Proliferating Immature Schwann Cells Contribute to Nerve Regeneration After Ischemic Peripheral Nerve Injury

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Abstract
Schwann cells exhibit a high degree of plasticity in adult peripheral nerves after mechanical injury; they have, therefore, been implicated in promoting nerve regeneration. However, Schwann cell behavior after ischemic injury has not yet been elucidated. To determine how Schwann cell plasticity may contribute to recovery from ischemic neuropathy, we used a rat model in which ischemia was induced in the tibial nerve by a 5-hour occlusion of the supplying arteries. Proliferation of immature Schwann cells that emerged in the injured nerve was evaluated by double immunostaining for the p75 neurotrophin receptor and proliferating cell nuclear antigen. The number of proliferating cell nuclear antigen/p75 neurotrophin receptor double-positive cells increased significantly in 1 to 2 weeks after ischemia and subsequently decreased by 4 weeks. During this time, the postmitotic Schwann cells differentiated into mature cells, as demonstrated with bromodeoxyuridine incorporation, which facilitated axon guidance and subsequent axon remyelination. These results suggest the emergence and proliferation of immature Schwann cells that contribute to nerve regeneration after ischemic injury. The manipulation of this population of proliferating immature Schwann cells may be a useful strategy for treating ischemic peripheral neuropathy.

Key Words: Cell dedifferentiation, Cell division, Myelin, Nerve regeneration, Neuroglia, Neurotrophin receptor p75, Peripheral nerve ischemia, Schwann cells.

INTRODUCTION
Ischemic peripheral neuropathy is a consequence of interruption of the blood supply resulting in axon disruption (1). Inadequate regeneration then results in irreversible neurologic dysfunction of the extremities. Nerve ischemia related to vascular abnormalities occurs in a variety of pathologic conditions. For example, capillary basement membrane thickening and endothelial cell hyperplasia are well-recognized components of the pathogenesis of diabetic neuropathy (2). Ischemic neuropathy associated with primary vasculitides and collagen vascular diseases is caused by occlusion of blood vessels due to inflammation in the walls of the vasa nervorum (3), and iatrogenic arterial occlusion after surgical procedures may result in ischemic monomelic neuropathy and critical limb ischemia (4). Thus, the pathophysiology of ischemic neuropathy due to various acquired conditions needs to be elucidated. Moreover, few specific therapeutic options have been established to alleviate ischemic nerve degeneration or to promote nerve regeneration in humans.

Animal models of ischemic neuropathy have provided insights into the mechanisms of nerve degeneration and regeneration after ischemic damage (5–18). After the arterial supply to the sciatic-tibial nerve is occluded in rodents, nerve fibers degenerate and subsequently regenerate, during which axon- and myelin-derived fragments are cleared and abundant regenerating thinly myelinated fibers subsequently emerge (12). Ischemic injury can also generate additional insults to both axons and Schwann cells through an inflammatory response and oxidative stress, resulting in apoptosis of Schwann cells (10, 11). However, it remains unknown whether changes in Schwann cell functions, including proliferation, differentiation, and remyelination, are related to the repair processes after nerve ischemia.

Mature Schwann cells retain a high degree of plasticity in adult peripheral nerves and have a crucial role in the repair of peripheral nerves after mechanical injuries such as nerve transection (axotomy) or crush injury. A sophisticated differentiation program, including proliferation, migration, upregulation of myelin proteins, and myelination of the axons, is required (19–23), but these phenotypic changes of Schwann cells after ischemic injury have not been well characterized. A member of the tumor necrosis factor receptor superfamily, p75NTR, is a low-affinity receptor of nerve growth factor and is highly expressed in immature Schwann cells (19). The major function of p75NTR in peripheral nerves is to myelinate axons via the myelin-promoting effect of brain-derived neurotrophic factor, a member of the neurotrophin family of growth factors that binds with low affinity to p75NTR (24–28). Thus, p75NTR can be used as a marker of dedifferentiated or immature Schwann cells, which are key to nerve regeneration (29).

