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Effects of axon degeneration on oligodendrocyte lineage cells: Dorsal rhizotomy evokes a repair response while axon degeneration rostral to spinal contusion induces both repair and apoptosis

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Abstract

Wallerian degeneration in the dorsal columns (DC) after spinal cord injury (SCI) is associated with microglial activation and prolonged oligodendrocyte (OL) apoptosis that may contribute to demyelination and dysfunction after SCI. But, there is an increase in OL lineage cells after SCI that may represent a reparative response, and there is evidence for remyelination after SCI. To assess the role of axonal degeneration *per se* in OL apoptosis and proliferation, we cut the L2-S2 dorsal roots producing massive axonal degeneration and microglial activation in the DC, and found no evidence of OL loss or apoptosis. Rather, the numbers of OL-lineage cells positive for NG2 and APC (CC1) increased, and BrdU studies suggested new OL formation. We then tested contusion SCI (cSCI) that results in comparable degeneration in the DC rostral to the injury, microglial activation, and apoptosis of DC OLs by 8 days. NG2+ cell proliferation and oligodendrogenesis was seen as after rhizotomy. The net result of this combination of proliferation and apoptosis was a reduction in DC OLs, confirming earlier studies. Using an antibody to oxidized nucleic acids, we found rapid and prolonged RNA oxidation in OLs rostral to cSCI, but no evidence of oxidative stress in DC OLs after rhizotomy. These results suggest that signals associated with axonal degeneration are sufficient to induce OL proliferation, and that secondary

injury processes associated with the central SCI, including oxidative stress, rather than axonal degeneration per se, are responsible for OL apoptosis.

Keywords

cell death; cell proliferation; progenitor cells; Wallerian degeneration; spinal cord injury; dorsal root; dorsal columns; oxidative stress

Introduction

Oligodendrocyte (OL) death and associated demyelination have been postulated to contribute to chronic deficits after SCI (Emery et al., 1998; Warden et al 2001; McTigue et al., 1998; Casha et al., 2001; Beattie et al, 2000). At a contusion SCI site, both neurons and glia die by necrosis and/or apoptosis soon after injury, and the spared rim of white matter exhibits demyelination (Beattie et al., 1997;; McTigue et al., 1998; Grossman et al., 2001). In addition, a longer term secondary injury process appears to affect OLs associated with axons that have been damaged and are undergoing Wallerian degeneration remote from the lesion site, and is particularly evident in the dorsal columns (DC) rostral to the lesion (Crowe et al, 1997; Shuman et al, 1997; Warden et al, 2000; Beattie et al, 2002). This apoptotic OL death can occur over many days or weeks at sites quite far from the injury (Crowe et al, 1997;; Warden et al 2000). This cell death appears to be associated with microglial activation, suggesting that inflammatory cytokines and the production of oxidative stress may be involved (Shuman et al., 1997; Abe et al., 1999; Casha et al., 2001; Beattie et al., 2002, 2004; Dong et al., 2003). Alternatively, developmental studies suggest that OL survival may depend upon signals from intact axons (Barres and Raff 1999; but see Ueda et al., 1999). Whatever the cause of the OL death, since each OL can myelinate numerous segments of different axons (Raine, 1984; Remahl and Hildebrand, 1990), this delayed OL death may contribute to additional demyelination of spared axons in distal tracts affected by Wallerian degeneration of neighboring axons (Shuman et al., 1997; Beattie et al., 2002; Kierstead et al, 2005). Apoptotic OL death has been reported after human SCI (Emery et al., 1998, Guest et al., 2005), as well as in multiple sclerosis (Barnett and Prineas, 2004; Matute and Perez-Cerda, 2005), and thus may be a secondary phenomenon that constitutes a target for neuroprotective therapy.. Recent studies have shown that neuroprotective, anti-inflammatory drugs like minocycline, can retard long tract OL apoptosis and enhance recovery after SCI in rodents (Stirling et al., 2004; Demjen et al., 2004).

However, several studies have questioned whether Wallerian degeneration alone results in long tract OL apoptosis and demyelination. After DC lesions, Li and Blakemore (2004) found no reduction in OL numbers when these cells were identified by the presence of proteolipid protein (PLP) message, although OL apoptosis was not examined. Siegenthaler et al (2007) reported less OL apoptosis in white matter tracts rostral to a hemisection versus a contusion. Further, several studies have shown that brain and spinal cord injury can induce reparative responses that include the proliferation and differentiation of adult oligodendrocyte progenitor cells (OPCs) that are capable, in some instances, of remyelination (e.g. Kierstead et al, 2005; Nielsen et al, 2006). Contusion SCI produces a proliferation of NG2+ cells in a marginal zone surrounding the lesion site, and many of these appear to be OPCs based on morphology and expression of Olig2 (Tripathi and McTigue, 2007). NG2+ OPC proliferation can also be induced by chemical activation of microglia (Schonberg et al, 2007). Moreover, in demyelinating lesions of the spinal cord, OPC proliferation and differentiation have been linked to inflammatory signals, and thus perhaps to microglial activation (Setzu et al, 2006). Clearly, the effects of injury and microglial activation on OL-lineage cells in SCI are complex. The effects of SCI on OPCs in

tracts away from the lesion center but exposed to Wallerian degeneration have not been examined.

To assess the role of intraspinal axonal degeneration *per se* in the production of OL apoptosis and proliferation, we induced pure axonal degeneration in the DC by cutting the L2-S2 dorsal roots while leaving the spinal cord intact. This produced massive axonal degeneration in the DC, and microglial activation, but no evidence of OL apoptosis or loss. Rather, there was an increase in the number of OL-lineage cells positive for NG2 and APC (CC1), and BrdU studies showed evidence for oligodendrogenesis. In a second experiment, we produced contusion SCI (cSCI) in the caudal thoracic cord. As shown previously, this resulted in massive axonal degeneration, microglial activation and apoptosis in DC OLs by 8 days after injury. However, we also saw evidence for proliferation of NG2 cells and formation of new CC1+ OLs, as in the rhizotomy experiment. The net result of this combination of proliferation and apoptosis was a reduction in DC OLs, confirming earlier studies. Reactive oxygen species (ROS) and oxidative stress have been implicated in cell death after SCI (e.g. Beattie 2004). Using an antibody to oxidized nucleic acids, we found evidence for rapid and prolonged RNA oxidation in OLs rostral to cSCI, which was absent in DC OLs after rhizotomy. These results suggest that signals associated with axonal degeneration are sufficient to induce OL proliferation, and that secondary injury processes associated with SCI, including ROS production, rather than axonal degeneration *per se*, are responsible for OL apoptosis.

