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Aspiration of a cervical spinal contusion injury in preparation for delayed peripheral nerve grafting does not impair forelimb behavior or axon regeneration

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Abstract

A peripheral nerve graft model was used to examine axonal growth after a unilateral cervical (C) contusion injury in adult rats and to determine if manipulation of an injury site prior to transplantation affects spontaneous behavioral recovery. After a short delay (7d) the epicenter of a C4 contusion was exposed and aspirated without harming the cavity walls followed by apposition with one end of a predegenerated tibial nerve to the rostral cavity wall. After a longer delay (28d) the aspirated cavity was treated with GDNF to promote regeneration by chronically injured neurons. In both groups forelimb and hindlimb locomotor scores decreased significantly 2d after lesion site manipulation, but by 7d, the forelimb score was not different from the pre-manipulation score. There was no significant difference in grid walking or grip strength scores for the affected forelimb in either group 7d after contusion vs. 7d after manipulation. Over 1500 brain stem and propriospinal neurons grew axons into the graft with either delay. These results demonstrate that a contusion injury site can be manipulated prior to transplantation without causing long-lasting forelimb or hindlimb behavioral deficits and that peripheral nerve grafts support axonal growth after acute or chronic contusion injury.

Keywords

spinal cord injury; cervical contusion; peripheral nerve graft; forelimb behavior; CNS regeneration

Introduction

Contusive spinal cord injury (SCI) results in damage to ascending and descending spinal pathways in addition to loss of interneurons, motoneurons and associated glial cells; all contributing to significant functional impairment. Shortly following injury, within hours to days, the lesion site begins to distend due to hemorrhage and accumulation of necrotic tissue (Beattie et al., 2000). Over the next few weeks to months the lesion transforms into a cystic cavity enclosed by glial scar tissue. While this scarring restricts further expansion of the injury, it also forms a physical and chemical barrier to growth of regenerating axons (McKeon et al., 1991; Davies et al., 1999; Fawcett and Asher, 1999; Yiu and He, 2006).

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A variety of therapeutic strategies designed to restore lost function have been implemented during acute and chronic stages of injury (for review see Schwab et al., 2006; Thuret et al., 2006), with transplantation being one of the principle approaches utilized to promote regeneration of damaged axons (Bregman, 1994; Murray and Fischer, 2001; Bunge and Pearce, 2003). Providing a supportive substratum is necessary to achieve extension of axons into and through an otherwise obstructive lesion site, and segments of predegenerated peripheral nerves are an excellent resource for growing axons of both peripheral and central nervous system origin (Richardson et al., 1980; David and Aguayo, 1981). Peripheral nerve grafts (PNG) have been used successfully in acute and chronic injury situations after creation of an intraspinal lesion cavity (Houle, 1991; Decherchi et al., 1996; Ye and Houle, 1997; Gauthier et al., 2002). Regenerating axons readily enter the graft and recently it has been shown that modulation of the extracellular matrix associated with the PNG-spinal cord interface allows for the extension of regenerating axons back into the spinal cord (Houle et al., 2006).

Since the properties of the lesion site evolve over an extended post injury period, it is unclear how manipulation of the injury site during different phases of secondary degeneration likely will affect the potential for recovery after injury. Conventional wisdom would suggest that manipulation of the injury site in preparation for transplantation should be limited to prevent damage of spared tissue, damage to sprouted or regenerated axons or exacerbation of functional impairment (Silver and Miller, 2004; Bakshi et al., 2006; Fawcett, 2006). Indeed, one topic of debate is whether aggressive removal of scar tissue at a chronic lesion site is necessary to achieve sufficient axonal extension across the scar-infested lesion site and whether such reinjury might be more harmful (in terms of functional loss) than beneficial. A recent study by Lu et al. (2007) suggests that a chronic spinal cord lesion does not form an impenetrable barrier; however, this may not be the optimal situation for regeneration, particularly when dealing with a contusion lesion that has ill-defined borders when the epicenter is examined without manipulation. The present study addressed the question of whether debris could be safely removed after a short (7d) or long (28d) post injury period without compromising the regenerative effort of injured propriospinal and supraspinal neurons. We also were interested in determining whether manipulation of a contusion lesion site in preparation for transplantation results in additional loss of functional capacity to an extent that would discourage adoption of this approach to repair the injured spinal cord in an acute or chronic situation.

Materials and Methods

Spinal cord injury, cavity preparation and peripheral nerve grafts

Adult (225–250 g) female Sprague-Dawley rats were anesthetized with a ketamine (60 mg/kg) and xylazine (6 mg/kg) mixture. A partial cervical level 4 (C4) laminectomy was prepared and the vertebral column stabilized by clamping the C3 and C5 vertebral bodies with forceps fixed to the base of an Infinite Horizon Impact Device (Precision Systems and Instrumentation, Lexington, KY). Animals were placed on the impactor and the 1.6 mm stainless steel tip positioned over the right side of the C4 spinal cord midway between the medial dorsal vein and the lateral edge of the spinal cord. The impactor tip was lowered to 3–4 mm above the dorsal surface of the spinal cord and the field flooded with sterile saline up to the impactor tip. A unilateral contusion injury was created by an impact force of 200 Kdynes and tissue displacement to a depth of 1600–1800 μ m. After injury, animals were released from the clamping forceps and overlying muscles closed with sutures. The skin incision was closed with wound clips. Animals were given ampicillin (100 mg/kg) and the analgesic buprenorphine (0.05 mg/kg) for 3 days post operatively. Animals receiving a peripheral nerve graft were given daily injections of cyclosporine A (CsA, 10 mg/kg, s.c., Sandimmune, Novartis Pharmaceuticals Corp., East Hanover, NJ) beginning 3 days prior to placement of the PN graft. After 2 weeks

of CsA injections animals were switched to oral administration (100 mg/kg in drinking water) for the remainder of the study. All procedures were performed in accordance with protocols approved by the Drexel University College of Medicine's Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines for the care and use of laboratory animals.

