

Impaired nerve fiber regeneration in axotomized peripheral nerves in streptozotocin-diabetic rats

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ABSTRACT

Aim/Introduction: Impaired nerve fiber regeneration is a salient feature of diabetic neuropathy. Its pathogenesis is still unclear. We attempted to characterize the structure of regenerated myelinated fibers after transection in streptozotocin-diabetic rats.

Materials and Methods: Streptozotocin-diabetic rats underwent transection of the sciatic nerve. Two and 4 weeks post-axotomy, regenerated myelinated fibers of the cut end and fibers at its proximal site were morphometrically examined. Non-diabetic control rats with axotomy were also examined for comparison.

Results: At 4 weeks post-axotomy, diabetic rats showed an increased myelinated fiber density and total fiber number with a trend toward reduced fiber size at the cut end compared with those in control rats. The average number of myelin lamellae relative to axonal size in regenerated fibers at the cut end was significantly reduced in diabetic rats compared with that in control rats. The proximal site showed a reduced size of fibers and axons in both diabetic and control rats to a similar extent compared with those in a non-axotomized state. At 2 weeks post-axotomy, these findings were less apparent.

Conclusions: The nerves of diabetic rats when axotomized undergo impaired regeneration characterized by increased fiber density with hypomyelination. (*J Diabetes Invest*, doi: 10.1111/jdi.12115, 2013)

KEY WORDS: Fiber atrophy, Myelination, Nerve regeneration

INTRODUCTION

Impaired nerve fiber regeneration is one of the salient pathological features in human diabetic neuropathy^{1,2}, and its correction is a major target for the therapeutic approach. In experimental diabetic animal models, extension of nerve bundles after transection and recovery of nerve function after crush or frozen injuries were delayed³⁻⁵. Underlying mechanisms for the impaired fiber regeneration in diabetes have been ascribed to hyperglycemia-related metabolic abnormalities^{5,6}, including the polyol pathway⁶⁻⁹, glycation¹⁰ and oxidative stress^{11,12}. Aldose reductase inhibitors and various neurotrophic factors have also been challenged for the treatment of human diabetic neuropathy, but are yet to be satisfactory¹³⁻¹⁵.

Despite the plethora of literature, little is known as to the mechanism of how the peripheral nerve fibers undergo abnormal regeneration and structural characterization of regenerative fibers in the nerves of diabetic patients. To evaluate the efficacy to prevent or correct the impaired regeneration, characterization of regenerated nerve fibers in the diabetic condition is essential. In previous preclinical studies, axonal transection^{3,9,16} and

crush injuries^{5,6,8,17} or freezing damage^{7,11} have commonly been employed using animal models. Results were not consistent, however, either in the case of transection^{3,9,16} or crush injuries^{5,6,8,17}.

In the present study, we sequentially examined by light microscopic and ultrastructural morphometric analyses on the peripheral nerve fiber regeneration after axotomy in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (Japan Clea, Tokyo, Japan) aged 8 weeks were rendered diabetic by intravenous injection of STZ (40 mg/kg; Sigma Co., St. Louis, MO, USA). Only rats with tail blood glucose levels over 22 mmol/L were used for the experiment. Age- and sex-matched normal control rats were used for comparison. Blood glucose was determined by a glucose reflectance meter (Glucoboy; Eiken, Kyoto, Japan). Glycated hemoglobin values were measured with a microassay (HbA1c Columntest; BioRad, Richmond, CA, USA)^{18,19}. A total of 4 weeks after the onset of diabetes, the left mid-sciatic nerve at the distal end of the femur was transected under isoflurane anesthesia in both diabetic and control rats. The transected site was marked with nylon suture thread. A sham operation was carried out on the contralateral side of the sciatic nerve. The cut end of the severed sciatic nerve was ensheathed with a low-density

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Received 8 March 2013; revised 7 May 2013; accepted 14 May 2013

polyethylene tube (1.2 mm internal diameter, #Cat-#1978A; SanplaTec, Osaka, Japan) in a 10-mm length to avoid exposure to the surrounding soft tissues. Before use, the end of the tube was heated to close the lumen, making a dead-end, and sterilized. Two and 4 weeks post-axotomy, the rats were killed by blood withdrawal from the heart under anesthesia with sodium pentobarbital (50 mg/kg i.p.; Abbot Ltd., Chicago, IL, USA) and the sciatic nerves were extirpated and processed for structural examination.