Material and methods

Experimental design

Experiment 1 evaluated axonal degeneration, microglial activation and OL apoptosis after either dorsal rhizotomy or cSCI. Six groups (n=4/group) were used for each injury type: sham, 8hr, 1d, 3d, 5d, and 8d survival. Experiment 2 examined the effect of rhizotomy or cSCI on OL proliferation and differentiation. Five groups were used: sham (n=6), 3d and 5d survival after rhizotomy (n=4/group), and 3d and 5d after cSCI (n=4/group). Experiment 3 evaluated the distribution of nucleic acid oxidation and OL cell death after cSCI using immunofluorescence. Seven groups were used (n=4/group): sham, 1h, 8h, 1d, 2d, 8d and 21d. Nucleic acid oxidation after rhizotomy alone was also evaluated using animals from experiment 1. Experiment 4 evaluated mRNA oxidation after cSCI using immunoprecipitation. Six groups were used: sham (n=6), 10min, 60min, 90min, 3h and 8h (n=3/group) post-injury.

Animals

Long Evans female rats (Simonsen Labs, Gilroy, CA), 78+/- 5 days old at study onset were housed two per cage with food and water available *ad lib*. All procedures complied with NIH guidelines and were approved by the university Institutional Laboratory Animal Care and Use Committee.

Surgery

Under pentobarbital anesthesia (50–60mg/kg; Ovation Pharmaceuticals Inc., Deerfield, IL), the surgical site was shaved and disinfected. For rhizotomy, a L2 laminectomy was performed, the dura cut, and the right L2-S2 dorsal roots were sectioned with irridectomy scissors. For cSCI, a T9-10 laminectomy was performed and a moderate injury (12.5 mm) was produced with the NYU/MASCIS device (Basso et al, 1996). The wound was closed in layers and the animals recuperated overnight in an incubator. Antibiotics (55mg/kg Kephlin-cephazolin; Apotex Corp. Weston FL) were administered postoperatively. Manual bladder expression was performed twice daily after SCI. No special post-operative care was required

for the rats receiving unilateral rhizotomies. All animals were inspected daily for wound healing, weight loss, dehydration, autophagia, and any discomfort. Appropriate veterinary care was provided when needed.

BrdU injection

For experiment 2, 50mg/kg 5-bromo-2'-deoxyuridine (BrdU; #280879; Roche Diagnostics Corp., Indianapolis, IN) was dissolved in saline and administered i.p. once daily on day 1 and day 2 after rhizotomy or contusion.

Tissue processing

For the histological studies, animals were perfused intracardially with 4% paraformaldehyde under deep anesthesia with ketamine and xylazine (ket, 80mg/kg; Hospira, Inc. Lake Forest, IL; xyl, 20mg/kg; VEDCO, Inc. St. Joseph, MO). The cord was removed, post-fixed in 4% paraformaldehyde overnight, and blocked as shown in Fig. 1. For animals with rhizotomies, the cord was cut into the entry zone of the cut roots (L2-S2), and successive 3 mm blocks rostrally. For animals with cSCI in experiments 1–3, the cord was blocked at the lesion center (6mm), and rostrally and caudally into 3mm blocks numbered for their location relative to the lesion. To examine axonal degeneration, for animals in 3d, 5d and 8d survival groups, 1mm blocks 6–7mm rostral to the most anterior root entry zone after rhizotomy, and 9–10mm rostral to the lesion center after cSCI, were prepared for semi-thin plastic sections (see below). All other cord blocks were cryoprotected in 30% sucrose for 3 days, frozen in dry ice and stored at –80 degrees C until cryostat sectioning frontally at 10um, or horizontally at 20um. For animals with cSCI in experiment 4 (RNA oxidation immunoprecipitation), after anesthesia, 30 mm of cord centered on the lesion site was exposed, removed and immediately embedded in dry ice, blocked into three 10 mm pieces (one rostral, one at, and one caudal to the lesion center) and stored at –80 degrees C until use. The rostral and lesion center blocks were used for the immunoprecipitation analysis.

Plastic sections and Toluidine Blue staining

1mm cord segments were rinsed with phosphate buffered saline (PBS, 0.1 M, pH 7.4), post-fixed in 1% osmium tetroxide in PBS for 2 hours, rinsed, stored at 4°C overnight in PBS with 0.1M sucrose, and dehydrated in graded ethanol (50%, 70%, 80%, 95%, 100%, 100% each for 10 min). The tissue was then immersed in propylene oxide for 20 min, propylene oxide/Spurr epoxy resin (1:1) for 2 hours, and propylene oxide/Spurr epoxy resin (1:2) overnight. The cords were infiltrated with Spurr resin for over 6 hours with 2–3 changes, and polymerized at 60°C for 20–24 hours. Semi-thin sections were cut at 0.6um on an ultramicrotome and mounted on microscope slides with a drop of water. The sections were dried on an 80–90°C hotplate, and stained with 1% toluidine blue with 0.5% Pyronin B for 15–30 seconds.

Immunofluorescence/immunohistochemistry

Tissue sections were rehydrated with PBS, placed in blocking solution (5% normal goat serum [NGS], 0.3% triton in 0.1 M PBS, pH 7.4) for 1 hour, and then incubated with primary antibody overnight at room temperature. Primary antibodies used were, CC1 for OLs (anti-APC, mouse monoclonal, 1:1000; Oncogene Research Products, San Diego CA), GFAP for astrocytes (rabbit polyclonal, 1:500; Sigma, Saint Louis MO), anti-CD11b (OX42) for microglia/macrophages (mouse monoclonal, 1:500; Serotec, Oxford, UK), anti-NG2 (rabbit polyclonal, 1:400; Chemicon International, Temecula, CA), anti-BrdU (G3G4, mouse monoclonal, 1:400; Hybridoma Bank, Iowa City, IA), anti-p75 (mouse monoclonal, 1:100; Chemicon International, Temecula, CA), anti-cleaved caspase-3 (rabbit monoclonal 9664, 1:200; Cell Signaling Technology, Danvers, MA), and 15A3 (anti-8OHdG) for RNA/DNA

oxidation (mouse monoclonal 12501-120996, 1:250; QED Biotechnology, San Diego, CA). The appropriate fluorescent secondary antibody was applied according to the primary antibody (1:1000 in 5% NGS in PBS; one of the following: Alexa Fluor 488 goat anti-mouse IgG; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 594 goat anti-mouse IgG; Alexa Fluor 594 goat anti-rabbit IgG; Molecular probes, Eugene, Oregon) and incubated for 2 hours at room temperature. The sections were then counterstained with Hoechst 33342 (5 mg/ml, Sigma, St. Louis, MO) for 10min, and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA).

