Ultrastructural Investigation of Nerve Regeneration in Vivo —Comparison with in Vitro Neurite Formation—

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Abstract

The process of regeneration of rat dorsal root ganglion cells was investigated by scanning and transmission electron microscopy and compared with the process of *in vitro* neurite formation. After disruption of the granular reticulum, numerous microtubules appeared in the perinuclear cytoplasm. Many thin, regenerating nerve fibers were found at the suture line adjacent to collagen fibers. Subsequently, the number of cytoplasmic neurofilament bundles increased and many of the regenerated nerves attached to the suture line. By the eighth week after experimental injury, the granular reticulum had reconstituted in the cytoplasm and the regenerated nerves resembled those of control animals, although the fiber arrangement was somewhat irregular. The process was almost identical to that of *in vitro* neurite formation, although the time course was much slower. Structural proteins, such as actin, tubulin, and neurofilament triplet, appeared to form similarly in neuronal regeneration and *in vitro* neurite formation. The results also suggest that microtubules are essential for nerve regeneration as well as neurite formation and that components of the neuronal microenvironment, such as collagen fibers and migrating cells, play crucial roles in nerve regeneration.

Key words: nerve regeneration, neuron ultrastructure, structural protein, microtubules

Introduction

Regeneration of nerve fibers^{16,18,19,36,53)} and their cell bodies^{6,17,40,42)} has been extensively investigated, in various models and with different techniques. The mechanism of nerve regeneration has been partially elucidated at the molecular level.^{27,54)} Studies involving culture and transplantation techniques^{2,5,21,22,29,32,39)} have made possible investigation of central nervous system plasticity^{4,7,15,50)} and regeneration,^{14,24,28,49,51)} and also application of peripheral nerve grafts in brain and spinal cord.^{1,10-12,37,45,47)} However, the mechanism of nerve regeneration is not yet clear. Earlier, in an attempt to better understand this process, we examined neurite formation in culture.²¹⁾ In the study reported here, we examined regeneration of dorsal root ganglion cells *in vivo* and compared these findings with those of our *in vitro* study. As in that investigation, regenerating nerve

fibers and cell bodies were observed serially by electron microscopy.

Materials and Methods

Male Wistar rats weighing 300–400 gm were anesthetized by intraperitoneal injection of 4 mg/100 gm pentobarbital. The sciatic nerve was transected at mid-thigh and the proximal and distal nerve stumps were sutured epineurally with 11-0 nylon monofilament under the operating microscope. At 3, 5, 7, 10, 14, 28, and 56 days after this procedure, rats were irrigated with Ringer's solution and perfused with a 2.5% glutaraldehyde solution. The sciatic nerve and L4, L5 dorsal root ganglia were excised and stored in a 1% glutaraldehyde, 3.5% formaldehyde solution at 4°C. The sciatic nerve was then cut longitudinally at the point of the suture line and postfixed with 2% osmium tetroxide. After dehydration, specimens were dried in a critical point dryer and coated with platinum for scanning elec-

tron microscopy (SEM). For transmission electron microscopy (TEM), the specimens were postfixed with osmium and embedded in Epon. Cultured neurons obtained from dorsal root ganglia of newborn rats were processed in the same way for SEM and TEM. Details of these methods, including the culture technique, were described previously.²¹⁾ In brief, dissociated neurons were seeded on polyornithine-coated glass cover slips and cultured with glioma-conditioned medium containing 5–10% fetal calf serum. The culture was maintained at 37°C in a CO₂ incubator. The cultured neurons were fixed with 2.5% glutaraldehyde solution and postfixed with 1% osmium tetroxide. After dehydration, the cells were dried and coated with platinum for SEM. For TEM the cells were embedded in Epon and the cover slips were removed with a 56% solution of hydrofluoric acid. Of the several types of neuron in the ganglia,^{43,48)} we focused on the changes of large neurons.

Results

I. Sciatic nerve changes at the suture line

Control sciatic nerve consisted of regularly arranged nerve fibers and a small amount of collagen fibers. Collagen fibers were attached to the nerve fibers and were also present in the spaces between nerve fibers (Fig. 1A). Three days postoperatively, the cut ends of the myelin sheaths were identified as vacant tubules interspersed with collagen fibers. After 5 days, the suture line was filled with many round cells and irregularly arranged collagen fibers.

