Noninvasive Monitoring of Diabetes-Induced Cutaneous Nerve Fiber Loss and Hypoalgesia in thy1-YFP Transgenic Mice

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Progressive loss of pain perception and cutaneous nerve fibers are frequently observed in diabetic patients. We evaluated the feasibility of using thy1-YFP mice that express the yellowish-green fluorescent protein (YFP) in all of their sensory/motor neurons for noninvasive monitoring of cutaneous nerve fiber loss during diabetes. Fluorescent fibers in skin sections from the leg of thy1-YFP mice stained positive for the neuron-specific protein gene product 9.5 (PGP9.5), indicating that the cutaneous fluorescent fibers are indeed nerve fibers. In diabetic thy1-YFP mice, significant small cutaneous nerve fiber loss in the leg was observed at 3 months following the onset of diabetes, but loss of heat-induced pain perception occurred as early as 1 month following the onset of diabetes, indicating that functional impairment of sensory nerves precedes cutaneous nerve fiber loss. Immunostaining of skin sections of mice killed at 6 months following the onset of diabetes showed that parallel to the loss of small fluorescent nerve fibers, there was a significant decrease in fibers stained positive for calcitonin gene–related peptide, substance P, and purinoreceptor subtype in diabetic thy1-YFP mice. These mice will be useful for noninvasive monitoring of cutaneous nerve fiber degeneration and loss of heat-induced pain perception during diabetes and for the assessment of efficacy of therapeutic treatment of diabetic neuropathy. Diabetes 54:3112–3118, 2005

Neuropathy is one of the most common and debilitating complications in diabetic patients. Peripheral sensory and motor nerve fibers, as well as autonomic nerve fibers, are affected (1). Lesions in the cutaneous sensory fibers may lead to hyperalgesia or hypoalgesia, depending on the stage or the severity of the disease. Hypoalgesia renders patients unaware of the wounds on their lower limbs and, together with impaired wound healing, often causes foot ulceration and gangrene, which may require amputation. There is evidence that the cutaneous nerve might be involved in wound healing as well. Impaired wound healing in diabetic mice was found to be associated with a reduced level of nerve growth factor at the wound site, presumably due to a reduced number of epidermal nerve fibers (2); nerve growth factor has been shown to accelerate wound healing (3,4). In addition to nerve growth factor, the cutaneous nerve may secrete other neurotransmitters and neuromodulators, including catecholamines, acetylcholine, substance P, calcitonin gene–related peptide (CGRP), α-melanocyte–stimulating hormone, and other agents (5). Some of these neuromodulators are known to regulate immune and inflammatory reactions (5). Thus, lesions in the cutaneous nerves contribute to lack of awareness to injuries, impaired wound healing, and impairment in skin immune defense, all key factors contributing to diabetic foot ulceration. Diabetic neuropathy is the leading cause of lower-limb amputation, underscoring the importance of monitoring the integrity of the cutaneous nerves during diabetes and during therapeutic treatment (6).

Different types of cutaneous sensory nerve fibers have been identified. They include the unmyelinated C-fibers, the thinly myelinated A-δ fibers, and the myelinated A-β fibers. C and A-δ fibers are thought to be involved in thermal and pain sensation, whereas A-β fibers are responsible for mechanical sensation. C-fibers are identified by immunostaining with CGRP and substance P (7); however, C, A-δ, and A-β fibers are distinguished only by electron microscopy. In addition to CGRP and substance P (7), immunostaining of purinoreceptor subtype (P2X3) (8) could also be used to identify cutaneous sensory C-fibers, whereas vasoactive intestinal peptide (9) and neuropeptide Y (10,11) staining were used to identify autonomic nerve fibers in skin biopsy samples of diabetic patients. However, most studies used PGP9.5, a neuronal-specific ubiquitin COOH-terminal hydrolase (12), to label all nerve fibers. These studies (9,13–18) indicated that nerve fibers in the epidermis, dermis, and sweat gland were significantly reduced in diabetic patients. Reduction of cutaneous innervation was also observed in animal models. The PGP9.5-positive cutaneous nerve profile, area fraction, and area density were found to be significantly lower in db/db compared with db/m mice (2,19). Furthermore, streptozotocin-induced diabetic mice displayed severely reduced cutaneous innervation in the flank and footpad, and intra-thecal treatment with glial cell line–derived neurotrophic factor or neurturin was able to stimulate axon regrowth and branching (20). Recently, similar examination of skin...
biopsies from 9-month diabetic mice also showed a reduction in cutaneous axons in the footpad, which was normalized by return of their blood glucose to near euglycemia (21). However, these assessment methods are cumbersome, and such invasive sampling is inappropriate for follow-up studies in live small animals because the small surface areas, like the foot and lower leg, do not allow for repeated sampling. Moreover, the wound created by biopsies may affect the course of the disease. Therefore, a more convenient and noninvasive monitoring of cutaneous nerve degeneration is desirable.

