

Effects of Peripheral Neuropathy on CTB-labeled Motor Neurons Following Ligation of the Tibial Nerve

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Abstract : Changes in CTB labeled motor neurons of the spinal cord were observed after the induction of peripheral neuropathy by ligation of the tibial nerve.

Rats were anesthetized and the tibial nerve was ligated with 3-0 silk. The rats were separated into three groups based on the length of time the tibial nerve was ligated (1, 2, or 4 weeks). After the ligation procedures were complete, the tibial nerve stumps were soaked in CTB solution. Tibial nerve segments and the spinal cord were then observed.

In the control and experimental groups, CTB-labeled neurons formed a discrete population that was concentrated primarily at the L5 level, while the contributions from L4 and L6 were minor. According to the distributions, CTB-labeled neurons were divided into rostral and caudal groups. A selective decrease of CTB-labeled neurons was observed only in the caudal group, extending from the rostral L5 to one-half of the rostral L6. The total numbers of CTB-labeled motor neurons were $2,160 \pm 169.3$, $1,002 \pm 245.1$, 587.5 ± 346.5 , and $1,728 \pm 402.6$ in the control group, 1 week group, 2 week group, and 4 week group, respectively. The selective decrease of CTB-labeled neurons in the caudal division was responsible for the decrease in the total number of labeled neurons in all groups.

Following peripheral neuropathy caused by ligation of the tibial nerve, CTB-labeled neurons in the spinal cord decreased selectively. These results may provide important neuroanatomical data regarding the effects of peripheral neuropathy by ligation of the tibial nerve.

Keywords : Peripheral neuropathy, CTB (cholera toxin B subunit), Motor neuron, Spinal cord, Tibial nerve

Introduction

Although peripheral neuropathy was first described over a century ago, there is little information about its pathogenesis (Campbell et al. 1988, Kajander et al. 1990). To understand the pathophysiological mechanisms of peripheral neuropathy, three experimental animal models for causalgia were developed using rats. The first model was produced by placing four loose ligatures around the sciatic nerve (Bennett and Xie 1988), the second model involves a tight ligation of the sciatic nerve (Seltzer et al. 1990),

and the third model involves complete ligation of the L5 or L6 spinal nerves (Kim and Chung 1992). Together, these three models mimic the major clinical symptoms of peripheral neuropathy. However, most of the experiments performed using these models have been physiological studies, and there have been only a few reports concerning the anatomical changes to the sensory neurons of experimental animal models (Kim et al. 2009). Thus, the present study may be the first descriptive study regarding the neuroanatomical changes in spinal motor neurons in a peripheral neuropathy model.

For this study, we selected the tight ligation model of Seltzer et al. (1990). The conditions specific to this model are easier to produce than ligation of the L5 or L6 spinal

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nerve, and the results are more definitive than those of the loose ligation model. We modified the Seltzer et al. model by selecting the tibial nerve instead of the sciatic nerve for two reasons: first, the sciatic nerve is composed of three branches, and ligation of a single branch is more effective than ligation of the entire sciatic trunk. Second, the tibial nerve is the largest branch of the sciatic nerve and is easier to distinguish and handle than are the other branches.

In studies of motor neurons, cholera toxin B subunit (CTB) has been shown to be a better qualitative labeling tracer than other neuronal tracers such as horseradish peroxidase (HRP), fluorescent tracers, or viral tracers (Kubek et al. 2004). CTB reveals an acceptable motor neuron count with satisfactory localization and identification of the cell bodies and dendrites.

The aim of our study, therefore, was to observe changes in CTB-labeled motor neurons of the spinal cord after tight ligation of the tibial nerve.

Materials and Methods

1. Animals

Male Sprague-Dawley rats (specific pathogen free, 9~10 weeks old; Damool Science, Taejeon, Korea) weighing between 290 and 330 grams at the beginning of the study were used. The experimental and control groups each consisted of six rats.

2. Peripheral neuropathy model

Animals were anesthetized (7% chloral hydrate, 0.5 mL/100 g body weight, intraperitoneal injection) and the right tibial nerve was exposed. In the rat, the sciatic nerve trifurcates into the tibial, peroneal, and sural nerves. Only the tibial nerve was ligated with 3-0 silk at the level of trifurcation of the sciatic nerve. The ligation was performed through the application of three tight ligatures spaced 1~2 mm apart. The rats were separated into three groups based on the length of time the ligation was maintained (1, 2, or 4 weeks). All experimental procedures and animal care were in accordance with the Chonbuk National University of Health guide for the care and use of laboratory animals.

