Published in final edited form as:

J Comp Neurol. 2006 October 1; 498(4): 525–538. doi:10.1002/cne.21065.

Fate of Endogenous Stem/Progenitor Cells Following Spinal Cord Injury

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Abstract

The adult mammalian spinal cord contains neural stem and/or progenitor cells that slowly multiply throughout life and differentiate exclusively into glia. The contribution of adult progenitors to repair has been highlighted in recent studies, demonstrating extensive cell proliferation and gliogenesis following central nervous system (CNS) trauma. The present experiments aimed to determine the relative roles of endogenously dividing progenitor cells versus quiescent progenitor cells in posttraumatic gliogenesis. Using the mitotic indicator bromodeoxyuridine (BrdU) and a retroviral vector, we found that, in the adult female Fisher 344 rat, endogenously dividing neural progenitors are acutely vulnerable in response to T8 dorsal hemisection spinal cord injury. We then studied the population of cells that divide postinjury in the injury epicenter by delivering BrdU or retrovirus at 24 hours after spinal cord injury. Animals were euthanized at five timepoints postinjury, ranging from 6 hours to 9 weeks after BrdU delivery. At all timepoints, we observed extensive proliferation of ependymal and periependymal cells that immunohistochemically resembled stem/progenitor cells. BrdU+ incorporation was noted to be prominent in NG2-immunoreactive progenitors that matured into oligodendrocytes, and in a transient population of microglia. Using a green fluorescence protein (GFP) hematopoietic chimeric mouse, we determined that 90% of the dividing cells in this early proliferation event originate from the spinal cord, whereas only 10% originate from the bone marrow. Our results suggest that dividing, NG2-expressing progenitor cells are vulnerable to injury, but a separate, immature population of neural stem and/or progenitor cells is activated by injury and rapidly divides to replace this vulnerable population.

Indexing terms

regeneration; NG2; gliosis; myelin; rat; proliferation; adult; oligodendrocyte; glia

Spinal cord injury results in gliosis and limited cellular regeneration (Horner and Gage, 2000). This is true despite the observation of multipotent spinal cord progenitor cells with significant potential for nervous system regeneration (Weiss et al., 1996; Shihabuddin et al., 1997). Little is known about the course of reaction of adult neural stem cells following trauma. Progenitor cells participate in the repair of demyelination lesions and, more recently, the glial

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progenitor has been implicated in the formation of scar tissue (Franklin et al., 1997; Hammang et al., 1997; Keirstead et al., 1998; Alonso, 2005).

Using a pinprick spinal cord injury (SCI) model, Adrian and Walker (1962) originally observed that cells (including oligodendrocytes, microglia, and astrocytes, as well as a substantial, unclassified population) incorporate [³H]-thymidine and comprise a population of rapidly proliferating glia. Recent studies demonstrated that a population of nestin+ cells abutting the central canal proliferates after SCI (Frisen et al., 1995; Mothe and Tator, 2005). These studies also showed that ependymal cells proliferate 50 times more rapidly in spinal cord-injured animals (24 hours after injury) than in uninjured animals, and that the dividing ependymal cell population differentiates into astrocytes that migrate toward the injury site and contribute to the formation of scar tissue over a period of several weeks (Johansson et al., 1999). Studies revisiting the Adrian and Walker experiments, with the addition of BrdU and cell-tracking dyes, have shown that ependymal cells do proliferate when a minimal lesion is made in the dorsal spinal cord (Mothe and Tator, 2005). In addition, dye-labeled cells near the ependyma were shown to divide within 48 hours, migrate from the central canal, and differentiate primarily into astrocytes. Alonso (2005) has shown that in a cortical stab wound there is also a very early (<24 hours) proliferative response. Using a combination of mitotic labels, Alonso speculated that local glial progenitor cells were at least in part responsible for the production of astrocytes. Yamamoto et al. (2001a,b) have shown that injury upregulates the total number of progenitor cells in ependymal but also nonependymal regions of the injured spinal cord. Proliferating progenitor cells in the subpial zone of the spinal cord have also been demonstrated (Yamamoto et al., 2001a; Shibuya et al., 2002, 2003) and significant cell proliferation and glial replacement occurs in regions of the spinal cord where the ependyma has been destroyed (Zai and Wrathall, 2005; Rosenberg et al., 2005). These latter studies challenge the idea that progenitor cells are generated exclusively from the subependymal region. A better understanding of when stem cells are activated following injury, their fate restriction, and site of origin is important in order to understand why the spinal cord does not significantly heal itself.

In this article we provide the first evidence that constitutively proliferating adult progenitor cells are vulnerable to injury. Their selective vulnerability is balanced by a second immature population of stem/progenitor cells originating in the subependymal region but also throughout the spinal cord parenchyma. Our findings indicate a proliferative burst of central nervous system (CNS)-derived progenitors occurs as early as 24 hours postinjury. This immature population exhibits massive proliferation and differentiates primarily into NG2-expressing glia and oligodendrocytes with no evidence for neuronal differentiation.

MATERIALS AND METHODS

Animals

Adult female Fisher 344 rats (160–185g), age 8–12 weeks, and adult female C57/BL-6 mice were used (n = 5 or 6 per group; Harlan Sprague Dawley, Indianapolis, IN). Their ad libitum diet consisted of water and chow (Teklad 4% rat diet 7001, Harlan Teklad, Madison, WI). All of the studies performed were conducted under the strict guidelines and consent of the institutional animal care and use committees of the Salk Institute and the University of Washington.

Anesthetic

Anesthesia was used for all surgical procedures and before perfusion. The animals received an intraperitoneal injection of 44 mg/kg ketamine (Ketaset, 100 mg/mL; Bristol Laboratories, Syracuse, NY), 4 mg/kg xylazine (Rompun, 20 mg/mL; Miles Laboratories, Shawnee, KS),

and 0.75 mg/kg acepromazine maleate (10 mg/mL; TechAmerica Group, Elwood, KS) diluted in 0.9% sterile normal saline.

Spinal cord injury model

Using appropriate aseptic technique, a partial dorsal laminectomy in the rat or mouse was performed at a single vertebral segment (T8) without disturbing the dura. Lidocaine (2%, 1–2 drops, Bimeda, Riverside, MO) was applied to the dura for 1–2 minutes to anesthetize the region to be transected. Then, using fine iridectomy scissors, a cut was made through the dorsal aspect of the cord (1 mm deep in the rat and 0.5 mm in the mouse). No tissue was resected. After hemostasis was confirmed, the overlying muscle layers were sutured and the skin was stapled closed.