All of the procedures in the present study followed the 'Principles of Laboratory Animal Care' (National Institutes of Health publication no.85-23, revised 1985), and the institutional guidelines of Hirosaki University Animal Experimentation for the care and use of laboratory animals. The experimental protocol was approved by the ethical committee of Hirosaki University (approval number: #M08020).

Preparation of Nerve Samples

Two portions of nerve samples – the cut end and the 5-mm site proximal to the transected portion – were processed for the light and electron microscopic observations. Samples from non-severed sciatic nerves adjusted to the site corresponding to 5-mm proximal to transection were also examined for comparison.

Nerve samples were fixed in 2.5% glutaraldehyde buffered with 0.05 mmol/L sodium cacodylate (pH 7.3) overnight at 4°C and postfixed in 1% osmium tetroxide for 4 h. Then they were dehydrated through an ascending series of ethanol and acetone. They were embedded in epon and polymerized.

Light Microscopic Morphometry

Semi-thin plastic transverse sections of the cut end of the nerve filled with regenerated fibers, and the proximal site of axotomized and non-axotomized nerve fasciculi were obtained with an RMC ultramicrotome (RMC, Tuscon, AZ, USA) and stained with 0.5% toluidine blue. First, the total fascicular area as a surface endoneurial area surrounded by the inner border of the perineurium was measured at $\times 100$ magnification on the cross-sections at both the cut end and proximal site by use of a computer-assisted image analyzing system (NIH image; Agfa-Arcus Scanner, Mortsel, Belgium)¹⁸. Then, myelinated fiber density (MFD; $\#/mm^2$) and myelinated fiber size (MFS; mm^2) were measured at $\times 400$ and $\times 2,000$ magnification on the sections of proximal sciatic nerves. MFD was obtained by measurement of myelinated fiber number per randomly selected four to six frames of the sciatic nerve fasciculi. The mean MFS was obtained by measurement of more than 300 nerve fibers delineated by an outer myelin border in each animal. The mean values of MFD and MFS in each group were then obtained by calculation using the mean value of each rat. MFD at the cut end in the operated group was obtained in a similar manner on the photomontages enlarged by $\times 2,000$. Only fibers with clear myelination stained with toluidine blue were counted. Total fiber number in the fascicle was calculated by multiplication of MFD by total fascicular

area in each rat, and the mean value was obtained in each group. The thickness of the perineurium was obtained by measurement of the thinnest width between the inner and outer border of the perineurium, and mean values were compared between the diabetic and control groups.

Electron Microscopic Morphometry

After light microscopic morphometric analysis, ultrathin sections were obtained from all groups. The sections were stained with uranyl acetate and lead citrate. Then they were observed by electron microscopy (JEOL 2000, Nihon-Denshi, Tokyo, Japan) and photographed. At the cut end, photographs were taken at a magnification of $\times 15,000$ – $20,000$. Proximal portions and samples of sham-operated rats were observed and photographed at $\times 3,000$ for the measurement of axonal size (AS), and then randomly selected myelinated fibers were enlarged to $\times 25,000$ – $30,000$ and photographed for counting the number of major dense lines of myelin sheath.

Measurements of MFS (mm^2), AS (mm^2) and the number of myelin lamellae (ML) were carried out by a computer-assisted image analyzing system as described earlier, and myelin axon ratio (ML/AS; $\#/mm^2$) was calculated accordingly²⁰. For this morphometry, at least 40–50 myelinated fibers were chosen, and the mean value of each rat was obtained. Then by using these mean values, the mean group values were calculated.

In the sections of proximal sites in the axotomized nerve and of the cut end, there were scattered fibers undergoing active axonal degeneration that were omitted for the morphometric analysis. Cross-sectioned fibers with a long axis and short axis ratio $<1:2$ were used for electron microscopic morphometric analysis. All morphometric analysis was carried out by two investigators unaware of the identity of the samples.

Statistical Analysis

Comparison of the mean values between groups was made using one-way ANOVA and Fisher's protected least significant methods. Significant difference was obtained when a *P*-value was <0.05 . Comparison of correlation lines was based on Dyck's methods²¹, and group comparisons at each point of ML were carried out by Mann–Whitney *U*-test.

