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Research report

The recovery of blood–nerve barrier in crush nerve injury—a quantitative analysis utilizing immunohistochemistry

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Abstract

The purpose of this study is to reveal whether the application of immunohistochemical examinations to the peripheral nervous system (PNS) can be a reliable method for the quantitative analysis of the blood–nerve barrier (BNB) and the relationship between restoration of BNB and nerve regeneration. Sciatic nerves in rats were examined after nerve crush. Immunohistochemical staining with anti-rat endothelial cell antigen-1 (anti-RECA-1) that recognizes endothelial cells and anti-endothelial barrier antigen (anti-EBA) for the detection of barrier-type endothelial cells were used. Neurofilament for staining axons was also performed. A quantitative analysis of the BNB was assessed using the ratio of EBA positive cells and RECA-1 positive cells. The ratio of EBA/RECA-1 decreased significantly 3 days postoperatively and reached its lowest level at day 7 in the segment 5 mm proximal and the entire distal stump. The ratio gradually recovered from the proximal and the regeneration of axons started a week earlier than BNB. The ratio of EBA/RECA-1 applied to the PNS can be a reliable method for the quantitative analysis of BNB. In crush injuries, the breakdown of BNB occurred simultaneously in the segment 5 mm proximal and the entire distal stump; restoration began from the proximal to distal and followed a week later to nerve regeneration.
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Theme: Development and regeneration
Topic: Regeneration

Keywords: Blood–nerve barrier; Endothelial barrier antigen; Rat endothelial cell antigen-1; Evans blue albumin; Peripheral nervous system; Sciatic nerve

1. Introduction

Blood–tissue barriers play an essential role in the maintenance and homeostasis of organs or tissue environments. Barriers such as the blood–brain barrier (BBB), blood–cerebrospinal fluid barriers, blood–nerve barrier (BNB), and blood–retinal barrier (BRB) consist of endothelial or epithelial cells, sealed by tight junctions. A selective transport system localized in the cells of the barrier provides substances needed by the cells inside the barrier [33].

In the central nervous system (CNS), BBB regulates the exchange of hydrophilic substances between the cerebral extracellular fluid and plasma [22]. In the peripheral nervous system (PNS), regulation is controlled by BNB, which limits entry of blood-borne, water-soluble substances into the endoneurial compartment and maintains the homeostasis of the endoneurial environment [37]. The environment of the nerve parenchyma is maintained by the combination of endoneurial blood vessels in the endoneurium (BNB) and the perineurium as the diffusion barrier. Tight junctions between the endothelial cells in the endoneurial blood vessels, and the junction between the perineurial cells in the perineurium are responsible for barrier functions.

These are lost when the system is altered. In experiments on nerve injuries, numerous investigators documented BNB function using a variety of tracers or techniques, focusing on the permeability of the barrier [15,18,19,25]. Such studies have usually been based on intravenous injection of tracers, for instance Evans blue albumin and horseradish peroxidase (HRP), which will pass out from microvessels at sites of

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barrier opening. But such procedures were difficult for quantitative analysis, so the relations between axonal regeneration and spatial recovery of the BNB have been unclear [10,14,28,29]. Thus a method for quantitative analysis was needed for detailed analysis of the barrier function. Anti-rat endothelial cell antigen-1(anti-RECA-1) is a mouse monoclonal antibody that recognizes rat endothelial cell surface antigen and is shown on all rat macrovascular and microvascular endothelial cells [5,36]. Anti-endothelial barrier antigen (anti-EBA) is a mouse monoclonal antibody to endothelial barrier antigen, which is specific to a rat endothelial protein found in areas with blood–nerve barriers [31,32].

The purpose of this study is to investigate the quantitatively temporal and spatial relations of the breakdown and restoration of BNB of peripheral nerves together with axonal regeneration using immunohistochemical study.

2. Materials and Methods

2.1. Animal experiments

The experiment protocol was carefully reviewed and approved by the animal studies subcommittee of Hamamatsu University School of Medicine.

Inbred female Sprague–Dawley (SD) rats weighting 200–250 g were used. The rats were anesthetized with 100 mg ketamine and 20 mg of xylazine in a doze of 1 ml/kg by intra-muscular injection. The left sciatic nerve was exposed through a gluteal muscle splitting incision. Then the nerve was elevated from its tissue bed from the level of the sciatic notch to the midthigh. The nerve was crushed with a vessel clip (AESCULAP, Germany) of 1.52 N for 5 min at the level just below the sciatic notch. This procedure induces Wallerian degeneration of the distal part of the nerve with complete nerve regeneration ascertained by walking track analysis and neurofilament staining (data not shown). The crush site was marked with 8–0 nylon epineural sutures. The muscle layer and the skin were closed with 4–0 nylon sutures. After the operation, the animals were held at standard laboratory conditions. The sciatic nerves from normal animals (control, $n=6$ nerves) were processed for comparison.

