Corneal confocal microscopy (CCM) has been used to evaluate small nerve fibers in the cornea. Such studies have revealed that the density of nerve fibers in the subbasal nerve plexus (SBNP) correlates with the severity of diabetic neuropathy in human patients, suggesting that such analyses can be used to assess diabetic nerve impingement. Animal models, especially rats and mice, are essential for studying diabetic peripheral neuropathy, as they can be used to replicate human-like corneal nerve lesions. Previous experimental studies assessing corneal nerves as a measure of the severity of diabetic peripheral neuropathy have yielded conflicting results; this may be due to the short duration of the induced diabetes.

METHODS. A total of 30 female ICR mice were divided into three groups: those with STZ-induced (STZ group) and AL-induced (AL group) diabetes, and a control group. Hyperglycemia was maintained in diabetic mice for 35 weeks. Animals were euthanized at 41 weeks of age.

RESULTS. Subbasal nerve plexus density (SBNPD) and terminal epithelial nerve density (TEND) in the cornea, as well as IENFD, were significantly lower, and mean sural nerve axon sizes were smaller in mice in the STZ and AL groups than in the control group. There were significant correlations between IENFD and TEND, and between IENFD and SBNPD.

CONCLUSIONS. These results indicate that the TEND and SBNPD of the cornea may be useful morphologic markers for diabetic peripheral neuropathy.

Keywords: cornea, diabetes, peripheral neuropathy, skin, sural nerve
conventional environment, with a temperature of 20°C to 26°C, and a relative humidity of 40% to 70%, under a 12/12-hour light/dark cycle. They were ventilated with filtered fresh air and allowed free access to tap water and to a widely used standard pelleted diet for experimental rats (Charles River Formula 1; Oriental Yeast, Tokyo, Japan). The animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research with the “Guide for the Care and Use of Laboratory Animals” of Setsunan University and the Japanese Association for Laboratory Animal Science. The Committee for Animal Experiments of Setsunan University approved the present study (approval number: K16-20).

Experimental Design

We performed a preliminary study to select the appropriate mouse strain and sex and to determine AL and STZ concentrations, using male and female ICR and C57BL/6 mice (Japan SLC, Inc., Hamamatsu, Japan). Female ICR mice and the concentrations of STZ and AL used in this study were selected so as to induce continuous glycosuria without moribund condition or death. A total of 30 female ICR mice were randomized based on body weight to minimize differences (initial mean body weight ± standard deviation [SD], 27.70 ± 2.45 g). Mice were divided into control, STZ, and AL groups (n = 10 per group) at 6 weeks of age (Fig. 1). Then, mice in the STZ group were treated with a single dose of STZ (Sigma-Aldrich Japan, Tokyo, Japan), via the tail vein, at 75 mg/kg body weight, while mice in the AL group were treated with a single dose of AL (Sigma-Aldrich), via the tail vein, at 200 mg/kg body weight. All mice were killed at 41 weeks of age. Mice in the control group were treated with saline.

Body Weight, and Blood and Urinary Glucose

Body weight, and blood and urinary glucose were measured monthly from 6 weeks of age until autopsy. Blood from the tail vein and urine samples were collected between 1:00 and 4:00 PM. Blood glucose levels were measured semiquantitatively using the glucose oxidase method (Glutest E; Sanwa Kagaku, Nagoya, Japan). Urinary glucose levels were measured semiquantitatively with urine test paper (Wako Pure Chemical Industries, Osaka, Japan).

Sensory Nerve Conduction Velocity (SNVC)

At the end of the experiment, SNVC was measured after mice had been anesthetized with ketamine (40 mg/kg, intramuscularly [IM]; Ketalar, Sankyo, Tokyo, Japan) and xylazine (2.0 mg/kg, IM; Seractal, Bayer, Tokyo, Japan). The SNVC was determined via distal supramaximal stimulation of the sural nerve at the ankle via bipolar electrodes. SNVC values were recorded at the fourth and fifth digits, using an electromyography system (Polygraph 560 System; Nippon-denki-sanei, Tokyo, Japan, and BioSignal Processing Program; Nihonsante-ku, Osaka, Japan). Values were calculated based on the onset latency and distance. The hindlimb skin temperature was maintained at 37°C during all measurements.

