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Influence of Electrical and Electromagnetic Stimulation on Nerve Regeneration in the Transected Mouse Sciatic Nerve: An Electron Microscopic Study

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Influence of electrical and electromagnetic stimulation on nerve regeneration was electron microscopically examined in the transected mouse sciatic nerve. Two days after the transection, several thin regenerating axons (daughter axons) were observed between the myelin sheath and basal lamina of Schwann cells in the proximal stump. Growth cones of the daughter axons contained several small round vesicles and mitochondria, and the shaft of them, neurofilaments, neurotubules and profiles of smooth-surfaced endoplasmic reticulum. The electrical and electromagnetic stimulation was applied to the proximal stump 2 days after the transection. At 3 and 6 hours after the electrical stimulation, some growth cones exhibited degenerative changes, such as swelling of mitochondria, rupture of small vesicles and formation of multivesicular bodies, but at 24 hours after the stimulation, these degenerative changes were hardly encountered. At 3 and 6 hours after the electromagnetic stimulation, most of the growth cones exhibited several multivesicular bodies, and at 24 hours after the stimulation, these degenerating growth cones were still encountered. These degenerative changes were not found in any axonal shaft of the daughter axons or parent axons encircled by myelin sheath. These findings suggest that the growth cones are very sensitive to the electrical and electromagnetic stimulation.

Key Words: Electrical stimulation, Electromagnetic stimulation, Nerve regeneration, Mouse sciatic nerve, Electron microscopy.

Introduction

Effects of electrical and electromagnetic stimulation on peripheral nerve regeneration have been still controversial. There are many reports that electrical and electromagnetic stimulation promotes regeneration of peripheral nerves[1-10], but some authors reported that

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they are not effective, or rather harmful for the regenerating axons[11-13]. In these studies, effects of the electrical and electromagnetic stimulation were assessed by clinical findings, such as contraction force and response of muscles to the nerve stimulation, and period necessary for functional recovery of the muscles. Morphologically, the effects were determined by the number and diameter of regenerating axons, and thickness of myelin sheath, and electrophysiologically, by the electromyogram of reinnervated muscles and conduction velocity of regenerating nerves. To date, however, the action site or action mechanism of the electrical and electromagnetic stimulation on nerve regeneration has not yet been fully elucidated. In the present study, therefore, the morphological changes in the regenerating axons after the electrical and electromagnetic stimulation were electron microscopically examined in the transected mouse sciatic nerve.

Materials and Methods

Animals

Twenty-four adult mice (ddY strain) weighing 35-44g, 12-20 weeks old, were used in the present study. The animals were anesthetized with intraperitoneal injection of Nembtal (pentobarbital sodium 50mg/kg body weight), the right and left sciatic nerves were exposed and transected with microdissection scissors at the mid-thigh level under an operation microscope. Soon after the transection, the skin wounds were closed with 4-0 suture.

Electrical stimulation

Two days after the operation, the electrical stimulation was applied to the right mid-thigh for 5 minutes by using an electric stimulator (SEN-3310, Nihon Kohden, Tokyo, Japan) and an isolator (SS-202J, Nihon Kohden, Tokyo, Japan) as follows. Two metal electrodes coated with electrode paste were placed on the skin covering the cut end of the proximal stump of the sciatic nerve under the anesthesia as described above. Rectangular wave pulses of 300 msec. duration, 0.5 mA, 1Hz were applied. With this electrical stimulation, enough muscle contractions corresponding to the electrical stimulation were observed in the thigh muscles.

Electromagnetic stimulation

Two days after the operation, the electromagnetic stimulation was applied to the right mid-thigh for 5 minutes by using an electromagnetic stimulator (Magstim 2000, Miyuki Giken, Tokyo, Japan). Small-size coils modified for small animals were put on the skin covering the proximal cut end of the sciatic nerve in the right mid-thigh, and the stimulation was carried out for 5 minutes every 5 seconds at 60%, 30%, and 10% levels of the maximum power. Distinct muscle contractions were noted in the thigh muscles at 60% level of stimulation, and faint contractions, at 30% level stimulation, but at 10% level, muscle contractions were hardly discerned.