For immunohistochemical labeling, goat anti-mouse IgG secondary antibody (1:400 in PBS) was used. Afterwards, tissue was processed using Elite ABC (Vector Laboratories, Burlingame, CA) for 1 hr, followed by either DAB or SG substrate (Vector Laboratories, Burlingame, CA) for 10 min. Sections were dehydrated and cover-slipped. When using primary antibodies from different species for double-labeling, simultaneous incubation was performed; for double labeling with primary antibodies derived from the same species, sequential labeling was used. Between labeling the two antigens of interest, thorough rinsing and blocking was performed to avoid cross-reactivity. For control sections, primary or/and secondary antibodies were omitted to assure specificity of the labeling. Additional steps were performed for some of the studies as follows. For labeling BrdU positive cells, tissue was treated with 2N HCl at 37°C for 30 min before blocking and incubation with anti-BrdU antibody. To test the specificity of the 15A3 antibody labeling, tissue was incubated with RNase (5 mg/ml, Boehringer Mannheim) for 30min at 37°C before blocking and incubation with 15A3.

Immunoprecipitation and cDNA synthesis

The methods were performed as previously described (Shan, et al., 2003). Briefly, Poly (A) mRNA was isolated from ~100 mg of spinal cord tissue using the QIAGEN total RNA isolation and mRNA isolation Kit (Qiagen, Valencia, CA). For immunoprecipitation, 1.5ug mRNA, 5.0ug 15A3 antibodies and 20ul Protein L gel (Pierce, Rockford, IL) was mixed in PBS and incubated at 4°C for 16 hrs. For control, 15A3 was incubated with 24 ng/ml of 8OHG (Cayman Chemical, Ann Arbor, MI) for 2 hour before mixing with mRNA samples. The protein L-immune complex was washed in PBS with 0.04% NP-40 three times and then cleaned by phenol extraction. The immunoprecipitated mRNA was precipitated in ethanol overnight at -20°C and resuspended in water. For cDNA synthesis, the precipitated mRNA was reverse transcribed into cDNA in a reaction of 30ul mixture containing heat denatured mRNA, 5xcDNA reaction buffer, 0.5mM each of dNTPs (dATP, dCTP and dGTP), 0.13mM of dTTP, 0.03mM of DIG-11-dUTP (Roche, Mannheim, Germany), 0.75ug of oligo-(dT)24-T7, 2.5U of RNase inhibitor (Invitrogen, Carlsbad, California) and 3.2U of AMV reverse transcriptase (Roche, Mannheim, Germany) at 42°C for 90 min. Second-strand synthesis was accomplished by adding 9ul of x DS buffer, 11U of E. coli DNA polymerase (United States Biochemicals, leveland, OH), 0.5U of E. coli DNA ligase (United States Biochemicals), 5U of RNase H (United States Biochemicals) and 3ul of 0.1M DTT in a final volume of 5ul and then by incubating the mixture at 16°C for 3 hrs. Double-strand cDNA was extracted by phenol-chloroform. 5ul out of 30ul digoxigenin-labeled cDNA was used to perform Southern blotting to compare the difference in cDNA quantities between injury and sham control.

Southern blotting

cDNA synthesized from the oxidized mRNAs was resolved in agarose gel and then transferred electrophoretically to a positively charged nylon membrane (Roche, Indianapolis, IN) using the transblot SD semi-dry transfer system (Biorad, Hercules, CA) according to the manufacturer's directions. Digoxigenin labeled cDNA was detected with a

Digoxigenin High Prime DNA labeling and Detection Starter Kit II (Roche, Indianapolis, IN).

Quantification of immunocytochemistry

We used previously published strategies to estimate microglial ‘activation’ (Popovich et al, 1997), numbers of OLs and OPCs, apoptosis (Crowe et al., 1997; Beattie et al, 2002) and to evaluate progenitor cell proliferation (McTigue et al., 2006; Tripathi and McTigue, 2007; Sellars et al., 2009). Evaluation of numbers of cells in the DC regions of interest (ROI) involved counting all cells present in the entire ROI in randomly selected sections. All quantification was performed blind to group inclusion. Sections of the spinal cord 3–6 mm rostral to the most anterior root entry zone after rhizotomy, and 7–9 mm rostral to the center of the contusion were used for analysis since these regions exhibited similar amounts of axonal degeneration. The 7–9 mm distance rostral to the lesions in the DC is also the area where OL apoptosis was shown to occur during the first 8 days after cSCI (Crowe et al., 1997; Shuman et al, 1997; Beattie et al., 2002).

For each animal, 4 sections were randomly selected, and digital photomicrographs of the DC were taken (see below) using the appropriate filter. Micrographs were all taken under the same conditions (exposure, brightness, contrast, etc) for each experiment and label. The sample site was the entire *ipsilateral* DC for rhizotomy, and the entire bilateral DC for cSCI as shown in Fig 2a and 2b.

For quantification of CD11b (OX42) immunoreactivity, MCID software (Imaging Research, St. Catharines, Ontario) was used. Images were digitized, sample areas outlined, and the threshold was set so that only the positively labeled structures were selected and quantified. The results are reported as density per unit area sampled (see Popovich et al., 1997).

To count apoptotic cells, sections were double labeled with Hoechst 33342 with one of the following phenotypic cell markers: CC1, OX42 or anti-NG2. The randomly selected sections were examined under 40x using a Zeiss fluorescence microscope. Apoptotic cells were identified by the presence of condensed or fragmented nuclei using the filter for Hoechst 33342 (see Crowe et al, 1997). The cross-sectional area of the DC was determined, and the results are presented as cell number/unit area. DC areas did not differ between sides. The phenotype of each apoptotic cell was identified by switching filters appropriate for each individual cell marker, and the results are presented as the percentage of total apoptotic cells. Potential apoptotic OLs after rhizotomy were examined in the same manner by using sections double-labeled with CC1, HO 33342, and also using antibodies to cleaved caspase-3 or p75.

For quantification of CC1 labeled OLs, labeled cells were counted using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA). A Metamorph journal segmented and identified individual cells. Only labeled cells with Hoechst labeled nuclei were counted. The DC ROI was entered by the operator, and the results are reported as cell number/unit area.

Putative OPCs were identified using NG2 immunostaining. Morphological features were used to confirm the likelihood that cells were OPCs. Double-labeling with OX42 was also used to eliminate false positives as previous studies reported staining of activated microglia or macrophages with this antibody (Nishiyama et al., 1997; McTigue et al., 2001). NG2-positive OPCs were counted manually using the following criteria: cells had the typical bipolar or multipolar OPC morphology, the nuclei were surrounded by NG2

immunoreactivity, and cells were not double labeled with OX42. Results are reported as cell number/unit area, again using the DC as the ROI.

For double labeling of CC1 or NG2 with BrdU, co-localization was confirmed in the DC ROI using a Zeiss 150 Meta confocal microscope, and the results are reported as cell number/unit area.

Cellular 15A3-immunoreactivity was quantified using the MCID software. The sample areas were outlined as before, and the threshold was set so that the labeling could be automatically picked up with minimum background. The results are reported as the proportion of the labeled area over the total area (i.e. percent labeled area), as for the analysis of OX42 labeling of microglia.