In this study, we created a rat model of ischemic neuropathy and evaluated the temporal profile of morphologic changes of ischemic nerve degeneration and regeneration. Our results may provide new insight into the possible therapeutic strategies for the modification of the Schwann cell response to ischemic neuropathy.
MATERIALS AND METHODS

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Animal Experimental Committee of Tokyo Medical and Dental University. We minimized the number of animals and used anesthesia appropriately to minimize suffering. We used male Wistar rats (n = 48; Sankyo Laboratory Animal Center, Tokyo, Japan), 10 to 15 weeks of age that weighed 200 to 300 g.

Surgical Technique

A previously described ischemia-reperfusion model was slightly modified (5, 6). The right iliac and femoral vessels were exposed through inguinal incision. The right common iliac artery, external iliac artery, femoral artery, and superficial epigastric artery supplying the right sciatic-tibial nerve were occluded with mini vascular clips (AS-1, clipping power 40 g/mm²; Natsume Seisakusho Co, Ltd, Tokyo, Japan) under 3% isoflurane anesthesia. During the surgery, a deep rectal thermometer monitored body temperature, which was maintained at 36.5 ± 0.5°C with a heating pad. The total occlusion time was 5 hours; reperfusion was then achieved by removing the clips. The rats were observed until they were awake in their cages. They were divided into 5 groups with reperfusion durations of 1, 4, 7, 14, and 28 days. Sham-operated rats were operated on in the same manner, but with no occlusion of arteries, and were killed 7 days after the sham operation.

Evaluation of Blood Flow in Rat Hind Limb

A laser Doppler flow meter (TBF-LN1; Unique Medical Company, Tokyo, Japan) and a laser Doppler probe (Type-CS; Advance Co, Inc, Tokyo, Japan) were used to monitor relative changes in the blood flow in the hind limbs. The probe was placed on the plantar aspect of the ischemic hind limb. The rats were placed on a heating pad to maintain constant body temperature at 36.5 ± 0.5°C during the entire procedure. The measurement of blood flow in each rat was performed before and after occlusion by the clips, before and after removal of clips, and at 7 days after the ischemia (7 days after the surgery for sham-operated rats). The blood flow was expressed as milliliters of blood flow per 100 g of tissue per minute and was reported as a percentage of the normal value before the ischemic surgery.

Evaluation of Neurologic Function in the Ipsilateral Hind Limb

We used the neurologic score previously reported by Iida et al (9) (Table). In brief, scores were based on gait, paw position, grasp, and pinch sensitivity, and the function was assessed by the total score of these 4 indices, ranging from 0 to 11. A larger score indicated better function.

5-Bromo-2′-deoxyuridine-5′-monophosphate Labeling

To label mitotic cells, 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU), a thymidine analog incorporated into the DNA of dividing (S-phase) cells, was administered intraperitoneally (50 mg/kg body weight; Sigma-Aldrich, St Louis, MO). We gave injections of BrdU once daily from Day 5 to Day 9 after surgery, which allowed us to measure the number of cells that incorporated BrdU during the 5 days. Thereafter, cellular profiles of postmitotic cells were quantified at 14 or 28 days after the ischemia.

Tissue Preparation

At 1, 4, 7, 14, and 28 days after ischemia, the rats were fixed by transcardiac perfusion with 4% paraformaldehyde under deep anesthesia (Fig. 1). The sciatic nerve in a rat divides into the tibial, peroneal, and sural nerves at the knee level. The tibial nerve splits into medial and lateral plantar nerves at the ankle level. The mid and distal nerve segments of the tibial nerve were harvested at 1, 4, 7, 14, and 28 days after ischemic injury for pathologic examination.