Postoperative care

Postoperatively, all animals were placed in cages on warming pads. Subcutaneous lactated Ringer's solution (5 cc) was given to the rats immediately postoperatively. Temperature and respiration were monitored until they awakened. Bladders were manually expressed 2 times per day until normal voiding reflexes returned, usually within 24 – 48 hours. (Bowel/bladder function and hindlimb function returned quickly after dorsal hemisection because the ventral motor fibers were spared by the injury.) An antibiotic (Baytril, 2.5 mg/kg) was given twice daily starting immediately before surgery until the return of bladder function and at any sign of bladder infection. In addition to their daily chow, all rats received a daily high caloric supplement, Nutracal (Evsco, Buena, NJ) for the first 3 postoperative days or until they regained their preinjury body weight. After the critical postoperative period, technical support personnel provided continuous care. All animal procedures were in accordance with the Department of Health, Education, and Welfare Publ. No. 85–23, “Guide for the Care and Use of Laboratory Animals.”

BrdU injection regimen

Three regimens were used to assess proliferation in the injured spinal cord. Group 1: For BrdU prelabeling experiments (to study the response of endogenously dividing neural progenitors to spinal cord injury), animals received one intraperitoneal (i.p.) injection of BrdU (50 mg/kg, Sigma, St. Louis, MO) each day for 10 days prior to injury and were injured on the eleventh day. Animals were euthanized at 1 day PI (postinjury, $n = 5$) and 1 month PI ($n = 5$) for histologic analysis. Group 2: To determine the peak of proliferation following spinal cord injury, animals were given a single pulse of BrdU (50 mg/kg i.p.) at 24 hours, 3, 6, or 9 days after injury ($n = 5$ per group). Animals were euthanized at 24 hours after BrdU injection for histologic analysis. Mitotic profiles were quantified in this group for the purpose of determining the peak proliferative period. This group was not processed for cell phenotype. Group 3: To determine the phenotype of cells which are dividing at 24 hours after injury, a single pulse of BrdU (50 mg/kg i.p.) was given at 24 hours after injury and animals were euthanized and processed for multiple label immunofluorescence at 6 hours, 1, 2, 3, or 9 days following the single BrdU injection ($n = 6$ per group).

Preparation of virus

Preparation of the green fluorescence protein (GFP)-retrovirus has been previously described (Palmer et al., 1999). The retrovirus was generously provided by Dr. Henriette van Praag (Salk Institute, La Jolla, CA) and is a vector based on the Moloney murine leukemia virus expressing GFP. GFP is driven by a cytomegalovirus (CMV) promoter to provide ubiquitous neural expression in dividing cells.

Injection of GFP retrovirus into spinal cord

For animals receiving viral injections, anesthesia and laminectomy were as above. To reduce movement due to respiration or reflex, which could have made the injection injurious, the animals were mounted on a spinal frame that secured the spinous processes immediately rostral and caudal to the exposed level. Then, using a glass pipette with an 80- μ m tip attached to a 5- μ l, 26 G Hamilton syringe mounted in a micromanipulator, 2 μ L of 1×10^8 cfu/mL were injected into the injured spinal cord (midline, 1 mm deep). Each injection occurred over 5 minutes (inject over 1 minute; wait 3 minutes; withdraw over 1 minute) to ensure optimal delivery. Over the first 1–2 hours after injection, cells in S phase incorporated the virus. In the experimental animals, the virus was delivered 24 hours before or after dorsal hemisection.

Bone marrow transplantation (BMT) in mice

Briefly, 20 C57/BL-6 female mice, 6–8 weeks of age, were lethally irradiated with 1,100 cGy and transplanted with $1\text{--}2 \times 10^6$ bone marrow cells from GFP+ transgenic donors. The level of long-term engraftment (>4 months posttransplantation) was $91 \pm 9\%$, as assessed by flow cytometry analysis of peripheral blood. Ten of the recipients underwent T8 dorsal hemisection and received a single injection of BrdU (50 mg/kg i.p.) 24 hours afterward. The other 10 were not injured but received BrdU along with the injured group.

Euthanasia/tissue preparation

On designated days, the animals were euthanized. For prelabeling studies (Group 1), rats were euthanized either 1 day or 1 month after injury. For postlabeling studies (Groups 2 and 3), rats were euthanized either 6 hours, 1 week, 2 weeks, 3 weeks, or 9 weeks after injection of the mitotic marker. Group 2 was processed for BrdU cyto-chemistry and quantification only, while Group 3 was processed for fluorescent immunophenotyping and quantification. The chimeric mice were sacrificed either 1 day or 1 month after BrdU injection. The rats received an overdose of anesthetic and were perfused intracardially with 200 mL saline followed by 400 mL fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The mice were perfused with 1/4 of the aforementioned volumes. The spinal cords were removed, postfixed in 4% paraformaldehyde for 1 hour, and then cryoprotected for 48 hours in 30% sucrose at 4°C. The spinal cords were then blocked into 1-mm cross sections (to best illustrate the ependyma in relation to the scar). Using the injury epicenter as the central point, 4–6 1-mm sections were obtained on either side of the injury. The 8–12 tissue sections were embedded in mounting medium (OCT, Tissue Tek, Sakura Fine Tek, Torrance, CA) in a base mold and then rapidly frozen on dry ice. All blocked cords were stored at -80°C until cut. Blocked spinal cord tissue was sectioned on a cryostat at 20 μ m thickness and stored on slides at -20°C .

Multiple immunofluorescence labeling

For determination of phenotype of BrdU+ or GFP+ cells, double or triple immunofluorescence was used. For BrdU detection, the tissue was pretreated with 2N HCl for 30 minutes at 37°C. Primary antibodies were used against BrdU (dividing nuclei, Accurate Chemical, Westbury, NY), nestin (stem/progenitor cells and reactive astrocytes, Pharmingen, San Diego, CA), vimentin (progenitor cells and reactive astrocytes, Chemicon, Temecula, CA), immature glia/macrophages (NG2, Chemicon), immature and mature astrocytes (S100 β polypeptide, Swant, Bellinzona, Switzerland), immature and mature astrocytes and oligodendrocytes (APC, Oncogene, Cambridge, MA), mature astrocytes (GFAP, Advanced Immunochemicals, Los Angeles, CA), mature oligodendrocytes (MBP, Hybridoma Bank, Iowa City, IA), immature neurons (TUJ1- β , Babco, Richmond, CA), mature neurons (NeuN, Chemicon), activated microglia and macrophages (OX42, Serotec, Bicester, UK), endothelial cells (RECA, Serotec), and fibroblasts (Fibronectin, Gibco, Gaithersburg, MD). As many as three compatible primary antibodies were placed on the tissue for 48 hours at 4°C in a solution of TBS, pH 7.5, containing

0.1% Triton and 5% donkey serum (TBS++). DAPI (1:100, Sigma) was added to a final wash to identify all nuclei. Slides were then coverslipped using PVA-DABCO and kept in the dark at -20°C until analysis. Antibodies are described in detail in Table 1.

