Degeneration of nociceptive nerve terminals in human peripheral neuropathy

Chun-Liang Pan, Yea-Hui Lin, Whei-Min Lin, Tong-Yuan Tai and Sung-Tsang Hsieh

Departments of 1Neurology and 3Internal Medicine, National Taiwan University Hospital, Taipei 10002; 2Department of Anatomy and Cell Biology, National Taiwan University College of Medicine, 1 Jen-Ai Road, Sec. 1, Taipei 10018, Taiwan

Received 6 December 2000; accepted 8 January 2001

Patients with peripheral neuropathy have symptoms involving small-diameter nociceptive nerves and elevated thermal thresholds. Nociceptive nerves terminate in the epidermis of the skin and are readily demonstrated with the neuronal marker, protein gene product 9.5 (PGP 9.5). To investigate the pathological characteristics of elevated thermal thresholds, we performed PGP 9.5 immunocytochemistry on 3 mm punch skin biopsies (the forearm and the leg) from 55 normal subjects and 35 neuropathic patients. Skin innervation was evaluated by quantifying epidermal nerve densities. Epidermal nerve densities were reduced in neuropathic patients compared to normal subjects. Epidermal nerve densities were variably correlated with thermal thresholds. The proportion of neuropathic patients with reduced epidermal nerve densities was larger than the proportion of neuropathic patients with elevated thermal thresholds. These results indicated that degeneration of epidermal nerve terminals preceded the elevation of thermal thresholds. Skin biopsy together with immunocytochemical demonstration of epidermal innervation offers a new approach to evaluate small-fiber sensory neuropathy.

Key words: Epidermal nerve densities; Immunocytochemistry; Nerve degeneration; Neuropathy; Quantitative sensory testing; Skin innervation; Unmyelinated nerves

INTRODUCTION

Patients with peripheral neuropathy frequently have sensory symptoms involving small-diameter nociceptive nerves. Because no neurophysiological test is clinically feasible for evaluating nociceptive nerves, quantitative sensory testing is the only tool for examining small-fiber sensory neuropathy at present [1]. Quantitative sensory testing measures the thresholds of various sensory modalities, including warm and cold stimuli [2–4]. Thermal sensations are conducted by small-diameter sensory nerves, which terminate in the epidermis of the skin, free nerve endings [5]. The extent of sensory deficits can be quantified according to changes in sensory thresholds compared to normative data. Thermal thresholds are altered in various types of neuropathies [6,7]. However, it remains unclear whether elevation of thermal threshold really reflects degeneration of small-diameter sensory nerves.

Pathological diagnosis of small-fiber sensory neuropathy has been a challenge to neurologists. Traditional evaluation of nerve biopsy specimens is a labor-intensive procedure because of the requirement for ultrastructural studies by electron microscopy. Small-diameter nociceptive nerves terminate in the epidermis of the skin as free nerve endings. Recently several groups including ours have demonstrated the rich innervation of the skin by immunocytochemistry with various neuronal markers, including protein gene product 9.5 (PGP 9.5) [8–11]. PGP 9.5, a ubiquitin carboxy hydrolase, is particularly enriched in small-diameter nerves [12]. Epidermal nerve terminals are depleted when nerve trunks are injured in Wallerian degeneration [13].

A critical issue is whether epidermal nerves degenerate in neuropathies patients with sensory symptoms, i.e. is epidermal innervation correlated with thermal thresholds? If so, what kinds of sensory modalities are best correlated with epidermal nerve densities? To address these issues, we compared epidermal nerve densities and thermal threshold of normal subjects and neuropathic patients.

MATERIALS AND METHODS

Normal subjects and neuropathic patients: Normal subjects were recruited from a cohort in the community and those visiting National Taiwan University Hospital, Taipei, Taiwan for a physical check-up [14]. They were evaluated by detailed questionnaires and neurological examinations to exclude any neurological disorder or clinical neuropathies [15]. Examinations consisted of laboratory tests (complete blood count, fasting blood glucose, hemoglobin A1C, liver and renal functions, serum protein electrophoresis, anti-nuclear antibody, and vitamin B12 level), nerve conduction studies, and quantitative sensory testing. There were 55 normal subjects (19 males and 36 females) aged 45.94 ± 12.95 years (range 26–82).

Neuropathic patients were regularly followed-up at the
Department of Neurology. They all had symmetrical sensory symptoms of the glove–stocking distribution in the upper and lower extremities. The neuropathy group consisted of 35 patients (17 males and 18 females) aged 47.3 ± 11.6 years (range 25–73). These patients had progressive sensory polyneuropathy. Nerve conduction studies showed reduction of sural sensory action potentials. Thus these patients all had neuropathy involving large-diameter nerves, and potentially had deficits of small-diameter sensory nerves. Seven patients underwent nerve biopsy and unmyelinated nerve densities were reduced. The etiologies included diabetes mellitus (5), vasculitis (2) and idiopathic neuropathy in the rest.

