

## A Correlative Study of Axonal and Perineurial Regenerations after Crush Nerve Injury in Rat Sciatic Nerve

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### Introduction

Recently, histological and functional studies have been done on the perineurium<sup>14,19</sup>. The perineurium protects the funiculus from mechanical stimuli and acts as a diffusion barrier to substances with specific permeability such as protein<sup>8,12,19</sup>. The interior of the funiculus is maintained in optimum condition by the perineurium and the peripheral nerve is protected from toxic or infectious factors<sup>8</sup>. Various theories have been published as to the genesis of perineurial cells, but the final conclusion is yet to be reached<sup>1,3,4,13</sup>.

Differing from the epineurium and the endoneurium, which also cover peripheral nerves, the perineurium has a specific structure and function. Further research on the regeneration process and the change in permeability will surely contribute to the development of surgery of the peripheral nerve.

The authors conducted the following study: Crush injury was produced on sciatic nerves of Wister rats; then chronological regeneration process of the perineurium was microscopically observed at the site of the lesion for up to 36 weeks; simultaneously, the changes in permeability of the perineurium was observed for the same period of time after crush injury using the fluorescence microscope technique of STEINWALL and KLANZO<sup>17</sup>

### Subjects and Method

The subjects were 40 Wister rats with an average weight of about 200 g. The sciatic nerve was exposed atraumatically using an operating microscope under intraperitoneal pentobarbital anesthesia. A 5 mm length of the nerve was circumferentially crushed for 10 seconds using Pean forceps of the Tajima Hand Surgery Kit. The rats were confined with sufficient space following the operation and compared with the normal control. Study methods 1 and 2 below were used for observation.

**Study 1:** 1, 2, 8 and 36 weeks after injury, the lesions were exposed using the above procedure. Following macroscopic observation and sampling, the samples were prefixed at 4°C for

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Key words: Crush nerve injury, Perineurium, Axonal regeneration.

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12 hours in a 0.1 M cacodyle buffer solution (pH 7.4) which contained 2% paraformaldehyde-2% glutaraldehyde. The samples were washed with the cacodyle buffer solution for one day and postfixed with a 2% osmic acid solution followed by dehydration with ethanol and were then embedded in epon resin.

An ultra-thin center section of the crushed sciatic nerve was prepared using an ultratome. The section was then double-stained using lead acetate and uranyl acetate. A JEM-100 type microscope was used for the observation.

**Study 2:** The lesions were allowed to develop for 1, 2, 8 and 36 weeks after crush injury and were then infiltrated with 0.5 ml of so-called "Evans Blue Albumin" (EBA), which is 5% bovine albumin (Sigma) labeled with 1% Evans Blue (Merk). A 2 cm section of the traumatized sciatic nerve was sampled and fixed with formal-dehyde solution for 24 hours. A 10  $\mu$ m frozen vertical section was prepared in a cryostat and embedded in 50% glycerin. The proximal and distal ends of the lesion was observed by fluorescence microscopy. Details of the above procedures were previously described by STEINWALL, KLANZO<sup>7)</sup> and OLSON<sup>12)</sup>.

## Results

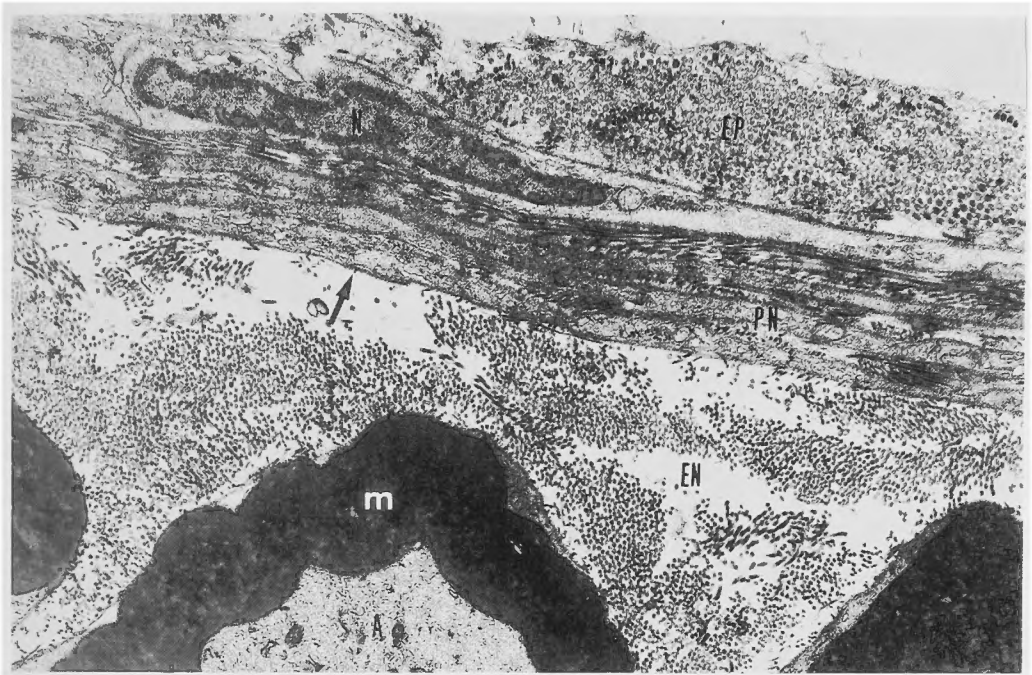
### 1. Study on axonal and perineurial regeneration