There were 4 groups of animals used in this study:

Group 1—Sacrificed 7d after C4 contusion (n=4).

Group 2—Sacrificed 28d after C4 contusion (n=4).

At the appropriate post injury period animals were given an overdose of Euthasol (390 mg/kg sodium pentobarbital and 50 mg/kg phenytoin, ip.) and perfused transcardially with 4% paraformaldehyde in 0.1M Sorenson's phosphate buffer. A length of spinal cord between C3 and C5 was removed, post-fixed in paraformaldehyde at 4°C for 4 h, and then submersed in 30% sucrose for 36 h at 4°C. Four series of alternating sections at 25 μ m were prepared with a cryostat in a transverse plane through the rostral to caudal extent of the lesion or in a horizontal plane through the dorsal to ventral extent of the lesion. Sections from each respective series were mounted on a glass slide and stained for Nissl-myelin histology or for immunocytochemical detection of astrocytes and phagocytic cells involved in the immune system response to SCI (see below).

Group 3—7d delay before manipulation of contusion epicenter and grafting of peripheral nerve segment (n=7). The contusion lesion epicenter was exposed after 7d by incising the overlying dura mater in a longitudinal plane. Aspiration through a glass micropipette was used to remove tissue debris and fluid from the contusion site to form a cavity capable of accommodating one end of a 15 mm long segment of pre-degenerated tibial nerve. Care was taken to restrict aspiration of tissue to the epicenter and not to expand the lesion in a rostral or caudal manner. The nerve graft was harvested from a donor animal 7d after it had been cut distal to its branch point from the sciatic nerve. Perineurium was stripped from one end of the graft and 10-0 sutures through perineurium and dura mater were used to secure the graft in apposition to the rostral cavity wall. The distal end of the graft was left free and unapposed to nervous or muscle tissue. Three weeks after placement of the PN graft the distal end was exposed (under isoflurane anesthesia), trimmed by 1 mm and covered by gel foam saturated with 2% True Blue (TB, Sigma, St. Louis, MO). Fresh TB-gel foam was provided at 20 minute intervals for 60 minutes of total labeling time. Muscles and skin were closed as before. Three days later animals were sacrificed with an overdose of Euthasol and perfused transcardially with 4% paraformaldehyde in 0.1M Sorenson's phosphate buffer. The entire brain and C1 to C5 spinal cord with attached PN graft were removed, post-fixed at 4°C for 4 h, and then submersed in 30% sucrose for 36 h at 4°C. Cryostat sections at 50 μ m in a frontal plane were prepared in serial order from the medulla through the cerebral cortex and sections in a sagittal plane were prepared in serial order through the spinal cord block, mounted on glass slides, air dried and cover slipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sagittal sections through the PN graft-spinal cord interface were stained for Nissl-myelin histology.

Group 4—28d delay before manipulation of contusion epicenter and grafting of peripheral nerve segment (n=7). The contusion lesion epicenter was exposed after 28d and the overlying dura mater opened in a longitudinal plane. Preparation of a lesion cavity to accommodate a PN graft was performed as described for group 3. Previous studies demonstrated poor axonal growth into a PN graft placed 28d after a cervical hemisection lesion (see Ye and Houle, 1997) compared to an acute or short delay (7d) graft. Consequently, to provide the best

opportunity to support axonal regeneration gel foam saturated with glial cell line-derived neurotrophic factor (GDNF, 10 µg/ml, R&D Systems, Minneapolis, MN) was placed into the lesion cavity to promote axonal growth in this chronic injury situation (as per Dolbeare and Houle, 2003). Fresh GDNF-gel foam was placed into the cavity 3 times at 20 min intervals then gel foam was removed and one end of a pre-degenerated donor graft was placed into the cavity. All other procedures for this group were performed as described for group 3.

Immunocytochemistry and image analysis

Brain and spinal cord sections were examined by fluorescence microscopy to detect the number and location of TB-labeled brain and propriospinal neurons that had grown axons into the PN graft. The total number of neurons labeled by retrograde transport of TB was calculated for each animal.

