Peri-pheral neuropathy is a major complication of diabetes mellitus. While sural nerve biopsy is widely used for morphologic assessment of diabetic peripheral neuropathy, this method is rather invasive, and many clinicians opt to perform minimally-invasive skin biopsies to investigate the small nerve fibers of the epidermis. Although this method allows quantitative evaluation of somatic unmyelinated intraepidermal nerve fiber (INF) density (IENFD), to our knowledge no completely noninvasive procedures for morphologic analysis have yet been developed in clinical practice. In the last decade, 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 represent an alternative, noninvasive marker of peripheral neuropathy in patients with diabetes.

Animal models, especially rats and mice, are essential for studying diabetic peripheral neuropathy, and corneal innervation is similar between mice and humans. Previous studies have investigated the severity of diabetic peripheral neuropathy by evaluating the corneal nerves within the SBNP in mouse and rat models of diabetes. However, such studies have yielded contradictory results, with some demonstrating significant decreases in fiber density and others reporting no abnormalities. Diabetic animal models, including those in rats and mice, cannot faithfully replicate the structural changes associated with microangiopathy of the peripheral nerve, kidney, and retina in patients with diabetes. While the lifespan of rodents is 1 to 3 years, the prevalence of human peripheral neuropathy increases as the duration of diabetes increases beyond 5 years. Thus, the shorter duration of diabetes in rodents deeply affects the development and progression of structural nerve injuries. Mice with streptozotocin (STZ)-induced type 1 diabetes do not exhibit any significant changes in the structure of the SBNP until hyperglycemia has been present for 20 weeks. We hypothesized that longer durations of hyperglycemia would induce corneal nerve lesions similar to those observed in patients with diabetes.

Therefore, we investigated whether increases in the duration of hyperglycemia result in the development of human-like corneal lesions in mice with diabetes induced by alloxan (AL) or STZ. In addition, we examined the relationship between corneal nerve morphology and nonocular standard diabetic neuropathy endpoints, including nerve conduction velocity, IENFD, and morphometric changes to the sural nerve in these animal models.

**METHODS**

**Animals and Housing Conditions**

Female ICR mice were supplied by Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in TPX cages in a
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 pelletized diet for experimental rats (Charles River Formula 1; Oriental Yeast, Tokyo, Japan). The animals were handled in accordance with 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 SLIC, 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 200 mg/kg; the AL group were treated with saline (Sigma-Aldrich Japan, Tokyo, Japan) via the tail vein, at 200 mg/kg, IM; Seractal, Bayer, Tokyo, Japan). 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 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 (nerv 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 fixed with water, immersed in 30% sucrose solution, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek Japan, 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. 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. 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-
epidermal junction were excluded. The data are presented as the number of fibers per millimeter.

**Histologic and Morphometric Analysis of the Corneal Nerve**

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 [SBNPD]) 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).

**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. PGP9.5–positive nerve fibers were detected in epithelial, subepithelial, and stromal layers in the cornea. In the control group, fine terminal epithelial nerves (TENs) derived 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). In the corneal stroma, large nerves divided into several branches and connected to the SBNP (Figs. 3l–p). In contrast, in the STZ and AL groups, the TENs 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 groups 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).

**Intraepidermal Nerve**

Significant differences in IENFs were observed between the STZ/AL and control groups (Fig. 6a, Table 1). The number of IENFs crossing the dermal–epidermal junction (i.e., the IENFD) was significantly lower in the STZ and AL than in the control groups (Fig. 6b). There was no significant difference between the STZ and AL groups. No significant increases in epidermal thickness were noted in the STZ and AL groups, and no ulceration or inflammation was detected in any group.

**Sensory Nerve Conduction Velocity**

SNCV was significantly lower in the STZ than in the control groups (Fig. 7). However, although slightly decreased, the SNCV in the STZ group did not differ significantly from that in the control group ($P = 0.11$).

**Sural Nerve**

Myelinated nerve fibers of the sural nerve exhibited slight axonal atrophy in the STZ and AL groups relative to those in the control group (Fig. 8). The endoneurium, including vessels of the sural nerves, exhibited normal structure in all groups. Mean axon sizes were lower in the STZ and AL than in the control groups, although there were no significant differences among the three groups (Table 2). The frequency histogram revealed a significant shift to smaller axon sizes in the STZ and AL groups compared to the control group ($P < 0.01$; Fig. 9).
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 or TEND in the central cornea ($r = 0.4977$, $P < 0.01$; $r = 0.5125$, $P < 0.01$). Although a slight correlation was observed for small myelinated axons and SBNPD or TEND of the central cornea, this result was not significant ($r = 0.4427$, $P = 0.075$; $r = 0.3899$, $P = 0.123$).

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 hyperglycemia.

### 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>
<th></th>
<th>Skin</th>
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<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>
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<td>55.41 (7.03)</td>
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<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>
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<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.

* $P < 0.05$.
† $P < 0.01$. 

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 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 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 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