### TABLE. Neurologic Scoring of Hind Limb Sensorimotor Function

<table>
<thead>
<tr>
<th>Score</th>
<th>Gait</th>
<th>Paw Position</th>
<th>Grasp</th>
<th>Pinch Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No function: drags limb, rigid hip, and knee</td>
<td>Contracted: no ankle or foot flexion</td>
<td>No grasp</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>Very impaired: minimal use of limb, rigid knee</td>
<td>Very impaired: curled digits</td>
<td>Very impaired</td>
<td>Slight</td>
</tr>
<tr>
<td>2</td>
<td>Slightly impaired: limb, toe-to-heel impairment</td>
<td>Slightly impaired: no toe spread</td>
<td>Slightly impaired</td>
<td>Present</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>Normal: full toe spread</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Schematic diagram of the rat tibial nerve, with the harvest site and times of harvest indicated. The sciatic nerve divides into the tibial, peroneal, and sural nerves at the knee level. The tibial nerve splits into medial and lateral plantar nerves at the ankle level. The mid and distal nerve segments of the tibial nerve were harvested at 1, 4, 7, 14, and 28 days after ischemic injury for pathologic examination.
to 3 small branches that innervate calf muscles at the upper calf level, and then splits into 2 terminal branches, the medial plantar and lateral plantar nerves, at the ankle level (30). The length of the nerve segment was dissected from the mid calf level distal to these small branches to the ankle level and was divided into mid and distal tibial nerve segments (Fig. 1). For semithin sections, these sections were fixed for 30 minutes in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer and then osmicated, dehydrated, dehydrated, and embedded in epoxy resin. One-micrometer-thick transverse sections were stained with 1% toluidine blue. For immunohistochemistry, the semi-thin sections, these segments were fixed for 30 minutes in 4% paraformaldehyde for 6 hours, incubated in 20% sucrose for 48 hours, and embedded in ice-cold OCT. Ten-micrometer-thick sections were obtained with a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and collected on gelatin-coated glass slides.

**Immunohistochemistry**

The primary antibodies used for the immunohistochemical staining were as follows: mouse anti-BrdU (1:100; Millipore, Billerica, MA), mouse anti-rat proliferating cell nuclear antigen (PCNA; 1:2000; Cell Signaling Technology, Danvers, MA) for detection of mitotic cells, rabbit anti-S100β (1:100; Abcam, Cambridge, MA) for detection of Schwann cells, rabbit anti-p75NTR (1:100; Millipore, Temecula, CA) for detection of immature Schwann cells, rat anti-myelin basic protein (MBP; 1:200; Abcam) for detection of myelinating Schwann cells, and mouse anti-SMI31 antibody (1:5000; Covance, Berkeley, CA) for detection of axons.

Single immunostaining was performed to evaluate S100β-positive Schwann cells. The 10-μm-thick sections were washed with PBS 3 times and blocked with 10% normal goat serum in PBS, followed by overnight incubation at 4°C in primary antibodies. After 3 washes in PBS, the sections were further incubated with a biotinylated anti-rabbit immunoglobulin G (1:200; Vector Laboratories, Burlingame, CA) at room temperature for 1 hour and then with streptavidin-biotin/ horseradish peroxidase complex (Vector Laboratories) for 30 minutes. Immunoreactivity was visualized with 3,3′-diaminobenzidine (Sigma-Aldrich).

To quantify cellular proliferation or myelination in immature Schwann cells, double immunohistochemical staining for p75NTR and PCNA, BrdU, or MBP was conducted. Immunostaining of PCNA required antigen retrieval by heating in 10 mmol/L citrate buffer, pH 6, in a microwave oven (5 × 5 minutes, 750 W). Immunostaining of BrdU required DNA denaturing. The 10-μm-thick sections were rinsed in PBS containing 0.1% Triton X-100 3 times for 5 minutes each at room temperature for permeabilization and then treated for 30 minutes with 2N HCl at 38°C. After the antigen retrieval for PCNA or the DNA denaturing for BrdU staining, the sections were rinsed in PBS containing 0.3% Triton X-100 for 30 minutes, preincubated with 10% normal goat serum, and then incubated with the 2 primary antibodies diluted with 10% normal goat serum overnight. Subsequently, sections were incubated in fluorescently labeled secondary antibodies (fluorescein isothiocyanate/rhodamine, raised in goat; Millipore) for 1 hour. After washes in PBS, the sections were mounted with VECTASHIELD (Vector Laboratories) and observed under a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