RESULTS

Laboratory Data

All diabetic rats showed marked hyperglycemia both at 2 and 4 weeks after axotomy (Table 1). At the time of killing, glycated hemoglobin levels were greater in the diabetic groups compared with the non-diabetic groups at both 2 and 4 weeks post-axotomy. Bodyweight was less in diabetic groups compared with control groups.

Light Microscopic Morphometric Data

Sham-Operated Groups (Non-Axotomized Condition)

Both at 2 and 4 weeks after axotomy, the mean values of total fascicular size were comparable between the diabetic and

Table 1 | Laboratory data of experimental animals at the time of killing

Group	No. animals	Bodyweight (g)	Blood glucose (mmol/L)	Glycated hemoglobin (%)†
C-2W	n = 6	289 ± 14	4.6 ± 0.6	1.4 ± 0.2
D-2W	n = 6	232 ± 11*	25.3 ± 0.1*	1.8 ± 0.2**
C-Ax-2W	n = 8	291 ± 12	4.4 ± 0.5	1.4 ± 0.1
D-Ax-2W	n = 7	230 ± 15*	25.4 ± 1.3*	1.8 ± 0.2**
C-4W	n = 6	301 ± 15	4.9 ± 0.5	1.4 ± 0.2
D-4W	n = 7	226 ± 19*	25.5 ± 0.8*	2.7 ± 0.4*
C-Ax-4W	n = 6	315 ± 14	4.5 ± 0.8	1.4 ± 0.2
D-Ax-4W	n = 6	219 ± 11*	25.9 ± 1.3*	2.7 ± 0.3*

Values are mean ± standard deviation. * $P < 0.01$ vs non-diabetic control, ** $P < 0.05$ vs non-diabetic control. †Glycated hemoglobin was measured by a microassay method (BioRad HbA1c Columntest; BioRad Lab)^{18,19}. C-2W, non-diabetic control rats 2 weeks after sham operations; C-4W, non-diabetic control rats 4 weeks after sham operations; C-Ax-2W, non-diabetic control rats 2 weeks after axotomy; C-Ax-4W non-diabetic control rats 4 weeks after axotomy; D-2W, diabetic rats 4 weeks after sham operations; D-4W, diabetic rats 4 weeks after sham operations; D-Ax-2W, diabetic rats 2 weeks after axotomy; D-Ax-4W; diabetic rats 4 weeks after axotomy.

control groups with or without axotomy. There was a significant reduction in mean MFS at proximal sites at 4 weeks after sham operations in diabetic rats (D-4W) compared with non-diabetic control rats (C-4W) ($P < 0.01$). The difference in MFS did not reach a significant level at the time of 2 weeks ($P = 0.052$; Table 2). By contrast, mean MFD and total fiber number in D-4W were greater than those in C-4W ($P < 0.01$), whereas those at 2 weeks were not different between the diabetic and control groups.

Post-Axotomy Groups

Total fascicular size of the proximal site or at the cut end was not significantly affected by axotomy in either the control or diabetic

groups at both 2 and 4 weeks. Two weeks post-axotomy, there were no significant differences in mean MFD or total fiber number at the cut end and its proximal site between the diabetic and control groups (Table 2). Mean MFS at the proximal site was smaller in the diabetic group than that in the control group at 2 weeks post-axotomy ($P < 0.01$). The cut end showed a thickening of perineurial membrane, but the mean thickness of the perineurium in diabetic rats (9.3 ± 4.1 mm) was not significantly different from those in control rats (9.2 ± 3.4 mm; $P > 0.15$). There were no significant differences in mean MFD and mean total fiber number at the cut end 2 weeks post-axotomy.

At 4 weeks post-axotomy, there was a significant increase (20%) in mean MFD and total fiber number at the cut end in the diabetic group compared with those in the control group ($P < 0.01$; Figure 1, Table 2). The mean thickness of the perineurium in diabetic rats (11.0 ± 2.6 mm) was not significantly different from those in control rats (12.1 ± 2.2 mm) ($P > 0.10$). At the proximal site, the mean MFD was similarly increased in the diabetic groups (24%) compared with those in the control groups. When compared with the values of the non-axotomized groups (C-4W and D-4W), both the diabetic (D-Ax-4W; 23%) and control (C-Ax-4W; 22%) groups showed a significant increase in MFD ($P < 0.05$ and $P < 0.01$, respectively). The mean MFS at the proximal site of axotomized nerves in the diabetic group was significantly smaller than that in the control group ($P < 0.01$). When compared with the values in the non-axotomized condition (C-4W and D-4W), the mean MFS in all axotomized groups was significantly smaller than the non-axotomized group ($P < 0.01$). A reduced rate of MFS or AS was comparable between the diabetic and control groups.

