

Original**Morphological Alterations of the Eccrine Sweat Apparatus in Amputated Feet from Diabetes Mellitus Patients**

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Abstract : Several physiological studies have demonstrated decreased or absent thermoregulatory sweating in the distal legs and feet of diabetes patients. Such hypohidrosis in diabetes patients is believed to be a clinical symptom of autonomic neuropathy. Thus, the present study sought to clarify the pathogenesis of structural alterations of the eccrine sweat apparatus in diabetes patients. For this study, we enrolled 17 patients with diabetic ulcers/gangrene who underwent amputation of the foot. Specimens were obtained 30 mm from the ulcer/gangrene after amputation using a 6-mm trepan, with 12 normal human skin samples obtained from areas adjacent to pigmented nevi or benign skin tumors on the legs or feet to serve as controls. Numbers and morphological abnormalities of eccrine sweat glands and ducts were assessed by light microscopy. The pathogenesis of morphological alterations was examined by electron microscopy and immunoelectron microscopy of type IV collagen. Rates of disappearance of the lumen, shrunken morphology, and irregular outlines of eccrine sweat glands and ducts were significantly higher or more abundant in diabetes patients than in controls ($P=0.0002 \sim 0.0001$). Ultra-structurally, we observed prominent thickening of the basement membranes in eccrine sweat glands, admixed cell debris, and narrowing of the luminal space. The thickened basement membranes resulted in the shrunken morphology and irregular outlines in eccrine sweat glands and ducts. Immunoelectron microscopy showed immunogold labeling for type IV collagen throughout the thickened basement membrane zone. These morphological alterations of the eccrine sweat apparatus in amputated feet from diabetes patients could be caused by diabetic and/or uremic neuropathy, and at least in part by angiopathy.

Key words : basement membrane, type IV collagen, eccrine sweat apparatus, microangiopathy, neuropathy, ischemia

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Introduction

Decreased or absent thermoregulatory sweating, termed hypohidrosis, has been reported in the distal legs and feet of diabetes patients^{1, 2)}, and is considered a clinical symptom of autonomic neuropathy^{3, 4)}. Diabetes patients also tend to show reduced hydration of the stratum corneum without any impairment of barrier function⁵⁾. In a physiological study, stratum corneum hydration increased in the frontal and zygomatic skin at the beginning of sweating or during excessive sweating, compared to the resting state⁶⁾. Hence, hypohidrosis of the lower extremities could contribute to xerosis with or without pruritus⁷⁾, followed by the formation of fissures, bacterial infection, and exacerbation of diabetic foot ulceration/gangrene. However, pathological approaches to elucidating the mechanism of hypohidrosis in diabetes patients have been limited to sympathetic nerve fibres^{4, 8)}. Therefore, this study aimed to clarify the pathogenesis of hypohidrosis in diabetes patients, focusing on structural alterations to the eccrine sweat gland.

Methods

Patients and specimens

Seventeen patients with diabetic ulcers/gangrene who underwent amputation of the foot participated in this study. All patients had diabetic and/or uremic neuropathy. Data for age, gender, HbA1c, skin perfusion pressure (SPP) or ankle brachial pressure index (ABI), and hemodialysis (HD) are shown in Table 1. The mean participant age was 70.4 ± 2.1 years, and all of patients receiving HD are resulting from diabetic nephropathy. SPP was measured on the affected foot alone, with a value of 50 or less (and/or ABI of 0.9 or less) considered to indicate peripheral arterial disease (PAD). Specimens were obtained 30 mm from the diabetic ulcer/gangrene within 15 minutes after amputation using a 6-mm punch biopsy device. Normal human skin adjacent to pigmented nevi or benign skin tumors on the legs or feet was obtained from 12 non-diabetic patients, who served as controls. The mean age of the controls was 36.9 ± 4.3 years. The Ethics Committee of our institution approved the study protocol.

Histology and quantitative analysis

Skin specimens fixed in 10 % formalin were routinely processed and embedded in paraffin. Numbers and morphological abnormalities of eccrine sweat glands and ducts such as disappearance of the lumen, shrunken morphology, and irregular outlines were quantified by light microscopy of hematoxylin–eosin-stained slides. The percentage of anomalous glands/ducts among all glands/ducts was calculated for each unit of the eccrine sweat apparatus, with 3 ~ 16 units per patient evaluated in total. Patient data are expressed as mean \pm standard deviation (SD). Student's t-test was used to evaluate differences between groups with equal SD values, while the Mann-Whitney U test was applied to groups with unequal SD values (Prism[®] 5, GraphPad Software Inc., La Jolla, CA, USA).

Table 1. Clinical data of the 17 diabetes patients

SPP, skin perfusion pressure ; ABI, ankle brachial pressure index, PAD, peripheral arterial disease ; HD : hemodialysis.