A line of transgenic mice has been generated using the neuron-specific thy1 promoter to drive the expression of the yellowish-green fluorescence protein (YFP) cDNA (22). All of the sensory/motor neurons in the thy1-YFP mice appeared bright yellowish-green when viewed by fluorescence stereomicroscopy. When the hair is removed, we found that cutaneous nerves are also visible as yellowish-green fluorescent fibers when viewed in this manner. In this report, we demonstrate that these thy1-YFP mice can be used for noninvasive assessment of small cutaneous nerve fiber degeneration induced by diabetes.

### RESEARCH DESIGN AND METHODS

#### Animal maintenance and genotyping
Male heterozygous thy1-YFP (line thy1-YFP16) mice backcrossed into C57BL/6 (22) were purchased from The Jackson Laboratory (Bar Harbor, ME). Thy1-YFP mice could be quickly distinguished from the nontransgenic counterparts by viewing the fluorescent nerves in the ear/footpad with fluorescence stereomicroscopy (MZFLIII; Leica, Bensheim, Germany) or by PCR (22). Mice were maintained in a 12/12-h light/dark cycle with food and water ad libitum. All studies involving animals were approved by the Animal Care and Use Committee of The University of Hong Kong.

#### Induction of diabetes
Diabetes was induced by a single intraperitoneal injection of streptozotocin (200 mg/kg; Sigma, St. Louis, MO) to 6-week-old mice. Control mice received an equal volume of vehicle (0.1 mol/l citrate buffer, pH 4.5). Tail blood glucose level was determined 3 days later (Glucoseometer Elite; Pymble, NSW, Bayer, Australia). Mice with blood glucose levels of ≥7.8 mmol/l were considered diabetic, and those with blood glucose levels of <7.8 mmol/l were considered nondiabetic (23). In addition to polyuria, diabetic mice exhibited body weight loss (Table 1).

#### Histological and immunocytochemical analyses
Sixty-nanometer cryostat sections (from the leg and footpad) were prepared according to the established protocol (22). Images of fluorescent nerve fibers in the cryostat sections were captured using a laser-scanning confocal microscope (LSM410; Carl Zeiss, Jena, Germany) at a magnification of 400X. The lengths of the skin sections were measured using the LSM410 software system. The number of small fluorescent nerve fibers present in the epidermis and upper layer of dermis perpendicular to skin surface was counted (2,16). The density of signals was expressed as linear density, i.e., number of immunoreactive profiles/millimeter (number/mm) (26).

For PGPI.5 immunohistochemistry, rabbit-PGPI.5 antibodies (1:500; Bio-genesis) were placed onto 10- or 60-μm skin cryosections and incubated overnight at 4°C. After washing, the tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit IgG (1:400; Molecular Probe, Eugene, OR) was then added and incubated with the sections for 1 h at room temperature. After another wash, images were obtained with the LSM410 system. Immunostaining with antibodies against antinociceptive P2X (1:100; Santa Cruz, Santa Cruz, CA), goat-CGRP (1:200; Santa Cruz), and goat–substance P (1:400; Santa Cruz) was performed using a Vectastain Elite ABC kit (Vector, Burlingame, CA) on 10-μm skin paraffin sections. The CGRP, substance P–, and P2X-positive nerve fibers were quantitated by counting the total numbers of each nerve type and dividing those numbers by the length of the skin section (26). All counting was performed with images taken with an inverted microscope (IX71; Olympus, Tokyo, Japan), which was connected to a charge-coupled device digital camera RT COLOR Spot (Diagnostic Instruments, Sterling Heights, MI) at a magnification of 40X. Lengths of skin sections were measured using Spot software system. The density of signals was expressed as linear density, i.e., the number of immunoreactive profiles/millimeters (numbers/mm) (26).

#### Statistical analysis
All data were expressed as means ± SE. Statistical analysis was performed by Student’s t test. P values < 0.05 were considered to be statistically significant.

### RESULTS

#### The cutaneous yellowish-green fluorescent fibers in the thy1-YFP mice are nerve fibers
After removal of a patch of hair from their legs a day earlier, anesthetized thy1-YFP mice were placed under a fluorescence stereomicroscope for examination of their cutaneous nerve fibers. Small and large fluorescent fibers were visible when the skin surface was in focus (Fig. 1A).

