SUPPLEMENTARY DATA

Study subjects

The study subjects were all patients with type 2 diabetic neuropathy as described before (1). All patients completed an initial evaluation that revealed no history of familial neuropathy or toxin exposure with normal results on blood tests to exclude other etiologies of neuropathy: electrolytes, liver function tests, complete blood count, thyroid function tests, autoimmune profiles, vitamin B_{12} and folic acid levels, and serum protein electrophoresis with an immunofixation analysis (1).

Nerve conduction studies

NCS following standardized methods were performed with a Nicolet Viking IV Electromyographer (Nicolet, Madison, WI). Studied nerves included sural and peroneal nerves. The results of the NCS were compared to normative data in our laboratory (2). An abnormal NCS was defined as having abnormalities in one or more nerves with reduced amplitude, prolonged distal latency, or slowed nerve conduction velocity.

Skin biopsy and immunohistochemistry

A skin specimen of 3 mm in diameter was taken with a biopsy punch from the lateral side of the distal leg under 2% lidocaine local anesthesia (3). Skin tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 48 h. Sliding microtome sections at 50 μm were quenched with 1% H_2O_2, blocked with 5% normal goat serum, and incubated with rabbit antiserum to PGP9.5 (UltraClone, Isle of Wight, UK, diluted 1:1000 in 1% normal serum/Tris) at 4 °C for 16~24 h. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (IgG) at room temperature for 1 h, followed by incubation with the avidin-biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated with chromogen SG (Vector) as the conventional PGP9.5-immunohistochemical staining and these sections were termed conventional sections. Additional conventional sections were further counterstained with 1% Congo red (Muto Pure Chemicals, Tokyo, Japan) for 5 min to reveal the territory of the sweat gland. These sections were designated counterstained sections. Sections were dehydrated with anhydrated alcohol and mounted using DPX (Sigma, St. Louis, MO), a non-aqueous synthetic resin.

Area-based morphometry of sweat gland innervation

The counterstained sections were employed for quantifying sweat gland innervation. All information on the slides was masked before quantitation and the examiner was blinded to the coding information. All clinical details were only retrieved after the completion of quantification procedures. Sections were observed under a 20x objective on a Leica DM2500 light microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC490 CCD camera (Leica Microsystems, Wetzlar, Germany). The image size captured by the camera was 2176 x 1632 pixels (on a resolution of 72 pixels/inch), equivalent to 76.76 x 57.57cm. A 10 μm scale appeared 1.23 mm on this image (one pixel = 353 μm), resulting in a final magnification of 123x. There was no any post-editing (including resizing) applied to the images. The acquired image was opened with Photoshop CS3 (Adobe Systems, San Jose, CA). The areas of sweat glands (in red) and nerve fibers (in dark blue) were measured separately in each section. For the measurement of sweat gland area, we used the “select/color range” command (under the “red channel”) of Photoshop CS3, which determined the margins of the sweat gland automatically without any manual tracing by the operator. According to this command, the software calculated the area of "non-highlights portion" for each image. Several factors might influence the pixel counts, including (1) the image intensity, (2) the variations of the intensities among different sections, and (3) photographing quality. We measured the intensity of stained structures on skin biopsies in preliminary studies (1) to minimize the aforementioned variations and (2) to exclude the possibility that high background staining of PGP9.5 might lead to over-estimation of sweat gland innervation. On 30 sweat glands, the intensities (on a scale

of 0~255) of background, sweat glands, and nerve fibers in appropriate channels were 210.4 ± 1.7, 239.8 ± 1.6, and 142.0 ± 5.8 respectively. Accordingly, two criteria were set for nerve fibers: (1) appropriate intensities as these pre-set values and (2) correct staining patterns with linear and varicose profiles. Only sections fulfilled this criterion were qualified for quantitation. The same "select/color range" command and parameters were applied on all images, i.e. parameters were not manually set by operators on each image. For measurement of the nerve fiber area, the PGP9.5 stained areas within the perimeter defined by Congo-red were included and the intervening unstained areas were excluded. We defined the sweat gland innervation index (SGII) of the selected sweat gland as the area of nerve fibers normalized to the area of a sweat gland, i.e. SGII = nerve fiber area/sweat gland area x 100%. The mean of all SGIIs (3~7 sweat glands from 3 different sections for each subject) was defined as the SGII of a given subject. This newly-designed approach had high correlation coefficient for the same investigator (r = 0.99, p < 0.0001) and for two independent examiners (r = 0.99, p < 0.0001) and validated with a stereology method (r = 0.96, p < 0.0001).

Quantification of epidermal innervation

The innervation of the epidermis was quantified on conventional sections following established protocols and slides were coded to ensure that measurements were blinded (4). PGP9.5-immunoreactive nerve fibers in the epidermis of each section were counted at 40x magnification with an Olympus BX40 microscope through the depth of the entire section. Each individual nerve fiber with branching points inside the epidermis was counted as one. For epidermal nerve fibers with branching points in the dermis, each individual nerve fiber was counted separately. The length of the epidermis along the upper margin of the stratum corneum in each section was measured using Image-Pro PLUS software (Media Cybernetics, Silver Spring, MD). The IENF density was derived and expressed as fibers/mm. In the distal leg, normative values of IENF density from our laboratory were 11.16 ± 3.70, 5.88, and 4.2 fibers/mm (mean ± SD, the 5th percentile value, and the 1st percentile value, respectively) for those aged < 60 years, and 7.64 ± 3.08, 2.50, and 2.2 fibers/mm for those aged ≥ 60 years.

Autonomic function tests

We performed beat-to-beat heart rate variability (HRV) tests during deep breathing, the most widely used cardiovagal function test for cardiac autonomic neuropathy (5,6). Impairment of the HRV was took as a clinical indicator of cardiac autonomic dysfunction (5). Each test was performed three times, and the mean value was compared with the normative values of the age-matched controls in our laboratory. Normative values during deep breathing were 19%~62% (20~29 years), 14%~48% (30~49 years), 11%~39% (50~59%), and 8%~28% (≥ 60 years) (7). Surrogate sudomotor activities were examined using the sympathetic skin response of the sole (SSR) (7,8). The results of the SSR was interpreted as present or absent, but was not evaluated quantitatively because of variations in the latencies and amplitudes of the SSR. If the responses could not be unequivocally elicited, the patient was considered to have an impaired SSR. Medications that interfered with sympathetic or parasympathetic functions were not administered before or during the test.

SUPPLEMENTARY DATA

Supplementary Table 1. Sweat gland innervation index (SGII) and autonomic symptoms

	Presence	Absence	*p* value
All autonomic symptoms	1.72% ± 1.54%	3.28% ± 1.86%	0.0053*
Cardiovascular system	0.82% ± 0.01%	2.62% ± 1.87%	0.18
Gastrointestinal system	1.90% ± 1.65%	2.82% ± 1.91%	0.14
Genitourinary system	2.06% ± 1.60%	2.60% ± 1.91%	0.55
Anhidrosis of the feet	0.82% ± 0.69%	3.00% ± 1.81%	0.0011*

* $p < 0.05$.

SUPPLEMENTARY DATA

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