The protocol was approved by the Institutional Review Board of National Taiwan University Hospital. Informed consent was signed before all biopsies.

Skin biopsy: Skin biopsy was performed following established procedures after local anesthesia with 2% lidocaine [15]. Punches 3 mm in diameter were taken from each site: (1) the forearm site: the extensor side of the distal forearm, 5 cm above the middle point of the line connecting the radial styloid process and the ulnar styloid process, and (2) the leg site: the lateral side of the distal leg, 10 cm above the lateral malleolus. All healthy controls and neuropathic patients had skin biopsies at both sites. All subjects tolerated the procedure with no obvious discomfort. No suturing was required, and the wounds were covered with a piece of gauze. Wound healing took 7–10 days, the same as a common abrasion wound.

Immunocytochemistry: For immunocytochemistry on freezing microtome sections, the skin tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 48 h [16]. After thorough rinsing in PB, samples were cryoprotected with 30% sucrose in PB overnight. Sections of 50 μm perpendicular to the dermis were cut on a sliding microtome (Microm 440E, Microm, Germany). Sections from each tissue were labeled sequentially and stored with antifreeze (30% glycerol, 30% ethylene glycol in PB) at –20°C. Sections were treated with 0.5% Triton X-100 in 0.5 M Tris buffer, pH 7.6 (Tris) for 30 min and processed for immunostaining. Sections were quenched with 1% H2O2 in methanol, and blocked with 5% normal goat serum in 0.5% non-fat dry milk/Tris. Sections were incubated with rabbit antiserum to PGP 9.5 (UltraClone, UK, diluted 1:1000 in 1% normal serum/Tris) for 16–24 h. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit IgG for 1 h, and the avidin–biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated by the chromogen SG (Vector, Burlingame, CA), and counterstained with eosin (Sigma, St. Louis, MO).

Quantitation of epidermal innervation: Epidermal innervation was quantified according to modified protocols in a coded fashion, with the examiners blinded to the coded information [11]. PGP 9.5 (+) nerves in the epidermis of each section were counted at a magnification of ×40 with an Olympus BX40 microscope (Shibuya-ku, Japan). Each individual nerve with branching points inside the epidermis was counted as one. For epidermal nerves with branching points in the dermis, each individual nerve was counted separately. The length of the epidermis along the upper margin of the stratum corneum in each section was measured with the Image-Pro PLUS (Media Cybernetics, Silver Spring, MD). Epidermal nerve density was therefore derived and expressed as the number of epidermal nerves per unit length of the epidermis (fibers/mm). For each tissue, there were 48–50 sections after sectioning, and all sections were sequentially labeled. Based on preliminary results of staining and quantifying all sections, the standard procedures were to immunostain and quantify the 13th, 19th, 25th, 31st, and 37th sections of each tissue. The mean of epidermal nerve densities on these sections was the epidermal nerve density of that tissue.

Quantitative sensory testing: Thermal sensory thresholds were measured by quantitative sensory testing with a Thermal Sensory Analyzer, TSA-2001 (Medoc Advanced Medical System, Minneapolis, MN) following established principles and protocols [17]. Testing modalities included warm sensation and cold sensation with the testing algorithms of level, which was independent of reaction time [1]. Thermal thresholds at the thenar area (the hand site) and the foot dorsum (the foot site) were measured. The procedures were carried out in a quiet room at a room temperature of 21–24°C. Skin temperatures were 31–34°C under such an environment. During the examination, the reference temperature was set at 32°C, and the temperature went up (warm stimuli) or went down (cold stimuli) according to the default algorithms. Normative data were generated previously in different cohorts, and there was no significant ethnic difference [18].

Statistical analysis: Epidermal nerve densities in the control group and in the neuropathy group were expressed as the mean ± s.d., and were compared by t-test after the normality of distribution was confirmed. Correlations between epidermal nerve densities and sensory thresholds were evaluated by linear regression analysis with SPSS for Windows (ver 6.1, SPSS, Chicago, IL) and GraphPad Prism (ver 2.01, GraphPad Software, San Diego, CA). The 95% confidence interval (95% CI) of Pearson’s coefficient was included. Sensitivity and specificity were calculated based on the fifth percentile value as the cut-off point. Any difference with p < 0.05 was considered statistically significant.

