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Numerical and comparative three-dimensional structural analysis of peripheral nerve fibers in epidermis of atopic dermatitis patients

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What's already known about this topic?

- Several reports indicate that the number of nerve fibers is increased in epidermis of atopic dermatitis (AD) patients, and pruritis in AD is suggested to arise from interaction between peripheral nerve fibers and epidermal keratinocytes. However, the three-dimensional distributions of nerve fibers in epidermis of AD skin and normal skin obtained from equivalent locations have not been compared in detail.

What does this study add?

- Precise three-dimensional imaging of nerve fibers in epidermis was carried out by two-photon microscopy with excitation in the near-infrared region, where tissue is most transparent.

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- Quantitative numerical analysis of images revealed that the density of nerve fibers in epidermis of AD patients is not increased, compared with that of skin samples from equivalent locations of normal subjects.

Abstract

Background

Previous studies indicate that the density of nerve fibers is increased in skin of atopic dermatitis (AD) patients, and the cause of pruritus in AD is proposed to be interaction between peripheral nerve fibers and epidermal keratinocytes.

Objective

To quantify and compare the three-dimensional structure of nerve fibers in back skin from healthy subjects and AD patients using two-photon microscopy.

Methods

We employed two-photon microscopy with excitation in the near-infrared region to construct sequential 10 μm cross-sectional images of nerve fibers in epidermis within 100- μm -thick skin specimens. We numerically quantified nerve fiber length from the basal membrane and nerve fiber density in epidermis. We also observed skin sequentially from the surface and constructed three-dimensional images of nerve fibers in epidermis.

Results

Nerve fibers extending from the basal membrane of epidermis were longer in AD than in healthy subjects, but the density of nerve fibers in epidermis was significantly lower in AD. Although thick nerve fibers were observed in the dermis of AD, the numbers of fibers that passed through the basal membrane were lower in AD than in healthy subjects.

Conclusion

Contrary to some previous reports, our quantitative numerical analysis indicates that the density of nerve fibers in epidermis of AD patients is not increased compared with that of normal subjects, when the skin samples from the same regions are compared.

Introduction

There is some evidence that the cause of pruritus in patients with atopic dermatitis (AD) is increased nerve fiber outgrowth in the epidermis. For example, itching requires the presence of epidermis, and although nerve fibers penetrate into the uppermost layer of the epidermis in healthy subjects^{1,2}, the number of nerve fibers in skin of AD patients is reported to be higher than in healthy controls^{3,4}. Also, levels of nerve growth factor (NGF) and its receptors in epidermis of AD patients are increased⁵, while

production of semaphorin 3A, which retracts NGF-sensitive neurons, is decreased⁴. Keratinocytes from AD patients express an increased level of NGF mRNA, and enhance outgrowth of calcitonin gene-related peptide immunoreactive neurites in a co-culture system of keratinocytes and neurons⁶. However, nerve fibers in epidermis of healthy subjects and AD patients have not been quantitatively compared.

Two-photon microscopy is superior to conventional microscopy, including confocal microscopy, for precise imaging of thick tissue sections because excitation in the near-infrared region minimizes tissue absorption and background autofluorescence. Here, we used two-photon microscopy to construct cross-sectional and three-dimensional images of nerve fibers in epidermis of healthy subjects and AD patients, and quantified their structural features in order to examine the validity of the idea that outgrowth of nerve fibers occurs in epidermis of AD patients.

Results

Representative immunofluorescence staining images (Fig. 1a, b, and c) and binary images (Fig. 1d, e, and f) of sequential 10 μ m cross-sectional images show that the epidermis is thickest in lichenified lesion (L), followed in order by unlichenified lesion (UL) and healthy skin (H) (Fig. 1a, b, and c). Although the number of nerve fibers in the epidermis was not significantly different among H, UL, and L, the length of nerve fibers was the longest in L, followed in order by UL and H (Fig. 1f, e, and d).

