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Bioengineering Neural Stem/Progenitor Cell-Coated Tubes for Spinal Cord Injury Repair

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The aim of this study was to understand the survival and differentiation of neural stem/progenitor cells (NSPCs) cultured on chitosan matrices in vivo in a complete transection model of spinal cord injury. NSPCs were isolated from the subependyma of lateral ventricles of adult GFP transgenic rat forebrains. The GFP-positive neurospheres were seeded onto the inner lumen of chitosan tubes to generate multicellular sheets ex vivo. These bioengineered neurosphere tubes were implanted into a completely transected spinal cord and assessed after 5 weeks for survival and differentiation. The in vivo study showed excellent survival of NSPCs, as well as differentiation into astrocytes and oligodendrocytes. Importantly, host neurons were identified in the tissue bridge that formed within the chitosan tubes and bridged the transected cord stumps. The excellent in vivo survival of the NSPCs coupled with their differentiation and maintenance of host neurons in the regenerated tissue bridge demonstrates the promise of the chitosan tubes for stem cell delivery and tissue regeneration.

Key words: Neural stem/progenitor cells; Chitosan; Tissue regeneration; Differentiation; Spinal cord injury

INTRODUCTION

Worldwide, an estimated 2.5 million people live with spinal cord injury (SCI), with more than 130,000 new injuries reported each year (International Campaign for Cures of spinal Cord Injury Paralysis website: http://www.campaignforcure.org/). In SCI, the primary mechanical damage is followed by a complex process of secondary damage due to inflammation, ischemia, free radical production, and apoptosis, among other processes. Massive disruption of the descending and ascending axonal networks, loss of neuronal and glial cells, myelin damage, and the formation of cysts and glial scars characterize the pathophysiology of the injured spinal cord (33,35). After SCI, many severed axons at the lesion site sprout at their cut ends, but then retract because central nervous system (CNS) axons have a poor intrinsic capacity for growth and encounter a series of inhibitory factors that are nonpermissive for growth such as myelin inhibitors (Nogo-A), myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (Omgp), as well as proteoglycans, such as chondroitin sulphate proteoglycans, which contribute to the glial scar (34,36).

Spinal cord injury is a complex problem. Currently there are no effective therapies for traumatic human SCI that are restorative; however, several repair strategies have been devised and tested in animal models of SCI (2,16,23,25,38,42). These have resulted in different degrees of regeneration of CNS axons within the injured spinal cord and include delivery of single or multiple neurotrophic factors including bFGF (17), BDNF (24), GDNF (6), NGF (26), and NT3 (32), neutralization of inhibitory molecules that are believed to inhibit axonal regeneration (3,18,43,45), and implantation of several types of cells and scaffolds, alone or in combination (4, 29,44).

Because the pathophysiology of SCI is complex and involves many destructive processes, combination strategies have begun to be pursued and include the provision of cellular scaffolds to replace necrotic tissue, the

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delivery of neuroprotective agents to limit secondary injury, and the delivery of biomolecules to limit and neutralize the inhibitory environment present after injury (2,16,23,25,38,42).

Our research team is developing an entubulation strategy for SCI repair, which comprises a polymeric tube, a localized drug delivery system (DDS), and neural stem/progenitor cells (NSPCs). The tubular construct presents a pathway for guided axonal growth and tissue regeneration in the completely transected injury model while at the same time providing a mechanism for local delivery of cells and therapeutically relevant biomolecules. The tubes can be used to create an environment that is permissive to regeneration while at the same time the tubes prevent in-growth of fibrous tissue that would otherwise block the path for regenerating axons. In previous studies, we have shown that implantation of tubular constructs alone or in combination with neurotrophic factors and/or peripheral nerve grafts provided some benefit in terms of tissue regeneration but were insufficient to restore function to the injured spinal cord (40, 41). With the objective of enhancing repair, NSPCs are included in our entubulation design where they may serve to replace damaged/necrotic cells and/or produce neurotrophic factors that enhance endogenous cell survival and axonal regrowth.

Towards this end, we screened a series of biomaterials that met a number of selection criteria, such as biodegradability, noncytotoxicity, moderate adhesion to cells, mechanical stability, and ability to form a tube that incorporates a drug delivery system (11,31). Based on a series of cell assays for survival, differentiation, and migration, chitosan was selected as the biomaterial of choice. Chitosan is *N*-deacetylated chitin, a naturally occurring polysaccharide that is the principal component of exoskeletons of crustaceans and insects (15). It is the second most abundant biomaterial and forms transparent tubular constructs, allowing easy visualization of cells and tissue within (8,11,31).