Electron Microscopic Morphometric Data

Regenerated Fibers at the Cut End

Two weeks post-axotomy, there was no significant difference in the mean MFS between the diabetic group at the cut end and

Table 2 | Light microscopic morphometric data on myelinated fibers in experimental animals

Group	n	Proximal site				Cut end		
		Total fascicular size (mm ²)	Myelinated fiber density (#/mm ²)	Myelinated fiber size (μm ²)	Total fiber number	Total fascicular area (mm ²)	Myelinated fiber density (#/mm ²)	Total fiber number
C-2W	n = 6	0.54 ± 0.08	6,090 ± 719	46.4 ± 1.5	3,522 ± 729			
D-2W	n = 6	0.56 ± 0.11	6,332 ± 530	44.5 ± 1.6	3,305 ± 471			
C-Ax-2W	n = 8	0.54 ± 0.08	6,055 ± 741	48.6 ± 3.9	3,396 ± 687	0.28 ± 0.09	6,468 ± 914	1,924 ± 690
D-Ax-2W	n = 7	0.53 ± 0.08	6,547 ± 1,146	43.2 ± 2.5*	3,498 ± 907	0.27 ± 0.06	6,760 ± 438	1,837 ± 432
C-4W	n = 6	0.73 ± 0.12	7,396 ± 1,352	53.4 ± 1.9	6,732 ± 1,067			
D-4W	n = 7	0.77 ± 0.16	9,270 ± 683**	47.6 ± 2.4**	7,685 ± 910			
C-Ax-4W	n = 6	0.74 ± 0.23	9,134 ± 337§	42.9 ± 1.5**	7,965 ± 1,150§	0.38 ± 0.12	10,008 ± 1,122	3,743 ± 1,101
D-Ax-4W	n = 6	0.79 ± 0.15	11,350 ± 621†¶	36.0 ± 2.7††	7,675 ± 1,518	0.35 ± 0.09	12,551 ± 480†	4,401 ± 1,285¶

Values are mean ± standard deviation. * $P < 0.01$ vs non-diabetic control rats 2 weeks after axotomy (C-Ax-2W), ** $P < 0.01$ vs non-diabetic control rats 4 weeks after sham operations (C-4W), § $P < 0.05$ vs C-4W, † $P < 0.01$ vs diabetic rats 4 weeks after sham operations (D-4W), ‡ $P < 0.01$ vs non-diabetic control rats 4 weeks after axotomy (C-Ax-4W), ¶ $P < 0.05$ vs C-Ax-4W. C-2W, non-diabetic control rats 2 weeks after sham operations; D-2W, diabetic rats 4 weeks after sham operations; D-Ax-2W, diabetic rats 2 weeks after axotomy; D-Ax-4W; diabetic rats 4 weeks after axotomy.

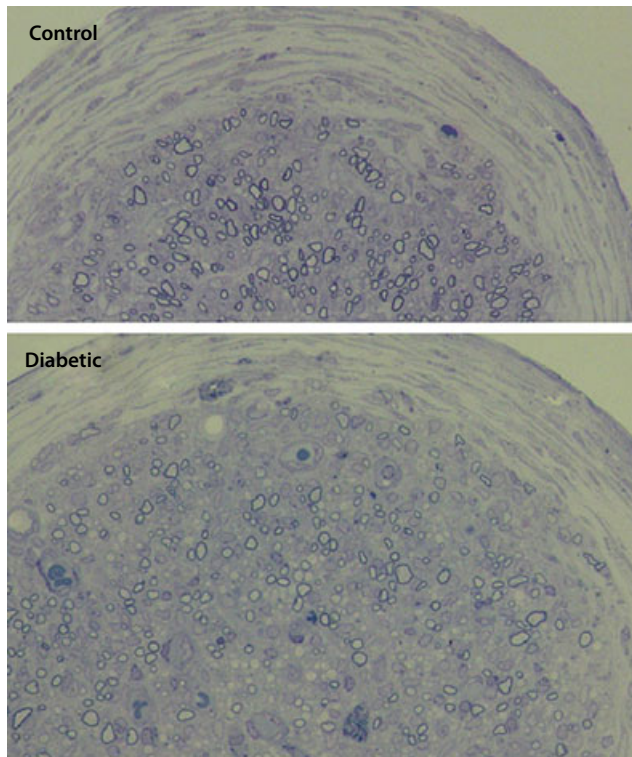


Figure 1 | Light microscopic view of regenerated fibers at the cut end 4 weeks post-axotomy in experimental animals.

