Regenerating Axons Enhance Differentiation of Perineurial-Like Cells Involved in Minifascicle Formation in the Injured Peripheral Nerve

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Abstract. The role of regenerating axons in the restructuring of the endoneurium into minifascicles after peripheral nerve injury was investigated. Endoneurial changes were studied in the control distal stumps of crushed rat sciatic nerves and in the distal nerve stumps in which, in addition to other cellular elements, the original perineurium had also been destroyed by freezing and thawing. Injured nerves in which axonal regeneration had been prevented were examined for comparison. The changes were observed at light microscopic and ultrastructural levels. Two weeks after nerve injury, incipient endoneurial restructuring by proliferating endoneurial fibroblast-like cells was observed in the distal nerve stumps in all groups of animals. By 4 weeks, the endoneurial changes in control regenerating nerves (crush only) displayed no progress. On the contrary, extensive minifascicular reorganization of the endoneurium was observed in the distal nerve stumps in which the original perineurial barrier had been eliminated. The cells that enclosed the minifascicles in the nerves with regenerating axons looked like differentiated perineurial cells, whereas in the absence of regenerating axons, the endoneurial cells that encircled the neurilemmal tubes mostly lacked typical features of the perineurial cells. Therefore, persistent perineurial damage is necessary to induce complete endoneurial compartmentalization. In this case, the regenerating axons enhance differentiation of the endoneurial fibroblast-like cells into the perineurial cells.

Key Words: Endoneurium; Minifascicles; Nerve injury; Nerve regeneration; Perineurium; Perineurial cells; Schwann cells.

INTRODUCTION

In the peripheral nerve (PN), perineurium separates endoneurium from the epineurium. It functions as a diffusion barrier which effectively limits access of various macromolecules and certain ions to the endoneurial space (1). Endoneurium surrounds the myelinated and unmyelinated axons with their respective Schwann cells (SC) enclosed in their basal lamina (BL) tubes. In normal PN, endoneurium contains collagen fibers, rare endoneurial cells, arterioles and capillaries. It has been reported that a nerve injury can lead to a local reorganization of the endoneurium into minifascicles at the lesion site. They consist of bundles of a few myelinated and unmyelinated axons surrounded by cells which have ultrastructural characteristics of perineurial cells: flattened cell processes covered by BL, numerous pinocytotic vacuoles and intercellular tight junctions (2–7). The prevailing opinion that the elongated cells surrounding the newly formed minifascicles derive from endoneurial fibroblasts, i.e. mesenchymal cells (4–7), has been supported by direct experimental evidence (8). Similar endoneurial reorganization also occurs in the course of some pathological processes in human PN (9–13).

Experiments with an isolated perineurial injury suggest that increased permeability of the perineurium may be responsible for the proliferation of the endoneurial cells and subsequent minifascicle formation which represents

formation of the substitute perineurium (14). Transection of a PN also results in loss of barrier function of the perineurium in the distal nerve periphery (15). However, if regenerating axons reinnervate the distal stump, the diffusion barrier function of its perineurium is restored (5).

Minifascicular reorganization of the endoneurium in the injured PN seems to be influenced by the regenerating axons, but their role is not clear. On one hand, after extraction of the intrafascicular content of a PN, minifascicles did not appear before the arrival of axons and the accompanying SC into the distal stump (4). Further, although the minifascicles were also observed in permanently denervated distal nerve stumps the authors concluded that they were not well formed (5, 16). This would suggest that regenerating axons enhance endoneurial reorganization into minifascicles. On the contrary, other studies showed that the incipient endoneurial reorganization in the distal stump regressed after successful axon regeneration, but persisted if axonal growth had been prevented (16, 17). These results indicate that the regenerating axons may actually inhibit endoneurial reorganization. The contradictions in conclusions of different studies may be a consequence of different experimental designs. Some experiments may have monitored direct effects of axonal regeneration on the endoneurial cells, while in other studies the axons may in addition have exerted effects via interactions with the original perineurium.