Histologic and Morphometric Analysis of the Sural Nerves

Mice were euthanized via exsanguination from the abdominal aorta under deep anesthesia with ketamine (40 mg/kg, IM; Ketalar, Sankyo) and xylazine (2.0 mg/kg, IM; Seractal, Bayer). The left sural nerve was removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After fixation, tissue samples were postfixed in 1.5% osmium tetroxide solution (pH 7.4) for 2 hours and processed in epoxy resin. Semithin 1-μm sections were cut and stained with toluidine blue. Semithin cross-sections of a distal portion of the sural nerve were used for morphometric analysis. One nerve section was analyzed for each animal. Sural nerve samples were obtained by selecting a terminal portion of the sural nerve, approximately 5 mm long, from immediately before the proximal to the terminal branching. Digital images (×40 objective lens, 3900 × 3090 pixels) were captured using a digital camera (DC450; Leica Microsystems, Wetzlar, Germany) attached to a light microscope (DM5500; Leica Microsystems). The following morphometric parameters were evaluated using image processing and analysis software (IP Lab version 4.0; BD Biosciences, Rockville, MD, USA): (1) total fascicular area; (2) numbers and sizes (cross-sectional area) of myelinated nerve fibers, myelin, and axons; and (3) mean fiber, axon, and myelin size (cross-sectional area). Fiber occupancy (nerve fiber area/fascicular area) was calculated by dividing the total area of myelinated fibers by the total fascicular area. Fiber density (number of fibers/mm²) was calculated by dividing the total number of myelinated fibers by the total fascicular area. Histograms for the size frequency of nerve fibers, axons, and myelin, which were separated into class intervals of 10 μm² (fibers and myelin) and 5 μm² (axons), also were constructed.

Histologic and Morphometric Analysis of Intraepidermal Nerves

Foot pads were collected from the plantar surface of the hind paw and fixed via immersion in 4% paraformaldehyde in a 0.1 M sodium phosphate buffer (pH 7.4). Samples were rinsed with water, immersed in 30% sucrose solution, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Tokyo, Japan), and frozen in hexane, cooled by liquid nitrogen. Sections (80 μm thick) then were cut at a cabinet temperature of −20°C using a cryostat (Leica CM3000; Leica Microsystems) and dried in cold air for 30 minutes. The slides were rinsed with 0.05 M Tris-buffered saline (TBS, pH 7.6), treated with 1% hydrogen peroxide in methanol, and again rinsed with TBS. The slides were incubated with 5% normal goat serum for 5 minutes, following which, they were incubated with a pan-neuronal marker, rabbit polyclonal anti-PGP9.5 antibody (dilution, 1:200; Dako, Santa Clara, CA, USA) overnight at 4°C.20 The sections then were exposed to Alexa Fluor 488-conjugated secondary antibodies for 1 hour (Invitrogen, Carlsbad, CA, USA) and mounted using mounting medium. As a negative control, mouse or rabbit isotype immunoglobulin, diluted to the same concentration, was substituted for the primary antibody. The IENFD was quantified in accordance with the European Federation of Neurological Societies guidelines.5 Five randomly chosen tissue sections from each animal were quantified. Only single IENFs crossing the dermal-epidermal junction were counted, while secondary branching and nerve fragments not crossing the dermal-

<table>
<thead>
<tr>
<th>experimental design</th>
<th>cont n=10</th>
<th>diabetes</th>
<th>AL n=10</th>
<th>diabetes</th>
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**Figure 1.** Experimental design.

- **Histologic and Morphometric Analysis of the Sural Nerves**
- **Histologic and Morphometric Analysis of Intraepidermal Nerves**
- **Sensory Nerve Conduction Velocity (SNVC)**
- **Body Weight, and Blood and Urinary Glucose**
analyses were performed using JMP Academic Suite 11.2 Pro.

To evaluate IENFD, the size frequency of sural nerve axons, and values. Spearman’s rank correlation coefficients were used to test was used to compare the differences among the mean variance was heterogeneous, the Kruskal–Wallis H-test (parametric) was performed. If a significant difference was observed among the groups, Tukey’s test (parametric) was performed to compare the differences among the mean values. When the variance was heterogeneous, the Kruskal–Wallis H-test (nonparametric) was performed. If a significant difference was observed among the groups, the nonparametric Steel–Dwass test was used to compare the differences among the mean values.

Bartlett’s test, followed by a 1-way ANOVA when the variance was homogeneous. Corneal TEND and SBNPD in the peripheral cornea did not differ significantly among the three groups (Table 1). Severe loss of nerve fibers was observed in the SBNP of the STZ and AL groups, among which whorl-like structures also were indistinct (Fig. 5a, Table 1). SBNPD also was significantly lower in the STZ and AL than in the control groups (Fig. 4b, Table 1). Severe loss of nerve fibers was observed in the SBNP of the STZ and AL groups, among which whorl-like structures also were indistinct (Fig. 5a, Table 1).