2.2. Evans blue albumin tracer

Evans blue albumin solution was prepared as a mixture of 5% bovine albumin and 1% Evans blue dye in saline. On days 1, 3, 7, 14, 21, 28 and 56 after nerve crush, five rats each were intravenously injected with 1 ml Evans blue albumin per 100 g body weight under anesthesia, according to previously reported methods [17,18,24]. Animals were killed 30 min after the injection and the left sciatic nerve specimens, 10 mm long proximal from the crushed site and 35 mm long distal, were harvested and divided into 5 mm segments each. All segments were immediately embedded in Tissue-Tek Optical Cutting Temperature Compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at $-80°$ until use. Transverse 4-μm-thick frozen sections were cut with a cryostat and mounted on MAS-coated glass slides (Matsunami, Tokyo, Japan). The sections were immediately viewed under florescence microscope with emission filters for 520–550 nm (AX-80; Olympus, Tokyo, Japan). Under these conditions the Evans blue albumin emits a brightly red fluorescence.

2.3. Immunohistochemistry

Five rats in each group were analyzed for immunostaining. The previously described frozen sections which were observed under florescence microscope were fixed in cold acetone for 10 min and washed with phosphate buffered saline (PBS). Sections were exposed to 0.3% hydrogen peroxide in 100% methanol for 20 min and 5% skim milk in PBS for 30 min at room temperature for blocking unspecific protein binding. The serial sections were then incubated overnight at 4° with anti-RECA-1 (1:5; MCA970; Serotec, UK), and anti-EBA (1:200; SM171; Sternberger Monoclonals, Lutherville, MD). For the assessment of nerve regeneration, a monoclonal antibody to neurofilament (1:50; 2763; Immunotech, Marseille, France) was used. Negative controls consisted of sections incubated without the primary antibody. After incubation with the primary antibody, the sections were washed in PBS three times (5 min), incubated for 60 min with the biotin conjugated anti-mouse IgG, $F(ab')_2$ (1:200; Jackson ImmunoResearch Laboratories) and washed in PBS three times (5 min). Incubation using horseradish peroxidase-streptavidin for 60 min was followed by washing three times in PBS (5 min). Binding was developed by the diaminobenzidine technique. Following a brief rinse in distilled water and dehydration, the sections were mounted for examination by light microscopy (LM).

2.4. Quantification

Positive endothelial cells were counted using a digital image analysis system Mac Scope 2.56 (Mitani, Japan) at 200× magnification on a Macintosh Computer (Apple Computer, CA, USA). The quantitative analysis of the BNB was assessed by the ratio of EBA positive cells and RECA-1 positive cells (EBA/RECA-1). With respect to the assessment of nerve regeneration, the number of axons positive for neurofilament staining per field was automatically counted using a digital image analysis system.

2.5. Statistical analysis

The ratio of EBA positive cells and RECA-1 positive cells and the number of immunopositive for neurofilament

per field were statistically analyzed by one-way analysis of variance followed by post-hoc analysis with the Man–Whitney U-test. Statistical significance was set at $p < 0.05$. All data is shown in all figures as the mean ± standard error.

3. Results

3.1. Evans blue albumin tracer

Normal rat sciatic nerve showed a bright red fluorescence confined to the lumen of endoneurial blood vessels and none appeared outside the vascular walls after intravenous injection of Evans blue albumin. And under normal circumstances the endoneurial blood vessels seem to be impermeable to Evans blue albumin (Fig. 1A). The segments from 10 mm proximal to the crush site to 35 mm distal were examined. One day after the crush injury, the sciatic nerve showed intense red fluorescence throughout the site of the crush and all the distal segments (Fig. 1B). The increased red fluorescence was also seen at 7 and 14 days after nerve crush and was uniform throughout the distal stump (Fig. 1C and D). At 21 days the red fluorescence decreased almost to normal level and at 56 days fluorescence was confined to the lumen of endoneurial blood vessels (Fig. 1E and F). These changes were uniform in all rats, but quantitative comparison of the intensity of the red fluorescence at 1, 7 and 14 days was not possible, as the evaluation had to be done on the leakage.

3.2. Immunohistochemical examination

Throughout the experiments all endoneurial blood vessels in control and injured nerves were immunostained with RECA-1 (Fig. 2-A1–4). Almost all endoneurial blood vessels in normal sciatic nerve were immunopositive for EBA (Figs. 2-B1), but on day 3 after nerve crush, a few endoneurial blood vessels were immunoreactive for EBA in the crushed sciatic nerve throughout the segments 5 mm proximal to 35 mm distal (data not shown). At day 7, there was also a severe reduction in immunostained vessels with EBA (Fig. 2-B2). At day 14, endoneurial blood vessels started to show restitution of immunoreactivity near to the crush site. On day 21, EBA positive vessels increased compared to day 7 (Fig. 2-B3), and on day 56, the EBA staining had almost returned to the control (Fig. 2-B4).