Normal perineurium control: Flattened perineurial cells have flat nuclei with many pinocytotic vesicles, but with poor cytoplasmic organellae. These cells were concentrically distributed and had tight junctions between the cellular processes. The total circumference was covered by a continuous basement membrane. Collagen fibrils ran between the layers. The diameter of the collagen fibril of the perineurium was smaller than that of the endoneurium (Fig. 1).

One week after injury: In the periphery of funiculus, cells which had flat nuclei and developed cytoplasmic organella arranged concentrically, composing 4 or 5 layers. There were no tight junctions between the cellular processes and the basement membrane was partially observed. There was an increase in collagen fibrils between the layers (Fig. 2). There were many Schwann cells surrounding the regenerated nerve fibers together with fibers which showed Wallerian degeneration. Myelinated nerve fibers were not observed, however fibroblast-like cells, which had ellipsoid nuclei and developed cytoplasmic organellae, formed protruding compartments around several Schwann cells (Fig. 3).

Two weeks after injury: There were more than 10 concentric layers of cells with ellipsoid nuclei and developed organellae in the periphery of funiculus. However, unlike the observation at first week, basement membrane was not observed in these cells (Fig. 4). Myelinated cells were observed in the funiculi but myelination was not complete. Those cells which formed compartments were more elongated than those of the first week and the organellae were less developed. The maximum number of layers of compartment was two (Fig. 5).

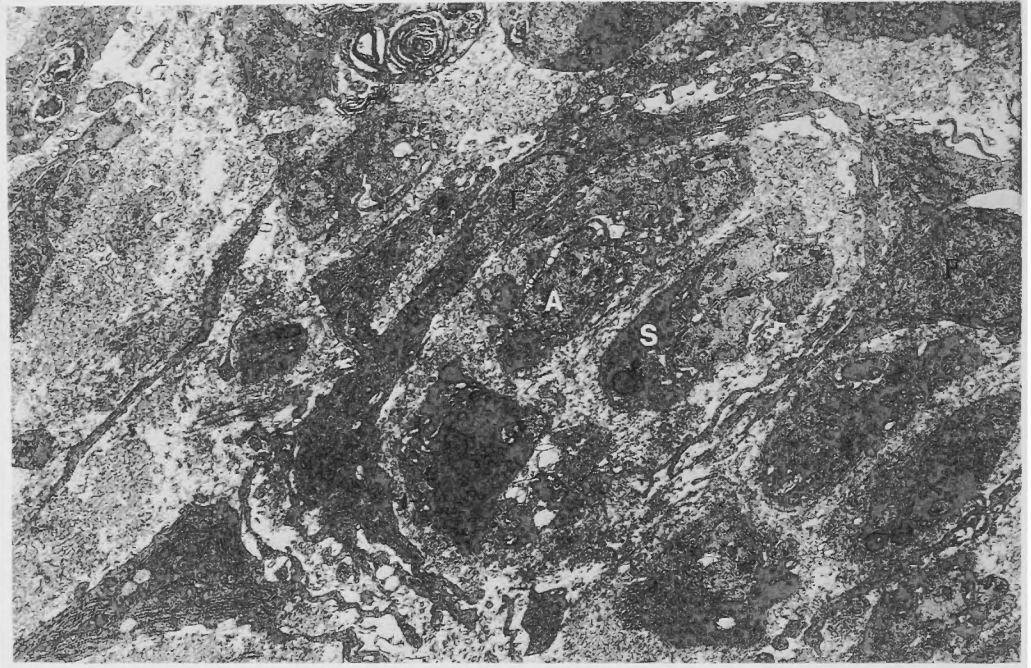
Eight weeks after injury: There were one or two layers of flat cells surrounding the circumference of the funiculus in the periphery of funiculus. These cells had a continuous basement membrane together with numerous pinocytotic vesicles. Morphologically, these cells was observed to be normal perineurium. Almost all the cellular processes showed to be combined