These results are quantified in figure 1g-n. For numerical analysis, we chose samples obtained only from back skin of males to minimize possible influences of different skin regions and sex on nerve fiber distribution. Details of the numerical processing are given in figure 2S, legend. The epidermis was thicker in group L than in the other groups (figure 1g). The asperity of the basal membrane was higher in group L than in the other groups (figure 1h). The length of nerve fibers extending from the basal membrane in group L was significantly greater than that in group H or UL (figure 1i). The percentages of nerve fibers within 0-15 degrees of vertical with respect to the skin surface are shown in figure 1j; the results indicate that nerve fibers in epidermis of groups UL and L were more vertically oriented towards the skin surface than those of group H. Length of nerve fibers in the epidermis per length of stratum corneum is shown in figure 1k. The density of nerve fibers was higher in group H than in the other groups (figure 1l). The number of nerve fibers per 1 mm basal membrane was also higher in group H than in the other groups (figure 1m). Further, the density of nerve fibers in the region to a depth of 50 μ m from the stratum corneum was also higher in group H than in the other groups (figure 1n).

Healthy subjects (H, figure 2a-d) showed many nerve fibers in the epidermis. In the unlichenified skin of AD patients, the epidermis was thickened, and thick nerve fibers were observed in the dermis, although the number of nerve fibers in the epidermis seemed to be lower than in group H (UL; figure 2e-h). These changes were more marked in lichenified skin (L; figure 2l). In particular, nerve fibers did not appear to penetrate far into the epidermis in group L (figure 4l). Next, nerve fibers within 50 μ m of the skin surface were examined. Figure 2m shows representative processed images in which nerve fibers are shown in black. Quantitative results are shown in figure 2n. The numbers of black pixels were lower in groups UL and L than in group H, in agreement with the findings in figure 1n.

Discussion

It has been suggested that an increase of nerve fibers in AD patients might be the cause of pruritus. However, when we analyzed the three-dimensional distribution by means of two-photon microscopy, we found that the density of nerve fibers was higher in healthy subjects than in AD (Figure 11,m,n). Although individual nerve fibers in L and UL were longer (stretched) than in H, the total amount of nerve fibers was lower in L and UL than in H, especially in the uppermost layer of the epidermis. These results are in contrast to previous findings in antecubital fossa and abdomen of healthy subjects and AD patients^{3,4}. Moreover, although stretched nerve fibers were observed in disease sites, they did not reach the upper layer of the epidermis (figure 2h,l). In addition, previous reports have indicated that epidermal nerve fiber density was decreased in the skin of nummular eczema⁷ and innervation of epidermis and dermoepidermal junction in lichen amyloidosis was diminished⁸. Overall, these findings indicate that pruritus is not necessarily associated with increased epidermal nerve fiber density.

It has been demonstrated that Langerhans cells elongate their dendrites within the granular layer just beneath the stratum corneum⁹. This is in striking contrast to our observation that epidermal nerves in AD lesional skin are not elongated in the region within a depth of 50 μ m from the stratum corneum. This difference may suggest that, unlike Langerhans cells, nerve endings are mainly stimulated by surrounding keratinocytes, but not by direct capture of external environmental stimuli. This is consistent with a recent report that release of calcium-dependent cytokine thymic stromal lymphoprotein (TSLP) from keratinocytes induced itching and inflammatory responses¹⁰. Keratinocytes functionally express many receptors, including modulators of intracellular calcium level¹¹, and examination of the interaction between keratinocyte receptors and neurons may throw new light on the mechanisms of itching in AD.

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Figure legends

Figure 1

Within 100- μ m-thick skin specimens, cross-sectional images (10- μ m thickness) in regions where the basal membrane appeared as a single distinct line (figure S1) were obtained by two-photon microscopy. Panels a,b,c: Multi-stained skin of a: H (healthy skin), b, UL (unlichenified lesion), and c: L (lesion). Nerve fibers appear in red (immuno-stained with anti-PGP9.5 antibody). Immuno-staining with anti-collagen type IV antibody appears in green. Yellow lines; skin surface. Blue arrowheads: coincidence of nerve fibers and blood vessels (yellow). White bars = 100 μ m. Panels d,e,f: Processed binary images showing epidermis in gray with nerve fibers in white. The region within 50 μ m of the skin surface is shown in light gray, with nerve fibers in white (d: H, e: UL, f: L). Image processing is described in detail in supplementary materials and methods.