In the experimental period, the corneas, which were divided into several branches and connected to the SBNP (Figs. 3i–k). In contrast, the TENDs exhibited markedly diffuse dispersion in the central cornea (Fig. 4a, Table 1). Moreover, TEND was significantly lower in the STZ and AL than in the control groups (Fig. 4b, Table 1). Severe loss of nerve fibers was observed in the SBNP of the STZ and AL groups, among which whorl-like structures also were indistinct (Fig. 5a, Table 1).

PGP9.5 is a panneuronal marker.5 PGP9.5–positive nerve fibers were detected in epithelial, subepithelial, and stromal layers in the cornea. In the control group, fine terminal epithelial nerves branches and connected to the SBNP (Figs. 3i–p). In contrast, in the control group, the TENDs exhibited markedly diffuse dispersion in the central cornea (Fig. 4a, Table 1). Moreover, TEND was significantly lower in the STZ and AL than in the control groups (Fig. 4b, Table 1). Severe loss of nerve fibers was observed in the SBNP of the STZ and AL groups, among which whorl-like structures also were indistinct (Fig. 5a, Table 1). SBNPD also was significantly lower in the STZ and AL than in the control group (Fig. 5b, Table 1). However, TEND and SBNPD in the peripheral cornea did not differ significantly among the three groups (Table 1). No obvious changes were detected in the corneal epithelium, and there was no significant difference in corneal epithelium thickness among the three groups (data not shown).

**Results**

**Body Weight, Glucosuria, and Glycemia**

Body weight gradually increased in the control group. Although slight increases in body weight were observed in the STZ and AL groups, no changes were observed between 26 and 41 weeks of age (Fig. 2). Severe hyperglycemia (>300 mg/dL) and glucosuria (>500 mg/dL) continued for 35 weeks in the STZ and AL groups. All control group mice were normoglycemic (<150 mg/dL) during the experimental period.

**Corneal Nerve**

PGP9.5 is a panneuronal marker.5 PGP9.5–positive nerve fibers were detected in epithelial, subepithelial, and stromal layers in the cornea. In the control group, fine terminal epithelial nerves stemmed from the SBNP innervated the epithelial cells of the cornea (Figs. 3a–h). Subbasal nerve bundles formed whorl-like structures or vortices in the subbasal zone. These subbasal bundles connected to each other to form the SBNP (Figs. 3i–k).

Whole eye globes were removed and fixed via immersion in 4% paraformaldehyde in a 0.1 M sodium phosphate buffer (pH 7.4). Corneas were excised and made transparent via immersion in optical cleaning agent (SCALEVIEW-A2; Olympus, Tokyo, Japan) for 4 weeks. The corneas then were rinsed with 0.05 M TBS (pH 7.6) for 1 day and incubated with the rabbit polyclonal anti-PGP9.5 antibody at 4°C (dilution, 1:200; Dako) for 1 week. The samples were exposed to Alexa Fluor 488-conjugated secondary antibodies for 1 hour (Invitrogen) on 2 consecutive days and stored at 4°C in TBS with azide until used. The corneas were mounted with mounting medium. Z-stack images of nerve fibers were taken in steps of 1 μm from the top of the epithelial zone to the stromal zone in the central and peripheral corneal regions using a confocal microscope (×400 magnification, FV1000; Olympus). The percent area of the terminal nerve fibers in the epithelial area (terminal epithelial nerve density [TEND]) and the percent area of nerve fibers in the subbasal area (subbasal nerve plexus density [SBNP]) were determined via computer-assisted image analysis (Photoshop CS4; Adobe, San Jose, CA, USA). The results are expressed as the density of nerve fibers within the area of interest.

**Statistical Analysis**

Data are presented as the mean ± SD. A multiple comparison test was performed to analyze the differences among the three groups. The homogeneity of variance was analyzed using Bartlett’s test, followed by a 1-way ANOVA when the variance was homogeneous. If a significant difference was observed among the groups, Tukey’s test (parametric) was performed to compare the differences among the mean values. When the variance was heterogeneous, the Kruskal–Wallis H-test (Wilcoxon test) was performed. If a significant difference was observed among the groups, the nonparametric Steel-Dwass test was used to compare the differences among the mean values. Spearman’s rank correlation coefficients were used to evaluate IENFD, the size frequency of sural nerve axons, and the corneal TEND/SBNP ratio.