We examined NSPC differentiation from the adult rat forebrain subependyma (7,21,30). Our objective was to use rat (not mice) cells because we wanted to limit the possibility of tissue rejection when implanted into rat models of SCI; however, in the past isolation of neural stem cells from adult rat forebrains and their long-term cultivation has proven challenging (10,37). In the present study, we describe the isolation, characterization, and long-term expansion (>20 passages) of multipotential NSPCs from the subependymal region of the lateral ventricles of adult enhanced green fluorescent protein (GFP) transgenic rat forebrains (13). GFP NSPC neurospheres (free-floating colonies of stem and progenitor cells) were seeded into the inner lumen of laminin-coated chitosan tubes to generate neurosphere-coated tubes. The tubes were implanted into completely transected spinal cord injured rats and the survival and differentiation of NSPCs was assayed 5 weeks postimplantation.

MATERIALS AND METHODS

Isolation and Culture of Adult Neural Stem/Progenitor Cells

NSPCs were isolated from the subependymal region of the lateral ventricles in the forebrain of adult enhanced green fluorescent protein (EGFP) transgenic male Wistar rats (Fig. 1) (21). Briefly, subependymal tissue was harvested from 8–12-week-old rats and subjected to papain dissociation (Papain Dissociation System; Worthington Biochemical Corporation, Lakewood, NJ, USA). The resultant cell suspension was centrifuged and the pelleted cells were subjected to a discontinuous density gradient to remove cell debris and resuspended in complete medium (CM) containing neurobasal medium (Gibco-Invitrogen, Burlington, ON, Canada), B27 neural supplement (Gibco-Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 100 μg/ml penicillin-streptomycin (Sigma-Aldrich), 20 ng/ml epidermal growth factor (recombinant human EGF; Gibco-Invitrogen), 20 ng/ml basic fibroblast growth factor (recombinant human bFGF; Gibco-Invitrogen), and 2 ng/ml heparin (Sigma-Aldrich). Cell numbers and viability were determined with a hemocytometer and trypan blue exclusion test. Dissociated cells were plated in complete medium and incubated at 37°C in an incubator with 5% CO_2. GFP-positive neurospheres (Fig. 1B) appeared in 2–3 weeks, after which cells were passaged every week.

In Vitro Differentiation of Neural Stem/Progenitor Cells

NSPCs (passage 4) were dissociated into a single cell suspension and seeded onto biocoat poly-D-lysine (PDL)/mouse laminin-coated eight-well culture slides (Becton Dickinson Biosciences, Mississauga, ON, Canada) in differentiation medium containing 1% fetal bovine serum (FBS) in the absence of mitogens at a cell density of 50,000 cells/per well. The medium was changed every 3 days for up to 7 days. NSPCs were fixed in 4% PFA in 0.1 M phosphate-buffered saline (PBS) for 30 min at 2 or 7 days posttreatment for immunocytochemical studies. The control cultures were seeded for only 4 h in complete medium on PDL/laminin-coated tissue culture slides to generate adherent NSPCs prior to fixation. Cells were stained with each of anti-β-III tubulin, GFAP, RIP, Ki67, and nestin antibodies. Immunostaining was carried out following the protocol described below. All sections were examined and photographed using a Zeiss-LSM 510 confocal microscope. Semiquantitative immunocytochemistry was performed by determining the total cell number of at least 200 cells per

