

Original paper

Repair of peripheral nerve defect by direct gradual lengthening of the distal nerve stump in rats –*Cellular reaction*

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Running title: Direct gradual lengthening of distal nerve

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Abstract

We investigated the effects of direct gradual lengthening of the distal stump of a peripheral nerve and subsequent nerve regeneration in rats. A segment 10 mm long was resected from rat sciatic nerve. The distal nerve stump was fixed to a ring and pulled directly at a rate of 1 mm/day using an original external nerve distraction device. After distraction for 10, 15, and 20 days, the lengthened nerves were evaluated macroscopically and immunocytochemically. At day 20, the mean (SD) distances from the ring to the 3 mm and 6 mm distal part, which were marked with sutures on the epineurium, were 7 (0.5) mm and 12.1 (0.5) mm, respectively, and the number of Schwann cells in the lengthening group had increased to twice that of control group. The distal stump of a peripheral nerve including the epineurium, endoneurium, and proliferation of Schwann cells can be lengthened directly. This method also made it possible to lengthen the nerve stump longitudinally and to control both the rate and distance. We think that this method may be used in the treatment of peripheral nerve injury.

Key words: peripheral nerve, lengthening, distraction, Schwann cell proliferation

Introduction

The current method of repair of segmental nerve defects that cannot be anastomosed directly is autogenous nerve grafting. However, nerve grafting has some disadvantages, such as the need to harvest a healthy nerve. Sensory nerves such as the sural nerve are usually used as a donor; however, sensory loss and occasionally a painful neuroma and the donor site morbidity (scarring) is possible. A nerve graft has no vasculature and the regenerated nerve has to cross two anastomoses to the end organ. In addition, the length of the nerve that is suitable for grafting is limited. Other techniques, for example, artificial or bioartificial nerve grafting, have been investigated, but the results of these techniques are not always satisfactory.

In recent years, nerve lengthening technique has been studied for the repair of segmental nerve defects. The lengthening of nerve stumps can be accomplished by two methods: one is by indirect lengthening using a tissue expander [1-10], and the other is by direct, longitudinal lengthening of the nerve stump using an external nerve distraction device [11-13].

The indirect lengthening using with a tissue expander has been tested for both proximal and distal nerve stumps. However, it does have some problems, in that, a tissue expander may press the nerve stump itself during expansion[2,4,5,9], and it is difficult to control the rate of lengthening.

However, there have been some reports about direct lengthening of a proximal nerve stump[11-14]. Direct lengthening using a nerve distraction device enables the control of the rate of longitudinal nerve lengthening. Saijilafu et al. reported that the degree of regeneration of the nerve after repair by direct of the lengthening proximal stump was equivalent to that achieved by nerve grafting [14].

Direct lengthening of a distal nerve stump has not previously been studied. After peripheral nerve injury, Wallerian degeneration occurs in a distal nerve stump. This is followed by the proliferation of Schwann cells inside the basal lamina that surrounds the original nerve fiber; these columns of Schwann cells that are surrounded by the basal lamina are known as

endoneurial tubes, which are also called the bands of Büngner. It can provide the environment for promoting axonal regrowth¹⁵.

The purpose of this study was to design an effective and simple peripheral nerve distraction device and to investigate the effects of direct gradual lengthening on a distal nerve stump.

Material&Methods

Animals

Fifty seven male 12-week-old Wistar rats (weighing 380 - 450 g) were used. They were housed in small groups in cages with solid floors and soft bedding, and maintained according to the guidelines of the Ethics Committee of the University of Tsukuba.

Surgical technique

The rats were anaesthetized by injection of pentobarbital sodium (40 mg/kg i.p.). A skin incision was made aseptically in the left thigh. The overlying gluteal muscles were exposed and retracted, and the sciatic nerve exposed. We prevented injuring the vasculature when exposing the nerve from its surrounding tissues. A nerve segment 10 mm long was resected from the mid thigh of each rat. Two stainless steel half-pins were inserted into the femur perpendicularly, and a nerve distraction device attached to them. To prevent the stump of the proximal sciatic nerve from retracting spontaneously, it was sutured to the adjacent muscle fascia. We placed 4/0 nylon sutures through the end of the distal nerve sciatic stump and fixed it to a ring. The traction suture (3/0 polyester) attached to the ring was passed through the soft tissues and skin through a small stainless steel pipe and then attached to the external nerve distraction device (Figure 1a). The distance between the sciatic nerve stumps was set at 10 mm. Marking sutures (10/0 nylon) were placed on the epineurium at 3 and 6 mm from the ring (Figure 1b). The wound was then closed with 4/0 nylon sutures. The rats were allowed to recover from the anesthetic and were then given a standard diet and allowed to move freely in their cages. These techniques were done under an operating microscope (Olympus Instruments, Japan).

