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Received, October 20, 2009.
Accepted, July 26, 2010.

Chitosan Channels Containing Spinal Cord-Derived Stem/Progenitor Cells for Repair of Subacute Spinal Cord Injury in the Rat

OBJECTIVE: We evaluated the survival and differentiation capacity of neural stem/progenitor cells (NSPCs) derived from the adult rat spinal cord and seeded on intramedullary chitosan channels that were implanted in a subacute rat spinal cord injury model.

METHODS: We implanted into the injured spinal cord a chitosan channel filled with NSPCs harvested from the spinal cord of adult transgenic rats expressing green fluorescent protein 3 weeks after extradural 35*g* clip compression injury at T8. The NSPC–chitosan channel group was compared with 2 control groups not receiving channels: 1 receiving a direct intramedullary injection of NSPCs into the lesion cavity and 1 receiving trauma alone. The survival and differentiation of NSPCs were evaluated with immunohistochemical and histopathological techniques, and functional improvement was assessed for 6 weeks with the Basso, Beattie, and Bresnahan locomotor score.

RESULTS: The NSPC–chitosan channel group showed enhanced survival of NSPCs compared with NSPCs transplanted directly into the lesion cavity, although there was no significant difference in functional recovery between the treatment and control groups. In addition, the intramedullary implantation of the chitosan channel did not worsen the functional deficit after the 35*g* clip injury.

CONCLUSIONS: Chitosan channels enhanced the survival of transplanted NSPCs in the subacutely injured spinal cord. Functional deficits were not exacerbated by the intramedullary transplantation of chitosan channels into the site of injury.

KEY WORDS: Adult neural stem/progenitor cells, Cell survival, Chitosan channel, Differentiation, Intramedullary implantation, Spinal cord injury

Neurosurgery 67:1733–1744, 2010 DOI: 10.1227/NEU.0b013e3181f9af35 www.neurosurgery-online.com

The majority of human spinal cord injuries (SCIs) are incomplete injuries, and the most common mechanism is impact-compression, which in the subacute or chronic state often results in considerable cavitation.[1] Loss of spinal cord tissue and cavity formation are major obstacles to repair of the injured spinal cord after severe injury. Attempts have been made to regenerate and bridge the lost tissue by grafting of tissues or cells[2,3] or to repair by using endogenous stem/progenitor cells. In the central nervous system of adult vertebrates, including humans, there are neural stem cells that self-renew and are multipotential with the capacity to differentiate into astrocytes, oligodendrocytes, and neurons. However, activation of endogenous stem/progenitor cells after SCI has produced minimal functional recovery, and it has been hypothesized that transplantation of large in vitro expanded populations of these cells is required to enhance recovery.[4,5] Several recent studies of transplanted neural stem/progenitor cells (NSPCs) in our laboratory and elsewhere showed cell survival, differentiation, and integration along injured axonal pathways in the spinal cord, suggesting their usefulness for repair by axonal regeneration and remyelination.[4-9] Furthermore, we, along with others, were able to

ABBREVIATIONS: **BBB**, Basso, Beattie, and Bresnahan; **GFAP**, glial fibrillary acidic protein; **GFP**, green fluorescent protein; **NSPC**, neural stem/progenitor cell; **SCI**, spinal cord injury

achieve functional improvement in experimental animals, although the improvement was modest.[6,8] Thus, additional strategies must be pursued, as in the present article, which describes transplantation of NSPCs within a biocompatible scaffold. There has been progress in the design of biocompatible materials for tissue engineering such as biopolymers and biocompatible grafts for surgical implantation. Combinatorial strategies involving biosynthetic conduits carrying extracellular matrix molecules and various cell lines have yielded promising results in experimental SCI.[10-12] In addition, tubular constructs may create a useful pathway for axonal regeneration.

The objectives of the present study were to compare transplantation into the injured spinal cord by direct injection of cells and transplantation of cells seeded into chitosan channels. Chitosan is derived from the naturally occurring polysaccharide chitin, a major component of crustacean exoskeleton. It is an increasingly popular material for biomedical use because of its biocompatibility and tunable degradation kinetics.[13] Nerve guidance channels made from chitosan have been shown to have good mechanical strength and support neurite outgrowth.[14] We previously used chitosan channels filled with peripheral nerve grafts and implanted directly into a spinal cord cavity and showed that these channels are safe and promote large numbers of axons to regenerate within its lumen.[15] In the present study, we measured the survival and fate of adult rat spinal cord–derived NSPCs transplanted directly into the spinal cord or within intramedullary chitosan channels. All transplantations were performed in the subacute stage after impact/compression. The present study also describes for the first time functional, histological, and immunohistochemical outcomes after intramedullary transplantation of chitosan channels containing NSPCs.

MATERIALS AND METHODS

Chitosan Channels

Chitosan channels were prepared as previously described with minor alterations.[14] Medical-grade chitosan chloride (5 g; Protosan UP CL 213, NovaMatrix, Brakeroya, Drammen, Norway) was dissolved in 500 mL distilled water and then precipitated with 40 mL of 4% wt/wt of sodium hydroxide (NaOH) solution. The remainder of the process was described previously.[16] Channels were cut into 6-mm lengths and dried. All channels used for implantation studies were sterilized by ethylene oxide gas. The channels were approximately 1.8 mm in outer diameter, and the wall thickness was 0.2 mm (Figure 1A).

Harvesting and Cell Culture of NSPCs

NSPCs were harvested from the spinal cords of transgenic adult Wistar rats (Wistar-TgN [CAG-GFP] 184ys) originally obtained from the YS Institute Inc, Utsunomiya, Tochigi, Japan.[17] NSPCs isolated from these rats stably express the transgene long term, both in vitro and in vivo.[18] Periventricular NSPCs were isolated with methods described previously.[19] Under sterile conditions, tissue segments from the cervical and thoracic spinal cord, including only the closely apposed ependymal, and some gray matter tissue immediately surrounding the central canal were dissected, cut into 1-mm^3 pieces, and then enzymatically dissociated in a solution containing 0.01% papain (Papain Dissociation System, Worthington Biochemical Corporation, Lakewood, New Jersey) and 0.01% DNase I for 1 hour at 37°C. Cells were seeded in free-floating culture in chemically defined serum-free media containing 20 ng/mL epidermal growth factor and 20 ng/mL fibroblast growth factor-2 (Sigma-Aldrich, Oakville, Ontario), and neurospheres were passaged every 7 days. Cells were assessed for self-renewal and multipotentiality as described previously.[19,20] Neurospheres were transplanted after 4 to 6 days in vitro at passage 3 or 4. We previously characterized the resulting spinal cord–derived neurospheres and determined that undifferentiated neurospheres express high levels of nestin (83%) and low levels of glial fibrillary acidic protein (GFAP; 0.4%), a marker for astrocytes; RIP (9%) a marker for oligodendrocytes; and β-III-tubulin (0.5%), a marker for neurons.[7,19]

Seeding of Spinal Cord-Derived NSPCs Into the Inner Lumen of the Channels

Previously, we used a laminin solution to coat the sterile chitosan channels to improve adherence of the cells to the channels.[21,22] In the present study, we did not use laminin because we wanted to examine cell survival and differentiation within the channels in the absence of an extracellular matrix. Spinal cord–derived NSPCs were inserted into the channels as neurospheres with a cell density of 1×10^6/10 μL complete medium. Chitosan channels were transferred into empty wells, and 10 μL cell suspension was pipetted into each channel. We attempted to maintain a uniform distribution of neurospheres within the channels containing the neurospheres by implanting them into the injured cord immediately after seeding.