3. Labeling technique

The tibial nerve was transected with scissors about 5

mm distal to the ligation site, and the proximal end of the tibial nerve stump was soaked for approximately 5 min in 5 μ L of CTB solution (List Biological Laboratories Inc., Campbell, CA, USA). Following exposure to CTB, the nerve was returned to its original anatomic position. In the control group, CTB was introduced at the level of the tibial nerve as in the experimental groups, approximately 10~15 mm distal to the trifurcation of the sciatic nerve.

4. Histologic preparations of the tibial nerve and the spinal cord

Rats were returned to normal conditions for 72 hours to allow for transport and accumulation of CTB in the spinal motor neurons. After the rats were sacrificed, a transcardial perfusion was performed with saline and 4% paraformaldehyde (Merck, Darmstadt, Germany). The tibial nerve was subsequently identified, dissected, and cut into three segments along its length: proximal tibial (superior to the proximal-most ligature), midtibial (within the ligature site) and distal tibial (inferior to the distal-most ligature). The tibial nerve segments were embedded in paraffin after dehydration and clearing. Transverse tissue sections (4 μ m thick) were stained with hematoxylin & eosin.

The spinal cord was exposed through a dorsal laminectomy, and a 1.5 cm portion was excised from the L3-6 region. This portion of the spinal cord was identified and submerged in 20% sucrose in phosphate buffer for 24 h before sectioning. Frozen sagittal sections (40 μ m thick; Cryostat; Leica Microsystems GmbH, Wetzlar, Germany) of the lumbar segment were obtained in serial longitudinal planes. The CTB in the sections was sequentially bound with primary antibody (1 : 1,000; Vector Laboratories, Inc., Burlingame, CA, USA), secondary antibody (1 : 200; Vector Laboratories), and avidin-biotin (Vectastain ABC kit; Vector Laboratories). Diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride (DAB) liquid substrate system; Sigma, St. Louis, MO, USA) was used as the chromogen. Sections were mounted onto gelatinized slides, dried, dehydrated, cleared, and mounted. In our experience, sections that are 40- μ m-thick are the most suitable for reconstructions of rat motor neuron populations. Thinner sections require too many drawings, and thicker sections contain too many overlapping cell profiles that obscure neuronal morphology.

5. Analysis of CTB-labeled neurons in the spinal cord

The counting criteria used for CTB-labeled motor neurons in the spinal cord in this study were modified from the criteria of Swett et al. (1986), in which HRP was used as the tracer. The criteria were as follows: (1) the majority of the cells in the labeled population (>90%) must exhibit a reasonably uniform density of reaction products such that dendrites of motor neurons are observed, (2) the labeling density must be sufficiently intense to obscure the nuclei in many cells, (3) there must be a small number of (<1% to 2%) weakly labeled cells outside the perimeter of the densely labeled population, and lastly (4) there must be a few (<5%) weakly labeled neurons in the central portions of a viewed labeled nucleus.

We collected and counted all sections. The total number of CTB-labeled neurons and the numbers of each spinal cord segment were assessed. This counting method can

overestimate the number of motor neurons, especially presumptive alpha motor neurons, which have a cell diameter of 40~60 μm and can easily appear on two adjacent 40 μm sections. To avoid this potential inaccuracy, we only counted the profiles of neurons that were associated with with dendrites or a nucleus. Student's t-test was used to evaluate the statistical significances of differences in the numbers of labeled cells in the spinal cord.

Results

1. Histological changes in the tibial nerve after ligation

When we exposed the ligated sites in the experimental groups, the silk ties were covered with new connective tissue, and local inflammatory reactions were observed

