cueto et al., 1998). Care was taken to minimize the inclusion of non-nerve fiber layer bulb tissue, and the pia was removed. The tissue was dissected in Leibovitz-15 (L-15) medium (Invitrogen, Gaithersburg, MD), washed twice with HBSS (Invitrogen), diced into small fragments, and incubated in HBSS with 0.25% trypsin (Worthington Biochemical, Lake Wood, NJ) and 50 μg/ml DNAase (Sigma) at 37°C for 60 min. Trypsinization was stopped by adding DMEM/Ham's/F-12 (Invitrogen) (1:1 mixture) supplemented with 10% FBS and 50 μg/ml gentamicin (df-10S). The dissociated cells were washed twice, resuspended in df-10S plus 2 μM forskolin and 20 μg/ml pituitary extract, and then plated onto poly-L-lysine-coated culture dishes. One week later, OEG were separated from other cell types by immunopanning using an antibody against p75, the low-affinity nerve growth factor receptor (gift of Dr. Eric Shooter, Stanford University), using a modification of the published protocol (Ramón-Cueto et al., 1998). Cells were detached with 0.05% trypsin and 0.02% EDTA (Invitrogen), centrifuged, washed two times, resuspended in L-15, and then plated on antibody-treated dishes for 30 min at 4°C. Before use, these dishes had been incubated overnight at 4°C with anti-mouse IgG A,M antibody (1:100 in Tris buffer) (0.05 M, pH 9.5; Jackson ImmunoResearch, West Grove, PA), washed three times with ice-cold L-15, incubated with antibodies against p75 (1:5 in L-15 medium with 5% FBS) for 2 hr at 4°C, and finally washed three times with ice-cold L-15 medium. The unattached cells were removed, and the dishes were washed gently five times with ice-cold L-15 medium. The attached cells were fed df-10S plus 2 μM forskolin and 20 μg/ml pituitary extract for 2 d at 37°C in 6% CO_2. Next, the cells were detached from the dishes with 0.05% trypsin and 0.02% EDTA and washed twice with df-10S. Finally, the cells were resuspended in DF-10S plus 2 μM forskolin and 20 μg/ml pituitary extract, plated onto poly-lysine-coated culture dishes, allowed to grow to confluency, and then harvested (in DMEM/F-12) for transplantation.

Animals. Adult female Fischer rats ($n = 68$; 160–180 gm; Charles River Laboratories) were housed according to National Institutes of Health and United States Department of Agriculture guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. Before surgery, the rats were anesthetized with 1–2% halothane in a mixture of 70% nitrous oxide and 30% oxygen. An adequate level of anesthesia was determined by monitoring the corneal reflex and withdrawal to painful stimuli for the hindlimbs. The back region was shaved and aseptically prepared with betadine. Bicillin (0.02 ml/100 mg body weight, 300 U/ml, i.m.) was administered before surgical procedures were performed. During surgery, the rats were kept on a heating pad to maintain the body temperature at 37 ± 0.5°C.

Contusion injury. Contusion injury was induced by the weight drop device developed at New York University (Gruner, 1992). Without disrupting the dura mater, the ninth thoracic (T9) spinal cord segment was exposed by removing the dorsal part of the T8 vertebra. The exposed cord was then moderately contused by a 10 gm weight that was dropped from a height of 12.5 mm. The contusion impact velocity and compression were monitored to guarantee consistency between animals. After injury, the muscles were sutured in layers and the skin was closed. The rats were returned to their partly warmed cages with *ad libitum* access to water and food. Bicillin was administered 2, 4, and 6 d after the contusion injury. The rats were maintained for a total of 12 weeks after injury.

Glia transplantation. One week after injury, the injury site was exposed, and a total of 2×10^6 SCs, OEG, or SC/OEG (1×10^6 each) in 6 μl DMEM-F12 medium (G. W. Plant, E. P. Cuervo, M. L. Bates, M. B. Bunge, and P. M. Wood, unpublished observations) was injected into the contused area using a 10 μl Hamilton syringe held in a micromanipulator. Control rats were injected with 6 μl of DMEM-F12 medium. After injections, the muscle layers and the skin were closed separately. Sixty-eight rats were transplanted with medium ($n = 21$), SCs ($n = 15$), OEG ($n = 21$), or SC/OEG ($n = 11$). The rats in each experimental group were randomly divided into subgroups for retrograde and anterograde tracing.

Anterograde axonal tracing and retrograde neuronal tracing. For anterograde axonal tracing, 9 weeks after contusion, the rats were anesthetized and biotinylated dextran amine (BDA; Molecular Probes, Eugene, OR) was injected stereotactically into the hindlimb area of the motor cortex (2×0.5 μl, bilateral), dextran coupled to fluorescein (D/Fl; Molecular Probes) into the reticular formation (2×0.3 μl, bilateral), and dextran coupled to rhodamine (D/Rho; Molecular Probes) into the lateral vestibular nuclei (2×0.2 μl, bilateral).

For retrograde neuronal tracing, 11 weeks after the contusion injury, the injury site was exposed, and a total of 0.6 μl of a 2% aqueous fast blue solution (FB; Sigma) was injected 7 mm distal to the distal laminectomy edge (in between vertebra T10 and T11) using a glass needle (diameter, 200 μm) attached to a 1 μl Hamilton syringe held in a micromanipulator (Takami et al., 2002). All injections was performed over a 3 min period, and the injection needle was kept in place for an additional 3 min to minimize leakage on withdrawal. After the injection, the muscles and skin were closed in layers.

Histological procedures. At 12 weeks after injury, all rats were anesthetized (2.57 mg ketamine, 0.51 mg xylazine, and 0.09 mg acepromazine per 100 gm body weight) and transcardially perfused with phosphate-buffered, 4% paraformaldehyde (0.1 M, pH 7.4) (Takami et al., 2002). The T7–9 thoracic segments, which included the contusion injury, were dissected and embedded in gelatin (Oudega et al., 1994). Using a sliding freezing microtome, the embedded cord segments were cut into 40-μm-thick horizontal sections. The T2, C6, and C2 spinal cord segments and the brainstem and cerebral cortex were cut into 40-μm-thick transverse sections. All sections were collected in PBS (0.1 M, pH 7.4) and kept at 4°C until further processing.

For electron microscopic analysis, a 6-mm-long piece of the spinal cord with the epicenter in the middle was dissected and divided into three 2-mm-long pieces. These pieces were prepared for analysis as described previously (Xu et al., 1995a).

Estimation of the number of myelinated axons and blood vessels in transplants. Estimated total numbers of myelinated axons and blood vessels as well as the density of myelinated axons in the contusion lesion–transplant area were determined by systematic random sampling (West, 1993). In toluidine blue-stained, 1-μm-thick transverse plastic sections, the injured–transplanted area was outlined and than scanned using a fractionator grid, which ranged in size from 35 to 70 μm^2. The objective lens used for all counts was 100× with oil immersion. In the center of the fractionator grid, a 15 μm^2 dissector probe was present. All myelinated profiles within this dissector probe were counted. In this way a fraction of the lesion–transplant area was analyzed (optical fractionator method) (West, 1993). For proper use of this technique, a minimum number of 150–300 myelinated axons is required per lesion–transplant area. In our counts, the number for myelinated axons was between 159 and 484, with the exception of 67 in a medium-injected control animal, which contained a very large cavity. The estimation of the total number of myelinated axons and blood vessels was done with the Stereoinvestigator software (MicroBrightfield, Inc., Colchester, VT).

Quantitative assessment of anterograde and retrograde tracing. For quantitative analysis of the anterograde tracing, BDA-labeled corticospinal axons were examined in every fifth section of the T7–9 cord segments. Within these sections, the labeled axons were visualized using a Ni-enhanced avidin–biotin–peroxidase staining (Oudega et al., 1999). The number of BDA-labeled corticospinal axons was determined by counting all labeled axons crossing an imaginary line placed perpendicular to the rostral–caudal axis of the spinal cord at 1 and 2 mm rostral to and at the lesion epicenter. The numbers of each section were summed per rat and

then multiplied by 5 to obtain the final number of labeled corticospinal axons. In addition, from the T7–9 segments, every fifth section was mounted onto gelatin-coated glass slides, coverslipped with Citifluor (UKC Chemical Laboratory) containing the nuclear dye, Hoechst (100 μM, Sigma), and used to examine the D/Fl-labeled reticulospinal axons and the D/Rho-labeled vestibulospinal axons.

