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Miki, Akinori

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Regenerating Outgrowth of the Proximal Axon Stump itself of Transected Myelinated Fibers in the Mouse Sciatic Nerve

Akinori Miki

Regenerating outgrowth of the proximal axon stump itself of myelinated fibers was examined in the transected mouse sciatic nerve by electron microscopy and with silver impregnation method. Attention was paid to the importance of the myelin sheath removal for survival and outgrowth of the proximal axon stump itself. Within one to two days after transection, myelin sheath degradation due to the injury extensively occurred near the cut end. It appeared that Schwann cells and macrophages were involved in the progressive demyelination in the proximal stump. Proximal axon stumps which became free from tight myelin sheath covering exhibited no morphological features indicative of an extensive degeneration such as cytoautolysis or increase of autophagosomes in the axoplasm. This reveals that demyelinated axon stumps can retain their viability. Owing to demyelination, axon stumps can make direct contact with the cytoplasm and basal laminae of Schwann cells. We found that these naked parent axons grew out, emanated some sprouts and formed mini-neuromas in the connective tissue compartment. These findings suggest that demyelination might be an indispensable requisite for the survival and outgrowing of the parent axon stump itself.

Key Words

Axotomy,
Nerve regeneration,
Myelinated fibers,
Sciatic nerve,
Parent axon stump,

INTRODUCTION

In the myelinated fibers in the peripheral nervous system, regenerating axons emerge from the proximal segment of the injured nerve and actively grow distally. It is well known that regenerative sprouts form at the node of Ranvier close to the injury in the proximal stump of the

traumatized axons. Cajal (6) observed in detail this process, and called the node of Ranvier a generative zone. Though not clearly stated in the text, his drawings suggest that the proximal axon stump itself also can grow as a regenerating sprout. Thus, regenerating axons emerged from the proximal segment might be of mixed population consisting of those from node of Ranvier and those of the proximal stump itself. Emergence of regenerating sprouts at the node of Ranvier in the proximal segment of injured axons has been extensively investigated by electron microscopy (10, 16, 20, 21, 25, 36). To date, however, regenerating outgrowth of the parent axon stump itself has not yet been fully documented at the ultrastructural level. Thus in the present

Faculty of Health Science, Kobe University
School of Medicine, Kobe, Japan.

study, the possibility of regenerating outgrowth of the proximal axon stump itself was investigated in the transected mouse sciatic nerve.

Previously, we examined the early reaction to injury in the proximal stump of the transected myelinated fibers in the mouse sciatic nerve, and found that the traumatic degeneration mainly occurred in the axon stumps which were confined by myelin sheath (23). These findings suggest that regenerating axons cannot extend vigorously through the myelin sheath tubes. On the other hand, it was reported that regenerating axons can grow along the surface of Schwann cells extending through the Schwann cell columns (11, 24, 32) or along the inner surface of basal lamina tubes when Schwann cells are not available as scaffolds (14, 15, 16, 18, 19, 27, 28, 29). Bearing these findings in mind, in the present study, attention was paid to the significance of removal of myelin sheaths from the proximal stump. It was suggested that myelin sheath removal might produce a favorable environment for the survival and outgrowth of the proximal stump of the parent axon.

MATERIALS AND METHODS

Light and Electron microscopy

Twenty adult mice (ddY strain), weighing 35-40 g, were used in the present study. The animals were anesthetized by intraperitoneal injection of Nembutal (pentobarbital sodium 50 mg/kg body weight), the left sciatic nerve was exposed and transected with microdissection scissors at the mid-thigh level under an operation microscope. At various

periods of 24, 32 and 40 hours, 2, 3 and 5 days after transection, these animals (3 or 4 animals at each period) were sacrificed by intracardiac perfusion initially with physiological saline followed by a fixative containing 2.0% paraformaldehyde and 2.5% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) under the anesthesia described above. Immediately after perfusion, proximal nerve stumps about 1 cm long including its distal tip were taken, and immersed in the same fixative for 2 hr at room temperature. After rinsing for 1 hr in the same buffer, specimens were then postfixed for 2 hr at 4°C in 1% osmium tetroxide dissolved in 0.1 M phosphate buffer (pH 7.4), dehydrated with a series of graded alcohols and propylenoxide, and embedded in an epoxy resin mixture (Quetol 812, Nissin EM, Tokyo, Japan). For light microscopy, semithin sections were cut with glass knives and stained with 1% toluidine blue. For electron microscopy, ultrathin sections were cut with a diamond knife, contrasted with uranyl acetate and lead citrate and examined with a JEM-100 SX transmission electron microscope (JEOL, Tokyo, Japan).