For immunocytochemical labeling free-floating sections were washed with 0.1M Sorenson's phosphate buffer before being permeabilized in triton phosphate buffered saline (T-PBS) for 15 minutes and blocked for non-specific reactivity with normal goat serum (NGS)/T-PBS, 1:20 for 15 minutes. Primary antibodies against glial fibrillary acidic protein to detect astrocytes (GFAP, 1:500, Dako, Carpinteria, CA) and antibodies against the lysosomal membrane of phagocytic cells (ED1 clone, 1:1000, Chemicon International, Temecula, CA) were applied to the sections and incubated overnight at room temperature. Sections were washed, incubated with either fluorescein-conjugated or rhodamine-conjugated secondary antibody for 90 minutes, washed and cover slipped with Vectashield mounting medium. Sections were examined under bright field or fluorescent light with a Zeiss Axioskop microscope. Montage images of the lesion epicenter at 7d or 28d after contusion and of the interface of the PN graft with spinal cord were created and assessed for extent of lesion, presence of cellular debris within the forming lesion cavity, continuity of graft apposition to the spinal cord and the presence of GFAP-ir astrocytes and ED1-ir macrophages/microglia.

Behavioral testing

All behavioral testing was completed between 08:00 and 11:00 AM. Animals were maintained on food and water *ad lib* with a 12 hour light-dark cycle (lights on at 07:00 AM). After acclimation to the testing apparatus over a 1 week period, baseline scores for forelimb and hindlimb open field locomotion, as well as grid walking and grip strength performances (see below) were established for each animal. Two days later, animals were subjected to the unilateral C4 contusion injury. Animals in group 3 and group 4 were evaluated at 2d and 7d after contusion injury and 2d and 7d after manipulation of the lesion site in preparation of the PN graft. Animals in the long delay group (group 4) also were evaluated at 28d after contusion, which was just prior to placement of the PN graft. Most animals had difficulty performing the grid and grip tasks at 2d after injury and at 2d after manipulation of the lesion, thus evaluation of these tests were performed only at 7d and 28d after contusion and 7d after lesion manipulation.

Open Field Locomotion—Forelimb and hindlimb function was evaluated in an open field measuring approximately 2.5 × 3 foot diameter and the rats were observed for 4 minutes by trained observers. The forelimb locomotor scale (FLS, Table 1), devised in our behavioral core facility from observation of recovery patterns of cervically injured rats, is a seventeen point scale that defines deficits based on range of motion, level of weight support, and whether the paw is parallel to the body (Cao et al., 2007). In principle it is similar in assessment style to the hind limb BBB (Basso et al., 1995) rating scale. Both the FLS and hindlimb BBB are qualitative assessments of locomotion in an open field that are scored by two experienced observers blinded to the treatment condition. Each animal was rated during direct observation in the open field for 4 minutes and videotaped for later referral if necessary.

Grid-walk Test—Paw placement for the forelimbs and hindlimbs was assessed as the animals walked on an elevated plastic coated wire mesh grid (36 cm × 38 cm with 3 cm² openings). The animals are placed on the grid for 2 min and allowed to walk freely across the space. Each limb was scored for the total number of steps and the total number of missteps. A misstep occurs when the entire foot falls through the grid. The total number of steps and missteps are added together to obtain the total number of placements. The percent correct is calculated by dividing the number of steps by the total number of placements (steps plus missteps). Grid-walk test has been validated as a sensorimotor assessment (Grill et al., 1997).

Grip Strength Test—The grip test assesses motor function by recording the amount of force (in Newtons) exerted by the affected forelimb prior to releasing grasp of the grip device (SDI Grip Strength System model DFM-10, San Diego Instruments, San Diego, CA). The animal is placed on the platform of the device and encouraged to grip the metal ladder attached to the force transducer with the affected forepaw. Once the grip is secured the animal is slowly pulled away from the bar. The transducer records the force just prior to grip release.

Statistical Analysis

A two-way ANOVA between the acute vs. delayed grafting groups and time, with time taken as a repeated measure, was used to analyze the behavioral data. The FLS and BBB scores covered a sufficient range over time to approximate a normal distribution, and therefore could be analyzed in this manner. However, post hoc analysis for the FLS and BBB data did not meet conditions of normality and therefore were analyzed using the Wilcoxon sign test to identify the effect of time and the Mann-Whitney U test to examine the differences between acute and delayed manipulation of the lesion site. Post hoc analysis for the grid and grip tests was performed using the Bonferroni test. All analyses were conducted using Stat View software (SAS Institute Inc., Cary, NC).

Results

Appearance of epicenter at short and long post injury periods

Tissue sections stained for Nissl-myelin provided information about the unilateral nature of the contusion lesion, the extent of damage to gray and white matter regions of the spinal cord and the presence of cysts and/or cellular debris within the forming cavity. At 7d post injury (group 1) the epicenter was comprised of a mix of fluid filled cavities and debris containing densely packed amorphous cells with dense nuclei and small clusters of red blood cells (Figure 1). This represented the bulk of the material that was aspirated from the lesion site just prior to placement of the PN graft and it was easily removed as a glob of tissue with a mucous-like consistency. At this early time point, the lesion epicenter measured 0.76 ± 0.12 mm in a rostral-caudal extent and was centered in the gray matter of the right side of the spinal cord, with no obvious involvement of the contralateral cord (Figure 2A). Dorsal white matter and medial portions of gray matter were spared in most animals of this group and portions of lateral and ventral white matter were intact although there were scattered vacuoles throughout the remaining white matter. Immunocytochemical staining for ED1 and GFAP demonstrated a clear distinction in the intensity of staining between the injured and non-injured sides of the spinal cord, as phagocytic cells were scattered in regions of spared gray matter but comprised nearly all of the cells within the cavity (Figure 2B) and reactive astrocytic cells and processes surrounded the evolving lesion cavity (Figure 2C). This was in contrast to the non-injured side that displayed normal appearing GFAP-immunoreactivity (IR) and only occasional ED1-IR cells in the gray matter.