**Morphometry of Myelinated Nerve Fiber Preservation**

We counted the number of myelinated axons in a randomly chosen field (0.090 mm²) in the mid tibial nerve segment in each rat at a magnification of 400× in transverse semithin sections on a light microscope coupled to a digital camera. We then calculated the density of myelinated nerve fibers (per squared millimeter).

**Cell Quantification**

To estimate the number of crescent-shaped S100β-positive cells, a single-stained transverse section from the mid tibial nerve segment was randomly chosen for each rat. These cells were examined by using light microscopy with a 40× objective and counted manually in the whole fascicle in each section.

To estimate the number of PCNA- or BrdU-positive Schwann cells, a double-stained transverse section of the distal tibial nerve segment was randomly chosen for each rat. Colocalization of PCNA and p75NTR or of BrdU and p75NTR was examined using a confocal laser scanning microscope with a 40× objective and was verified by scanning the cell in its entirety within the section by focusing through the z axis. The distal segment is composed of 2 comparably sized fascicles that bifurcate into the medial and lateral plantar nerves; the number of double-positive cells was counted manually in the 2 fascicles in each section and reported as the number per fascicle.

**Statistical Analyses**

Unpaired Student t-test or one-way analysis of variance with post hoc Bonferroni/Dunn test was used to analyze most data. Repeated-measures analysis of variance with post hoc Bonferroni/Dunn test was used to analyze the data of the blood flow in the hind limb. The threshold for statistical significance was set at p < 0.05. All values are mean ± SEM.

**RESULTS**

**Changes of Blood Flow in the Hind Limb After Induction of Ischemia in the Experimental Rats**

The mean percent blood flow in the hind limb relative to the preoperative baseline was examined after occlusion, before the release of occlusion, and at 5 minutes after the release of occlusion in the ischemic rats (n = 8) and was compared with the blood flow in sham-operated rats (n = 8). In the sham-operated rats, the hind limb blood flow (LBF) transiently decreased to 45.3% ± 11.1% of baseline after the exposure of the vessels, recovered to 113.6% ± 12.8% of baseline before the incision was closed, and was 101.7% ± 11.2% after the sham operation was complete (Fig. 2A). Limb blood flow was maintained at 114.0% ± 12.9% at 7 days after the sham operation. In the ischemic rats, the ipsilateral LBF was significantly decreased during the 5 hours of ischemia to 6.7% ± 1.2% of baseline immediately after occlusion (p < 0.01 vs sham-operated rats) and to 10.3% ± 12.2% before occlusion.
release (p < 0.01 vs sham-operated rats). Limb blood flow had recovered to 86.3% ± 11.9% of baseline at 5 minutes after release and was 133.2% ± 17.2% at Day 7 after ischemia (Fig. 2A).

Functional Recovery of the Ipsilateral Hind Limb After Ischemia

At 1, 4, and 7 days after the sham operations, all sham-operated rats showed a maximal neurologic score of 11 (Fig. 2B). In ischemic rats, the scores were markedly decreased to 2.9% ± 0.4% at Day 1 after the ischemia. The neurologic scores gradually recovered and reached 10.4% ± 0.3% at Day 28, suggesting that the nerve functions of the ischemic hind limb had been restored within 1 month after reperfusion.

