Degeneration of Myelinated Efferent Fibers Prompts Mitosis in Remak Schwann Cells of Uninjured C-Fiber Afferents

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The factors inducing normally innervated Schwann cells in peripheral nerve to divide are poorly understood. Transection of the fourth and fifth lumbar ventral roots (L4/5 ventral rhizotomy) of the rat is highly selective, sparing unmyelinated axons and myelinated sensory axons; Wallerian degeneration is restricted to myelinated efferent fibers. We found that L4/5 ventral rhizotomy prompted many normally innervated nonmyelinating (Remak) Schwann cells to enter cell cycle; myelinating Schwann cells of intact (sensory) axons did not. Three days after L4/5 ventral rhizotomy, [³H]thymidine incorporation into Remak Schwann cells increased 30-fold. Schwann cells of degenerating efferents and endoneurial cells also incorporated label. Increased [³H]thymidine incorporation persisted at least 10 d after ventral rhizotomy. Despite Remak Schwann cell proliferation, the morphology of unmyelinated nerve (Remak) bundles was static. Seven days after L5 ventral rhizotomy, Remak Schwann cells in the L5-predominant lateral plantar nerve increased slightly; endoneurial cells doubled. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling-positive nuclei increased dramatically in peripheral nerve after L5 ventral rhizotomy; many of these were macrophage nuclei. In summary, we find that the degeneration of myelinated motor axons produced signals that were mitogenic for nonmyelinating Schwann cells with intact axons but not for myelinating Schwann cells with intact axons.

Key words: apoptosis; C-fiber; degeneration; mitosis; myelin; nerve; Schwann cell; ventral rhizotomy; TUNEL; neuregulin

Introduction

Schwann cell division is tightly regulated. Vigorous proliferation during development is followed by infrequent cell division in adulthood. A variety of Schwann cell mitogens have been identified in tissue culture, including contact with neurites and with axolemmal fragments (Wood and Bunge, 1975; Salzer and Bunge, 1980; Salzer et al., 1980a,b; Baichwal et al., 1988; Baichwal and DeVries, 1989). The best defined Schwann cell mitogens are neuregulins, including neuregulin-1 family members. These neuregulins interact with the Schwann cell tyrosine kinase receptors of the erbB family (Cohen et al., 1992; Jin et al., 1993; Morrissey et al., 1995). The importance of these factors in development is demonstrated by the paucity of Schwann cells and nerve fibers in genetically engineered animals lacking neuregulin-1 or its heteromeric receptor components erbB2 and erbB3 (Riethmacher et al., 1997; Morris et al., 1999; Garratt et al., 2000). Later in development, however, neuregulins may act to suppress proliferation, as indicated by the increase in Remak Schwann cell (RSC) 5-bromo-2-deoxyuridine incorporation in animals with a

dominant-negative defect in the erbB2 neuregulin receptor (Chen et al., 2003).

During development, some Schwann cells associate with axons in a one-to-one relationship and proceed to initiate PNS myelination (Asbury, 1967; Webster et al., 1973). Other Schwann cells do not produce myelin but continue to ensheath one or more axons in unmyelinated nerve fiber (Remak) bundles (Murinson and Griffin, 2004). In the normal adult nerve, there is no evidence that myelinating Schwann cells (MSCs) enter cell cycle, and the division of nonmyelinating (Remak) Schwann cells is infrequent (Griffin et al., 1987, 1990). Nerve disease and injury, including nerve transection and demyelination, prompt Schwann cells to enter cell cycle (Friede and Johnstone, 1967; Pellegrino and Spencer, 1985; Griffin et al., 1987, 1990; Oaklander et al., 1987b; Cheng and Zochodne, 2002). The factors that signal this proliferation are not fully understood (Pellegrino et al., 1986; Oaklander and Spencer, 1988; Clemence et al., 1989). The Schwann cells of individual degenerating nerve fibers divide (Pellegrino et al., 1986; Oaklander et al., 1987a), and an attractive speculation has been that axonal degeneration results in either degradation or release of axolemmally bound neuregulins (Ratner et al., 1986). However, Wallerian degeneration produces a variety of other changes throughout the nerve distal to the site of injury, including opening of the blood–nerve barrier (Weerasuriya, 1988; Bouldin et al., 1990, 1991) and recruitment of circulating monocytes (Beuche and Friede, 1984; Stoll et al., 1989; Avelino et al., 1995). Thus, several potential mitogens may be increased after injury, including circulating factors, molecules synthesized by Schwann cells, and products of macrophages.

