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Epidermal innervation in healthy children and adolescents

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Abstract

Introduction—Epidermal nerve fiber (ENF) density, morphology, and epidermal innervation patterns were examined in children using 2 different techniques, punch biopsy and suction blister.

Methods—Healthy children without symptoms or history of peripheral neuropathy and normal by neurologic examination were studied. Punch biopsy and suction blister specimens were collected from the lateral thigh and distal leg. ENFs were traced from confocal images of immunohistochemically stained samples. Statistical methods included repeated measures analysis of covariance.

Results—Blister and biopsy nerve counts were associated. ENF density in children was dense, lower for older children ($P < 0.01$), with no difference between boys and girls ($P = 0.92$). Many ENFs appeared multi-branched and elongated.

Discussion—Epidermal innervation in the pediatric population is dense and age dependent. Blister specimens are less invasive and may provide an alternative to punch biopsy for determining ENF density in children at risk for neuropathy.

Keywords

Children; Epidermal Nerve Fibers; Skin Biopsy; Immunohistochemistry; Peripheral Neuropathy

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The first two authors contributed equally to the study

Introduction

Epidermal nerve fiber (ENF) density and morphology have been well studied in the healthy adult population as well as in a variety of peripheral neuropathies.^{1,2} Although both children and adolescents develop neuropathies associated with diabetes, HIV infection, or chemotherapy, our knowledge of patterns of epidermal innervation from childhood to adolescence is limited. Reduced ENF density is the main diagnostic feature of small-fiber sensory neuropathies and can be abnormal even when the neurological examination, nerve conduction studies, and sural nerve biopsy results are within the normal range.^{3–5} Skin punch biopsy is the present gold standard diagnostic method for evaluation of small fiber neuropathies. An alternative minimally invasive technique, the suction skin blister, is suitable to study epidermal nerve fibers (ENFs) in a pediatric population because it can be performed without pain, bleeding or scarring.^{6–9} Our goal was to analyze and compare ENF morphology and density in tissue obtained by punch skin biopsy and suction skin blister in 20 healthy children and adolescents.

Materials and Methods

Subjects

Twenty healthy Hispanic children age 7 to 17 years (M/F = 10/10) participated in the study. The children were part of the control group in a larger cohort study of pediatric HIV neuropathy at the University of Puerto Rico Medical Sciences Campus.¹⁰ The study was approved by the Institutional Review Board of the University of Puerto Rico. All subjects and their legal guardians provided informed consent. All procedures were in accord with the standards of the Committee on Human Experimentation of the institution in which the experiments were done.

None of the children had a history of neuropathy, diabetes mellitus, HIV infection, exposure to neurotoxic drugs, or other known causes of neuropathy. All subjects underwent a neurologic examination, including sural sensory and fibular motor nerve conduction studies, assessment of tendon reflexes, muscle strength, sensitivity to vibration, pin prick, and light touch. Vitamin B12 and fasting blood glucose were within normal limits, and hepatitis C virus antibodies were negative for all participants. Subject heights and weights were recorded.

Specimens

Two 3 mm suction blister specimens were acquired simultaneously⁹ from standardized areas on the lateral thigh and distal leg.¹¹ The time required for suction blister formation was recorded. A 3 mm punch skin biopsy specimen was acquired from an area adjacent to the blisters.⁹

Immunofluorescent staining

All specimens were fixed with Zamboni solution and processed for immunofluorescent localization of nerve and tissue antigens as described in Table 1.^{9,12} Intact blister roofs were double immunostained with rabbit anti PGP 9.5 and mouse anti CD1a. Sectioned skin

biopsies were immunostained with rabbit anti PGP 9.5, mouse anti type IV collagen and lectin *Ulex europaeus* agglutinin type. Non-immune controls for antibody specificity were performed.

Confocal microscopy and quantification of ENFs

Confocal image acquisition was performed using methods described previously.⁹ To reduce sampling bias, tissue selection for image acquisition was determined by viewing only CD1a staining to assess tissue integrity with the viewer blind to the ENF staining. For punch biopsy specimens, nerve quantification was performed on 8 epidermal areas, 2 images from each of 4 different sections. This provided a skin surface area of approximately 160,000 μm^2 (20,000 $\mu\text{m}^2 \times 8$). In blister roofs, 2 areas were chosen randomly, providing a skin surface area of 285,120 μm^2 (142,560 $\mu\text{m}^2 \times 2$).