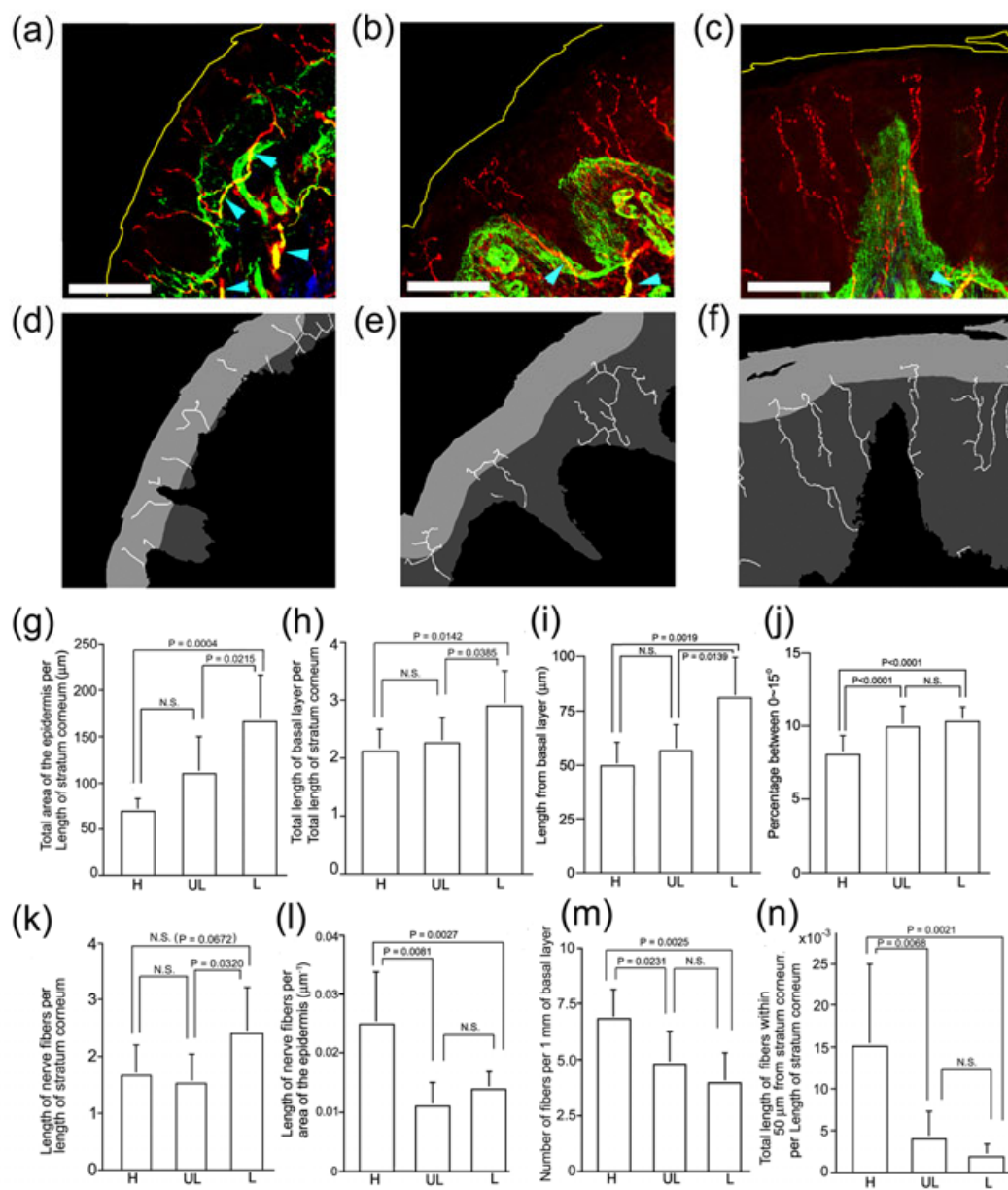
Results of quantification of 10 μ m cross-sectional images (6 healthy subjects and 6 AD patients). Thickness of the epidermis (panel g). Asperity of basal membrane (panel h). Length of nerve fibers extending from the basal membrane (panel i). Percentage of nerve fibers within 0-15 degrees of vertical with respect to the surface of the skin (panel j). Length of nerve fibers in the epidermis per length of