At 28d after injury (group 2) there was significantly less gray matter present compared to the 7d condition and numerous vacuoles (Figure 3A) were present in all regions of remaining white

matter. Small, densely packed ED1-IR cells occupied most of the lesion cavity (Figure 3B), GFAP-IR cells were sparse within the debris of the cavity but immunoreactivity was intense in surrounding portions of injured tissue (Figure 3C). There persisted a distinction in the intensity of the immunoreactivity of astrocytes and phagocytic cells on injured and uninjured sides of the spinal cord, suggesting limited spread of the tissue response across the midline after a contusion injury. At this later post injury time point, the lesion epicenter measured 1.2 ± 0.12 mm in a rostral-caudal extent. Horizontal sections encompassing the lesion site prepared from animals injured for 28d (Figure 3E) provided information about the rostral-caudal extent of the injury, as well as on the arrangement of astrocytes and phagocytic cells at the rostral end of the lesion (the site of PN graft apposition). With Nissl-myelin staining there were large cavities in the lesion epicenter surrounding a central mass of cellular debris. Numerous vacuoles were present throughout the lateral white matter rostral and caudal to the lesion, and the white matter was obviously more cellular when compared to the non-injured contralateral white matter. ED1 immunoreactive cells comprised most of the cellular debris within the cavity and similarly reactive cells were scattered in both gray and white matter areas around the lesion cavity (Figure 3F). Healthy appearing neurons were adjacent to the cavity at both ends and there was no marked accumulation of scarring around the lesion site even though immunocytochemical staining showed GFAP-IR astrocytes clustered in spinal cord tissue surrounding the lesion cavity, with greatest intensity of staining close to the lesion boundaries (Figure 3G).

Assessment of forelimb activity

1. Qualitative examination of forelimb locomotion was investigated with the FLS. Two days following contusion animals in both groups had extensive movement of one joint or slight movement of two forelimb joints (FLS= 2.5 ± 0.3 ; 28d delay group mean FLS= 2.7 ± 0.47) indicating the consistency of the injury (Figure 4A). At 7d after injury, animals in each group were able to make dorsal and/or plantar placements of the affected paw (short delay group mean FLS= 10.4 ± 1.5 ; 28d delay group mean FLS= 9.3 ± 1.4) and at 28 days the long delay group displayed further improvement (FLS 12.1 ± 0.7) with 4 out of 7 rats exhibiting continuous plantar stepping with parallel paw positioning (FLS ≥ 13). Two days after manipulation of the lesion site and placement of the PNG, previous recovery of extensive movement around three joints and the ability to plantar place without weight support was lost (7d delay group mean FLS= 6.1 ± 1.8 ; 28d delay group mean FLS= 7.9 ± 0.6). By 7d, both the short and long delay groups had regained a level of forelimb locomotor activity that was not significantly different from their score prior to removal of scar tissue and grafting (Figure 4A). After the recovery phase between 2d and 7d, the FLS scores remained stable for at least 21d in both groups (data not shown).

ANOVA displayed no interaction between groups indicating that the removal of scar tissue and apposition of the PNG at 7d or 28d post injury periods did not affect recovery or cause additional impairment across time points. As expected, the main effect of time was significant for each group [$F(3, 36) = 25.9$, $p < 0.0005$], indicating a deficit followed by spontaneous recovery. When the behavioral recovery at 7d post injury was compared between the first and second procedures, a transient deficit (Wilcoxon test, $p < 0.05$) was found that recovered to pre-grafting scores. The 28d delay group exhibited a similar transient deficit (Wilcoxon test, $p < 0.05$) and recovery [$F(5, 30) = 8.1$, $p < 0.0005$].

2. The grid walk test examined sensorimotor function by evaluating foot placement over a challenging terrain. Data collection for this and the grid test (below) was limited to 7d after contusion injury and 7d after lesion site manipulation since animals were too impaired at 2d after either surgical procedure. There was no significant difference in grid performance of affected forelimbs for either the 7d or 28d delay treatment groups at 7d or 28d post contusion

(Figure 4B). There was considerable variability in the score of individual animals in each group, which may have contributed to the absence of significance. The scores at 7d post placement of the PNG were comparable to those prior to manipulation of the lesion site.

3. Motor function of the injured forelimb as assessed by the grip test was not significantly changed at 7 days after contusion in either the 7d or 28d term delay animals (Figure 4C). The average force exerted by each group significantly decreased 7d post contusion and persisted in the chronic group until 28 days post contusion.