Statistical analysis

One-way ANOVAs were used to compare study variables. Post-hoc comparisons were performed using Tukey analysis. Significance was set at $p < 0.05$.

RESULTS

Dorsal rhizotomy produces axonal degeneration and microglial activation similar to that seen after cSCI

Unilateral L2-S2 rhizotomies resulted in pronounced axonal degeneration in the DC after five days evident in toluidine blue-stained semithin plastic sections. Axonal degeneration occupied most of the ipsilateral DC 6–7 mm rostral to the most anterior root entry zone (Fig 2A&C), while contralateral axons and tissue were unaffected (Fig. 2A&E). Degenerating axons could be identified by dark, collapsed axoplasm surrounded by thickened myelin (arrows, Fig 2C). Empty myelin sheaths were also present (asterisks, Fig 2C). Similar axonal degeneration was observed rostral to contusion injury (Fig. 2B&D), except the degeneration was bilateral. Examination of the margins of the degenerating axon tracts in these sections from both the rhizotomy and the contusion cases showed no evidence of demyelination of intact axons (see supporting material).

Axonal degeneration produced by rhizotomy also induced microglial activation, as evidenced by a change in cell morphology and increased CD11b (OX42) immunostaining (Fig. 3). In normal white matter, microglial cells have elongated, fine, ramified processes, and small cell bodies (Fig. 3A). Three days after rhizotomy, a significant increase in OX42 labeling was seen ipsilaterally; the cell processes shortened and thickened, and the cell bodies became rounder (Fig. 3B). By day 5, many cells continued rounding up, adopting a phagocytic morphology (Fig. 3C and D). These morphological changes were reflected in an increase in the density of OX42 staining on days 5–8 (Fig. 3E). This increase in microglial labeling, and presumptive activation (Fig. 3E) was similar to that after cSCI in the area of axonal degeneration (Popovich et al., 1997, Shuman et al, 1997).

DC OL apoptosis occurred after cSCI, but not after dorsal rhizotomy

As previously reported, cSCI resulted in large numbers of CC1-positive cells containing apoptotic nuclei in the DC 7–9 mm rostral to the lesion. Dorsal rhizotomy produced microglial activation that looked similar to that seen after cSCI (see Shuman et al, 1997; Popovich et al, 1997). However, rhizotomy failed to produce significant apoptosis of OLs at 8 days, when apoptosis peaks after cSCI (Crowe et al., 1997; Shuman et al, 1997; see below and Figure 4). Although numerous apoptotic cells could be identified by their fragmented or condensed nuclei at day 8 post-rhizotomy (5.75 ± 0.54 /section), very few of these (<1%) were positive for the OL marker CC1; most apoptotic cells after rhizotomy were Cd11b (OX42)+

microglia or macrophages (about 67%, Fig 4A). Consistent with this result, OLs also lacked other apoptotic indicators such as cleaved caspase-3 and the low affinity neurotrophin receptor p75 (which we previously reported increased in apoptotic OLs after cSCI, Beattie et al., 2002) (data not shown). This lack of apoptosis of CC1+ OLs was observed at all time points up to 3 weeks after rhizotomy. In contrast, OLs underwent apoptosis after cSCI, as previously shown. CC1+ OLs comprised a significant percent ($24 \pm 3.8\%$) of the total apoptotic cells ($7.5 \pm 0.50/\text{DC}$ section, Fig. 4A&B) after SCI. This result is similar albeit slightly lower than that reported in Shuman et al. (1997), where a greater injury severity was used (25 mm injury vs 12.5 mm used here). NG2+ cells were also examined under both injury situations to search for apoptotic OPCs. NG2+ cells with apoptotic profiles (condensed or fragmented nuclei) were not seen after rhizotomy for at least 3 weeks. After cSCI, a few NG2+ cells with apoptotic profiles were found 8 day post-injury, but it is uncertain whether they were OPCs, since they were shrunken and did not have the typical OPC morphology (see below).

Rhizotomy induces increased OL cell number, OPC proliferation and new OL generation

Although OL apoptosis was not found after rhizotomy, we counted total OL cell numbers to confirm that cell loss seen after cSCI was not present after rhizotomy. First, a statistical analysis of the size of the sampled area across survival times was performed to assess tissue shrinkage, and showed no change; therefore the results are presented as cell number per unit area (mm^2). The results showed that OL cell numbers (CC1+ cells) on the rhizotomy side did not differ from sham control at 1d or 3d after rhizotomy, but there was a significant increase at 5 days and 8 days (Fig 5). We postulated that this increase in CC1+ OLs might result from proliferation and differentiation of the resident OPCs, which are widely distributed in the mature CNS (Chang et al., 2000; Horner et al., 2000; McTigue and Tripathi, 2008). These cells express the proteoglycan NG2, and can be identified by their typical morphology (small bipolar or multipolar cell bodies and large nuclei; Fig. 6A). NG2+ OPCs were significantly increased within 1 day of rhizotomy (Fig. 6B&E). Interestingly, at day 5, when CC1+ OLs were increased (Fig. 5), the numbers of NG2+ OPCs had declined to the control level, although many cells showed more complex processes than at day 3, possibly indicating further differentiation (Fig. 6C&E). At day 8, NG2+ OPC numbers increased again, and their morphology was more similar to day 3 than day 5 (Fig. 6D&E). This early rise in NG2+ OPCs followed by increased CC1+ OLs suggests that many OLs present at day 5 may be newly differentiated from proliferating NG2+ OPCs.

To test this hypothesis, BrdU was injected on day 1 and day 2 after rhizotomy, and the animals were sacrificed on day 3 or day 5. Thus, by tracing BrdU+ cells, we could determine whether the day 5 CC1+ OLs were new and derived from cells dividing on days 1 or 2. As expected, few BrdU+ cells were present in normal tissue. Three days after rhizotomy, a dramatic increase in BrdU+ cells was seen ipsilaterally, but not contralaterally (Fig. 7M). At this time, many of the BrdU+ cells were NG2+ OPCs. None of BrdU+ cells were CC1+ OLs revealing that, as expected, mature OLs present at 3d had not taken up BrdU on days 1 or 2 (Fig. 7A–F&N). Five days after rhizotomy, the overall BrdU+ cell number and distribution appeared similar to that of day 3 (data not shown). The number of BrdU+ NG2 cells was approximately half that present at 3d; however, a significant increase in BrdU+ OLs was detected at this time (Fig. 7G–L&N), suggesting that a portion of the NG2+ OPCs proliferating on days 1 and 2 differentiated into more mature CC1+ OLs by 5d after axotomy.