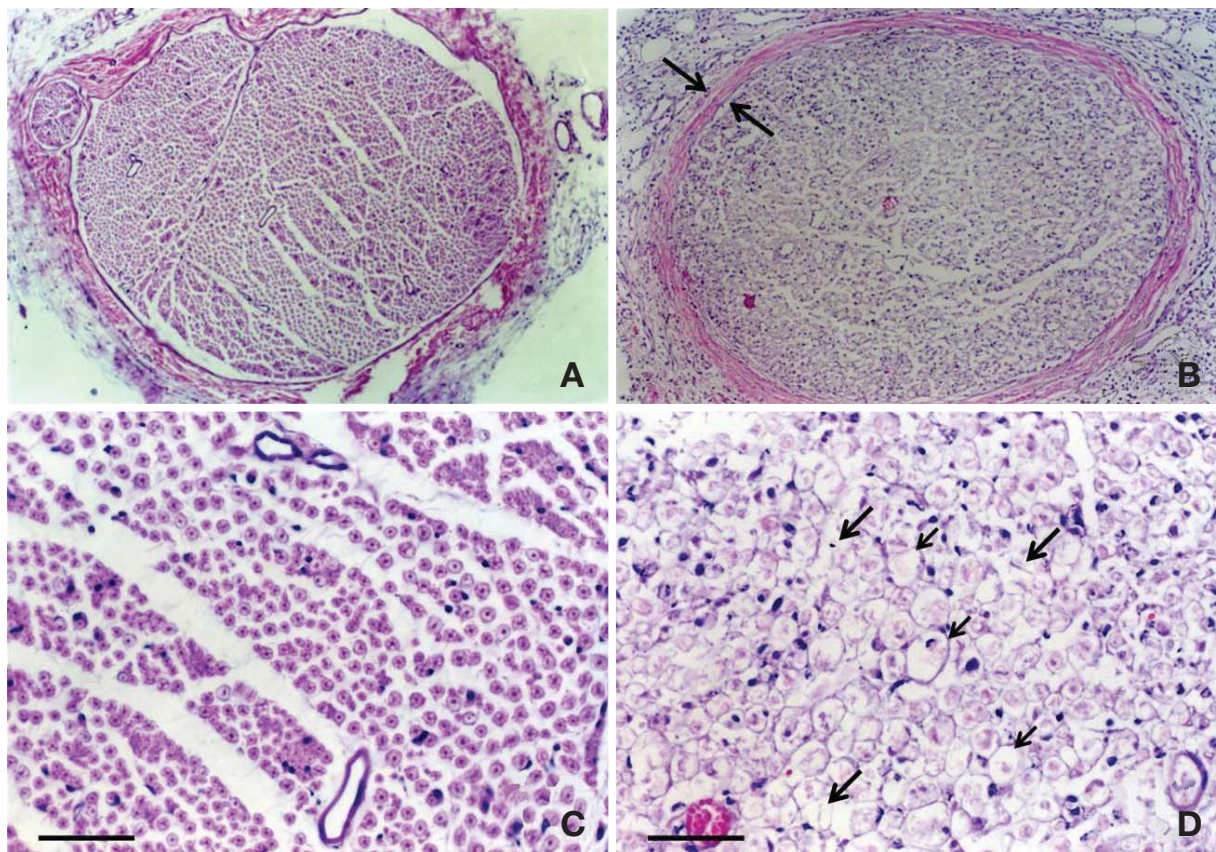


Fig. 1. Cross-section of the tibial nerve with hematoxylin and eosin stain. A, Low magnification of the tibial nerve in the control group; B, Low magnification of the tibial nerve in the 1 week group. Arrows indicate the thickening of the epineurium; C, High magnification of the tibial nerve in the control group (scale bar=100 μm); D, High magnification of the tibial nerve in the 1 week group (scale bar=100 μm). Extensive disruption of the neurilemma (large arrows) and edema (small arrows) are observed.

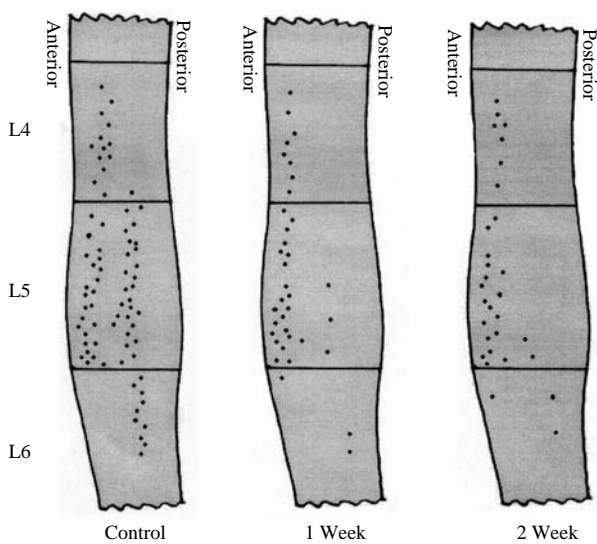


Fig. 2. Distribution of the CTB-labeled neurons in the spinal cord. Every dot represents 30 CTB-labeled motor neurons.

around the nerves. In cross sections of the ligated tibial nerves, the central area of the nerves exhibited a paler appearance than the peripheral portions (Fig. 1B).