Although some immunohistochemical labels are not specific to a single cell type, we were also able to use morphology to aid in determining phenotype. For example, NG2+ oligodendrocyte progenitors (OPCs) were distinguished from NG2+ macrophages by morphology. NG2+ cells are small cells with multiple processes, whereas macrophages are large, amoeboid cells without processes. Macrophages were not included in the NG2+ cell counts.

GFP enhancement

In cases where GFP expression was reduced or in colabeling techniques requiring acid pretreatment, the GFP signal produced by retrovirus-infected cells was enhanced with the primary polyclonal antibody rabbit α GFP (1:1,000, Santa Cruz Biologicals, Santa Cruz, CA) in TBS++ for 2 hours, followed by donkey α rabbit biotin (1:250, Jackson Immunochemicals, West Grove, PA) overnight, and then streptavidin-FITC (1:250, Jackson).

Quantitative analysis of mitotic cells

Three random 20- μm sections spanning $\sim 400\text{ }\mu\text{m}$ through the region of interest (the sections closest to the injury epicenter with a distinguishable ependymal cell layer and dorsal spinal cord tissue that retained the majority of its structural integrity) were studied per animal. Three sections per animal were counted in the preinjury labeling studies, in which a *P*-value was calculated. The density and distribution of BrdU+ cells were measured with a BH-2 microscope (Olympus, Lake Success, NY) equipped with a mechanical stage, a video camera (Dage, Michigan City, IN), and Stereo Investigator software (v. 3, MicroBrightfield, Colchester, VT). In 20- μm cross sections of tissue containing the area of interest (i.e., injury epicenter), a digitized tracing was made over a live, $10\times$ microscopic image of the tissue section. Any regions devoid of tissue (usually due to artifact or to the presence of a cyst) were excluded from the tracing. The tracings, which measured surface areas of the circumscribed regions, were used to calculate numbers of cells/area, which was then converted to numbers of cells/volume by dividing the amount by the 20- μm tissue thickness. In the majority of analyses, all BrdU+ cells in the spinal cord sections were counted. The BrdU+ cell counts in the preinjury labeling studies did not take regional distribution into account.

In the prelabeling study we compared BrdU+ cell density at the injury epicenter to BrdU+ cell density 3 mm rostral and 3 mm caudal to the epicenter to determine whether BrdU+ cell loss is a function of distance from the injury. In this analysis, we counted the total number of BrdU+ cells in the epicenter and in the rostral and caudal regions (2–3 sections per region per animal) in injured and in control animals.

In some of the PI labeling studies, regional analysis of BrdU+ cells was performed. In these cases, outlines of the injury zone, uninjured gray and white matter areas, and dorsal and ventral areas and ependyma were traced. The specific goals of the regional analyses were to determine the degree of incorporation of BrdU cells into the “injury zone,” the origin of the BrdU+ cells (6 hours after BrdU delivery), the patterns of survival and/or migration of BrdU+ cells over time (6 hours vs. 3 weeks after BrdU delivery), and the dorsal versus ventral distribution of BrdU+ microglia.

The dorsal region was distinguished from the ventral region by drawing a line through the central canal, from the left lateral to the right lateral side of the cord. White matter was easily distinguished from gray matter by visualization through the light microscope. Injured tissue was distinguished from uninjured tissue by changes visualized under light microscopy as well

as fluorescence. Under light microscopy, disturbances in the normal connective tissue architecture were noted. Under fluorescence, two major changes were noted in the injured tissue: hypercellularity, as shown by an increased number of DAPI+ nuclei, and autofluorescence. Using these cues, stereologic zones were established. The digitized tracing was then overlaid onto a live image of the tissue and the tissue was examined under fluorescence. Each BrdU+ cell was counted using a 40×–60× oil lens (in each region of interest, when applicable) and was examined for double labeling when indicated.

To be considered BrdU-positive, nuclei were required to be uniformly labeled and devoid of punctate staining, with the following exceptions. If the antibody signal was higher in the periphery of the nucleus than in the center, this was considered a technical artifact, and the nucleus was included in the count. Also, we recognized that punctate BrdU labeling resulted from dilution upon multiple cell divisions. This phenomenon was particularly notable in the 9-week timepoint of the PI labeling study. The nucleus was considered intact if it colabeled with DAPI.

Confocal microscopy

For phenotypic analysis of BrdU+ cells in the tissue labeled PI, sections were imaged with confocal microscopy (Bio-Rad, Hercules, CA). Two to three random sections through the T9 region (closest section to the injury epicenter with a distinguishable ependymal cell layer and dorsal spinal cord tissue that retained the majority of its structural integrity) were studied per animal. To analyze the phenotype of BrdU+ or GFP+ cells, at least 100 parenchymal cells per injured group (five or six animals) were counted, using k40× and 60× oil lenses. Because of reduced numbers of mitotic cells in the uninjured animals, approximately eight 20-μm midthoracic sections per animal were examined, each spaced 1 mm apart. Using this approach, ~50 BrdU+ cells per control group were analyzed.

Statistical analysis

All computed statistics were considered statistically significant at $P < 0.05$. Analysis of variance was used to determine significant differences between the experimental groups. Levels of significance were determined by Dunnett's *t*-test (i.e., comparison to control group).

Image processing

Confocal images were collected on a BioRad radiance microscope and images were imported into Volocity (Improvision, Lexington, MA) to generate compressed z-stacks as well as 3D and orthogonal views. Images were exported to PhotoShop for cropping and to create figure montages (Adobe Systems, San Jose, CA). In some cases, individual color channels were selected to adjust brightness and/or contrast levels.

RESULTS

Response of constitutively dividing cells to spinal cord injury

To study the PI response of cells that divided over a 10-day period prior to injury, 21 adult female Fischer 344 rats were given prolonged systemic administration (10 days) of the mitotic indicator BrdU. It has previously been shown that over 50% of the endogenously dividing cells labeled by this method are NG2+ oligodendrocyte progenitors (Horner et al., 2000). As before, we observed a large number of BrdU-labeled cells throughout the parenchyma at 1 day after BrdU (Fig. 1A) or 1 month after BrdU pulsing, with cells rarely labeling in the central canal region and a higher density of labeled cells observed in the lateral aspects of the spinal cord. The primary phenotype observed in these studies was also that of the NG2-expressing progenitor cell (Fig. 1B, arrows). One day after 10 daily pulses of BrdU, 10 rats underwent a

midthoracic (T8) dorsal hemisection. The rats were euthanized either 1 day or 1 month later (5 control, 5 injured at 1 day; 6 control, 5 injured at 1 month).