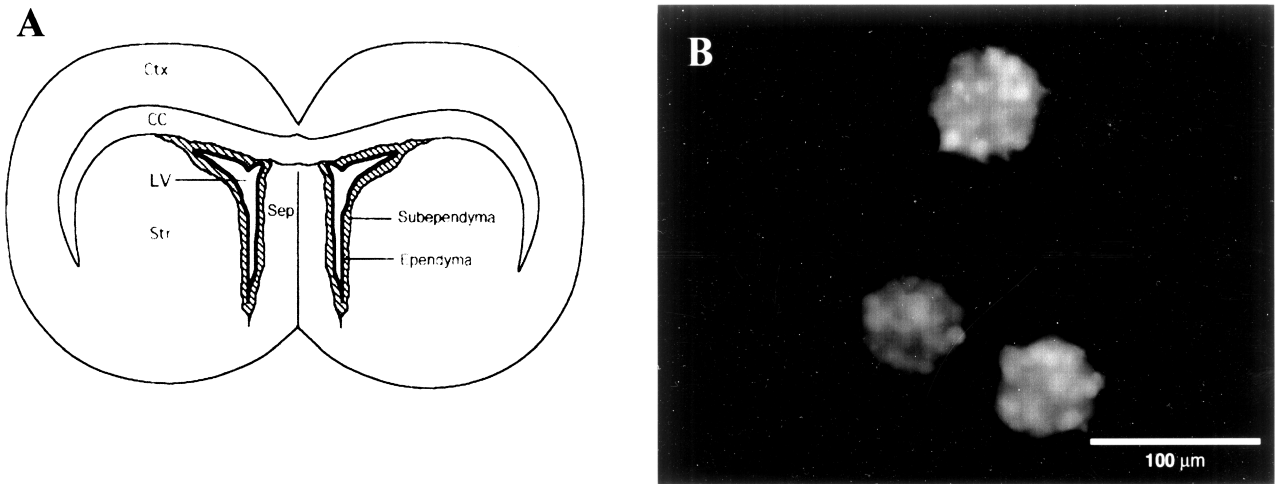

Figure 1. NSPCs were isolated from (A) subependyma of adult rat forebrains and (B) grown in vitro as neurospheres. NSPCs were harvested from (A) the subependymal region of the lateral ventricles of adult EGFP transgenic rat forebrains, in the presence of EGF and bFGF and (B) maintained as GFP-positive neurospheres in complete medium.

well in five randomly selected fields using DAPI. Each determination was performed in triplicate ($n = 3$), and each experiment was repeated at least three times.

Chitosan Tube Preparation

Chitosan tubes were prepared as previously described (11). Briefly, highly purified chitosan chloride (Protosan UP CL213, NovaMatrix, Brakeroya, Drammen, Norway) was dissolved in water and then precipitated with a 4% NaOH solution, filter washed, and freeze-dried. The dried chitosan was made into a 3% solution in 2% acetic acid, followed by 50:50 (v/v) dilution in ethanol. This chitosan solution was stored at 4°C. Tubes were prepared by addition of acetic anhydride to the chitosan solution and injection into a tubular glass mold. Tubes were washed then immersed in 40% (w/v) sodium hydroxide solution for two consecutive 2-h cycles at 110°C, after which they were washed again and air dried. The degree of deacetylation of the chitosan tubes was 89 ± 2% ($n = 3$) as determined by proton nuclear magnetic resonance ^{1}H NMR spectroscopy. All tubes used for implantation studies were cut into 8-mm lengths and sterilized by ethylene oxide gas.

Seeding of Adult Neural Stem/Progenitor Cells Into Chitosan Tubes

Chitosan tubes (length = 8 mm, inner diameter = 3.7 mm) were coated with 5 μg/ml of laminin solution (Gibco-Invitrogen) at room temperature overnight and incubated in complete medium for 2–4 h, prior to cell seeding. Next, these tubes were transferred into empty wells and the medium inside the tubes replaced with 100 μl of medium containing GFP-positive neurospheres (passage 4); cell density was estimated at 1.5×10^6/100 μl, based on cell counts of a neurosphere suspension that was dissociated into single cells prior to counting. For the in vivo study, the tubes containing the neurosphere suspension were rotated manually every 15 min for 1 h to get uniform cell seeding. The seeded tubes were then transferred to new wells containing complete medium and incubated for 2 days prior to implantation into rats with transected spinal cords. After 2 days the neurosphere-seeded chitosan tubes were either implanted or cryoprotected with 30% sucrose in 0.1 M PBS at 4°C and then frozen and embedded in frozen section medium compound (Stephens Scientific, Riverdale, NJ, USA). Cross sections (20 μm thick) were cut on a cryostat and mounted on cold (–20°C) Platinum Line™ microscope slides. Cells were visualized by counterstaining with the nuclear dye, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) present in vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Tube Implantation

Chitosan tubes with NSPCs seeded within were implanted into female Sprague-Dawley rats ($n = 4$) as described previously (22). Rats were deeply anesthetized with 2% halothane and then 1:2 nitrous oxide to oxygen. After laminectomy from T7–9, the facets at the same levels were removed, and the dura mater was longitudinally incised in the midline. The spinal cord at T8 was then completely transected with microscissors. The adjacent dorsal and ventral nerve roots were also completely transected to allow the stumps to be mobilized for insertion into the 8-mm-long tubes. Next, the rostral and caudal cord stumps were inserted approximately 3 mm into

the rostral and caudal ends of the tube, respectively. In all animals, the tube was positioned symmetrically so that equal lengths of rostral and caudal stumps were present in the tube; the remaining gap between the stumps in the tube was approximately 2 mm. A spinal fusion was then performed from T6 to T10 with No. 5 surgical steel (Ethicon Inc., Somerville, NJ, USA) and 2-0 silk (Johnson and Johnson, Peterborough, ON, Canada) as previously reported (22). Forty microliters of the fibrin sealant Beriplast P (Beriplast, Gift from ZLB Behring GmbH, Marburg, Germany) was applied to both rostral and caudal stump–tube interfaces, and a synthetic expanded poly(tetrafluoroethylene) (ePTFE®) membrane (Preclude Dura Substitute, Gore, Gift from Gore & Associates, Inc., Flagstaff, AZ, USA) was placed between the tube and the wire used for fusion to prevent extradural scarring and invasion of fibrous tissue. The incision was then closed with 3-0 Vicryl sutures (Johnson and Johnson) in the paravertebral muscles and Michel clips (Fine Science tools, North Vancouver, BC, Canada) in the skin. All animals received buprenorphine (0.03 mg/kg) subcutaneously for 3 days postinjury, and manual bladder compression three times daily for the duration of the study. To aid transplant survival, all animals were immunosuppressed with a daily SC injection of cyclosporine (15 mg/kg, Sandimmune, Nacartis Pharma Canada Inc., Dorval, QB, Canada) until they were sacrificed.