The main pathological findings were thickened epineuria (Fig. 1B), extensive disruption of the neurilemma, and edema (Fig. 1D). Fibers were considered to represent axonal degeneration. In some cases, the axon was swollen or shrunken, and typically observed secondary myelin changes included attenuation, collapse, or breakdown (Fig. 1D).

2. Topographic distribution of CTB-labeled motor neurons in the spinal cord

In the control group, the motor neuron population of the tibial nerve was distributed longitudinally in the dorso-lateral portion of the ventral horn from L4 to the rostral half of L6, a distance covering slightly less than three complete spinal segments. The most typical arrangement is shown in Fig. 2, which also shows an accurately scaled longitudinal reconstruction of the right side of the lumbar spinal gray matter in which the motor neurons of each major subdivision of the tibial nerve can be differentiated. In control animals, the cells formed a discrete population concentrated primarily in the L5 segment, while the contributions from L4 and L6 were minor.

Tibial motor neurons formed two large, distinct subnuclear divisions (the rostral and caudal divisions). The rostral

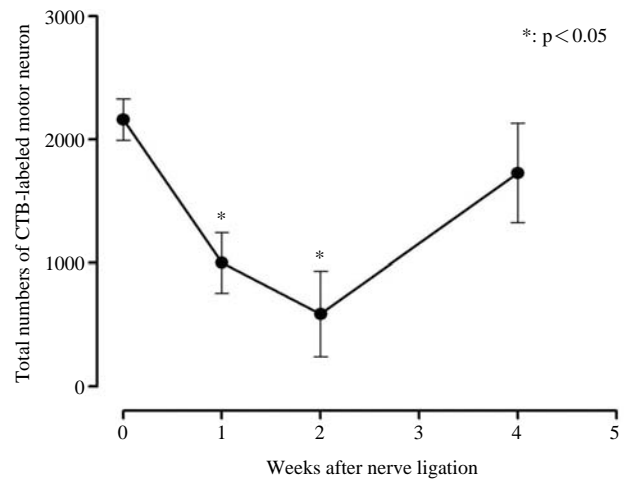


Fig. 3. Total number of CTB-labeled neurons in the control and experimental groups. The values of the 1 week and 2 week groups were significantly different from that of the control value but not from one another ($p < 0.05$). The 4 week group was not significantly different from the control group or either of the other experimental groups.

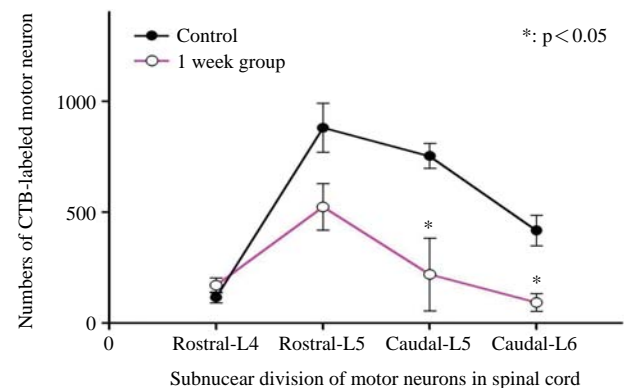


Fig. 4. Number of CTB-labeled neurons of the subnuclear division in the control and 1 week group. The control and 1 week groups were not significantly different in the rostral division but were in the caudal division ($p < 0.05$). The number of CTB-labeled neurons in the caudal division of L5 and L6 in the 1 week group was significantly decreased compared to that of the control group ($p < 0.05$). Rostral 4; L4 segment of the rostral division in the CTB-labeled motor neurons, Rostral 5; L5 segment of the rostral division in the CTB-labeled motor neurons, caudal L5; L5 segment of the caudal division in the CTB-labeled motor neurons, caudal L6; L6 segment of the caudal division in the CTB-labeled motor neurons.

division (Fig. 5A) was less compactly organized, laid rostral to the caudal division, and extended from L4 to L5. Further, the rostral division (Fig. 5A, B) formed a narrower longitudinal column of cells than did the caudal division

