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感覺及自主神經病變之診斷及其應用

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中文摘要

負責傳遞冷、熱、痛等刺激的感覺神經末梢位於皮膚的淺層，這些感覺神經通常非常的微小，一般的研究必須借助電子顯微鏡，也因如此，過去數十年來，有關這方面的研究非常少。在電子顯微鏡的層次要研究這些神經，通常就須施行神經切片，神經切片是一侵襲性甚高的檢查，因此病人的接受度不太高，在這種情況下，要研究或是診斷有關周邊神經的病變就是一個很大的盲點。本研究試圖建立以皮膚切片為基礎的病理檢查，於一般光學顯微鏡層次就可以評估位於表皮層之感覺神經末梢的退化。直徑3釐米之皮膚切片，以 protein gene product 9.5 (PGP 9.5)的免疫化學染色，經過影像分析系統定量，正常人的表皮神經密度 (epidermal nerve density)為 12.92 ± 5.33 fibers/mm，明顯降低 ($P < 0.001$)。本研究顯示皮膚切片合併免疫化學染色及表皮神經之定量分析，可用以診斷小纖維感覺神經病變。

關鍵詞: **神經傳導檢查，皮膚切片，感覺神經病變，神經末梢**

ABSTRACT

Pathological diagnosis of small-fiber neuropathy has traditionally depended on ultrastructural examinations of nerve biopsy specimens. To investigate the potential of epidermal nerve evaluation as a diagnostic approach, we performed 3-mm punch skin biopsies on healthy controls and patients with sensory neuropathy. Epidermal nerves were demonstrated by immunocytochemistry with the axonal marker, protein gene product 9.5. Patients with sensory neuropathy had both qualitative and quantitative features suggestive of cutaneous nerve degeneration. Degenerated dermal nerves exhibited a characteristic beaded appearance. Epidermal nerve density in the distal leg of healthy controls was 12.92 ± 5.33 fibers/mm (mean ± SD), and the value was markedly reduced in patients with sensory neuropathies, at 1.80 ± 3.16 fibers/mm ($p < 0.001$). The results provide pathological criteria to interpret skin biopsies and also suggest that evaluation of epidermal innervation by skin biopsy is a feasible diagnostic approach with minimal invasiveness.

Keywords: Epidermal nerves, Skin biopsy, Small-fiber neuropathy, Skin innervation, Nerve degeneration

INTRODUCTION

The pathology of nociceptive nerves has traditionally been examined by nerve biopsy. However, visualization of C- and Aδ-fibers usually requires time-consuming electron microscopic examinations, and the ultrastructural technique precludes large-scale evaluation of unmyelinated nerves at the light microscopic level. Small-diameter sensory nerves terminate in the epidermis of the skin as free nerve endings. Epidermal nerves are readily demonstrated by immunocytochemical staining of the skin with various axonal markers, particularly protein gene product 9.5 (PGP 9.5).[1-4] PGP 9.5 is a ubiquitin carboxy hydrolase, and is enriched in epidermal nerves.[5-7] Epidermal innervation evaluated by skin biopsy provides an opportunity to examine the pathology of cutaneous nerve terminals in small-fiber neuropathy.[8-15]

However, critical and basic questions remain which must be resolved before skin biopsies can become a routine diagnostic approach for small-fiber neuropathy.[16-19] For example, in patients with symmetric sensory polyneuropathy, what are the optimal sites for skin biopsy? What are the sensitivity and specificity of skin biopsies in diagnosing small-fiber neuropathies in different sites?

To address these issues, we compared epidermal nerve densities in normal subjects and in patients with small-fiber sensory neuropathy.

MATERIALS AND METHODS

Subjects. Normal controls were recruited from a cohort, including those in the community and those visiting National Taiwan University Hospital, Taipei, Taiwan for a physical check-up.[20] These subjects were evaluated by staff neurologists including detailed questionnaires and neurological examinations to exclude any neurological disorder and clinical neuropathy.[8,21] Examinations consisted of laboratory tests (complete blood count, fasting blood glucose, hemoglobin A_{1C}, liver and renal functions, serum protein electrophoresis, anti-nuclear antibody, and vitamin B_{12} 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: 26-78).

Patients with symmetric sensory polyneuropathy were regularly followed-up at the Department of Neurology, and had symmetrical sensory symptoms in the upper and lower extremities following the glove-stocking distribution. The neuropathy group consisted of 35 patients (17 males and 18 females) aged of 47.3 ± 11.6 years (range: 25-73). These patients

had progressive sensory or sensorimotor polyneuropathy of axonal type with elevated thermal thresholds by quantitative sensory testing. The evidence of axonal degeneration included reduced amplitudes of sural sensory action potential on nerve conduction studies, or reduced unmyelinated nerve densities in sural nerve biopsy. The etiologies included diabetes mellitus (5), vasculities (2) and the rest were idiopathic neuropathy. 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 the established procedures after local anesthesia with 2% lidocaine.[8] Punches of 3 mm in diameter were taken from each site: (1) 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 lateral side of the distal leg, 10 cm above the lateral malleolus. All healthy controls and neuropathic patients had skin biopsy 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 usual abrasion wound.