Few BrdU-labeled cells could be found at the site of injury at 1 day PI (Fig. 1C). In addition, BrdU+ debris in the injury epicenter, consistent with the ongoing death of prelabeled BrdU+ cells, was observed at 24 hours PI. A limited number of BrdU/NG2-immunopositive nuclei could be identified at the lesion epicenter compared to noninjured controls (Fig. 1D, arrow) and there were fewer intact BrdU+ nuclei (evenly stained, oval shaped, smooth edges) in regions 3 mm rostral and 3 mm caudal to the epicenter (data not shown). BrdU-colocalized cells were primarily NG2 colabeled but had abnormal morphologies that differed from that of normal, stellate-shaped NG2 cells observed in the intact spinal cord (Fig. 1D, arrow). At 1 month PI, the density of BrdU-labeled cells increased toward normal levels and the return of more typical stellate-shaped morphologies was observed.

The numbers of surviving BrdU+ cells (all dividing cells) and BrdU+/NG2+ cells (oligodendrocyte progenitors) in the injury epicenter were quantified by measuring the total number of immunopositive cells per volume of tissue in cross sections of the injury epicenter. The numbers of BrdU+ cells/ μm^3 , number of BrdU+/NG2+ cells/ μm^3 , and percentage of BrdU+ cells that were NG2+ in the injury epicenter and in control tissue are shown in Figure 1E. These data show a significant loss of prelabeled BrdU cells at 1 day after injury, with an increase toward normal levels by 1 month (Fig. 1E).

To confirm that the decreased BrdU signal was an indicator of cell loss, we also measured cell proliferation using a retroviral reporter (MLV-pCMV-GFP). The reporter virus was injected 0.5 mm lateral to midline. One week after injection of the retrovirus into control, uninjured animals, we observed a total of 5–10 GFP+ cells/section (15–20 cells total) at the injection site (Fig. 1E, $n = 6$). The majority of the GFP-expressing cells colocalized with NG2 (Fig. 1G). When the retrovirus was injected into the spinal cord 24 hours before injury and the tissue processed 1 week later, we observed fewer GFP-immunoreactive cells at the injection site (Fig. 1H). The remaining GFP signal was found in swollen cells lacking stellate morphologies found in the controls (Fig. 1I). Amplification of the GFP signal using immunohistochemistry did not reveal cells with lower expression. These data confirm the BrdU-labeling method and demonstrate that progenitor cells that are proliferating before injury either die or do not activate a proliferation program to a significant extent following spinal cord injury.

Cell division and survival after spinal cord injury

We next sought to determine when cells proliferate following injury and when the peak of progenitor cell activation occurs. We injected BrdU as a single dose at 24 hours, 3, 6, or 9 days following hemisection. BrdU-labeled nuclei were counted at 24 hours after the administration of BrdU in the region of the dorsal columns using the optical fractionator method. Injury induced a significant increase in BrdU-labeled cells within the dorsal columns, particularly when the injection of BrdU was made at 24 hours PI. More BrdU-labeled cells were concentrated in the dorsal columns and neighboring gray matter at the lesion epicenter (Fig. 2A), as well as the central canal (Fig. 2B, inset from A). BrdU incorporation was also elevated in segments 3 mm rostral or caudal to the lesion site (Fig. 2C,D). A quantitative analysis of BrdU-labeled cell density in the dorsal columns at the epicenter revealed a spike of proliferation at 24 hours. Proliferation was rapidly attenuated by 9 days PI but remained significantly above noninjured control (Fig. 2E) levels. We further quantified the early proliferative response at 24 hours by counting BrdU-labeled cells in each of four readily identifiable spinal compartments (dorsal or ventral gray and dorsal or ventral white matter). Counts were made at 30 hours or 3 weeks following injury. These data show that proliferation is highest in the gray matter adjacent to the lesion soon after injury (Fig. 2F). By 3 weeks, many of the BrdU-labeled cells persist, with a larger proportion of cells detected in the ventral gray region. These

data suggest that cells are likely derived from the gray matter, with fewer proliferating cells originating in white matter.

CNS or blood as the source for proliferating cells

To determine whether the dividing cells are derived from the parenchyma or peripheral blood, we analyzed cell proliferation in a subset of mice that had received a bone marrow transplant from a GFP mouse donor before injury. To determine whether any of the BrdU+ cells originated from the bone marrow, we used a chimeric mouse model. We first confirmed that the mouse spinal cord responded to injury with the same proliferative response that we observed in the rat. In the uninjured female adult C57/BL-6 mouse, we detected 5–10 BrdU+ cells 1 day after BrdU delivery (Fig. 2G). When BrdU was injected at 24 hours after injury, we observed over 100 BrdU+ cells per 20- μ m section of tissue in animals sacrificed 1 day later (Fig. 2H). To make chimeras, C57/BL-6 mice were lethally irradiated and transfused with bone marrow from GFP+ mice. After 16 weeks, bone marrow in the C57/BL-6 mouse was almost fully reconstituted with GFP+ cells ($91 \pm 9\%$). The chimeras received dorsal hemisections at T9 and were sacrificed either 2 days or 1 month after injury. At both timepoints the uninjured mouse cords contained ~20–40 GFP+ cells per section; these cells appeared to be localized to the meninges and along blood vessels (Fig. 2I). Of note, BrdU+ cells were observed in the injured and uninjured irradiated mice at levels not noticeably different from those in matched nonirradiated animals; the radiation that the mice received 8 weeks prior to the spinal cord injury did not eradicate dividing cells in the spinal cord. By 2 days after injury, however, the GFP-labeled bone marrow cells were primarily located in the central portion of the cord, within the gray matter region (Fig. 2J). By 1 month PI, most of the GFP-labeled cells appeared in the dorsal half of the cord or the injury epicenter (Fig. 2K). At 2 days after injury, only 10% of BrdU+ cells were GFP+ ($n = 2$, $SD = 0.05$, Fig. 2L). By 1 month after injury, 6% of the BrdU+ cells were GFP+ ($n = 2$, $SD = 0.009$, Fig. 2M–P) and had unique morphologies compared to virally labeled cells (presented below). Many of the bone marrow-derived cells were vacuolated and had macrophage morphologies (Fig. 2M). In some cases, GFP-labeled cells contained neural antigens that appeared to be within vacuole compartments but not within the nucleus (Fig. 2O). When the BrdU/GFP analysis at 1 month was confined to the dorsal, injured half of the cord, 11% of the BrdU+ cells were GFP+ or derived from the bone marrow ($n = 2$, $SD = 0.03$). These data indicate that BrdU predominantly labels cells entering cell cycle that are derived from the spinal cord parenchyma and not from the blood.