RESULTS

Skin innervation in small-fiber sensory neuropathy: In the leg skin of normal subjects, PGP 9.5(+) nerves arose from the dermis, subepidermal nerve plexuses, and ascended vertically in the epidermis (Fig. 1a). In the dermis, PGP 9.5(+) dermal nerves were occasionally in groups with a dense and continuous pattern of staining (Fig. 1). In patients with sensory neuropathy, the abundance of PGP 9.5(+) was reduced, and the staining in the subepidermal nerve plexuses became faint and discontinuous (Fig. 1b). The immunoreactive pattern of dermal nerves in neuropathic patients became fragmented and swollen, reflecting Wallerian-like degeneration (Fig. 1d).

Quantitative characteristics of epidermal innervation:
Epidermal nerve densities were reduced in neuropathic patients, particularly in the distal leg. Epidermal nerve densities of the distal forearm were 17.360 ± 6.194 fibers/mm in normal subjects. In neuropathic patients, epidermal nerve densities of the distal forearm were reduced to 5.631 ± 4.955 fibers/mm (p < 0.001, Fig. 2a). In the distal leg of normal subjects, epidermal nerve densities were 12.970 ± 5.284 fibers/mm, and these values were 2.160 ± 2.185 fibers/mm at the same site of neuropathic patients (p < 0.001, Fig. 2a).

In normal subjects, epidermal nerve densities in the forearm and leg were correlated (slope = 0.916 ± 0.111, r = 0.75, p < 0.0001, Fig. 2b). Densities in the forearm were significantly higher than those in the leg (Y-intercept: 5.720 ± 1.535 fibers/mm, p < 0.001). In neuropathic patients, the loss of epidermal nerves in the forearms and in the legs was variable, which reflected the pathophysiology of different underlying neuropathies.

The ratios of epidermal nerve densities (the forearm vs the leg) were 1.488 ± 0.569 (95% CI: 1.328, 1.647) in normal subjects and were independent of age (p = 0.86, Fig. 2c). These ratios were significantly elevated in neuropathic patients compared to normal subjects (3.670 ± 4.532, p < 0.002), reflecting the nature of length-dependency.

**Epidermal nerve densities and sensory thresholds:** The correlations between epidermal nerve densities and sensory thresholds at the same site were evaluated by linear regression analysis. In normal subjects and neuropathic patients, correlations between epidermal nerve densities and sensory thresholds varied among different sites and different modalities (Fig. 3). Epidermal nerve densities were better correlated with warm thresholds than with cold thresholds. For example, correlations between epidermal nerve densities of the distal leg and warm thresholds of the foot dorsum (r = 0.483; 95% CI: –0.6819, –0.2168, p < 0.001) were higher than those between epidermal nerve densities and cold thresholds of the foot (r = 0.381, 95% CI: 0.094, 0.609, p = 0.01) in normal subjects. The correlations were more obvious in the neuropathy group than in the control group, e.g. the correlation coefficient for epidermal nerve densities and warm thresholds in the foot dorsum

---

*Fig. 1.* Skin innervation. Skin was immunocytochemically stained with protein gene product 9.5 (PGP 9.5). (a,c) are from normal subjects, and (b,d) are from neuropathic patients. (a) In normal skin, PGP 9.5 (+) nerves are in the epidermis (epi) and in the dermis (derm), forming subepidermal nerve plexuses (arrow, snp). PGP 9.5 (+) epidermal nerves (arrowheads) ascend in the epidermis perpendicularly to the epidermal–dermal junction. Individual epidermal nerves are difficult to show in this low-magnification figure, but their details, particularly varicosities, are clearly demonstrated at high magnification (inset). (b) In a neuropathic patient, there was a marked depletion of epidermal and dermal nerves. The staining pattern of subepidermal nerve plexuses (arrow, snp) has become faint and fragmented. (c) In a normal subject, individual dermal nerves are grouped in bundles with a pattern of dense and continuous staining. (d) Dermal nerves in a neuropathic patient have become fragmented with a beaded appearance. Bar = 100 μm (a,b); 50 μm (c,d); 20 μm (inset in a).
were $-0.483$ (95% CI: $-0.6819$, $-0.2168$) in the control group and $-0.705$ (95% CI: $-0.8713$, $-0.3922$) in the neuropathy group. The proportion of neuropathic patients with reduced epidermal nerve density was larger than the proportion of neuropathic patients with elevated thermal thresholds (Table 1).

**DISCUSSION**

This report provides rationales for using skin biopsy and quantitative sensory testing in clinical practice. In neuropathic patients, epidermal nerve density was reduced and the immunoreactive patterns of dermal nerves become fragmented. These provide objective and quantifiable parameters for evaluation of small-diameter sensory nerve degeneration.