Tissue Preparation

Five weeks after channel implantation, the rats were deeply anesthetized by an IP injection of 1.0 ml sodium pentobarbital. After an intracardiac injection of 1 ml of 1000 U/ml heparin, the animals were perfused through the ascending aorta with 500 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). A 2-cm length of spinal cord encompassing the implanted channel and transection site was carefully removed. The spinal cords were cryoprotected with 30% sucrose in 0.1 M PBS at 4°C and then frozen and embedded in frozen section medium compound. Parasagittal sections (20 μm thick) were cut in a 1:9 series on a cryostat and mounted on cold (–20°C) platinum line™ microscope slides. Note that the chitosan tubes were removed prior to tissue processing.

Immunohistochemistry

The following antibodies were used for the immunohistochemical studies: mouse anti-β-III tubulin protein monoclonal antibody (β-III tubulin; 1:500, Chemicon, Temecula, ON, Canada) for neurons; mouse anti-glial fibrillary acidic protein monoclonal antibody (GFAP; 1:200, Chemicon) for astrocytes; mouse anti-CC1/APC monoclonal antibody (CC1; 1:1000, Calbiochem, San Diego, CA, USA) for oligodendrocytes; mouse anti-neurofilament 200 monoclonal antibody (NF200; 1:500, Sigma, St. Louis, MO, USA) for neurons and axons; mouse anti-Ki67 antibody (Ki67; 1:100 dilution, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) for proliferating cells; mouse anti-microtube associated protein 2 monoclonal antibody (MAP2; 1:1000 dilution, Chemicon) for neurons and dendrites; NeuN, (NeuN; 1:100 dilution, Chemicon) for neurons; mouse anti-rat nestin monoclonal antibody (nestin; 1:100 dilution, BD Biosciences Pharmingen, Mississauga, ON, Canada) for neuronal progenitor cells; and mouse anti-RIP monoclonal antibody (RIP; 1:5, Development Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA) for oligodendrocytes. For all immunohistochemistry procedures, appropriate negative controls were obtained by omission of the appropriate primary antibody.

For immunohistochemistry in frozen sections, anti-NF200, GFAP, CC1, MAP2, and nestin antibodies were used. Sections were thawed and washed three times with PBS for 10 min. After being rinsed with PBS, the sections were blocked for nonspecific antibody binding at RT for 1 h with the following: 10% heat-inactivated serum in PBS containing 0.3% Triton X-100 for GFAP and NF200, and 2% normal goat serum (NGS) containing 0.3% Triton X-100 for CC1, MAP2, and nestin. Then all sections were incubated overnight in a solution of primary antibody diluted in blocking solutions at 4°C. After being washed three times with PBS, the specimens were incubated with goat anti-mouse Alexa Fluor™ 568 (1:500 dilution in PBS; Molecular Probes, Eugene, OR, USA) for 1 h. The sections were counterstained with the nuclear dye DAPI present in vectashield mounting medium and coverslipped.

Ten sections per animal for each antibody were examined and photographed using a Zeiss-LSM 510 confocal microscope. Z-stack images were taken, collecting 20–40 optical sections, at 0.5 μm in height per section. Then the entire thickness of the section was visualized using ImageJ software system (a public domain Java image processing program inspired by NIH Image for the Macintosh).

Stereological Analysis of Cell Survival and Differentiation

For stereological cell counts of GFP-positive cells to determine cell survival, the ninth equidistant frozen section of each series was selected from all of the spinal cord tissues per animal ($n = 3$) and counterstained with the nuclear dye DAPI present in vectashield mounting medium and coverslipped. To observe cell differentiation of NSPCs at 5 weeks post-tube implantation, five frozen spinal cord tissue sections per animal containing

the middle part of the tissue bridge in the tubes were selected for each of the following antibodies; anti-nestin, GFAP, CC1, MAP2, and NeuN antibody, and immunostained and counterstained with DAPI as described above. These spinal cord sections were then observed with a Leica DMRB light microscope under a 100× objective magnification, and the cell counts were performed using the Stereo Investigator Software (Version 7, MBF Biosciences, Williston, VT, USA).