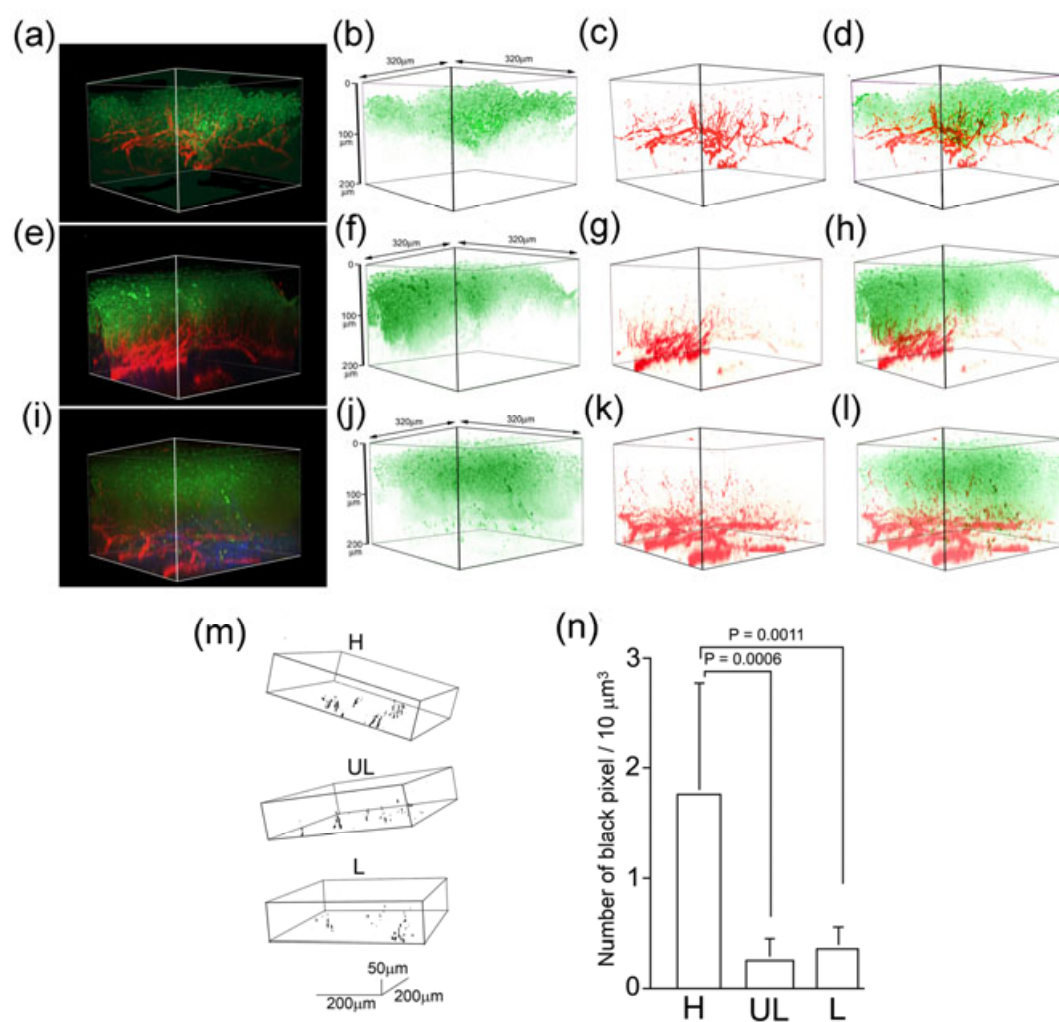
stratum corneum (panel k). Density of nerve fibers (panel l). Number of nerve fibers per 1 mm of basal membrane (panel m). Density of nerve fibers in the region within a depth of 50 μ m from the stratum corneum (total length of fibers / length of stratum corneum) (panel n). The number of samples in each group was 6.

Figure 2

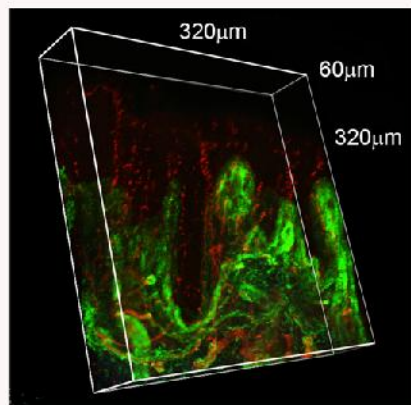
Three-dimensional representations of representative nerve fibers. Panels a-d: group H. Panels e-h: group UL. Panels i-l: group L. Panel a, e, and i are the original multi-stained image. Nerve fibers appear in red (anti-PGP9.5 antibody) and epidermal keratinocytes appear in green (Hoechst33258). Panels b, f, and j pick out epidermal keratinocytes. Panels c, g, and k pick out nerve fibers. Panel d, h, i: merged images of b and c, f and g, and j and k, respectively.