For quantitative analysis of the retrograde tracing, every third horizontal section of the T7–9 cord was mounted on gelatin-coated glass slides and coverslipped with Citifluor (UKC Chemical Laboratory). For the T2, C6, and C2 cord segments, every 5th transverse section and, from the brainstem and cerebral cortex, every 10th transverse section was mounted onto gelatin-coated slides and coverslipped with Citifluor (UKC Chemical Laboratory). For each rat, the number of FB-labeled neurons was determined in each section. These numbers were summed per rat and multiplied by 3 (T9-T7), 5 (T2, C6, and C2), or 10 (brainstem and cerebral cortex) to obtain the final number of labeled neurons.

Assessment of spinal tissue sparing. Every fifth horizontal section from the T7–9 segment was mounted onto gelatin-coated glass slides, stained with cresyl violet, dehydrated, and coverslipped in Pro-Texx (Baxter Diagnostics, Deerfield, IL) to determine the volume of spared spinal tissue using an image analysis computer system (Universal Imaging, West Chester, PA). In each section, the total area of a 5-mm-long cord segment with the lesion epicenter in the middle and the area of damaged spinal tissue were determined. The border of the damaged area was defined as an obvious discontinuity in density of small cells, i.e., transplanted cells and inflammatory cells, and the absence of healthy-looking spinal neurons. Occasionally, this area contained small cysts. The measurements of each section were summed per rat and multiplied by 5 (every fifth section was analyzed) to give the total area of the 5-mm-long cord segment and of damaged spinal tissue. The volumes were then calculated by means of numerical integration. The volume of spared spinal tissue within the 5-mm-long segment was determined by subtracting the volume of damaged tissue from the volume of the whole segment. Finally, this value was expressed as the percentage of the total volume of a 5-mm-long segment from the same cord level from normal, uninjured rats ($n = 4$).

Immunohistochemical procedures. Every sixth horizontal section was immunohistochemically stained with mouse monoclonal or rabbit polyclonal antibodies, or both, following an earlier described protocol (Takami et al., 2002). The polyclonal antibodies used were anti-serotonin (5-HT, 1:200; Incstar Corp., Stillwater, MN), anti-dopamine-β−hydroxylase (Dβ H, 1:200; Incstar Corp.), anti-calcitonin gene-related peptide (CGRP; 1:100; Cappel, Aurora, OH), and anti-glial fibrillary acidic protein (GFAP, 1:400; Dako, Carpinteria, CA). The monoclonal antibodies used were anti-low-affinity nerve growth factor receptor [p75 (192IgG), 1:1; gift from Dr. E. Shooter, Stanford University], anti-neurofilament (RT-97, 1:10; Developmental Hybridoma Bank), anti-chondroitin sulfate proteoglycan (CS-56, 1:100; Sigma), and anti-growth associated protein-43 (GAP-43, 1:500; Boehringer Mannheim, Mannheim, Germany). The secondary antibodies used were Alexa 488-conjugated goat anti-rabbit (1:200; Molecular Probes) and Alexa 594-conjugated rabbit anti-mouse (1:200; Molecular Probes). The sections were mounted onto gelatin-coated glass slides and coverslipped in Citifluor (UKC Chemical Laboratory) containing 100 μM Hoechst dye (Sigma).

Open field locomotor test. Hindlimb performance was evaluated using the open field locomotor test developed by Basso et al. (1995, 1996). Two observers, unaware of the experimental procedures, performed the test once a week for 9 weeks (for rats that received anterograde tracing) or 10 weeks (all other rats).

Statistical analysis. One-way ANOVA followed by Fisher's protected least-significant difference test was used to determine statistical differences between the average number of axons and neurons as determined for each group. However, in case of unequal variance (F test), a nonparametric analysis (Kruskal–Wallis test followed by Mann–Whitney U test) was used. The latter test was also used to determine whether differences between the average Basso, Beattie, and Bresnahan (BBB) score per group were statistically significant. A statistically significant difference was accepted at $p < 0.05$.

RESULTS

SC and OEG transplants promote tissue sparing

In medium-injected animals, 12 weeks after a moderate contusion injury, large cysts were present extending in the rostral–caudal direction (Fig. 1*A*). These cysts were widest at the lesion epicenter. In transplanted animals, the glial grafts were continuous with host spinal tissue (Fig. 1*B–D*), although some small cavities could be observed (Fig. 1*C*,*D*). The effect of SC and OEG grafts on tissue sparing was assessed by subtracting the volume of damaged tissue (including cavities and transplant) within a 5-mm-long cord segment (with the epicenter in the middle of the section) from the total volume of this segment. This value was expressed as a percentage of the volume of the comparable (T9) segment in uninjured rats. The volume of a 5-mm-long spinal cord segment of a normal, uninjured rat at the T9 level was 21.2 ± 0.9 mm^3 (SEM; $n = 4$). In control medium-injected animals, the volume of spared tissue was 6.9 ± 0.7 mm^3 ($n = 8$), or 33 ± 3% of the volume of the comparable segment of an uninjured rat (Fig. 1*E*). With an SC or OEG graft, the volume of spared tissue was 10.8 ± 1.5 mm^3 ($n = 5$) and 9.1 ± 0.8 mm^3 ($n = 9$), or 51 ± 7 and 43 ± 4%, respectively, of the volume of the comparable segment in a normal, uninjured cord (Fig. 1*E*). These values were significantly greater than the volume of spared tissue in medium-injected animals. Grafting the combination of SCs and OEG resulted in a volume of 9.4 ± 1.1 mm^3 ($n = 5$) of spared tissue, or 44 ± 5% of the comparable segment in an uninjured cord (Fig. 1*E*), which was also significantly ($p < 0.05$) greater than in the medium-injected control animals. These results demonstrate that implantation of SC or OEG grafts, or both, into a 1-week-old moderate contusion lesion promotes spinal tissue sparing.

All grafts contain myelinated axons

At 12 weeks after injury, i.e., 11 weeks after implantation, toluidine blue-stained, 1 μm plastic cross-sections of the lesion epicenter were prepared to examine the presence of myelinated axons within and around the transplants. In medium-injected control animals, some tissue usually was found within the large cavities (Fig. 2*A*). This tissue contained axons surrounded by typical peripheral-type (SC) myelin (Fig. 2*B*). The criteria to recognize peripheral-type myelin in plastic cross sections were the presence of a signet ring configuration (myelin ring with the proximate SC nucleus) and the presence of space between the myelinated axons, which is caused by the formation of extracellular matrix (Bunge et al., 1994). In contrast, central-type myelin profiles do not exhibit adjacent nuclei and have little or no space between them. In controls as well as in grafted animals, a rim of preserved spinal white matter that contained axons with typical central-type (oligodendrocyte) myelin was observed. In the dorsal aspect of this rim, both peripheral and central types of myelin were found, indicating the presence of SCs that had migrated from the dorsal roots into the damaged spinal cord.