BrdU cell phenotypes in the parenchyma and ependyma

At both 30 hours and 3 weeks after injury in animals receiving a single pulse of BrdU at 24 hour PI, we detected labeled cells that colocalized with progenitor and glia cells markers but never neuronal antigens. Numerous BrdU-labeled cells were detected at the central canal at both timepoints that expressed either S100 β or nestin (Fig. 3A–D). Outside of the central canal, BrdU-labeled cells colocalized with the oligodendrocyte marker Adenomatosis polyposis coli (APC or CC1) at 3 weeks but not 24 hours postinjection (Fig. 3E,F). The most abundant phenotype at both early and late timepoints was reactive for NG2 (Fig. 3H–J) and, to a lesser extent, OX-42 (Fig. 3K–M). These cells were dispersed throughout the injury region. Cell phenotype was quantified in rats that received a single 24-hour pulse of BrdU at 30 hours, 1, 2, 3, and 9 weeks following injury. BrdU-labeled cells near the ependyma primarily localized with nestin and S100 β at early time-points (Fig. 3O,P). Nestin colocalization diminished and S100 β colocalization disappeared over time. Many ependymal zone cells also labeled with GFAP, particularly after 1 week PI. Outside the ependymal region, the predominant phenotype expressed NG2 early after injury (Fig. 3Q,R). Up to 50% of all BrdU-labeled cells were also NG2-colabeled until 9 weeks postinjection, when the number decreased significantly. By 1 week, a significant number of BrdU-labeled cells were APC (CC1) colabeled and this population was maintained until 9 weeks PI (Fig. 3O,P). S100 β and GFAP-labeled astrocytes

comprised a relatively small population of cells that are BrdU-labeled within the parenchyma. S100 β labels immature and mature astrocytes as well as Schwann cells that may invade from the dorsal or ventral roots (Bhattacharyya et al., 1992). By 1 week after injury, only 2% of the BrdU+ cells were S100 β + in the injured group and they were concentrated in the injury zone. They may have migrated outward from the ependyma, where they were highly concentrated, or inward from the dorsal root ganglia. At 2 weeks after injury, BrdU/S100 β expression peaked at 12% (Fig. 3Q,R). At 9 weeks, no S100 β -labeled cells were observed in either control or lesioned animals, but the percentage of BrdU+/GFAP+ mature astrocytes reached 15%. This finding strongly suggests that the S100 β + cells present at 1–3 weeks were immature astrocytes that matured into GFAP+ astrocytes by 9 weeks.

OX-42 labeled microglia/macrophages were rarely seen at 30 hours or 1 week postinjection but they increased in number by 2 weeks to between 42–54% (Fig. 3Q,R). These data indicate that the primary phenotype of the dividing progenitors that enter cell cycle at 24 hours postinjection are NG2-expressing progenitor cells. As NG2-colocalization declines, there is an increase in the number of cells that express oligodendrocyte markers and, to a lesser degree, astrocyte antigens. Interestingly, OX-42 cells, which do not comprise a significant population of the BrdU-labeled cells at 30 hours or 1 week, increase significantly by 2 weeks postinjection.

None of the BrdU+ cells expressed neuronal markers at any timepoint in the injured or control group. This finding is consistent with our understanding that the spinal cord is not a neurogenic region in either the injured or uninjured state (Horner et al., 2000; Yamamoto et al., 2001a).

Phenotype of virally labeled cells

BrdU labeling indicates that cells proliferate rapidly in response to injury (<24 hours) and are primarily derived from parenchymal sources. BrdU labeling, particularly at the injury epicenter, can be hard to interpret due to the reliance on immunodetection in the injury setting, where significant labeling issues can arise. In addition, BrdU is subject to dilution effects, where the absence of labeling or decreased labeling over time can be misinterpreted. Lack of BrdU-labeled phenotypes can indicate that a particular cell type is either not dividing or its proliferation rate is very high and BrdU is being diluted with successive progeny. We thus also utilized a retroviral method to label cells and their progeny, a technique that is not affected by the number of cell divisions. Another advantage in the use of a retroviral approach is the ability to direct the location of injection into a particular spinal compartment. In Figure 4 we show that virally labeled cells express high levels of GFP and are primarily localized to the NG2 progenitor population. In Figure 4, virus was injected into the spinal cord of rats just lateral to the central canal at 24 hours following injury. Spinal cords were analyzed at 2 days, 1, 2, or 3 weeks postinjection. At 2 days postinjection, numerous GFP-labeled cells were detected throughout the dorsal spinal cord (Fig. 4B). By 1 week, the number of virally labeled cells increased dramatically (Fig. 4C), indicating significant cell proliferation compared to progenitors labeled before injury (Fig. 1F,H). GFP-labeled cells were found largely to localize to the dorsal and ventral gray matter near the lesion epicenter bilaterally. GFP-labeled cells were never seen at the ependymal region. By 3 weeks PI, the GFP-labeled cells became undetectable. This outcome is potentially due to the shutdown of the viral promoter or cell death. Due to the lack of GFP signal by 3 weeks PI, we restricted our analysis to the 1- and 2-week timepoints. GFP-labeled cells exhibited elaborate morphologies at early timepoints (Fig. 4D,E). These data indicate that the cells form a complex network within the injury environment. By 1 week PI, the spinal cord contained a large amount of OX-42 labeled cells with phagocytic morphologies. However, similar to the BrdU labeling, GFP-labeled cells were integrated among the phagocytic cells but rarely colocalized (Fig. 4F,G). Within the injury epicenter, GFP cells clustered within the dorsal columns and most of these cells colocalized with NG2 (Fig. 4H,I). The majority of cells had stellate morphologies, but some had a few processes and

resembled beads on a string; they are likely vascular pericytes (Fig. 4K–P). Although rare, we found evidence of cells which committed to an astrocyte fate and expressed GFAP (Fig. 5A–C). These cells were found at the lesion epicenter and had the typical morphology of reactive astrocytes, including a small number of large processes. Similar to the BrdU data above, we found that some of the virally labeled cells expressed oligodendrocyte markers such as APC (data not shown) but very few expressed the myelin proteins MBP or Rip (Fig. 5D). Myelin tubes that were clearly MBP-immunoreactive and colocalized with GFP were found only outside the scar zone (Fig. 5D–G). Most virally labeled myelin profiles were found in the adjacent, intact gray matter. The lesion zone contained diffuse myelin debris, making it a challenge to colocalize myelin profiles and virally labeled cells definitively. Hence, the number of myelinating cells derived from virally labeled cells which divide early after injury may be underestimated due to methodological considerations. Nonetheless, at least a portion of these early injury-induced progenitor cells participate in myelin genesis.

DISCUSSION

Response of constitutively dividing (prelabeled) cells to spinal cord injury

To our knowledge, these data are the first to demonstrate that within 24 hours after injury most prelabeled dividing progenitor cells die or remain postmitotic. A small proportion of NG2-labeled cells survive and divide, but the response of these is minor in comparison to the robust proliferative response of dividing cells at 24 hours after injury. Importantly, our findings are confirmed using a secondary labeling technique (retrovirus).