RESULTS

Adult Rat Forebrain-Derived NSPCs Are Multipotent and Self-Renewing In Vitro

NSPCs were dissociated into a single cell suspension and cultured on laminin-coated tissue culture slides in the presence of EGF/bFGF/heparin (EFH) for 4 h (undifferentiated group) or 1% FBS for 7 days. Immunocytochemical staining was used to determine if the undifferentiated cells expressed markers for neural precursors and if they were capable of multilineage differentiation. A vast majority of NSPCs (95.9 ± 2.1%) in the undifferentiated group were nestin positive, while very few cells stained positive for differentiation markers (β-III tubulin for neurons, GFAP for astrocytes, and RIP for oligodendrocytes). In contrast, after 7 days of FBS treatment most of the NSPCs differentiated into one of the three phenotypes (Fig. 2): neurons (10.7 ± 2.9%) (Fig 2A–C), astrocytes (12.6 ± 3.6%) (Fig. 2D–F), and oligodendrocytes (40.6 ± 4.4%) (Fig. 2G–I), and a subset continued to express nestin (23.1 ± 3.4%). We assayed for self-renewal by passaging the neurospheres and showing that neurosphere-derived cells replated in mitogenic growth factors could generate neurospheres for up to 20 passages. Hence, the rat NSPCs displayed the stem cell properties of self-renewal and multilineage differentiation.

Cell Seeding Into the Inner Lumen of Chitosan Tubes

Several techniques were tested to achieve optimal cell seeding for the generation of neurosphere-coated tubes including seeding neurospheres on substrate-coated (laminin or fibronectin) or uncoated chitosan tubes, varying the cell densities (5×10^5 to 1.5×10^6), and manual versus mechanical rotation of tubes for uniform seeding. Optimal cell seeding was achieved when chitosan tubes were coated with laminin, seeded with 1.5×10^6 cells, and rotated manually four times in 1 h (Fig. 3). As shown in Figure 3A and C, the cells adhered and grew on the surface of the inner lumen of the nonporous tubular conduit. The chitosan tubes were autofluorescent (depicted by arrows), requiring DAPI labeling of nuclei to easily distinguish GFP-positive cells from the tube (Fig. 3B).

The NSPCs were also assayed for precursor and differentiation marker expression after being cultured on chitosan for 48 h, to ensure that their cell potency had not changed during this time interval prior to implantation. The majority of cells continued to express nestin (88.8 ± 2.9%) and 5–10% of the cells expressed differentiation markers: β-III tubulin for neurons (1.4 ± 1.04%); GFAP for astrocytes (4.4 ± 1.9%); and RIP for oligodendrocytes (5.5 ± 2.3%). After 48 h in vitro, the cell density increased to $2.25 \pm 0.15 \times 10^6$ cells per tube.

In Vivo Survival and Differentiation of NSPCs

At 5 weeks postimplantation, a thick tissue bridge filled the original gap between the rostral and caudal stumps in the NSPC-coated tubes in all four animals (Fig. 4A, B). Fluorescent microscopy on frozen spinal cord tissue sections showed excellent cell survival of GFP-positive cells in the tissue bridge (Fig. 4C). Stereological cell counts on these sections showed $2.09 \pm 0.35 \times 10^6$ GFP-positive cells in the tissue bridges within the chitosan tubes. Immunohistochemistry showed that 23.2 ± 6% of GFP-positive cells were CC1-positive oligodendrocytes (Fig. 4D–F), 9.9 ± 1.2% were GFAP-positive astrocytes (Fig. 4G–I), and 18.3 ± 3.6% were nestin positive (Fig. 4J–L). There were many NF200-positive axons in the tissue bridges that were host derived (i.e., not expressing GFP). Indeed, none of the GFP-positive cells colocalized with the neuronal markers, MAP2 and NeuN. We observed GFP-positive cells running in close proximity to host axons and at times they appeared to wrap around the axons, resulting in a partial overlap, but there was no complete colocalization between the NF200- and GFP-positive cells (Fig. 4M–O). These data are summarized graphically in Figure 4P.

DISCUSSION

In this study, the survival and differentiation of adult rat NSPCs were tested both in vivo and in vitro. When seeded onto the inner lumen of laminin-coated chitosan tubes implanted into the completely transected spinal cord of adult rats, NSPCs survived 5 weeks postimplantation and were seen within the newly generated tissue bridge that formed between the two severed stumps inside the tubular construct. Because cell survival following transplantation is a concern in developing therapeutic approaches, the long-term cell survival that was observed is significant and may reflect the protective environment provided by the chitosan tube. Previously, polymer/progenitor cell composite grafts have been shown to be an effective means of increasing cell survival when transplanted into sites within the central nervous system compared to progenitor cell injections alone (39). The poor cell survival that was previously observed in the cell injection-alone group has been corroborated in the present study. We found less than 1% NSPC survival was observed at 7, 14, and 28 days after transplantation