Nerve distraction

In the lengthening group, The distal sciatic nerve stump was distracted from the next day after the operation at the rate of 1 mm/day. Under sodium pentobarbital (20 mg/kg intraperitoneally) anesthesia, the nerve was distracted at the same time daily. In the control group, nerves were not lengthened after the operation. To prevent infection, attachments such as external fixators were sterilized, and gauze bandages were changed daily.

Macroscopic assessment of lengthened nerve

At 10, 15, and 20 days after the initiation of nerve lengthening, the sciatic nerve was exposed under sodium pentobarbital anesthesia. To evaluate the lengthening distance, the nerves were observed macroscopically (n=6 for each). The distances between the ring and each marking suture and both marking sutures were measured (Figure 1b (a, b)). The overlapping length of both sciatic nerve stumps was also measured (Figure 1b (c)).

Electron microscopic analysis

Twenty days after the initiation of lengthening, of the nerve, it was exposed under sodium pentobarbital anesthesia. After perfusion with 4% paraformaldehyde to the left ventricle, 15 mm portions of the lengthened sciatic nerve were harvested just distal to the ring (n=3). The specimens were further fixed in 10% formalin containing 1% glutaraldehyde and 1 % sucrose overnight at 4°C. The next day the tissue was postfixated in 1% osmium tetroxide for two hours, dehydrated in a graded ethanol series, and embedded in Epon 815 (TAAB, Aldermaston, England). Ultra-thin cross sections were cut and viewed using a Hitachi H-7000 electron microscope after staining with uranyl acetate and lead citrate solutions

Immunocytochemical analysis

At 10, 15, and 20 days after the initiation of lengthening of the nerve, it was exposed under sodium pentobarbital anesthesia. After perfusion with 4% paraformaldehyde to the left ventricle, 15 mm portions of the lengthened sciatic nerves were harvested just distal to the ring. The specimens were immediately fixed in 4% paraformaldehyde overnight at 4°C. The next day, the

specimens were dehydrated in a graded sucrose series and quickly frozen in a Tissue-TeK[®] optimal cutting temperature (OCT) compound (Miles, USA). Serial sections of 8 μ m thick were cut longitudinally using on a Cryostat and mounted on gelatin-coated slides and stored at -20°C . The sections were rinsed in phosphate buffered saline (PBS) and incubated in cold acetone for 10 minutes, and endogenous peroxidase activity was inhibited by incubating the sections in 0.3 % hydrogen peroxide in methanol for 30 minutes.

Immunocytochemical identification of Schwann cells

To find out whether the whole sciatic nerve, including the epineurium and endoneurium, could be lengthened, the distance from the distal end of the ring to the tip of a Schwann cell was measured at 10, 15 and 20 days after nerve lengthening (n=6 for each).

Schwann cells were visualized by immunocytochemical staining for S-100 protein using the avidin-biotin complex method. The sections were then incubated with goat serum for 20 minutes to prevent non-specific staining. The sections were incubated at room temperature with a polyclonal rabbit anticow antibody for S-100 (Dako, Japan) for one hour. The antibody was diluted at 1:600 in 0.01 M PBS with a pH of 7.2. The sections were washed in PBS and incubated with the second antibody (biotinylated goat antirabbit IgG (1:150)) for 30 minutes. After an additional washing in PBS, peroxidase conjugated avidin-biotin complex (Elite ABC kit, Vectastain[®], Vector laboratories, USA) was applied for 30 minutes. The sections were washed in PBS, stained with diaminobenzidine (DAB) (Pierce, USA) for one minute, washed again, contrast-stained in Mayer HTX, dehydrated with ethanol, and coverslipped with EUKITT[®] (O, Kindler, Germany). Negative controls were prepared by omitting primary antibodies in the parallel sections.

Proliferation of Schwann cells

To elucidate the cellular mechanisms of nerve lengthening, we analyzed the proliferation rate of Schwann cells at 10, 15 and 20 days after nerve lengthening. Six rats at each time point were killed and longitudinal sections of sciatic nerve were used for the immunocytochemical staining

for S-100, proliferating cell nuclear antigen (PCNA), and cell nuclei.