Table 3 | Electron microscopic data of myelinated fibers at cut end and its proximal site

	Myelinated fiber size (μm^2)	Axonal size (μm^2)	No. myelin lamellae (#/fiber)	Myelin axon ratio (#/ μm^2)
Cut end				
C-Ax-2W $n = 5$	6.4 ± 1.0	3.9 ± 0.5	14.9 ± 1.2	6.1 ± 1.1
D-Ax-2W $n = 5$	6.2 ± 0.5	3.5 ± 0.4	14.7 ± 0.8	5.5 ± 0.7
C-Ax-4W $n = 5$	6.5 ± 1.0	3.5 ± 0.5	21.6 ± 2.3	6.4 ± 0.8
D-Ax-4W $n = 5$	5.6 ± 0.5	3.2 ± 0.4	$17.1 \pm 2.7^{**}$	$5.4 \pm 0.3^{**}$
Proximal site				
C-Ax-2W $n = 5$	47.9 ± 5.2	15.8 ± 2.4	75.9 ± 5.4	6.3 ± 1.3
D-Ax-2W $n = 5$	49.9 ± 2.0	16.7 ± 0.4	77.6 ± 4.8	6.0 ± 0.9
C-Ax-4W $n = 5$	49.4 ± 3.4	14.8 ± 0.8	80.1 ± 5.6	5.4 ± 0.3
D-Ax-4W $n = 5$	$43.9 \pm 1.4^{**}$	$11.8 \pm 2.2^*$	79.9 ± 1.2	$7.4 \pm 0.8^{**}$

Values are mean \pm standard deviation. * $P < 0.01$ vs non-diabetic control rats 4 weeks after axotomy (C-Ax-4W), ** $P < 0.05$ vs C-Ax-4W. C-2W, non-diabetic control rats 2 weeks after sham operations; C-4W, non-diabetic control rats 4 weeks after sham operations; C-Ax-2W, non-diabetic control rats 2 weeks after axotomy; D-2W, diabetic rats 4 weeks after sham operations; D-4W, diabetic rats 4 weeks after sham operations; D-Ax-2W, diabetic rats 2 weeks after axotomy; D-Ax-4W, diabetic rats 4 weeks after axotomy.

the control group (Table 3). The mean number of myelin lamellae (ML) per fiber was not different between the diabetic and control groups.

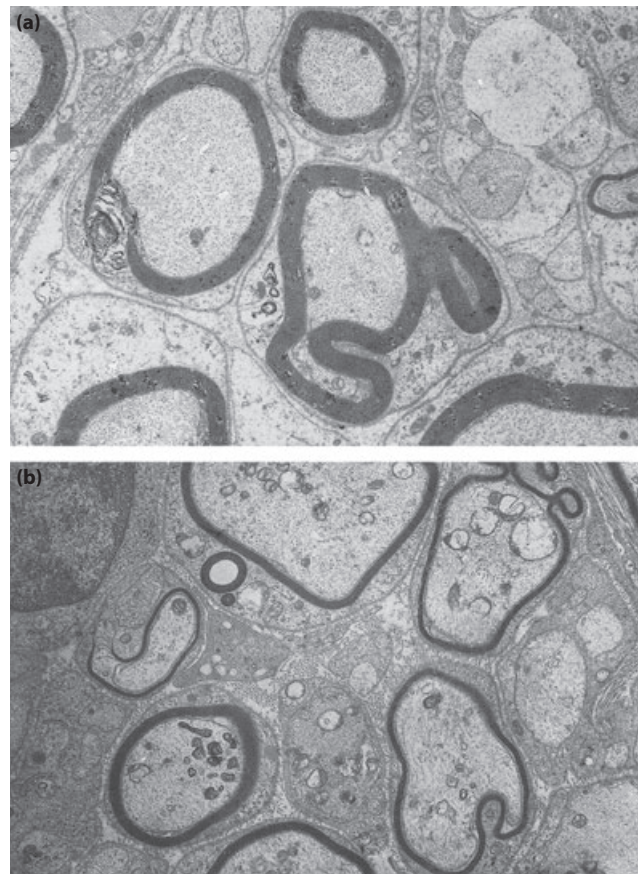


Figure 2 | Ultrastructure of regenerated fibers at the cut end. Compared with (a) control rats, (b) diabetic rats showed many thinly myelinated regenerated fibers.