All types of grafts contained axons surrounded by peripheral-type myelin (Fig. 2*B*,*D*,*F*). However, the SC graft clearly contained more axons than the other types of grafts (Fig. 2, compare *D*, *F*; Table 1). We estimated the total number of myelinated axons and blood vessels in the contusion lesion area by image analysis using the optical fractionator method (West, 1993) (Stereoinvestigator program). In SC-injected animals (Fig. 2*C*,*D*), the number of myelinated axons was 5212 ± 1783 (mean ± SEM; $n = 2$) compared with 2125 ± 697 ($n = 3$) in medium-injected animals (Table 1). In OEG-injected animals (Fig. 2*E*,*F*), the number of axons with peripheral-type myelin was 2965 ± 1110 ($n = 3$), and in SC/OEG grafts, 3884 ± 711 ($n = 3$). The density of myelinated axons was 75.8 ± 31.9 myelinated axons per 0.1 mm^2 in SC grafts, 32.3 ± 7.01 in OEG grafts, 40.2 ± 21.5 in SC/OEG grafts, and

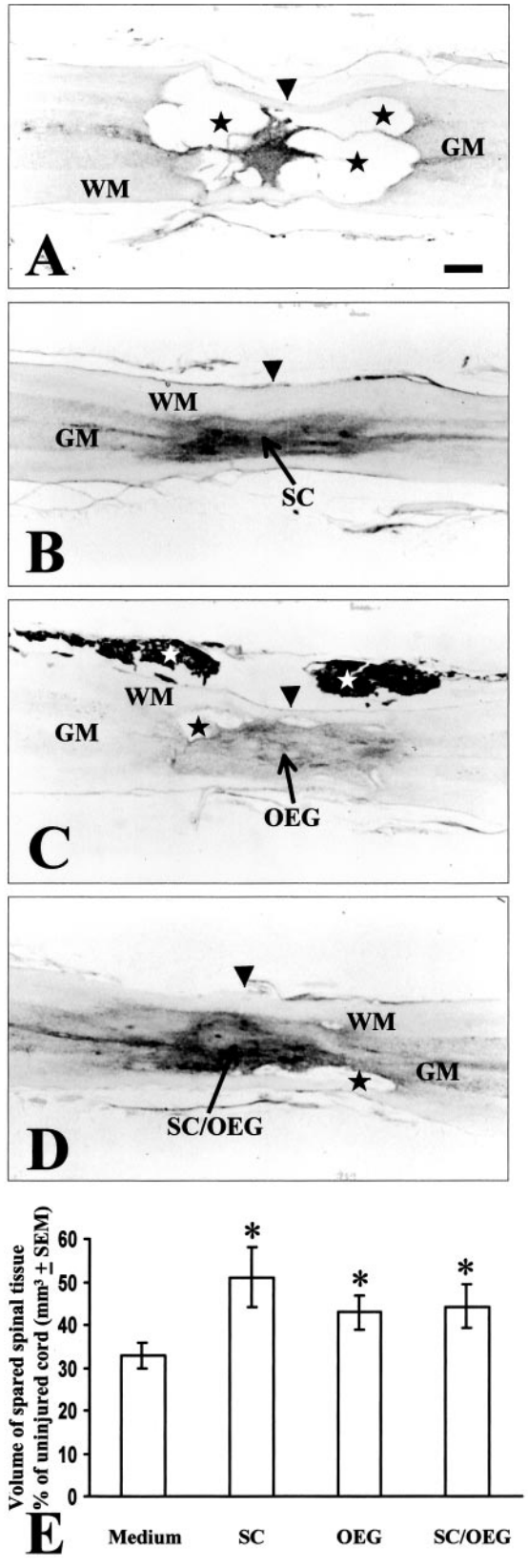

Figure 1. Glial grafts limit contusion-induced spinal tissue damage. Cresyl violet-stained horizontal sections of T9 thoracic spinal cord 3 months after a moderate contusion injury and injection of medium (*A*), SCs (*B*), OEG (*C*), or SCs/OEG (*D*) 1 week after injury. *Arrowheads* indicate the contusion impact site. *Black stars* indicate cystic cavities, and *white stars* indicate coagulated blood between the dura mater and the spinal cord. *GM*, Gray matter; *OEG*, olfactory ensheathing glia transplant; *SC*, Schwann cell transplant; *SC/EOG*, transplant of Schwann cells and olfactory ensheating glia; *WM*, white matter. Scale bar, 50 μm. *E*, Bar graph showing the volume of spared spinal tissue represented as the percentage (±SEM) of a 5-mm-long analyzed spinal segment (T9 cord segment) in an uninjured spinal cord. *Asterisks* indicate a significant difference ($p < 0.05$) from the medium-injected control animals.

26.9 ± 12.2 in medium-injected animals. The number of blood vessels was similar in all groups (Table 1).

In electron micrographs, peripheral-type myelin is recognized by the presence of an external collar of SC cytoplasm, a basal lamina, and collagen fibers that run parallel to the myelin sheath. Central-type myelin does not exhibit these features. The electron micrograph in Figure 3*A* illustrates central- and peripheral-type myelin in tissue next to a cavity. Although in this area from a control animal there is little glial limiting membrane evident, in other areas it was much more prominent at the interface between central tissue and invading SCs. A wide range in degree of glial limiting membrane (basal lamina and highly filamentous astrocytic processes) was observed also in grafted animals. Fasciculation and perineurium formation were not evident in the SC area in Figure 3*A*. In all animals examined, perineurium was only minimally developed at 12 weeks. This was surprising in comparison with other lesion models such as the photochemical lesion, where fasciculation and perineurial development in SC areas are prominent (Bunge et al., 1994). SC-related axons were often clustered around blood vessels. Occasional macrophages were seen in all samples. Near the lesion or transplant, thin central myelin was observed, suggestive of remyelination by oligodendrocytes.

Figure 3*B* illustrates at the electron microscopic level the presence of many axons with typical SC myelin in the middle of an SC graft. Both myelinated and unmyelinated axons were present, and the SCs were surrounded by basal lamina. Axons varied considerably in diameter. In both SC- and SC/OEG-grafted animals, the myelin in the grafts was of the peripheral type. In most SC/OEG- and OEG-grafted animals, it was not possible to identify the OEG. Moreover, in OEG grafts, the myelin was so typical of SCs that it was not possible to determine whether the myelin had been formed by OEG or host SCs. In one area of an SC/OEG graft, however, cells considered likely to be OEG were observed. They exhibited patches of basal lamina and extended processes that meandered among clusters of axons but did not encircle them as SCs do (Fig. 3*C*). These cells were found only in an area of central myelinated axons near the site of implantation and were not seen in SC-only grafts.

Transplanted glia survive and support axonal sparing/regeneration

The glial transplants were identified using immunostaining for p75, the low-affinity nerve growth factor receptor, which stains both SCs and OEG. In control rats, p75 immunoreactivity was found in strands of tissue within the damaged area (Fig. 4*A*). In animals with an SC (Fig. 4*B*), OEG (Fig. 4*C*), or SC/OEG graft, p75 immunoreactivity was observed within the transplanted area. Generally, the p75 staining appeared more intense and extensive in SC grafts than in OEG or SC/OEG grafts.

The presence of reactive astrocytes within the transplanted cord segment was evaluated using antibodies against GFAP. In control animals, a rim of GFAP staining along the surrounding spinal cord tissue delineated the injury area. Similarly, GFAP staining was observed within the spinal cord surrounding the SC