ENF density, nerve length, and nerve branch quantification in the punch biopsy specimens were performed manually using NeuroLucida 5.05.4 nerve-tracing software (MBF Bioscience, Williston, VT) according to standardized rules.¹ Only ENFs that were observed to penetrate the basement membrane were counted. Free epidermal nerve fragments were not counted. ENF quantification in blisters was performed using the same tracing software and following similar general rules altered for blister analysis as described previously.⁹ Because the blister separated immediately superficial to the dermal/epidermal boundary,^{6,7} the distinct basement membrane staining used to delineate ENFs in biopsies was not present. The nerve fibers were traced through confocal image stacks from origins, perceived as enlargements occurring where the nerve stump severed from the underlying dermal nerve fiber at the basal aspect of epidermis. Only ENFs whose entering point into the basal epidermal layer was contained in the acquired image were counted.

ENF density was expressed as the number of nerve fibers per epidermal surface area (ENFs/mm²) for both the punch biopsy and the suction blister methods.⁹ Epidermal surface area for the punch biopsy sections was calculated as epidermal length included in the image multiplied by the examined thickness of the section; and for the blister roofs as the area of the image used for ENF quantification (142,560 μm^2). Results for biopsy sections include nerve density, ENFs/mm², followed by number of fibers per section length shown in parentheses (ENFs/mm). Length of nerve fibers was determined for the punch biopsy specimens.

Statistical Analysis

Analysis of ENF density used mixed linear models. The random effect was a subject; depending on the analysis, fixed effects included method (blister vs. biopsy), location (leg vs. thigh), gender, age, or location within blister (center vs. periphery). Analyses used the restricted likelihood method in either JMP (v. 8) or SAS (v. 9.2; both SAS Institute Inc, Cary NC). Preliminary analysis of ENF density showed a distribution skewed to the right, suggesting that analysis of square-root or log ENF density was indicated. Analysis of the original ENF density measurements and these 2 transformations of ENF density measurements gave very similar results, so we present analyses of the original ENF density measurements.

Nerve length per nerve, branches per nerve, and time for blister formation were analyzed using the method described for ENF density.

To analyze variation between images in a specimen, a “specimen” was defined for biopsies as a biopsy section (4 sections per biopsy, 1 biopsy per subject and location) and for blisters as a blister (2 blisters per subject and location). Each specimen, a biopsy section or a blister, had 2 images (except for a very few having only a single image). For each specimen, we computed the absolute difference between images in ENF density, i.e., image 1 ENF density minus image 2 ENF density with any minus signs stripped off. This absolute difference was the measure of variability between images and was the dependent variable in analyses related to variability. Analyses used mixed linear models as described above.

Results

Morphology and distribution of ENFs

Normal epidermis was highly innervated both in biopsy and blister specimens (Figures 1A, 1B). ENFs varied in length and complexity from simple single short fibers ending in the basal layer of the epidermis to multibranched fibers extending to the surface of the epithelium. Within the same specimen, ENFs were either homogeneously distributed across the epidermis or grouped (Figure 1C, 1D). Occasionally, some ENFs within a blister or punch biopsy specimen were oriented in parallel arrays (Figures 1E, 1F). Some ENFs were very long and gave rise to many branches. Because these samples were innervated densely, the branches of several ENFs often overlapped (Figure 2A). The full lateral extent of all branches that arose from these complex ENFs was better observed in the “bird’s eye” view afforded by the blister roofs than in the vertical-biopsy sections (Figure 2B), where the 60 μm thickness of the sections sometimes limited the visualization of entire nerve endings. Crawlers (horizontally-oriented ENFs within the *stratum basale*¹³) were observed in both the punch biopsy and the suction blister specimens. In some cases, 1 or more nerve fiber branches arose from a single crawler and ascended in the epidermis (Figure 2C). Nerve swellings were rare and small in size.