Silver impregnation

At 14 days after transection, the proximal stump of the sciatic nerve was fixed for 2 days in a mixture of 80% ethanol (90 ml), 100% neutral formalin (5 ml) and acetic acid (5 ml), dehydrated with alcohol and embedded in paraffin. Longitudinal sections, about 20 μ m thick, were cut, mounted on glass slides, deparaffinized and rinsed with distilled water. These sections were processed for sil-

ver impregnation according to the Bodian's method modified by Otsuka (30).

RESULTS

Axons, myelin sheaths and Schwann cells in the proximal stumps underwent complicated changes within a few days after transection. It is not within the scope of the present study to describe the variety of cellular reactions. Only the changes relevant to survival and outgrowth of the axon stump have been described in the present study.

Demyelination

Within 1 to 2 days after transection, myelin sheath degradation due to the injury occurred near the cut end. Light microscopic observation revealed that this kind of early traumatic degradation of myelin sheath tended, in most cases, to be confined within 0.1-0.2 mm from the cut end, demyelination proceeded in some fibers further proximally up to 0.3-0.5 mm from the cut end.

Electron microscopically, Schwann cells and macrophages appeared to be involved in the progressive demyelination at the proximal stump (Figs. 1

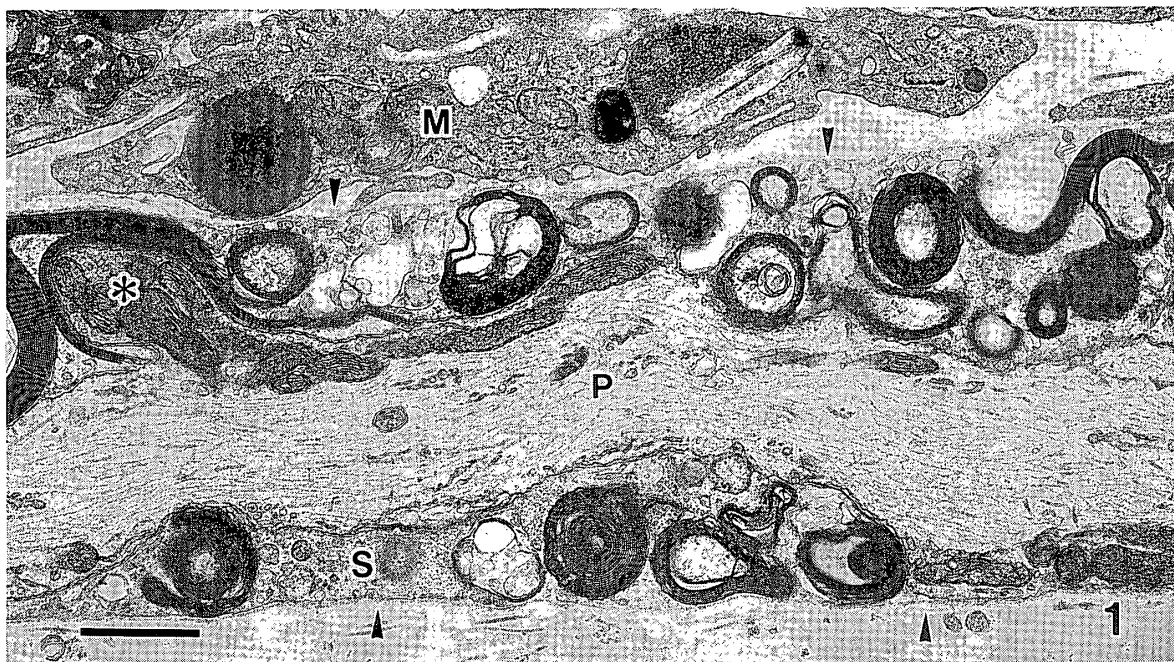


Figure 1. Two days after transection. Proximal stump of a demyelinating nerve fiber. Myelin balls are seen in the cytoplasm of the Schwann cell (S). The parent axon stump (P) appears to be almost normal. A nascent axonal sprout (*asterisk*) containing some round vesicles and mitochondria is formed. Outside the basal lamina tube (*arrowheads*) of the Schwann cell, a macrophage (M) containing lipid droplets and lysosomes is seen. Bar = 1 μ m.

and 2). If Schwann cells were alive, myelin sheaths could be disorganized into myelin balls first within Schwann cells, which subsequently might be released from the Schwann cell and phagocytosed by macrophages. When Schwann cells were dead, macrophages were directly involved in the disorganization as well as digestion of myelin sheaths. In this manner, the proximal axon stump near the cut end might become naked. It appeared that myelin sheath degradation stop-

ped at the node of Ranvier, thus the node of Ranvier near the cut end remained as "a half node".