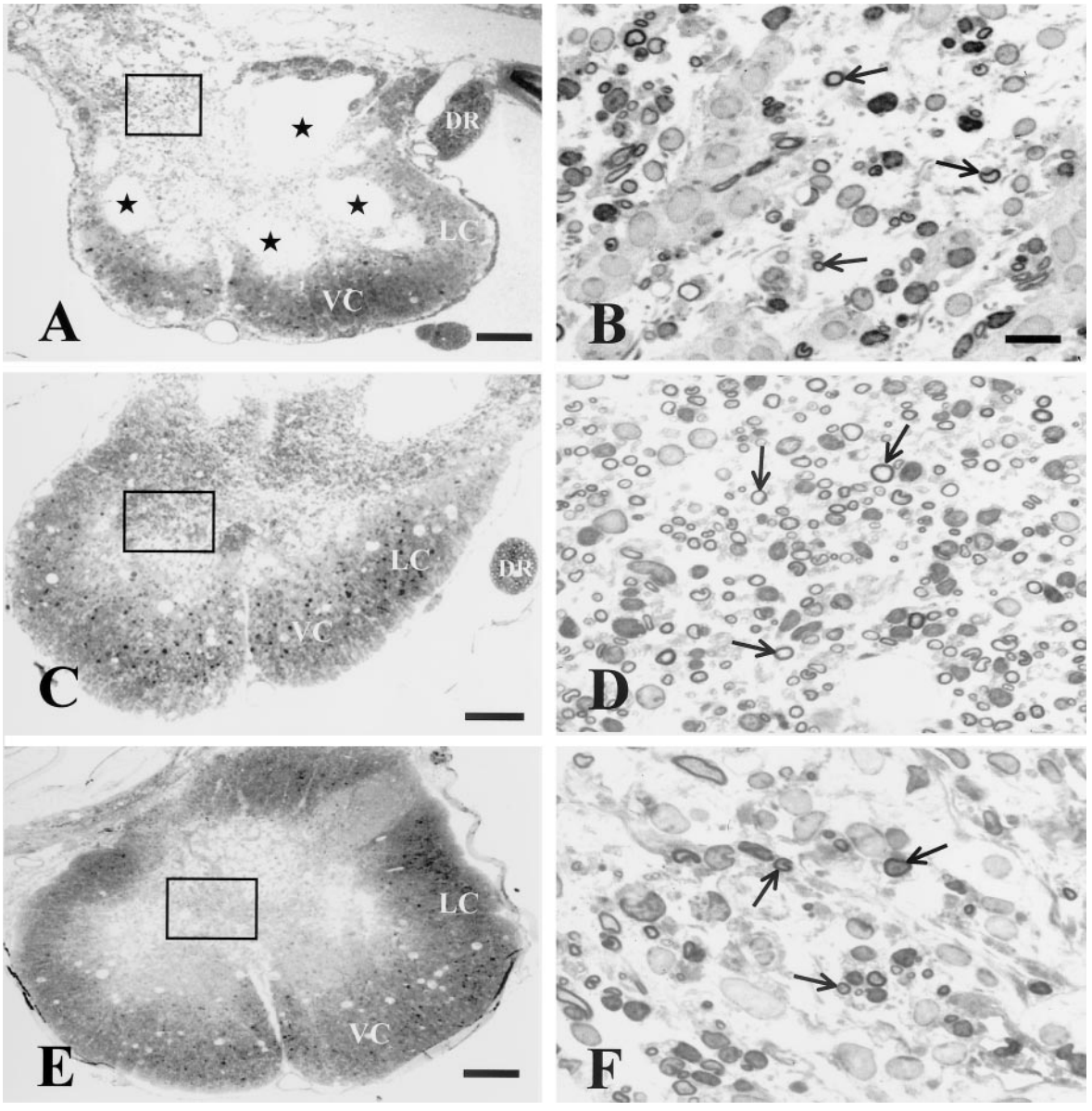

Figure 2. Schwann cell transplants contain numerous myelinated axons in the middle of the graft. All *panels* show toluidine blue-stained, 1-μm-thick plastic sections. *A*, Control, medium-injected animal. *Stars* indicate cystic cavities. *B*, Higher magnification of *boxed area* in *A*, demonstrating that SCs have migrated into the lesion area, as evidenced by the presence of peripheral-type myelin (*arrows*). *C*, SC-transplanted animal. *D*, Higher magnification of *boxed area* in *C*. Numerous myelinated axons (*arrows*) are present within the SC transplant. *E*, OEG-transplanted animal. *F*, Higher magnification of *boxed area* in *E*, demonstrating a few myelinated axons (*arrows*) within the OEG transplant. *DR*, Dorsal root; *LC*, lateral white column; *VC*, ventral white column. Scale bars: *A*, *C*, *E*, 300 μm; *B*, *D*, *F*, 20 μm.

(Fig. 4*D*), OEG (Fig. 4*E*), and SC/OEG grafts (Fig. 4*F*). Some GFAP reactivity was found within the transplants. Generally, GFAP staining was more intense in SC-containing grafts (Fig. 4*G*,*I*) than in OEG grafts (Fig. 4*H*).

The presence of chondroitin sulfate proteoglycan (CSPG) within the grafted cord segment was studied using the CS-56 antibody. In control animals, some CSPG staining was found within the injury area. All three types of glial grafts and the surrounding spinal tissue displayed CSPG immunoreactivity. Generally, SC grafts (Fig. 4*J*) displayed more intense CSPG immunostaining than OEG (Fig. 4*K*) or SC/OEG grafts (Fig. 4*L*).

Intense neurofilament (Fig. 4*M*) and GAP-43 immunostaining, indicating the presence of numerous axons, was present in SC (Martin et al., 1991, 1996), OEG, and SC/OEG grafts. Different antibodies were used to examine phenotypes of these axons. Anti-CGRP antibodies revealed that ascending sensory axons (Gibson et al., 1984) had grown into and across, but not beyond, the grafts (Fig. 4*N*). CGRP-positive axons were also found within the surrounding white matter. Anti-5-HT antibodies revealed that some serotonergic axons, from the raphe nuclei in the brainstem (Newton and Hamill, 1988), had grown into the grafts (Fig. 4*O*). 5-HT-positive axons were also present in the rostral gray matter and in the lateral white matter (Fig. 4*O*). Adrenergic axons, most likely originating from locus ceruleus neurons and identified with anti-DβH-antibodies (Newton and Hamill, 1988), were found in spared white matter but not in the transplants or beyond the distal interface. These observations indicate that the transplants

Table 1. Estimated numbers of myelinated axons and blood vessels in contusion lesion areas

Transplant[a]	Myelinated axons[b]	Surface area[c]	Density[c]	Blood vessels[b]
Medium				
(1)	3442	0.76	45.3	121
(2)	1863	0.59	31.6	100
(3)	1072	2.69	3.99	128
Mean ± SEM	2125 ± 697	1.35 ± 0.67	26.9 ± 12.2	116 ± 8
SC				
(1)	6991	0.65	107.6	174
(2)	3433	0.78	44	67
Mean ± SEM	5212 ± 1783	0.72 ± 0.07	75.8 ± 31.9	120 ±54
OEG				
(1)	3390	0.83	40.8	133
(2)	866	0.47	18.4	87
(3)	4639	1.23	37.7	196
Mean ± SEM	2965 ± 1110	0.84 ± 0.22	32.3 ± 7.02	138 ± 32
SC/OEG				
(1)	5222	0.63	82.9	189
(2)	2800	1.82	15.4	272
(3)	3632	1.62	22.4	176
Mean ± SEM	3884 ± 711	1.36 ± 0.37	40.2 ± 21.5	212 ± 303

[a] The total number of injected cells was 2×10^6 in 6 μl of medium. All animals received a moderate contusion 1 week before transplantation.

[b] The numbers of myelinated axons and blood vessels in the contusion lesion area were estimated by image analysis using the fractionator method of the Stereoinvestigator program. The numbers of all animals counted are presented as well as the means ± SEM. One micrometer plastic sections were used.

[c] The surface area is expressed in millimeters squared. The density is expressed as the number of axons per 0.1 mm^2.

contain some sensory and serotonergic axons, but most of the axons appear to originate from spinal cord neurons.

More axons are present beyond the lesion level with SC-containing than OEG grafts

Retrograde neuronal tracing analysis

Axonal sparing/regeneration of propriospinal and supraspinal projections was examined using retrograde FB neuronal tracing. Figure 5, *A* and *B*, shows representative examples of FB-labeled neurons in the T8–7 cord segment in control and SC-grafted rats, respectively. The number of FB-labeled neurons in the T8–7, T2, C6, and C2 cord segments in SC and SC/OEG transplanted animals, but not in OEG-only grafted animals, was significantly higher ($p < 0.05$) than in controls (Fig. 5*C*). Figure 5, *D* and *E*, demonstrates FB-labeled cells within the reticular formation of control and SC/OEG-grafted animals, respectively. Within the brainstem, the total number of FB-labeled neurons was significantly higher ($p < 0.05$) in animals receiving SC or SC/OEG, but not OEG, grafts compared with controls (Fig. 5*F*). Although FB-labeled neurons were also found within the cerebral cortex of control (Fig. 5*G*) and grafted (Fig. 5*H*) animals, no significant difference was found in the total number of FB-labeled neurons in any of the groups (Fig. 5*I*). These results indicate that SC and SC/OEG grafts but not OEG grafts placed within a 1 week moderately contused thoracic spinal cord promote the sparing/regeneration of propriospinal and supraspinal axons. None of the grafts appeared by this measure to affect sparing/regeneration of corticospinal axons.