Assessment of hindlimb activity

1. Qualitative assessment of hindlimb locomotion was determined by hindlimb BBB open field scoring. Both groups achieved similar BBB scores for the right hindlimb 2d following contusion (short delay group mean BBB = 10.7 ± 0.3 ; 28d delay group mean BBB = 9.8 ± 0.6), which again displayed consistency with the severity of the injury among groups (Figure 5A). By 7d post contusion both groups recovered to a score of 13, indicating the ability to consistently plantar place with weight support and make coordinated steps. In the 28d delay group a mean improvement of 1.5 points was observed from day 7 to day 28 after the initial injury. In both groups there was a significant decrease in BBB score 2d after manipulation of the lesion epicenter, with animals exhibiting weight support in stance only (score of 8–9). This deficit persisted for at least 7d in the short delay group but animals in the 28d delay group had recovered by 7d to near pre-manipulation scores, with frequent coordinated stepping (mean BBB = 12.86 ± 0.55) (Figure 5A).

Statistical analysis was applied to the hindlimb BBB data in the same manner as for the FLS. ANOVA revealed a significant interaction between groups [$F(3,36) = 3.1$, $p < 0.05$], demonstrating that delaying manipulation of the lesion until 28 days when animals had more fully recovered from spinal contusion injury resulted in less severe deficits after a second injury. Both groups displayed a main effect of time [short delay group $F(3, 18) = 6.9$, $p < 0.005$ / long delay group $F(5, 30) = 8.1$, $p < 0.0005$], which demonstrates a significant deficit after each surgery followed by a rapid recovery.

2. Collection of data for hindlimb grid walking was performed at 7d and 28d after contusion injury and 7d after lesion site manipulation, but not at 2d since animals were too impaired shortly after the surgical procedures. While there was a decline in success of foot placement at 7d and 28d after contusion injury there was no significant difference in grid performance of affected hindlimbs for either the 7d or 28d delay treatment groups (Figure 5B). There was considerable variability in the score of individual animals in each group, which may have contributed to the absence of significance. The scores at 7d post placement of the PNG were comparable to those prior to manipulation of the lesion site.

Integration of PNG and axonal ingrowth

The goal of the grafting experiment was to provide a substratum for axonal growth by apposing one end of the PN segment to the rostral end of the cavity. While there often remained a gap between the caudal end of the cavity and the PNG, this did not affect the overall efficiency of the grafting procedure as all grafts were fully apposed to the rostral wall of the spinal cord lesion cavity (Figure 6). The only obvious interruption at the graft-host interface occurred where blood vessels extended from the spinal cord into the graft. There was no detectable difference between graft appositions with white or gray matter regions and the ventral extent of the graft included at least part of the ventral white matter in most animals. There was an obvious linear orientation of Schwann cells along the length of the grafts.

After a delay of 7d between contusion injury and grafting, retrogradely labeled propriospinal neurons were detected throughout the cervical spinal cord rostral to the injury and labeled neurons were found in 5 brainstem regions (reticular formation, RF; raphe; vestibular nuclei, VN; locus coeruleus, LC; red nucleus, RN) (Figure 7). The mean total number of neurons retrogradely labeled from the distal end of the PNG was 1948 ± 683 (mean $\pm$ SD) with 43% of these being propriospinal neurons in the spinal cord (range of 450 to 1264) and 57% in the brainstem (range of 655 to 1656). The distribution of labeled neurons in the spinal cord was 68% in the ventral half and 32% in the dorsal half. In the brainstem 67% of labeled neurons were located in the RF, 13% in the raphe, 8% in VN, 5% in LC and 7% in RN.

After a 28d delay between contusion and grafting, the distribution and total number of labeled neurons in the cervical spinal cord and brainstem was comparable to that detected in the 7d delay group. The number and distribution of regenerating brain stem neurons was similar with a 7d or 28d delay, but there was greater variability between rats of the 28d delay group particularly related to the number of propriospinal neurons contributing to the graft. There was a 50% reduction in the mean number of contributing propriospinal neurons after the longer delay (Figure 7) but this was not at a level of statistical significance. While the mean total number of brainstem neurons in the 28d delay group was comparable to the 7d delay group (1018 ± 553 vs. 1113 ± 372 respectively) there was much greater variability in axonal growth into the PNG after a longer delay, with a range of 264 to 2108 compared to the 7d delay group (666 to 1656). A large range of propriospinal neurons also was observed (174 to 1046) in the 28d delay group and there was an association between the amount of labeling in the brain stem and labeling in the spinal cord, i.e. a high number of labeled propriospinal neurons appeared in rats with a high number of labeled brainstem neurons or a low number of labeled propriospinal neurons appeared in rats with a low number of labeled brainstem neurons. This likely reflected moderate differences in the quality of nerve graft apposition due to persisting scar tissue and not a diminished potential for axonal growth after a 28d delay. The distribution of labeled propriospinal and brain stem neurons was similar to that observed in the 7d delay group, with 75% in the ventral half and 25% in the dorsal half of the spinal cord and 61% of labeled cells in RF, 15% in raphe, 12% in VN, 2% in LC and 10% in RN respectively (Figure 7).