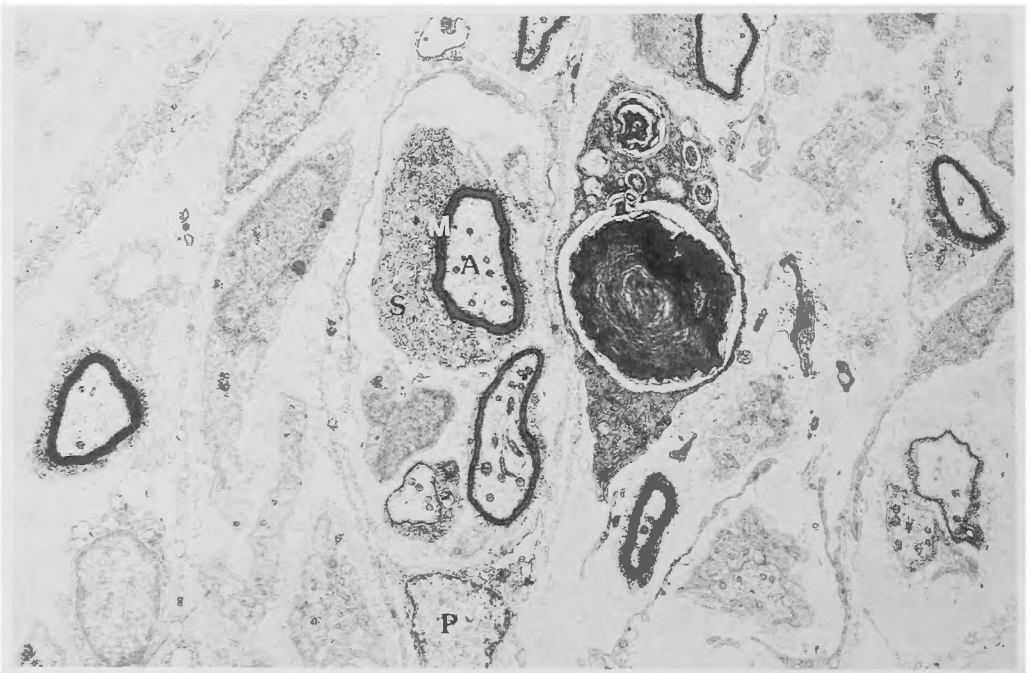
**Fig. 1** Normal perineurium (PN). Perineurial cells which contain a flat nucleus (N) and have basement membranes (arrow) show a concentric arrangement consisting of 5 layers. EP: epineurium, EN: endoneurium, M: myelin, A: axon. ( $\times 5000$ )



**Fig. 2** One week after injury. Fibroblast-like cells (F) show a concentric arrangement consisting of 5 layers in the periphery of funiculus. EN: endoneurium, EP: epineurium. ( $\times 2700$ )



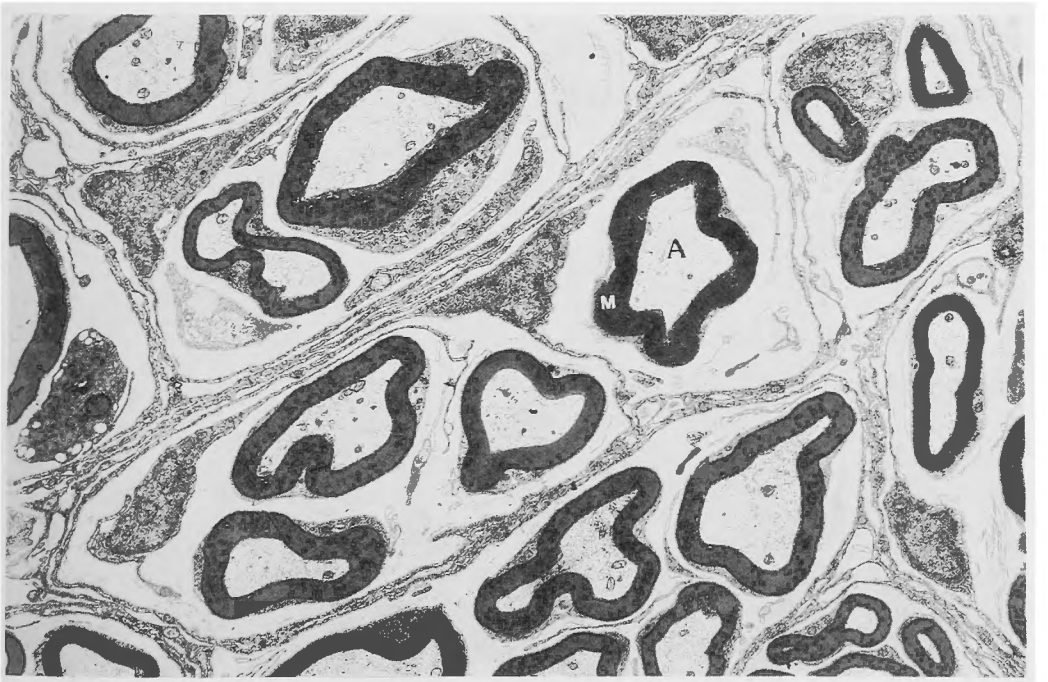
**Fig. 3** One week after injury (in the funiculus). Fibroblast-like cells (F) begin to form the compartment which surrounds the regenerating nerve fibers. A: axon, S: Schwann cell. ( $\times 2700$ )