Temporal Profile of Morphologic Changes of the Tibial Nerve in the Ischemic Hind Limb

Semithin transverse sections of the mid tibial nerves showed myelinated fibers with normal morphology in sham-operated rats (Fig. 3A). In ischemic rats, 1 and 4 days after the ischemia, myelinated fiber densities were not significantly decreased (Day 1, 14,994 ± 1,164/mm², n = 4; Day 4, 13,034 ± 1,002/mm², n = 4) versus those of sham-operated rats (14,563 ± 949/mm², n = 4). At Day 1 after ischemia, the number of myelinated nerve fibers was preserved, but some myelinated fibers showed “empty” or “dark” axons, similar to the morphologic changes in ischemic axons (31) (Fig. 3B). At Day 4, a few myelin ovoids appeared (Fig. 3C). At Day 7, the injury was characterized by abundant myelin degradation products and ovoids, indicating progressive axonal degeneration (Fig. 3D). Along with these findings of axonal degeneration, some crescent-shaped swollen cells were located prominently around intact axons at 7 days (Fig. 3D). At

FIGURE 2. Changes of blood flow and neurologic score in the hind limb after ischemic surgery in rats. (A) The blood flow in the hind limb during occlusion of vessels decreased to less than 5% of the baseline value, a significantly greater reduction than after sham operation (group effect, F1,14 = 13.8, p = 0.0023; group × trial interaction, F3, 42 = 13.0, p < 0.0001). After release of the occlusion, the blood flow returned to the baseline level. The blood flows are expressed as a percentage of the preoperative values (% blood flow), n = 8 for each group; *, p < 0.01 versus the sham-operated rats by analysis of variance for repeated measures followed by the Bonferroni post hoc test. (B) Neurologic scores in ischemic rats gradually increased and reached almost the maximal score of 11 after ischemic surgery. Sham controls maintained maximal neurologic scores for 7 days. Data represent mean ± SEM.

FIGURE 3. Temporal profile of morphologic changes of the tibial nerve after ischemia. (A–F) Toluidine blue–stained semithin transverse sections show normal structures of myelinated nerve fibers in a sham-operated rat (A); the degeneration phase of nerve fibers at Day 1 (B), at Day 4 (C), and at Day 7 (D) and the regeneration phase of nerve fibers at Day 14 (E) and at Day 28 (F) after ischemia. At Day 1 (B), some myelinated fibers showed “dark” axons (red arrows) or “empty” axons (red arrowheads). At Day 4 (C), there are a few myelin ovoids (yellow arrowheads). At Day 7 (D), there are more numerous myelin degradation products and ovoids (yellow arrowheads). Note the crescent-shaped swollen Schwann cells around morphologically intact axons (yellow arrows). At Day 14 (E), there are abundant naked axons. At Day 28 (F), there are many thinly myelinated fibers. (G) Time course of myelinated fiber density in the tibial nerve after ischemia compared with that of sham-operated rats (Control). Myelinated fiber density decreased at Days 7 and 14 and then recovered by Day 28. *, p < 0.01 for the indicated comparisons by one-way analysis of variance with the Bonferroni post hoc test. Scale bars = (A–F) 20 μm.
The numbers returned to the baseline level at Days 14 and 28 for controls (17.8 ± Day 7, 68.5 ± 2012 American Association of Neuropathologists, Inc. Copyright © 2012 by the American Association of Neuropathologists, Inc. Unauthorized reproduction of this article is prohibited.

**Temporal Profile of Crescent-Shaped S100β-Positive Schwann Cells After Ischemia**

Crescent-shaped cells around morphologically intact axons, which were characteristic structural changes seen in semithin sections (Fig. 3D), showed S100β positivity, indicating that they are Schwann cells (Figs. 4A, B). At Days 4 and 7 after the ischemia, the number of these cells was significantly increased (Day 4, 48.8 ± 5.0 per fascicle, n = 4; Day 7, 68.5 ± 6.7 per fascicle, n = 4) versus that of the sham controls (17.8 ± 3.1 per fascicle, n = 4, p < 0.01 for both days). The numbers returned to the baseline level at Days 14 and 28 (Fig. 4C).