ENF density in punch biopsy and suction blister specimens

ENF density from the punch biopsy specimens was higher compared to ENF density from the suction blister method for both body locations, thigh ($P < 0.0001$) and leg ($P < 0.0001$). Specifically, for the punch biopsy method the average ENF density was significantly higher on the thigh -779 ± 50 SE ENFs/ mm^2 (39.1 ± 3.0 ENFs/mm) than on the leg -685 ± 50 ENFs/ mm^2 (34.3 ± 3.0 ENF/mm). For the suction blister method the ENF density was not significantly different between the thigh -403 ± 53 ENFs/ mm^2 and the leg -447 ± 53 ENFs/ mm^2 (Figure 3A). The ENF density results from these 2 methods were highly correlated for the thigh ($r = 0.65$) and leg ($r = 0.91$) (Figure 3B).

ENF density did not differ between girls and boys when both locations were analyzed together --respective adjusted averages 582 ± 60 ENFs/ mm^2 and 573 ± 60 ENFs/ mm^2 , $P = 0.92$. ENF density decreased with increasing age (Figure 3C). Specifically, the ENF density was decreased by 65 ± 16 ENFs/ mm^2 (3.2 ± 0.8 ENF/mm) for each additional year of age

when the punch biopsy was used [59.1 ENF/mm^2 (3.0 ENFs/mm) for thigh and 70.2 ENF/mm^2 (3.5 ENFs/mm) for leg]; and $33 \pm 9 \text{ ENFs/mm}^2$ when using the suction blister [19.2 ENF/mm^2 for thigh and 46.1 ENF/mm^2 for leg]. Children's height, weight, and BMI correlated positively with each other and also strongly correlated with subject age; therefore no further analysis was performed to assess their association with the ENF density in this sample size population.

Nerve length and branching pattern for the punch biopsy specimens

Average ENF length per nerve was calculated for each subject from biopsy sections. The average nerve fiber length for leg was $55.22 \pm 2.01 \mu\text{m}$ and for thigh was $55.74 \pm 2.45 \mu\text{m}$ (Figure 3D). Average nerve length was not associated with gender, age, or body location. The number of branches per nerve fiber was similar for girls and boys (respective adjusted averages 0.47 ± 0.04 and 0.39 ± 0.04 branches per axon, $P = 0.14$) and was higher on the thigh than on the leg (respective adjusted averages 0.47 ± 0.03 and 0.39 ± 0.03 branches per axon, $P = 0.017$).

Intrabiopsy and intrablister variation of ENF density

Intrabiopsy ENF density variation between different images from punch biopsies was on average 3 times larger than the variation between images for blisters (respective average absolute difference 273 ± 15 and 90 ± 21 , $P < 0.0001$). This is not surprising, given that epidermal surface area represented in each biopsy image was one-seventh of the area represented in each blister image. Considering each method (suction blister and punch biopsy), the intraspecimen ENF density variation was not different on the thigh versus the leg (respective average absolute difference 191 ± 18 and 173 ± 18 , $P = 0.93$) and was not associated with the gender (average absolute difference for boys, 180 ± 19 , and girls, 184 ± 19 , $P = 0.95$). To determine if ENF density differences were induced by the suction effect during blister formation, we compared the ENF density estimates obtained from the center and the periphery of the blister roofs and found no difference (respective average ENF density 413 ± 34 and 431 ± 32 , $P = 0.28$).

Characteristics of the suction blister method

ENF density estimates in adjacent blisters collected from the same body location of each volunteer were very highly correlated (leg $r = 0.92$, thigh $r = 0.90$). The time for blister formation was less on distal thigh (average 85, range 42 – 129 min) than on distal leg (average 115, range 63 – 193 min) ($P = 0.0002$). Blister formation time was not associated significantly with estimated ENF density (thigh, $r = 0.08$, $P = 0.72$; leg, $r = 0.10$, $P = 0.68$), or with subject ages ($r = 0.34$, $P = 0.14$) or gender (average, boys 102 ± 8 , girls 97 ± 8 , $P = 0.72$).