OPC proliferation and new OL generation also occurred in the DC tracts rostral to a lesion after cSCI, but OL cell numbers still decreased after cSCI

The number of NG2+ OPCs increased after cSCI as well as after rhizotomy. NG2+ OPC numbers increased by day 1, and returned toward control levels by day 8. The secondary increase observed at day 8 after rhizotomy was not seen after cSCI (Fig. 8A). We then repeated the same BrdU pulse injection protocol used in the rhizotomy experiments. Numerous NG2+ OPCs were BrdU+ at 3 day after SCI (Fig. 8B). Furthermore, a number of CC1+ cells with BrdU+ nuclei were present at day 5 after cSCI (Fig. 8B&C), indicating the presence of new OLs. Despite the apparent generation of new OL lineage cells, the total OL cell number did not increase after cSCI at the time points examined, and in fact declined to levels significantly less than control at day 8 after injury (Fig. 8D), consistent with previous findings (Beattie et al., 2002; Dong et al., 2003). Comparison of the cSCI and rhizotomy results suggests that a stimulus induced by trauma overrides the reparative response initiated by axonal degeneration and results in OL apoptosis and a significant decline in OL numbers after cSCI.

Oxidative stress/RNA oxidation increased in OLs after cSCI but not after rhizotomy

A number of previous studies suggest that oxidative stress could be an important stimulus mediating the deleterious effect of trauma on OLs. In addition, nucleic acid oxidation has been suggested to be a potential factor in cell loss in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (Nunomura et al., 1999; Shan et al., 2003; Chang et al., 2008; Kong et al., 2008). We therefore examined nucleic acid oxidation as a marker for oxidative stress in both the rhizotomy and contusion models. Immunostaining with the 15A3 antibody was used as in previous studies; this antibody specifically recognizes markers of oxidative damage to DNA and RNA, nucleosides 8-hydroxydeoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHG), respectively (Nunomura et al., 1999; Shan et al., 2003). In normal animals, 15A3 immunoreactivity (IR) was very low (Fig. 9A&D). After rhizotomy, it remained at this level through all time points studied (Fig. 9B&C; Fig. 10A). After cSCI, however, a notable increase in 15A3-IR was observed in the DC rostral to the lesion epicenter where axonal degeneration and OL apoptosis have been previously demonstrated (Crowe et al., 1997; Beattie et al., 2002; present study, Fig. 2 & 4). 15A3-IR increased in cells by 1 hour after cSCI and was dramatically increased at 8 hours (Fig. 9D–G). On horizontal sections, these 15A3+ cells exhibited a linear pattern in the white matter reminiscent of OLs; the 15A3-IR was primarily located in the cytoplasm, a distribution pattern similar to RNA (Fig. 9H). When the sections were treated with RNase, or the primary antibody was pre-incubated with 8-OHG, the labeling was mostly eliminated, indicating that the oxidized nucleic acid was mainly RNA rather than DNA (Fig. 9I). Quantification of the 15A3-IR showed that RNA oxidation peaked at 8 hours and then lasted for weeks after cSCI (Fig. 10B). The lesion site exhibited a transient increase in 15A3-IR within 3 days after injury (data not shown). Notable changes in 15A3-IR were not found in other areas after cSCI.

Since mRNA oxidation could result in impaired translation as suggested by prior studies (Shan et al., 2003; Tanaka et al., 2007; Shan et al., 2007), we further examined whether mRNA was a component of the total oxidized RNA at its peak level (8 hour). Poly(A)+ mRNAs were isolated from the rostral region and oxidized mRNAs were separated from non-oxidized mRNAs by immunoprecipitation with 15A3 antibodies. The isolated oxidized mRNAs were then reverse transcribed to cDNAs. DIG labeled-dUTPs were incorporated into cDNAs to facilitate analysis by Southern blotting. As shown in Fig. 9N, oxidized mRNA was dramatically increased at 8 hour post injury in this distal area. The signal was eliminated when the primary antibody was omitted or pre-incubated with 8OHG, indicating the specificity of this method for detecting oxidized mRNA (Fig. 9N).

To identify the cell types exhibiting RNA oxidation in the distal area, sections were double labeled with 15A3 and the following cell markers: CC1 for OLs, GFAP for astrocytes, CD11b (OX42) for microglia/macrophages, and NG2 for OPCs. Double labeling revealed that most cells with RNA oxidation were immunopositive for CC1, indicating that they were OLs (Fig. 9J). In contrast, few double labeled cells were GFAP or OX42 positive (Figs 9J and K). Occasionally, NG2+ OPCs could be found with RNA oxidation at 8hrs after injury, but not afterward (Fig. 9M).

To investigate the relationship between RNA oxidation and apoptosis, we examined whether OLs expressing 15A3 IR also expressed the early apoptotic marker cleaved caspase-3. Cleaved caspase-3 was increased in cells with OL morphology in a time course similar to previous reports (Springer et al., 1999; Beattie et al., 2002); it did not reach significant levels until 8 days after cSCI (Fig. 11B, E & H). At this time, some cells positive for RNA oxidation were also cleaved-caspase-3 positive (Fig. 11G, H & I).

Discussion

The initial hypothesis driving these studies was that OL apoptosis in long tracts undergoing Wallerian degeneration is due to events initiated by degeneration *per se*. Such events could include withdrawal of trophic factors (Barres and Raff, 1994, 1999), as well as microglial activation (e.g. Beattie, 2004). Many DC OLs undergo delayed apoptosis in association with microglial activation after cSCI (Shuman et al., 1997). However, extensive rhizotomies that produced similar amounts of axonal degeneration and microglial activation, failed to produce OL apoptosis or loss, and instead stimulated proliferation and apparent differentiation of resident NG2+OPCs. After cSCI, we also detected proliferating and differentiating OPCs in long tracts. This response may account for the fact that overall OL numbers remained constant for several days despite RNA oxidation peaking at 8h after cSCI. That is, new OLs may have replaced some pre-existing OLs lost to oxidative damage and apoptosis (Horky et al, 2006; Rabchevsky et al, 2007). However, this process clearly was not sufficient to replace all lost cells since overall OL numbers declined significantly by 8d after cSCI. Since axonal degeneration was present in both models, but OL death occurred only after central lesions, some aspect of the central lesion in combination with axonal degeneration, induced OL death. The presence of oxidative stress indicators early after SCI, preceding the increase of cleaved caspase-3 and apoptotic nuclei, suggests that combining axonal degeneration with oxidative stress may produce OL apoptosis.