Regenerative outgrowth

One to 2 days after transection, axon stumps which had recovered from the traumatic injury displayed various features of accumulation of round or tubular vesicles (50-100 nm in diameter) together with a small amount of mitochondria at their terminal end. Proximal to the terminal

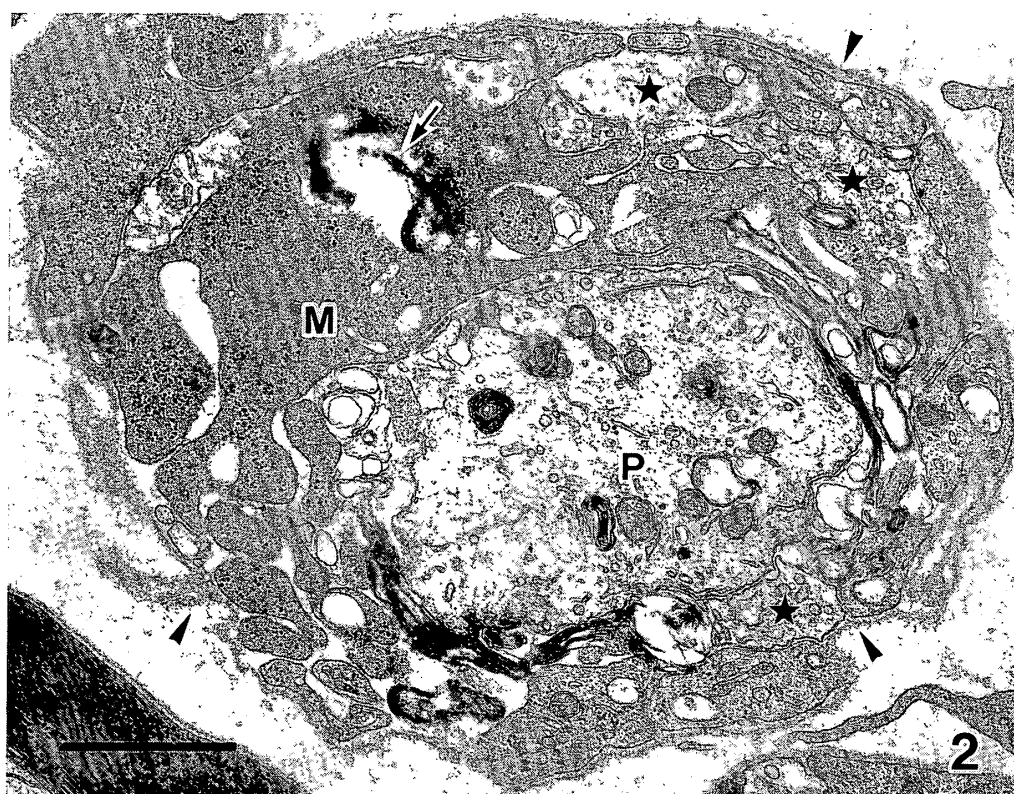


Figure 2. Three days after transection. Transverse section of a myelinated fiber seen near the cut end of the proximal stump. The parent axon (*P*) is still partly enclosed by an extensively split myelin sheath. Several growth cones and daughter axons (*stars*) are seen in the vicinity of the basal lamina (*arrowheads*). A macrophage (*M*) which had invaded the nerve fiber extends cytoplasmic processes among the axons, and phagocytoses myelin debris (*arrow*). Bar = 1 μ m.