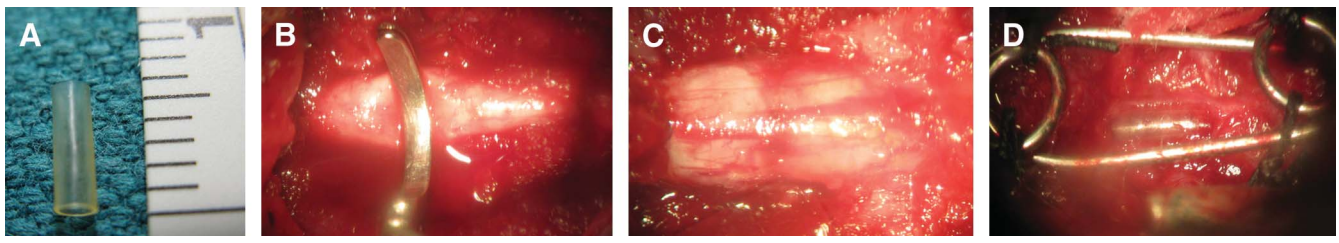

FIGURE 1. *Photo of chitosan tube (A). Operative photographs: clip compression injury with the 35g clip in place on the cord (B); dorsal aspect of the compressed spinal cord after the clip was removed (C); and implantation into the injured spinal cord of a chitosan tube seeded with neural stem/progenitor cells 3 weeks after spinal cord injury (D). After tube implantation, a spinal stabilization was performed from T6 to T10 with No. 5 surgical steel.*

Animals

Twenty-seven wild-type adult female Sprague-Dawley rats (280 ± 335 g; Charles River, St Constant, Quebec, Canada) were used in this study. All animal procedures were performed in accordance with the *Guide to the Care and Use of Experimental Animals* (Canadian Council on Animal Care) and approved protocols from the Animal Care Committee of the Research Institute of the University Network, Toronto, Ontario, Canada. Sprague-Dawley rats with clip compression injury (described below) were randomized into 3 groups of 9 rats each. In group 1, the channel plus NSPC group, spinal cord-derived NSPCs were administered into the damaged spinal cord within chitosan channels. In group 2, the NSPC group, NSPCs were injected directly into the injured spinal cord. In group 3, the control group, a durotomy was performed at the injury site at 3 weeks, and no channel or cells were implanted. A channel-only group was not included in the present study because it was included in our previous study.[15] All animals were maintained for 6 weeks after transplantation.

Spinal Cord Clip Compression Injury

Anesthesia was induced with 2% halothane with 1:2 nitrous oxide to oxygen. An injection of 5 mL saline was given subcutaneously before surgery, and the rats were placed prone on a heating pad at 36°C throughout the operation and securely positioned in a stereotaxic frame. A 3-level laminectomy was performed at T7 to T9 and a clip compression injury was made at a 35*g* force for 1 minute according to the method of Rivlin and Tator[23] (Figure 1B). The dorsal aspect of the compressed spinal cord was then covered by a synthetic expanded polytetrafluoroethylene membrane (Preclude Gore-Tex Dura Substitute, Gore & Associates, Inc, Flagstaff, Arizona) to prevent extradural scarring and invasion by fibrous tissue; the muscles were sutured with 3-0 Vicryl sutures (Johnson & Johnson, Peterborough, Canada); and the skin was closed with Michel clips (Fine Science Tools, North Vancouver, Canada). All animals received buprenorphine subcutaneously before awakening and then every 12 hours for 3 days after operation. Bladders were evacuated 3 times daily until spontaneous voiding was established, and hematuria or urinary tract infection was treated with Clavomax (62.5 mg PO BID for 5 days). The rats were housed singly in a temperature-controlled room at 26°C with a 12-hour light/dark cycle. Water and food were provided ad libitum.

Transplantation

All rats underwent a second operation 3 weeks after SCI. For implantation of the chitosan channels containing NSPCs (group 1), the rats were anesthetized and positioned as described above, the previous operative site was reopened, and after removal of the Gore-Tex membrane on the dorsal surface of the injured spinal cord, the dura mater was incised longitudinally with microscissors from T7 to T9. There was focal atrophy of the cord and cavitation from the clip injury. A dorsal midline myelotomy 5 mm in length was created longitudinally at the SCI site with a No. 11 surgical blade (Feather, Ohyodo-Minami, Osaka, Japan) and microscissors, avoiding the midline dorsal spinal vein. A small piece of absorbable gelatin sponge (Surgifoam, Ethicon Inc, Soeberg, Denmark) was inserted temporarily into the cavity to control hemorrhage. Chitosan channels (length = 6 mm, inner diameter = 1.6 mm) were seeded with 10 μL medium containing 1×10^6 NSPCs 30 minutes before intramedullary transplantation. One channel was then implanted into the cavity of each rat in group 1 (Figure 1D). For direct injection of NSPCs without a channel (group 2), a durotomy was performed as above, and then a dorsal midline myelotomy 0.2 mm long was performed at the SCI site with a No. 11 surgical blade and microscissors, avoiding the dorsal spinal vein. With the surgical microscope, 10 μL containing 10^6 NSPCs was injected into the injury cavity with a Hamilton syringe. To decrease leakage from the needle tract or myelotomy defect, the cells were slowly delivered into the cavity, after which the needle was left in place for at least 2 minutes before being slowly withdrawn. The control rats (group 3) underwent a similar operative procedure, and after removal of the Gore-Tex membrane, only a durotomy was performed as above. In all 3 groups, a spinal stabilization was then performed from T6 to T10 with No. 5 surgical steel (Ethicon Inc, Somerville, New Jersey) and 2-0 silk (Johnson and Johnson, Peterborough, Ontario, Canada) using a method we reported previously[24] (Figure 1B through 1D). The dorsal aspect of the spinal cord was then covered with 40 μL of the fibrin sealant Beriplast P (Beriplast, a gift from ZLB Behring GmbH, Marburg, Germany), and the Gore-Tex membrane was repositioned to cover the exposed spinal cord, after which the muscles and skin were closed. To aid transplant survival and to decrease the extent of graft rejection, all animals received cyclosporine (15 mg/kg, Sandoimmune, Novartis Pharma, Canada Inc, Dorval, Quebec, Canada) injected subcutaneously daily from the second operation until death.