Immunocytochemistry. For immunocytochemistry on freezing microtome sections,[8,14] the skin tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 48 h. 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 Laborgerte, 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. Briefly, sections were quenched with 1% H_2O_2 in methanol, and blocked with 5% normal goat serum of appropriate species 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 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, and the examiners were blinded to the coded information.[4,8,22] PGP 9.5-immunoreactive nerves in the epidermis of each section were

counted at a magnification of 40x 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. Epidermal nerve abundance was defined as the number of total epidermal nerves in that section, i.e., fibers/section. The length of the epidermis along the upper margin of the stratum corneum in each section was measured with the Image-Pro PLUS system (Media Cybernetics, Silver Spring, MD). Epidermal nerve density was therefore derived and expressed as epidermal nerve abundance 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 34th sections of each tissue. The mean of epidermal nerve densities on these sections was the epidermal nerve density of that tissue.

Statistical analysis. Data of epidermal nerve densities in the control group and in the neuropathy group were expressed as the mean ± SD, and were compared by t-test. Sensitivity and specificity were calculated based on the fifth percentile value as the cut-off point. The correlations between epidermal nerve densities and sensory thresholds were evaluated by linear regression analysis with SPSS for Windows (version 6.1, SPSS, Chicago, IL) and GraphPad Prism (version 2.01, GraphPad Software, San Diego, CA). Any difference with $p < 0.05$ was considered statistically significant.

RESULTS

Skin innervation. In normal human skin, there were abundant epidermal nerves, subepidermal nerve plexuses, and dermal nerves immunoreactive for PGP 9.5. Individual epidermal nerves ascended perpendicularly in the epidermis after arising from the subepidermal nerve plexuses, which paralleled the junction between the epidermis and the dermis. Epidermal nerves had typical varicosities, and terminated in the upper portion of the granular layer of the epidermis. Occasionally, epidermal nerves had branches in the supra-basal layer of the epidermis. In the skin of neuropathic patients, there was a significant reduction in epidermal innervation. The extent of epidermal nerve loss was variable among different patients.

Quantitation of epidermal innervation. To evaluate the abundance of epidermal nerves, we quantified the epidermal innervation by calculating epidermal nerve density, i.e., the number of epidermal fibers per millimeter of epidermis.[8,14,17] Epidermal nerve densities were

significantly higher in the forearm than in the distal leg with 17.07 ± 6.51 fibers/mm in the distal forearm, and 12.92 ± 5.30 fibers/mm in the distal leg ($p < 0.001$). There was a significant correlation between densities at both sites ($r = 0.55$, $p < 0.0001$).

Epidermal nerve density was reduced in patients with sensory neuropathies, with 5.82 ± 6.50 fibers/mm in the distal forearm ($p < 0.01$ compared to healthy controls). The reduction in epidermal nerve density was more robust in the distal leg, at 2.40 ± 2.30 fibers/mm ($p < 0.001$ compared to normal subjects).

DISCUSSION

Currently there are two major methods of quantifying epidermal nerves. Studies by Kennedy et al. and Periquet et al. employed 100-μm sections, immunofluorescence, and confocal microscopy.[11,12] Studies by McArthur et al. used 50-μm sections, and were based on the immunoperoxidase method and regular light microscopy [8,13] All results were correlated with stereological studies on the total length of epidermal nerves.[11,23] Normative data for the distal leg are available for both methods, but there are some differences because of different thickness of sections and rules of counting epidermal nerves.[11,12,16,21] The quantitative method in the present report is the same as that of McArthur et al., and the normative data are similar.[21] Either method of epidermal nerve quantitation requires imaging analysis systems, which are not necessarily available in every laboratory. The present report therefore proposes modified strategies based on certain assumptions, which are easily fulfilled. The findings suggest that quantitative evaluation of epidermal innervation can be made based on epidermal nerve abundance and crude epidermal nerve density by counting the number of epidermal nerves per section in appropriate sections.

The other major finding in this report is that the distal-leg biopsy has a higher sensitivity than the distal-forearm biopsy. Previous studies have generated normative data in the thigh and other sites.[16,21] The present report also examined the distal forearm because many neuropathic patients had sensory symptoms following glove-stocking distribution. Patients with reduced epidermal nerve densities in the distal forearm always had reduced epidermal nerve densities in the distal leg. The reverse condition was not true. These findings are consistent with the nature of length-dependent neuropathy, and suggest that a single biopsy site in the distal leg is sufficient for evaluation of symmetric sensory polyneuropathy.