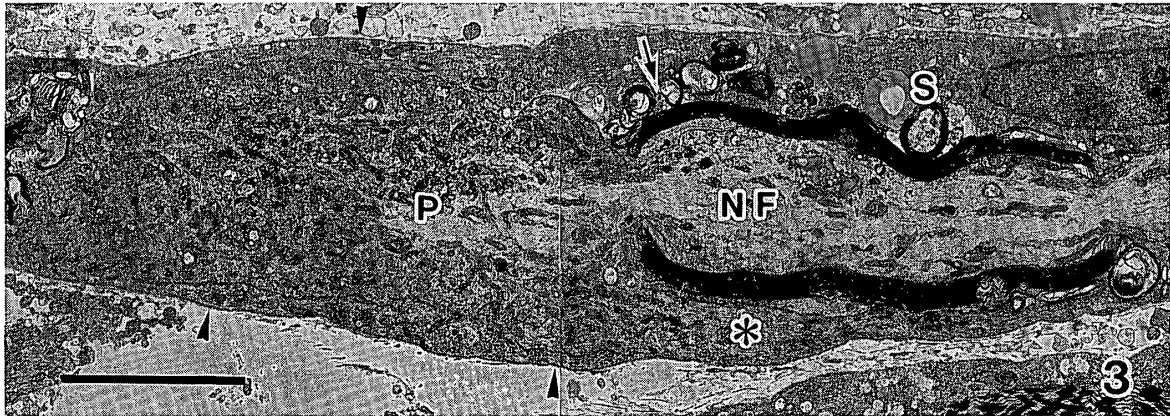


Figure 3. Two days after transection. A regenerating parent axon stump. The demyelinated terminal end of the proximal stump (*P*) swells extensively due to an accumulation of round or tubular vesicles and mitochondria, and is directly in contact with the basal lamina (*arrowheads*) of Schwann cell. A sprout (*asterisk*) extends proximally between the myelin sheath and basal lamina. Proximal to this area is neurofilament-predominant area (*NF*) and still enclosed by a degenerating myelin sheath. Myelin balls (*arrow*) are seen in the cytoplasm of a Schwann cell (*S*). Bar = 5 μ m.

end, the axon contained an abundance of neurofilaments, mitochondria and smooth endoplasmic reticulum (SER). The myelin sheaths of these axons disintegrated, so that parts of the axon became naked (Fig. 3), and in direct contact with the Schwann cell or Schwann cell basal laminae.

Demyelinated terminal axon ends swelled extensively probably due to dense accumulation of the vesicles and mitochondria (Fig. 3). There were no features indicative of an extensive degeneration of organelles including cytoautolysis or increase of autophagy. Within 0.2-0.3 mm from the cut end, besides the parent axon stump itself, several thin axons and small growth cones were also found

within the basal lamina tube of Schwann cell (Fig. 4). They were always in contact with the inner surface of the basal lamina tube. Many of them were presumed to be daughter axons which emerged from the first or second proximal node of Ranvier as suggested by Blümcke and Niedorf (4).

With advancement of the regenerative outgrowth, the parent axon stumps appeared to grow distally beyond the end of the basal lamina tube (Fig. 5). While their shafts were predominated with longitudinally-aligned neurofilaments, distal part of these axons somewhat swelled and occasionally ramified, containing mitochondria, SER and



Figure 4. Two days after transection. Another example of regenerating parent axon stump. Myelin degeneration stops at the node of Ranvier (*N*), thus the node exhibits a half nodal configuration of myelin sheath. The demyelinated parent axon (*P*) swells extensively, and appears to extend distally. Growth cones (*stars*) and daughter axons (*asterisk*) are always in contact with the basal lamina (*arrowheads*) of a Schwann cell. Proximal to the node of Ranvier is a neurofilament-predominant area, and enclosed by a normally appearing myelin sheath. Bar = 2 μ m.

variously-oriented neurofilaments in the center, and a number of round or tubular vesicles in the periphery. These axons together with numerous growth cones balled up forming a mini-neuroma in the connective tissue compartment.

At 3 and 5 days after transection, several mini-neuromas formed at the tip of the proximal segment. Between 0.1 and 0.5 mm proximally from the distal end, some axon bundles were seen which consisted of one naked parent axon and its sprouts (daughter axons). They were included in a common basal lamina tube (Fig. 6). The parent axon was thick and exhibited conspicuous neurofilaments, mitochondria and SER. These find-

ings suggested that some axon stumps denuded of myelin sheaths could retain their viability and grow out as regenerating axons. Some daughter axons were filled with dense bodies (Fig. 7), suggesting that they might be destined to degenerate.