### TABLE 1
Body weight and blood glucose

<table>
<thead>
<tr>
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<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>6 months</th>
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<tbody>
<tr>
<td><strong>Body weight</strong> (g)</td>
<td>27.0 ± 0.7</td>
<td>28.4 ± 0.7</td>
<td>29.0 ± 0.6</td>
<td>32.1 ± 1.1</td>
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<tr>
<td><strong>Blood glucose (mmol/l)</strong></td>
<td>7.7 ± 0.2</td>
<td>8.0 ± 0.3</td>
<td>7.8 ± 0.2</td>
<td>7.7 ± 0.2</td>
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<tr>
<td><strong>Body weight</strong> (g)</td>
<td>19.1 ± 0.9*</td>
<td>21.4 ± 1.2*</td>
<td>22.3 ± 1.6</td>
<td>26.1 ± 1.2†</td>
</tr>
<tr>
<td><strong>Blood glucose (mmol/l)</strong></td>
<td>31.9 ± 0.7*</td>
<td>31.4 ± 0.5*</td>
<td>30.6 ± 1.1*</td>
<td>27.4 ± 1.9*</td>
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Data are means ± SE. *P < 0.001 by Student’s t test; †P < 0.01. n = ~15–20 for each group. STZ, streptozotocin-induced diabetic mice.
fibers on the footpad were also visible, their image was blurred due to the thickness of the skin, making it difficult to count individual fibers. Besides cutaneous nerves, all other nerve fibers of thy1-YFP mice also appeared bright yellowish-green when viewed by fluorescence stereomicroscopy. Examples of fluorescent nerve fibers innervating the abdomen (Fig. 1B), diaphragm (Fig. 1C), and the sural nerves in the lower leg (Fig. 1D) indicate that these nerve fibers could be readily identified without immunostaining for nerve-specific markers.

To confirm that the cutaneous fluorescent fibers are nerve fibers, cryostat sections of the skin on the leg were prepared and stained with antibodies against PGP9.5. Although PGP9.5 is a nerve-specific marker, it could not be used for classifying the specific type of nerve fiber as in the case of YFP nerve fibers. As shown in Fig. 2, the fluorescent fibers also expressed PGP9.5, indicating that they were indeed nerve fibers.

**Noninvasive monitoring of cutaneous nerves in diabetic thy1-YFP mice**

Thy1-YFP mice were made diabetic by streptozotocin injection, and small cutaneous YFP nerve fibers on six defined areas on their legs were counted after 1, 2, 3, and 6 months. As shown in Fig. 3, there was no significant change in the density of cutaneous small fibers after 1 and 2 months of diabetes. However, there was a significant decrease in the density of primary fibers in the 3-month diabetic group compared with the nondiabetic group (P < 0.001), and there was a trend toward a decrease in secondary fiber density in this diabetic group compared with the nondiabetic group. After 6 months of diabetes, both primary and secondary fiber densities were significantly decreased (P < 0.01 for both primary and secondary fibers).

**Reduced perception for heat-induced pain in diabetic mice**

Thermal hypoalgesia is one of the characteristics of diabetic small unmyelinated or thinly myelinated fiber neuropathy. Along with monitoring the cutaneous small YFP nerve fibers, hot-plate tests were also conducted after 1, 2, 3, and 6 months of diabetes. The results showed that the delay in response to heat-induced pain was evident at as early as 1 month of diabetes (P < 0.05), the first time point in the study (Fig. 4). Hypoalgesia persisted throughout the 6 months of diabetes (2 and 3 months: P < 0.05, 6 months: P < 0.01).

**Verification of cutaneous nerve fiber loss**

After 6 months of diabetes, mice were killed, and 60-μm cryostat sections of the skin on the legs and footpads were prepared for examination of fluorescent YFP fibers. Counting of small YFP fibers perpendicular to the skin surface, which were mainly located in the upper part of the dermis and epidermis, showed that there was a significant reduction in the density of small YFP fluorescent fibers (Fig. 5). Immunostaining with PGP9.5 antibodies revealed that all the nerve fibers that stained positive for this antigen also}

![Image](image-url)
showed fluorescent yellowish-green color regardless of their sizes (online appendix [available at http://diabetes.diabetesjournals.org]), indicating that the reduction in the number of YFP fluorescent fibers in diabetic mice is not the consequence of inhibition of the expression of the YFP transgene but the result of nerve fiber degeneration.