In this study, we asked whether the Schwann cells of intact

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axons might enter the cell cycle. Such an observation would suggest that diffusible mitogens, capable of signaling Schwann cells that neighbor degenerating axons, might be important. Previous studies of demyelinating nerve injury showed division of Schwann cells associated with uninjured Remak bundles, but a direct effect on Remak Schwann cells could not be excluded (Griffin et al., 1987, 1990). In this study, we used ventral rhizotomy as a model of selective nerve injury in the rat. In rat, the ventral roots of lumbar segments 4 and 5 (L4 and L5) each contain thousands of myelinated axons but fewer than 50 unmyelinated axons (Coggeshall et al., 1977). Thus, ventral rhizotomy at L4 and L5 results in degeneration of the myelinated motor fibers in the sciatic nerve, whereas almost all of the unmyelinated axons (>99%) remain intact. This allowed us to investigate the effects of Wallerian degeneration on nearby Schwann cells that were directly associated with intact axons. We found that myelinating and nonmyelinating Schwann cells differed dramatically in the response to degeneration of nearby myelinated motor axons.

Materials and Methods

Surgical approach. Animals were managed using an approved protocol and were housed in plastic cages with bedding. Five-week-old male Sprague Dawley rats were anesthetized with chloral hydrate. Under deep anesthesia, a hemi-laminectomy exposed the cauda equina under the L3–L5 vertebrae. A lateral approach (ventral to the dorsal root) was used to gain access to the ventral roots. In turn, the L4 and L5 ventral roots were carefully transected using iris scissors, and a 2 mm piece of each root was removed. The utmost care was taken not to damage the dorsal roots; any damage to the dorsal roots during this procedure precluded further use of the animal. The successfully operated animals were sutured and allowed to recover with suitable postoperative care. After L4/5 ventral rhizotomy, the animals were mobile but had no movement of the lower leg on the side of the surgery. Sensation to pinch and light touch was preserved in the experimental limb. In an additional series of experiments, animals underwent transection of the L5 ventral root alone. The procedure was as described above, except the anesthesia was with pentobarbital and chloral hydrate together.

To evaluate the selectivity of these procedures, we analyzed by electron microscopy the ventral roots rostral to the site of ventral rhizotomy, the ventral, dorsal, and mixed spinal roots caudal to the site of ventral rhizotomy, and the sciatic nerves and the plantar nerve branches.

[³H]thymidine administration, tissue preparation, and autoradiography. The L4/5 ventral rhizotomy and sham-operated control animals received intraperitoneal injections of [³H]thymidine (40–60 Ci/mmol; 6 μ Ci/g per injection; Amersham Biosciences, Arlington Heights, IL). Two labeling schedules were used. Schedule 1 consisted of [³H]thymidine injections 2, 4, and 6 h before fixation. This schedule labeled cells that were in the S-phase at some time during the 6 h. Because our autoradiograms rarely contained labeled mitotic figures or adjacent labeled cells (suggesting that, by the time of fixation, they had completed mitosis), we presumed that most of the labeled cells were still premitotic (Bradley and Asbury, 1970). To ensure that each labeled cell was premitotic and to be certain of its location at the time of [³H]thymidine incorporation, in a second series of experiments, we used a single injection with a postinjection interval of 2 h (schedule 2). As expected, fewer cells were labeled by schedule 2, but the results of the two protocols were otherwise similar. All results below are for schedule 1.

Groups of animals were killed by perfusion fixation (0.9% sodium chloride, followed by 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) 0, 1, 2, 3, 4, 7, 10, and 14 d after axotomy. The sciatic nerves from the lesioned sides and the contralateral sides were removed, and segments were taken 5–10 and 20–25 mm distal to the L5 DRG. Samples were osmicated and embedded in Epon. One micrometer sections were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), exposed for 26 or 41 d, and developed in Kodak D19 developer.

The labeled nuclei in the resulting autoradiograms were quantified for each time point, using Bioquant (Nashville, TN) software to analyze

unstained sections in dark field. Nuclei with more than five silver grains were considered to be labeled. The studies of total [³H]thymidine incorporation were performed in triplicate animals for each of eight time points, and the results are reported as the average of these values. In the experiments that characterized the uptake of [³H]thymidine by each of the individual cells in an entire sciatic nerve cross section, thick sections from representative control animals and animals 3 and 10 d after surgery were studied in detail as described below. The results from a representative sciatic nerve 3 d after ventral rhizotomy are reported. The autoradiograms were stained lightly with toluidine blue. Each of the labeled cells was photographed under Nomarski optics and printed at a total magnification of 320–500 $\times$. These micrographs allowed unique identification of individual labeled cells in the subsequent electron microscopy.

After photography, the coverslips were removed, the 1 μ m section was hardened to fresh Epon by means of an inverted Beam capsule, and resulting block with the section at the face was removed from the glass slide by immersion in liquid nitrogen. Thin (80 nm) sections were cut from the 1 μ m sections and examined by electron microscopy, specifically identifying at the EM level individual labeled cells that were evaluated previously by image analysis (Griffin et al., 1990). The sciatic nerves 5–10 and 20–25 mm distal to the lesion were assessed separately by these methods. The 20–25 mm segment was evaluated in detail as described below.