Quantitative evaluation of length of nerve fibers within 50 μ m of the surface of the skin in the three groups. Panel m: representative images of upper layer of the skin (within 50 μ m from the skin surface). Panel n: quantitative evaluation of total nerve fibers (including spots). The vertical scale shows the mean value (6 subjects each for H, L and UL) and error bars indicated standard deviation. The total count of black pixels (nerve fibers and spots) was significantly lower in groups UL and L than in group H, in accordance with the results in figure 3f

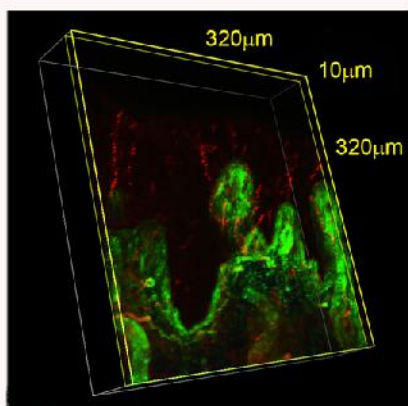




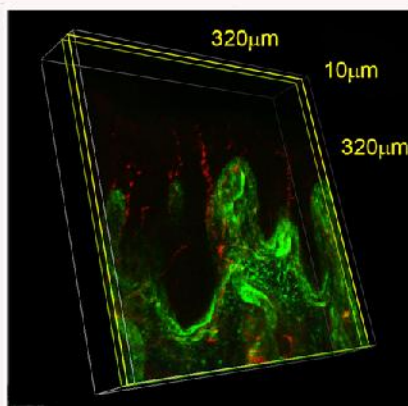
(a)



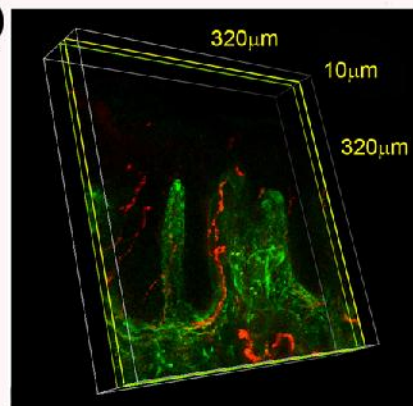
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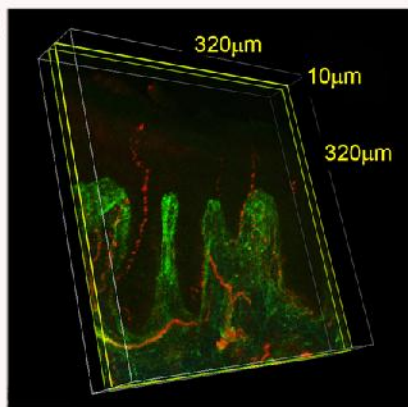
(c)



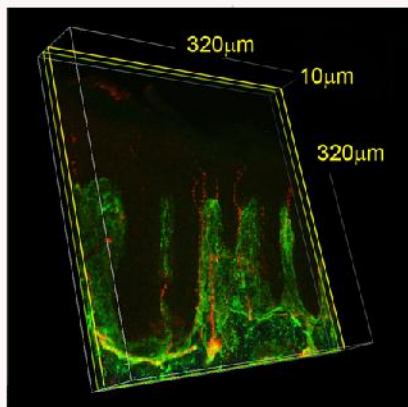
(d)



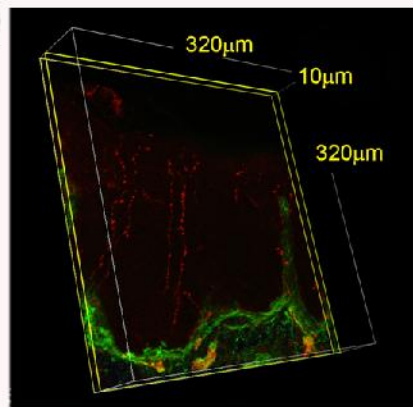
(e)