Discussion

Contusion injury as a model for regeneration

The report of a unilateral cervical contusion injury (Gensel et al. 2006) served as the impetus for this study in which we questioned whether resection of a lesion cavity prior to transplantation would adversely affect behavioral recovery and/or the potential for axonal regeneration in acute and chronic injury situations. Development of a spinal contusion injury model was predicated on the perception that this type of injury more closely approximates the majority of human SCI situations. While important information regarding potential neuroprotective strategies has been derived from use of this injury model, there are underlying concerns that manipulation of the lesion site (e.g. in preparation for neurotransplantation) would be deleterious to spared or regenerating axons, leading to additional behavioral dysfunction. Indeed, most previous studies attempting to replace lost neural tissue at the epicenter have used a cell or tissue suspension microinjected into the epicenter without otherwise disturbing the original lesion site (Reier et al., 1992; McDonald et al., 1999; Tarasenko et al., 2007). In the one study that mentions resection of glial scar prior to placing short segments of peripheral nerve between ends of a thoracic contusion injury cavity (Rasouli et al., 2006), it is not easy to determine the extent of cavity modification or the actual growth of axons into these grafts. The present study provides critical evidence that spontaneous recovery of forelimb activity is not diminished by manipulation of the injury site weeks after

the initial injury, and that several thousand axons regenerate into a peripheral nerve graft even when grafting is delayed for as long as 28d after SCI. The results indicate that the concept of “do no harm” can be safely adhered to without compromising the ability to promote anatomical regeneration with the capacity to lead to an improvement in functional recovery when combined with other treatment strategies designed to enhance outgrowth from the PN graft (see Houle et al., 2006).

Debate about the disruptive nature of glial scarring arises when designing the best approach to address the question of whether removal of scar tissue is necessary to achieve axonal growth into and through a cell or tissue graft. There is little doubt that reactive gliosis represents at least a partial physical and chemical barrier to the advancement of regenerating axons (Reier and Houle, 1988; Fitch and Silver, 1999; Fawcett, 2006). Yet it does not appear to be totally impermeable, as recent studies have indicated growth of axons into autologous bone marrow stromal cell grafts made 6 weeks after a mid-cervical injury (Lu et al., 2007). In the study by Lu et al., there was no attempt to resect the chronic scar and the extent of axonal ingrowth improved when grafted cells were genetically modified to express neurotrophin-3. It is possible that the presence of these bone marrow stem cells or invading Schwann cells affected the integrity of the established scar, similar to an observation that fetal spinal cord tissue grafts placed into a chronic lesion without disruption of the scar resulted in physical gaps in astroglial scarring surrounding the transplant site (Houle, 1992). While the addition of exogenous neurotrophic factors potentiates axonal growth by chronically injured neurons if scar tissue is excised (Ye and Houle, 1997), it is not known whether treatment with these factors might influence the integrity of an established scar thereby enhancing the overall regenerative effort.

There is at least one negative outcome to the aggressive removal of scar tissue as a treatment strategy. Chronically injured neurons appear to be extra sensitive to the resulting second injury with increased cell death, although treatment of the re-lesion site with BDNF prevents further significant loss of reinjured neurons (Houle and Ye, 1999). In addition to neuron loss, resection may cause tissue degeneration or remodeling through heightened inflammatory or astroglial responses (Tuszynski et al., 2003), leading to worsening behavioral activity. Results from the present study demonstrate that manipulation of a chronic cervical contusion lesion is well tolerated, resulting in no significant diminishment of gross motor activity of either forelimb or hindlimb after 7d. Minor deficits observed at 2d after manipulation did not persist, suggesting a compensatory mechanism or spontaneous recovery similar to that observed after the initial injury. Aspiration of the injury site had no adverse effect on the number or type of regenerating axons that penetrated the PN graft. The number of regenerating neurons was comparable with either a 7d or a 28d delay between contusion injury and nerve grafting, although it was necessary to provide GDNF to the 28d lesion site to attain such a high level of regeneration (Dolbeare and Houle, 2003). With our chronic hemisection lesion model, placement of a PN graft without resection of scar tissue, even in the presence of neurotrophic factors, was not sufficient to support axonal regrowth. This suggests some fundamental differences in the arrangement and/or continuity of glial scarring between a penetrating lesion and a contusion injury and indicates that possibilities for promoting functional recovery after a chronic injury may best be studied with a contusion injury model.

Given the positive results of the present study regarding extensive axonal growth and absence of lasting behavioral deficit after aspiration of a chronic contusion lesion site, new experiments are underway to test whether behavioral recovery results when this injury approach is combined with enzyme treatment to counteract inhibitory components of the extracellular matrix (Houle et al. 2006).