Silver impregnation

By silver impregnation method, the distribution of regenerating axons was examined at the proximal stump 14 days after transection. The site where the sciatic nerve had been cut could be recognized as the end of the perineurial sheath. Proximal to the transection site, axons (2-5 μ m in diameter) run in an orderly fashion parallel with the long axis of the

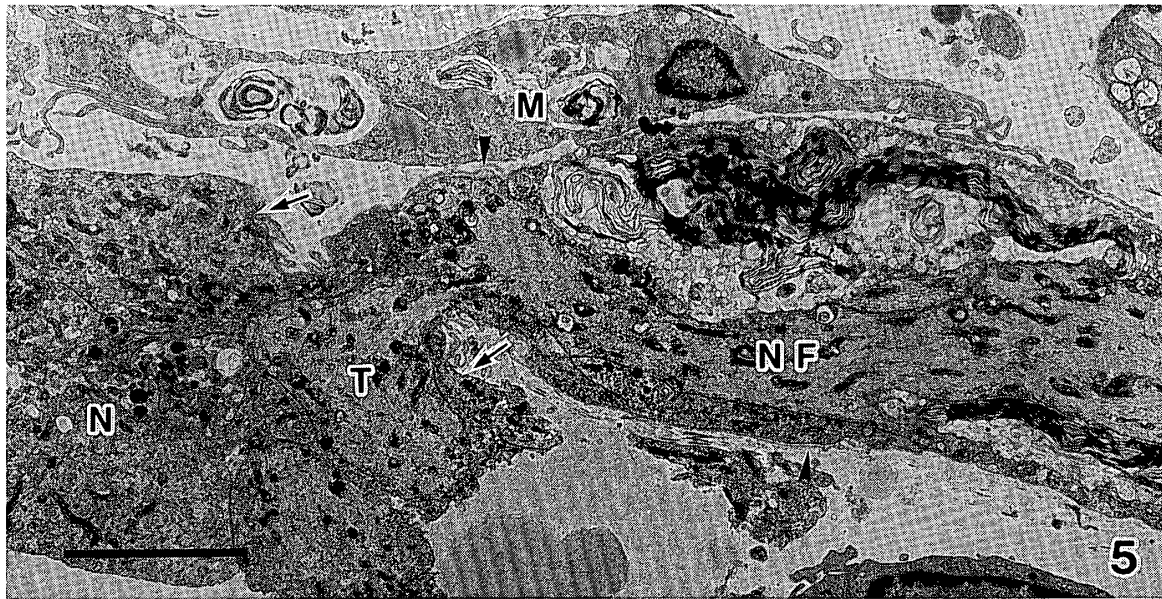


Figure 5. Forty hours after transection. A demyelinated axon stump showing densely packed neurofilaments (NF) extends distally beyond the end (arrows) of the basal lamina (arrowheads) of Schwann cell. The proximal portion is still enclosed by an extensively split myelin sheath, indicating that this is a parent axon stump itself. The distal tip (T) of this axon stump somewhat swells and ramifies. Around the tips, a neuroma (N) consisting of several growth cones is formed in the connective tissue compartment. A macrophage (M) containing myelin debris and lipid droplets is seen. Bar = 5 μ m.

nerve (Fig. 8). In the mini-neuromas, numerous axons were found running in various directions; most of them were thinner (less than 2 μ m in diameter) than normal axons, and some were thick measuring 2 to 5 μ m in diameter. The former were considered to be regenerating daughter axons, and the latter were presumed to be elongated parent axon stumps themselves. Some thin daughter axons were found to emerge from the elongated parent axon stump (Fig. 9), the finding consistent with the

electron microscopic features of Figs. 5 and 6.

DISCUSSION

The present study was performed to demonstrate the morphological evidence which supports regenerating outgrowth of the proximal stump itself of the transected myelinated fibers in the mouse sciatic nerve. Electron microscopic observations revealed that when the terminal ends of the proximal axon stump were freed from