Since the YFP transgene labeled all sensory and motor neurons, to further determine which subtypes of the cutaneous nerve fibers were lost in the 6-months diabetic mice, paraffin-embedded sections of the skin from the legs of the mice were immunolabeled with antibodies against CGRP (13,20), substance P (13,27), or P2X3 (8). The densities of all three types of nerve fibers in the skin were significantly reduced in the diabetic mice (CGRP: \( P < 0.01 \), substance P: \( P < 0.05 \), and P2X3: \( P < 0.05 \)) (Fig. 6).

**Reduction of MNCV after 6 months of diabetes**

The MNCV, an indicator of functional integrity of peripheral motor neurons, was significantly reduced in the sciatic nerves of the 6-month diabetic mice (Fig. 7A, \( P < 0.01 \)). However, linear regression analyses showed that there was no correlation between MNCV deficits and cutaneous nerve fiber reduction (Fig. 7B and C). Moreover, there was also no correlation between increased heat-induced pain sensation latency and small cutaneous YFP fiber loss after 6 months of diabetes (Fig. 7D and E).

**DISCUSSION**

Loss of cutaneous nerve fibers is thought to be partly responsible for the diabetes-induced impairment in the

**FIG. 3.** The histograms showing that primary and secondary cutaneous YFP fiber densities were unchanged after 1 (A) and 2 (B) months of diabetes. The primary cutaneous fibers were reduced after 3 (C) and 6 (D) months of diabetes, while the secondary cutaneous fibers were reduced after 6 months of diabetes (D). \( n = 15-20 \) for both diabetic and nondiabetic groups. CTL, control; STZ, streptozotocin-induced diabetic mice; Primary, primary fibers; Secondary, secondary fibers. *\( P < 0.01 \), **\( P < 0.001 \) by Student’s \( t \) test.

**FIG. 4.** The histogram showing that the heat-induced pain thresholds (withdrawal latencies) were delayed after 1, 2, 3, and 6 months of diabetes. \( n = 15-20 \) for both diabetic and nondiabetic groups. CTL, control; STZ, streptozotocin-induced diabetic mice. *\( P < 0.05 \), **\( P < 0.01 \) by Student’s \( t \) test.

**FIG. 5.** Photomicrographs showing the cutaneous small YFP fibers in skin biopsy of thy1-YFP mice. Cutaneous small fibers were significantly less in the leg (A) and footpad (B) in 6-months diabetic mice. The arrowheads indicated the nerve fibers. \( n = 5 \) for both diabetic and nondiabetic groups. The boundary between epidermis and dermis was shown. ep, epidermis; h, hair shaft; d, dermis; kl, thick keratin layer; lf, large fibers. CTL, control; STZ, streptozotocin-induced diabetic mice. *\( P < 0.05 \) by Student’s \( t \) test.
skin's immune defense, wound healing, and pain perception that may lead to foot ulceration and gangrene. The mechanism leading to this diabetic neuropathy is still unclear. We demonstrated that thy1-YFP mice provide a convenient noninvasive method of monitoring cutaneous small nerve fiber loss induced by diabetes. We showed that the fluorescent fibers stained positive for the nerve-specific PGP9.5 antigen, confirming that they were indeed nerve fibers. We also showed that the reduction in the number of small cutaneous YFP fluorescent fibers in the 6-months diabetic mice was not due to downregulation of the YFP transgene, as all the PGP9.5 fibers were yellowish-green fluorescent. However, the present noninvasive method of counting the YFP nerve fibers by viewing from the top of the skin only distinguishes small versus large fibers. Electron microscopic analysis of cutaneous axons would be required to classify the types of small YFP fibers. Alternatively, a combined method of immunocytochemical analysis using antibodies specific for the particular subtypes of fibers and quantitation of fibers colocalized with YFP would be useful. However, the drastic reduction in intensity of YFP due to extensive processing of the tissue for immunocytochemical staining would preclude this possibility. The major advantage of using thy1-YFP mice for small-fiber loss is that a larger area of the skin can be assessed for changes in the small nerve fibers and that the same skin areas can be monitored over long periods of diabetes. In the future, development of transgenic mice expressing YFP under the control of a C-fiber–specific promoter may allow the noninvasive technique to distinguish the C-type–specific cutaneous nerve fibers from other types of nerve fibers.