Discussion

The ENF density in these 20 healthy children, as determined by punch biopsy and suction blister, was more than 3 times higher than that found in similar studies of normal adults.⁹ Similar to normal adults, ENF density did not differ between genders and was lower in older subjects.^{9, 11, 14} ENF density in these children correlated inversely with increasing body

surface area, which is consistent with a model based on nerve fibers decreasing by half for every doubling of body surface area. More studies in larger and more varied datasets of healthy children are needed to establish associations of distinct body measurements with ENF density.

In normal adults, ENF density is higher in the thigh than in the calf.⁹ In our 20 children, ENF density was significantly higher in the thigh than the leg when the punch method was used, but not when the blister method was used.

These healthy children had many multi-branched and long ENFs. We also noticed crawlers that gave rise to more than 1 nerve fiber branch. The suction blister method was advantageous for studying the morphology, distribution, spatial analysis, and structure of complex and long ENFs, because they are wholly included in the blister roof but not in a typical single 60 μ m thick vertical punch biopsy section. Orientation of groups of ENFs in the same direction was noticed both in punch biopsy and suction blister specimens. This could be a chemotaxis phenomenon related to the expression of growth factors at a particular site during development, since it has also been noticed in nerve regeneration studies.¹⁵

ENF density estimates from the blister method were lower than those from the punch biopsy method. This disparity is most likely related to modifications of the standardized biopsy ENF counting rules. Though similar in most respects, the rules differ for blister and biopsy, especially in identification of the “origin” where a nerve fiber enters into the epidermis. For biopsies, this is based on the intersection of the nerve fiber with the dermal-epidermal junction. For blisters, it is based on the appearance of an enlarged distal stump where the nerve fiber was severed from the underlying parent nerve during blister formation. Determining whether 2 very closely located nerve fibers are either 2 branches of a single ENF or just separate ENFs is sometimes difficult in a blister and could account for the disparity. Also, very short ENFs may retract and remain attached to the underlying dermal neural plexus. Other possible sources of the disparity were investigated, as explicated in the following text and were found to be unassociated with estimated ENF density. The disparity between blister and biopsy ENF density estimates for an individual is not uniformly high or low in either lower leg or thigh, suggesting that it is not specific to the child. The disparity is greater in the thigh than the leg, but it is not related to age, gender, estimated ENF density, or time for blister formation.

Regardless of the difference between blisters and punch biopsies in estimated ENF density, the blister method ENF density estimates are reproducible and show minimal variation between or within blisters from a given subject. The bloodless and painless removal of epidermis creates a superficial wound that heals rapidly. Absence of scar formation or need for injection of local anesthesia increase the appeal of the suction blister method. These characteristics may be especially useful for pediatric studies or for studies that require multiple specimens to study ENF changes over time or ENF distribution at multiple body locations. Although the blister method has many advantages, several limitations must be considered when deciding which method to use to study cutaneous innervation. Studies of subepidermal neural plexus and innervation of dermal structures such as sweat gland and

arrector pillorum muscle require a full thickness punch biopsy. Also, blister formation time is a limiting factor. The longer time for blister formation in children compared to adults could be due to ethnic differences or to unknown qualitative differences of skin integrity.⁹

Punch biopsy, a well-established method, provides information on both epidermal and dermal innervation and is already used in clinical settings. It is invasive, results in scar formation and does not facilitate study of the whole distribution of each ENF nor the association of each ENF with adjacent ones. On the other hand, the suction blister method facilitates visualization and observation of more complex and elongated ENFs as well as their distribution across the epidermis, but it does not provide information regarding dermal structures.

This study showed ENF morphology and distribution pattern in childhood and adolescence using 2 different skin sampling methods. Additional studies with a larger sample size are needed to establish the normal range of ENF density for each age category from childhood through puberty, and to study possible associations of ENF density with height, weight, and gender. The punch biopsy and the suction blister methods should also be used to study children with small fiber neuropathy, such as diabetic, HIV, or chemotherapy-induced neuropathy to better understand pathologic alterations that occur and to further test the usefulness of the 2 methods for diagnosis, staging, and follow up of children with these disorders.