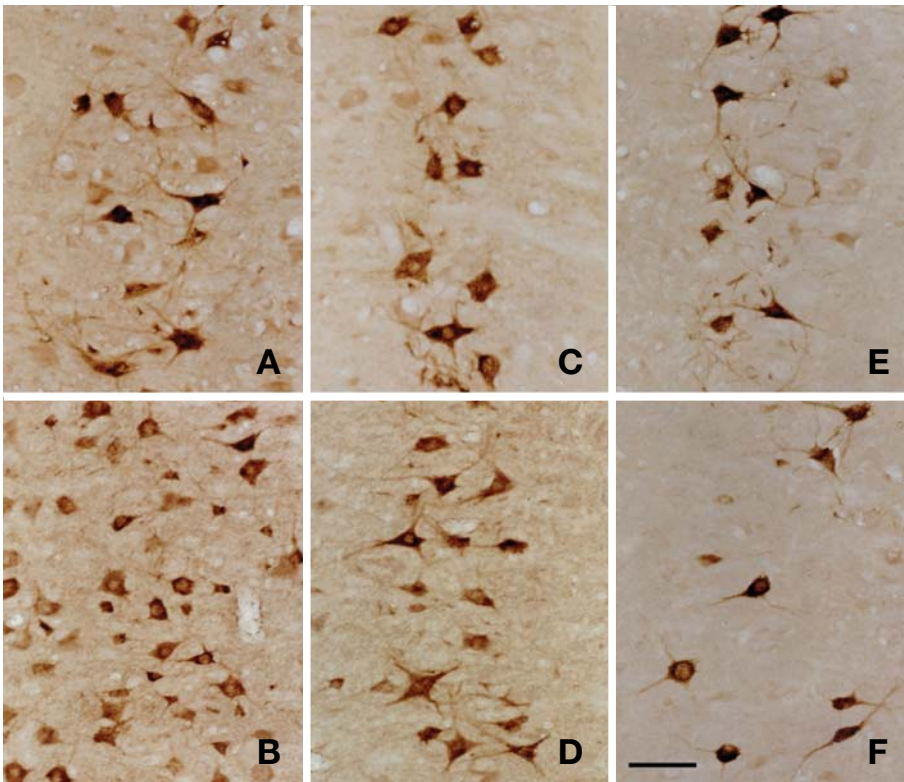


Fig. 5. Light micrographs of CTB-labeled neurons (scale bar=50 μ m). A, L4 segment of the rostral division in the control group; B, L5 segment of the caudal division in the control group; C, L4 segment of the rostral division in the 1 week group; D, L5 segment of the caudal division in the 1 week group; E, L4 segment of the rostral division in the 2 week group; F, L5 segment of the caudal division in the 2 week group.

(Fig. 5B), which was compactly organized, approximately one and one-half segments in length, and extended from the rostral aspect of L5 to the rostral half of L6. The caudal division was maximally developed in L5. The rostral division of L5 lay immediately anterior to the caudal division of L5 as a part of the same longitudinal column of cells. There were no sharp boundaries between the rostral and caudal divisions at the L5 level.

A selective decrease of CTB-labeled neurons was observed in the experimental groups (Fig. 2). Specifically, the rostral division of the motor neurons was labeled from L4 to L5, as shown in the control group (Fig. 5C, 5E). In the caudal division, however, >80% of the CTB-labeled neurons were absent (Fig. 5D, 5F). The changes in the distribution of CTB-labeled neurons after ligation of the tibial nerve are shown in Fig. 2.

3. Number of CTB-labeled motor neurons in the spinal cord

The number of CTB-labeled motor neurons in the spinal cord was determined by counting. Fig. 3 shows the mean \pm standard error of the total number of labeled motor neurons in the spinal cords of the control and experimental

groups. The total numbers of CTB-labeled motor neurons were $2,160 \pm 169.3$, $1,002 \pm 245.1$, 587.5 ± 346.5 , and $1,728 \pm 402.6$ in the control group, 1 week group, 2 week group, and 4 week group, respectively. The 1 week group exhibited a motor neuron loss of 54% compared to that of the control group, while the 2 week group showed a 73% loss. The 1 week and 2 week groups were significantly different from the control group ($p < 0.05$) but not from each other, while the 4 week group was not significantly different from any of the other groups.

Fig. 4 shows the changes in the mean number of labeled motor neurons in the subnuclear division. In the rostral division, there was no difference between the control and 1 week groups. In the caudal division, however, there was an 82% decrease in the number of labeled motor neurons between the control and 1 week groups, and this difference was statistically significant ($p < 0.05$). The number of motor neurons in the caudal divisions of L5 and L6 were significantly decreased in the 1 week group compared to those in the control group ($p < 0.05$). This selective decrease in the caudal division was responsible for the decrease in the total number of labeled neurons in the experimental group.