Anterograde axonal tracing analysis

The presence of corticospinal axons in the grafted area was evaluated using anterograde axonal tracing. At 12 weeks after injury, most of the corticospinal axons were present rostral to the grafted area and had formed axonal end bulbs, indicating ongoing degeneration (Tator, 1995). In all groups, corticospinal axons had extended thin sprouts into the nearby gray matter and into the transplants (Fig. 6*A*). Quantification revealed that a significantly higher number of corticospinal sprouts was present at 2 mm rostral to the lesion epicenter in grafted animals compared with control animals (Fig. 6*B*). The numbers found at 1 mm rostral to the epicenter and at the epicenter did not significantly differ between groups (Fig. 6*B*).

Axonal sparing/regeneration of reticulospinal and vestibulospinal fibers was examined using anterograde axonal tracing. In control and grafted rats, reticulospinal (Fig. 6*C*) and vestibulospinal (Fig. 6*D*) axons were observed in the peripheral spinal white matter but rarely in the grafts.

Transplantation of SC or SC/OEG 1 week after moderate contusion improves hindlimb performance

All animals exhibited a gradual improvement in hindlimb locomotor function during the 11 week period after cell transplantation (Fig. 7). In all experimental groups, most rats recovered to a BBB score of 10–11; they exhibited occasional (10) or frequent to consistent (11) weight-supported plantar stepping. Frequent to consistent weight-supported plantar stepping with occasional to frequent forelimb–hindlimb coordination (BBB score of 12) was observed only in SC-only grafted animals. Statistical analysis indicated that the open field locomotor scores of the SC-transplanted group at 8, 9, 10, and 11 weeks after injury and of SC/OEG at 8 weeks after injury were significantly higher ($p < 0.05$) than the control group.

DISCUSSION

The axonal growth-promoting effects of SCs (for review, see Bunge, 2001; Plant et al., 2001) and OEG (for review, see Franklin and Barnett, 2000; Kleitman and Bunge, 2000; Ramón-Cueto, 2000; Plant et al., 2001; Raisman, 2001) have been studied in different adult rat spinal cord injury models. Here, we have investigated and compared the restorative abilities of SC and OEG transplants grafted into a 1-week-old moderate contusion lesion in the adult rat thoracic spinal cord at 12 weeks after injury. All grafts diminished injury-induced cavitation. The results indicate that a SC graft is more effective in promoting spinal tissue sparing and SC-containing grafts are more effective in promoting sparing/regeneration of spinal and supraspinal axons than an OEG graft. All grafts promoted axonal growth, but SC grafts exhibited the greatest number of myelinated axons. Moreover, with an SC graft but not with an OEG graft, hindlimb performance of the contused rats was significantly improved 8 weeks after transplantation.

Retrograde neuronal tracing demonstrated that with SC and SC/OEG grafts, but not with OEG-only grafts, more propriospinal and brainstem neurons projected distal to the graft. We do not know whether the grafts promoted axonal sparing, i.e., reduced injury-induced axonal dieback, or axonal regeneration, or both. Certainly, some regeneration had occurred because axons were present in the lesion epicenter where the glia had been injected 1 week after injury. Also, because a contusion injury generally obliterates the corticospinal tract (Hill et al., 2001), the presence of corticospinal tract fibers in the graft implies regeneration, with

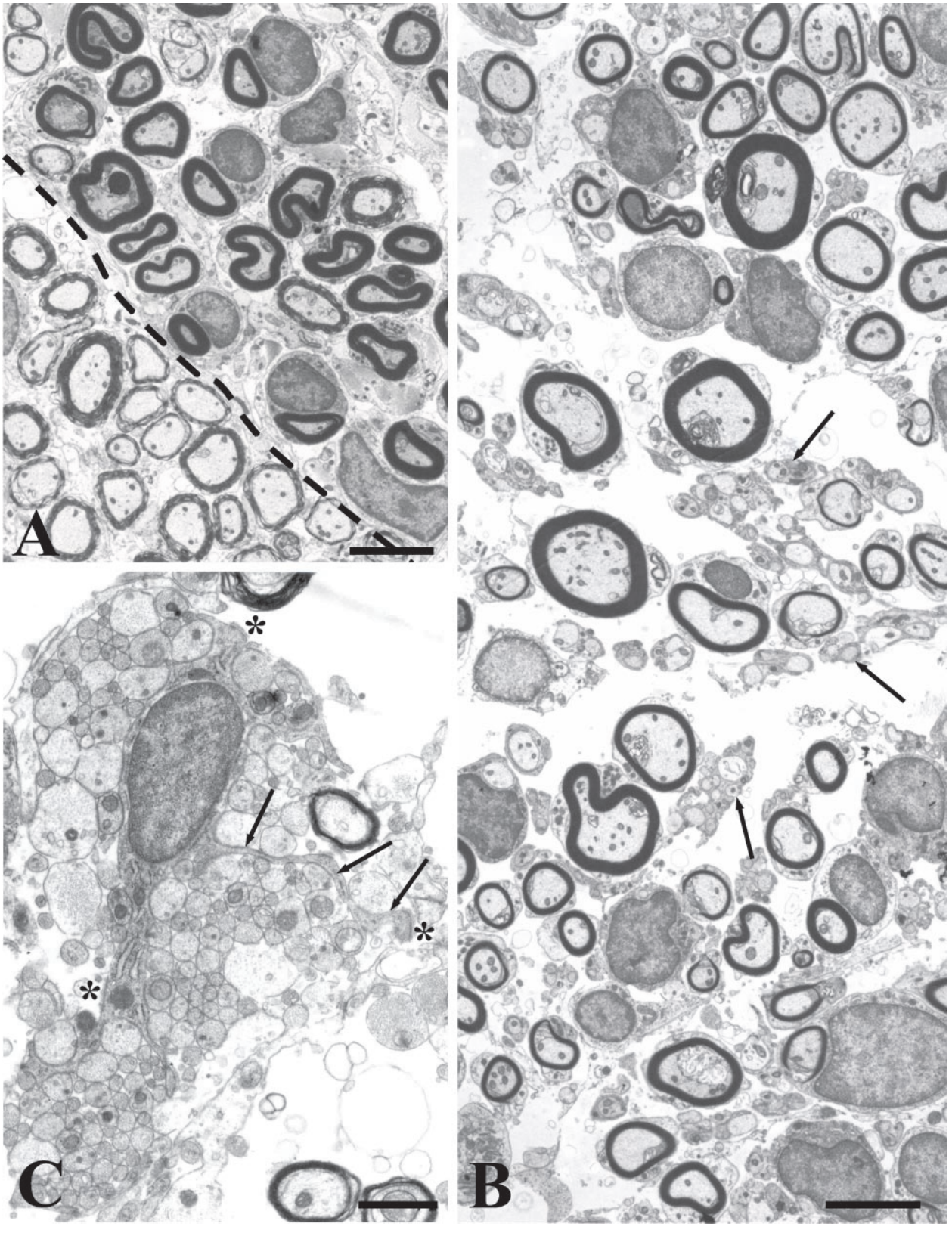

Figure 3. SC-containing grafts contain more myelinated axons than OEG-only grafts. *A*, SC-myelinated axons (at the *right* of the *dashed line*) are found in the cord near the cavity in a control animal, showing that host SCs migrated into the spinal cord as well as into the lesion site. *B*, After grafting of SCs, numerous SC-myelinated axons were observed in the lesion area at 12 weeks. In addition, many axons of small diameter (*arrows*) were typically ensheathed by nonmyelinating SCs. *C*, A few cells that resemble OEG were found after SC/OEG grafting in areas near the lesion where central myelin was evident. These are tentatively identified as OEG because, despite numerous naked axons nearby, only meandering (*arrows*) rather than clearly ensheathing processes extended from the cells. The cells exhibited patches of basal lamina (*asterisks*) indicating that these cells were not oligodendrocytes. All *panels* show electron micrographs. Scale bars: *A*, *B*, 4 μm; *C*, 1 μm.