Tissue Preparation and Immunohistochemistry

Six weeks after channel transplantation and 9 weeks after SCI, the animals were transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline at pH 7.4 and/or 10% neutral buffered formalin (EMD Chemicals Inc, Gibbstown, New Jersey). A 2-cm segment of the spinal cord encompassing the injury site was carefully removed and postfixed in neutral buffered formalin. Some of these tissues were processed through alcohol and chloroform, embedded in paraffin blocks, and sectioned transversely into 8-μm parasagittal serial sections. Every eighth section was stained with hematoxylin-eosin and Luxol fast blue for general morphology, and other sections were stained with Masson trichrome for collagen. The spinal cords for frozen sections were cryoprotected in 30% sucrose for at least 24 hours and then frozen and embedded in Shandon Cryomatrix compound (VWR Laboratories, Mississauga, Ontario, Canada), and serial 20-μm parasagittal sections were cut.

The immunohistochemistry was performed as previously reported.[21,22,25] For immunohistochemical assessments, the following antibodies were used: mouse anti-GFAP monoclonal antibody (GFAP; 1:200; Chemicon, Temecula, Ontario, Canada) for astrocytes, mouse anti-CC1/APC monoclonal antibody (CC1; 1:1000; Calbiochem, San Diego, California) for mature oligodendrocytes, mouse anti-NeuN monoclonal antibody (NeuN, 1:500; Chemicon) for neurons, mouse anti-rat nestin monoclonal antibody (nestin; 1:100; BD Bioscience Pharmingen, Mississauga, Ontario, Canada) for neuronal precursor cells and reactive astrocytes, Ki-67/MM1 (1:100; Novacastra, Newcastle, United Kingdom) for proliferating cells, and ED-1 (1:2000, Serotec, Raleigh, North Carolina) for macrophages/microglia. Immunofluorescent tissue was examined with a Nikon Eclipse TE 300 microscope and a Zeiss LSM-510 confocal microscope. Immunoperoxidase staining was performed as described previously.[26] Mouse monoclonal anti-GFAP antibody (1:200; Chemicon), green fluorescent protein (GFP; Chemicon), ED-1 (1:200, Serotec), and Ki-67/MM1 (1:100, Novacastra) were used with biotinylated anti-mouse secondary antibodies (Vector Laboratories, Burlington, Ontario, CA) and avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit Standard, Vector Laboratories). Diaminobenzidine (Vectastain Elite

ABC Kit Standard, Vector Laboratories) was applied as the chromogen. Sections were pretreated in a microwave oven to enhance immunoreactivity for the GFAP, GFP, ED-1, and Ki-67 immunostaining. After deparaffinization and blocking of endogenous peroxidase with 1% methanol peroxidase for 30 minutes, slides were placed in a pressure cooker with citrate buffer (pH 6.0) and treated in a household microwave oven for 35 minutes at the highest power (1.40 kW). After heating, slides were left in the microwave oven for 30 minutes and then rinsed twice for 10 minutes in phosphate-buffered saline, and immunostaining was performed according to standard protocols for immunoperoxidase. Negative controls were obtained by omission of the primary antibody.

Quantitative Analysis of Cell Survival and Differentiation of NSPCs

Our method of quantitative analysis of transplanted cells in the injured cord has been described previously.[8,21,22,27,28] Fifteen sections were cut from each rat spinal cord in the parasagittal plane at 20-μm thickness 140 μm apart, counterstained with the nuclear dye DAPI (4[prime], 6-diamidino-2-phenylindole dihydrochloride) present in Vectashield mounting medium, and coverslipped. All $GFP^+/DAPI^+$ cells in each of the 15 sections were counted. Cells were defined as any nonautofluorescent object expressing GFP with an average diameter of a cell body and typical cell morphology with clearly delineated cell borders, and only GFP-positive cells containing DAPI-positive nuclei were counted. Because cell counts were taken from every eighth section, cell counts were multiplied by eight and then adjusted using the Abercrombie factor to obtain an estimate of the total cell count for the entire cord thickness.[29] The average cell survival was calculated for the 3 rats in each group. To observe cell differentiation of NSPCs at 6 weeks after implantation, 9 sections per animal containing new tissue formation in the intramedullary channels or direct injection groups were selected for each of the following antibodies: anti-nestin, GFAP, CC1, and NeuN. Five sections per rat containing the central portion of new tissue in the channel or direct injection groups were selected for each of the following histochemical reactions: anti-nestin, GFAP, CC1, and NeuN. The percentage of double-labeled cells was calculated relative to the total number of GFP-positive NSPCs.

Behavioral Analysis

For the behavioral analysis, 3 blinded observers analyzed hind-limb motor function and functional recovery weekly for 9 weeks after SCI using the Basso, Beattie, and Bresnahan (BBB) open-field locomotor test, and all animals were scored for 4 minutes every week.[30] The score of each hind limb was recorded; the averages are presented.

Statistical Analysis

All data are presented as mean ± SE. Differences in cell survival and comparison of percentage of transplanted cells that were double-labeled between groups were analyzed with 2-way analysis of variance, followed by pairwise multiple comparisons with the Bonferroni method. The BBB locomotor scores of each rat were averaged to obtain 1 score for each weekly test session, and the scores of all animals in the same experimental group in the same week were averaged. Analysis was performed with 2-way repeated-measures analysis of variance and pairwise multiple posthoc comparison using the Bonferroni method.[31] Statistical significance was determined at the $P < .05$ level.