**Emergence of Immature Schwann Cells and Their Proliferation After Ischemia**

Immature Schwann cells were detected by immunostaining for p75NTR. In sham-operated rats, there were few p75NTR-positive cells (Fig. 5A). After ischemia, the numbers of p75NTR-positive cells dramatically increased for 14 days (Fig. 5A). To quantify the proliferation of p75NTR-positive immature Schwann cells, we examined sections doubly stained with an anti-PCNA antibody and anti-p75NTR antibody. The number of PCNA-positive cells in ischemic rats significantly increased at Day 7 (177.8 ± 29.2 per fascicle, n = 4) and Day 14 (181.3 ± 58.1 per fascicle, n = 4) versus the controls (16.8 ± 2.7 per fascicle, n = 4, p < 0.01 for both days) and decreased to the level of the sham control at Day 28 (Fig. 5A). Many PCNA-positive cells were also positive for p75NTR (Fig. 5B); the numbers of these PCNA/p75NTR double-positive cells in ischemic rats was also significantly increased at Day 4 (37.5 ± 2.2 per fascicle, n = 4, p < 0.05), at Day 7 (80.8 ± 12.0 per fascicle, n = 4, p < 0.01), and at Day 14 (52.4 ± 3.5 per fascicle, n = 4, p < 0.01) versus numbers at Day 1 after the ischemia (13.5 ± 1.7 per fascicle, n = 4); the numbers decreased by Day 28 (14.0 ± 3.5 per fascicle, n = 4; Fig. 5C).

**Immmature Schwann Cells Survive and Sort and Remyelinate Axons After Ischemia**

To investigate the fate of p75NTR-positive cells, we administered BrdU for 5 days (Fig. 6A) and examined the cells doubly labeled with an anti-BrdU antibody and an anti-p75NTR or anti-S100β antibody (Fig. 6B–D). BrdU-positive/p75NTR-positive cells were found at Day 14 (99.1 ± 27.0 per fascicle, n = 4) and at Day 28 (50.4 ± 13.3 per fascicle, n = 4; Fig. 6B). The mean number of these cells at Day 28 was approximately half that at Day 14 (Fig. 6C), although this difference was not significant (p = 0.156). We observed some BrdU-positive/S100β-positive cells at Day 28; the cytoplasm of these S100β-positive Schwann cells formed rings around individual axons (Fig. 6D). These results indicate that postmitotic immature Schwann cells survive and differentiate into mature cells by Day 28 after ischemia. To investigate whether immature Schwann cells contribute to axon regrowth and remyelination, we performed double labeling with the anti-p75NTR antibody and an anti-SMI31 or anti-MBP antibody at Days 14 and 28 after ischemia. We detected several SMI31-positive small-caliber axons engulfed by p75NTR-positive cytoplasm at Day 14; the p75NTR positivity seemed to disappear together with the emergence of an abundance of large-caliber axons at Day 28 (Fig. 7A). A few ring-shaped MBP-positive myelin formations surrounded by p75NTR-positive cells were detected at 14 days, and ring-shaped myelin formations without p75NTR positivity were abundant at Day 28 (Fig. 7B). These results suggest that immature Schwann cells sort and remyelinate axons after they have proliferated.

**DISCUSSION**

In this study, we have shown that Schwann cells undergo phenotypic changes during nerve degeneration and regeneration in a rat model of ischemic neuropathy. Our results indicate that there is a repair process in which immature Schwann cells emerge, proliferate, survive, and remyelinate axons after ischemic injury.