Behavioral outcomes with injury site manipulation

Our unilateral contusion injury with the Infinite Horizon device led to expanding tissue loss over many weeks that appeared to be more extensive than the moderate lesions resulting from use of a modified NYU/MASCIS weight drop device (Gensel et al., 2006). We detected persisting forelimb and hindlimb deficits that remained relatively stable from 7d to 28d post injury despite the progressive loss of gray matter at the contusion epicenter and the progressive disruption of vestibulospinal, reticulospinal and rubrospinal pathways. This was in contrast to the transient nature of hindlimb deficits observed after more moderate injuries (Gensel et al., 2006), but it does suggest that the majority of functional deficits observed with a contusion injury result from the initial tissue damage and not to any secondary degeneration that occurs. It may be that the continued loss of tissue limits the extent of spontaneous recovery that would be possible over a longer post injury period, although we and others have shown that behavioral recovery assessed by basic locomotor rating scales progresses through deficit, recovery, and plateau phases by 3–4 weeks in untreated rats regardless of the severity of the initial lesion (Basso et al. 1995; Basso et al., 1996; Soblosky et al., 2001; Shumsky et al., 2003; Gensel et al., 2006). Several reports have indicated spontaneous reorganization of tissue in the injury site that includes Schwann cell invasion, slow formation of astroglial scarring (Brook et al., 1998) and sprouting of descending axons within 14–21 days of the contusion injury (Hill et al., 2003). It is not clear how (or if) spontaneous recovery is related to the pattern or extent of tissue reorganization that occurs, but it is important to emphasize that the behavioral deficit remains fairly stable after recovery plateaus, despite the disruption of the reorganized tissue within the contusion epicenter in preparation for the PN graft. In contrast to a previous study (Shumsky et al., 2003) that noted a reduction in plateau recovery following debridement of the scar in a surgical cervical hemisection model, this observation also suggests that there is little detectable functional consequence to this endogenous repair effort or to the removal of these fibers during early stages of the repair effort in a cervical contusion model.

The behavioral tasks for study after a unilateral cervical injury were chosen because they demonstrate significant deficits in gross motor skills followed by a significant level of spontaneous recovery. It is likely that more sensitive assessment criteria will be needed to detect finer aspects of recovery, particularly as therapeutic interventions are applied at acute and/or delayed periods after injury. For more sensitive measures such as the assessments of forelimb reaching (Stackhouse et al., 2007) or gait analysis using CatWalk or TreadScan systems (Hamers et al., 2001; Hamers, 2006; Magnussen et al., 2007) it may be necessary to perform daily testing after SCI to determine the window of time where the change from a significant behavioral deficit to recovery of baseline function occurs. In several of our tests (grid and grip) animals were too severely impaired at 2d after injury to perform the task, but by 7d had recovered sufficient joint and distal digit movement to perform the grid and grip tasks. Some of the early behavioral deficits may be associated with the anesthesia and physical manipulation of back and paravertebral musculature during exposure of the spinal cord, yet these resolve during the 7d post operative period when more task specific analysis can be accomplished.

GDNF use for neuroregeneration

An axon growth promoting factor was necessary in the 28d group because untreated chronically injured neurons fail to show a strong regenerative effort (Ye and Houle, 1997). Co-expression of the GDNF receptor complex of a ligand-binding component (GFR α 1) and the transmembrane Ret receptor tyrosine kinase (Airaksinen et al., 2002) by most neurons involved directly or indirectly with motor function, including those that contribute to descending spinal pathways (Glazner et al., 1998; Trupp et al., 1997), makes GDNF a strong candidate as an agent for studying neuroprotection and/or neurorepair after SCI. Our previous studies demonstrate multiple roles for GDNF at both acute and chronic stages after SCI. GDNF

provided to an acute lesion site decreased the extent of retraction of rubrospinal and reticulospinal tract axons (Dolbeare and Houle, 2003) and promoted reversal of neuron atrophy (Storer et al., 2003). In a chronic injury, axonal regeneration of supraspinal neurons into a PN graft was promoted by GDNF treatment of an SCI site (Dolbeare and Houle, 2003). In the present study a single exposure to GDNF at 28d after injury probably was not involved in limiting behavioral changes associated with cavity manipulation, since neuroprotective features (sparing of white matter and overall decrease in lesion volume) of GDNF which might contribute to a behavioral effect appear to transpire only after acute treatment (Iannotti et al., 2004). Promoting regeneration of chronically injured reticular formation neurons, as well as rubrospinal, vestibulospinal, and raphespinal tract neurons, is potentially clinically important because of their prominent role in rat locomotor activity (Jordan, 1991; Loy et al., 2002; Martin et al., 1985; Muir and Whishaw, 2000; Noga et al., 1991).

Conclusion

There are two important aspects to this study involving contusion injury of the spinal cord and the potential for axon regeneration. First is that there is remarkable stability of both forelimb and hindlimb movement at 7d after manipulation of the lesion site, indicating no additional gross motor deficit results from clearing of cellular debris from the lesion cavity. This opens up the possibility for study of more chronic injury situations where the fear has been that debridement of the lesion would result in additional functional impairment and should be avoided. The notion of doing more harm greatly limited the treatment strategies that could be considered for the chronic injury situation, but now it appears that this apprehension can be lessened. Secondly, this study demonstrates that regeneration of axons after a contusion injury is possible after sub-chronic (7d) or chronic (28d) manipulation of the lesion site and that the use of a PN graft offers a better chance for long distance regeneration than other strategies used to fill a lesion cavity. The issue of regenerated axons remaining in a pathophysiological state, in part due to ineffective axon interaction with myelinating cells in the lesion site (Tan et al., 2007) seems to be easily overcome with the PN graft as Schwann cell tubes are provided by the graft and there is extensive myelination of axons in the graft (see Houle, 1991). PN grafts also have the advantage of being able to target axonal growth towards a specific target region.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1. Histological appearance of cellular debris in contusion lesion cavity. Small densely stained cells were tightly packed in the lesion cavity forming at 7d after SCI (**A**), in contrast to the more loosely arranged cells in the cavity at 28d after injury (**B**). More spindle-shaped cells and amorphous debris were observed at the longer post injury period. Adjacent to the epicenter is spared spinal cord tissue with intact myelin to the right of dashed line in (**A**).