The ratio of EBA/RECA-1 in the injured nerve was not statistically different between the normal control and 1 day postoperatively (Fig. 3A). The ratio of EBA/RECA-1 decreased significantly 3 and 7 days postoperatively throughout the crush site and all the distal segments. The ratio also declined in the segment 5 mm proximal to the crush site. The ratio reached its lowest level on days 3 and 7 (Fig. 3B and C). At day 14, the ratio of EBA/RECA-1 gradually started to recover at the crushed site. The ratio was significantly low at the segments 25 and 35 mm distal, but was not statistically different at the segments 5 mm, 15 mm distal, and 5 mm proximal (Fig. 3D). The ratio of EBA/RECA-1 gradually tended to recover and there were no

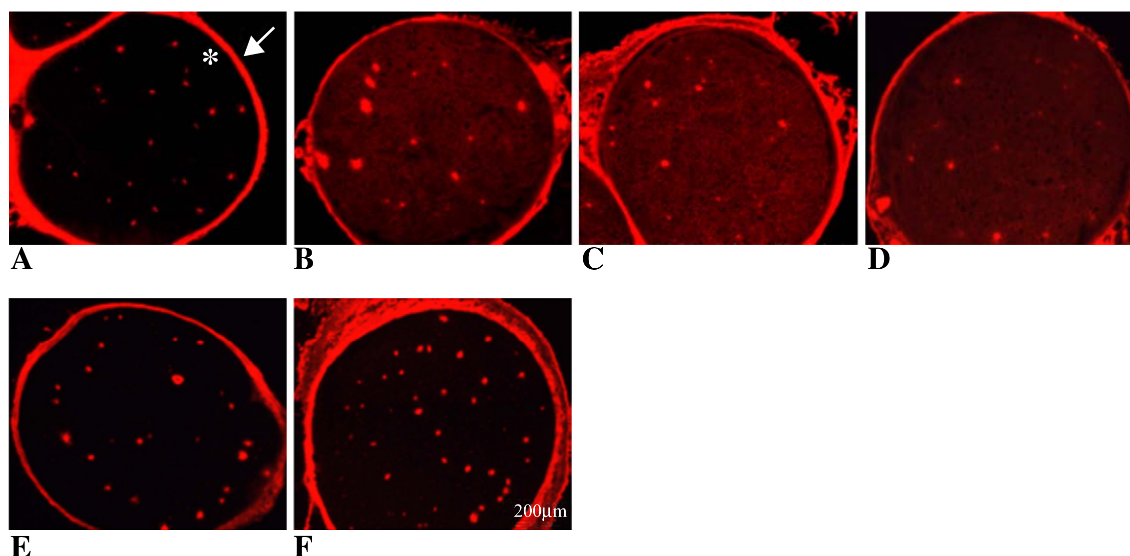

Fig. 1. Temporal change of permeability for Evans blue albumin tracer in the rat sciatic nerve after crushing. Each is at 20 or 25 mm distal to the crushed site of the nerve injury. (A) In the control, fluorescence was confined to the lumen of the blood vessels and none appeared in or outside the vascular walls. (B) One day after the crush injury. The sciatic nerve showed an intense red fluorescence in endoneurium and throughout the crush site and the distal all segments. (C) Seven days after the crush injury. (D) Fourteen days after the crush injury. (E) Twenty-one days after the crush injury. The red fluorescence decreased to almost normal level. (F) Fifty-six days after the crush injury. The fluorescence was confined to the lumen of endothelial blood. Scale bar = 200 μm. The arrow indicates perineurium and the asterisk indicates endoneurium.