Therefore, we designed the present study to clarify the role of axons in the restructuring of the endoneurium in the injured PN into minifascicles. In addition to studying the endoneurial changes in control crushed nerves, we investigated the endoneurial reorganization in the distal nerve stumps in which, in addition to cellular elements
of the endoneurium, the original perineurium had been destroyed. In the absence of perineurium, therefore, direct effects of regenerating axons on endoneurial cells could be analyzed. Under these conditions, extensive endoneu-
rial compartmentalization by fibroblast-like cells occurred regardless of the presence of the regenerating axons. However, the axons were required for differentiation of these cells into perineurial-like cells.

MATERIALS AND METHODS

Experiments were performed on male Wistar albino rats weighing 200–250 g at the time of the initial surgery. All sur-
gical procedures were done under pentobarbital anesthesia (Nembutal, Abbott Lab., IL, USA; 50 mg/kg, i.p.).

Experimental Design

Four types of sciatic nerve injury were performed, two of which served as controls. In all animals, the right sciatic nerve was exposed and crushed in the upper thigh with a 3 mm-wide serrated hemostat which was held in place for 30 seconds.

In the first experimental group, i.e. regenerating frozen-thawed nerve, the nerve was cut 15 mm distal to the crush site. The whole segment between the crush and the cut was then frozen in situ by applying dry ice to the nerve. This was done on a 2 mm-thick plastic bed to protect the surrounding tissues from injury. The frozen segment was rinsed with ice cold Ring-
er's solution after 20 seconds to thaw. Freezing and thawing was repeated several times for a total of 5 minutes. The distal end of the frozen segment was then loosely sutured to the neighboring muscle. In the control regenerating nerve, the nerve was left lying on the plastic bed for 5 minutes without freezing. It was covered with tissue paper pads soaked in Ring-
er's solution to prevent drying.

In the second experimental group, i.e. non-regenerating frozen-thawed nerve, the nerve segment distal to the crush was frozen–thawed as described above. Then, the nerve was cut 3 mm proximal to the crush site and both ends of the nerve segment were loosely sutured to the neighboring muscle. In this way axon regeneration was prevented, while the short non-frozen segment proximal to the crush site was left as a potential source of viable SC and other non-neuronal cells to repopulate the acellular distal segment. Control non-regenerating nerves were prepared in the same way except that freezing–thawing was omitted.

There were six animals in every group. Nerves were obtained from three animals from each group for immunohistochemical analysis and three for electron microscopy. Animals were sacri-
ficed by exsanguination in deep anesthesia 14 and 28 days after treatment.

Processing of Tissue Samples

Nerve segments distal to the crush site were isolated, gently stretched and fixed in Bouin's solution for 2 hours. After de-
hydration in ethanol and paraffin embedding, 5 μm-thick cross-
sections were cut for immunohistochemical processing. The polyclonal rabbit antibody against laminin was a gift from Dr.
Fred Bosman, Rotterdam, The Netherlands. Tissue sections were rinsed in phosphate-buffered saline (PBS) and incubated

with anti-laminin serum diluted 1:10 in PBS overnight at 4°C. Thereafter, the slides were rinsed with PBS and incubated for 30 minutes at room temperature with a secondary fluorescein-conjugated swine anti-rabbit IgG antibody diluted 1:20 in PBS, rinsed with PBS and covered with an antifading medium. The slides were examined with an Opton-Axiophot fluorescent mi-

Nerve samples for electron microscopy were fixed in McDowell's fixative (18) for at least 24 hours, postfixed with 1% osmium tetroxide, dehydrated in alcohol, and embedded in Epon 812. Thin sections (40 nm) were mounted on copper grids using collodium foils, contrasted with uranyl acetate and lead citrate, and examined under a Jeol 100Cx electron microscope.

RESULTS

Figures shown in this section represent typical findings observed in all nerve samples from the experimental an-
imals in a single group, treated in the same way. We observed no qualitative differences between the nerve samples from different animals belonging to the same experimental group.

Immunohistochemical Visualization of Laminin in the Distal Nerve Segments

The perineurium in normal PN as well as in control (crush only) distal nerve segments of the injured nerves, either with or without regenerating axons, contained abundant laminin, as demonstrated by intense immuno-

reaction to this glycoprotein (Fig. 1A, B, C). On the other hand, the immunofluorescent reaction with the same anti-

body on sections from the frozen–thawed distal nerve segments did not yield a visible product in places of former perineurium for up to 4 weeks after injury (Fig. 1D, E). Two weeks after injury, laminin immunohistochem-

stry revealed no reorganization of the endoneurium in the distal nerve segments in all groups. At the light micro-

scopic level, none of the control crushed nerves showed endoneurial restructuring even at 4 weeks after nerve injury, regardless of whether axonal regeneration took place (Fig. 1B, C). However, at that time, the min-

ifascicular restructuring of the endoneurium was very pronounced in the frozen–thawed regenerating nerves (Fig. 1D), but not in the non-regenerating ones (Fig. 1E).