Four weeks post-axotomy, there was no difference in mean MFS or AS between the two groups, although there was a trend toward a decrease in the diabetic group ($P = 0.09$, $P = 0.12$, respectively; Table 3). The mean number of ML per fiber was significantly reduced in the diabetic group (21%) compared with the control group ($P < 0.05$). Electron microscopic features typically showed thinly myelinated regenerated fibers in the diabetic rats compared with the control rats (Figure 2).

Myelinated Fibers at the Proximal Site

Electron microscopic morphometric analysis on myelinated fibers in the proximal site in the axotomized condition confirmed a significant decrease in mean MFS (12% reduction of MFS) and mean AS (20% reduction of AS) in the diabetic group (D-Ax-4W) compared with the control group (C-Ax-4W) at 4 weeks ($P < 0.05$, $P < 0.01$, respectively; Table 3). There was no significant difference in the number of ML among groups.

Relationship Between Number of ML and AS

There was a significant correlation between AS and the number of ML in regenerated myelinated fibers at the cut end in all

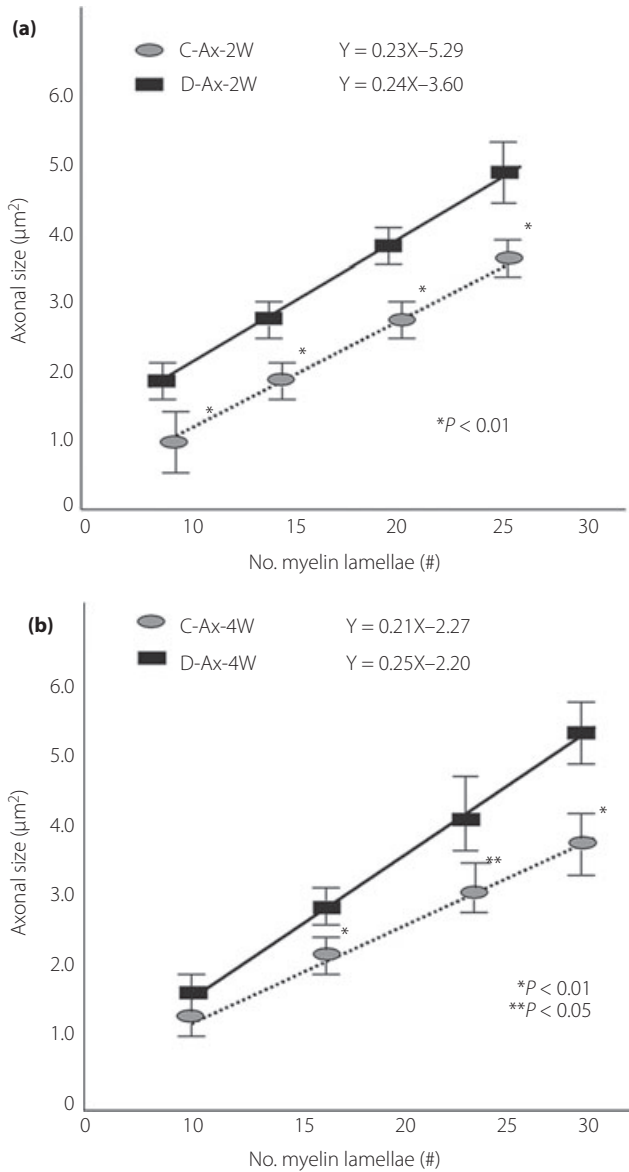


Figure 3 | Relationships between the number of myelin lamellae and axonal size of myelinated fibers at the cut end in axotomized experimental animals. There was a significant correlation between the number of myelin lamellae and axonal size in both (a) 2 weeks post-axotomy and (b) 4 weeks post-axotomy. In relation to the axonal size (y-axis), the number of myelin lamellae (x-axis) was constantly smaller in diabetic rats compared with control rats, suggesting the presence of hypomyelination in diabetic rats. Bar stands for standard deviation.

groups (Figure 3). In relation to each point of AS, the number of ML was consistently smaller in the diabetic group, indicating hypomyelination of regenerated fibers in diabetic rats. The relationship between AS and the number of ML in the myelinated fibers at the proximal site showed a significant reduction of AS at 4 weeks in relation to the respective number of ML, but not at 2 weeks (Figure 4).