OL death and proliferation in response to axonal degeneration

There are many reports of delayed OL apoptosis in the degenerating DC (Crowe et al., 1997; Abe et al., 1999; Casha et al., 2001; Warden et al., 2002). The death receptor Fas and the low affinity neurotrophin receptor p75 are also increased in OLs along degenerated axons (Casha et al., 2001; Beattie et al., 2002). When axonal degeneration was delayed in Wld transgenic mice, OL apoptosis was also delayed (Dong et al., 2003). Thus, it was assumed that OL apoptosis was due to axonal degeneration *per se*. However, the notion that axonal degeneration can induce mature OL apoptosis is controversial. Evidence from optic nerve transection studies by Barres and Raff (1999) supports this hypothesis, whereas those of Ueda et al (1999) do not. Further, work by Neilsen et al (2006) showed that axon degeneration caused by perforant path transection induced NG2 cell proliferation and OL genesis. In the present study, dorsal rhizotomies produced axonal degeneration, yet without CNS injury we found no evidence of OL death. Instead, we found proliferation and differentiation of NG2+OPCs, resulting in new CC1+OL production and increased cell numbers. Thus, despite the apparent association, OL loss and apoptosis after SCI was likely not induced by axonal degeneration alone.

Following rhizotomy, we saw an apparent reduction of CC1+ cells between 5 and 8 days; but again, no apoptosis of OLs or OPCs was found. It is possible that the cells could have undergone a form of death not detected by the methods used herein. Perhaps more likely, the decrease in CC1+ cells might have been due to changes in antigen expression rather than cell death. It is notable that the number of NG2+OPCs and CC1+OLs showed an alternating pattern, when CC1+OL number decreased at day 8, NG2+OPCs again increased. Possibly, newly produced CC1+cells 5 days after rhizotomy were not mature OLs, but OLs at an intermediate stage having lost NG2 antigenicity and starting to express CC1, but not yet expressing genes for myelination such as PLP as suggested by the study of Li and Blakemore (2004). These cells might have completed the differentiation process had the environment been suitable and contained abundant bare axons, e.g. as observed after demyelination (Watanabe et al., 2002). While CC1 and other antibodies like Rip have been used to identify mature OLs, these antibodies also may recognize less mature, premyelinating OLs, (Li and Blakemore, 2004; Zai and Wrathall, 2005; and see below). Thus, the newly differentiated CC1+ cells induced by rhizotomy and SCI might be a population of non-myelinating OLs, especially given the fact that intact viable axons were lacking in this lesion.

Phenotypic markers of OLs and OPCs

The proteoglycan NG2 has been considered as a marker for OPCs but it is recognized that NG2 can be expressed by a heterogeneous population of CNS cells, as well as by peripheral monocytes/macrophages (see Nishiyama et al, 2009; McTigue and Tripathi, 2008). Injury may induce a non-OL-lineage, non-GFAP-positive NG2 expressing cell that actively phagocytoses myelin at the center of a spinal cord hemisection lesion (Sellars et al, 2009). We considered NG2+/OX42- cells most likely to represent OPCs in the regions of the DC away from the lesion center. These cells proliferated after both rhizotomy and cSCI as evidenced by BrdU incorporation. Further, BrdU-labeled NG2+ cells preceded the appearance of CC1+BrdU+ cells in the regions of Wallerian degeneration, suggesting that OLs were being produced by proliferation and differentiation of NG2+ OPCs. The CC1+phenotype has been used by many investigators to identify mature OLs *in vivo*, as in the present study. However, Li and Blakemore (2004) questioned the use of this marker, showing no reduction in the number of PLP positive OLs after DC lesions. They did not look for apoptosis, but rather counted intact cells expressing PLP mRNA. It is possible in the current work that a portion of those OLs undergoing apoptosis represent pre-myelinating CC1+OLs. Therefore, it remains to be seen whether the CC1+OLs killed by cSCI but spared when various anti-inflammatory treatments are given after injury (e.g. minocycline- Sterling et al., 2004; anti-CD95- Demjen et al., 2004; reviewed by Beattie 2004) can successfully myelinate axons.

There is substantial evidence showing that apoptosis after cSCI is associated with demyelination, which supports the idea that sparing of CC1+OLs is associated with sparing of myelination (Guest et al., 2005; Keirstead et al., 2005). Nevertheless, the issue remains unresolved whether remote long tract demyelination and remyelination (as opposed to demyelination at the site of injury, see McTigue et al., 2001, Zai et al., 2005; Tripathi and McTigue, 2005) contribute significantly to loss and recovery of function after SCI. The limited data in the present study relevant to this question come from our examination of the borders of the degenerating ascending DC tracts (supporting material), where no apparent demyelination was observed. This does not preclude the possibility that more extensive studies using techniques aimed at quantifying demyelination at this and other time points after injury, might yield different results. Nevertheless the clear evidence for demyelination at the lesion borders in SCI (McTigue et al., 2001; Zai et al., 2005; Tripathi and McTigue, 2005), and the presence of a substantial response of the NG2+, presumably OPC population,

suggests that in SCI, as in models of multiple sclerosis, there may be a failure of successful remyelination even in the presence of a vigorous progenitor cell response (e.g. Franklin, 2002)

Microglia/macrophage effects on OL cell death, proliferation, and protection

Apoptotic OLs are often found in contact with activated microglia after SCI (Shuman et al., 1997), suggesting a role for microglia in OL apoptosis, but there is also evidence that microglia may protect OLs (Frei et al., 2000). Recent work from our laboratory shows that even activated microglia can reduce OL apoptosis in mixed cultures (Miller et al, 2007). In the present study, we used the antibody OX42, which recognizes Cd11b expressed on microglia and peripheral macrophages, to evaluate microglial activation (Popovich et al, 1997) and apoptosis. Morphological changes in OX42+ cells that have been associated with microglial 'activation' were seen beginning three days after rhizotomy in the DC in association with degenerating axons and the time course for 'activation' was similar to that reported after cSCI (Popovich et al, 1997; Shuman et al, 1997). However, only cSCI produced OL apoptosis. It seems likely that microglia 'activated' by different stimuli may express different profiles of secreted molecules, and thereby differentially affect neighboring cells (McKimmie and Fazakerley, 2005). Neighboring cells could also respond differently to microglial activation depending upon their phenotype or status (Streit, 2005). Thus, when OLs are susceptible to apoptosis as may occur subsequent to RNA oxidation, activated microglia may trigger cell death. When OLs are less susceptible, the products secreted by neighboring microglia could be less toxic or even protective (Miller et al., 2007). Microglia are highly heterogeneous and cells exhibiting the same morphological features of activation can apparently be detrimental, protective or can induce differentiation, depending upon the presence of different environmental stimuli (Arnett et al., 2001; Nicholas et al., 2002; Carbonell et al., 2005). It is possible that some of the OX42+ cells in the contusion could be a macrophage subtype that is not present after rhizotomy alone (such as a monocyte-derived macrophage) and this could be responsible for differences in OL death. However, evidence suggests that most of the macrophage-like cells seen away from the lesion center are microglia-derived (Popovich and Hickey, 2002; Popovich et al, 2002). Separating the presence and effects of peripheral macrophages that invade the lesion from phagocytic, activated microglia has been problematic, and the roles of each cell type on injury and repair are a matter of some controversy (see Popovich et al, 1999; Popovich et al, 2002; Donnelly and Popovich, 2008; Schwartz, 2003; Schwartz et al., 2006). In the absence of frank trauma, the effects of 'activated' microglia after rhizotomy may be supportive, i.e. they might facilitate proliferation and differentiation of OPCs in contrast to their assumed role in OL apoptosis after SCI (see below), while OX42+ microglia/macrophages seen after contusion injury may support NG2+ cell proliferation but also lead to cell death. Indeed, injection of inflammatory substances into the spinal cord that activate microglia can stimulate NG2+ cell proliferation and differentiation (Schonberg et al, 2007), and the demyelinated margins of a contusion lesion are populated with numerous newly divided NG2+ OPCs and CC1 positive OLs (Tripathi and McTigue, 2007).