Electron Microscopic Analysis of Endoneurial Reorganization in the Distal Nerve Stump

Control Crushed Nerves: Two weeks after lesion, the endoneurium of the distal segments of the control (crush only) regenerating nerves contained sparse fibroblast-like cells, some of which partially surrounded axon-SC units with their elongated cell processes. These cells did not possess continuous BL, but some had an occasional small fragment of BL apposed to their plasmalemma (Fig. 2A). Their cytoplasm often contained electronlucent phago-

ycytic vacuoles representing lipid droplets, very similar to those found in the cells inside the BL tubes. Regenerating
Fig. 1. Distribution of laminin, visualized by indirect immunofluorescence (fluorescein-labeled secondary antibody), in the normal sciatic nerve (A), in the distal segment of control nerves, crushed 4 weeks earlier, with (B) or without (C) regenerating axons, and in the distal nerve segments which had been frozen–thawed 4 weeks earlier and through which axonal regeneration had been either allowed (D) or prevented (E). Note the presence of laminin in the perineurium of the normal and control crushed nerves (arrows), and its absence in the place of former perineurium in the previously frozen–thawed nerve segments. ×124.

Fig. 2. Electron micrographs of the distal nerve segments of the injured sciatic nerves. (A) Control crushed nerve with regenerating axons 2 weeks after injury; (B) the same as (A) except that the nerve sample was collected 4 weeks after injury; (C) control crushed nerve without the regenerating axons 2 weeks after injury; (D) the same as (C) but collected 4 weeks after nerve injury. SC = a cell within the basal lamina tube, likely a Schwann cell, but may also be a macrophage in the early samples; EFC = endoneurial fibroblast-like cell; Ax = axon. Note that the thin processes of the endoneurial cells tend to encircle the BL tubes in the distal nerve segment 2 weeks after injury but not 4 weeks after injury. (A) ×10,200; (B) ×6,250; (C) ×9,000; (D) ×6,000.
axons could be observed in the distal segments of control crushed nerves but never in the non-regenerating nerves.

Four weeks after crush, the distal segments of the control regenerating nerves still contained sparse endoneurial fibroblast-like cells but they displayed little tendency to wrap the regenerating axon-SC units with their processes (Fig. 2B).

In the distal segments of the control non-regenerating nerves, proliferation of the endoneurial fibroblast-like cells was also observed at 2 weeks after nerve injury (Fig. 2C). Cells with many cell processes, containing lipid droplets, were filling the BL tubes. The latter were partly or sometimes completely surrounded, and therefore separated from each other by the slim elongated cytoplasmic processes of the endoneurial fibroblast-like cells around the BL tubes. The cytoplasm of these cells also contained lipid droplets. The cytoplasmic processes of the endoneurial cells displayed little overlapping and did not have perineurial cell characteristics.

By 4 weeks after nerve injury, the endoneurial cell processes became shorter and showed less tendency to encircle BL tubes (Fig. 2D). The numerous endoneurial cells resembled fibroblasts and displayed no features of perineurial cells. There were many cytoplasmic profiles of SC processes inside the BL tubes and they no longer contained lipid droplets.

**Crushed Nerves with Frozen–Thawed Distal Segments:**

Two weeks after lesion, the distal segments of the frozen–thawed regenerating nerves displayed incipient endoneurial reorganization into minifascicles of variable intensity. Endoneurial cells that partly encircled small groups of axon-SC units resembled fibroblasts (not shown).

At 4 weeks, the distal segments of the frozen–thawed regenerating nerves displayed complete minifascicular reorganization (Fig. 3A). Each minifascicle contained several axon-SC units which were separated from the surroundings by one to three layers of flattened cell processes. These processes were coated by long continuous stretches of BL on one or both aspects of the process. Their cytoplasm contained numerous pinocytotic vacuoles. Tight junctions could be observed between the processes (Fig. 3C). Thus, the endoneurial cells encircling the minifascicles in these nerve segments had all the ultrastructural characteristics of perineurial cells.