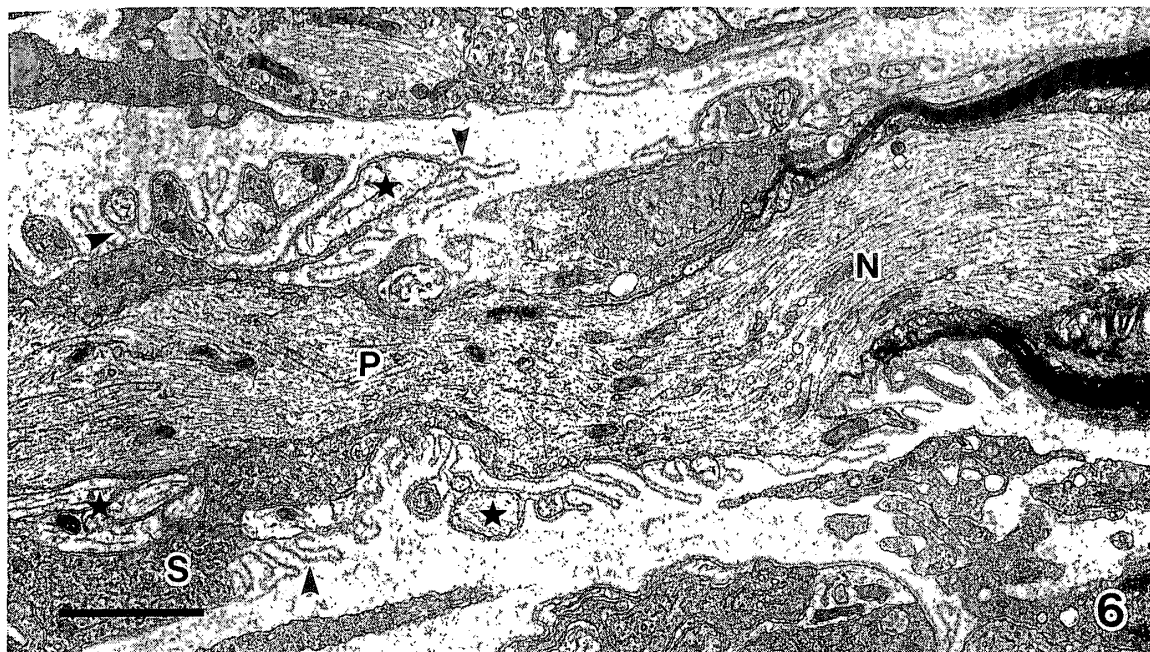


Figure 6. Five days after transection. The parent axon stump (*P*) distal to the node of Ranvier (*N*) is completely demyelinated, while proximally enclosed by a normally appearing myelin sheath. The parent axon stump is evidently thicker and displays more densely packed neurofilaments than the daughter axons (*stars*) which are always in contact with the basal lamina (*arrowheads*) of Schwann cell or the cytoplasm of Schwann cell (*S*). Bar = 2 μ m.

the myelin sheath confinement, they could grow out as regenerating sprouts. With the silver impregnation method, it was clearly demonstrated that the parent axon stumps grew out into connective tissue compartment with some daughter axons further emanating from their growing tips. These findings suggest that demyelination might be an indispensable requisite for survival and outgrowth of the parent axon stump itself.

Demyelination

An extensive degradation of myelin

sheath occurred at the proximal end of the injured nerve. If Schwann cells are damaged at the site of injury, macrophages appeared to invade the Schwann cell compartment and phagocytose myelin sheaths. Whereas, if Schwann cells remained alive, myelin sheaths would disintegrate into myelin balls in Schwann cells. Crang and Blakemore (8) reported that during Wallerian degeneration, myelin sheaths were fragmented and liberated into the endoneural space in a culture system containing no macrophages. It appears that Schwann