Apoptotic OLs after cSCI

After contusion, apoptosis results in significant OL loss. After severe contusion, there are 50% fewer CC1+OLs than in controls (Beattie et al., 2002). After milder injuries as in the present study, there is less cell loss, but still significantly less CC1+OLs than in controls. This indicates the death of pre-existing CC1+OLs. As seen in the present study, an early increase in RNA oxidation might initiate degenerative and even apoptotic events in OLs. Newly generated CC1+cells are also likely to be included in the population of OLs undergoing apoptosis after cSCI. As demonstrated above, NG2+OPC proliferation was similar after SCI and rhizotomy, and a portion of these cells gave rise to new (BrdU+) OLs,

albeit many fewer were present after cSCI than after rhizotomy (Fig. 7 & 8). Despite ongoing local oligogenesis in the DC after contusion injury, the overall number of OLs remained constant and then declined below control levels indicating that both pre-existing and newly formed OLs are lost. This suggests that after cSCI, intrinsic repair mechanisms are activated for OL regeneration but are overwhelmed by the degenerating cascades, resulting in significant cell loss.

RNA oxidation and OL apoptosis

Oxidative stress has been reported to increase after injury and is suggested to contribute to secondary cell death (Azbil et al., 1997; Liu et al., 1999; Kamencic et al., 2001; Leski et al., 2001; Xu et al., 2001; Lucas et al., 2002; Vaziri et al., 2004). However, despite strong *in vitro* evidence that OLs are highly susceptible to oxidative stress (Richter-Landsberg et al., 1998; Bhat et al., 1999; Laszkiewicz et al., 1999; Mouzannar et al., 2001; Baud et al., 2004), the role of oxidative stress in OL death after SCI is unclear. Increased RNA oxidation in OLs observed in the present study suggests that oxidative stress may play a role in delayed apoptotic OL death as well as in more acute neuronal cell death after cSCI. Our finding of RNA oxidation is similar to previous studies in other neurological conditions where it occurs early in vulnerable but otherwise apparently normal cells (Nunomura et al., 1999; Zhang et al., 1999; Liu et al., 2002; Shan et al., 2003; Shan et al., 2007; Chang et al., 2008).

This indicates that RNA oxidation is an early event rather than a result of endstage cell degeneration. More importantly, oxidative damage to RNA, especially mRNA, may profoundly affect cellular function. Our previous work (Shan et al., 2007) demonstrated that oxidized mRNA can not be translated properly, leading to reduced protein expression and consequently, loss of normal protein function, which could result in cell death. In the present study, significant levels of mRNA oxidation were found in OLs well before the onset of apoptosis, suggesting that mRNA oxidation could be an important factor initiating the cascade of delayed OL death. This possibility is currently under investigation.

Possible mechanisms inducing oxidative stress and delayed OL apoptosis after SCI

Currently it is not clear which traumatic component(s) induced the oxidative stress in OLs, but a comparison between contusion injury and rhizotomy may provide some clues. A prominent difference between these models is the integrity of the blood brain barrier (BBB). Contusion and other forms of SCI, e.g. cord transection, result in BBB breakdown (Popovich et al., 1996) while rhizotomy does not (Liu et al., 1998; but see Ling, 1979). Recent studies suggest that barrier breakdown could distinctly alter inflammatory responses; e.g., after rhizotomy complement reaction was absent (Liu et al., 1998), while after contusion, complement was remarkably activated within a day, and could be seen centimeters away both in white and gray matter (Anderson et al., 2004). This shows a dramatically rapid global inflammatory response to injury. Anti-inflammatory treatments such as minocycline reduce OL apoptosis, and one of minocycline's proposed effects is blockade of oxidative stress (see Beattie 2004). BBB breakdown could also result in abnormal cytokine transport and glutamate release (Pan et al., 2003); these all could work additively or synergistically with axonal degeneration to induce oxidative stress and OL degeneration. Alternative stimuli could include hypoxia induced by ischemia or the diffusion of injury-induced elements from the lesion center such as glutamate and inflammatory cytokines (Park et al., 2004). These might diffuse along the axons in the DC, a frequent site for lesion expansion. In this distal area, although Wallerian degeneration does not produce OL death, it might prime OLs to succumb to these other insults. Our original hypothesis was that activated microglia or macrophages may have contributed to oxidative stress in long tract OLs (Shuman et al., 1997). However, the time course of microglia/macrophage increased staining was similar in both the rhizotomy (present results) and

previous studies of cSCI (Popovich et al, 1997). In contrast, evidence of RNA oxidation in DC OLs away from the lesion occurred within hours after cSCI, and was not seen at all after rhizotomy, even in the presence of morphologically activated OX42 cells.

These results reinforce a growing view that secondary injury after CNS trauma is a complex, time-dependent and multifaceted cascade that is accompanied by secondary processes that promote endogenous repair. Further understanding of the signaling events and multiple dynamic cell-cell interactions that induce cell death, proliferation, and differentiation after CNS injury should help to generate better strategies for intervention. In the case of white matter OL damage after SCI, we clearly need to know more about the multiple interactive facets of OL and OPC signaling and microglial activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Tissue sampling. A) After rhizotomy (RZ), the cord was blocked into the entry zone (EZ) of the cut roots and two 3 mm blocks rostral (R1&R2). B) For cSCI, the cord was blocked into a 6 mm segment containing the lesion (LC), and two rostral 3 mm blocks (R1&R2). 1mm segments were embedded in plastic for toluidine blue stained semi-thin sections to identify axon degeneration in both models.

Fig. 2.

Axonal degeneration in the dorsal funiculus induced by dorsal rhizotomy and SCI. Schematics demonstrate the injury models, rhizotomy (upper left) and SCI (lower left). After rhizotomy, pronounced axonal degeneration occupied most of the medial dorsal funiculus ipsilaterally (A). Similar axonal degeneration was observed rostral to a contusion injury, except the degeneration was bilateral (B). High magnification micrographs taken from the boxed areas in A and B are shown in C, D and E. Degenerating axons exhibited dark, collapsed axoplasm surrounded by thickened myelin sheath (arrows; C&D). Myelin sheaths that contained debris or were empty, were also present (asterisks; C&D). Contralateral to rhizotomy, axons and tissue were unaffected (A&E). Semithin plastic section stained with toluidine blue. Day 5 post-injuries. Scale bar =20um in C and applies to D&E.