Cell division after spinal cord injury

Adrian and Walker (1962) originally showed that glial cells incorporate ^3H -thymidine soon after injury. In a contusion model of spinal cord injury, multiple daily BrdU injections demonstrated that cell proliferation is highest during the first week PI and steadily declines to an insignificant level by 4 weeks (McTigue et al., 2001; Zai and Wrathall, 2005). Using single pulses of BrdU following a cortical stab lesion, Hampton et al. (2004) have shown a relatively steady level of BrdU incorporation between 2 days and 2 weeks following cortical injury when cells were analyzed at the lesion zone. Using a similar lesion paradigm and BrdU protocol, Alonso (2005) detected a peak in proliferation at 2 days PI within a border zone of the lesion but a similar, constant mitotic number to that of the Hampton study in a region more than 100 microns lateral to the lesion. Overall, the density of BrdU-incorporating cells in the border zone showed remarkable concordance between these two studies. In the present work, we studied proliferation over time using two methods. We first gave single pulses of BrdU at 24 hours, 3, 6, and 9 days PI and then analyzed the tissue at 1 week after the delivery of BrdU. We found a clear peak of proliferation at 24 hours. These data are in agreement with previous studies but indicate that proliferation is initiated earlier than previously reported. In a second approach, we labeled dividing cells by pulsing BrdU only at 24 hours and then examined the tissue at multiple timepoints after injury. The results of these studies show that cells that incorporate BrdU as early as 24 hours after injury persist for up to 9 weeks, indicating that they become integrated into the spinal parenchyma.

Multiple origins of a dividing cell population

We propose that there is no single origin of the cells that divide 24 hours after injury. Cells residing near the spinal cord ependyma as well as the dorsal horns of the gray matter appear to be the major populations that acutely divide in response to injury. Our chimeric mouse experiments revealed that only 10% of the BrdU+ cells found in the spinal cord after injury are bone marrow-derived. Bone marrow-derived cells do not comprise a significant portion of the early dividing progenitor cells described in these studies and do not appear to participate directly in the generation of glia.

Phenotypes of BrdU+ cells in the parenchyma

NG2—NG2 appears to be expressed by the majority of cells that divide very soon after spinal cord injury (Zai and Wrathall, 2005). The formation of new myelin begins 10–14 days after injury (Harrison and McDonald, 1977; Keirstead et al., 1998; McTigue et al., 2001; Zai and Wrathall, 2005), a timeframe that is associated with the time when BrdU+ oligodendrocytes begin to mature and NG2-colocalization declines. Hence, it is likely that many cells that divide in response to trauma and express NG2 are destined to become oligodendrocytes. In demyelinating lesions, NG2-expressing cells have been shown to proliferate, coexpress the PDGFr+ and, by temporal association of markers, undergo differentiation into oligodendrocytes (Watanabe et al., 2002). In trauma studies, a decline in NG2 cells with a corresponding increase in oligodendrocytes was interpreted to validate the NG2 population as an oligodendrocyte progenitor (McTigue et al., 2001; Watanabe et al., 2002; Rosenberg et al., 2005).

However, a surprising number of NG2 cells that divide after injury persist as long as 9 weeks PI and a subpopulation of NG2-expressing cells in the adult CNS may represent a differentiated glial phenotype not directly serving as a reservoir for the replacement of other macroglia (Levine and Nishiyama, 1996; Horner et al., 2002). An alternative explanation is that newly generated cells may be replacing killed NG2 cells or differentiating into an as yet undescribed injury-associated glial phenotype. For example, Alonso (2005) interpreted BrdU/NG2-labeled cells that decline after injury to be glial progenitors destined to participate in scar formation and the production of astrocytes. A high density of NG2 chondroitin sulfate proteoglycan appears to inhibit neuronal growth in vitro (Fidler et al., 1999), and adding antibodies against NG2 reverses this effect (Chen et al., 2002). But the presence of NG2 alone is not sufficient to characterize newly generated NG2-cells as inhibitory to growth. Recent studies from postnatal oligodendrocyte progenitor cells suggest that the presence of NG2 leads to the promotion of axon attachment and growth (Yang et al., 2006). Hence, the molecular phenotype of newly generated NG2 cells after injury needs to be more directly assessed to see if they play a supportive or antagonistic role in regeneration. In addition, NG2-cell genesis may be important for the newly hypothesized role of NG2-expressing cells at the Nodes of Ranvier or at synaptic connections (Butt et al., 2002; Lin and Bergles, 2002; Horner et al., 2002; Berry et al., 2002; Nishiyama et al., 2002). These functions could be essential for the restoration of conduction. Collectively, these data demonstrate the complexity of the NG2-expressing progenitor response to trauma and argue for further studies.

Gliosis—Over time, GFAP-reactive astrocytes become hypertrophic and form a barrier at the site of lesion (Reier and Houle, 1988). Few studies have shown that mature astrocytes divide acutely after injury, despite the almost universal formation of an astrocyte-rich scar at the site of CNS lesions by 1 week. Most data support the observation that astrocytes do not divide until 2 days or even 4 weeks after injury (Wagner et al., 1978; Miyake et al., 1988; Di Prospero et al., 1998; Norton, 1999). Labeling dividing cells at the earliest timepoints after cortical stab injury showed very limited colocalization of BrdU with GFAP (Hampton et al., 2004). Exceptions to these findings have been reported at the ventricular zones and at the spinal central canal. Utilizing DiI or BrdU labeling has produced a small but significant finding of GFAP-colocalized cells that are only briefly prefaced by vimentin/NG2-positive, GFAP-negative astrocyte progenitor cells following spinal cord injury (Frisen et al., 1995; Hampton et al., 2004; Mothe and Tator, 2005; Zai and Wrathall, 2005). We observed an initially high number of BrdU+/S100 β + cells that diminished before an increase in BrdU+/GFAP+ cells emerged over the 9-week period. S100 β is expressed by immature astrocytes and glial progenitor cells in the adult CNS (Deloulme et al., 2004; Hachem et al., 2005). Together, our data suggest that dividing S100 β + or NG2+ cells present at 1–3 weeks could be immature astrocytes that mature into GFAP+ astrocytes by 9 weeks.