In the diabetic mice, loss of cutaneous nerve fibers did
not occur until the 3rd month of diabetes, while functional impairment of the sensory nerve, as indicated by the delayed heat-induced pain response, occurred in the 1st month of diabetes. This finding indicates that functional impairment preceded the loss of small cutaneous YFP nerve fibers, although we could not be certain these YFP fibers are C-fibers. Interestingly, the secondary fibers, which were distal to the main fibers, did not preferentially degenerate before the primary fibers. This suggests that the secondary fibers and the primary fibers, from which they bifurcated, degenerated at the same time. Immunohistochemical analyses revealed that nerve fibers immunoreactive to CGRP, substance P, and P2X3 were all reduced in the skin of the mice that were diabetic for 6 months. The parallel loss of these small sensory fibers and YFP fluorescent fibers further confirmed that the reduction in the number of fluorescent fibers in diabetic mice was due to fiber loss rather than inhibition of expression of the YFP transgene. The CGRP and substance P are markers for sensory fibers involved in nociception and neurovascular dilatory response (9,28,29). The decreased number of these nerve fibers may lead to reduced blood flow to the skin, thereby further exacerbating the degeneration of these nerves. The P2X3-expressing nerves are involved in the pain processing pathway (30). Lacking P2X3-positive sensory nerve fibers may lead to hypoalgesia.

Linear regression analyses revealed that reduction of MNCV did not correlate with cutaneous nerve fiber loss. This is consistent with previous observations (13,16) that functional changes, including reduction in MNCV in diabetics, did not correlate with cutaneous fiber changes, confirming that small-fiber deficit does not necessarily reflect lesion in the large fibers.

The YFP transgene is expressed in all sensory and motor neurons in thy1-YFP mice, and, as shown in our figures, both small and large nerve fibers are YFP fluorescent. Whereas only the small YFP fibers in the epidermis and upper part of dermis were quantitated in the present study, it is not possible to identify them as C-type fibers. Such a limitation may explain why there is no close correlation between the delayed heat-induced pain response with the reduction of small cutaneous YFP fiber density. In addition, using these mice to monitor cutaneous nerve fiber loss cannot reveal if there is a differential rate of degeneration among different types of nerves. However, the advantage of this noninvasive monitoring of cutaneous nerves is that the fate of individual nerve fibers during the course of diabetes can be followed. There is evidence that degeneration, as well as regeneration, of nerves occurs during diabetes (31,32). Sampling the population of nerve fibers as we have done here only shows the net effect of these two processes. Further studies monitoring a large number of individual small nerve fibers may determine the timing of degeneration. It would also be interesting to determine whether the primary and secondary fibers degenerate at the same time and if the new fibers sprout from the same locations as that of the degenerated fibers. With appropriate markings on the skin, the structure of individual axons can be examined repeatedly over a period of time. The changing structure of the nerve fibers can be recorded by confocal microscope with three-dimensional reconstruction software. Our study demonstrates that thy1-YFP mice will be a useful animal model for convenient noninvasive monitoring of cutaneous nerve fiber degeneration during diabetes and that they can also be used to assess the efficacy of drugs for the treatment of diabetic neuropathy. Since all the neurons in these mice are clearly visible with fluorescence stereomicroscopy without the need for immunohistochemical identification, this approach provides a convenient model to study diabetes-induced nerve fiber loss in other tissues.

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REFERENCES

19. Underwood RA, Gibran NS, Muffley LA, Usui ML, Olender JE: Color subtractive-computer-assisted image analysis for quantification of cutane-
20. Christianson JA, Riekof JT, Wright DE: Restorative effects of neurotro-
spontaneous recovery: is there irreparable damage? Diabetes 54:830–837, 2005
23. Song Z, Fu DTW, Chan YS, Leung S, Chung SSM, Chung SK: Transgenic
mice overexpressing aldose reductase in Schwann cells show more severe
nerve conduction velocity deficit and oxidative stress under hyperglycemic
24. Crawley JN: What’s Wrong With My Mouse? Behavioral Phenotyping of
25. Yagihashi S, Yamagishi SI, Wada R, Baba M, Hohman TC, Yabe-Nishimura
C, Kokai Y: Neuropathy in diabetic mice overexpressing human aldose
27. Unger JW, Klitzsch T, Pera S, Reiter R: Nerve growth factor (NGF) and
diabetic neuropathy in the rat: morphological investigations of the sural
28. Grant AD, Pinter E, Salmon AM, Brain SD: An examination of neurogenic
1015, 2000
31. Behse F, Buchthal F, Carlsen F: Nerve biopsy and conduction studies in
history of acute painful neuropathy in diabetes mellitus. J Neurol Neuro-
surg Psychiatry 46:491–499, 1983