Fig. 3.

Rhizotomy induced microglial activation. In control tissue, microglia with small cell bodies and fine processes are homogeneously distributed (A and inset). After rhizotomy, OX42 labeling ipsilateral to the lesion dramatically increased. Cell processes thickened (B and inset), and cell bodies became progressively rounder as they adopted phagocytic morphology (C & D and insets). The boxed area in the spinal cord schematic outlines the location of the photomicrographs shown in A–D. Quantitative analysis of OX42 immunostaining as a function of time is shown in E (** $P < 0.01$, compared with uninjured control animals; error bars represent SEM). Scale bar in A = 30 μ m and applies to B, C and D. Scale bar in inset = 10 μ m and applies to all insets.)

Fig. 4.

OL apoptosis was observed after SCI but not after rhizotomy. Apoptotic cells with condensed or fragmented nuclei in Hoechst-stained sections were observed after rhizotomy and after SCI. After rhizotomy, <1% of total apoptotic cells were CC1+ OLs; most cells were OX42+ microglia/macrophages. In contrast, after SCI, about one fourth of the apoptotic cells were CC1+ OLs. Arrow in B shows an apoptotic CC1+ OL after SCI. (Error bars represent SEM. Scale bar in B=10um.)

Fig. 5.

OL cell number increased after rhizotomy. In uninjured control animals, CC1+ OLs were equally distributed in the dorsal funiculus bilaterally (A). After rhizotomy, there was a significant increase in OL number on the lesion side vs the contralateral control side by 5 days post-lesion (B–D). The boxed area in the spinal cord schematic outlines the location of the photomicrographs shown in A–D. Quantitative analysis of the changing number of CC1+ OLs ipsilateral to the rhizotomy vs the contralateral control number is shown as a function of time in E (** $P < 0.01$, compared with uninjured control animals; error bars equal SEM). Scale bar in A=30um and applies to B–D.)

Fig. 6.

OL progenitor cells (OPCs) increased after rhizotomy. NG2+ OPCs have bipolar or multipolar morphology with relatively symmetrical and ramified processes (A, and inset). Three days after rhizotomy, the number of NG2+ OPCs dramatically increased ipsilaterally (B, and inset). At day 5, the numbers of NG2+ OPCs declined to control levels, but cells showed more complex processes than at day 3 (C, and inset). At day 8, NG2+ OPC number increased again, and their morphology was more similar to day 3 than day 5 (D, and inset). The boxed area in the spinal cord schematic outlines the location of the photomicrographs shown in A–D. Quantitative analysis of the changing of NG2+ OPC cell number as a function of time is shown in E (** $P < 0.01$, compared with uninjured control animals; error bars represent SEM). Scale bar in A=30um and applies to B–D; scale bar in inset in A=10um and applies to all insets.

Fig 7.

Rhizotomy induces NG2+ OPC proliferation and new CC1+ OL generation ipsilateral to the injury. At day 3 after injury, numerous BrdU+ cells were observed ipsilaterally, few were found contralaterally (M; the boxed area in the schematic outlines the location of the photomicrographs shown in M). At this time, many BrdU+ cells were NG2+ OPCs (arrows, A–C), but none were CC1+ OLs (DF). Five days after rhizotomy, fewer NG2+ OPCs were BrdU+ (arrows, G–I), but significantly more double-labeled cells were CC1+ (arrows, J–L), even though the number of BrdU+ cells remained the same as day 3. Quantitative analysis of the double-labeled BrdU+ NG2+ OPCs and BrdU+ CC1+ OLs is shown in N (** $P < 0.01$, compared with non-injury control animals; error bars represent SEM). Scale bar in A=20µm and applies to B–L; Scale bar in M=20µm.

Fig. 8.

The response of NG2+ OPCs and CC1+ OLs after SCI. NG2+ OPCs increased after SCI; cell numbers were significant from day 1–5 (A). NG2+ OPCs with BrdU incorporation increased at day 3 and 5 whereas CC1+ OLs with BrdU incorporation were increased at day 5. In CC1+ OLs, the incorporated BrdU was mostly found throughout the nucleus (a&b in C). Occasionally, the distribution of BrdU in the nucleus had a punctate pattern (c in C). In spite of new OL generation, the total number of OLs after SCI did not increase, and by day 8, was significantly less than the control level (D; ** $P < 0.01$, * $P < 0.05$, compared with uninjured control animals; error bars represent SEM). Scale bar in a = 20 μm and applies to B&C.

Fig. 9.

RNA oxidation increased after SCI but not after rhizotomy (RZ). 15A3 antibodies were used to detect nucleic acid oxidation. 15A3 immunoreactivity (IR) was very low in control animals (A&D). After rhizotomy, it remained at this level through all time points studied (B&C). After SCI, however, 15A3-IR increased in the dorsal funiculus in the sampled region (7–9mm rostral to the lesion epicenter) at 1 hour (E), 8 hrs (F), and stayed elevated for at least 8 days (G). Horizontal sections of the dorsal funiculus showed that cells with increased 15A3 IR were arranged linearly similar to OLs (H). IR was prominent in the somatic cytoplasm and was diminished greatly by the RNase treatment, indicating cytoplasmic RNA is a major site of nucleic acid oxidative damage (I). Double labeling (J–M, 8hrs after SCI) showed that the primary cells undergoing RNA oxidation were CC1+ OLs (arrows, J); few of the cells were GFAP+ astrocytes (K) or OX42+ microglia (L). Occasionally, NG2+ OPCs could be found with RNA oxidation (arrow, M). mRNA oxidation was also increased at 8hrs while sham injury produced very low signal (N). When the 15A3 antibody was pre-incubated with 8OHG, the signal was diminished, indicating specificity for oxidized mRNA (N). Scale bar in A=30um and applies to B–G; in H=20um and applies to I; in J=20um and applies to K–M.

Fig. 10.

Quantification of 15A3 immunoreactivity after rhizotomy or SCI. After rhizotomy, 15A3-IR was not increased at least for 8days (A), while after SCI, 15A3-IR was significantly increased at all time points tested (B; ** $P < 0.01$, * $P < 0.05$, compared with uninjured control animals; error bars represent SEM).

Fig. 11. RNA oxidation preceded the presence of cleaved caspase-3 after SCI. Double labeling in the dorsal funiculus from control, and spinal injured cases surviving for 1 and 8 days. 15A3-IR (A, D&G) and cleaved caspase-3 IR (B, E&H; Merged in C, F&I) are shown. Scale bar in A=20um and applies to all.