RESULTS

General Histological Appearance

As we reported previously,[32] for injuries of this magnitude, there was a major loss of spinal cord tissue in all 3 groups at 9 weeks (Figure 2A) with extensive rostrocaudal cavitation. Most cavities were compartmented by septae. The tissue loss was most

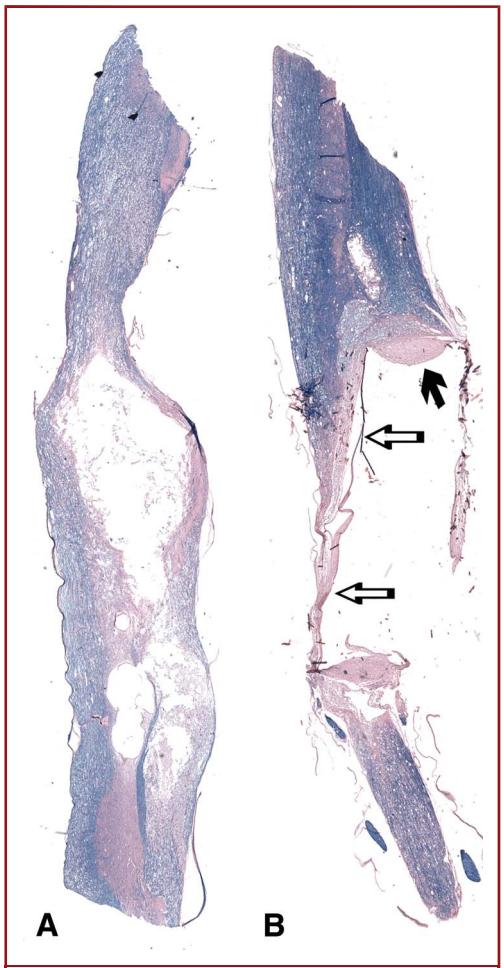

FIGURE 2. *Sagittal sections (hematoxylin-eosin and Luxol fast blue) obtained from the epicenter of the injured spinal cord 9 weeks after clip compression injury. There was progressive loss of spinal cord tissue, together with a thin rim of spared white matter and inflammatory reaction.* **A,** *control rat.* **B,** *rat from the channel plus neural stem/progenitor cell group 6 weeks after transplantation. Most of the chitosan channel disintegrated during sectioning of the paraffin embedded tissue. Remnants of the tube walls are indicated by the open arrows. The transplanted cells are clustered within the lumen of the channels and adjacent spinal cord tissue at its rostral and caudal ends indicated by the solid arrow. There was no continuous tissue bridge in the channels.*

marked centrally and was well demarcated from a surrounding thin rim of persisting white matter containing some inflammatory reaction. The pia mater of the injured segment was intact, but the ependymal cells at the epicenter of the lesions were completely lost. There was less tissue loss in the NSPC plus channel group compared with the other 2 groups. There was no collapse of the chitosan channels, and they demonstrated excellent biocompatibility with the transplanted NSPCs and injured spinal cord.

In the NSPC plus channel group, some tissue was present in the channels, but there was not a continuous tissue bridge spanning the entire length of the channels. External to the channel walls, there was a major loss of spinal cord tissue (Figure 2B). The new tissue formation in the channels was of varying size and shape and was composed of the transplanted cells protruding into the rostral and caudal ends of the channels adjacent to the host tissue. These protrusions were more pronounced caudally, and their convex surfaces faced into the channel. In general, the pattern of the cells in the protrusions was perpendicular to the long axis of the channels and spinal cord. The protrusions extending into the channels displayed 2 different shapes in the 9 rats: broad based and cone shaped in 6 rats and broad based with linear extensions parallel to the inner sides of the channels in 3 rats. The maximum length of these protrusions was approximately one-fifth the length of the channel (Figure 3). There was a mild inflammatory reaction in the cord immediately adjacent to the protrusions of transplanted cells, and beyond that there was host tissue, mainly white matter, containing some preserved myelin and generally infiltrated with macrophages. The direct NSPC injection group and the trauma-only control group were similar in overall appearance without any significant difference in the maximal area of cavitation (data not shown), cavity volume, or area of spared tissue.

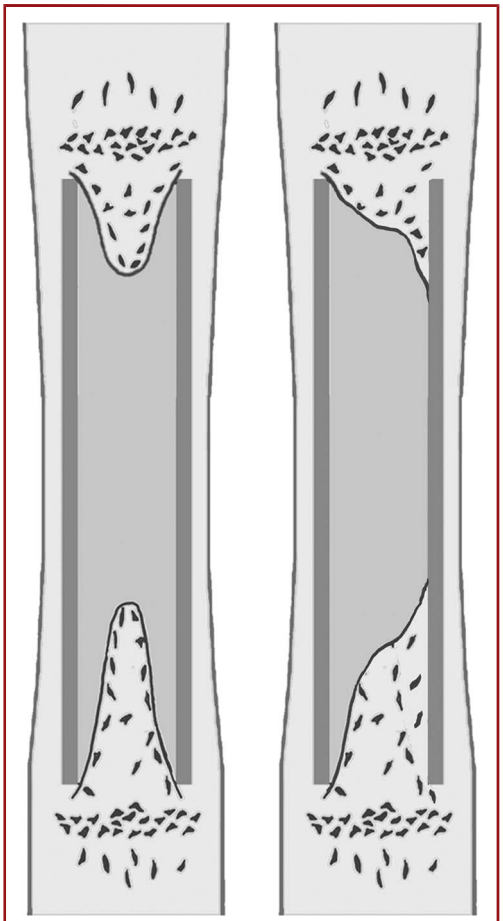

FIGURE 3. *Diagrams showing 2 different patterns of the protrusions of transplanted cells extending into the channels displayed with hematoxylin-eosin and Luxol fast blue staining, immunofluorescence, and immunohistochemistry in the channels plus neural stem/progenitor cell group. Black markings represent transplanted cells.*

Immunohistochemistry

The arrangement of the GFP$^+$ cells in the new cell formations protruding into the channels was different from the cell pattern in the normal spinal cord. Although there were cells oriented in a rostrocaudal direction along the walls of the channels, the cells in the protruding segments were usually oriented transversely (Figures 4 and 5). The protruding segments at each end of the channel appeared to be trying to form a bridge. The number of GFP$^+$ cells in the protrusions was greater than the number extending into the adjacent spinal cord or along the inner walls of the channels. The GFP$^+$ cells that migrated into the spinal cord tissue were often located adjacent to reactive astrocytes and macrophages. There were no GFP$^+$ cells along the external surfaces of the channels or in the subarachnoid space. After direct injection, most of the GFP$^+$ cells were located adjacent to less damaged tissue along the margin of the cavities at the epicenter of the lesions (data not shown).

The sections stained with ED-1-DAB and GFAP-DAB in the channel group showed that most of the GFAP$^+$ and ED-1$^+$ cells were located along the external surface of the channels, and only a small number of GFAP$^+$ and ED-1$^+$ cells were located near the GFP$^+$ cells (Figures 4 and 5). In the control rats, there were large numbers of GFAP$^+$ and ED-1$^+$ cells throughout the epicenter and periphery of the lesions.