Two weeks after nerve injury, the distal stumps of the non-regenerating frozen–thawed nerves mostly contained BL tubes still filled with myelin whorls or cells ingesting myelin. Many BL tubes were empty and collapsed. Endoneurial fibroblast-like cells were still sparse, usually without long cell processes, and only occasionally partly wrapped around BL tubes (not shown).

Four weeks after nerve injury, the BL tubes in the distal segment of the non-regenerating frozen–thawed nerve were filled with cytoplasmic profiles of SC processes. Individual BL tubes were completely surrounded and separated from each other by the slim elongated cytoplasmic processes of the endoneurial fibroblast-like cells (Fig. 3B). Often, the cell processes did not overlap; they displayed only a few short BL fragments and contained rare pinocytotic vacuoles and an occasional intercellular tight junction (Fig. 3D). An unusual example of a cell that was located inside a BL tube and extended its cell process through the hole in BL into a newly formed endoneurial cell barrier is shown in the same figure.

**DISCUSSION**

The present study was performed in order to distinguish between the direct effects of regenerating axons on endoneurial restructuring in the injured PN and the indirect effects via the indigenous perineurium. Endoneurial morphology of the distal nerve segments with undamaged perineurium was compared to that in the distal nerve segments in which the perineurium had been destroyed at the time of injury by freezing–thawing. Laminitin is abundant in the perineurium of a normal peripheral nerve and could also be demonstrated in the perineurium of the distal nerve segments in the control crushed nerves. The striking absence of immunoreactivity to laminitin in place of former perineurium in the frozen–thawed nerve segments 4 weeks after injury indicates that the perineurium and its diffusion barrier in these nerve segments have not regenerated during the period of observation. This is in accordance with findings of others who reported that the perineurium destroyed at the site of nerve injury did not regenerate for up to 6 weeks after injury (19).

We found that 2 weeks after nerve injury the distal nerve segments in all groups of animals displayed proliferation of the endoneurial fibroblast-like cells and signs of incipient endoneurial restructuring. The cells at this

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**Fig. 3.** Electron micrographs of the previously frozen–thawed distal nerve segments of the crushed sciatic nerves 4 weeks after nerve injury. Nerves in which axon regeneration had been either allowed (A, C) or prevented (B, D) are presented. Note complete endoneurial reorganization in the distal segments of both nerves. The endoneurial cells in the nerve segments containing regenerating axons (C) look like perineurial cells with long continuous stretches of BL (arrows), tight junctions (double arrows) and numerous pinocytotic vacuoles (arrowheads). In the nerve segments without regenerating axons (D), the endoneurial cells remained undifferentiated with only occasional short BL segments (arrow) and few pinocytotic vacuoles (arrowheads). A cell lying within the BL tube and extending its process through the breach in the tube wall is denoted by an asterisk. (A) \( \times 3,750 \); (B) \( \times 4,000 \); (C) \( \times 24,000 \); (D) \( \times 15,000 \).
stage showed virtually no features of perineurial cells. By 4 weeks, the proliferation of the endoneurial cells as well as extension of their cell processes showed no progress in the control regenerating nerves with intact perineurium. On the contrary, in the frozen–thawed nerve segments with damaged perineurium, endoneurial cell proliferation and compartmentalization were much more pronounced at 4 weeks than they had been at 2 weeks after nerve injury. The endoneurial cells that encircled the minifascicles of axon-SC units in the nerve segments with regenerating axons looked like differentiated perineurial cells. In the frozen–thawed segments of the nerves in which axonal regeneration had been prevented, endoneurial compartmentalization became extremely extensive. Individual BL tubes rather than small groups of them were surrounded and separated from one another by the endoneurial cell processes. These cells, contrary to those in frozen–thawed nerve segments with regenerating axons, however, did not display typical features of differentiated perineurial cells. In contrast, in the non-regenerating nerves with undamaged perineurium no such compartmentalization of the endoneurium developed up to 4 weeks after nerve injury because most of the endoneurial fibroblast-like cells, although still very numerous, retracted their cell processes and showed little tendency to wrap the BL tubes.