Criteria for classification of cells by electron microscopy. Schwann cells of myelinated axons were divided into those associated with intact fibers (MSC-I) and those of degenerating myelinated fibers (MSC-D). The Schwann cells of degenerating fibers were distinguished from non-Schwann cells in the endoneurial space by their tightly applied basal lamina and their cytologic features. At three days after ventral rhizotomy, most of the MSC-D were easily identified by the presence of myelin ovoids within the same section as the nucleus. In some MSC-D, the ovoids and myelin debris were not present in the transverse section containing the Schwann cell perikaryon; in such fibers, the Schwann cell perikaryon was “hypertrophied” and occupied the whole cross-sectional area of the fiber. This characteristic appearance was frequently encountered between 2 and 4 d after transection and was easily recognized in transverse sections as a large round perikaryon with tightly applied basal lamina and cytologic features characteristic of Schwann cell nucleus and cytoplasm (Pellegrino et al., 1986; Stoll et al., 1989). These hypertrophied Schwann cell, perikarya usually also contained clear lipid droplets. In the present model, we did not have to distinguish Schwann cells of degenerating myelinated fibers from those of degenerating unmyelinated fibers because, as demonstrated below, there were almost none of the latter. It is known that under certain conditions, including demyelination, macrophages can enter the Schwann cell tube. Using the criteria established here, these macrophages might be classified as MSC-D. However our detailed analysis was performed at three days after L4/5 ventral rhizotomy, a time when few macrophages should be present inside the Schwann cell tubes. The Schwann cells of Remak bundles were recognized by their ensheathment of one or more unmyelinated axons. Endothelial cells comprised a fourth category. A final cell class was termed “endoneurial cells.” This group includes both endoneurial fibroblasts, mast cells, resident macrophages, and, at least by day 4, macrophages derived from the circulation. They were grouped together because ultrastructural criteria for distinguishing these cell types are imprecise.

Assessment of the proportion of total cells incorporating [³H]thymidine. To assess the proportions of the labeled cells that were comprised by each of the cell types, the whole cross-sectional area of a 3 d schedule 2 test nerve was photographed in the electron microscope, and each labeled nucleus present in the prints was classed by its cell type. These percentages were compared with the proportions of each cell type that was labeled. The proportions of each cell type were calculated by counting the cells of each type in 14–20 uniformly sized regions in the sciatic nerve, the placement of which was determined by the EM grid bar intersections.

These numbers were used to construct an estimated proliferation index that was calculated as follows: number of cell type A labeled by [³H]thymidine/(number of cell type A in the sample population/fraction of the total cross sectional area counted for the sample). This index approximates the fraction of the population entering cell cycle because all

[³H]thymidine incorporating cells in the sciatic nerve were counted, and the nuclear numbers were obtained through systematic sampling of the nerve. Systematic sampling was necessary because substantial portions of the sciatic nerve were obscured by the supporting EM grid. Calculating the number of cells in the sciatic by extrapolating from the systematic sample for each transverse section makes allowance for the edema that follows ventral rhizotomy.

Subsequently, we undertook the plantar nerve analysis below. These small anatomically invariant nerves arise predominantly from axons of the L4 and L5 dermatomes. The cellular contents of these nerves were counted in their entirety, and, in this way, errors arising from systematic sampling were eliminated.

Morphology and morphometry of Remak bundles. To assess the impact of ventral rhizotomy on Remak bundle structure in the peripheral nerve distal to the sciatic nerve, three preterminal branches of the tibial nerve in the hindpaw were dissected at the level of the tarsal bones: the lateral plantar, middle plantar (a mixed nerve between the third and fourth digits), and medial plantar branches (Li et al., 2000). These nerve branches are anatomically stable and yet small enough that all of the unmyelinated axons in the nerve can be counted, avoiding sampling problems. Most important to this work, these three nerves represent a sampling of the L4 and L5 dermatomes: in the lateral plantar branch, L5 axons predominate, and, in the medial branch, L4 axons predominate; the middle plantar branch receives nearly equal contributions from L4 and L5 axons. This study focused on the events in the lateral plantar nerve because this branch is most affected by L5 ventral rhizotomy.

For this study, entire plantar nerves were studied quantitatively in transverse section using electron microscopy. All unmyelinated axons and all nucleated cells in these nerves were included in the analysis. A Remak bundle was defined as an assembly of one or more unmyelinated axons associated with a nonmyelinating Schwann cell and contained within a continuous basal lamina. The number of axons in each Remak bundle was determined by direct observation of the material with the electron microscope at 50,000 $\times$ magnification. For each plantar nerve, the number of axons in the Remak bundles was represented in a histogram; the mean, median, and SD of the number of axons per Remak bundle was computed (Murinson and Griffin, 2004). Myelinated axons were enumerated using Nikon (Tokyo, Japan) NH2-B.