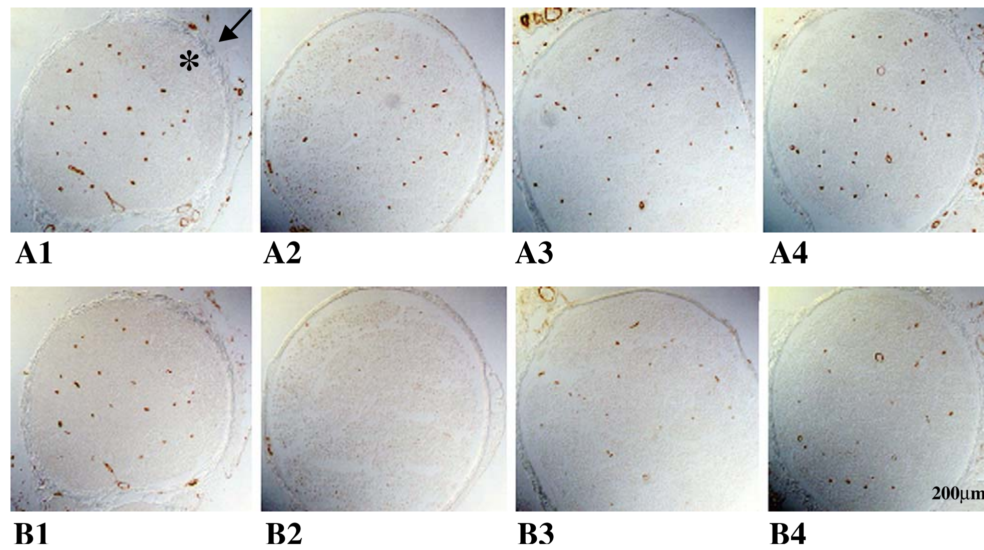

Fig. 2. Immunostaining with anti-rat endothelial cell antigen-1 (anti-RECA-1; A1-4) and anti-endothelial barrier antigen (anti-EBA; B1-4) at the segment 20 mm distal to the crush site. A1 and B1: control. All endoneurial blood vessels in both normal sciatic nerve and crushed sciatic nerve were immunostained for RECA-1. Almost all endoneurial blood vessels in normal sciatic nerve were immunopositive for EBA. A2 and B2: 7 days after the crush injury. The endoneurial blood vessels in crushed sciatic nerve were unreactive or only slightly reactive for EBA. A3 and B3: 21 days after the crush injury. EBA-positive vessels increased. A4 and B4: 56 days after the crush injury. Scale bar = 200 μm. The arrow indicates perineurium and the asterisk indicates endoneurium.

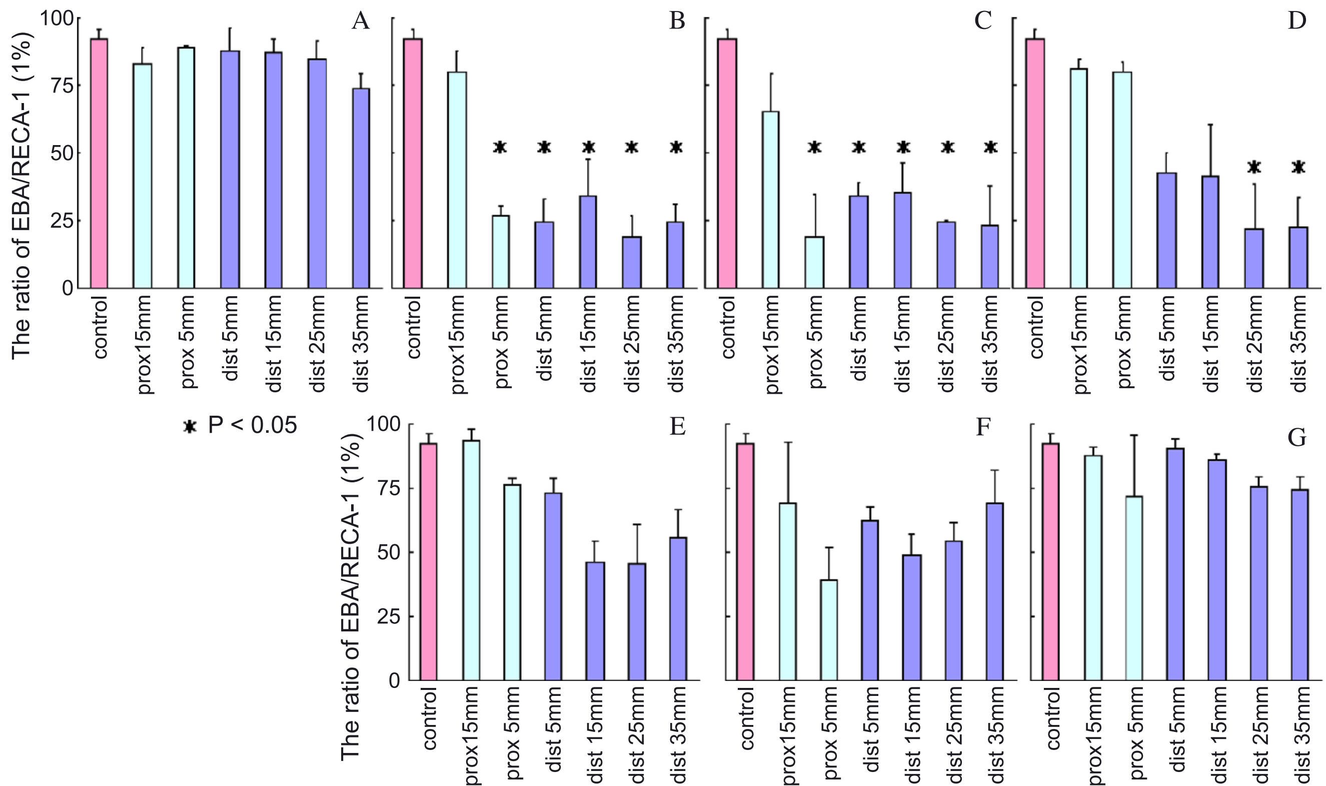

Fig. 3. Temporal change of the ratio of EBA/RECA-1 after the crush injury. (A) The ratio of EBA/RECA-1 was not statistically different between the normal control and 1 day postoperatively. (B and C) The ratio decreased significantly 3 and 7 days postoperatively throughout the crushed site and all the distal segments. The ratio also declined in the segment 5mm proximal to the crush site. (D) The ratio gradually started to recover at day 14. (E, F, G) The ratio was significantly lower at the segments 25 and 35 mm distal, but was not statistically different at the segments 5 mm, 15 mm distal, and 5 mm proximal. The ratio of EBA/RECA-1 gradually tended to recover but did not change significantly 21, 28 and 56 days postoperatively compared with the control segments.