**Fig. 4** Two weeks after injury (in the funiculus). Cytoplasm of perineurial-like cells (P) which form compartment becomes flat. S: Schwann cell, M: myelin, A: axon. ( $\times 2700$ )

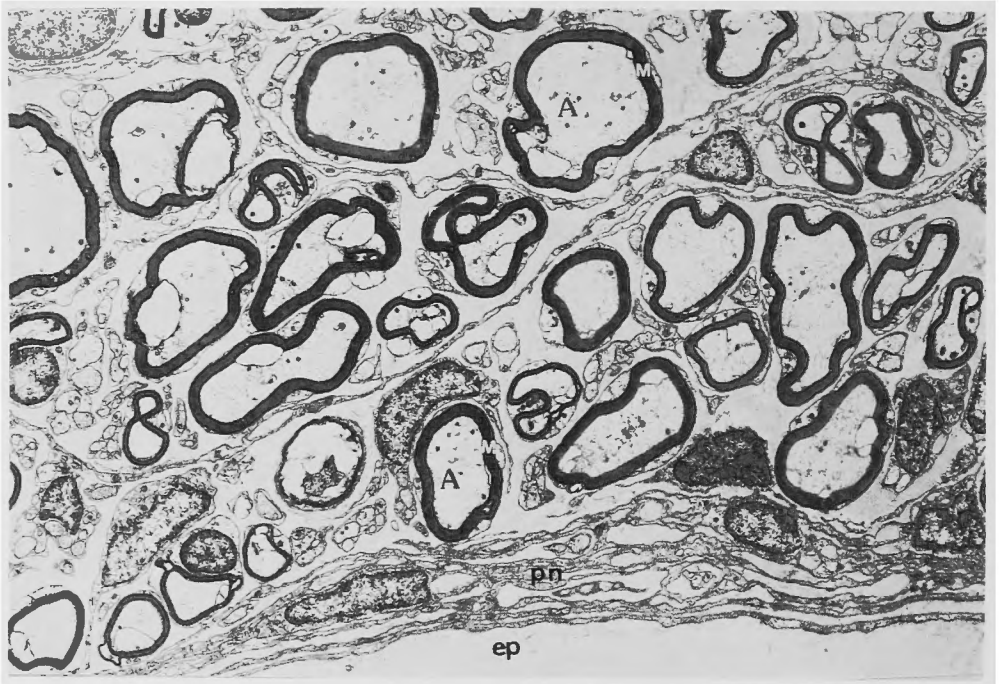


**Fig. 5** Two weeks after injury (higher magnification of Fig. 4). Perineurial-like cell (P) which forms compartment contains poorly developed cytoplasmic organelles. M: myelin, A: axon, S: Schwann cell. ( $\times 5000$ )