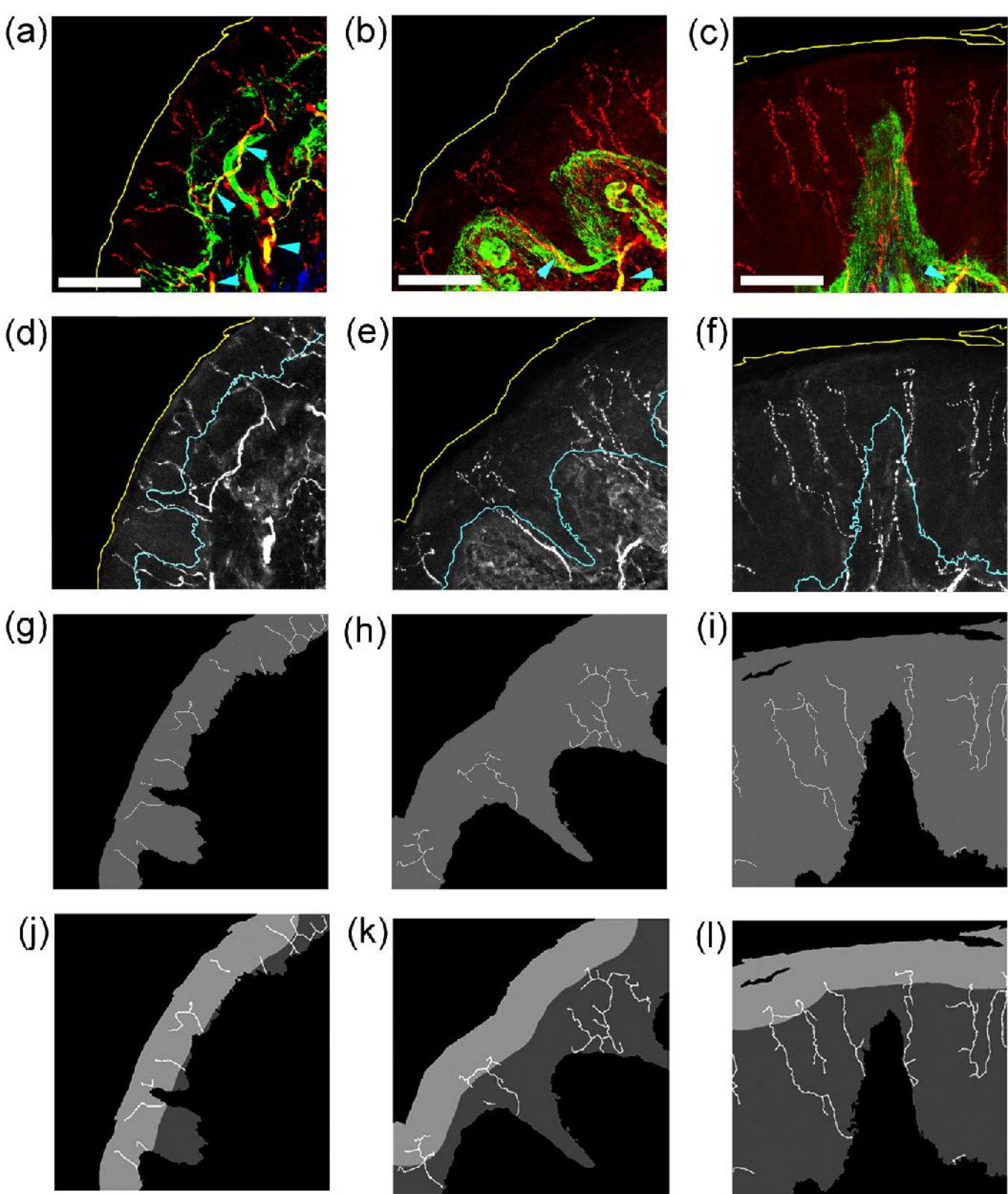


(f)



(g)





Materials and Methods

Patients and biopsies

Subjects were Japanese healthy individuals (6 males) and 9 AD patients (6 males and 3 females) with lichenification (20-37 years; mean age 24 years), selected on the basis of clinical history and diagnostic observation by a dermatologist at Tohoku University Hospital. Under local anesthesia with lidocaine, tape stripping was performed 10 to 30 times to accelerate antibody penetration and reduce autofluorescence due to stratum corneum, and 3-mm punch biopsies of back skin were taken (from both lichenified lesions and non-lichenified lesions in AD patients). Specimens were immediately immersed in 4% paraformaldehyde overnight at 4 °C. The study protocol was fully discussed with each patient and informed consent was obtained. The protocol was approved by the ethics committees of Shiseido Research Center and Tohoku University Hospital, and was in accordance with the National Institute of Health guideline and Declaration of Helsinki principles.