Survival of NSPCs After Transplantation

GFP$^+$ cells were easily identifiable in the spinal cord 6 weeks after transplantation (Figure 3), and there was a significant increase in cell survival in the chitosan channel-NSPC group compared with direct injection ($P < .05$) (Figure 6). The number of surviving cells in the channel group was 10 995 $\pm$ 1361, and the percentage of surviving cells was 1.1%. The number of cells surviving rostrally vs caudally in the channels did not differ ($P > .05$). In contrast, in the direct injection group, there were 1824 $\pm$ 454 GFP$^+$ cells at 6 weeks, and the percentage of surviving cells was 0.18%.

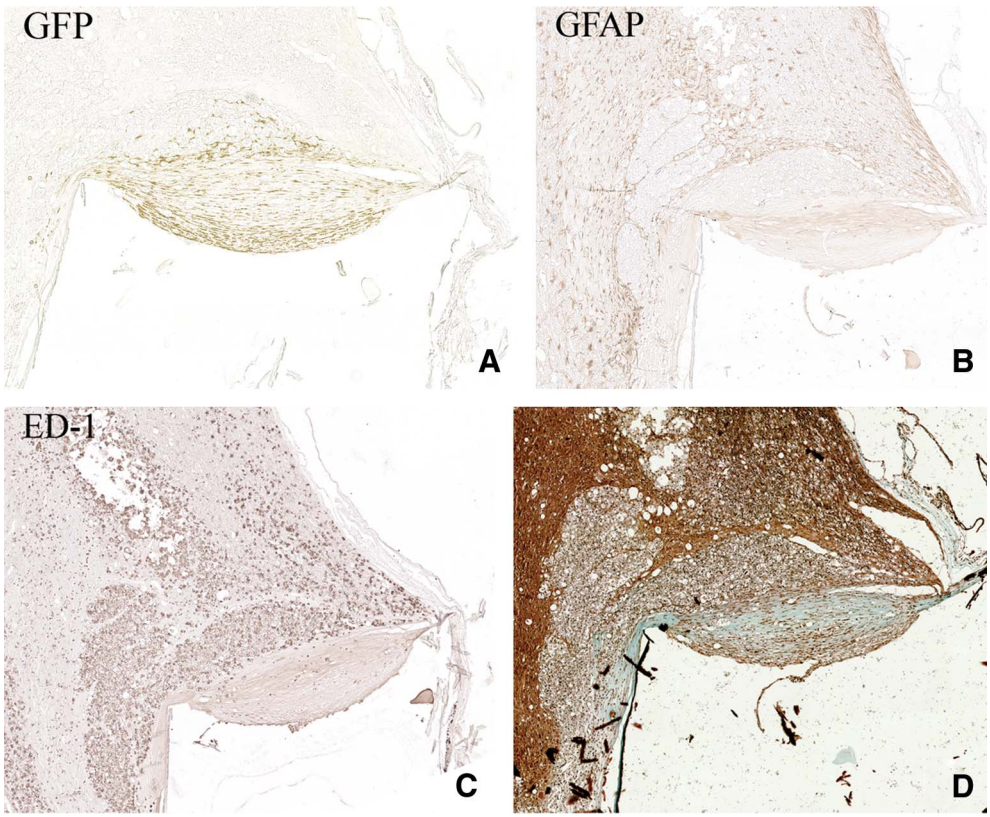

FIGURE 4. *Sagittal sections stained with green fluorescent protein (GFP; A), glial fibrillary acidic protein (GFAP; B), ED-1-DAB (C), and Masson trichrome (D). The protruded segments are seen containing formations of neural stem/progenitor cells extending into the ends of the channels at 6 weeks after transplantation. The GFP$^+$ cells (dark brown) are seen in the convex parts at the tube ends extending transversely. Some of the transplanted cells have migrated into the host tissue (A). Some GFAP$^+$ cells (brown) are in the new formation, but the most of the GFAP$^+$ cells are in the host spinal cord distant from the channels (B). ED-1$^+$ cells (brown) are adjacent to the host tissue and in the less damaged spinal cord tissue. A small number of reactive astrocytes and macrophages are in the protrusions within the channels (C). Masson trichrome staining showed some collagen in the protruding tissue within the channels and around the channels (blue; D).*

Phenotype and Cell Division of Transplanted NSPCs

In the channel plus NSPC group, the transplanted NSPCs expressed the following markers in the proportions indicated: GFAP, 3.7%; CC1, 49.7%; NeuN, 0.7%; and nestin, 46.0% (Figures 7 and 8). In contrast, in the direct injection group, the proportions were as follows: GFAP, 4.7%; CC1, 65.3%; NeuN, 0.7%; and nestin, 12.40% (Figure 8). Thus, NSPCs differentiated into mature astrocytes and oligodendrocytes in both groups. A higher proportion of oligodendrocytes progeny was noted in the direct injection group, although the differences were not significant ($P > .05$). The percentage of undifferentiated nestin$^+$ cells in the channel group was significantly higher than in the direct injection group ($P < .05$), and they were located mostly in the protrusions.

NSPCs did not colocalize with ED-1, a marker for macrophages and microglia, and no NSPCs colocalized with the Ki-67 antibody, indicating that the transplanted cells did not continue to proliferate 6 weeks after transplantation.

Locomotor Recovery

The average BBB scores for both hind limbs recorded weekly for 9 weeks after SCI are shown in Figure 9. There was some functional improvement in all groups, which reached a plateau by 9 weeks. There was no significant difference in ultimate recovery between the 3 groups of rats ($P > .05$). However, at the fourth week, there was a temporary decline in the BBB score in the chitosan channel group, suggesting that the implantation procedure at week 3 may have caused the decline.

DISCUSSION

Major SCI causes significant tissue loss and interruption of axons, resulting in permanent neurological deficits, including loss