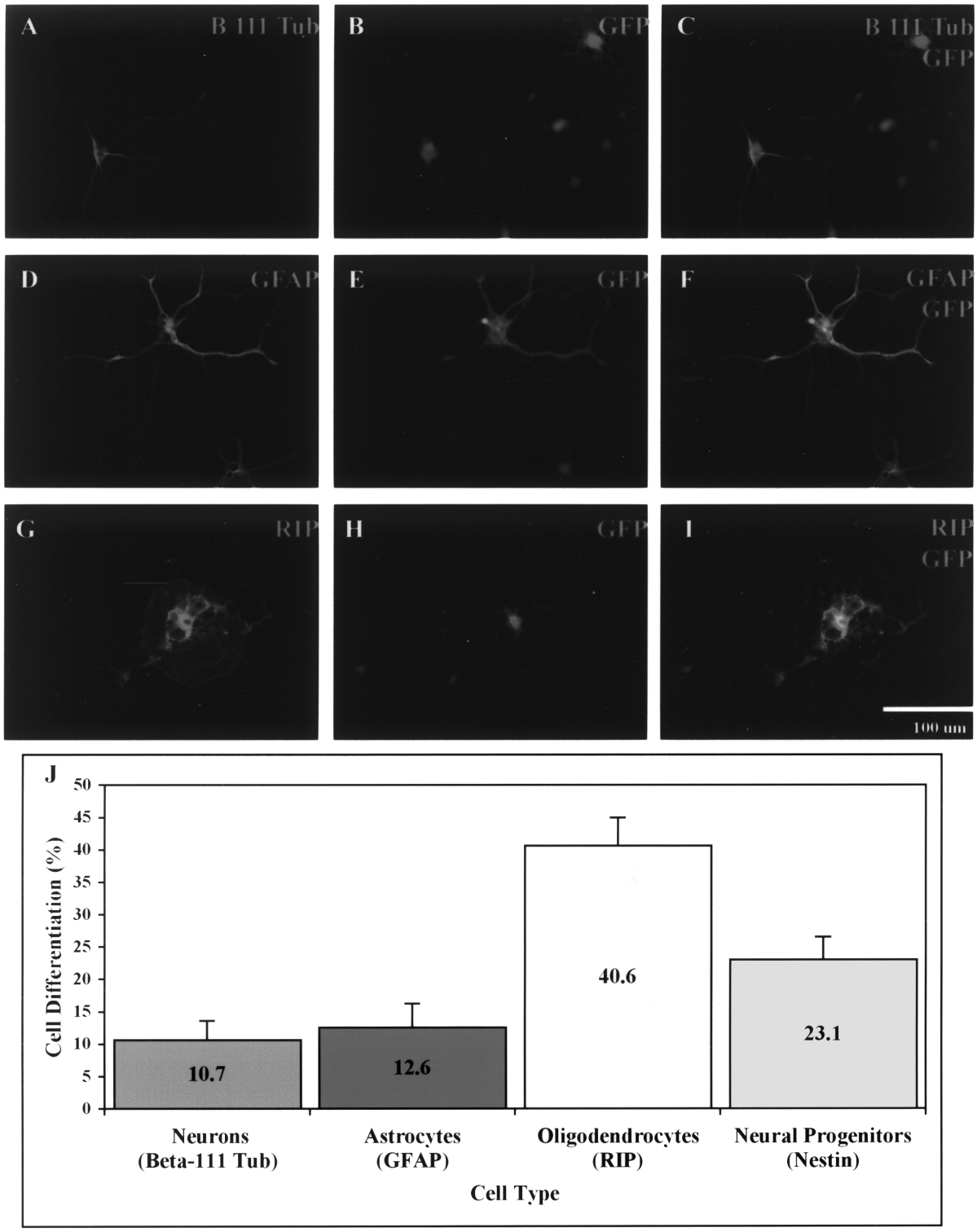

Figure 2. NSPCs isolated from the subependyma of adult rat forebrain exhibit multipotency in vitro. (A–I) Representative micrographs of adult NSPCs grown in 1% FBS for 7 days differentiate into neurons (β-III tubulin), astrocytes (GFAP), and oligodendrocytes (RIP). (J) Quantitative data of the proportion of in vitro differentiated NSPCs to neurons, astrocytes, and oligodendrocytes (mean ± SD; $n = 9$).