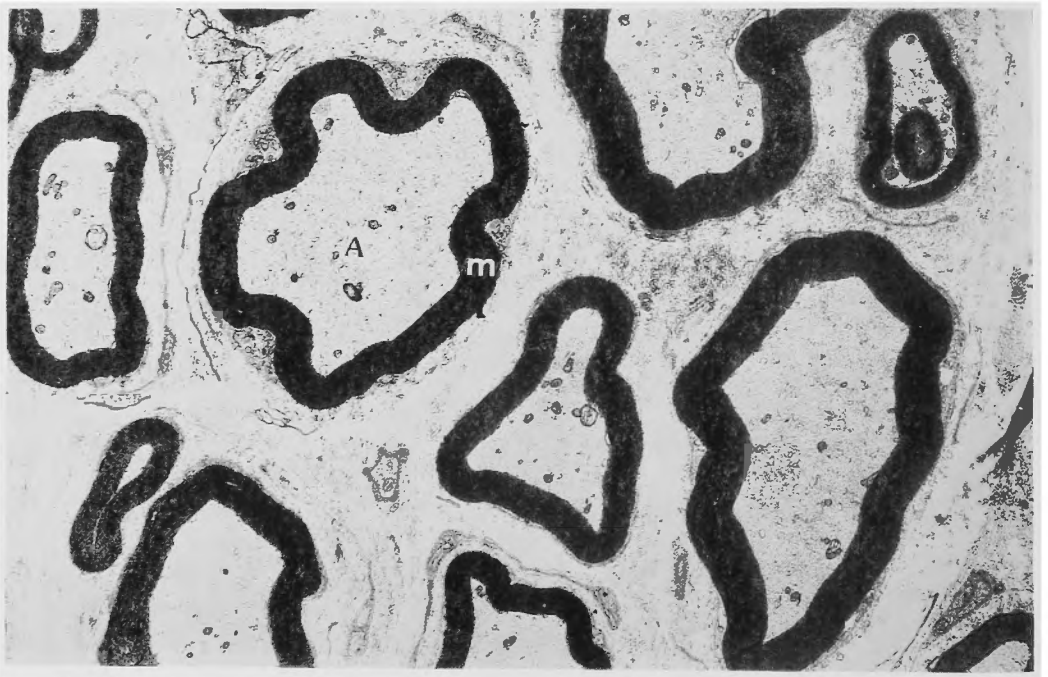


**Fig. 6** Eight weeks after injury. Perineurial-like cells which form compartments have morphological characteristics of perineurial cells. Number of myelinated axons and grade of myelination recover normally. A: axon, M: myelin, P: perineurial cell, S: Schwann cell. ( $\times 2700$ )

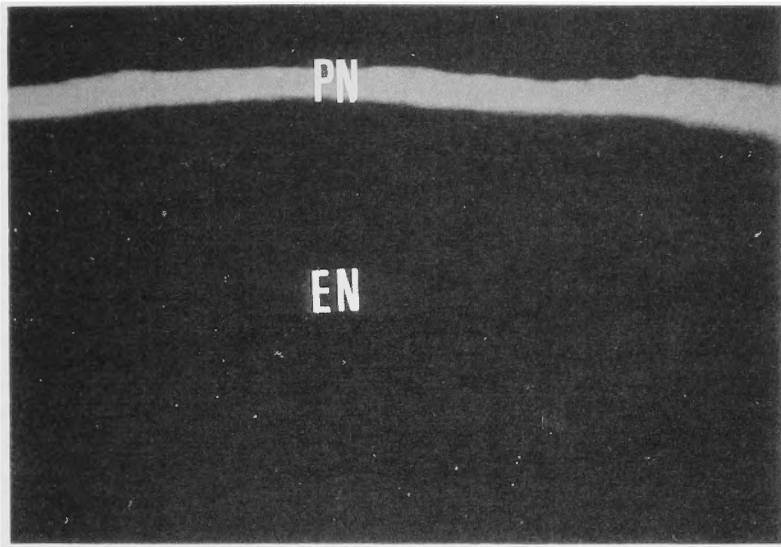




**Fig. 7** Thirty-six weeks after injury. Perineurium cells show a concentric arrangement consisting of approximately 6 layers surrounding the funiculus. Compartments which have various size show a degenerative appearance. EP: epineurium, PN: perineurium, M: myelin, A: axon. ( $\times 2000$ )



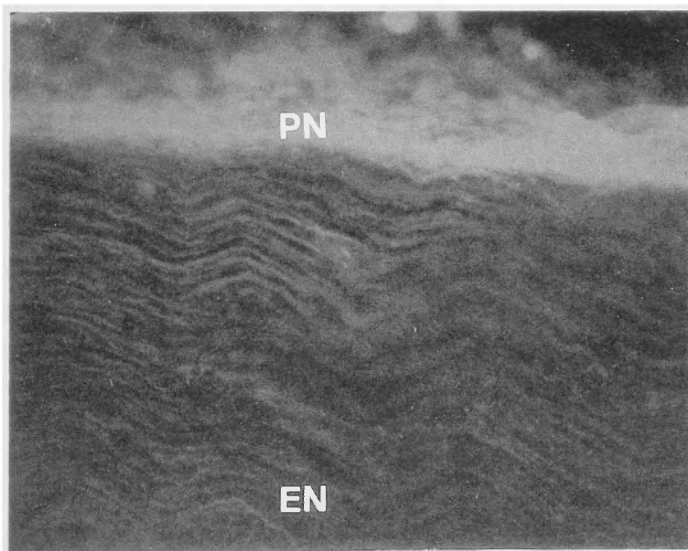
**Fig. 8** Thirty-six weeks after injury. Compartment formation is scarcely seen in the center of funiculus. A: axon, M: myelin. ( $\times 2700$ )