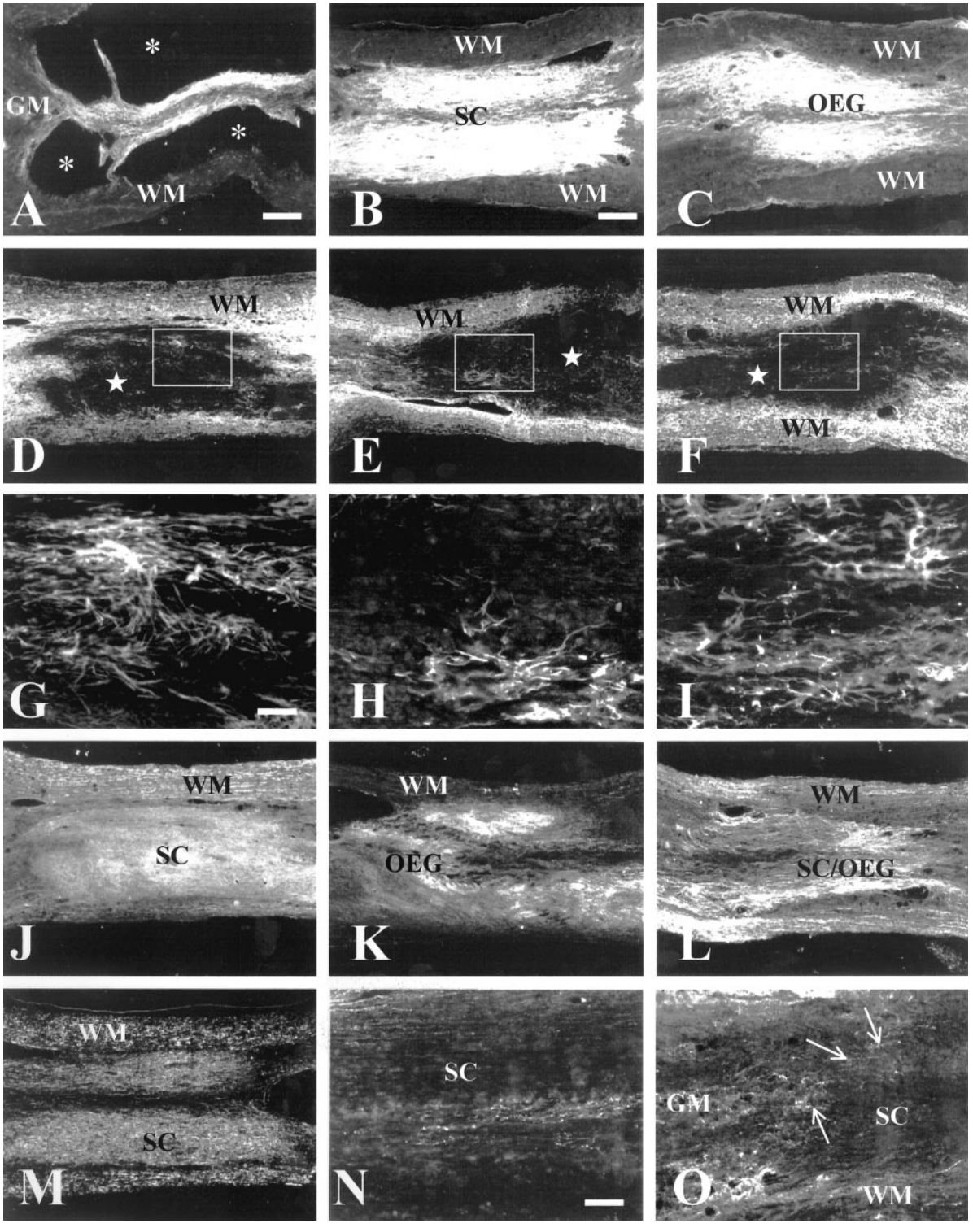

Figure 4. Transplanted glia survive, express CSPG, and promote sparing/regeneration of axons. All *panels* show photographs taken from horizontal sections of the T9 cord segment 3 months after injury. The presence of p75-positive cells is shown in medium- (*A*), SC- (*B*), and OEG- (*C*) injected animals. Note the presence of p75-positive cells in the trabecula of spinal tissue in the cavity (*asterisks*) in the medium-injected animal. A rim of GFAP-positive reactive astrocytes was present within the spinal cord tissue surrounding the SC (*D*), OEG (*E*), or SC/OEG (*F*) graft. *Stars* indicate the transplants. GFAP staining was also present within the SC (*G*), OEG (*H*), and SC/OEG (*I*) grafts. *G*, *H*, and *I* are higher magnifications of the *boxed areas* in *D*, *E*, and *F*, respectively. Generally, GFAP staining was more intense in SC-containing grafts than in OEG-only grafts. The presence of CSPG is demonstrated in an SC (*J*), OEG (*K*), or SC/OEG (*L*) graft but was most intense in the SC graft. Also, CSPG was present in the surrounding spinal tissue in animals receiving SC-containing grafts. All transplants contained many neurofilament-positive axons as demonstrated in *M*, an SC graft. Some of the axons within the grafts were CGRP positive as shown in an SC transplant (*N*). Serotonergic axons were present within gray matter just rostral to an SC graft (*O*), with some penetrating the transplant (*arrows*). *GM*, Gray matter; *WM*, white matter; *OEG*, olfactory ensheathing glia transplant; *SC*, Schwann cell transplant; *SC/OEG*, transplant containing Schwann cells and olfactory ensheathing glia. Scale bars: *A*, 350 μm; *B–F*, *J–M*, 400 μm; *G–I*, 100 μm; *N*, *O*, 200 μm.