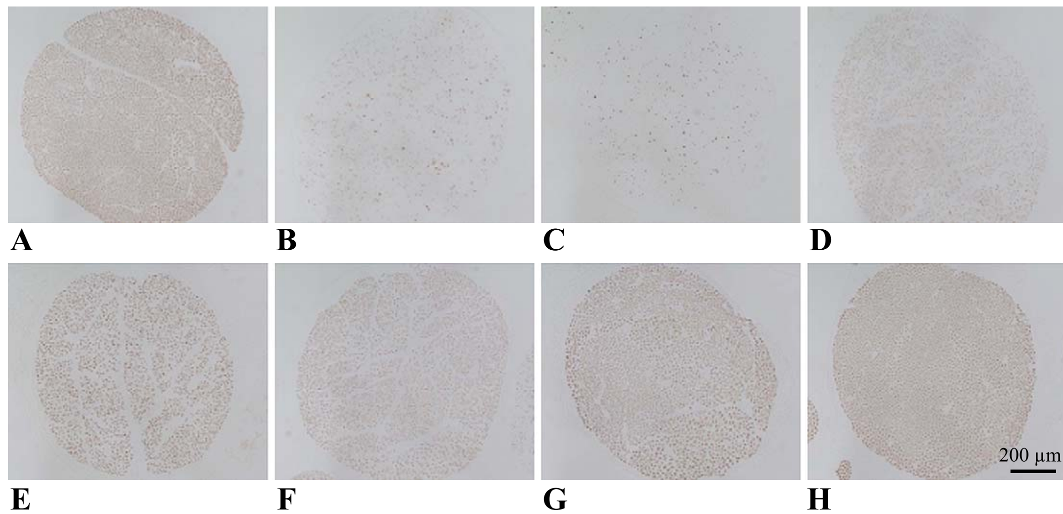

Fig. 4. Immunostaining with anti-neurofilament antibody in crushed sciatic nerve and normal sciatic nerve. (A) At day 1, the immunoreactivity for neurofilament remained normal. (B, C) From day 3 to day 7, axons positive for neurofilament decreased dramatically. (D) On day 14, a number of small axons exhibited immunoreactivity showing signs of regeneration. (E) On day 21, the section was filled with small axons positive for neurofilament. (F) Twenty-eight postoperative days. (G) By day 56, normal mixture of large and small axons is seen. (H) Normal sciatic nerve. Scale bar = 200 μm.

differences 21, 28 and 56 days postoperatively compared with the control segments (Fig. 3E–G). Fifty-six days postoperatively the ratio reached more than 80% compared with the normal control in all segments.