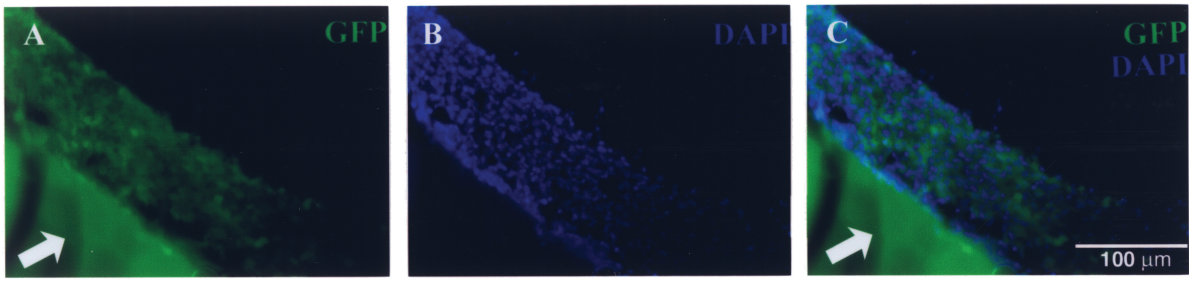

Figure 3. Seeding of adult NSPCs into chitosan tubes. GFP-positive NSPC neurospheres were seeded at a high cell density (1.5×10^6 cells per tube) into the inner lumen of laminin-coated chitosan tubes and rotated mechanically every 15 min for 1 h at 37°C prior to 48-h incubation to generate neurosphere-coated chitosan tubes. After 48 h the neurosphere-seeded chitosan tubes were frozen and cross sectioned (A–C). Because the chitosan tubes autofluoresced (depicted by arrows), cells were visualized by counterstaining with DAPI (B, C) on an epifluorescent microscope.

at the site of lesion in an acute compression injury model of SCI (27). Most (if not all) cell transplantation studies (except encapsulated cell therapy studies) administer an immunosuppressant, such as cyclosporine, to avoid phagocytosis of the implanted cells by macrophages. While we did not specifically examine the effect of cyclosporine on cell survival or proliferation, a recent study by Guo et al. demonstrated that cyclosporine inhibited the proliferation of neural stem cells in a dosage-dependent manner, increased astrocyte genesis, and decreased neuron differentiation (12).

In the present in vivo study, we found differentiation of NSPCs into astrocytes and oligodendrocytes, yet no neurons. Because the NSPC were grown as adherent cultures on laminin for 48 h prior to implantation and not as free-floating spheres, we stained them with various markers to ensure that the lack of neurons in vivo was not due to a change in their potency ensuing from our distinct culture system. While we observed that 5–10% of NSPCs expressed differentiation markers prior to implantation, these cells were not restricted to astroglial lineages. Importantly, the majority of cells (almost 90%) continued to express nestin and remained in an undifferentiated state prior to implantation. We also observed nestin-positive cells in vivo, indicating that undifferentiated NSPCs persist for at least 5 weeks following implantation. To gain greater insight into the proliferative potential of these cells, they were immunostained with Ki67, a prototypic cell cycle-related nuclear protein, expressed by cells in all phases of the active cell cycle (G_1, S, G_2, and M phase) (9). NSPCs stained negative for Ki67 at 5 weeks postimplantation, excluding any possibilities of tumor formation. Similar to other transplantation studies (14), a large fraction of the GFP-positive cells that survived in vivo did not stain for any of the neural cell-specific markers in the present study. We speculate that these cells may be immature neural phenotypes, in preliminary stages of differentiation that were not detected by the differentiation markers used in this study.

Importantly in the in vivo study, many host-derived NF200-positive fibers were detected in the tissue bridge between the stumps of the completely transected spinal cord, suggesting that regenerating axons had grown into the chitosan tube. This is a striking observation given that our SCI model, the complete spinal cord transection model, is the most severe of the injury models and, unlike partial injury models, there is no ambiguity between regenerating axons and spared lesioned axons (16). Given that neuronal differentiation appears to be limited in the injured spinal cord in models of transplantation and endogenous stimulation (19), it is encouraging that the transplanted cells within the tubular constructs are able to enhance axon elongation through the lesion.

Previous studies have shown enhanced axonal regeneration after transplantation of Schwann cells (SCs) into the contused spinal cord (1,28). The transplanted SCs formed a substantive graft within the injury site, which in turn supported the in-growth of numerous axons. Many of the axons that were found within regions of grafted SCs were myelinated, demonstrating the myelination potential of these transplanted cells in the injured CNS. However, the Schwann cells failed to migrate from the lesion site, thus spatially limiting their therapeutic potential.