Electron microscopy

At 3, 6 and 24 hours after the electrical and electromagnetic stimulation, the animals were anesthetized and sacrificed by intracardiac perfusion initially with Millonig's phosphate buffer (pH 7.4), followed by a fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde dissolved in the phosphate buffer. Immediately after the perfusion, proximal stumps of the sciatic nerve about 1 cm long including the cut end were taken, and further immersed in the same fixative for 12 hours at 4℃. After rinsing for 1 hour in the same buffer, specimens were then postfixed for 2 hours at 4℃ in 1% osmium tetroxide dissolved in the phosphate buffer, dehydrated with a series of graded alcohols 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 toluidine blue. For electron microscopy, ultrathin sections were cut with a diamond knife, and contrasted with uranyl acetate and lead citrate, and examined with a JEM-1220 transmission electron microscope (JEOL, Tokyo, Japan) at 80kV.

In each experiment, the proximal stumps of the left sciatic nerve, which did not receive any stimulation, were used as control specimens.

Results

Nerve regeneration in the mouse sciatic nerve

Two days after the transection, numerous thin regenerating axons were observed in the proximal stump. These regenerating axon arose from nodes of Ranvier locating near the cut end of the proximal stump, and extended distally through narrow spaces between the myelin sheath and basal lamina of Schwann cells (daughter axons). The distal tip of the daughter axons (growth cones) contained several small round vesicles (approximately 50 nm in diameter), and a few small oval mitochondria, but not any neurofilament or neurotubule (Fig. 1a). The proximal portion of the daughter axons (axonal shafts) contained some longitudinally oriented neurofilaments and neurotubules, and a few profiles of smooth-surfaced endoplasmic reticulum (Fig. 1b).

Three days after the transection, some regenerating axons extended distally beyond the cut end of the basal lamina of Schwann cells, and formed mini-neurinomas in the connective tissue. At the cut end of the proximal stump, numerous macrophages containing several electron-light vacuoles and small myelin balls were scattered between and within the nerve fibers. They had a relatively abundant cytoplasm with several thin cytoplasmic processes, and a dark nucleus. Numerous Schwann cells having an abundant cytoplasm and round pale nucleus were also found along the nerve fibers (activated Schwann cells).

On both days, daughter axons seen between the myelin sheath and basal lamina of Schwann cells, and in the basal lamina tube of Schwann cell, appeared to be normal (Fig. 2). On the

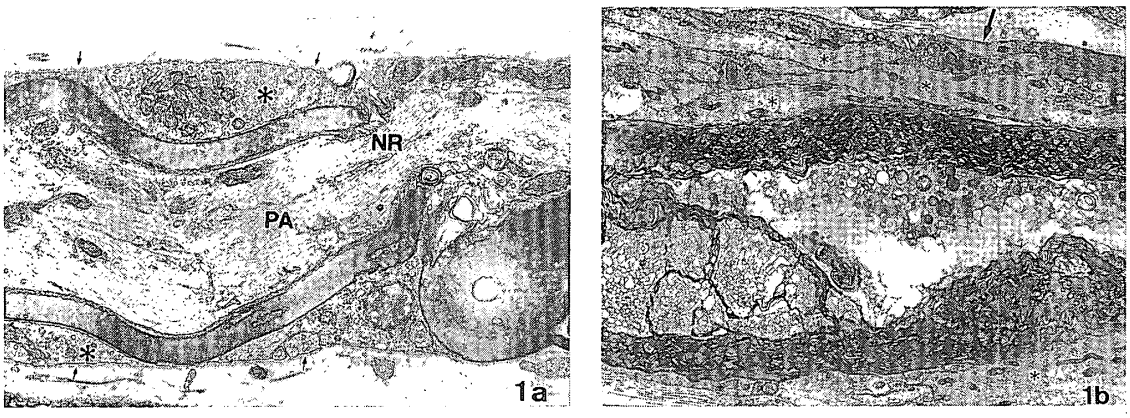

Figure 1. Sciatic nerve of mouse two days after the transection. (a) Growth cones (asterisks) containing many small round vesicles and mitochondria are seen between the myelin sheath and basal lamina of Schwann cells (arrows). PA: parent axon, NR: node of Ranvier x 20,000. (b) Axonal shafts of the regenerating daughter axons (asterisks) containing some longitudinally oriented neurofilaments and neurotubules extend distally between the myelin sheath and basal lamina of Schwann cell (arrows). X 8,000

other hand, some growth cones seen in the mini-neuroma exhibited degenerative changes, such as swelling of mitochondria, rupture of small vesicles and formation of multivesicular bodies. In the present study, therefore, influence of electrical and electromagnetic stimulation on nerve regeneration was examined in the regenerating daughter axons extending between the myelin sheath and basal lamina of Schwann cells and in the basal lamina tubes.