In the few studies that address migration of dividing, GFAP-expressing cells derived from the central canal, there is evidence that these cells contribute to scar formation by migrating toward the lesion site (Johansson et al., 1999; Mothe and Tator, 2005). The degree of migration is likely dependent on the type of spinal cord lesion. In the case where the central canal is not directly injured, migration is limited to less than 50 μm (Mothe and Tator, 2005), but in a larger surgical lesion migration may be more substantial (Johansson et al., 1999). Cortical lesions result in significant numbers of BrdU-labeled astrocytes over 1 week PI, which are likely to be derived locally and not from the ventricular zone (Alonso, 2005). Hence, paraventricular or central canal progenitors may contribute to astrogliosis, but there is also a substantial localized source for newborn astrocytes. This source could be meningeal or local glial progenitor cells (Shibuya et al., 2003; Hampton et al., 2004; Wu et al., 2005). It is notable that in our viral reporter studies no ependymal infection was detected at 2 days postinjection. This could be explained by the possibility that ependymal cells were not infected due to the injection being outside the central canal region or that ependymal cells divided and migrated away from the central canal by 2 days. The former is a favored hypothesis since asymmetric cell division is thought to occur at the ependymal zone, predicting that a parent stem cell should remain at the ependymal zone. Since this was not observed, we conclude that viral labeling of parenchymal progenitors not directly derived from the ependymal zone yield a rapidly expanding population that contributes to extensive PI gliogenesis. The relative contributions of parenchymal versus ependymal zone progenitor cells to astrocyte formation and the molecular phenotype of these newly generated astrocytes is a key area for future study.

Microglia/macrophages—Our data identify a population of early dividing cells that can be separated from the later responding macrophage/monocytes. Macrophage and microglia activation and infiltration peak at 3–8 days PI and diminish by 1 month after injury (Popovich et al., 1997; Schnell et al., 1999; Sroga et al., 2003). Monocytes make up the majority of dividing cells after spinal cord injury when BrdU is dosed over multiple days during the first week PI. But morphologic and histologic indicators of macrophage/microglial activation show that these cells are relatively quiescent until 24 hours after spinal cord injury. Few cells in our acute 24-hour BrdU pulse were macrophages. These data indicate that there is a narrow window of 24 hours following injury in which neural progenitor cells are dividing before local or peripheral macrophages enter the cell cycle. By 2 weeks and beyond, OX-42-reactive cells comprise as much as 30% of the dividing population, indicating that either dividing microglia/macrophages do not express OX-42 at 24 hours or that BrdU is labeling peripheral monocytes within the blood system. The results of the viral reporter support the latter contention, since no virally labeled microglia were detected using this method, which only labels cells within the spinal parenchyma.

Neurogenesis—Neurogenesis has not been detected in the adult spinal cord; indeed, there has been some suggestion that the adult spinal cord may contain inhibitors to neurogenesis (Song et al., 2002). Together with other reports, we conclude that neuronal phenotypes are not generated following spinal cord injury (Frisen et al., 1995; Mothe and Tator, 2005; Zai and Wrathall, 2005).

CONCLUSIONS

Multiple populations of stem/progenitor cells exist in the spinal cord. An active progenitor population involved in spinal cord homeostasis is sensitive to injury and is lost or significantly reduced in response to trauma. This vulnerable population is rapidly replaced by a second, quiescent stem/progenitor population that divides as early as 24 hours PI and is localized throughout the spinal cord. Robust proliferation at 24 hours PI leads to the production of new glial cells involved in astrogliosis and the generation of new myelin. These studies provide a

spatial and temporal roadmap for the design of future strategies to manipulate the gliogenic cascades that follow CNS injury.

Acknowledgements

We thank Bob Summers, Linda Kitabayashi, and Steve Forbes for excellent technical assistance, and Dr. Henriette van Praag for constructing the GFP retrovirus.

Grant sponsor: Neuroplasticity of Aging training grant from the National Institutes of Health (NIH) (to L.H.); Grant sponsor: Paralysis Project, Paralyzed Veterans of America; Grant sponsor: the Glaucoma Research Foundation; Grant number: NS046724 (to P.J.H.); Grant sponsor: Christopher Reeve Foundation, Project ALS (to F.H.G.); Grant number: NIH AG21876 (to F.H.G.); Grant number: NS050217 (to F.H.G.).

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Fig. 1.

BrdU pulse labeling of constitutively dividing cells before dorsal hemisection. BrdU was pulsed for 10 days and rat tissue was analyzed at 1 day or 1 month after BrdU. At 1 day after 10 daily BrdU-injections there were many BrdU-labeled cells (~100/section) throughout the spinal sections (A). The majority of cells expressed the proteoglycan NG2 and resembled polydendrocytes (B, arrows). A spinal hemisection was performed at T8 after the last of 10 BrdU injections and the lesion epicenter sectioned and reacted for BrdU immunocytochemistry (C). At the lesion center, very few BrdU-labeled cells were detected, indicating either significant cell loss and/or a decrease in proliferative potential dividing progenitor cells. The few BrdU-labeled cells at the lesion epicenter were often NG2-immunoreactive (D, arrow). A quantitative assessment of total BrdU at the lesion epicenter and BrdU/NG2 colabeled cells revealed a significant decline in the number of BrdU labeled cells at 1 day after injury compared to control (E, $*P < 0.01$ compared to 1-day control). By 1 month after injury, NG2 expression was slightly increased over injury controls indicating significant mitotic recovery (E, $**P < 0.05$ compared to 1 month control). To confirm that dividing progenitor cells are lost early after injury, a second mitotic label was employed. A single injection of a retroviral reporter was made at 0.5 mm lateral to midline at T8 (F). At 1 week postinjection, spinal cord sections near the injection site had an average of 5–10 virally labeled cells that expressed GFP (F). Virally labeled cells primarily colocalized with NG2 (G, inset panels show separate GFP and NG2 channels). In injured animals 1 week postvirus injection there was a significant decrease in the number of virally labeled cells indicating cell loss (H, circles indicate virally labeled cells). In addition, cell morphology indicated significant cell swelling and necrosis of virally

labeled cells at the injury site (**I**, inset panels show separate GFP and NG2 channels). Scale bars = 200 μm in A,C; 20 μm in B,D; 150 μm in F,H; 20 μm in G,I.

Fig. 2.

The peak of postinjury proliferation is at 24 hours, with the majority of cells derived from parenchymal sources. Injury induced a significant increase in BrdU-labeled cells within the dorsal columns, particularly when the injection of BrdU was made at 24 hours postinjury and the tissue analyzed 6 hours later (A). BrdU-labeled cells were concentrated in the dorsal columns and neighboring gray matter at the lesion epicenter as well as 3 mm rostral (C) or 3 mm caudal (D). Significant BrdU labeling was also noted at the central canal within the lesion epicenter (C). A quantitative analysis of BrdU labeling demonstrates that peak proliferation occurs at 24 hours and BrdU incorporation is rapidly attenuated over time (E, $*P < 0.01$ compared to noninjured control). BrdU labeling is highest in gray matter adjacent to the lesion,

with accumulation of cells at 3 weeks particularly in the dorsal and ventral gray matter. In a subset of mice, bone marrow chimeras were created to assess the contribution of the blood-derived cells to the proliferating population. BrdU labeling after bone marrow transplantation was similar in number and pattern at 24 hours (**G**) or 3 weeks (**H**) postinjection of BrdU compared to control rat tissue. In uninjured chimeric mice, the distribution of GFP-labeled cells was localized to the meningeal and vascular compartments, with limited labeling within the spinal parenchyma (**I**). The chimeras received dorsal hemisections at T9 and were sacrificed either 2 days or 1 month after injury. At both timepoints, the uninjured mouse cords contained ~20 – 40 GFP+ cells per section; these cells appeared to be localized within injured white matter at 2 days (**J**), with an increase in gray matter at 1 month (**K**) the meninges and along blood vessels (Fig. 3I). When BrdU was pulsed at 24 hours, 10% of the GFP-labeled bone marrow cells were colocalized with BrdU (**L**). The GFP-labeled cells had morphologies distinct from NG2-expression progenitor cells and many of the GFP-labeled cells had vacuoles containing neural antigens (**M–P**). Scale bars = 100 μ m in A,C,D,G–K; 25 μ m in B; 50 μ m in L–P.

