EFFECTS OF AGING ON HUMAN SKIN INNERRATION

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INTRODUCTION

Detection of thermal stimuli begins from receptors in the skin [1]. This information is conveyed via the spinothalamic tract to the cerebral cortex. It is generally believed that the sensitivity to thermal and noxious stimuli is reduced in the elderly [2]. Reduced sensitivity can be due either to reduced quantities of sensory nerve terminals at the level of the skin or to defective processing of sensory inputs at the level of the CNS [3]. However, the former issue has not been thoroughly examined.

Sensory nerve terminals are localized in the most superficial layer of the skin. Morphologically, the unmyelinated nerves are 1–2 μm in diameter and are traditionally studied in sural nerve biopsies at the level of electron microscopy. Recently, nerve endings of small myelinated and unmyelinated fibers (i.e., small-diameter sensory nerves) in the epidermis of the skin can be demonstrated by immunohistochemistry using various neuronal proteins, particularly protein gene product 9.5 (PGP 9.5) [4]. PGP 9.5 is a ubiquitin C-terminal hydrolase, is enriched in unmyelinated axons, particularly the terminals [5].

Previous pathological studies of aging effects have mainly focused on large-diameter nerves by measuring nerve conduction and evaluating nerve biopsies [6]. There are only rare reports exploring the effect of aging on unmyelinated nerve fiber densities in sural nerve biopsies [7]. The interpretation of these studies, however, is a complicated issue because autonomic nerve fibers were present in the sural nerves [8]. Thus, it is not clear whether the abundance of small-diameter sensory nerve fibers changes with aging.

To address these issues, we studied the effect of aging on cutaneous innervation. By immunohistochemical staining of the skin with the neuronal marker, PGP 9.5, and quantification of IENF densities, we were able to demonstrate an age-dependent reduction in skin innervation.

MATERIALS AND METHODS

Study population: Healthy subjects of different ages were evaluated by staff neurologists using detailed questionnaires and neurological examinations to identify any neurological disorder or clinical neuropathy [9]. Examinations consisted of laboratory tests (complete blood count, fasting plasma glucose, hemoglobin A1C, liver and renal function, serum protein electrophoresis, anti-nuclear antibody, and vitamin B12 level), nerve conduction studies, and quantitative sensory testing. Only subjects free from sensory symptoms and neurological signs and with normal results on nerve conduction studies and quantitative sensory testing were included for this study. There were 87 healthy subjects (36 males and 51 females; aged 19–78 years) with skin biopsy from the distal leg. Skin biopsies from the distal forearm were also taken in 62 subjects (27 males and 35 females; aged 19–78 years). Among these, there were 56 subjects (25 males and 31 females; aged 19–78 years) with skin biopsies from both sites. The Ethics Committee of National Taiwan University Hospital, Taipei approved this study, and informed consent was obtained before skin biopsy.

Skin biopsy: Skin biopsy was performed following established procedures after local anesthesia with 2% lidocaine [10]. Punches 3 mm in diameter were taken from the following locations: (1) the extensor side of the distal forearm, 5 cm above the middle point of a 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 subjects tolerated the procedure with no obvious signs of discomfort. No suturing was required, and the wounds were covered with a piece of gauze. Wound healing took 7–10 days, the same as would a typical abrasion wound.

**Immunohistochemistry:** For immunohistochemistry on microtome sections [10], skin tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 48 h. Sections of 50 μm were quenched with 1% H2O2, blocked with 5% normal goat serum, and incubated with rabbit antiserum to PGP 9.5 (UltraClone, 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 IgG at room temperature for 1 h, followed by incubation with avidin–biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated with chromogen SG (Vector, Burlingame, CA), and counterstained with eosin (Sigma, St. Louis, MO).

**Quantitation of epidermal innervation:** Epidermal innervation was quantified following established protocols, and slides were coded to ensure that measurements were blinded [11]. PGP 9.5-immunoreactive nerve fibers in the epidermis of each section were counted at a magnification of ×40 with an Olympus BX40 microscope (Tokyo, Japan) 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 (Media Cybernetics, Silver Spring, MD). Intraepidermal nerve fiber density (IENF density) was derived and expressed as fibers/mm. For each tissue, there were 48–50 sections, and all sections were sequentially labeled. Every fifth section was immunostained and quantified. The mean of values from these sections was considered the IENF density of the tissue specimen.

**Statistical analysis:** IENF densities in different age groups (the young adult group aged 19–39 years and the elderly group aged ≥60 years) were expressed as the mean ± SEM, and were compared by t-test after the normality of the distribution was confirmed.