Electrical stimulation

At 3 and 6 hours after the electrical stimulation, many of the growth cones seen between the myelin sheath and basal lamina of Schwann cell appeared to be morphologically normal. Only some growth cones contained swollen

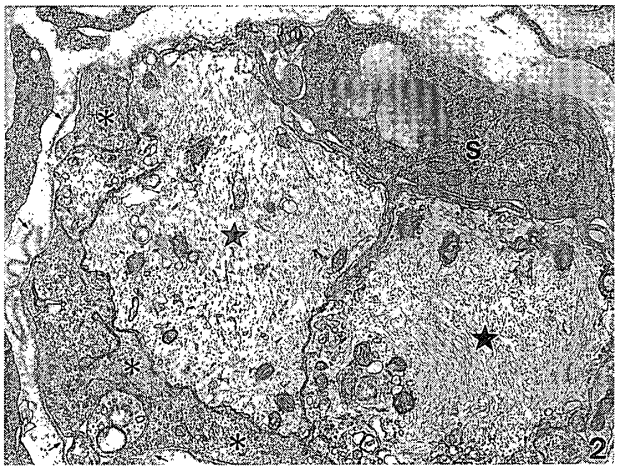

Figure 2. Three days after transection. Some growth cones (asterisks) and normal shafts (stars) of the regenerating daughter axons encircled by a common basal lamina of Schwann cell (arrows) are seen. Degenerative changes are not found in any regenerating axons. S: Schwann cell x 16,000

mitochondria, ruptured small vesicles and multivesicular bodies (Fig. 3). The axoplasm of the growth cones containing multivesicular bodies was slightly darker than that of the normal growth cones. At 24 hours after the stimulation, growth cones exhibiting these degenerative changes were hardly observed in the proximal stump (Fig. 4).

At 3, 6 and 24 hours after the stimulation, distinct degenerative changes were not found in any axonal shaft of the daughter axons or parent axons encircled by myelin sheath. Morphological changes were not noted in any macrophages or activated Schwann cells in the proximal stump.

Electromagnetic stimulation

Influence of the electromagnetic stimulation on daughter axons was evident, when the stimulation was performed at

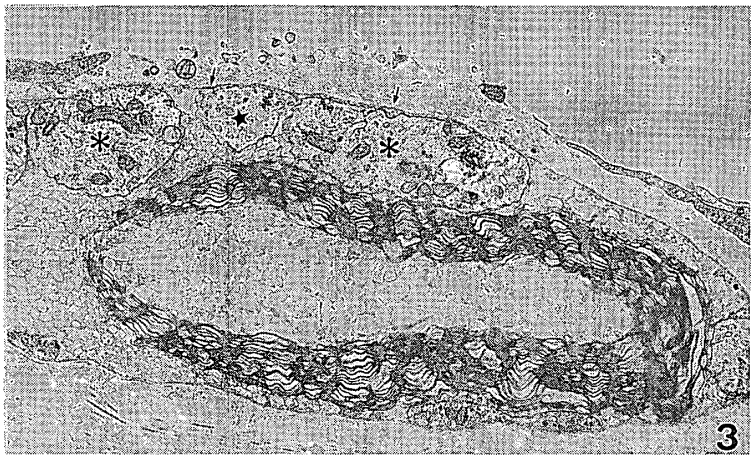

Figure 3. Three hours after the electrical stimulation. Growth cones (asterisks) are seen between the basal lamina of Schwann cell (arrows) and myelin sheath. Some of them contain swollen mitochondria, ruptured small vesicles and multivesicular bodies. On the other hand, degenerative changes are not found in any axonal shaft of the regenerating daughter axons (stars). X 1,2000