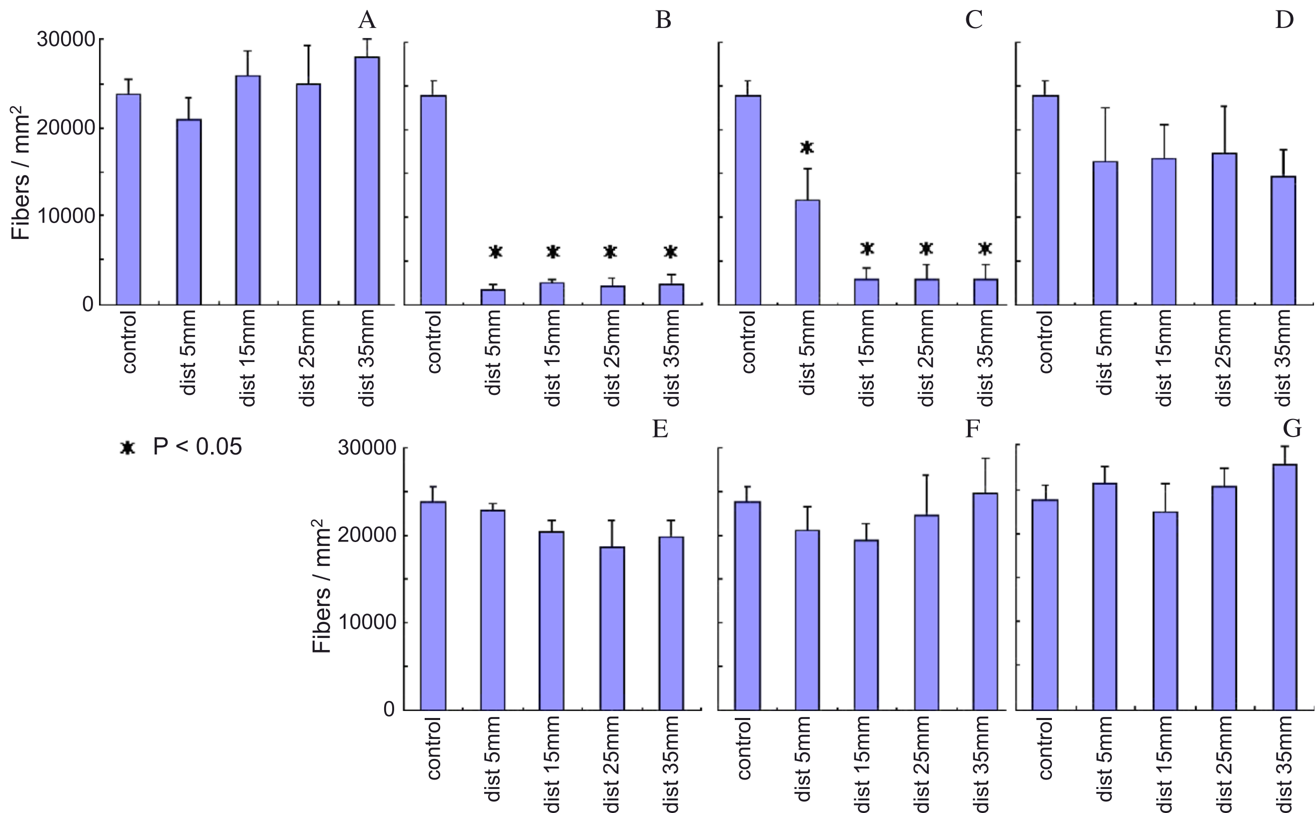

Fig. 5. Temporal change of the number of axons immunopositive for neurofilament after the crush injury. (A) The number of axons immunopositive for neurofilament was not statistically different from the normal control 1 day postoperatively. (B) At day 3, the number of axons immunopositive for neurofilament was significantly lower in all the segments distal to the crush site. (C) At day 7, the number increased at the segment 5 mm distal but was still statistically lower in all distal segments compared to the control. (D) By day 14, the axons showed recovery compared with the control subjects in all the segments. (E, F, G) At 21, 28 and 56 days, the immunoreactivity did not changed compared with the control.

The immunoreactivity for neurofilament remained normal at day 1 (Fig. 4A). From day 3 to day 7, axons positive for neurofilament decreased dramatically (Fig. 4B and C). On day 14, a number of small axons exhibited immunoreactivity showing signs of regeneration (Fig. 4D). On day 21, the section was filled with small axons positive for neurofilament (Fig. 4E). By day 56, normal mixture of large and small axons is seen (Fig. 4G). The number of axons was counted based on a micrograph at 200 × magnification. At day 1, the number of axons immunopositive for neurofilament was not statistically different compared with the normal control in all segments (Fig. 5A), but at day 3, it was significantly lower in all the segments distal to the crush site (Fig. 5B). At day 7, the number increased in the segment 5 mm distal but was still statistically low in all distal segments compared to the control (Fig. 5C). By day 14, the axons showed significant recovery compared with the control subjects in all the segments (Fig. 5D). At 21, 28 and at 56 days, the immunoreactivity for neurofilament was similar compared with the control (Fig. 5E–G).

4. Discussion

In particular parts of the body, free exchange of substances between blood and tissue cells is hindered by the presence of a barrier cell layer. Specialized milieu of the compartments provided by these "blood–tissue barriers" seems to be important for specific functions of the tissue cells guarded by the barriers.

In the PNS, the environment of the nerve parenchyma is maintained by the combined impermeability of endoneurial blood vessels and the perineurium. Waksman presented the first experimental investigation for BNB and demonstrated that several animals species possess a "blood–nerve barrier" comparable to those of the BBB [37].

Olsson showed that under normal circumstances the endoneurial blood vessels seem to be impermeable for labeled albumin with Evans blue and fluorescence isochipcyanate. In short, tracers failed to pass through the endothelium of the blood vessels within the nerve due to the BNB when intravenously injected [18,19]. On the other hand, when a nerve has been injured, by crushing or cutting, the endoneurial blood vessels become permeable to labeled albumin, which leak into the extracellular space throughout the segment of nerve distal to the site of injury [18]. This phenomenon has been based on the breakdown of the BNB. After the event, numerous investigators focused on the permeability of the barrier and morphological studies on barrier dysfunction and have documented evaluation of BNB function using a variety of tracers or techniques [15,18,19,25]. Such studies have usually been based on intravenous injection of tracers, such as Evans blue albumin and HRP, which will pass out from microvessels at sites of barrier opening. But in studies using these tracers, it was not possible to evaluate the state of BNB quantitatively and they showed only the permeability of BNB qualitatively.