Evaluation of epidermal innervation by skin biopsy is a new approach to diagnose small-fiber sensory neuropathy, and is particularly suitable for investigating temporal changes because of the small wound after a punch biopsy, for example, in clinical trials for small-fiber sensory neuropathy. It is impossible to achieve this goal by routine nerve biopsy, which can only be performed once. In addition, skin biopsy is also useful for side-to-side or site-to-site comparisons.

REFERENCES

1. Wilkinson KD, Lee K, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 1989; 246:670-673.
2. Wang L, Hilliges M, Jernberg T, Wiegleb-Edstronom D, Johansson O. Protein gene product 9.5-immunoreactive nerve fibers and cells in human skin. Cell Tissue Res 1990; 261:25-33.
3. Kennedy WR, Wendelschafer-Crabb G. The innervation of human epidermis. J Neurol Sci 1993; 115:184-190.
4. Chiang H-Y, Huang I-T, Chen WP, Chien H-F, Shun CT, Chang YC et al. Regional difference in epidermal thinning after skin denervation. Exp Neurol 1998; 154:137-145.
5. Hilliges M, Wang L, Johansson O. Ultrastructural evidence for nerve fibers within all vital layers of the human epidermis. J Invest Dermatol 1995; 104:134-137.
6. Hedge AN, Inokuchi K, Pei W, Casadio A, Ghirardi M, Chain DG et al. Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. Cell 1997; 89:115-126.
7. Johansson O, Wang L, Hilliges M, Liang Y. Intraepidermal nerves in human skin: PGP 9.5 immunohistochemistry with special reference to the nerve density in skin from different body regions. J Peripher Nerv Syst 1999; 4:43-52.
8. McCarthy B, Hsieh ST, Stocks EA, Hauer P, Macko C, Cornblath DR et al. Cutaneous innervation in sensory neuropathies: evaulation by skin biopsy. Neurology 1995; 45:1848-1855.
9. Holland NR, Stocks A, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Intraepidermal nerve fiber density in patients with painful sensory neuropathy. Neurology 1997; 48:708-711.
10. Scott LJ, Griffin JW, Luciano C, Barton NW, Banerjee T, Crawford T et al. Quantitative analysis of epidermal innervation in Fabry disease. Neurology 1999; 52:1249-1254.
11. Kennedy WR, Wendelschafer-Crabb G, Johnson T. Quantitation of epidermal nerves in diabetic neuropathy. Neurology 1996; 47(4):1042-1048.
12. Periquet MI, Novak V, Collins MP, Nagaraja HN, Erdem S, Nash SM et al. Painful sensory neuropathy: prospective evaluation using skin biopsy. Neurology 1999; 53(8):1641-1647.

13. Holland NR, Crawford TO, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Small-fiber sensory neuropathies: clinical course and neuropathology of idiopathic cases. Ann Neurol 1998; 44:47-59.
14. Hsieh ST, Chiang H-Y, Lin W-M. Pathology of nerve terminal degeneration in the skin. J Neuropathol Exp Neurol 2000; 59(4):297-307.
15. Kennedy WR, Nolano M, Wendelschafer-Crabb G, Johnson TL, Tamura E. A skin blister method to study epidermal nerves in peripheral nerve disease. Muscle Nerve 1999; 22(3):360-371.
16. Lauria G, Holland N, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Epidermal innervation: changes with aging, topographic location, and in sensory neuropathy. J Neurol Sci 1999; 164:172-178.
17. Herrmann DN, Griffin JW, Hauer P, Cornblath DR, McArthur JC. Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. Neurology 1999; 53(8):1634-1640.
18. Barohn RJ. Intraepidermal nerve fiber assessment: A new window on peripheral neuropathy. Arch Neurol 1998; 55:1505-1506.
19. Kennedy WR, Said G. Sensory nerves in skin: answers about painful feet? Neurology 1999; 53(8):1614-1615.
20. Lin YH, Huang MH, Chang YC, Tai TY, Chen WH, Yang CC et al. Quantitative sensory testing: normative values and its application in diabetic neuropathy. Acta Neurol Taiwan 1998; 7:176-184.
21. McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency [see comments]. Arch Neurol 1998; 55:1513-1520.
22. Hsieh ST, Lin W-M. Modulation of keratinocyte proliferation by skin innervation. J Invest Dermatol 1999; 113:579-586.
23. Stocks EA, McArthur JC, Griffin JW, Mouton PR, Griffen JW. An unbiased method for estimation of total epidermal nerve fiber length. J Neurocytol 1996; 25:637-644.