The cell profiles containing a nucleus in the nerve were evaluated using the criteria described above and classified as myelinating Schwann cell (intact or degenerating axon, MSC), Remak Schwann cell, endoneurial cell, or endothelial cell. Every cell containing a nucleus within the nerve transverse sections was classified by cell type. The number of nuclei associated with each cell type was normalized by the number of unmyelinated axons in the nerve for comparison of the three branches. After ventral rhizotomy, the cellular composition changed systematically across the three nerve branches, and this was evaluated using an ANOVA (regression of two populations, comparison of slopes) (Dixon and Massey, 1983).

Immunocytochemistry. Three animals underwent L5 ventral rhizotomy, as described above. Eight days later, these animals were deeply anesthetized and perfused with PBS, followed by 4% paraformaldehyde in Sorenson's buffer. After 2 h of postfixation, the sciatic nerves were transferred to graded sucrose solutions overnight, and 50 μ m sections were prepared with a freezing sliding microtome (Microm, Walldorf, Germany). Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using the DeadEnd Fluorometric System (Promega, Madison, WI) modified for free-floating sections, and immunocytochemical staining was performed immediately after the TUNEL staining procedure. Briefly nerve sections were washed twice in PBS, incubated with blocking buffer [5% normal goat serum (Vector Laboratories, Burlingame, CA), 0.1% Tween 20 (Sigma, St. Louis, MO), and 0.1% Triton X-100 (Sigma)] for 1 h, and then transferred to antibody solution (1% NGS, 0.1% Tween 20, and 0.1% Triton X-100) with primary antibody for 4°C overnight. After rinsing two times with antibody solution, the sections were incubated with fluorescence-conjugated secondary antibody or fluorescence-conjugated streptavidin as appropriate in antibody solution for 1 h at room temperature before rinsing and mounting with 4',6-diamidino-2-phenylindole-containing mounting

media (Vector Laboratories). Reagents were used at the following dilutions: biotinylated ED1 (Serotec, Oxford, UK), 1:300; biotinylated ED2 (Serotec), 1:300; rabbit anti-PPGP9.5 (protein gene product 9.5) (Biogenesis, Poole, UK) 1:750; Cy3 goat anti-rabbit, 1:300 (Amersham Bioscience); and Cy3 streptavidin (Jackson ImmunoResearch, West Grove, PA), 1:300. Slides were viewed and quantitated using a Nikon E600 and photographed with a Zeiss (Oberkochen, Germany) LSM510 meta confocal microscope.

Results

The effects of ventral rhizotomy

Examination of the mixed spinal roots immediately distal to the DRG confirmed that Wallerian degeneration was limited to axons arising from the ventral root (Fig. 1A). Assessment of the ventral root in normal animals and in sham-operated control animals confirmed that unmyelinated axons were rare in the L4 and L5 ventral roots (Coggeshall et al., 1977). Transection of these roots resulted in the degeneration of approximately one-third of the myelinated axons in the sciatic nerve. No evidence of unmyelinated axon degeneration was observed, as detailed below. The distribution of degenerating myelinated axons in the sciatic nerve was non-uniform (Fig. 1B) (Montoya et al., 2002) so that there were islands of degenerating myelinated fibers in transverse sections, but, within and around these islands of degenerating motor nerve fibers, there were intact nerve fibers.

Transection of the L5 ventral root alone produced a pattern of Wallerian degeneration in the distal hindlimb consistent with the established myotomal and dermatomal anatomy. One week after L5 ventral rhizotomy, the lateral plantar nerve branch showed a reduction in the number of myelinated axons; myelinated fibers undergoing Wallerian degeneration were a prominent feature (Fig. 1C). The other two plantar nerve branches demonstrated myelinated fiber loss reflecting the relative contribution of L5 axons to those nerves: the middle plantar nerve was modestly affected, and the medial plantar nerve was minimally affected. These experiments were conducted in parallel to behavioral and electrophysiological studies on the effects of L5 ventral rhizotomy that were reported previously (Sheth et al., 2002; Wu et al., 2002). The spontaneous behavior of the animals in this study, e.g., mild weakness of hindlimb flexion, matched that seen in the parallel studies.

Incorporation of [³H]thymidine

After L4/5 ventral rhizotomy, incorporation of [³H]thymidine into the sciatic nerve increased dramatically in the first 3 d, as shown in Figure 2. [³H]thymidine incorporation increased successively from day 1 to day 4, and, on day 4, the number of cells incorporating [³H]thymidine was 30-fold above the mean control level. By day 7 after rhizotomy, the incorporation of label fell to one-half of the day 4 level. The incorporation on day 14 remained sixfold above the mean control level, indicating that increased [³H]thymidine incorporation was sustained for at least 2 weeks. In the control nerve, administration of [³H]thymidine labeled a number of cells in the whole sciatic nerve cross-section, ranging from 1 to 17, with a mean of 8 and an SD of 6.