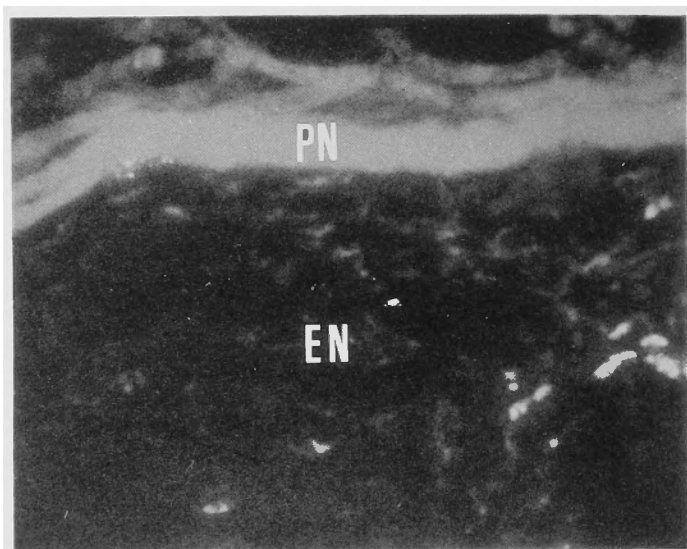
**Fig. 9** Normal rat sciatic nerve. Diffusion of the fluorescent tracer into endoneurium (EN) is prevented by perineurium (PN). ( $\times 100$ )

with tight junctions. The cells composing compartment in the funiculus had numerous pinocytotic vesicles and continuous basement membrane. Morphologically they also appeared to be normal perineurial cells. The number of myelinated fibers and the degree of myelination had recovered to those of the normal control (Fig. 6).

Thirty-six weeks after injury: Concentrically distributed cells which had the characteristics of perineurial cells arranged in about 6 layers. Tight junctions were observed between the



**Fig. 10** Eight weeks after injury. Fluorescent tracer spreads well into endoneurium (EN). PN: perineurium. ( $\times 100$ )



**Fig. 11** Thirty-six weeks after injury. Fluorescent tracer spreads into endoneurium (EN), but the amount of tracer is less than that of 6 weeks. PN: perineurium. ( $\times 100$ )

cellular processes. There were compartments of various sizes within the funiculi and the number of confined nerve fibers in several amounted to more than ten. The size of the compartments tended to increase as they came closer to the center of the funiculus. Among the cells which formed compartments, several cellular processes which had not had any junction with other processes were observed (Fig. 7, 8).

## 2. Study on permeability of perineurium

This method is based on the fact that blue EBA emits red fluorescence and nerve cells emit green auto-fluorescence under ultraviolet ray.

Normal nerve control: EBA red fluorescence can only be seen in the epineurium and perineurium and not in nerve fibers which emit green auto-fluorescence (Fig. 9).

One and two weeks after injury: The proximal and distal ends of the lesion showed dissolution of the green fluorescence towards the center of the lesion. On the contrary the red fluorescence emitted by EBA increased. The central area of the lesion emitted only red fluorescence. At the area proximal and distal to the lesion, there was red fluorescence emitted by EBA only on epineurium and perineurium. Green fluorescence emitted from the nerve fibers was regular proximal to the lesion, but vague and irregular distally. This obscurity was most prominent one week after injury.

Eight weeks after injury: At the site of the lesion, red fluorescence was not limited only to the perineurium but spread into the green fluorescence area emitted from the nerve fibers. At an area proximal to the lesion, red fluorescence emitted from EBA was not observed inside the funiculi, but was observed along with the green fluorescence, however very slightly (Fig. 10).

Thirty-six weeks after injury: Red fluorescence from the EBA was observed along with



green fluorescence from the nerve fibers at the location of the lesion, however the total area of red fluorescence decreased in comparison to the state observed at eighth week. At the area proximal and distal to the lesion, red fluorescence was observed only at epineurium and perineurium, and not along with the green fluorescence of the nerve fibers (Fig. 11).

## Discussion

### 1) Genesis of perineurial cells

There have been many theories forwarded as to the genesis of perineurial cells, but there is as yet no unified theory. DENNY-BROWN<sup>39</sup> considered fibroblast tissue originating from the mesoblast to be the origin. SHANTHAVEERAPPA<sup>14)</sup> showed from electronmicroscopic observations that the perineurium is the extension of the arachnoid of the spinal cord and came to the conclusion that it must be epithelial tissue if its morphology and functions are taken into account. THOMAS<sup>18)</sup>, AHMED<sup>1)</sup>, LUNDBORG<sup>9)</sup> and SCARAVILLI<sup>13)</sup> demonstrated, based on electron-microscopic observation of the regeneration process of the perineurium, that fibroblast-like cells with developed organelles were first observed, then, with the reduction of organelles, pinocytotic vesicles began to appear in these cells and finally, basement membrane formed and the morphology of the cell became that of the perineurium. GAMBLE<sup>4)</sup> observed the peripheral nerve of an embryo and demonstrated that embryonic perineurial cells had fibroblast-like morphology. From their observations, these researchers concluded that perineurial cells generated from the fibroblast.