The cut ends of the nerve sheath were also covered with these materials (Fig. 1B). After 7 days, abundant collagen fibers were found in the sutured space, covering many of the round cells (Fig. 1C). Both proximal and distal nerve sheaths exhibited irregularities, such as varicosities near the suture line. After 10 days, the proximal sheaths appeared smooth and many thin nerve fibers were observed at the point of transection (Fig. 1D). The surfaces of the distal sheaths, however, were still irregular. Two weeks postoperatively, the gap between the cut ends had completely disappeared and the area was filled with numerous regenerated nerves and collagen fibers. Four weeks postoperatively, regenerating nerves of various sizes were abundant, and migrant round cells were not seen. There were fewer collagen fibers and they were connected by small, spherical structures. By 8 weeks, the nerve fibers and collagen fibers appeared identical to those of control specimens, although their arrangement was somewhat irregular.

II. *In vivo* cell body changes

Examination of large neurons in the dorsal root ganglia 3 days postoperatively revealed perinuclear disruption of the granular endoplasmic reticulum and dissociation of ribosomes from their membranes. Multivesicular and dense bodies were sometimes observed (Fig. 2A, B). After 5 days, disruption of the granular reticulum had progressed to the peripheral regions and small Golgi complexes were occasionally seen. Seven days postoperatively, many microtubules were observed in the perinuclear

Fig. 1 Scanning electron micrographs of sciatic nerves. A: A normal sciatic nerve with regularly arranged nerve fibers (NF) interspersed with collagen fibers (arrows). B: A sutured sciatic nerve on postoperative day 5. The gap at the suture line is filled with migrant round cells (RC) and irregularly arranged collagen fibers. C: At day 7, the wound area is occupied by abundant collagen fibers (CF), which cover the migrant cells. D: Many thin, new nerve fibers (arrows) are present at the suture line after 10 days. Bars = 5 μ m.

Fig. 2 Transmission electron micrographs of dorsal root ganglia. A: A normal large neuron of the dorsal root ganglion, containing well organized granular reticulum (GR). B: A ganglion showing disruption of granular reticulum and multivesicular and dense bodies (arrows) in the cytoplasm 3 days after injury. C: Many microtubules (arrows) are present in the perinuclear region after 7 days. D: Numerous small Golgi complexes (G) are also seen in the cytoplasm 7 days after injury. Bars = 1 μ m.

region (Fig. 2C) and the number of small Golgi complexes appeared to have increased (Fig. 2D). Neurofilament bundles were somewhat irregular and not abundant. After 10 days, microtubules appeared infrequently. Two weeks postoperatively, neurofilaments were numerous and distributed throughout the cytoplasm. By 4–6 weeks postoperatively, the granular endoplasmic reticulum had reconstituted at the periphery of the cell and bundles of neurofilaments had become regular and clear. By week 8, the granular reticulum and the arrangement of neurofilaments had returned to normal, but microtubules and multivesicular bodies were sometimes still found.

III. Comparison with the process of neurite formation *in vitro*

The process of *in vivo* nerve regeneration was almost identical to that of neurite formation in culture,²¹⁾ although the time course of the former was longer than that of the latter. The results are summarized in Table 1.

Discussion

Many electron microscopic studies have revealed in detail the changes that occur during axonal regeneration^{18,19,36,53)} and elucidated the importance of axonal transport in nerve regeneration.^{16,25)} In the peripheral nervous system, as opposed to the central nervous

Table 1 Comparison of nerve regeneration *in vivo* and neurite formation *in vitro*

Nerve regeneration (<i>in vivo</i>)			Neurite formation (<i>in vitro</i>) ²¹⁾		
Time (days)	Nerve	Cell body	Neurite	Cell body	Time (hours)
3	vacant space	disruption of granular reticulum, vesicular bodies	microspike (stages 1–2)	disruption of granular reticulum	
5	migrant cells	small Golgi complexes	veil (stage 3)	Golgi complexes	2
7	collagen fibers	microtubules	growth cone (stage 4)	microtubules	3–4
10	regenerating fibers		neurite elongation (stage 5)		8
14		neurofilaments		neurofilaments	
30–60	normalization	reorganized granular reticulum	network of neurite (stage 6)	reorganized granular reticulum	24–48

system, the role of the basal lamina and Schwann cells has been emphasized.^{3,20,31,41)} In the search for a good model of nerve regeneration, silicone and bioresorbable tubes have recently been applied,^{9,23,24,33)} and neurite-promoting factors in the tubes have been examined by bioassay as well as by measurement of the activity of the nerve extracts.^{30,44)} In addition, new clinical suturing and grafting methods are under development.^{13,34)}

In this study, using SEM, we examined regenerating nerve fibers in three dimensions and compared the changes to neurite formation in culture. Our results support those of other investigators. In addition, we found marked early changes in collagen fibers and migrating round cells at the suture line, which may be important in nerve regeneration, at least in the initial phase, in terms of mechanical support and promotion of regrowth. In the culture system, we did not detect large growth cones in the sciatic nerve. This is true of other culture systems involving the use of collagen,³⁸⁾ so that the shape and size of growth cones may be influenced by collagen fibers *in vivo* as well.