Classification of cell types in the normal nerve

Cells in the endoneurium that contained a nucleus at the level of cross section were classified by morphologic characteristics at the EM level. Five categories were defined: MSC-I, MSC-D, RSC (all of which were innervated), endothelial cells, and endoneurial cells, including both fibroblasts and macrophages (Fig. 3). In the intact sciatic nerve, MSC-I represented 50% of the nucleated cells

Figure 1. Appearance axons in the rat lumbar outflow 7 d after ventral rhizotomy. *A*, Transverse section through the L5 DRG demonstrating Wallerian degeneration of axons in the ventral root; all of the axons in this root were involved. The axons in the dorsal root section (left portion of figure, asterisk) were unaffected. *B*, Transverse section of the sciatic nerve 7 d after L4 and L5 ventral rhizotomy demonstrating numerous myelin ovoids, increased nuclear figures, and minimal edema throughout the nerve. The degenerating fibers were scattered in islands but were in close juxtaposition to normal axons. The majority of myelinated fibers were normal in appearance. *C*, Lateral plantar nerve 7 d after L5 ventral rhizotomy showed decreased density of myelinated axons, some myelin ovoids, and an increased number of nuclear figures. Axons undergoing degeneration were scattered throughout the nerve (black arrowheads). The majority of myelinated axons were normal in appearance. One micrometer plastic sections were stained with toluidine blue. Scale bars: *A*, *B*, 100 μ m; *C*, 12 μ m.

in the endoneurium, RSCs 25%, endoneurial cells 18%, and endothelial cells 7%. In the plantar nerves of control animals, MSCs represented 35% of the nucleated cells in the endoneurium, RSCs 28%, endoneurial cells 27%, and endothelial cells 9%.

Figure 2. The number of [3 H]thymidine-labeled cells in the sciatic nerve at intervals after L4/5 ventral rhizotomy. The number of cells with label on the operated side is shown in light gray, and the number on the contralateral side is shown in dark gray. The number of cells incorporating [3 H]thymidine increased dramatically over the first several days and did not return to baseline 2 weeks after surgery. The values on the side contralateral to surgery were indistinguishable from control. The data are from animals labeled with schedule I (see Materials and Methods).

Identification of cell types that incorporated [3 H]thymidine

Individual cells incorporating [3 H]thymidine identified by post-autoradiography electron microscopy are illustrated for each cell type in Figure 3. The incorporation of [3 H]thymidine into cells of MSC-I, MSC-D, RSC, endoneurial, and endothelial type was assessed under control and post-rhizotomy conditions on days 3 and 10. Under control conditions, MSC-I did not incorporate [3 H]thymidine, and MSC-D were not seen. RSCs, endoneurial cells, and endothelial cells only rarely incorporated [3 H]thymidine. On day 3 after ventral rhizotomy, [3 H]thymidine incorporation increased dramatically in all cell types except the MSC-I, in which incorporation was never seen.

MSC-D accounted for all of the [3 H]thymidine incorporation by the myelin-associated Schwann cell population (no incorporation by MSC-I). RSCs incorporated [3 H]thymidine vigorously at 3 d, a greater than 30-fold increase over baseline, with a lower level on day 10. Endoneurial cells incorporated [3 H]thymidine at a rate >30-fold above baseline and continued to incorporate [3 H]thymidine on day 10, albeit at much lower levels. Endothelial cells displayed a modest increase in the incorporation of [3 H]thymidine, which was not sustained at 10 d.

Proliferation index

Interpretation of [3 H]thymidine incorporation into specific cell types necessitated consideration of the rate of incorporation relative to the number of that cell type present. We estimated the proliferation index as described in Materials and Methods. The RSCs had a proliferation index after ventral rhizotomy approaching that of the MSC-D (Fig. 4). The proliferation index of endoneurial cells also increased after rhizotomy. The proliferation index of endothelial cells was highest at baseline relative to the other cell types and increased modestly after rhizotomy.

Identification of mitotic figures

Schwann cells undergoing mitosis were identified in the sciatic nerve after L4/5 ventral rhizotomy. These cells were identified by characteristic condensation of chromatin and complete absence of a nuclear membrane. For the myelinating Schwann cells, mitosis was only seen in cells associated with degenerating axons, shown in Figure 5A. In contrast in the Remak Schwann cells,

Figure 3. Typical electron micrographic appearance for each of the four cell types: MSC-D, the endoneurial cell, the endothelial cell, and the Remak bundle Schwann cell. The cells shown here were labeled by [^3H]thymidine in rat sciatic nerve 3 d after ventral rhizotomy. In each panel, an inset image shows a light autoradiograph of the same cell that is shown in the electron micrograph, confirming [^3H]thymidine uptake. *A*, MSC-D seen at the level of the perikaryon of a degenerating myelinated fiber. Scale bar, 1 μm . The labeling of this nucleus by [^3H]thymidine is shown in the bottom right inset. Scale bar, 5 μm . This transverse section is taken through the nucleus and corresponds to a level indicated by the arrow in the teased fiber preparation in the bottom left inset. Scale bar, 3 μm . This illustrates the appearance of degenerating fibers in which the myelin debris has been displaced from the perikaryal region. *B*, Labeled endoneurial fibroblast. Scale bars: *B*, 1.5 μm ; inset, 7 μm . The endoneurial cell class also included macrophages (example not shown). *C*, Labeled endothelial cell. Scale bars: *C*, 1.5 μm ; inset, 7 μm . *D*, Labeled RSC. The nucleus that has incorporated label has a normal appearance of the Schwann cell during this premitotic phase. Scale bars: *D*, 1.5 μm ; inset, 7 μm .

mitosis occurred even as the cells continued to ensheath a full complement of axons (Fig. 5*B*).