Sample preparation

Following 4% paraformaldehyde treatment, samples were divided into two parts for observation of 100 μ m sections and for whole-mount observation and placed in 30% sucrose until they sank to the bottom. Then, half of each sample was embedded in optimal cutting temperature compound (OCT, TissueTek; Sakura Finetek, Torrance, CA, USA) and cryosectioned at 100 μ m for immunostaining and at 8 μ m for routine HE histochemistry. The 100- μ m sections were treated with 10% H₂O₂/0.3% Triton X in PBS for 10 min at room temperature (20-25 °C). The remaining skin samples were cut into quarters and treated with 10% H₂O₂/0.3% Triton X in PBS for 12 hr at 4 °C for whole-mount immunostaining. Samples were immersed in blocking solution, i.e. 3% bovine albumin PBS solution containing 10% normal goat serum and 0.4% Triton X for 2 hr at room temperature before being treated with first antibodies.

Immuno-staining of 100- μ m skin sections and numerical analysis of nerve fibers in epidermis.

Rabbit polyclonal anti-PGP9.5 antibody (Enzo, NY, USA) was diluted 1:2,000 and mouse monoclonal anti-collagen type IV antibody (abcam, Cambridge, UK) was diluted 1:500 with blocking solution. Sections were incubated with the appropriate solution for 2 to 3 days at 4 °C, washed with PBS including 0.25% Tween (PBST) for 30 min 3 times, and rinsed with PBS. Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Life Technologies) were diluted 1:1000. Skin sections were incubated with the appropriate solution for 2 hr at room temperature, washed with PBST for 1 hr 3 times, and rinsed with PBS. Skin sections were mounted on glass slides with Fluoromount Plus, and observed with a two-photon laser-scanning microscope (A1R MP, Nikon, Tokyo, Japan) equipped with a Ti-sapphire laser, Mai Tai DeepSee (Spectra-Physics, California) using a x40 water immersion objective lens (N.A. = 1.15). Excitation: 800 nm. Emission: 500-550 or 601-657 nm band pass filter.

Generation and selection of 10- μ m cross-sectional images from 100- μ m-thick skin specimens

For numerical analysis, we constructed sequential 10- μ m cross-sectional images of nerve fibers in epidermis from the observations of 100- μ m-thick skin specimens. Since the presence of overlapping lines of basal membrane in the transverse view of 100- μ m-thick skin specimens interfered with the numerical evaluations of nerve fiber length, structure and orientation to the basal membrane (Supplementary Figure S1a), we numerically analyzed epidermal nerve fibers in 10- μ m cross-sectional images in which the basal membrane appeared as a single line (Supplementary Figure S1b-g). The selection of 10- μ m cross-sectional images from 100- μ m-thick skin sections was carried out in an

observer-blinded fashion, and a key consideration was the clarity of basal membrane visualization by collagen type IV immunostaining. This approach was chosen in order to avoid collection of data from peripheral areas of specimens, where tissue damage might have occurred during the process of sample preparation. We obtained six 10- μ m cross-sectional images for each sample, and the average of the data is presented for each individual, as described in the main manuscript.

Process of numerical analysis

Samples from 6 healthy males and 6 male AD patients were analyzed. We constructed sequential 10- μ m cross-sectional images of nerve fibers in epidermis from the data obtained with 100- μ m-thick skin specimens as described above (Figure S1). We obtained three images from each 10 μ m cross-sectional image, each of which was made by superposition of 30 images of samples stained with anti-PGP9.5 antibody (image A), with anti-collagen type IV antibody (image B), and with collagen SHG in dermis (image C). That is, images A, B and C are the projections of the three-dimensional structures in a space whose depth is 10 μ m. First, we extracted the epidermal region by setting a suitable threshold in the images B and C. Next, PGP9.5-positive nerve-fiber-like structures were extracted from image A in the region of the epidermis defined above. In this process, the extracted nerve-fiber-like structures were not continuous, and the connections between them had to be estimated. To do this, we calculated the distances from one structure to the other structures, and we connected the two structures with the smallest distance under the constraint conditions that two structures separated by more than 16 μ m cannot be connected, and that one structure can be connected to at most three other structures. Then, we measured the area of epidermis, the lengths of stratum corneum and basal membrane, the number and length of neurons and the angle of neurons from the direction of the boundary between epidermis and stratum corneum. In these processes, we performed noise removal and smoothing in order to eliminate errors arising from threshold setting and discretization by using a plugin running in ImageJ (NIH). We repeated these processes for at least six 10- μ m cross-sectional images of each sample, and the average of these data is presented for each individual.