These results demonstrate that the conclusions reached by earlier studies (4, 5, 16, 17) in regard to the role of axons in endoneurial restructuring in an injured PN are only seemingly contradictory. Namely, the presence of regenerating axons has dual, and contrasting, effects on the endoneurial cells in the nerve segment distal to the lesion site depending on the integrity of the indigenous perineurium. Temporary breakdown of the perineurial as well as the blood–nerve diffusion barrier also occurs in the distal nerve stump not immediately adjacent to the nerve lesion site (15), but the barrier function recovers after axonal regeneration (5). Under such conditions, the results obtained in our control crushed nerves (with undamaged perineurium in the distal segments) confirmed earlier observations (16, 17) that an incipient proliferation of the endoneurial fibroblast-like cells did occur but regressed thereafter, and the minifascicles did not form. On the other hand, we demonstrated that, under conditions of long-term perineurial damage, very pronounced endoneurial cell proliferation took place in the previously frozen–thawed distal nerve segments. Progressive extension of the endoneurial cell processes subsequently led to complete endoneurial compartmentalization regardless of the presence or absence of regenerating axons. Therefore, under the conditions of permanent perineurial damage, the presence of the regenerating axons did not prevent compartmentalization. On the contrary, the axons enhanced differentiation of the endoneurial fibroblast-like cells into the perineurial cells. Endoneurial compartmentalization in grafted PN, taking place in the absence of the regenerating axons, has been described in the nerve samples taken close to the grafting site where the perineurium had been permanently damaged by the nerve lesion itself (5). The authors observed that the minifascicles “were not so well formed” as in the nerves with the regenerating axons, but did not specifically point out the differentiating influence of the regenerating axons on the endoneurial cells involved in compartmentalization.

Besides perineurium, freezing and thawing also destroyed the microcirculation in the distal segment of the injured PN. Microvessels at the lesion site of an injured PN normally regenerate together with the axons (1, 5). The blood–nerve barrier in regenerated blood vessels is transiently leaky (5, 15), which might, in addition to the breakdown of the perineurial barrier, favor diffusion of putative mitogenic factors into the distal nerve segments which had been frozen–thawed previously. However, no significant role could probably be ascribed to microcirculation damage in regard to differentiation of the proliferated endoneurial cells because identical blood vessel injury occurred in frozen–thawed nerve segments either with or without regenerating axons.

In order to explain all these changes in an injured PN, we assume that mitogenic, growth-promoting (trophic) and differentiating factors must act in concert to induce complete endoneurial restructuring in the injured PN, giving rise to minifascicles encircled by differentiated perineurial cells. Mitogenic substances probably gain access to the endoneurial space during the breakdown of the perineurial diffusion barrier. This breakdown is most complete and long-lasting at the site of traumatic or disease-induced nerve lesion (5, 12–14), and only transient more distally in the nerve segments undergoing Wallerian degeneration before axonal regeneration (5). Accordingly, endoneurial cell proliferation was very extensive in our nerve segments with permanent perineurial damage. It was less extensive in non-regenerating distal nerve segments with undamaged perineurium in which a functional defect of the perineurial diffusion barrier persists but the blood–nerve barrier recovers (15), and it did not show any progress in regenerating control nerves in which the perineurial function recovers (5). The requirement of a trophic environment for endoneurial cell persistence is indicated by the observed long-term regression of the proliferated endoneurial cell population in the distal nerve segments after axonal regeneration and its persistence in the absence of the regenerating axons (16).

Finally, differentiation factor(s) is(are) required in order to transform the proliferating endoneurial fibroblast-like cells to become phenotypically indistinguishable from the perineurial cells. The differentiating signal is either produced by the regenerating axons or the latter induce its production in some other cells in the distal stump. If the axons are the source of such a signal, the
effect is probably mediated via some diffusible substance considering that the endoneurial cells are not in direct contact with the axons. In this respect it is interesting that axons also stimulate the differentiation of SC, and they seem to do this by means of a diffusible substance (20). However, it is also conceivable that SC, stimulated by the regenerating axons, become a source of the differentiating signal which transforms the endoneurial fibroblast-like cells into the perineurial cells.

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