P < 0.05 was considered statistically significant. Statistical analyses were performed using JMP Academic Suite 11.2 Pro software (SAS Institute, Tokyo, Japan).

**Corneal Nerve Lesions in Type 1 Diabetes Mice**

Human-like corneal nerve lesions in type 1 diabetes mice.

**FIGURE 2.** Changes in body weight in the three groups. Data are expressed as the mean ± SD. Cont, control, nondiabetic mice; AL, alloxan-induced diabetic mice; STZ, streptozotocin-induced diabetic mice.
Correlation of SBNPD, TEND, or IENFD, With the Sural Nerve Axon Size

Correlation coefficients were determined for IENFD, SBNPD, TEND, and sural nerve axon size. Significant correlations were observed for IENFD and SBNPD in the central cornea ($\rho = 0.4977$, $P < 0.01$; $\rho = 0.5125$, $P < 0.01$). Although a slight correlation was observed for small myelinated axons and SBNPD or TEND of the central cornea, this result was not significant ($\rho = 0.4427$; $P = 0.075$; $\rho = 0.3899$; $P = 0.123$). There was no correlation between IENFD and small-sized axons.

**DISCUSSION**

Our results demonstrated that mice with STZ- or AL-induced type 1 diabetes exhibit significantly lower small nerve fiber density in the SBNP of the central cornea than nondiabetic mice. Yorek et al. reported that mice with STZ-induced type 1 diabetes exhibit no significant changes in the SBNP until after 20 weeks of hyperglycemia, while others have reported that such mice exhibit loss of fibers within the SBNP after only 12 weeks of hyperglycemia. We observed significant reductions in corneal nerve fibers in mice with STZ- and AL-induced diabetes after 35 weeks of hyperglycemia, suggesting that a longer duration of hyperglycemia induces corneal nerve lesions similar to those observed in patients with type 1 diabetes. Thus, shorter hyperglycemia may cause contradictory results and lead to data misinterpretation.

In patients with diabetes, CCM can detect loss of corneal nerve fibers in the SBNP of the central cornea, which has been associated with the severity of peripheral neuropathy, suggesting that this method can be used as an alternative marker of diabetic peripheral neuropathy. A previous experimental study using CCM and immunohistochemical staining revealed that the SBNP exhibits no changes in type 1 diabetes, but is reduced in type 2 diabetes. These data suggest a significant correlation between CCM and immunohistochemical staining findings. Although loss of corneal nerve fibers in the SBNP is similar in rats, mice, and humans, mice with STZ-induced type 1 diabetes do not exhibit significant changes in the SBNP until after 20 weeks of hyperglycemia, as assessed by using CCM and histochemical staining. Our results suggested that long-term hyperglycemia is required for the reduction of small nerve fiber density in mice with STZ- and AL-induced diabetes, as in humans. Previous research also has indicated that TEND is reduced in the cornea of rats and mice with type 1 or 2 diabetes. However, similar to findings observed for the SBNP in mice with STZ-induced diabetes, no significant changes in TEND can be observed after 20 weeks of hyperglycemic conditions.

**TABLE 1. Morphometric Analysis of the Cornea and Skin**

<table>
<thead>
<tr>
<th>Group</th>
<th>Central Cornea</th>
<th></th>
<th>Peripheral Cornea</th>
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<th>Skin</th>
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<tbody>
<tr>
<td></td>
<td>TEND</td>
<td>SBNPD</td>
<td>TEND</td>
<td>SBNPD</td>
<td>IENFD</td>
</tr>
<tr>
<td>Control, mean (SD)</td>
<td>6.09 (1.91)</td>
<td>17.10 (6.51)</td>
<td>3.70 (1.08)</td>
<td>16.98 (5.39)</td>
<td>55.41 (7.03)</td>
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<tr>
<td>STZ, mean (SD)</td>
<td>4.49* (1.77)</td>
<td>10.05† (3.65)</td>
<td>2.91 (1.42)</td>
<td>12.89 (5.31)</td>
<td>30.11† (5.98)</td>
</tr>
<tr>
<td>AL, mean (SD)</td>
<td>3.52 † (1.77)</td>
<td>9.22‡ (5.23)</td>
<td>2.35 (1.14)</td>
<td>11.68‡ (3.93)</td>
<td>35.68§ (15.21)</td>
</tr>
</tbody>
</table>

AL, alloxan-induced diabetic mice; STZ, streptozotocin-induced diabetic mice.
$^*$ $P < 0.05$.
$^†$ $P < 0.01$.
$^‡$ $P < 0.001$.
$^§$ $P < 0.0001$. 