Mellick and Cavanagh performed a quantitative investigation of the permeability in endoneurial blood vessels using I^{131} labeled albumin in chicken sciatic nerves after the nerve was crushed. Intravenous injection of I^{131} labeled albumin was carried out over several periods after the nerve was crushed, and the weight of the sciatic nerve was measured and was compared with a control. At day 1 there was a great increase of albumin outside the blood vessel at the distal stump and 6 mm proximal part of the injury. Between day 1 and day 4 there is a temporary decrease, followed at day 7 by a marked increase in vascular permeability. By 14 days, a dramatic increase in permeability occurs in the same areas and continues to day 21. Between day 21 and day 32, there is an overall decrease of albumin outside the blood vessel, which nevertheless remain significantly higher than the control value [15]. There appeared to be two waves of increased permeability after injury. As it is well known that the permeability of BNB differs in species, it may not be appropriate to compare the results between the study described herein and Mellick and Cavanagh's as their experiment was performed on chickens. However, the results are in agreement when the barrier is broken at the distal stump and adjacent proximal part of the injury and the early recovery course of BNB was almost the same as that observed in our study. Even so, several problems were noted. For example, it was unclear whether the weight of the sciatic nerve could reflect the dysfunction and recovery of the BNB. We therefore aimed to investigate both temporal and spatial relations of the breakdown and restoration of BNB together with axonal regeneration using immunohistochemical study.

The monoclonal antibody, anti-EBA, was first introduced by Sternberger et al. [31,32]. The antibody recognizes a protein triplet of 30, 25 and 23.5 kDa and reacts with the luminal surface of endothelial cells that have a selective permeability barrier in the CNS and PNS. Anti-EBA is localized in the plasma membrane of endothelia that have a BBB but not in vessels of circumventricular organs, which lack barrier function. In the CNS, many authors indicated anti-EBA as an effective marker to study barrier dysfunction and recovery in different types of disorders [1,2,4,6,7,12,13,16,17,21,23,38]. On the other hand, few reports have been made on the presence of anti-EBA in the PNS [3,11,20]. It was stated in the literature that the sciatic nerve showed trace labeling for anti-EBA. Furthermore, no reports have been made on the restoration of BNB using anti-EBA.

In our study we were able to stain the sections of rat sciatic nerve using anti-EBA. We thought that an immunohistochemical study using anti-EBA could reflect the process of dysfunction and recovery of the BNB. Our study noted almost all endoneurial blood vessels in uninjured nerves were immunopositive for anti-EBA. On the other hand, the reactivity of anti-EBA reduced after the nerve was

crushed and recovered gradually. The whole process after the nerve was crushed proved to be similar to the result obtained by Evans blue albumin tracer, as the leakage of BNB occurs after the nerve crush and recovers with axonal regeneration. This means that anti-EBA could be used as a BNB marker in PNS. We also carried out an immunohistochemical examination using anti-glucose transportor-1 (anti-Glut-1), which reacts specifically with the blood–tissue barrier (data not shown). Anti-Glut-1 is localized not only in endoneurial blood vessels, which have barrier function, but also in the perineurium of the PNS [30,33]. In our result, anti-Glut-1 reacted both to endoneurial blood vessels and perineurium in the normal control. The immunoreactivity to the endoneurial blood vessels and perineurium decreased when the nerve was injured. However, it reacted also to the background such as myelin debris. For this reason, we chose to use anti-EBA for evaluation of the barrier. Previous study shows that occludin, a tight junction protein is expressed in the perineurium and the blood vessels of the endoneurium in uninjured nerve. The immunoreactivity for occludin disappears with nerve injury in the perineurium and the endoneurium. The expression may be closely linked to the barrier function of endothelial cells in peripheral nerve [8].

Anti-EBA combined with anti-RECA-1 could be a useful marker for determining the breakdown and recovery of BNB and the ratio of EBA/RECA-1 can be utilized for the quantitative analysis of BNB. The comparison between the results obtained using Evans blue albumin and the ratio of EBA/RECA-1 showed a similar pattern. The great advantage with this immunohistochemical method was that the process of recovery could be studied in detail. The ratio of EBA/RECA-1 enabled a detailed examination of the restorative process from the temporal and spatial points of view. For example, 14 days after nerve crush the leakage of Evans blue albumin decreased but still remained in the endoneurial space. This reflects the recovery of the barrier but the degree of recovery is difficult for objective evaluation. On the contrary, the utilization of the ratio of EBA/RECA-1 enabled observation of the degree of recovery of the BNB in detail and quantitatively at different times and places. However, there were some problems. Leakage of Evans blue albumin was seen on day one, but the reduction of immunoreactivity for anti-EBA was significant at day 3 compared with the control. We believe that the dysfunction of BNB occurred within 24 h, but this cannot be shown by anti-EBA as the immunoreactivity still remains at this period. It was concluded that this staining is not useful in the early period after nerve injury and both anti-EBA and anti-RECA-1 are restricted to use only in rats.