The results of Study 1 show that fibroblast-like cells with developed cytoplasmic organelles began to form compartments at first and second weeks, and continuous basement membrane and pinocytotic vesicles began to appear from eighth week. It was also observed in the periphery of the funiculus that fibroblast-like cells gradually took the characteristics of perineurial cells.

From the present research, it can be concluded that perineurial cells are originated from the fibroblast.

### 2) The formation of compartments in the regeneration process of the nerve.

There are many theories on the formation of so-called compartments (perineurial cells surround several regenerated nerve fibers and form small funiculi) in the regeneration process of nerves: 1. THOMAS<sup>18)</sup> cut the sural nerves of rats, separated the two cut ends by 0.5 cm and observed their regeneration. 2. HUDSON<sup>6)</sup> conducted autografting of the nerves of rabbits and observed the suture line. 3. AHMED<sup>1)</sup> conducted autografting on rats and observed the suture line. 4. LUNDBORG<sup>9)</sup> cut the sciatic nerves of rats, bridged the two ends with pseudo-synovium tube and observed the changes in the lumen. 5. IDE<sup>7)</sup> transplanted frozen nerve grafts into mice and observed the changes. 6. SCARAVILLI<sup>13)</sup> utilized plastic tubing in grafting and observed the changes in the grafts. In all six experiments, compartments were reported to have been formed. These experiments indicate that in the regeneration process of nerves, where normal Schwann cells and perineurium are lost, compartments will be formed.

THOMAS<sup>18)</sup>, in his experiments with rabbits, removed all contents of the funiculus while preserving the perineurium and ligated the proximal side in order to prevent the invasion of Schwann cells. In that case, the perineurium could not maintain its normal structure, and

presented corrugation and finally collapsed. If not ligated, the normal structure of the perineurium was maintained with the invasion of Schwann cells, and at the same time, compartments were formed despite the existence of normal perineurium. Based on these findings, THOMAS emphasized the importance of Schwann cells in maintaining the structure of the perineurium.

NESBITT<sup>10)</sup> indicated in his report concerning the regeneration of excised perineurium of the sciatic nerve, that the normal structure of the perineurium could be regenerated if the contents of the funiculus were not damaged, but compartments would be formed if the funiculus was damaged at the time of excision.

From the above findings, it can be considered that preservation and regeneration of the perineurium are controlled by Schwann cells and compartments will be formed only when nerves with damaged Schwann cells are regenerated.

Why is the formation of compartments necessary? Denny-Brown<sup>3)</sup> demonstrated that when nerves were regenerated within normal perineurium, swelling of the area was rarely observed. HIRASAWA<sup>5)</sup>, in his experiments on nerve grafts in dogs, reported that regenerating nerve fibers are spreading out radially from the amputated stump to nerve defect, which suggested pressure existed within the intact funiculus surrounded by perineurium. SPENCER<sup>16)</sup> speculated that the perineurium preserved the optimum pressure within the funiculus from the fact that nerve fibers herniated when the perineurium was fenestrated. On the other hand, Ask<sup>2)</sup> demonstrated the importance of the perineurium in adjusting the osmotic pressure. From these findings, it can be assumed that the perineurium functions to create and maintain an optimum environment within the funiculus.

As shown in the results of Study 1, compartment formation had already started one week after injury when Schwann cells and regenerated nerve fibers appeared. The formation of compartments proceeded with the progress of nerve fiber regeneration and myelination. Eight weeks after injury, regeneration of nerve fibers, myelination and compartment formation by perineurial cells had almost been completed, however there were only one or two layers of perineurial cells surrounding the funiculus at this stage. At thirty-six weeks after injury, the compartments became degenerative and approximately six layers of perineurial cells were observed around the funiculus.

The following conclusions can be obtained from the results of the present experiments: the so-called compartments create a small funiculi within the funiculus in order to provide optimum conditions for the regeneration of nerve fibers. However, when myelination is completed, the perineurial cells recover to form six layers around the funiculus, compartments become unnecessary and eventually degenerate.

### 3) Changes in permeability of the perineurium

The perineurium of rat sciatic nerve is composed of flattened cells with flat nuclei. Six layers of perineurial cells are arranged concentrically covered by basement membrane on both sides. Tight junctions were observed between the cellular processes. KRISTENSSON<sup>8)</sup> demonstrated that because of the lack of tight junctions between the perineurial cells of immature rats and

mice, the perineurium lacked the role of diffusion barrier. Sima<sup>15)</sup> demonstrated that long-term malnutrition leads to damage of the tight junctions between cellular processes and at the same time impedes the function of diffusion barrier.