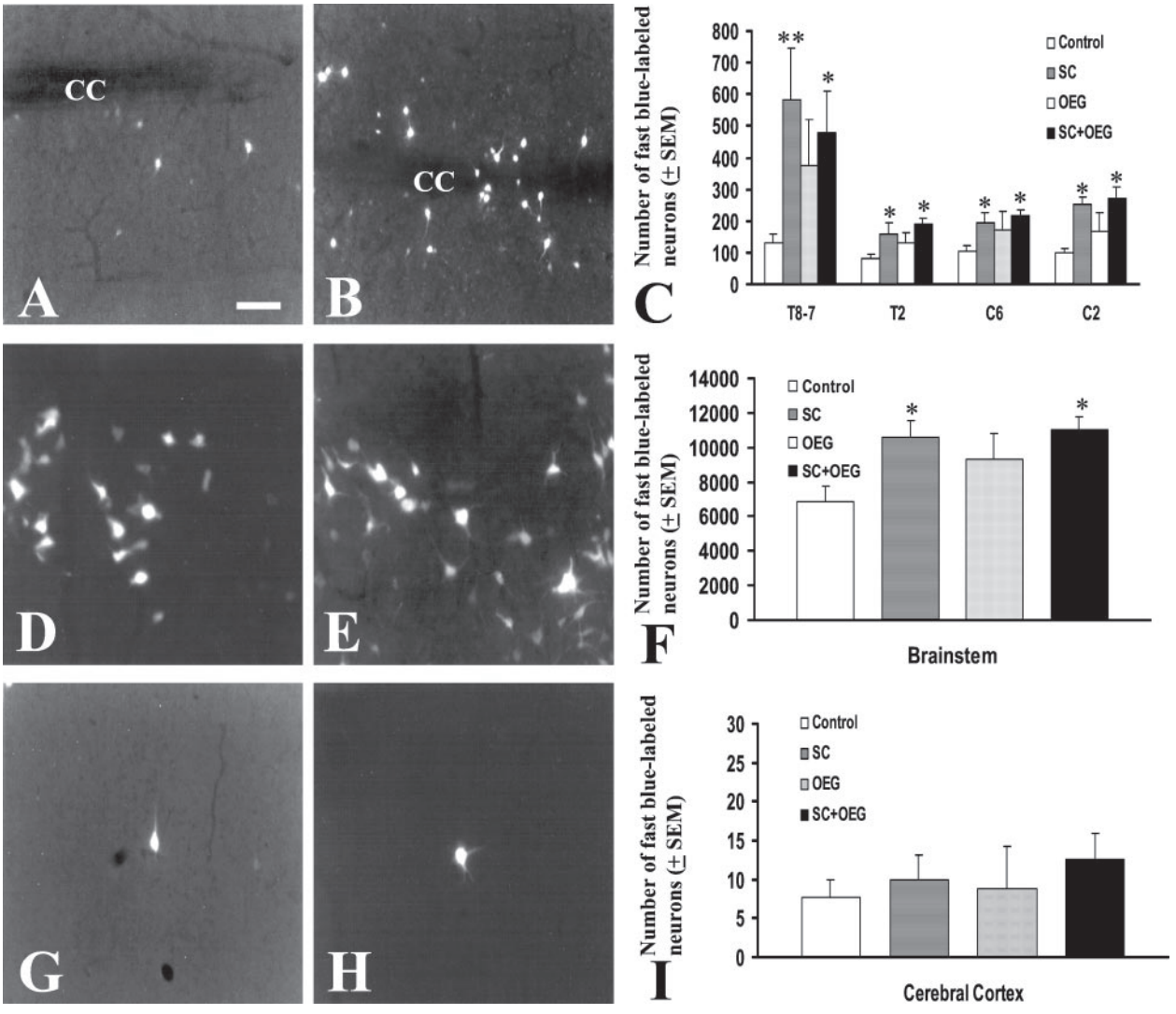

Figure 5. SC-containing grafts promote the most sparing/regeneration of propriospinal and supraspinal axons. FB-labeled neurons were present in the T8–7 cord segment in medium- (*A*) and SC- (*B*) injected animals. *C*, Bar graph showing the total number of FB-labeled neurons (±SEM) in different levels of the spinal cord. FB-labeled neurons in the reticular formation in medium- (*D*) and SC/OEG- (*E*) injected animals. *F*, Bar graph showing the total number of FB-labeled neurons (±SEM) in the brainstem. FB-labeled neuron in the cerebral cortex in medium- (*G*) and OEG- (*H*) injected animals. *I*, Bar graph showing the total number of FB-labeled neurons (±SEM) in the cerebral cortex. *Single asterisks* indicate a significant difference of $p < 0.05$ from the medium-injected control animals; *double asterisks* indicate a significant difference of $p < 0.01$ from the medium-injected control animals. *CC*, Central canal. Scale bar: *A*, *B*, 100 μm; *D–H*, 50 μm.

the caveat that there could be sprouting from spared lateral and ventral corticospinal fibers.

All three types of implants were found to promote spinal tissue sparing at 12 weeks after injury. The SC graft appears to be more effective in reducing spinal tissue damage than the SC/OEG or OEG-only graft. SC-induced tissue sparing may have led to less damage to spinal white matter, which could explain the presence of more propriospinal and brainstem projections distal to the graft. SCs produce various growth factors such as nerve growth factor (Bandtlow et al., 1987), brain-derived neurotrophic factor (Acheson et al., 1991; Meyer et al., 1992), ciliary neurotrophic factor (Friedman et al., 1992; Meyer et al., 1992; Rende et al., 1992), and glial cell line-derived neurotrophic factor (Widenfalk et al., 2001). Although OEG share many properties with SCs, they are believed to be a distinct cell type (Lakatos et al., 2000). At present, convincing evidence that OEG harvested from the adult olfactory bulb produce neurotrophic factors is meager (Ramón-Cueto and Avila, 1998; Kleitman and Bunge, 2000). Although reports have differed on the production of neurotrophic factors by OEG (for review, see Kleitman and Bunge, 2000), it is now clear that these cells express several factors and their receptors (Boruch et al., 2001; Woodhall et al., 2001). We speculate that in the present experiment, SCs produced more supportive factors than OEG, which may have caused the observed difference in tissue sparing.

The secretion of various axonal growth-promoting factors has made the SC a widely used successful cellular substrate for repair strategies in the adult spinal cord (Martin et al., 1991, 1996; Paino et al., 1994; Xu et al., 1995a,b, 1997, 1999; Chen et al., 1996; Montgomery et al., 1996; Guest et al., 1997; Oudega et al., 1997). OEG have also been shown to promote regeneration in different injury models in the adult rat spinal cord (Ramón-Cueto and Nieto-Sampedro, 1994; Li et al., 1997, 1998; Ramón-Cueto et al., 1998, 2000). With OEG implanted in the cord beside a transection

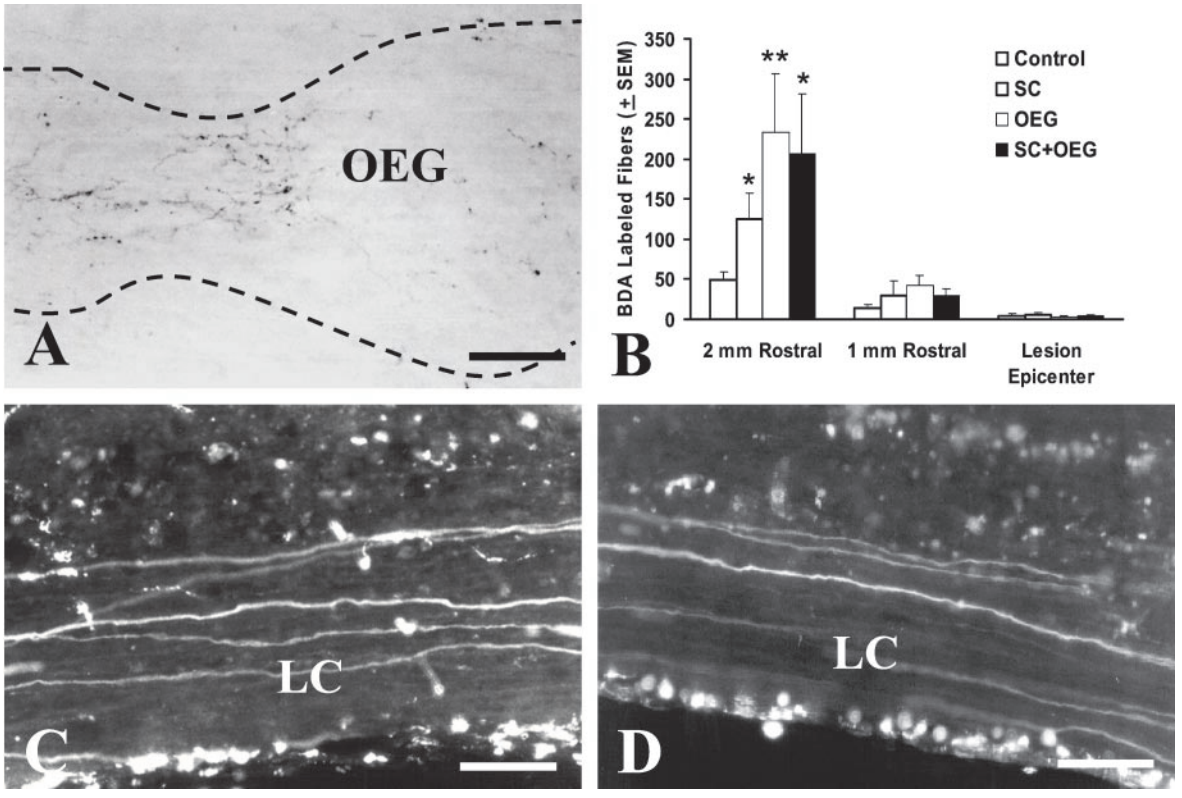

Figure 6. Glial grafts promote corticospinal axon sparing/regeneration. All *panels* except *B* show photographs taken from horizontal 40-μm-thick sections. *A*, BDA-labeled corticospinal axons in an OEG transplant (*outlined*). *B*, Bar graph showing the number of BDA-labeled axons (±SEM) at different levels within the glial transplants. *Single asterisks* indicate a significant difference of $p < 0.05$ from the medium-injected control animals; *double asterisks* indicate a significant difference of $p < 0.01$ from controls. *C*, Dextran-rhodamine-labeled vestibulospinal axons in the lateral white matter columns (*LC*) at the level of the lesion epicenter. *D*, Dextran-fluorescein-labeled reticulospinal axons in the lateral white matter columns (*LC*) at the level of the lesion epicenter. Scale bars, 100 μm.