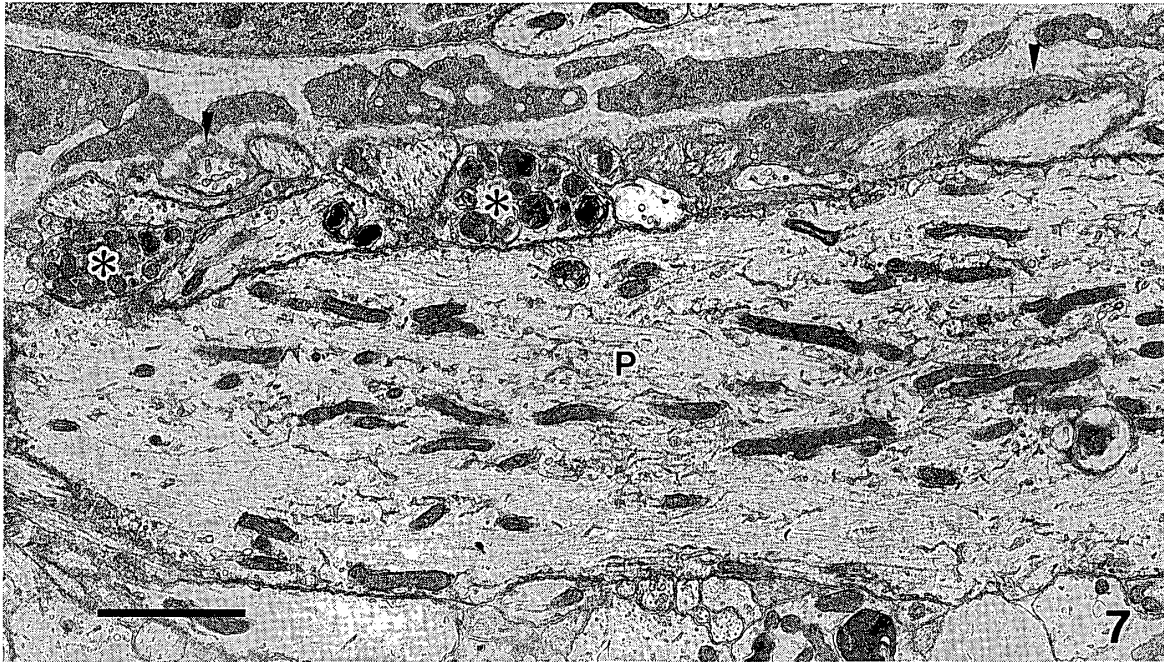


Figure 7. Five days after transection. A demyelinated parent axon stump (*P*) and its daughter axons extend through a common basal lamina tube (*arrowheads*). The parent axon appears normal, while some of the daughter axons (*asterisks*) exhibit autophagosomes, suggesting that they are degenerating. Bar = 2 μ m.

cells can disintegrate the myelin sheath into myelin balls, but not digest them by lysosomes (1, 8). Myelin balls, discharged from Schwann cells, and in turn phagocytosed by macrophages, were degraded eventually by lysosomal digestion. Thus, macrophages are the main scavengers of degenerated myelin sheaths (12, 17). Demyelination might stop at the first or second node of Ranvier, because degradation of myelin sheath tended, in most case, to be confined 0.1-0.2 mm from the cut end. The length of demyelinated axon stumps might be related to the distance between the cut end and the

first or second node of Ranvier.

Regenerating outgrowth

In the case of axonal sprouting at the node of Ranvier, sprouts extend along the inner side of the Schwann cell basal lamina (10, 16, 21, 25, 36). Thus it was suggested that the Schwann cell basal lamina is an effective scaffold and conduit for growing axons (14, 15, 16, 18, 27, 28, 29, 33, 35). Previously, we reported that as far as the terminal ends of the proximal axon stumps were ensheathed by myelin lamellae, they are considered, without exception, to undergo degenerative changes (23).