In 1981, Sparrow and Kiernan reported that endoneurial vascular permeability to rhodamine B isothiocyanate (RBA) in the crushed region of rat sciatic nerves increased greatly and the fluorescence extended distally from the crush site, which advanced along the nerve at the same rate as that of the most rapidly regenerating axons. The increase in permeability may assist the regenerative process [29]. This result is interesting as it showed association between the recovery of BNB and the regenerating axons, which differs from result obtained in our study.

Seitz et al. indicated that in mice, using single intraperitoneal injections of biotinylated human albumin, transferrin, IgG, and complement components as tracers, the BNB becomes leaky for serum proteins during nerve degeneration and is restored in parallel with nerve regeneration irrespective of their molecular size by electrophysiology and morphology. When regeneration potentials could first be elicited from the small foot muscles, and when thinly myelinated nerve fibers were present, the BNB gradually regained its

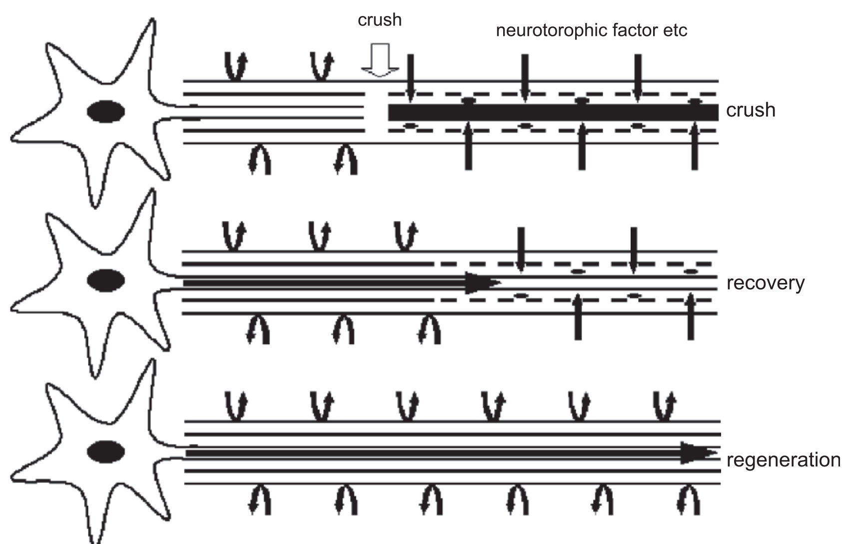

Fig. 6. The model of BNB breakdown for nerve regeneration. Once the peripheral nervous system is broken down, endoneurial vascular permeability increases simultaneously at all the distal segments and neurotrophic factors flow in the endoneurium. In regenerating nerves, endoneurial blood vessels become impermeable. Increased endoneurial vascular permeability after peripheral nerve injury is required for axonal regeneration.

barrier function and was nearly intact on day 30 after being crushed [28]. Our result was much closer to Seitz's but their experiment lacked the observation of the BNB in different parts of the injured nerve in detail.

From our result we can say that the BNB breakdown simultaneously at all the distal segments and the recovery is followed by the regeneration of the axons occurring from proximal to distal. In our study, the regeneration of axons started a week earlier than BNB. We speculate that in peripheral nerve injury, the essential event for successful regeneration is the preparation of the most suitable environment in the endoneural space. The breakdown and restoration of BNB is one of the most important reactions for regenerating nerve. The purpose of the breakdown of BNB is for the acceptance of the cytokines and various growth factors within the endoneural space. Once the nerve has been regenerated, BNB restores and endoneurial blood vessels become impermeable as the environment has been prepared for nerve regeneration (Fig. 6). Recent reports show that the source of increased permeability may be due to tumor necrosis factor (TNF)-alpha and interleukin (IL)-beta produced by recruiting macrophages from the circulation [24,26,27,34,35]. Macrophages are not only scavengers during Waller degeneration, but also play an important role for nerve regeneration and growth factor production. They regulate inflammation by secreting several pro-inflammatory cytokines including IL-1, IL-6, IL-12 and TNF-alpha. They also contribute to nerve regeneration via the termination of inflammation by promoting T-cell apoptosis and expressing anti-inflammatory cytokines including transforming growth factor (TGF)-beta 1 and IL-10 [9]. Our result supports this data concerning cytokines. In our experiment, the breakdown of BNB occurred simultaneously with allowing the introduction of substances needed for nerve regeneration in the entire endoneural space of the degenerating nerve. The arrival of regenerating axons allows the recovery of BNB. This recovery parallels the growth of the axons but occurs with a delay of one week. The ratio of EBA/RECA-1 applied to the peripheral nerve can be a reliable method for the quantitative analysis of BNB and the study of the PNS. We demonstrated that in nerve injury, BNB breaks simultaneously and the restoration of BNB begins from proximal to distal.

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