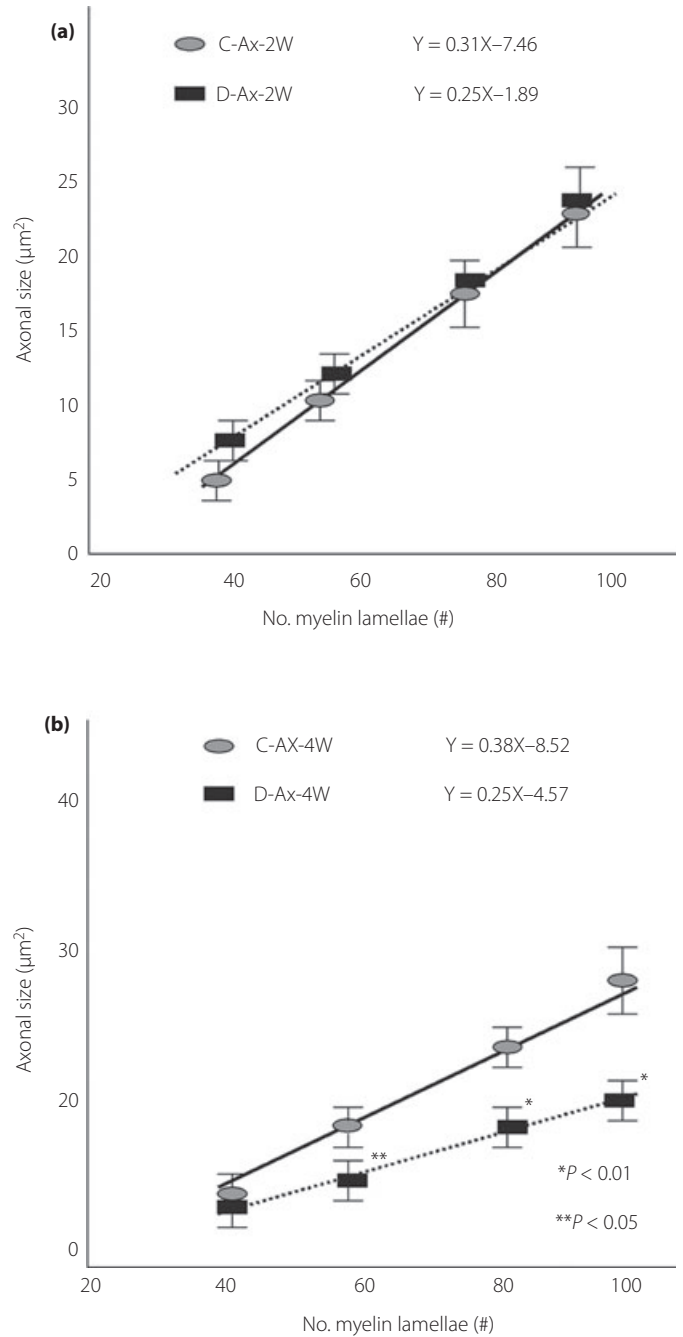


Figure 4 | Relationships between the number of myelin lamellae and axonal size of myelinated fibers at the proximal site in axotomized animals. (a) At 2 weeks post-axotomy, there was no difference in the correlation lines of diabetic and control animals. (b) In contrast, 4 weeks post-axotomy, there was a constant decrease in axonal size, indicating the presence of axonal atrophy. Bar stands for standard deviation.