Previous studies indicated that the influence of age on pathologic and physiologic parameters are not always linear [12]. For example, there was a non-linear decline in nerve conduction velocities and myelinated nerve fiber densities with age [6]. To determine the best-fit model for analyzing the influence of age on skin innervation, we assessed IENF density with various curve estimation regression models using SPSS for Windows (SPSS, Chicago, IL). These included linear, logarithm, exponential, quadratic, cubic, inverse, growth, compound and power. From that analysis, we determined that linear regression model was the most optimal model for analysis and was used in the current report.

The effects of age and gender on IENF densities of the upper and lower extremities were further evaluated by multiple linear regression model. The 95% confidence interval (95% CI) of Pearson’s coefficient was included. Any difference with \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Skin innervation:** In human skin, there were abundant PGP 9.5-immunoreactive nerves in the dermis and in the epidermis (Fig. 1a). Epidermal nerves arose from the subepidermal nerve plexuses, penetrated the basement membrane, and had a typical varicose appearance (Fig. 1b). The number of epidermal nerves was variable among different subjects and required quantification for comparison.

![Fig. 1. Skin innervation. Skin tissues from normal subjects were immunostained with protein gene product 9.5 (PGP 9.5). (a) PGP 9.5 (+) nerves are in the epidermis, in the dermis, and around sweat glands (sg). (b) Typical epidermal nerves arise from the subepidermal nerve plexus (SNP) and have a varicose appearance (arrow). Brown melanin pigments in the basal keratinocytes delineate the epidermis from the underlying dermis. Bar = 670 μm (a), 27 μm (b).](image-url)
of epidermal nerve densities in the elderly compared to the young adults.

To further investigate the effect of age on epidermal innervation, we analyzed IENF densities by linear regression analysis on the entire study population. IENF density of the distal leg was linearly correlated with age (Fig. 2b) with a slope of $-0.1463 \pm 0.0273$ (95% CI = $-0.2007$ to $-0.0919$, $p < 0.0001$) and a Y-intercept of $17.47 \pm 1.35$ (95% CI = $14.78$–$20.16$). Because gender plays an important role in determining physiological parameters, we further analyzed the data using a multiple linear regression model with age and gender as independent variables. The standardized coefficient for age was $-0.462$ ($p < 0.001$). Male subjects tended to have lower IENF densities (standardized coefficient of $-0.166$ for gender), but the difference did not reach statistical significance ($p = 0.84$).

### Age-related changes in IENF densities in the upper extremity:
In the distal forearm, IENF densities were also reduced in the elderly (11.67 ± 1.55 fibers/mm, $n = 11$) compared with the young adults (19.39 ± 1.60 fibers/mm, $n = 14$, $p < 0.001$, Fig. 2a). IENF densities of the distal forearm were linearly correlated with age with a slope of $-0.1692 \pm 0.054$ (95% CI = $-0.2772$ to $-0.0612$, $p = 0.0028$) and a Y-intercept of $23.52 \pm 2.61$ (95% CI = $18.30$–$28.74$; Fig. 2c). On multiple linear regression analysis with age and gender as independent variables, the standardized coefficient for age was $-0.335$ ($p = 0.005$). Male subjects had lower epidermal nerve densities than did female subjects with standardized coefficients of $-0.275$ ($p = 0.020$) for IENF density.

### Regional differences in epidermal innervation:
To understand whether there is a regional difference in age-dependent reduction of epidermal nerve densities, we analyzed the differences and ratios of epidermal nerve densities between the upper and lower extremities. Epidermal nerve densities were higher in the upper extremities than in the lower extremities as demonstrated by the difference in IENF density (4.78 ± 0.80 fibers/mm different from 0, $p < 0.0001$) and the ratio of IENF density (1.503 ± 0.079 different from 1, $p < 0.0001$). The difference in IENF density between the forearm and the leg, however, was not correlated with age (Fig. 3a, slope = $-0.0641 \pm 0.0605$, 95% CI = $-0.1855$ to $0.0574$, $p = 0.29$). Ratios of IENF densities were independent of age as well (Fig. 3b, slope = $-0.0033 \pm 0.0065$, 95% CI = $-0.0154$ to $0.0088$, $p = 0.58$). These findings indicate that age-dependent reductions in epidermal innervation were similar in the upper and lower extremities.