Quantitation of nonmyelinating Schwann cells and unmyelinated axons after rhizotomy

The number of unmyelinated axons in the lateral plantar nerve ranged from 283 to 587 under control conditions. The number of myelinated axons ranged from 143 to 285. In the plantar nerve branches, the number of myelinated axons was highly correlated with the number of unmyelinated axons in the nerves ($r = 0.97$). The number of unmyelinated axons in the lateral plantar nerve was not reduced by ventral rhizotomy, a result that was expected because there are so few unmyelinated axons in the L5 ventral

root in rat. We quantitatively evaluated the RSCs and unmyelinated axons of the lateral plantar nerve 7 d after ventral rhizotomy.

Because RSCs rapidly incorporated [^3H]thymidine in the first several days after ventral rhizotomy, we hypothesized that the division of Schwann cells could result in an increased number of Remak bundles, each with a smaller number of axons, or could result in an unchanged number of axons per Remak bundle, with an increased proportion of Remak bundles with nuclei (if the Schwann cells were separating along the axis of the nerve). We found that the total number of Remak bundles in the nerve was unchanged, as was the quantitative relationship between axons and RSCs. The mean number of axons per bundle was 3.37–4.57 under control conditions and 3.65–4.75 after rhizotomy. The number of axons in individual Remak bundles ranged from 1 to 13 in both control and ventral rhizotomy conditions. The distribution of axons into Remak bundles did not change quantitatively after L5 ventral rhizotomy, as measured by comparison of the histogram of axons per Remak bundle, by mean, or by range. The number of Schwann cell nuclei associated with Remak bundles showed a statistically insignificant increase after ventral rhizotomy (0.048 ± 0.02 vs 0.063 ± 0.01).

To assess the effects of ventral rhizotomy on various cell types, the nucleated cells of the lateral plantar nerve were classified by cell type, using the same criteria applied to the sciatic nerve. In control animals, there was some variation in the cellular composition of each nerve branch, but no discernible trend was observed. In the control lateral plantar nerve, MSC nuclei were present at 18 per 1000 unmyelinated axons, RSC nuclei were present at 21 per 1000 unmyelinated axons, and endoneurial cells were present at 15 per 1000 unmyelinated axons. After L5 ventral rhizotomy, the number of endoneurial cells in the lateral plantar nerve was nearly

twice the number in the control lateral plantar nerve; 27 versus 15 per 1000 unmyelinated axons. Regression analysis showed that the number of endoneurial cells in the lateral nerve branch was significantly increased 1 week after ventral rhizotomy (ANOVA; $p < 0.01$). The changes in cell types for the control and ventral rhizotomy lateral plantar nerve are shown in Figure 6. The increase in MSCs did not reach significance. The population of RSCs was stable in size.

Assessment of apoptosis after ventral rhizotomy

Fluorescence immunochemistry of the sciatic nerve 8 d after L5 ventral rhizotomy demonstrated several important changes. Consistent with our other results, the overall number of nuclei

Figure 4. Proliferation index of the different cell classes in the sciatic nerve 3 d after ventral rhizotomy. There was no proliferation in MSC-I. The MSC-D and the endoneurial cells (EN) attained a high rate of [3 H]thymidine incorporation, the RSC showed a substantial increase in rate of proliferation after ventral rhizotomy, and the endothelial cell population (ET) showed a minimal increase. The proliferation indices are shown for contralateral nerve (dark gray) and for post-rhizotomy (light gray).

increased by 70%. The TUNEL-positive nuclei increased nearly fivefold so that 13% of nuclei in the sciatic nerve were TUNEL positive on the 8th postoperative day. The majority of these were nuclei of cells positive for macrophage markers. Because it is likely that cell-specific markers are downregulated by proliferating RSCs, this population was identified through association with bundles of small PGP9.5-positive axons. TUNEL-positive cells associated with PGP9.5-positive axons were present in the nerve but were much less frequent than TUNEL-positive macrophages.