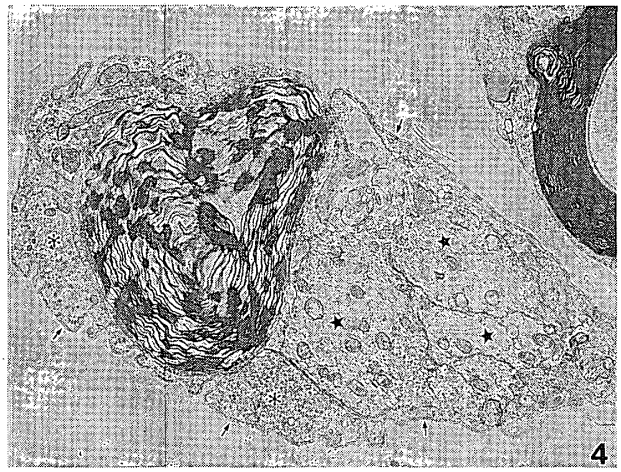

Figure 4. Twenty-four hours after the electrical stimulation. A number of growth cones (asterisks) and regenerating daughter axons (stars) encircled by the basal lamina of Schwann cell (arrows) are seen. They appear to be morphologically normal. X 10,000

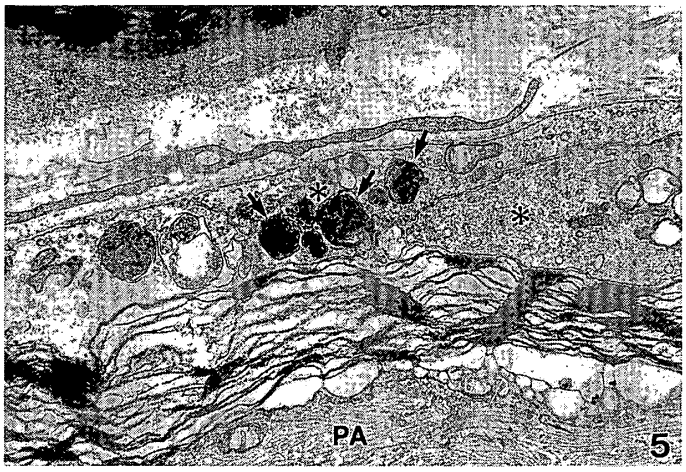

Figure 5. Three hours after the electromagnetic stimulation at 10% level of the maximum power. Growth cones (asterisks) seen between the myelin sheath and basal lamina of Schwann cell contain many multivesicular bodies (arrows). PA: parent axon x 16,000

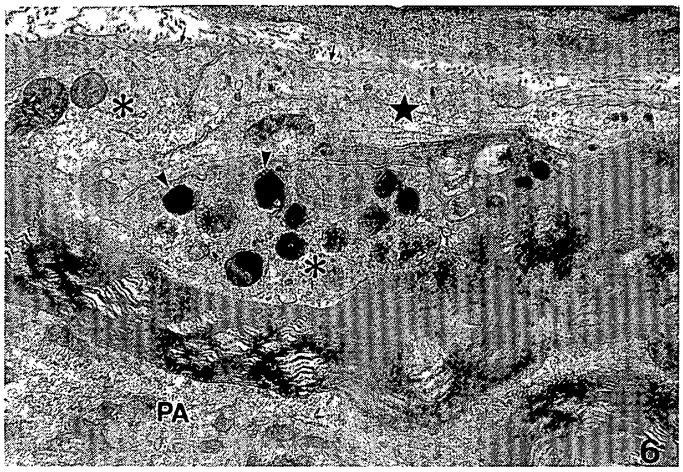

Figure 6. Six hours after the electromagnetic stimulation at 60% level. Growth cones (asterisks) and axonal shafts (arrows) of the regenerating axons are seen between the myelin sheath and basal lamina of Schwann cell. The electron density of the multivesicular bodies (arrowheads) in growth cones becomes higher than that 3 hours after the electromagnetic stimulation. Degenerative changes are not noted in any axonal shaft of the regenerating daughter axons. X 16,000

60% and 30% levels of the maximum power. At 10% level of the maximum level, light morphological changes were detected only in a few growth cones.

At 3 hours after the stimulation, most of the growth cones exhibited electron-light multivesicular bodies, but mitochondria and small vesicles appeared to be almost normal (Fig. 5). At 6 hours after the stimulation, the number of the growth cones containing multivesicular bodies did not change, but the electron density of the multivesicular bodies became higher (Fig. 6). At 24 hours after the stimulation, several growth cones still contained electron-dense multivesicular bodies (Fig. 7), but there were also many growth cones, which appeared to be morphologically normal.