PCNA is widely used as a marker of proliferative activity with the endogenic protein appearing from the their develops DNA synthesis period. The cell nucleus was seen by immunocytochemical staining with Sytox green. Triple immunohistochemical staining for consistency was done using the secondary antibodies, namely, Alexa Fluor[®] 546 goat antirabbit IgG (H+L) conjugate (Molecular Probes, Inc., USA) for S-100, Cy5 for PCNA, and fluorescein isothiocyanate (FITC) for the cell nucleus. The sections were incubated with goat serum to prevent non-specific staining and incubated for one hour at room temperature with a polyclonal rabbit anti-cow antibody for S-100 (DAKO, Japan). The antibody was diluted at 1/600 in 0.01M PBS with a pH of 7.2. The sections were washed in PBS and incubated with the second antibody, Alexa Fluor[®] 546 (1:150), for 30 minutes. The sections were then incubated for 20 minutes with horse serum to prevent non-specific staining, and incubated for one hour at room temperature with a monoclonal mouse antiproliferating cell nuclear antigen antibody for PCNA (DAKO, Japan). The sections were washed in PBS and incubated with the second antibody (biotinylated horse anti-mouse IgG (1:150)) for 30 minutes. After additional washing in PBS, the peroxidase conjugated avidin-biotin complex (Elite ABC kit, Vectastain[®], Vector laboratories, USA) was applied for 30 minutes. The sections were washed in PBS, and stained with Cy5 for 10 minutes. Finally, the sections were washed in PBS and counterstained with Sytox green for 10 minutes. The stained sections were mounted using the Slow Fade[®] Antifade kit (Molecular Probes, USA).

The proliferation rate of Schwann cells was expressed as (the amount of PCNA immunohistochemical activity in the Schwann cell nuclei) $\times 100 /$ (total number of Schwann cell nuclei). In the 3 and 6 mm portions from the distal end of the ring, we divided the series of 50 sections equally on ten slides and selected one of the sections randomly. The widest sections on each slide were used to count the number of S-100-stained, PCNA-stained, and nuclei-stained Schwann cells. The sections were examined using a Leica TCS SP2 confocal laser

scanning microscope. Under the same magnification, one photomicrograph was each taken at the centers of the 3 and 6 mm portions from the distal end of the ring.

Statistics analysis

All data are presented as mean (SD). We used a functional analysis of variance (ANOVA), and the significance of differences between groups were calculated using the post hoc test according to Fisher's protected least significant difference (PLSD), which allowed the assessment of individual differences. A probabilities of less than 0.05 were accepted as significant.

Results

Macroscopic assessment of lengthened nerve

At each time point, a lengthened sciatic nerve was loosely stuck to the underlying muscles with a transparent connective tissue, but it was easy to separate the nerves from the surrounding connective tissue. The distance between the distal end of the ring and each marking suture was increased in proportion to the period of distraction (Figure 2a). At 20 day, the distances from the ring to the 3 mm and 6 mm marking sutures were 7.1 (0.5) mm and 12.1 (0.5) mm, respectively. The overlapping lengths of the proximal nerve and the distal nerves were also increased in proportion to the period of distraction. At day 20, the distal nerve stump overlapped to the proximal nerve stump by 6.8 (0.6) mm (Figure 2b).

Electron microscopic analysis

The basal lamina of Schwann cells remained intact and the cells retained their original tubular configuration after lengthening of the nerve for 20 days (Figure 3).

Immunocytochemical analysis

Immunocytochemistry of Schwann cells

At each period of nerve lengthening, Schwann cells were stained longitudinally up to the end of the ring (Figure 4).

Schwann cell proliferation

Triple immunofluorescent staining and confocal microscopy were used to analyze the rate that Schwann cells proliferated. These were distinguished as cells with the cytoplasm stained blue for S-100 and the cell nucleus stained orange for PCNA and with Sytox green (Figure 5a, b). As shown in Figure 6, proliferating Schwann cells were seen in both groups on days 10 and 15. On day 20, the rates of proliferation of Schwann cells in the lengthening group were higher (41.5 (13.1)% in the 3 mm portion and in the 6 mm-portion 37.1 (8.6%)) than that in the control group (21.4 (7.3)% in the 3 mm portion and 17.5 (4.5)% in the 6 mm portion); both differences were significant ($p < 0.05$).

Discussion

We have developed an original device for distracting nerves. We attached a ring to the distal sciatic nerve stump, then bound distraction threads to the ring and distracted the nerve using an external fixator. This method made it possible to lengthen the nerve stump longitudinally and to control the rate and distance of lengthening. Our pilot study has shown that when only the epineurium of the nerve stump was sutured to the ring, the whole nerve stump (including the perineurium and endoneurium) could not be lengthened; only the epineurium. We therefore sutured the entire distal nerve stump to the ring including its epineurium and endoneurium. S-100 positive Schwann cells were stained throughout the lengthened nerve and the basal lamina of Schwann cell remained intact after lengthening of the nerve. These results showed that the whole nerve, including the epineurium and endoneurium, had been lengthened.

In this study we set the lengthening rate at 1 mm/day. The clinical safety rate of nerve lengthening for a human peripheral nerve is 1 mm/day for leg lengthening. Our previous study has shown that changes of deterioration have not been seen in femoral lengthening in rats at 1 mm/day [16].

We tried to clarify whether direct distraction of the distal nerve stump could be possible, and

investigated the influence of nerve lengthening on the Schwann cells of the distal nerve stump.