Discussion

1. Histological changes in the tibial nerve after ligation

The main pathologic findings were thickening of the epineurium, local inflammatory reaction, disruption of the neurilemma, and edematous changes. These findings were similar to the results of partial sciatic nerve transection as reported by Lindenlaub and Sommer (2000). Observations from loose ligation of the sciatic nerve (Bennett and Xie 1988) showed a profound loss of large myelinated fibers and a relative preservation of small myelinated and unmyelinated fibers at and below the lesion site (Coggeshall et al. 1993).

The results of the present study showed that, in a cross section of the ligated tibial nerve, the central portion of the nerve was paler than the peripheral portion. A previous report describing the difference between the center and peripheral portions of the ligated sciatic nerve found that in the loose ligation model (Bennett and Xie 1988), there was an early increase in nerve blood flow within the lesion with persistent hyperemia, followed by ischemia at the lesion edge. Further, their study found that nerve blood flow increased at early time points (2 and 14 days) within the ligatures, while at the lesion edge, measured in close proximity to the ligature, nerve blood flow was reduced to < 50% of normal after 14 days, indicating the presence of nerve ischemia. Presumably, nerve blood flow just underneath the ligature would be even more reduced. A second possible mechanism is that of edge ischemia, which develops between days 2 and 14, and presumably is responsible for ischemic fiber degeneration. The combination of edge ischemia, endoneurial edema, and leukocyte adhesion suggests a role for increased oxidative stress in the mediation of hyperalgesia. Prominent fiber degeneration occurs with the development of edge ischemia and > 90% of fibers show axonal degeneration in the loose ligation model (Bennett and Xie 1988).

2. Topographic distribution of CTB-labeled motor neurons in the spinal cord

In the control group, tibial motor neurons formed a discrete population concentrated primarily at the L5 level, with minor contributions from L4 and L6, when using the CTB tracing method. Our results are in agreement with

those reported by Swett et al. (1986), who used HRP, but differ from the results reported by Kaizawa and Takahashi (1970). Swett et al. (1986) found that sciatic motor neurons were situated in L4, L5, and L6, but they did not observe labeled motor neurons in the caudal half of L6, as did Kaizawa and Takahashi (1970). The other studies related to the tibial nerve did not examine only the tibial nerve, but the entire sciatic nerve.

Our data showed that there was a selective decrease of the caudal division of CTB-labeled motor neurons in the experimental group, the presence of which was contrary to our initial expectations. Specifically, there was no significant change in distribution, cell number, or morphology in the rostral areas between the control and experimental groups; however, in the caudal area, there was a selective decrease with no morphological changes to the CTB-labeled neurons of the experimental group. Kim et al. (2009) reported changes of the sensory neurons in the peripheral neuropathy of rat tibial nerve using WGA-HRP tracing method. In their study, the abundance of sensory neurons was decreased in all levels after ligation of the tibial nerve, but no selective decrease was observed.

Such a selective decrease may be associated with the changes observed to the tibial nerve in the experimental group, such as the paler appearance of the central portion of the tibial nerve as compared to that of the peripheral portion. The ability to selectively block only the peripheral portion of the tibial nerve may confirm the relationship between the peripheral portion of the tibial nerve and the selective decrease of CTB-labeled neurons. Thus, these results may be the first report of changes to motor neurons in the peripheral neuropathy by tight ligation, and may provide neuroanatomical data regarding the neurons of the tibial nerve of the rat.

3. Number of CTB-labeled motor neurons in the spinal cord

The total number of CTB-labeled motor neurons in the spinal cord was $2,160 \pm 169$ in the control group. Swett et al. (1986) found that the total number of motor neurons of the tibial nerve was 982 ± 36 using HRP. Using biotin-dextran and HRP, Todorova and Rodziewicz (1995) reported that the total numbers of motor neurons in the tibial nerve were 962 ± 51 and $1,077 \pm 219$, respectively.