(Ramón-Cueto et al., 2000) or a transection filled with an SC graft (Ramón-Cueto et al., 1998), axons were found to cross the injury site and grow into the spinal cord. This finding may be related to the ability of OEG to migrate away from the injury site into the spinal nervous tissue. SCs do not have such migratory ability (Xu et al., 1995a, 1997; Iwashita et al., 2000).

In our transplantation model, the SCs and OEG were injected into a 1 week moderate contusion injury. Ongoing cytotoxic processes within the contusion environment may be harmful to the grafted cells. These processes include excitotoxic, inflammatory, proteolytic, and anoxic events, which are all components of progressive secondary injury (Tator and Fehlings, 1991; Anderson and Hall, 1993; Amar and Levy, 1999). Our results suggest that OEG may have been more susceptible to these cytotoxic events than SCs. We were not able to assess survival of OEG by morphological means because of the difficulty in definitively identifying OEG without a specific label. Both SCs and OEG are stained by p75 antibody. OEG have been reported to closely resemble SCs when they remyelinate axons in demyelinated cord (Imaizumi et al., 1998, 2000). We found only a few cells that might be identified as OEG on the basis of the pattern of ensheathment of axon bundles observed in the electron microscope. At 12 weeks after injury, SC transplants were generally in better continuity with the spinal tissue than OEG transplants. These observations may explain why SC transplants were more effective in limiting injury-induced tissue damage, in promoting axonal sparing/regeneration, and in forming more myelin than an OEG

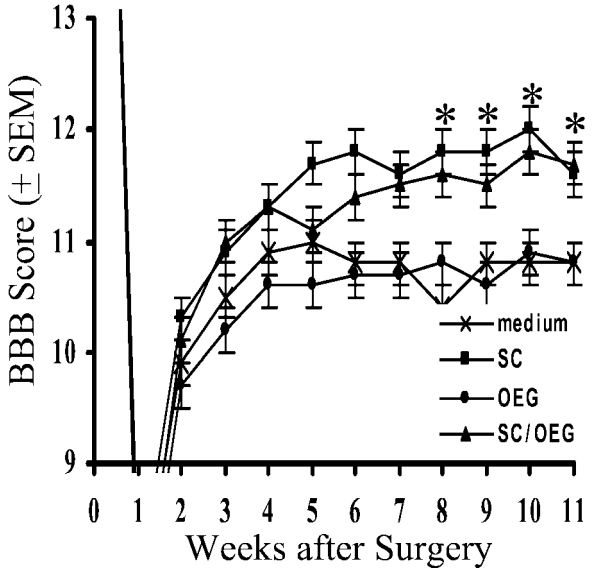

Figure 7. Transplantation of SC or SC/OEG 1 week after moderate contusion improves hindlimb performance. *Bar graph* shows the average open-field BBB score at different times after injury. Locomotor function of the hindlimbs was evaluated blindly once a week for 11 weeks. Data are presented as means ± SEM. *Single asterisks* indicate a significant difference ($p < 0.05$) from the medium-injected control animals. Compared with the control group, the SC-transplanted group had a significantly higher BBB score at 8–11 weeks after grafting. The SC/OEG group had a significantly higher score only at one time point, 8 weeks, after grafting.

transplant. Also, it should be kept in mind that the SC graft contained twice as many SCs as the SC/OEG graft.

It was not possible to decide whether the axons with peripheral-type myelin in the transplant area were myelinated by SCs or OEG. Axons with peripheral myelin were found in the nontransplanted animals in this study as well as in several previous studies conducted in our laboratory as well as others, showing that SCs migrate into lesion areas and myelinate axons there. In SC/OEG transplants, cells located in areas of central-type myelin near the implantation site exhibited patches of basal lamina and extended processes that meandered among clusters of axons but did not encircle them as SCs typically do (Fig. 3*C*). This type of ensheathment more closely resembles that of OEG *in vivo* (for review, see Kleitman and Bunge, 2000). Possibly, these cells are OEG that migrated into spinal tissue. Many papers have reported myelination by OEG *in vivo* (Franklin et al., 1996; Li et al., 1997, 1998; Imaizumi et al., 1998, 2000; Barnett et al., 2000; Kato et al., 2000). Possibly, less myelin was formed in OEG transplants in our study because the environment of the contusion cavity had damaged them. However, a recent study from our laboratory has shown that OEG do not form myelin under the same culture conditions that are favorable for SC myelination (Plant, Cuervo, Bates, Bunge, and Wood, unpublished observations). In future studies, transplantation of labeled OEG is needed to prove the ability of OEG to produce myelin.

Both types of SC-containing grafts improved hindlimb function, but the SC-only graft was the only group consistently statistically greater than controls. This finding is in agreement with our observation that SC grafts resulted in a higher number of spinal and supraspinal axons reaching spinal segments distal to the grafted area compared with OEG grafts. It was shown previously that hindlimb recovery after an incomplete SCI depends on the number of spared and regenerated descending axons from brainstem nuclei and cerebral cortex (Saruhashi and Young, 1994; Basso et al., 1996) and from local propriospinal axons (Goldberger, 1988a,b; Helgren and Goldberger, 1993). Interestingly, it appears that only a small percentage of the descending brainstem axons is needed to drive the segmental circuits involved in the generation of basic locomotion patterns (Helgren and Goldberger, 1993; Basso et al., 1996; Ribotta et al., 2000). Thus the increased sparing of tissue seen in the present study could explain the observed improvement in functional recovery. Alternatively, the improvement in behavioral outcome may have been caused, at least in part, by remyelination of spared axons by the transplanted SCs. It was suggested previously that functional recovery seen in rats transplanted with embryonic stem cells was caused by remyelination of axons by oligodendrocytes deriving from the grafted stem cells (McDonald et al., 1999). In the present study, it was not possible to address this question because the transplanted cells were not prelabeled; even if the cells had been prelabeled, it would not have been possible to identify the myelinated axon as spared or regenerated.

Both SCs and OEG have emerged as important candidates for future cell transplantation strategies in the injured adult spinal cord because of their axonal growth-promoting and myelinating properties. The present study directly compared these cell types individually and in combination and shows that SC grafts have the greatest effect on protecting contused spinal tissue. Another clinically relevant advantage is that SCs can be obtained from a spinal cord injured person's peripheral nerve and produced in very large numbers in culture, which might allow autologous transplantation into the injured human cord. A potential limitation of SC grafts is that they do not promote reentry of axons into the spinal cord, which is necessary for reestablishing axonal circuits that are involved in locomotion. OEG have shown promise in improving such reentry, but we show here that they may be less effective in promoting axonal growth and myelination than SCs when transplanted into the contusion lesion. An alternative approach that would take advantage of the regeneration-promoting characteristics of both cell types is to graft SC into the contusion lesion and OEG into the adjacent spinal tissue. The restorative ability of this combination strategy in promoting axonal regeneration/sparing and improving hindlimb function is currently being investigated in our laboratory.

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