**FIGURE 3.** Innervation in the cornea. **Left:** Diagram of innervation (green lines) of the corneal epithelium, subbasal layer, and stroma. **Right:** Representative confocal microscopic images of PGP9.5-positive nerve fibers in the corneal epithelium, subbasal layer, and stroma in the central cornea of the control (Cont) group. (a–h) Fine TENs are derived from the subbasal nerve plexus and innervate epithelial cells. (i–k) Subbasal nerve bundles form whorl-like structures or vortices in the subbasal zone. These subbasal bundles connect with one another to form the SBNP (l–p) Large nerves divide into several branches in the stroma.
Human-Like Corneal Nerve Lesions in Type 1 Diabetes Mice

FIGURE 4. Significant reductions in TENs in the central cornea of the STZ and AL groups. (a) Representative confocal microscope images of PGP9.5-positive TENs in the corneal epithelium. (b) TEND is significantly lower in the AL and STZ groups than in the control (Cont) group. Data are expressed as the mean ± SD. *P < 0.05, **P < 0.01.

FIGURE 5. Significant losses in the SBNP of the central cornea in the STZ and AL groups. (a) Representative confocal microscope images of PGP9.5-positive cells in the SBNP of the cornea. (b) SBNPD is significantly lower in the AL and STZ groups than in the control (Cont) group. Data are expressed as the mean ± SD. **P < 0.01.
FIGURE 7. Significantly lower SNCV in the STZ and AL groups than in the control (Cont) group. Data are expressed as the mean ± SD. *P < 0.05.

FIGURE 8. Representative semithin sections of sural nerves in the control, STZ, and AL groups. Myelinated nerve fibers in the STZ and AL groups exhibit slight axonal atrophy when compared to those of the control (Cont) group.
mia, and Cai et al. reported that loss of TENs is associated with the animal’s age, regardless of the presence of diabetes. However, in our study, we observed significant decreases in TEND in the AL and STZ groups. These findings further supported the notion that long-term hyperglycemia is required for the reduction of small nerve fiber density in TENs and the SBNP of the central cornea, and that loss of nerve fibers progresses more rapidly in the central than in the peripheral cornea. Furthermore, our results indicated that the AL-induced diabetic mouse is an adequate model for investigating diabetic neuropathy in the corneal nerve.

We used immunohistochemical staining following in vitro tissue clearing of the cornea. This method allowed detailed analysis of the corneal nerves from the epithelial to the stromal layers. For immunohistochemical analysis of nerve fibers, we used PGP9.5, a pan neuronal marker. As A-delta and C nerve fibers distributed in the cornea were stained by PGP9.5, we could not demonstrate which type of nerve fiber was affected by hyperglycemia. In future studies, specific markers for A-delta and C nerve fibers may clarify the type of nerve fiber loss.

The density of single IENFs crossing the dermal–epidermal junction is decreased in patients with types 1 and 2 diabetes. Punch skin biopsy is a safe and reliable technique for quantifying IENFD, which has been proposed as a standard morphologic marker for peripheral neuropathy. Decreases in IENFD also have been observed in various rodent models of diabetes, including STZ-induced type 1 diabetic models. Thus, our data were consistent with the findings of these previous studies. Our study provided novel evidence, as to our knowledge no previous studies have investigated fiber density in the skin of mice with AL-induced diabetes. We showed that these mice exhibited decreased IENFD similar to those treated with STZ. These findings suggested that IENFD can be used as a morphologic marker of diabetic neuropathy in mice with AL-induced diabetes.

In accordance with our findings, previous morphologic or morphometric studies on the sural nerve have reported significant axonal atrophy, but no fiber loss in rats and mice after STZ or AL administration. In our study, mice with STZ- and AL-induced type 1 diabetes, subjected to 35 weeks of hyperglycemia, simultaneously demonstrated axonal atrophy of the sural nerve, decreased IENFD, and reduction of small nerve fibers in the cornea. There were significant correlations among IENFD, SENFD, and TEND. These findings also were consistent with those of previous human and animal studies, reporting correlations between IENFD and SENFD/TEND.

Overall, our results suggested that diabetic peripheral neuropathy can be evaluated via morphologic examination of the small nerve fibers in the cornea and skin. In addition, our results indicated that the AL-induced diabetic mouse may be a suitable animal model for diabetic peripheral neuropathy.

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References