Fig. 3. Confocal image of BrdU-labeled cells. Cells associated with the ependymal region at 6 hours after BrdU injection commonly coexpressed S100 β or nestin (A–D, C and D are separate nestin and BrdU channels of the BrdU cluster on the left). At 7 days postinjury, BrdU/APC-labeled oligodendrocytes (E–G, F and G are separate BrdU and APC channels of the cell on the left) as well as BrdU/NG2-labeled cells were present (H: 20 μ m confocal montage with orthogonal views taken at the center of one cell; I, J: 1 μ m, single channel confocal images of BrdU and NG2 channels, respectively). A minor part of the early BrdU-labeled population was OX-42 colabeled (K, 18- μ m thick confocal montage with orthogonal views taken at the center of one cell; L, M: 1- μ m thick single channel confocal images of BrdU and OX42, respectively). BrdU-labeled ependyma coexpressed nestin and S100 β at 6 hours after BrdU injection (O, Q). S100 β expression was transient and decreased by 1 week in both ependymal and parenchymal regions (O, R). The percentage of BrdU-labeled ependyma that expressed GFAP was constant over time (O) but the total number of ependymal cells that coreacted for GFAP increased at 3 and 9 weeks (P). Within the dorsal columns at the site of injury, NG2 and BrdU colocalization was the most common cell label at 6 hours (Q, R). By 1 week, the appearance of oligodendrocytes throughout the parenchyma increased significantly, whereas the number of

OX-42-positive phagocytes increased later at 2 weeks (Q,R). GFAP-labeled astrocytes were a minor population appearing outside of the ependymal region at 1 week (Q,R). The immature neuronal marker Tuj-1 was never found to colocalize with BrdU (Q,R). Scale bars = 40 μm in A–E,H–M; 20 μm in F,G.

Fig. 4.

Phenotype of retrovirally labeled cells 1 and 2 weeks postinjury. At 1 week following virus injection, an average of 8–10 cells per section is labeled using this method in an intact spinal cord (A). When the spinal cord received a dorsal hemisection, numerous cells are GFP-labeled, indicating a rapid expansion at 1 week (B) and 2 weeks postinjury (C). Confocal z-stack images of GFP+ cells reveal elaborate and varied cellular morphologies 2 weeks after spinal cord injury (D,E). Note that some cells exhibited multipolar, stellate processes reminiscent of normal NG2 cells (D), whereas others exhibited larger processes and resembled reactive glia (E). NG2 colocalized with both cellular morphotypes. At 1 week postinjury the lesion site is infiltrated by a large population of macrophages/microglial cells that express OX-42 (F). Despite the robust labeling of the injury site with OX-42, GFP-labeled cells rarely colocalized with this population (F and 3D reconstruction and rotation in G). Note the unique morphologies of the GFP-labeled cells and intimate but separate relationship with OX-42-labeled cells. Most GFP-labeled cells are NG2-immunoreactive (H) at 2 weeks and exhibit stellate morphologies (H,I, 15 µm Z-stack with orthogonal views at the point of a single cell). Some NG2-colabeled cells had the morphology of a pericyte, with no branched processes around the soma and cylindrical shaped lumen (J, 12 µm confocal stack with orthogonal views taken at the point of a single cell). GFP- and NG2-colabeled cells appear as beads on a string (K–P). Single black and white confocal images of NG2 (L,O) and GFP (M,P) illustrate a typical lumen structure. Scale bars = 200 µm in A–C; 10 µm in D,E; 20 µm in F–I; 5 µm in J; 10 µm in K–P.

Fig. 5.

Differentiation of virally labeled cells at 3 weeks postinjury. The predominant phenotype of virally labeled cells expressed NG2. Nonetheless, a small number of cells could also be classified as either astrocytes or oligodendrocytes. Cells with broad processes were GFAP-immunoreactive especially within the lesion epicenter (**A–C**, **B** and **C** are confocal stacks showing GFP and NG2 as individual channels). In rare cases, cells with oligodendrocytes morphologies and limited membrane staining were observed outside of the scar zone. In **D** a cell with a bifurcating process (white arrowhead) connects with at least one of two myelin tubes (green arrow heads). One of the myelin tubes appears to be sectioned at the internode

(box). A 3D reconstruction of this myelin profile (**E,F**) shows a GFP-expressing tube colocalized with MBP (white arrowhead). Scale bars = 5 μm in A–C; 15 μm in D; 2 μm in E,F.

TABLE 1. Antibodies

Antibody	Specificity	Epitope/Immunogen	Lot #	Source	Catalog #
GFP	Green fluorescent protein	27-kDa protein originally isolated from jellyfish	41723A	Invitrogen	A6455
NG2	Oligodendrocyte precursor cells, pericytes	High molecular weight integral membrane chondroitin sulfate proteoglycan	24080225	Chemicon	A85320
APC	Oligodendrocytes, perkinje cells and low-level expression in astrocytes	312 kDa APC protein	D11780-6	Oncogene Research	OP80
MBP	Oligodendrocytes	Human myelin Basic protein from brain	23010307	Chemicon	AB980
GFAP	Astrocytes	Glial fibrillary acidic protein	04/06-AS-3GF	Advanced Immunochemical	O31223
RIP	Oligodendrocytes from early in differentiation into adulthood	Rat olfactory bulb	Made in-house	Hybridoma Bank	N/A
BrdU	Nucleated cells in S phase that have incorporated BrdU	Bromodeoxyuridine	H7786	Accurate	OBT0030
OX-42	CD11b, macrophages, dendritic cells and granulocytes	Peritoneal macrophages	702	Serotec	MCA275R
S1003	Astrocytes	S1003 polypeptide	1177383	Swant Ab	37A
Nestin	Neuroepithelial stem cells early in embryogenesis	200 kDa Nestin protein	22914	BD Pharmingen	556309
Tuj-1	Neurons	Neuronal Class III beta tubulin	14813102	Covance	PRB435P