Ultrastructural changes in ganglia have also been observed in many models of nerve regeneration.^{6,17,40,42)} Disorganization of granular reticulum and proliferation of free polyribosomes suggests an increase in the synthesis and turnover of ribosomal ribonucleic acid. Changes in Golgi complexes and agranular reticulum may support expansion of the membrane. The increase in microtubules and neurofilaments indicates accelerated polymerization and assembly and suggests axonal elongation. Our results are similar to those previously reported; the time required for regeneration, however, appears to depend on the model used. Since we examined axons and cell bodies in the same rats, we were able to observe the temporal relationship between axonal and cell body changes. Several mechanisms for the initiation of cell body changes have been proposed, including electrophysiological, chemical, pharmacological, and physical processes.^{8,46)} After initiation, we found a dramatic increase in the number of microtubules during both *in vivo* nerve regrowth and *in vitro* neurite development. This strongly suggests that microtubules are essential for nerve regrowth and elongation, as we noted previously.²¹⁾

The sequence of neurite formation or regeneration can be readily observed in culture systems.⁵²⁾ Also, the effects of nerve growth factor and similar substances are easily detected, so that new substances may be tested for possible clinical use. Furthermore, study of culture systems contributes to the understanding of the basic processes and mechanisms of

nerve regeneration.^{5,29,32)} Recently, these mechanisms have been studied at the molecular level⁵⁴⁾ as well as in a culture system.²⁷⁾

Using our culture system, we found that neurons undergo dramatic changes during neurite formation, and we were able to divide the process into six stages. As mentioned, we concluded that microtubules are essential for initiation and elongation of neurites.²¹⁾ During microspike and veil formation in stages 1–3, changes took place in the granular reticulum and Golgi complexes in the cell body. Microfilaments and agranular reticulum were present in the microspike and the veil. Since actin and membranous materials are thought to be important in neuronal surface changes, synthesis and polymerization of actin may increase, and the membrane may expand, in this stage. In stages 4 and 5 (growth cone formation and neurite elongation), microtubules proliferated, probably as a result of tubulin and synthesis polymerization. In stage 6, neurofilaments, granular reticulum, and other organelles were arranged regularly in the cytoplasm.

The process of sciatic nerve regeneration was almost identical to that of neurite formation, except that the time course of the former was much longer. The changes from days 3 to 5 in nerve regeneration roughly corresponded to stages 1–3 in culture. During days 7–10, *in vivo* microtubule formation and nerve regrowth resembled the events of stage 4 in culture. Weeks 2–8 *in vivo* were similar to stages 5–6 in culture and consisted mainly of reorganization. As in the culture system, *in vivo* process seems to progress from actin filaments and membranous materials to microtubules and then neurofilaments. These results correspond to the findings obtained with radiolabelling techniques in studies of axonal transport of structural proteins, actin, tubulin, and neurofilament triplet in regenerating nerves.^{26,35)}

Figure 3 illustrates the process of nerve regeneration and neurite formation, as indicated by our results. After axonal injury, free polyribosomes proliferate in the cell body and actin may be produced. The membrane expands at almost the same time. The injured axon tip forms fine cell process, microvilli, and protrusions, which are composed of microfilaments and agranular reticulum. Subsequently, tubulin may be synthesized in the cytoplasm and then polymerized. Many microtubules appear in the cell body and nerve fibers regrow at the tip of the injured axon, forming the growth cone. Later, neurofilament triplets may form, appearing as neurofilaments in the elongating axons. This is a model of sensory nerve regeneration; some differences may exist in other types of neurons. However,

Fig. 3 Diagrammatic representation of nerve regeneration and neurite formation. Changes in organelles in the cell body and growing nerve fibers are shown, as well as nerve structures related to structural proteins. G: Golgi complex, R: ribosomes, Mt: microtubules, NF: neurofilaments, GR: granular reticulum.

understanding this process in the peripheral nervous system will contribute to our knowledge of regeneration in the central nervous system.

In conclusion, these results indicate that nerve regeneration *in vivo* is an orderly process identical to that of neurite formation *in vitro* and that structural proteins, such as actin, tubulin, and the neurofilament triplet, may be synthesized in the same way during nerve regeneration and neurite formation. Microtubules appear to be essential for nerve growth both *in vitro* and *in vivo*, and certain other components of the neuronal microenvironment, such as collagen fibers and migrating cells, also may play crucial roles in nerve regeneration.

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