**Skin innervation in small-fiber sensory neuropathy:** The 3-mm punch skin biopsy is a simple procedure with minimal invasiveness, and provides ample information regarding the pathological basis of small-fiber neuropathy. Epidermal innervation is reduced in small-fiber neuropathy, particularly in the lower extremities, consistent with the notion of length-dependent neuropathy. These pathological changes are correlated with changes in sensory thresholds, and probably preceded the elevation of sensory thresholds. This carries an important implication that evaluation of epidermal innervation is feasible as a clinical diagnostic parameter.

Nerve terminals are particularly vulnerable in toxic and mechanical damage to nerves of various types [19]. Epidermal nerves are depleted within 24–48 h after nerve transec-

---

**Fig. 2.** Epidermal nerve densities. (a) Comparison of epidermal nerve densities between normal subjects and neuropathic patients at different sites (filled symbols for normal subjects and open symbols for neuropathic patients; squares for the forearm and circles for the leg). Epidermal nerve densities are reduced in neuropathic patients compared with normal subjects in the forearm ($5.631 \pm 4.955$ vs $17.360 \pm 6.194$ fibers/mm, $p < 0.001$) and in the leg ($2.160 \pm 2.185$ vs $12.970 \pm 5.284$ fibers/mm, $p < 0.001$). (b) Correlation between epidermal nerve densities of the forearm and those of the leg (closed squares for normal subjects and open circles for neuropathic patients). In normal subjects, epidermal nerve densities of the forearm and the leg are highly correlated (slope: $0.916 \pm 0.111$, $p < 0.0001$). Epidermal nerve densities of the forearm are higher than those of the leg (Y-intercept: $5.720 \pm 1.535$ fibers/mm, $p < 0.001$). (c) Relationship between age and ratios of epidermal nerve densities (the forearm vs the leg). Closed squares are for normal subjects and open circles are for neuropathic patients. In normal subjects, the ratio of epidermal nerve densities is $1.488 \pm 0.569$ and is independent of age (slope: $-0.001 \pm 0.007$, $p = 0.86$). The ratios are increased in neuropathic patients ($3.670 \pm 4.332$) compared with normal subjects ($p < 0.002$).
tion in rodents. At that time point, the organization of nerve trunks remains normal [11]. Degenerating motor nerve terminals become swollen before the degeneration of motor nerve trunks in acrylamide intoxication [19]. The mechanisms of early degeneration in nerve terminals remain largely unknown. Calcium influx and free radicals contribute to the process [20]. All these suggest the early degeneration of nerve terminals, and offer a rationale for examining epidermal nerve terminals in small-fiber sensory neuropathy [21,22].

**Diagnostic approaches to small-fiber sensory neuropathy:** Evaluation of epidermal innervation by skin biopsy offers pathological evidence of nerve degeneration at the level of nociceptive terminals. Traditional evaluation of small-fiber sensory neuropathy relies entirely on measurements of various psychophysical tests, for example, sensory thresholds and current perception thresholds [23]. Thresholds of warm sensation, cold sensation, and current perception are elevated in diabetic neuropathy, uremic neuropathy, and various hereditary sensory neuropathies [24]. Quantitative sensory testing is particularly useful for series follow-up of clinical progression and therapeutic responses, as well as group comparison in field studies [24]. An important drawback of quantitative sensory testing is its dependence on the attention and cooperation of subjects [1]. There are physiological alternatives for measuring the conduction of small-diameter sensory nerves, such as with laser-evoked stimulation [25]. However, the discomfort caused by stimulating nociceptive nerves precludes large-scale clinical applications. Studies by laser-evoked stimulation of nociceptive nerves are mainly restricted to research purpose.
CONCLUSION
Skin biopsy and quantitative sensory testing are complementary for evaluation of small-diameter sensory nerve functions. The elevation of sensory thresholds can be caused by disorders of the central or peripheral nervous systems, for example, the elevation of sensory thresholds in a diabetic patient with a history of stroke. Quantitative sensory testing, with the advantage of simplicity and non-invasiveness, provides no information regarding anatomical localization. Skin biopsy, on the other hand, can assess the pathological substrate of elevated sensory thresholds [21,22]. The present study proposes that both skin biopsy and quantitative sensory testing should be an integrated package for examining small-fiber sensory neuropathy.

REFERENCES

Acknowledgements: We are indebted to Drs CP Tsai, JW Griffin, JC McArthur, and WR Kennedy for critical discussion and encouragement. This work was supported by the National Health Research Institute, Taiwan (NHRI-GT-EX89S727Cs), and National Taiwan University Hospital, Taipei, Taiwan (90M002).