There are several possible explanations for these differences. First, differences in motor neuron counts can result

from the use of different neural tracers. CTB is known to be a more sensitive tracer of motor neurons than others, and the present study is the first report in which CTB was used as a tracer in the tibial nerve. The second possibility involves the "articular branch." Motor neuron pool counts for the common peroneal nerve reported by Todorova and Rodziewicz (1995) differed considerably from those of Swett et al. (1986). Todorova and Rodziewicz (1995) explained these differences by noting the morphology of the sciatic nerve in the mid-thigh. In some cases in the present study, we observed a fourth branch near the branching of the tibial, common peroneal, and sural nerves. As suggested by Todorova and Rodziewicz (1995), application of a tracer at points above and below this branch may account for some of the apparent variability in motor neuron counts. Because there was no information regarding the branch point structure described by Swett et al. (1986), we may have selected a different site for tracer application. The final possibility is that different counting criteria were used. Swett et al. (1986) and Todorova and Rodziewicz (1995) used criteria that were stricter than were the criteria in the present study.

In our study, the 1 and 2 week groups showed a significant difference from the control group, but there was no significant difference between the 4 week group and control group. These results may indicate that there was recovery in the CTB-labeled motor neurons of the ligated tibial nerve, even though ligation of the tibial nerve was maintained for four weeks. Kim et al. (2009) reported that the sensory neurons in the peripheral neuropathy of rat tibial nerve were decreased significantly in the first, second, and fourth weeks and that there were no recovery in CTB-labeled sensory neurons. In the loose ligation model of the sciatic nerve, Sasaki et al. (1997) reported recovery in spite of the persistence of the ligature. They reported that fiber regeneration occurred through the lesion, as indicated by the large number of small myelinated fiber sprouts observed, especially at day 45. Specifically, while there was a relative preservation of unmyelinated fibers at all time points, the abundance of which was highest at day 45.

There have been a few reports about the changes in motor neurons in peripheral neuropathy (Wu et al. 2007, Bril et al. 2010). However, these reports do not consider the exact site of the lumbar segment of the spinal cord. Thus, our study may provide important neuroanatomical data concerning the peripheral neuropathy caused by tibial nerve

ligation.

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정강신경결찰에 의한 말초신경병증이 CTB에 표지되는 운동신경세포체에 미치는 효과

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간추림 : 흰쥐 정강신경결찰로 말초신경병증 모델을 만들고, 신경추적자인 CTB를 정강신경에 묻힌 후 척수에서 나타나는 CTB표지 운동신경세포의 변화를 관찰하였다.

흰쥐의 정강신경을 묶은 후 1주, 2주, 4주간 묶은 실험군을 만들고, 각 기간이 끝난 후에 정강신경에 CTB를 묻혔으며, 척수에서 CTB에 표지된 신경세포체의 개수와 분포의 변화를 관찰하였다.

정강신경을 각각 1주, 2주, 4주간 묶은 실험군과 정상군 모두에서 CTB에 표지된 신경세포체는 주로 척수의 5번째 허리분절에서 관찰되었으며, 4번째 허리분절과 6번째 허리분절에 걸쳐서 관찰되었다. CTB에 표지된 정강신경의 운동신경세포체는 분포부위에 따라서 입쪽부위와 꼬리쪽부위로 나눌 수 있었는데, 입쪽부위는 4번째와 5번째 허리분절에서, 꼬리쪽부위는 5번째와 6번째 허리분절에 분포하였다. 실험군에서 CTB에 표지된 신경세포체의 수는 입쪽부위에서는 정상군과 유사하였지만, 꼬리쪽부위에서는 선택적으로 감소하였는데, 이로 인해 실험군에서 CTB에 표지된 전체 운동신경세포체의 수가 감소하였다.

정상군의 경우 CTB에 표지된 신경세포체의 전체 평균개수는 $2,160 \pm 169.3$ 개였으며, 1주군의 경우 $1,002 \pm 245.1$ 개, 2주군의 경우 587.5 ± 346.5 개, 4주군의 경우 $1,728 \pm 402.6$ 였다. 1주군과 2주군은 정상군에 비해 평균개수가 유의하게 감소하였는데, 이는 꼬리쪽부위의 선택적 감소로 인한 것이었다.

정강신경결찰에 의한 말초신경병증 모델에서는 CTB에 표지되는 신경세포체가 선택적으로 감소한다는 것을 알 수 있었고, 이러한 결과는 신경결찰에 의한 말초신경병증 모델을 사용하는 연구의 신경해부학적인 자료로 사용될 수 있을 것으로 사료된다.

찾아보기 낱말 : 말초신경병증, 정강신경, 운동신경세포, CTB, 척수