Discussion

Normally innervated Remak Schwann cells enter the cell cycle after ventral rhizotomy

Wallerian degeneration of myelinated nerve fibers promptly induced a variety of cell types in the nerve to enter into the cell cycle. Within 3 d, the Schwann cells of uninjured Remak bundles reached a high proliferative index, almost equal to that of the Schwann cells associated with degenerating myelinated fibers. The proliferation of the Remak Schwann cells in the absence of unmyelinated axon degeneration implicates a diffusible Schwann cell mitogen arising directly or indirectly from degeneration of neighboring myelinated fibers. Previous studies of nerves undergoing demyelination reached similar conclusions. In the models of lysophosphatidyl choline application (Griffin et al., 1990) and 3,3'-iminodipropionitrile (Griffin et al., 1987) administration, internodal and paranodal demyelination, respectively, were associated with entry of RSCs into the cell cycle. In those models, it was difficult to exclude a degree of injury to the axons of unmyelinated fibers. In the present model, the near absence of Remak bundles in the L5 ventral roots precludes direct injury to these fibers.

The nature of the diffusible mitogenic stimulus is speculative. It could represent diffusion of neuregulins normally bound to the proteoglycans in the axolemma of the myelinated motor axons and released by breakdown of the axolemma during degeneration of these fibers. An important caveat is that neuregulin signaling

Figure 5. Electron micrographs of mitotic Schwann cells in sciatic nerve after L4/5 ventral rhizotomy. *A*, Myelinating Schwann cell associated with axon undergoing Wallerian degeneration is characterized by highly condensed chromatin and the absence of a nuclear membrane. Scale bar, 3 μ m. *B*, In a Remak Schwann cell ensheathing 14 unmyelinated axons while undergoing mitosis, nuclear contents are within nanometers of the Schwann cell membrane. Scale bar, 1.2 μ m.

via erbB receptors may suppress proliferation of Remak Schwann cells in adult mice (Chen et al., 2003). This is in contrast to the strong mitogenic effects of neuregulins on Schwann cells during development and indicates that myelinating Schwann cells, Remak Schwann cells, and Schwann cell progenitors may have distinct responses to specific neuregulins. The observation that disruption of erbB signaling led to excessive proliferation of Remak

Figure 6. The number of cells of each type in the endoneurium of the lateral plantar nerve branch 7 d after L5 ventral rhizotomy. The columns are labeled as follows: MSC, myelinating Schwann cells; RSC, Remak (nonmyelinating) Schwann cells; EN, endoneurial cells. The data from control animals are shown as dark gray bars, and the ventral rhizotomy data are shown as light gray bars. The number of MSCs did not increase significantly. Despite evidence that ventral rhizotomy prompts vigorous proliferation among RSC, the number of these cells showed no significant increase. The numbers of endoneurial cells in the lateral plantar nerve branch doubled 1 week after L5 ventral rhizotomy.

Schwann cells (Chen et al., 2003) implies that there are juxtacrine, axon-derived signals inhibiting proliferation, but this cannot be invoked to explain our findings because the ventral rhizotomy spared unmyelinated axons from degenerating. Products released by the activated Schwann cells of the degenerating motor fibers could promote RSC proliferation. Activated Schwann cells in the denervated distal stump make a variety of cytokines, chemokines, and growth factors; these might, directly or indirectly, stimulate entry into the cell cycle by RSCs (Heumann et al., 1987; Lindholm et al., 1988; Lisak, 1989). It has been shown that neuregulin is upregulated in the distal nerve stump after sciatic axotomy (Carroll et al., 1997) and that Schwann cells and fibroblasts produce distinct forms of neuregulin (Rosenbaum et al., 1997). This raises the possibility of both paracrine and autocrine neuregulin signaling by Schwann cells (Rosenbaum et al., 1997). The explosive increase in Schwann cell proliferation between 2 and 3 d after ventral rhizotomy certainly favors an autocrine (positive feedback) mechanism, but the intimate comingling of myelinated and unmyelinated axons in peripheral nerve makes it difficult to distinguish autocrine, paracrine, and juxtacrine signaling pathways *in vivo*. By 48 h after axotomy, circulating monocytes have entered the degenerating nerve, facilitated by MCP-1 (monocyte chemoattractant protein-1) elaborated by the denervated Schwann cells of degenerating myelinated fibers (Toews et al., 1998; Taskinen and Roytta, 2000). The products of these macrophages might be mitogenic (Mason et al., 2003). We demonstrate that there was a large increase in endoneurial cells after L4/5 ventral rhizotomy. Based on immunochemical staining, many of these were macrophages. The blood–nerve barrier opens all along the distal stump of the nerve early after axotomy (Bouldin et al., 1990, 1991). Circulating materials could conceivably be the diffusible mitogens. Finally, in this model, the unmyelinated axons

are still connected to the cell bodies in the DRG so that the neurons themselves might express mitogens.