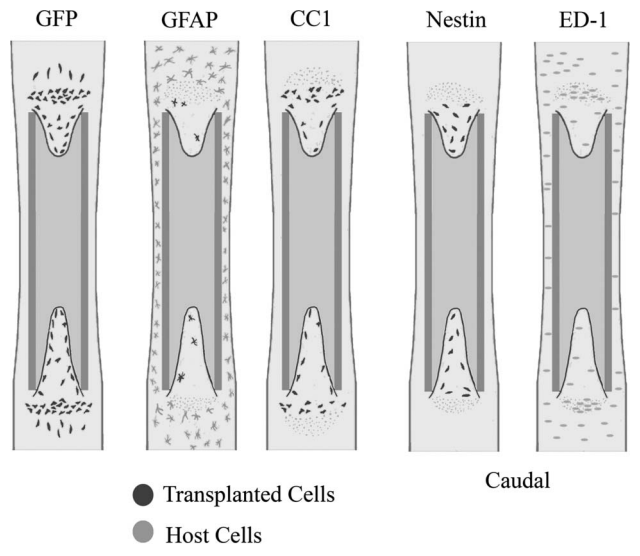

FIGURE 5. *Diagrams summarizing localization of green fluorescent protein (GFP)–, glial fibrillary acidic protein (GFAP)–, CC1-, ED-1–, and nestin-positive cells after differentiation in the channel plus neural stem/progenitor cell group at 6 weeks.*

of sensory, motor, and reflex functions. Although most experimental and clinical studies have been directed toward the acutely injured spinal cord, subacute and chronic injuries are receiving increasing attention because of their higher prevalence.[33,34] The present study used the clip impact-compression injury model, a reproducible, clinically relevant SCI model that has given reliable and consistent results for studying all phases of SCI.[23] With this model, it has been shown that several histological and functional outcome measures correlate with the force of clip compression injury.[32,33,35] We studied subacute SCI, a more stable injury than acute SCI in which there are progressive changes during the first week.[36] Our group has examined the survival of stem cells after transplantation at various times after injury and found that survival was optimal in the subacute stage 9 days after SCI.[25] The 21-day interval between trauma and treatment in the present study is considered to be subacute, a relevant interval for examining regeneration.[34]

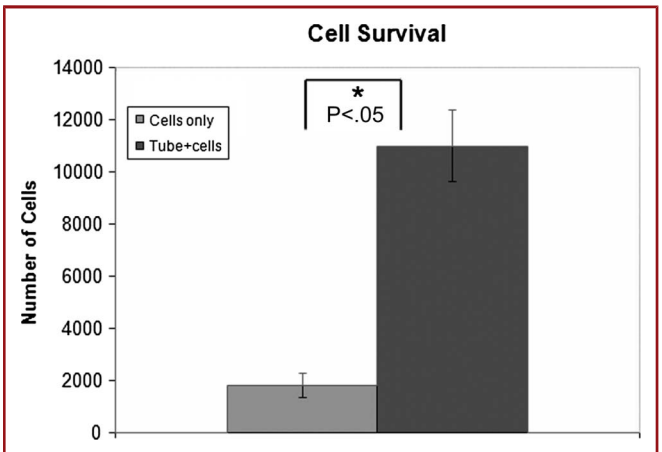

FIGURE 6. *Histogram showing the much greater survival ($P < .05$) of the neural stem/progenitor cells transplanted in the intramedullary channels vs direct injection at 6 weeks after transplantation.*

A cascade of secondary changes after SCI results in the formation of a cystic cavity surrounded by an astrocytic scar and an increasingly inhospitable environment for regeneration. Axons do not grow across cavities, and the surrounding scar imposes a physical and chemical barrier to regenerating axons.[37] Also contributing to regeneration failure is the loss of trophic factors derived from axons, harmful cytokines secreted by infiltrating cells, and apoptotic death of oligodendrocytes.[36] It is generally believed that the adult mammalian central nervous system has limited intrinsic regenerative capacity.[38] However, our group has found promising regenerative features in the endogenous ependymal region stem/progenitor cells that proliferate, migrate, and differentiate after either clip compression or minimal needle injury in adult rats.[26,39] We also showed that intrathecally delivered epidermal growth factor and fibroblast growth factor-2 enhanced the response to injury of these NSPCs and produced a mild improvement in functional recovery after SCI.[40,41] However, cell transplantation has greater potential as a regenerative strategy than stimulation of endogenous NSPCs because more NSPCs can be delivered than are present endogenously after SCI.[36,38,42]

Beginning with the pioneering grafting of peripheral nerves, many other neural and nonneural tissues and cells have been transplanted into the injured spinal cord, including olfactory ensheathing cells, embryonic stem cells, and Schwann cells.[16,36] Neural stem cells generated from embryonic cells or from fetal or adult brain and spinal cord may be ideal for spinal cord repair.[5,42,43,44] We have used neural stem cells from the adult spinal cord and brain rather than embryonic or fetal stem cells because of their similarities to the injured cells they replace, their lower oncogenic potential, and minimal ethical issues.[44-46] We have used both adult spinal cord- and brain-derived stem/progenitor cells for treatment of experimental SCI models.[7,8,21] Adult spinal cord-derived NSPCs differentiate into a high percentage of oligodendrocytes in vivo without the addition of growth factors or mitogenic agents that could enhance any oncogenic potential.[7,8,27,28] In the present study, 3 weeks after SCI, these NSPCs transplanted in chitosan channels or by direct injection showed stability of the fluorescent signal and retained expression of GFP, consistent with our previous studies.[18] There have been other studies of transplantation of adult spinal cord-derived stem cells into the adult rodent spinal cord after SCI, and in most there was differentiation mainly into astroglial and oligodendroglial lineages.[5,9,47,48]

The efficacy of neural stem cell transplantation into the injured spinal cord depends on a grafting method that optimizes the