### DISCUSSION

**Reduced epidermal innervation of the skin with aging:**
The present study demonstrates a linear reduction of epidermal innervation in the elderly. Previous studies have indicated that age could influence various morphological and physiological parameters in different manners. Epidermal nerves are the peripheral processes of dorsal root ganglion neurons. The effect of aging on epidermal nerve terminals has been rarely explored because of the technical difficulties in demonstrating unmyelinated nerve fibers in the skin. This issue can now be resolved using the sensitive...
immunohistochemistry of neuronal proteins including PGP 9.5, substance P, and calcitonin gene-related peptide [13]. Several lines of evidence suggest that PGP 9.5 (+) epidermal nerves are sensory nerve endings. For example, epidermal nerve fibers are depleted by ganglionectomy of dorsal root ganglia, and epidermal nerves remain intact after specific lesions of sympathetic ganglia [5]. The loss of epidermal nerves is associated with ultrastructural evidence of nerve degeneration [13]. In human small-fiber sensory neuropathies, such as diabetic neuropathy, epidermal innervation is reduced [14,15].

Previous studies on sciatic nerves comparing young and aged mice indicate that the loss of unmyelinated nerve fibers is more robust than that of myelinated nerve fibers with aging [16]. Unmyelinated nerve degeneration is associated with an increase in denervated Schwann cell pockets, accumulation of collagen and lipid droplets, and infiltration of mast cells and macrophages. Because sciatic nerves consist of motor, sensory, and autonomic nerves, it is not clear whether the reduction in unmyelinated nerve fiber density really reflects the degeneration of small-diameter sensory nerves. IENF densities might decrease with aging, for example, the normative data of epidermal nerve fiber densities were divided into < 60 and ≥ 60 years of age [15,17]. The present study provides direct evidence of a significant reduction in cutaneous nerve terminals with aging, and there is linear reduction of IENF densities starting from the adulthood.

Regional differences in skin innervation: The effects of aging on cutaneous innervation of different body parts appear to be similar. Epidermal nerve densities vary among different regions of bodies, for example, distal vs proximal body parts and the glabrous vs hairy skin [17–19]. A major factor in determining IENF densities apparently is related to the distance between neuronal cell bodies and nerve terminals; for example, epidermal innervation densities are higher in the trunk than in the distal leg [18]. In this study the lower IENF densities in the lower extremity compared with the IENF densities in the upper extremity is consistent with this notion. Of course, there may be local differences in IENF densities for the same extremity, for example, the medial side vs the lateral side of the distal leg.

The degree of reduction in epidermal nerve densities with aging is similar in both the upper and lower extremities. This may indicate that age plays a universal role in influencing sensory neurons regardless of the localization of sensory neurons. The ratio of epidermal nerve densities turns out to be a useful parameter for evaluating length-dependent peripheral nerve disorders [20]. Peripheral neuropathy tends to start from the most distal part of nerves due to either metabolic insufficiency or toxic insult. Therefore, patients with peripheral neuropathy have a higher ratio of epidermal nerve densities [20]. This ratio remains useful clinically for evaluating aged people.

Mechanisms of reduced skin innervation and cutaneous sensitivity in the elderly: Mechanisms for reduced skin innervation in the elderly remain obscure. One potential candidate is trophic factor. In aged animals, the production of nerve growth factor is reduced, and this is associated with increased cell death [21]. In diabetic neuropathy, the abundance of epidermal nerves is reduced, and the content of nerve growth factor in diabetic skin is reduced [22]. Epidermal nerves depend on various neurotrophins for survival, maintenance, and regeneration, including nerve growth factor and glial cell line-derived growth factor. In the hippocampus of young animals, the content of nerve growth factor increases after neuron injury. In contrast, the post-injury up-regulation of nerve growth factor is not observed in aged animals [23]. The application of nerve growth factor to aged sensory neurons restores neurite growth and neuronal survival [24]. Thus the study of skin innervation provides a new system to test this hypothesis.

CONCLUSION

Detection of thermal stimuli begins from receptors in the skin. Age presumably influences the perception of thermal and nociceptive stimuli. There is, however, lack of direct evidence. We investigated the effect of age on sensory nerve terminals in the skin. In the elderly (≥ 60 years of age), there is 40–42% reduction of epidermal nerves in the distal leg and distal forearm, compared to young adults (19–39 years of age). On the entire study population (age range 19–78 years), epidermal nerve densities of both the distal leg and
the distal forearm are negatively correlated with age according to multiple linear regression analysis. These findings suggest a significant and linear reduction of epidermal nerves with age starting from adulthood.

REFERENCES


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