Although the use of NSPCs is a promising therapy for neuronal replacement in the injured spinal cord, the issues of NSPC replacement of neurons are complex because the injured spinal cord may be restrictive to mature neuronal differentiation of even neuronal precursor cells (5,14). This fact has been reaffirmed in the present in vivo study where the transplanted NSPCs failed to generate neurons. More recently, greater understanding of disease states and the role of oligodendrocytes in remyelination have sparked tremendous interest in oligodendrocytes/oligodendrocyte precursors. As remyelina-

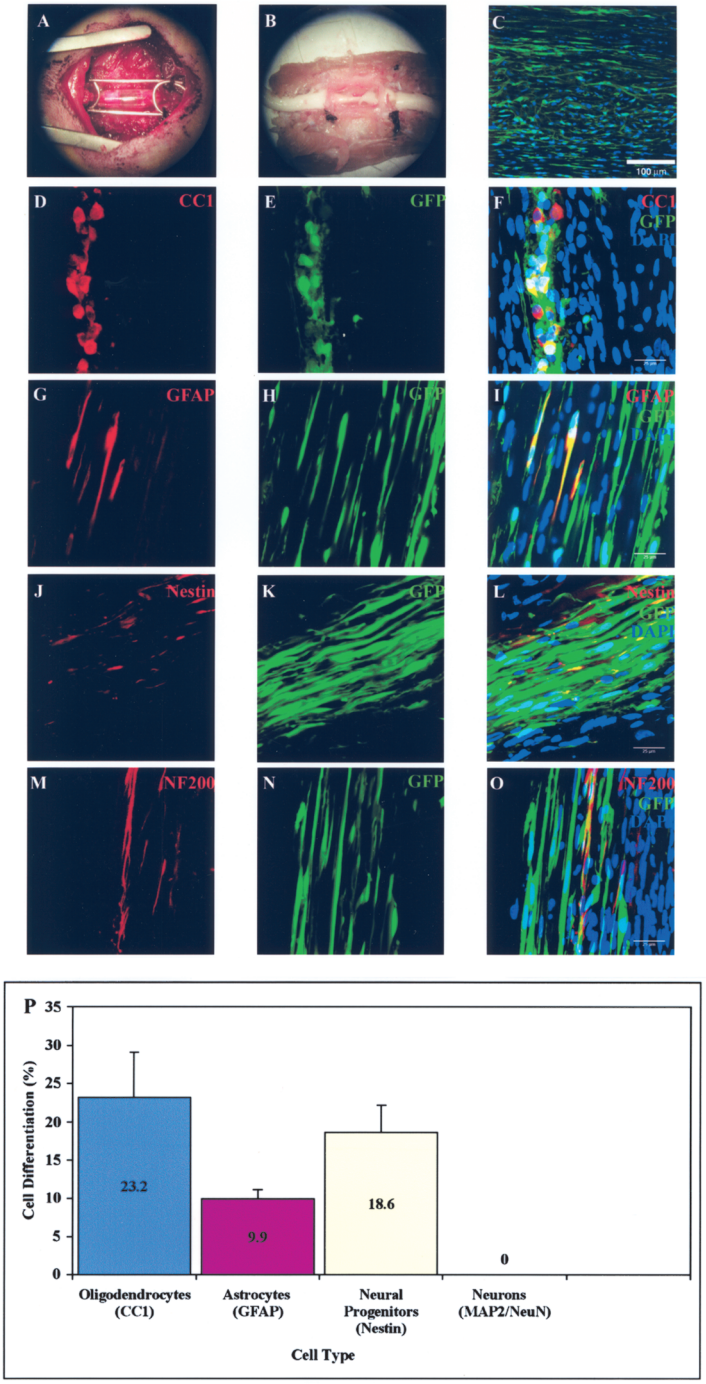

Figure 4. In vivo survival and differentiation of adult NSPCs into specific cell types (A–O). NSPC-coated chitosan tubes were implanted into completely transected spinal cords of adult rats (A). A thick tissue bridge formed between the rostral and caudal stumps at 5 weeks postimplantation (B). Implantation of NSPC-coated chitosan channels allowed very good in vivo survival of NSPCs at 5 weeks postimplantation (C). The surviving NSPCs expressed oligodendrocyte primarily in the inner periphery and center of the bridge (CC1; D–F), astroglial (GFAP; G–I) primarily in the outer periphery of the tissue bridge and neural progenitor (nestin; J–L) markers, but not neuronal markers (NF200; M–O). (P) In vivo differentiation of NSPCs into oligodendrocytes and astrocytes at 5 weeks, in the proportions indicated in the histograms (mean ± SD; $n = 3$).

tion appears to be one of the most feasible neuroprotection strategies for spinal cord injury repair (20), oligodendrocytes are important near-clinical therapeutic targets for restoring function after SCI. In the present in vivo study we demonstrated that the transplanted rat NSPCs primarily differentiated into oligodendrocytes.

In conclusion, we have tested the in vivo utility of the first two components of our entubulation strategy: the implantation of a chitosan tubular construct and its delivery of neural stem/progenitor cells for spinal cord injury repair. We have demonstrated that NSPCs seeded in chitosan tubes survive well, differentiate, and allow axonal regeneration through the tubular construct in a severe, complete spinal cord transection injury model. We are examining axonal regeneration and the potential for functional recovery using these bioengineered neurosphere-coated tubes in long-term studies.

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