Normally innervated myelinating Schwann cells do not divide after ventral rhizotomy

The Schwann cells associated with normal myelinated nerve fibers of the sciatic nerve, representing predominantly sensory fibers, did not incorporate [^3H]thymidine after ventral rhizotomy. This observation contrasts with the proliferation of the Schwann cells of the degenerating motor fibers, which promptly entered S-phase, confirming observations in whole nerve transection studies (Bradley and Asbury, 1970; Oaklander et al., 1987b). This leads to the conclusion that Wallerian degeneration of a myelinated axon stimulates the proliferation of the Schwann cell myelinating that axon but cannot induce proliferation in those adjacent myelinating Schwann cells that are normally innervated. These cells cannot respond to the mitogen that drives RSCs. Even at baseline, Remak Schwann cells show low levels of DNA synthesis that are absent in myelinating Schwann cells, indicating that RSCs preserve the capacity for proliferation as part of their “differentiated” phenotype. In Figure 5B, we show a Remak Schwann cell undergoing mitosis. Interestingly, this cell maintains many of the cytoplasmic processes that wrap around unmyelinated axons even when the nuclear membrane has dissolved. Although the mechanisms that determine the ensheathment of axons by Remak Schwann cells are not fully understood, we interpret this to mean that the cell maintains at least some of its differentiated characteristics during proliferation.

Remak Schwann cells do not measurably increase in numbers

Several parameters were used to evaluate Remak Schwann cell numbers: the number of RSC nuclei per nerve, number of RSC nuclei per bundle, number of Remak bundles per nerve, and number of axons per Remak bundle. Multiple approaches were used to achieve greater sensitivity to change. As RSC nuclei separate after mitosis, it is possible that they migrate apart along the nerve axis, thereby increasing the number of nuclei per Remak bundle (Peyronnard et al., 1973). Observations of nonmyelinating Schwann cell division *in vitro* indicated that this is the typical pattern of cell separation in cultured cells (Fernandez-Valle et al., 1995). An alternative is that the separation of daughter Schwann cells occurs in such a way as to create new Remak bundles, side-by-side division. To assess this possibility, we counted the number of Remak bundles in the nerves after ventral rhizotomy and the number of axons per Remak bundle. This type of division would be expected to produce an increased number of Remak bundles, each with a reduced number of axons.

The only measure that showed any indication of variation was the number of nuclei per Remak bundle, a measure of cell division and separation along the nerve axis, and that increase did not reach statistical significance. Possible alternative fates of RSCs incorporating [^3H]thymidine include the following: failure to complete cell division, cell death, entrance into the endoneurial space, and/or migration (Abercrombie et al., 1959). Widespread failure to complete cell division is unlikely, and our observation of mitotic figures in Remak Schwann cells argues against this (Fig. 5B). Cell death cannot be excluded, but we did not see EM features suggesting Schwann cell apoptosis. TUNEL staining showed that apoptotic nuclei, although prevalent in the nerve 8 d after ventral rhizotomy, were infrequently associated with bundles of small axons. Thus, apoptosis was very frequent among macrophages and present at low levels among Remak Schwann cells. The possibility that cell division results in one cell remaining

with the Remak bundle and the other leaving the bundle is consistent with conclusions from studies of Remak Schwann cell division during demyelination (Griffin et al., 1987, 1990). The Remak Schwann cell entering the endoneurial space unassociated with an axon might be difficult to distinguish from the Schwann cell of a Bungner band. In summary, despite vigorous [3 H]thymidine uptake, the number of nuclei associated with Remak bundles did not increase substantially, suggesting to us that RSC division may be asymmetric.

Relevance to Remak bundle responses in partial nerve injury models of neuropathic pain

Partial nerve injuries are of increasing interest because many forms result in hyperalgesia and neuropathic pain. Experimental manipulations used in pain research include transection of the mixed spinal nerve, dorsal root ganglionectomy, chronic constriction injury of the nerve, and ventral rhizotomy (Kim and Chung, 1992; Munger et al., 1992; Sheth et al., 2002). In each of these models, some of the axons in the peripheral nerve are undergoing Wallerian degeneration, whereas other axons remain intact. Recent data indicates that degeneration of the L5 ventral root fibers in the sciatic nerve induces spontaneous electrical activity in the intact L4 C-fibers (Wu et al., 2002). This spontaneous activity originates in the distal region of these C-fibers and presumably reflects a change induced by the degeneration of neighboring fibers. In addition, L5 ventral rhizotomy induces persistent hyperalgesia similar to that seen after mixed spinal nerve transection, indicating that degeneration of myelinated motor axons is a sufficient stimulus for behavioral change (Sheth et al., 2002).

Our study indicates that the Schwann cells of the Remak bundle respond actively to degeneration of neighboring myelinated fibers, reflected by their entry into the cell cycle. Whether RSC mitosis has a direct effect on axons-of-passage remains to be explored. Thus, alternative explanations for spontaneous activity in the intact C-fibers might include interruption of neurotrophic support from RSCs undergoing mitosis as well as the production of new synthetic products by Remak Schwann cells and other cells in the endoneurial space. Tumor necrosis factor α has been postulated to contribute to spontaneous C-fiber activity, and NGF has been thought to contribute to the development of peripheral and central sensitization. Both might be potential products of the responsive Remak Schwann cell.

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