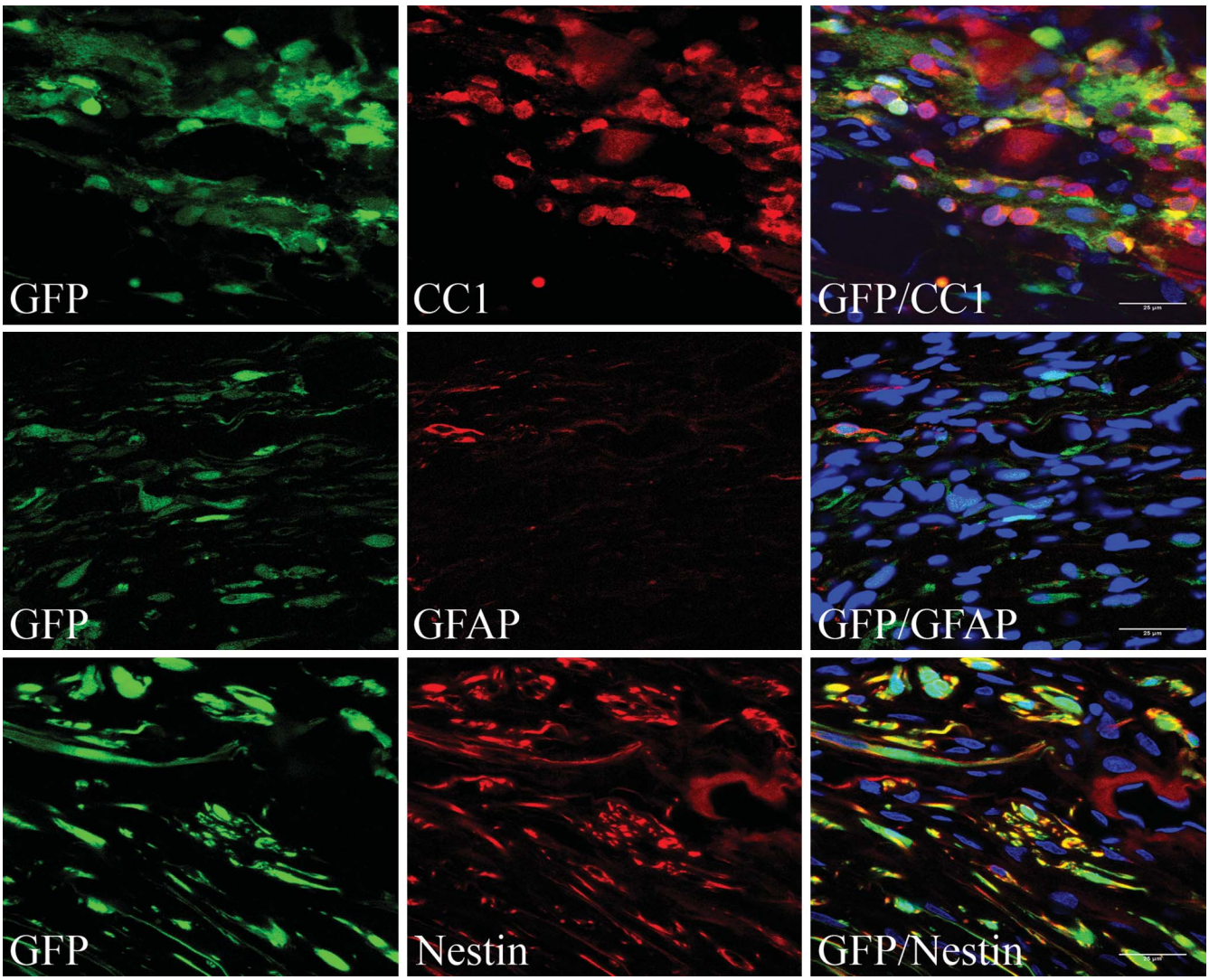

FIGURE 7. *Immunohistochemistry of transplanted neural stem/progenitor cells (NSPCs) for CC1 (oligodendrocytes), glial fibrillary acidic protein (GFAP; astrocytes), and nestin (undifferentiated cells) 6 weeks after transplantation in the channel plus NSPC group (× 63, confocal microscope). Green shows enhanced green fluorescent protein (GFP) transplanted cells; red shows GFAP, CC1, and nestin; and the merged panel demonstrates yellow double-labeled cells. In the merged panels, the sections have been DAPI stained.*

survival of the transplanted cells and minimizes host trauma. There is evidence that survival and differentiation of neural stem cells transplanted immediately after injury are compromised by the acute inflammatory response in the posttraumatic environment.[49] Survival of transplanted cells is increased after subacute or chronic transplantation, although in the chronic stage after injury, the glial scar surrounding the lesion inhibits axonal regeneration.[50,51] In the present study, the intramedullary implantation of the channel into the injured spinal cord through a dorsal myelotomy did not permanently exacerbate functional deficits.

Many strategies have been attempted to enhance the viability of transplanted NSPCs, including growth factors, immunosuppressants,[6,52,53] and tissue engineering strategies to bridge the lesion site.[3,11,12] The rationale for these strategies includes provision of a pathway for guided axonal growth, reduction of the barrier caused by scar tissue, production of neurotrophic factors to enhance endogenous cell survival or axonal regrowth, and delivery of cellular and noncellular agents to limit or neutralize secondary injury or inhibitory agents. Tissue engineering strategies may also enhance survival or differentiation of stem/progenitor cells.[15,21,22,54-63] Various degradable or

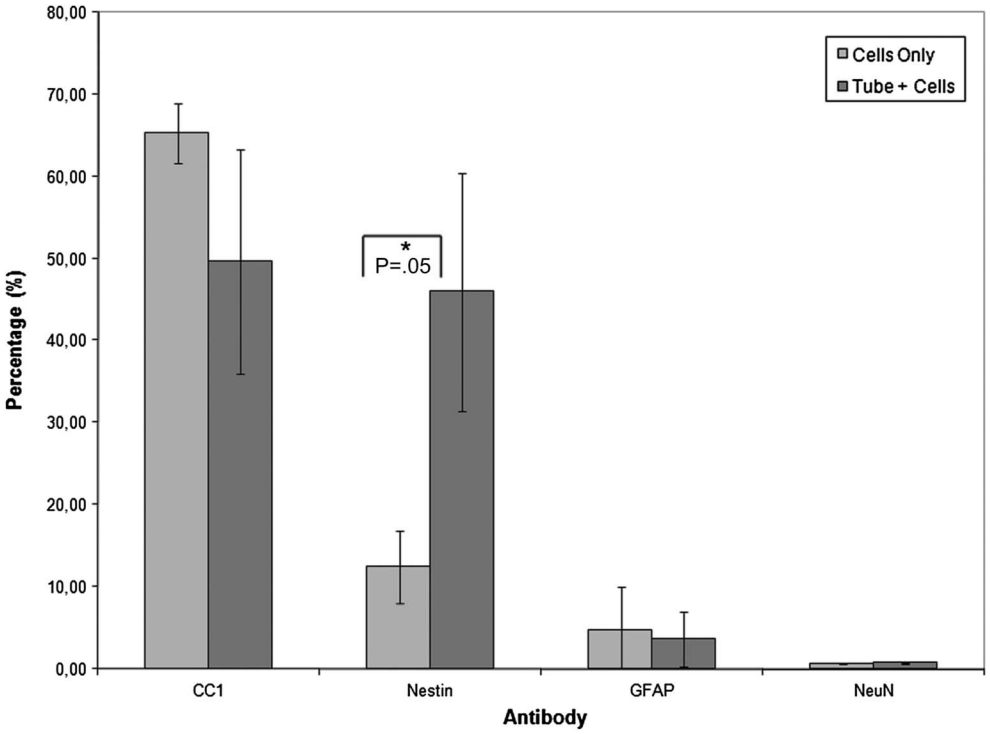

FIGURE 8. *Histogram showing in vivo differentiation of neural stem/progenitor cells (NSPCs) transplanted into the injured spinal cord 6 weeks after clip compression injury in the channel plus NSPC vs the direct injection of NSPC groups. Only the percentage of nestin⁺ cells was different between the 2 groups (*$P < .05$*).*