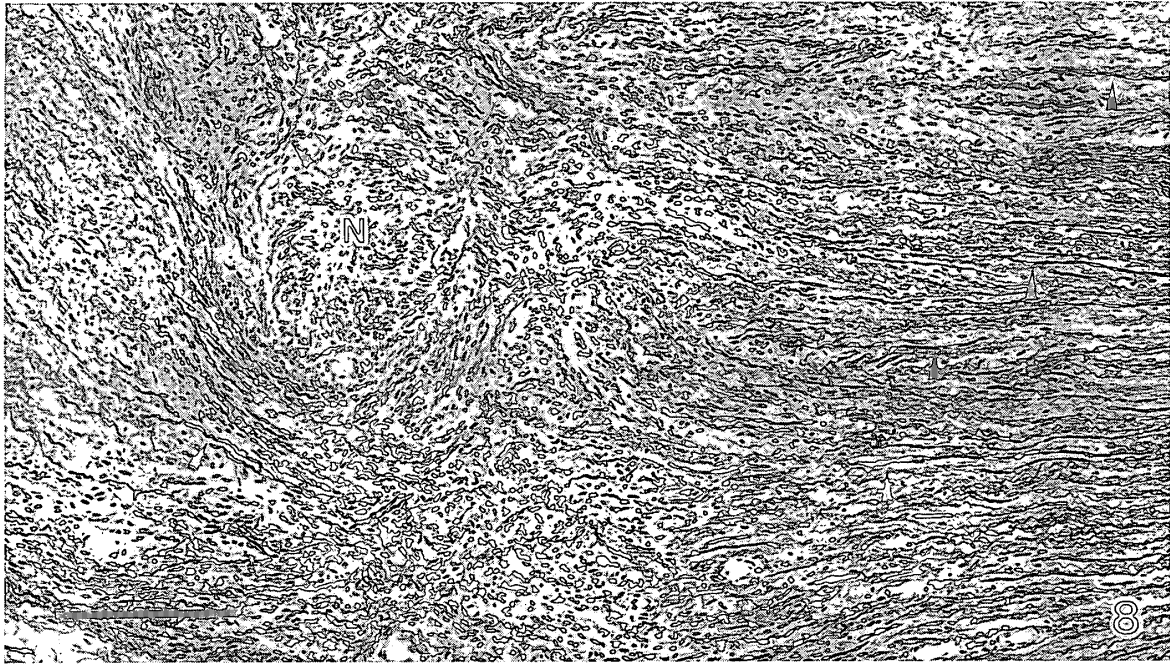


Figure 8. Fourteen days after transection. Silver impregnation. General view at the end of the proximal stump. Proximal to the transection site (right half of the picture), several axons measuring 2-5 μm diameter (*arrowheads*) run orderly being in parallel with the long axis of the nerve. In the neuroma (*N*: left of the picture), several thin axons and some thick axons (*arrowheads*) run in various directions in a wavy fashion. Bar=0.2mm.

In contrast, as demonstrated in the present study, the terminal ends of the proximal axon stumps which were freed from the tight myelin sheath covering exhibited no morphological features indicative of an extensive axonal degeneration including cytoautolysis or increase of autophagosomes. Owing to demyelination, the proximal axon stumps can make direct contact with the plasma membrane and basal lamina of Schwann cells. It is possible that the direct contact with the extra-myelin sheath

tissues might be very important for regenerating axons, especially for the growing tips (growth cones), to sustain their viability and to grow out as regenerating sprouts.

It has been demonstrated that laminin is a potent promoter for neurite growth in vitro (2, 5, 31). Tohyama and Ide (34) reported that laminin is distributed mainly on the inner surface of the basal lamina of Schwann cells. On the other hand, Okajima et al. (26) reported that protein kinase C (PKC) was strongly expressed in

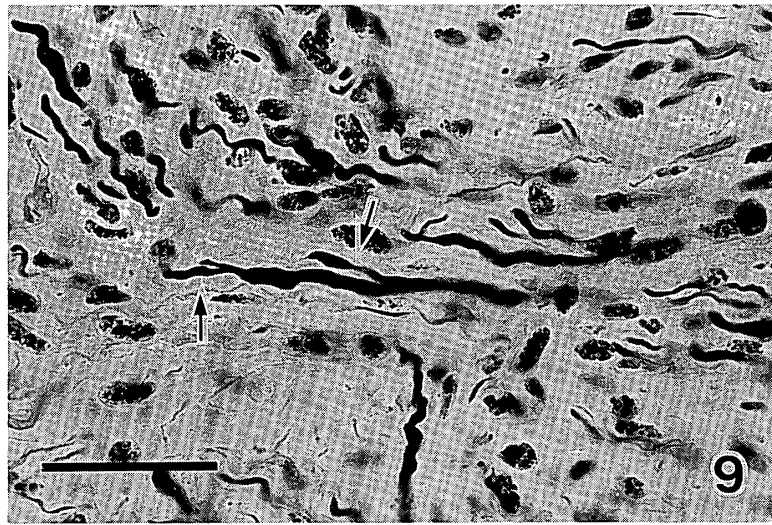


Figure 9. Fourteen days after transection. Silver impregnation. In the neuroma, thick and thin axons running in various directions in a wavy fashion were noted. The former are presumed to be elongated parent axons themselves, and the latter to be daughter axons. Daughter axons emerging from the elongated parent axons were often found (*arrows*). Bar = 25 μ m.

the regenerating myelinated axons, especially at the growing tips (growth cones). It was suggested that PKC may regulate the response to extracellular matrix molecules, cell adhesion molecules and growth factors through different intracellular mechanism including by affecting integrine interactions with laminin (2, 3, 7, 9, 13,22). These findings further support the thesis that Schwann cell plasma membranes and Schwann cell basal laminae are favorite scaffolds for regenerating axons, as well as suggest that the functional contact with the inner surface of basal lamina or Schwann cell plasma membranes of the naked axon stumps might be an essential requisite for axonal elongation, movement, spread-

ing and pathfinding during the course of nerve regeneration.

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