At each time point of nerve lengthening, a distracted nerve was usually loosely adhered to the underlying connective tissue that was easily separated although the distal nerve stump adhered to the ring. These results suggest that the ring and nerve stump adhered sufficiently to withstand the distraction force.

To evaluate the lengthening rate of the distal nerve stump, we marked the nerve stump at 3 and 6 mm distal to the ring using marking sutures. The distance between the distal end of the ring and each marking suture and that between both marking sutures, were measured. Each distance increased proportionally according to distraction period. At day 20, the distances from the ring to the 3 mm and the 6 mm marking sutures were 7.1 (0.5) mm and 12.1 (0.5) mm, respectively. This suggests that the lengthening rate could be controlled by distraction period. The overlapping length between the proximal nerve stump and the distal nerve stump was also increased in proportion to distraction period. At day 20, the total lengthening distance of the distal nerve stump was 16.8 (0.6) mm, so that the distal nerve stump and the proximal nerve stump had an overlap length 6.8 (0.6) mm. This indicates that the technique could sufficiently elongate the nerve stumps for us to be able to anastomose them to each other directly without tension.

At day 20, the rate of proliferation of Schwann cells was significantly higher in the lengthening group than in the control group. Previous reports have shown that the rate of proliferation of Schwann cells and the appearance of Schwann cell migratory promoting activity in extracellular fluid were increased considerably at 7~14 days post-transection, and returned to the control level later [17, 18]. Our study has shown that the number of proliferating Schwann cells increased significantly in a lengthened nerve at day 20. We speculate that Schwann cell proliferation might have occurred not only as the result of Wallerian degeneration but also of nerve lengthening. The increase in both the distance from the marking suture to the ring and the rate of Schwann cell proliferation may indicate that histological neogenesis occurred in response

to distraction of the nerve.

We anticipate that the lengthening of a nerve during Wallerian degeneration by direct lengthening is a new procedure for the repair of a segmental nerve defect. Further studies are required to apply the method for the treatment of a peripheral nerve segmental defect in rats.

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Figure legends

Figure 1. (a) The original design of our nerve distraction device. The end of the distal nerve stump was fixed to a ring with a 3/0 nylon suture (asterisk). The traction suture (arrow) was inserted through the stainless pipe (arrow head), and then fixed to distraction device. The silver rectangular structure (double arrow head) was the scale 10mm long for nerve gap. (b) Diagram of the nerve lengthening technique. Distance between ring and suture at 3 mm mark (a) and distance between both marking sutures (b). Overlapping length of proximal and distal nerve stumps (c).

Figure 2. Open bars = 0-3 mm segment; Solid bars = 3-6 mm segment. (a) Relation between distraction periods and lengthening distance for each marking suture. The distance between the distal end of the ring and each marking suture was increased in proportion to the period of distraction. The distance from the ring to the 3 mm marking suture increased significantly compared with the distance from the 3 mm marking suture to the 6 mm marking suture at both days 15 and 20 ($p < 0.05$). Bars indicate SD. (b) Overlapping length of distal and proximal nerve stumps. At day 10 both nerve stumps were lengthened near each stump and overlapping length was then increased in proportion to the period of distraction. $*p < 0.05$

Figure 3. Electron microscopic image showing transverse section of the lengthened nerve (6 mm away from stump). At day 20, the basal lamina (arrow) remained in the lengthened nerve (scale bar: 4 μm).

Figure 4. Distance from the distal end of ring (black line) to tip of the Schwann cells (arrow). At day 20, the Schwann cells were stained longitudinally up to the ring (Scale bar: 200 μm).

Figure 5. Triple immunohistochemical staining of Schwann cells, proliferating cells, and nuclei in the lengthened nerve at day 20 after distraction. (a) lengthened group, (b) control group. Schwann cells stained blue for S-100. The nucleus of proliferating cells was stained red for proliferating cell nuclear antigen (PCNA) and the nuclei of all cells were stained with Sytox green. Proliferating Schwann cells were labeled for PCNA and distinguished as cells with an

orange nucleus. Many more orange nuclei were seen in the lengthening group compared with the control group at day 20 after distraction (Scale bar: 40 μm).

Fig. 6: Relation between distraction periods and rate of proliferation of Schwann cells. Schwann cell proliferation rate was expressed as $(\text{the number of Schwann cell nuclei that stained for proliferating cell nuclear antigen (PCNA)}) \times 100 / (\text{total number of Schwann cell nuclei})$ (a) 3 mm part, (b) 6 mm part. In each group, proliferating Schwann cells were seen during each period. At day 20, there were significantly more proliferating Schwann cells in the lengthening group than in the control group ($p < 0.05$). Open bars = control group; Solid bars = lengthening group.