**Rat Model of Ischemic Neuropathy**

Mitsui et al (6) used a model of ischemic neuropathy in which the abdominal aorta, right iliac, and femoral arteries and all identifiable collateral vessels are ligated for 3 or 5 hours followed by reperfusion. Muthuraman et al (15) used a model in which only the femoral artery is clamped for 2 to 6 hours followed by reperfusion. The first method can
produce prominent axonal degeneration in the sciatic-tibial nerve, but it requires invasive intraperitoneal surgical techniques to clamp the aorta; the second method may not produce sufficient ischemia to result in severe axonal degeneration (15). Therefore, we established a less invasive method that does not require clamping the aorta but produces prominent axonal degeneration in the rat tibial nerve. In addition, we adopted a method of measuring blood flow in the plantar...

FIGURE 5. Emergence and disappearance of p75 neurotrophin receptor (p75NTR)-positive/proliferating cell nuclear antigen (PCNA)-positive cells in the tibial nerve after ischemia. (A) Low-magnification images of p75NTR (red in soma) and PCNA (green in nucleus) double-positive cells in representative rats at 1, 4, 7, 14, and 28 days after ischemia and in a sham-operated control. Note that double-positive cells peaked at Day 7 or 14 and disappeared by Day 28. (B) Orthogonal reconstruction from confocal Z-series shown as if viewed in the x-z (top) and y-z (right) planes, showing the colocalization of p75NTR (red in soma) and PCNA (green in nucleus) signals in the tibial at Day 7 after ischemia. (C) The number of p75NTR and PCNA double-positive cells after ischemia increased until Day 14 and then decreased. *, $p < 0.05$; **, $p < 0.01$ versus the sham-operated (Control) rats by one-way analysis of variance with the Bonferroni post hoc test. Scale bars = (A) 20 μm; (B) 10 μm.
aspect of the ischemic hind limb using a laser Doppler flow meter that provided a useful index of ischemia during surgical procedures.

Temporal Amelioration of Morphologic Changes and Neurologic Functions in Ischemic Neuropathy

The ischemic nerve fibers underwent axonal degeneration during the 2 weeks after the ischemic episode and subsequently underwent regeneration. Myelinated nerve fiber density decreased during the first 2 weeks and then recovered to normal levels by 4 weeks, although the myelin thickness of the regenerated fibers had not completely recovered that time. Neuronal functions were markedly decreased at Day 1 after the ischemia and then gradually recovered almost fully by 4 weeks. Thus, the improvement on the neurologic score and the amelioration of pathologic changes in our model are consistent with the improvements seen in the model described by Iida et al (10).

Crescent-Shaped Schwann Cells in the Early Stage After Ischemia

We detected the emergence of crescent-shaped Schwann cells surrounding intact axons in the first week after ischemia, preceding the maximum numbers of proliferating PCNA-positive/p75NTR-positive cells. The crescent-shaped Schwann cells were observed in only a subpopulation of normal myelinated fibers (Fig. 4A). Schwann cells in the mid part of the internode appear crescent shaped in the morphology of myelinated nerve fibers on cross sections (32). The increased numbers of these cells is likely due to Schwann cell proliferation after ischemic injury. These cells also appeared enlarged (Figs. 3D and 4B), although size measurements were not performed; they closely resemble cells in other rodent neuropathy models and human neuropathies (10, 33, 34). In another rodent ischemic neuropathy model, some cells positive for 8-hydroxydeoxyguanosine or terminal deoxynucleotidyl transferase dUTP nick end labeling had the appearance of hypertrophic Schwann cells, exhibiting crescent shapes and surrounding axons (10). Chronic nerve compression in a rodent model induced Schwann cell proliferation and concurrently produced morphologic changes in Schwann cells, including large vacuolar cytoplasmic inclusions and condensed chromatin, but axons and the myelin sheath appeared intact (33). These Schwann cell changes support the idea that the earliest