Supplementary figure S2 a,b,c (typical images from groups H, UL and L, respectively) shows 10- μ m cross-sectional images of multi-stained skin, in which nerve fibers (immuno-stained with anti-PGP9.5 antibody) appear in red, and anti-collagen type IV antibody staining visualizing basal membrane appears in green. Yellow lines indicate the surface of the skin. Blue arrowheads show locations where nerve fibers and blood vessels (yellow) coincide. These images were successively processed as shown in figure 2d-f (nerve fibers in white, skin surface yellow, dermo-epidermal junction of the epidermis in blue) and figure 2g-i (epidermal area in gray, nerve fibers in white), respectively. Finally, as shown in figure 2j-l (H, UL and L, respectively), we obtained binary images in which the region from the skin surface to a depth of 50 μ m is highlighted in light gray and nerve fibers in this region are shown in white.

Immunostaining of whole-mount skin samples and quantification of peripheral nerve fiber density in upper epidermis

Rabbit polyclonal anti-PGP9.5 antibody (Novus Biologicals, LLC, Co., USA) was diluted 1:10000 with blocking solution. Samples were incubated with this solution for 1 week at 4 °C, washed with PBST for 1 to 2 hr 3 times, and rinsed with PBS. Fluorescent secondary antibody Alexa Fluor 594 goat anti-rabbit (Life Technologies Japan (Tokyo, Japan) was diluted 1:1000 and Hoechst33258 was diluted 1:2,000. Samples were incubated with the appropriate solution overnight at 4 °C, then washed with PBST for 1 hr 3 times, and rinsed with PBS. The samples were placed on glass slides and surrounded with silicone, then mounted with Fluoromount Plus (Diagnostic Biosystems, Pleasanton, CA, USA). They were observed with a two-photon laser scanning microscope and a x40 water immersion objective lens

(excitation: 800 nm; emission: 500-550 or 601-657 nm band-pass filter). Cross-sectional and 3D images were constructed from A1R MP confocal microscope images using NIS-Elements software (Nikon, Tokyo, Japan).

Upper epidermis (50 μm depth, 200x200 μm area) was extracted from each image of a whole-mount preparation with NIS Elements. Samples from 6 healthy males and 6 male AD patients with lichenification were used for numerical analysis. Images were processed to binary using Adobe Photoshop, and black pixels (representing nerve fibers) was counted using Image J. Fragments were not counted. The vertical axis in figures is the pixel number in a cube (10x10x10 μm). For 100- μm sections, the epidermal region was defined from the combined images of collagen type IV and collagen SHG in dermis. Next, PGP9.5-positive nerve fiber structures were extracted from the PGP9.5 immuno-staining image in the epidermal region. The length of neurons and the angle of neurons from the vertical to the epidermis/stratum corneum boundary were calculated using ImageJ (NIH).

Statistics

The statistical significance of differences was determined by ANOVA with Fisher's protected least significant difference. P-values less than 0.05 were considered significant.

Supplementary Figure Legend

Supplementary Figure S1

Typical cross sectional images of 60 μm (a) and 10 μm (b,c,d,e,f,g) thickness from a 100- μm -thick skin specimen are shown (area is 320 x 320 μm in each case). The orientations of basal membrane (green) in the cross-sectional images of the thick slice (60 μm thickness) are too complex to be analyzed by numerical methods. On the other hand, the cross-sectional images of the thin slices (10 μm thickness) show a simpler, single-line basal membrane, which enabled us to numerically analyze the orientation of basal membrane (green) and neuron fibers (red).

Supplementary Figure S2

Cross-sectional images (10- μm thickness) obtained by two-photon microscopy. Panels a,b,c: Multi-stained skin of a: H, b: UL, and c: L. Nerve fibers appear in red. Immuno-staining with anti-collagen type IV antibody appears in green. Yellow lines; skin surface. Blue arrowheads: coincidence of nerve fibers and blood vessels (yellow). White bars = 100 μm . Panels d-f: Processed images of nerve fibers appear in white (d: H, e: UL, f: L). Blue lines: the basal membrane of the epidermis. Panels g-i: Processed binary images showing epidermis in gray with nerve fibers in white (g: H, h: UL, i: L). Panels j-l The region within 50 μm of the skin surface is shown in light gray, with nerve fibers in white (j: H, k: UL, l: L).