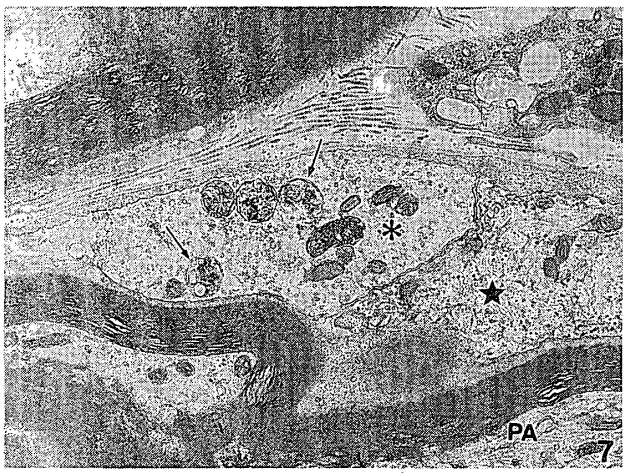

Figure 7. Twenty-four hours after the electromagnetic stimulation at 30% level. Several growth cones exhibiting electron dense multivesicular bodies (arrows) are still encountered. Almost all the axonal shafts of regenerating duaghter axons (stars) appear to be normal. X 16,000

At all stages examined, degenerative changes were not observed in any axonal shaft of the daughter axons or in the parent axons. Macrophages and activated Schwann cells seen in the proximal stump appeared to be almost normal.

Discussion

In the present study, distinct degenerative changes such as swelling of mitochondria, rupture of small vesicles and formation of multivesicular bodies induced by the electrical and electromagnetic stimulation were noted exclusively in the growth cone of the regenerating axons. Axonal shafts of the regenerating axons, normal parent axons, macrophages or activated Schwann cells seen in the proximal stump of the transected sciatic nerve did not show any morphological change after the electrical and electromagnetic stimulation. These findings suggest that the growth cone of the regenerating axons is very sensitive for the electrical and electromagnetic stimulation. Since the growth cones play important roles for elongation and pathway finding of the regenerating axons[14], destructive changes induced by the electrical and electromagnetic stimulation should be crucial for nerve regeneration.

It is said that both electrical and electromagnetic stimulation applied to the skin produces the electrical field, magnetic field and electrical current in the subcutaneous tissues[15]. It was also reported that the electrical current, electrical field and magnetic field affect permeability of various kinds of ions through plasma membrane[16]. Of these, Ca^{2+} might be very important, because Ca^{2+} regulates various kinds of important events in nerve regeneration such as elongation through addition of new membranes and pathway finding through adhesion molecules in the regenerating axons[17]. Thus, in the regenerating axons, especially in the growth cones, the intracellular Ca^{2+} level

should be accurately controlled[18,19]. It was reported that the density of Ca^{2+} channels in the growth cones is extremely higher than that in the axonal shaft of the regenerating axons and normal myelinated axons[20], and also that the distribution of voltage-dependent Ca^{2+} channels was affected by the electrical and electromagnetic stimulation[16,20]. It is possible that the excessive influx of Ca^{2+} activates the Ca^{2+}-dependent proteases existing in the axons, by which the components of the growth cones are degraded. This might be one of the reasons why the growth cones are very sensitive for the electrical and electromagnetic stimulation.

To determine the effects of the electrical and electromagnetic stimulation on functional recovery of the denervated muscles after the peripheral nerve injury, not only the effects of electrical and electromagnetic stimulation on nerve regeneration, but also the effects of the stimulation on denervated muscles should be assessed. Because the functional recovery of the denervated muscles is largely depend the degree of muscle atrophy that takes place during the nerve degeneration and nerve regeneration. Several authors reported that the muscle contraction induced by the direct electrical stimulation on denervaed muscles is useful to prevent the muscle atrophy and to preserve the normal properties of the muscles[21-26]. By the clinically applied electrical stimulation (electrical current: 50mA, wave form: rectangular, pulse rate: 1 pulse/sec, pulse duration: 300msec), used also in the present study, enough contraction was observed in the mouse thigh muscles. While, the destructive changes occurred in the regenerating axons after the electrical stimulation was relatively mild and transient. In contrast, after the electromagnetic stimulation even at 30% level, by which only faint muscle contractions were induced, the destructive changes occurred in the regenerating nerve were much severer than those after the electrical stimulation. At 10% level, although distinct destructive changes were not detected in the growth cones, enough muscle contractions were not induced. These findings suggest that the electromagnetic stimulation at the level which can produce efficient muscle contraction might be much more harmful for the nerve regeneration than the electrical stimulation Further animal experiments are necessary to establish the effective parameters and treatment method of the electrical and electromagnetic stimulation for clinical application in the physical therapy.

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