From these findings, it can be stated that due to the tight junctions between perineurial cell processes, the perineurium is able to function as a diffusion barrier.

As can be seen from the results of Study 2, one week after injury, EBA infiltrated into the funiculus because the perineurium had not regenerated. This is also clear from the results of Study 1. However, despite the fact that perineurial cells surround the funiculus, although there are only one or two layers, EBA freely infiltrated into the funiculus. Why?

WAGGENER<sup>19)</sup> electronmicroscopically observed that when infiltrated with ferritin, the ferritin migrated to the outer two or three layers. This indicated that pinocytotic vesicles are related to the transfer of ferritin. Oldfors<sup>11)</sup> demonstrated electronmicroscopically that pinocytotic vesicles of perineurial cells transport ferritin from the outside to the inside of the funiculus at peripheral nerves having one or two layers of perineurial cells.

These reports and the results of Studies 1 and 2 indicate that the perineurium will function as a diffusion barrier when the structure has four to six layers.

Olson<sup>12)</sup> reported that the function of diffusion barrier, following crush injury on peripheral nerves, would not recover by 16th week after injury. By 36th week after injury, the layer structure of the perineurium recovered to 6 layers. The infiltration of EBA into the funiculus decreased from eighth week. Therefore, it is considered that the recovery of perineurial function, although gradual, coincides with that of cell morphology.

## Conclusion

The followings will be concluded according to this experimental study.

In the periphery of funiculus, fibroblast-like cells were observed in concentric arrangement by second week after injury. Eight weeks after injury, one or two layers of perineurial cells surrounded the funiculus and the structure of the perineurium recovered to six layers at thirty-sixth week.

In the interior of the funiculus, fibroblast-like cells formed compartments from one week after injury. At eighth week, cells forming compartments already had the characteristics of perineurial cells. Toward thirty sixth week, compartments started to degenerate. From these microscopic observations, it is considered that perineurial cells originate from the fibroblast.

In normal sciatic nerve, EBA did not infiltrate into the funiculus. Eight weeks after crush injury, EBA freely infiltrated into the funiculus, however at thirty-sixth week, the rate of infiltration decreased.

It has been indicated that perineurial cells, by forming compartments within the funiculus, were able to produce optimum conditions for early-stage of nerve fiber regeneration, and at the same time showed specific permeability which protected mature nerve fibers. This permeability was impaired by crush injury but gradually recovered.

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## 和文抄録

## 神経周膜の再生に関する実験的研究

—末梢神経の圧挫損傷を中心に—

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神経周膜は神経束を囲むように同心円状に配列する扁平な周膜細胞と、その間を縦走する膠原原線維からなる強靱な結合組織である。周膜上皮を形成する細胞の両面には基底膜があり、線維芽細胞と大きな違いがある。周膜上皮は中枢神経系の被膜のうち広義の軟膜と連続すると考えられている。神経周膜の機能の特徴についてみると以下のようにある。(1)神経束を機械的刺激から保護する機能をもつ。(2)神経周膜は神経束内の圧を一定に保ち、軸索の機能のために適した環境を保つ。(3)周膜は固有の透過性を持ち、蛋白などの物質に対して一種の diffusion barrier としての機能をもつ。また神経は周膜によって中毒性因子や感染性因子から防御されている。

最近末梢神経損傷時の神経再生メカニズムの研究が進み、軸索や Schwann 細胞の働らきに加えて、神経周膜の機能の再生についての研究もクローズアップされるようになった。われわれは成熟ラットの坐骨神経に圧挫損傷を作成し、神経周膜の再生過程も電子顕微鏡学的に観察するとともに、透過性の変化についても蛍光顕微鏡を用いて観察した。

その結果についてまとめると以下のようである。(1)細胞内小器官の発達した線維芽細胞様の細胞が次第に基底膜および飲小体をもつようになり、周膜細胞の特徴を有してくる。神経損傷初期より神経線維の再生にとって最適な環境をつくるために神経束内に小さな神経束をつくるいわゆるコンパートメントの形成がみられる。しかし神経再生および髄鞘化が完了し神経束全周をとり囲む周膜細胞の層が6層前後に回復するに従い、コンパートメントは不要となり退化していく。(2)周膜の層構造が1, 2層しかない時期には本来の機能は認められず、徐々に4層から6層になり周膜細胞突起間が緊密に接着することによって、はじめて diffusion barrier としての機能を十分に果たすようになる。

以上の神経周膜の再生所見は Schwann 細胞による制御、再生軸索の有無などによって大きく影響されていると考えられる。神経周膜はその再生過程と透過性の変化に特有な所見が認められ、末梢神経の被膜でありながら、神経上膜や神経内膜と異なった特異な構造と機能を有していると推察された。