2.

GFAP and ED1 immunocytochemistry at lesion epicenter, 7d after contusion injury. **A)** A thin rim of lateral white matter and dorsal and ventral medial gray matter remains at this short post injury period. The unilateral nature of the injury is obvious here. The * indicates densely packed cells that partially fill the lesion cavity which are ED1 immunoreactive (**B**). These comprise the majority of cells observed in Figure 1A. **C)** Intense staining for GFAP surrounds the lesion cavity and is spread throughout the spared gray and white matter areas of the spinal cord ipsilateral to the lesion. The astrocytic reaction contralateral to the lesion is less intensely stained, especially in the gray matter. **D)** Merged images of astrocyte and phagocytic cell response to contusion injury. Bar = 500 μ m.

3.

GFAP and ED1 immunocytochemistry at lesion epicenter, 28d after contusion injury. **A–D** are in a transverse plane and **E–H** are in a horizontal plane, midline to the left of image. At this long post injury period more extensive tissue damage is obvious compared to the 7d survival period. There is loss of most gray matter within the lesion epicenter and only a thin rim of spared white matter ventrally and laterally (**A and E**). Cellular debris that partially fills the cavity is comprised primarily of phagocytic cells (**B and F**) with substantial aggregation of astrocytes in gray and white matter surrounding the lesion (**C and G**). Merged images (**D and H**) indicate little migration of phagocytic cells into adjacent spinal cord tissue. Bar = 500 μ m.

4.

Forelimb behavioral assessment. **A)** The forelimb locomotor scale (FLS) scores were comparable in the 2 groups at 2d and 7d with a significant increase in performance from 2d to 7d after contusion injury, indicative of spontaneous recovery from slight movement of all 3 joints to frequent plantar stepping. Animals in the 28d group showed further improvement beyond the 7d group. At 2d after manipulation and PN grafting to the lesion epicenter there was a significant decrease in FLS score compared to the 7d or 28d post-contusion score respectively, but by 7d post grafting animals had recovered to their pre-manipulation scores. **B)** Grid testing at 7d post contusion showed no significant decrease in forelimb performance in either group, with a trend towards improvement with a longer survival period. Manipulation

and grafting at the epicenter did not significantly change foot placement when tested 7d later. C) Forelimb grip was not significantly impaired at any of the post-contusion intervals nor did manipulation of the epicenter lead to greater loss of function. ◇ indicates significantly greater than 2d post contusion injury; * indicates significantly less than 7d post contusion; ▲ indicates significantly less than 28d post-contusion injury.

A **B** **5.**

Hindlimb behavioral assessment. **A)** Hindlimb open field locomotor performance was not significantly different between 2d and 28d post injury. Manipulation of the lesion epicenter resulted in a significant decrease in BBB score (loss of frequency of weight support and no FL/HL coordination) that persisted through day 7 in the 2d group but not the 28d group. **B)** Grid testing at 7d post contusion showed no significant decrease in forelimb performance in either group, with a trend towards improvement with a longer survival period. Manipulation and grafting at the epicenter did not significantly change foot placement when tested 7d later. * indicates significantly less than 7d post contusion; ▲ indicates significantly less than 28d post-contusion injury.

6. Peripheral nerve graft interface with injured spinal cord. Spinal cord tissue rostral to the lesion is to the right of this figure. Arrows indicate the interface of spinal cord and peripheral nerve graft that extends from the dorsal surface (top arrow) ventrally to include a portion of the ventral funiculus. Some spared tissue (*) in the epicenter below the PN graft prevented full apposition to the ventral funiculus. There was no obvious difference in the extent of apposition with a 7d or 28d delay between contusion injury and nerve grafting.

True Blue Labeled Neurons: Brainstem & Spinal Cord

7.

Number and distribution of regenerating neurons. Delaying the PN graft for 7d or 28d had no significant effect on the number or distribution of neurons that regenerated their axon into the graft. The majority of regenerating neurons were of brain stem origin, being led by those in the reticular formation and raphe regions. While there appeared to be fewer propriospinal neurons contributing to the graft with a 28d delay, this was not significantly less than with a 7d delay because of the large variability within the groups.

Table 1**Forelimb Locomotor Scale (FLS)**

0. No movements of the forelimb (shoulder, elbow or wrist joints)
1. Slight movements of one or two joints of the forelimb
2. Extensive movement of one joint and slight movement of another joint of the forelimb
3. Slight movement of all three joints of the forelimb
4. Extensive movement of one joint and slight movement of two joints of the forelimb
5. Extensive movement of two joints and slight movement of one joint of the forelimb
6. Extensive movement of all three joints of the forelimb
7. Plantar placement of the forelimb with no weight support
8. Dorsal stepping only
9. Dorsal stepping and/or occasional plantar stepping
10. Frequent plantar stepping
11. Continuous plantar stepping
12. Continuous plantar stepping with paw position rotated (either at initial contact, lift off or both)
13. Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both)
14. Continuous plantar stepping with paw position rotated (either at initial contact, lift off or both) and occasional toe clearance
15. Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and occasional toe clearance
16. Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and frequent toe clearance
17. Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and continuous toe clearance