FIGURE 6. Dividing Schwann cells after ischemic injury survived and differentiated into mature cells. (A) Experimental scheme for labeling dividing cells. 5-Bromo-2-deoxyuridine-5'-monophosphate (BrdU) was injected intraperitoneally on Day 5 through Day 9; tibial nerves were harvested on Days 14 and 28. (B) Representative images of double staining with anti-BrdU (green) and p75 neurotrophin receptor (p75NTR) (red) antibodies in the tibial nerve at Days 14 and 28 after ischemia. (C) Quantification of BrdU-positive/p75NTR-positive cells after ischemia. The number of double-positive cells was larger at Day 14 than at Day 28, but this difference was not statistically significant by an unpaired Student t-test (p = 0.156). (D) Orthogonal reconstruction from a confocal Z-series represented as if viewed in the x-z (top) and y-z (right) planes showing the colocalization of S100β (red in soma) and BrdU (green in nucleus) signals in the tibial nerve at 4 weeks after ischemia. Note that the S100β-positive Schwann cell cytoplasm formed rings. Scale bars = (A) 20 μm; (B) 10 μm.

FIGURE 7. Immature Schwann cells contribute to axonal sprouting, regrowth, and remyelination during the 2 to 4 weeks after ischemia. (A) Double staining with anti-SMI31 and p75 neurotrophin receptor (p75NTR) antibodies shows small caliber axons engulfed by the p75NTR-positive cytoplasm of Schwann cells (arrows) at Day 14 and mature large-caliber axons without p75NTR positivity at Day 28. (B) Double staining with anti-myelin basic protein (MBP) and anti-p75NTR antibodies shows ring-shaped MBP positivity surrounded by p75NTR-positive cytoplasm (arrows) at 14 days and an abundance of ring-shaped MBP positivity without p75NTR positivity at Day 28. SMI31 (axons, green in A), MBP (myelin, green in B), p75NTR (red), and Hoechst (nucleus, light blue) images were obtained with confocal laser microscopy. Scale bars = (A) 10 μm; (B) 10 μm.
Immature Schwann Cells Proliferate After Ischemia

The numbers of p75NTR-positive/PCNA-positive cells increased 1 to 2 weeks after ischemia and then decreased until 4 weeks. In traumatic crush nerve injury or axotomy models, axons degenerate in nerve segments distal to the site of injury, and Schwann cells concurrently dedifferentiate, begin to proliferate and then act as guideposts for axonal regrowth (19–23, 35). In the earliest phase after such injuries, Schwann cells acquire the expression of surface molecules, including p75NTR, a marker of immature Schwann cells in developing nerves. By 5 to 7 days after injury, there is an initial wave of Schwann cell proliferation (35). As in the trauma models, we also observed that ischemic injury can promote p75NTR expression and Schwann cell proliferation.

Immature Schwann Cells Subsequently Differentiate into Mature Schwann Cells

We demonstrated an appreciable number of BrdU-positive/p75NTR-positive cells at 2 weeks and even at 4 weeks after ischemia, indicating that some postmitotic Schwann cells survived at 4 weeks, although some might have undergone cell death. We also detected BrdU-positive/S100β-positive cells with the morphology of myelinating Schwann cells at 4 weeks (36). We also demonstrated the presence of myelin or sprouting axons surrounded by immature Schwann cells at 2 weeks and an abundance of myelinated axons with diminished p75NTR positivity at 4 weeks. Thus, some of the proliferating immature Schwann cells differentiated into mature Schwann cells. Because p75NTR activation promotes the differentiation of Schwann cells into mature cells capable of remyelinating axons after crush injury (27, 37, 38), our findings indicate that similar events likely occur in our model of ischemic neuropathy.

In summary, we provide evidence that ischemia increases the number of immature Schwann cells in the peripheral nervous system. These immature cells first proliferate and then undergo differentiation into mature Schwann cells during the process of nerve repair. Therefore, the manipulation of proliferating, immature Schwann cells may provide a new therapeutic approach for the treatment of ischemic neuropathy.

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