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Abbreviations

ENF	epidermal nerve fiber
ENFs	epidermal nerve fibers
SE	standard error

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Figure 1. Epidermal nerve fiber (ENF) distribution in normal children – Confocal microscopic image projections

A. Uniform distribution of ENFs in leg (7 year-old girl). 50 μ m thick cross biopsy section immunostained for ENFs with rabbit anti-PGP9.5 (green), basement membrane with mouse anti-type IV collagen (red), and endothelial and epithelial cells with the lectin *Ulex europaeus* agglutinin type I (blue).

B. Leg blister (same child as in Figure 1A) providing a “bird’s eye” view of the ENFs. Blister immunostained (without sectioning) for ENFS with rabbit anti-PGP 9.5 (green) and Langerhans cells with mouse anti-CD1a (red).

C. Thigh biopsy specimen (17 year-old girl); uniformly distributed ENFs with lower density than seen in Figure 1A. Staining as in Figure 1A. D. Same thigh biopsy specimen as in Figure 1C, displayed areas of epidermis with clustered ENFs. Staining as in Figure 1C.

E. Occasionally, ENFs were oriented in parallel, at acute angles to the dermal-epidermal junction; leg biopsy specimen (14 year-old girl). Stained as in Figure 1A, but without lectin staining, therefore the blue color is absent.

F. This acutely-angled fiber orientation appeared as parallel arrays of ENFs in the blister specimen from the same subject as illustrated in Figure 1E. Staining as in Figure 1B.

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Figure 2. Nerve fiber morphology

A. Blister specimens facilitate better visualization of complex ENFs; the branches of this long multi-branched ENF (thigh, 12 year-old boy) traversed over 100µm from the point where it crossed the dermal-epidermal junction to enter the epidermis, to the free endings in the superficial layer of the epidermis. Immuno-localization of ENFs with rabbit anti-PGP 9.5 (green). The arrow indicates where the nerve fiber crossed the dermal-epidermal junction.

B. Punch biopsy cross section (thigh, 17 year-old girl); highly branched ENFs clustered around their epidermal entry point. Because fibers may be transected during the sectioning procedure, long fibers may be truncated with fragmented endings appearing in adjacent section. Staining as in Figure 1A.

C. Blister specimen (thigh, same subject as in 2B). ENFs sometimes “crawled” along the superficial aspect of the dermal/epidermal junction giving off branches that ascended into the squamous epidermis. This was viewed in blister specimens as in this sample; three branches (vertical fibers) arose from the parent fiber situated at the base of the blister (horizontal fiber at the bottom of the image as pointed by the dashed line). The arrow indicates where the nerve fiber crossed the dermal-epidermal junction. Staining as for Figure 2A.

Figure 3. Epidermal nerve fiber density and length in children

A. ENF density in thigh and leg blisters and biopsies from children. Box plot depicts group values for each site and type of specimen. Box surrounds the first and third quartiles with medians (green triangle), maximum (black x) and minimum (red square) values shown for each group.

B. Correlation of ENF density for blister and biopsy specimens of individual subjects. Regression lines are shown for leg (blue) $r = 0.91$, thigh (red) $r = 0.65$ and thigh with single outlier omitted (black) $r = 0.77$.

C. Correlation of age and ENF density. ENF density was inversely correlated with age to 12 years for both leg and thigh biopsy specimens.

D. Correlation of age and ENF length in biopsy specimens. No association between age and ENF length was found in either leg or thigh biopsies.

Table 1

Antibodies

Primary Antibody	Source	Dilution	Secondary Fluorophore	Reactivity
Rabbit anti PGP 9.5	AbD Serotec, Raleigh, NC	1/1000	Cy3	Pan-neuronal marker
Mouse anti type IV collagen	Chemicon International, Temecula, CA	1/6000	Cy2	Basement membrane Dermal/epidermal junction
Ulex europaeus agglutinin type I	Vector Laboratories, Burlingame, CA	1/1000	Cy5	Vasculature and superficial epidermis
Mouse anti CD1a	Biodesign International, TN	1/2000	Cy2	Langerhans cells