nondegradable polymer biomaterials and various device designs, including gels, sponges, and channels, have been tested as scaffolds, guidance channels, or delivery systems for both cellular and noncellular neuroprotective or neuroregenerative agents in experimental SCI.[11] Our group has focused on the implantation of guidance channels in combination with NSPCs. We chose a tubular, 3-dimensional biodegradable material composed of chitosan, which is noncytotoxic, mechanically stable, and capable of forming a channel that is nonadhesive for cells.[14,64-66] Others have shown that chitosan substrates are highly biocompatible with neural and mesenchymal stem cells.[67,68] Initially, we seeded the NSPCs on the chitosan channels in an attempt to increase their survival after SCI,[21] and we recently reported 2 studies in which chitosan channels seeded with NSPCs were implanted.[21,22] We found that brain-derived NSPCs seeded onto the inner surface of laminin-coated chitosan channels and implanted after complete spinal cord transection survived for at least 5 weeks after implantation and generated a tissue bridge between the 2 severed stumps placed inside the chitosan channel with good survival of the transplanted cells. Brain- or spinal cord–derived NSPCs seeded on laminin-coated chitosan channels produced a substantial tissue bridge and long-term survival.[21] However, laminin coating caused deposition of large amounts of collagen in the channels and changed the differentiation pattern of the NSPCs.

After transplantation of adult spinal cord–derived NSPCs in the present study with or without the chitosan channels, only a small number of NSPCs migrated into the adjacent host spinal cord tissue in which there were abundant reactive astrocytes, and perhaps this hostile environment prevented further migration. In contrast, with fetal spinal cord tissue transplanted into adult rats with chronic hemisection injury, there was good graft survival, differentiation, and integration with the host spinal cord.[69,70] The lack of a complete tissue bridge in the present study may be related to poor transplant survival; thus, additional strategies are required to overcome this problem. Recently, a PLGA-based scaffold seeded with mouse embryonic NSPCs was implanted into the injured area in a spinal cord hemisection model in the rat, and there was significant motor recovery.[48]

In the present study, we used uncoated chitosan channels filled with spinal cord-derived NSPCs in a nontransection subacute model to avoid the effects of coating. We found that although cell survival was higher in the channel group than in the direct injection group, the maximum percent survival was only 1.1%. NSPC survival in the channel group was 6 times higher than in the direct injection group ($P < .05$). Oligodendrocytes

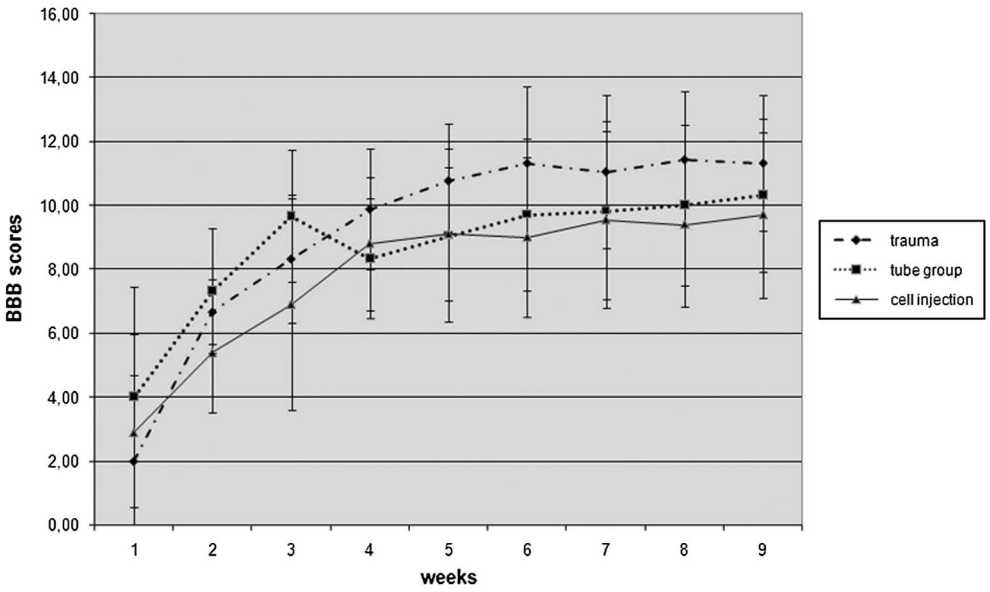

FIGURE 9. *Weekly locomotor testing for neurological recovery. No significant difference in functional recovery ($P > .05$) was seen in open-field locomotor testing between rats receiving neural stem/progenitor cells by channel or direct injection and those receiving injury only.*

predominated in both the channel and direct injection groups, and the only significant difference in differentiation was fewer differentiated cells in the channel group (Figure 9). However, the uncoated chitosan channels showed a much higher percentage of oligodendrocytes and a much lower percentage of astrocytes than in our previous studies with laminin-coated seeded chitosan channels in which astrocytes predominated.[21] Therefore, laminin coating and preseeding altered the differentiation of NSPCs toward astrocytes. Other favorable aspects of the present strategy were the absence of double labeling with Ki-67, of any mass lesions, or of additional inflammation. These results are considered promising, and future trials of uncoated chitosan channels are being planned.

CONCLUSION

The use of bioengineered, biodegradable, biocompatible, and nontoxic 3-dimensional chitosan scaffolds combined with adult spinal cord-derived NSPCs is a promising therapeutic strategy for enhancing regenerative potential and restoring spinal cord function after SCI. Adult spinal cord-derived NSPCs transplanted in uncoated chitosan channels after subacute spinal cord trauma retained their progenitor features, but only a small number migrated into the spinal cord and integrated with the host tissue. There was improved survival of NSPCs after intramedullary implantation in chitosan channels compared with direct injection. However, NSPC survival was less than previously found with laminin-coated, preseeded chitosan channels, and the cells did not completely bridge the damaged spinal cord, suggesting that cell attachment/adherence to the scaffold may play a crucial role in enhancing survival of the transplanted cells. In addition, the intramedullary implantation of the channel into the injured spinal cord through a dorsal myelotomy did not cause permanent loss of function. Additional modifications of the channel are necessary to enhance transplant survival and to improve bridging such as the concomitant administration of growth factors and coating of the channels with matrixes or provision of intraluminal scaffolding.

Disclosure

This study was supported by the Natural Sciences and Engineering Research Council of Canada, Canadian Institutes of Health Research NET, Canadian Paraplegic Association (Ontario Branch), and a studentship from the Ontario Neurotrauma Foundation. The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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Acknowledgment

We thank Rita van Bendegem and Linda Lee for excellent technical assistance and Dr Armand Keating, Princess Margaret Hospital, Toronto, for providing the GFP rats. We also thank Dr Cindi Morshead, Terrace Donnelly Centre for Cellular and Biomolecular Research, for helpful discussions.