DISCUSSION

The present study confirmed the presence of impaired nerve fiber regeneration in STZ-diabetic rats, characterized by thinly-myelinated regenerated fibers at the cut end 4 weeks

post-axotomy. The differences in overall morphometric data investigated in the present study between the diabetic and control groups were not significant at 2 weeks post-axotomy. Regenerated fibers were evaluated at the cut end right next to the transection site, where the examined tissues were considered to consist only of regenerated fibers derived from pre-existing fibers; some of which might undergo axonal degeneration. We found increased MFD at the cut end in the diabetic group compared with the non-diabetic controls 4 weeks post-axotomy. Increased MFD in the diabetic group at the cut end 4 weeks post-axotomy was associated with increased total fiber number in the present study, indicating an increased population of small regenerated fibers in diabetes. It has previously been shown that regenerated fibers grew slowly in STZ-diabetic rats to the extent of approximately 2 mm at 3 weeks, whereas they reached more than 6 mm in non-diabetic control rats³. The results were consistent with a previous report showing increased MFD at 5 mm proximal to the crushed site with a mixture of degenerated and regenerated fibers in STZ-diabetic rats¹⁷.

In contrast to the changes at the cut-end, the effects of diabetes on MFD and MFS in the proximal site 4 weeks after axotomy might require careful interpretation. Even without axotomy, reduced MFS is characteristic in diabetic rats, suggesting the impaired maturation of fibers in diabetes (Table 2)²². Comparison of the data showed a more severe reduction of fiber size (75% in diabetic and 80% in control) at the proximal site of transected nerves compared with unaxotomized conditions. Ultrastructural quantitative analysis further disclosed a significant reduction of fiber and axonal sizes in the diabetic group. Thus, the changes of the transected nerves at the proximal site detected in the present study might possibly result from the combination of impaired nerve fiber maturation and the influence of diabetes. The data of reduced MFS in the proximal site are comparable to the results of morphometric data of post-axotomized sciatic nerves in a rat^{23,24} or feline model until 2 years post-axotomy²¹. In spontaneously diabetic biobreeding rats, the increase in MFD of regenerated fibers was transient for the initial 6 weeks and returned to normal at 4 months post-axotomy⁹. The trend of increased fiber density was also detected in the peripheral nerves of STZ-diabetic rats 18 weeks after crush injury, although the number of animals examined in the present study was too small for statistical comparison¹⁵.

Regenerated nerve fibers at the cut end are small, so the precise morphometry on fiber size and axonal size, as well as the myelin sheath, was only feasible at electron microscopic levels. At 4 weeks post-axotomy, there was no significant difference in mean MFS of regenerated fibers between diabetic and control rats. This could indicate that the somato-fugal fiber and axonal atrophy of the peripheral sensory nervous system previously shown in long-term STZ-diabetic rats^{25,26} might not influence the size of regenerated fibers in diabetic groups at this stage.

The present study showed impaired myelination of regenerated fibers showing a reduced number of myelin lamellae in diabetic rats. Local metabolic abnormalities might be responsible for the

defect in myelination. Myelination could largely be dependent on Schwann cell metabolism, in which membrane synthesis is impaired by hyperglycemia through activation of the polyol pathway^{26–29}. Alternatively, excessive breakdown of the myelin membrane once produced might be accelerated in an injured site of the cut end. In STZ-diabetic rats, alterations in membrane lipid composition can also cause reduced myelination³⁰ or vulnerability of the myelin sheath to environmental factors³¹, as lipoprotein metabolism is perturbed in diabetes³⁰. Recent studies showed that excessive oxidative stress and neurovascular dysfunction are involved in the impairment of myelination of regenerated fibers^{11,12,32}. We did not specifically address the microvessel changes, because newly-derived vessels at the cut end were not easily identified because of their immaturity. Future investigations of microvessels with the aid of immunohistochemistry in a systematic manner might therefore be important to elucidate the role of microangiopathy in the regeneration defects in diabetes.

Impaired regeneration attributes to progressive fiber loss with functional deficits with sensory loss, abnormal sensation in diabetic neuropathy and leads to a poor prognosis^{1,2}. The loss of nerve fibers correlates well to the severity of human diabetic neuropathy^{33,34}. The current results suggest there needs to be a multifaceted approach to the amelioration of impaired regeneration in diabetes; one way to promote regeneration by improving neuronal metabolism and correcting the supply of neurotrophic factors^{14,15}, and another to improve myelination by correcting oxidative stress as well as impaired vascular circulation^{12,32}. Precise analysis of a variety of environmental factors will further elucidate other factors involved in impaired regeneration in diabetes³⁵.

ACKNOWLEDGEMENTS

This study was supported in part by a grant-in-aid from the Japanese Ministry of Science, Culture, Education and Sport to SY. The authors also thank Ms Saori Ogasawara and Ms Hiroko Mori for excellent technical assistance. All authors listed declare no conflict of interest in this study.

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