No of Case	Age/Gender	HbA1c	SPP	ABI	PAD	dialysis
1	70 F	7.2	15		+	HD
2	63 M	11.2	ND	ND	+	
3	66 M	5.5	50		+	HD
4	59 F	6.4	25	1.25/0.22	+	
5	70 M	7	30		+	HD
6	80 F	6.5	30		+	HD
7	79 M	11.5		0.75/0.62	+	
8	75 M	6.5		1.06/1.00	-	
9	82 M	7.9		1.05/1.10	-	
10	65 M	6.1		0.50/1.00	+	HD
11	52 F	12.0		1.17/1.21	-	
12	74 F	5.3	30	0.99/0.94	+	HD
13	75 M	5.7	15		+	HD
14	75 M	9.0	25	0.72/0.63	+	
15	71 M	8.5	25	0.54/0.67	+	
16	59 F	7.2	30	0.89/0.77	+	HD
17	82 F	ND	30		+	

Conventional ultrastructure

For electron microscopic observation, the specimens were immediately fixed in 3% glutaraldehyde at 4°C followed by osmium tetroxide, dehydrated through a graded series of ethanol solutions, and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Hitachi H-7000 electron microscope.

Post-embedding immunoelectron microscopy

Skin specimens were immediately fixed in 4% paraformaldehyde overnight, dehydrated through a graded series of dimethylformamide (DMFA) solutions, and then embedded in LR White (London Resin, Berkshire, UK), which was polymerized at a wavelength of 366 nm with a DSK TUV-200 ultraviolet polymerizer for 48 h (Dosaka EM, Osaka, Japan). Ultrathin sections were cut with an ultramicrotome (LKB Instruments, Melbourne, Australia) and then mounted on nickel grids. After incubation in normal goat serum to prevent non-specific background labeling, the grids were placed on droplets of a mouse anti-type IV collagen monoclonal antibody (Dako Japan, Tokyo), diluted 1:1000 in phosphate-buffered saline (PBS), and then incubated at 4°C for 24 h. Normal mouse IgG was used instead of anti-type IV collagen antibody as a control. The specimens were washed twice in PBS and then incubated with a 15-nm gold-conjugated goat anti-mouse antibody (BB International, Cardiff, UK). After immunostaining, grids were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 min, washed in distilled water, and then dried. The grids were stained with uranyl acetate and examined under a Hita-

chi H-7600 electron microscope (Hitachi, Tokyo, Japan).

Results

Histology and quantitative analysis

In the majority of eccrine sweat glands and ducts from the diabetes patients, light microscopy showed the disappearance of luminal spaces, shrunken morphology, and irregular outlines. More specifically, the tissues showed atrophy and degeneration of the epithelium with hyalinization, an absence of clear cells, and stromal edema. In some cases, glands and ducts in the secretory portion were difficult to distinguish by light microscopy (Fig. 1).

There were no significant differences in the total numbers of glands and ducts per eccrine sweat apparatus between diabetes patients and controls, with the morphometric analysis results shown in Fig. 2. Disappearance of the lumen in glands and ducts was significantly more frequent in diabetes patients than in controls, as was the incidence of shrunken morphology in glands and ducts ($P < 0.001$). Similarly, irregular outlines in glands and ducts ($P < 0.001$) were significantly more frequent in diabetes patients than in controls.

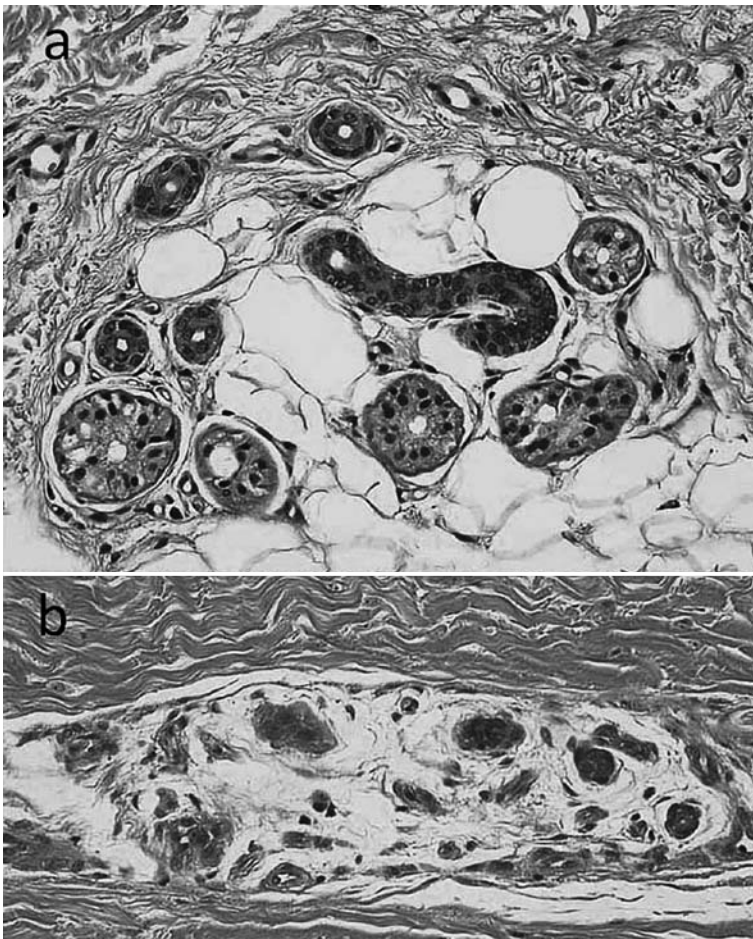


Fig. 1. Light microscopy of the eccrine sweat apparatus

a. Non-diabetic control. b. Diabetes patient. Eccrine sweat glands and ducts in the diabetes patient showed disappearance of luminal spaces, shrunken morphology, and irregular outlines.

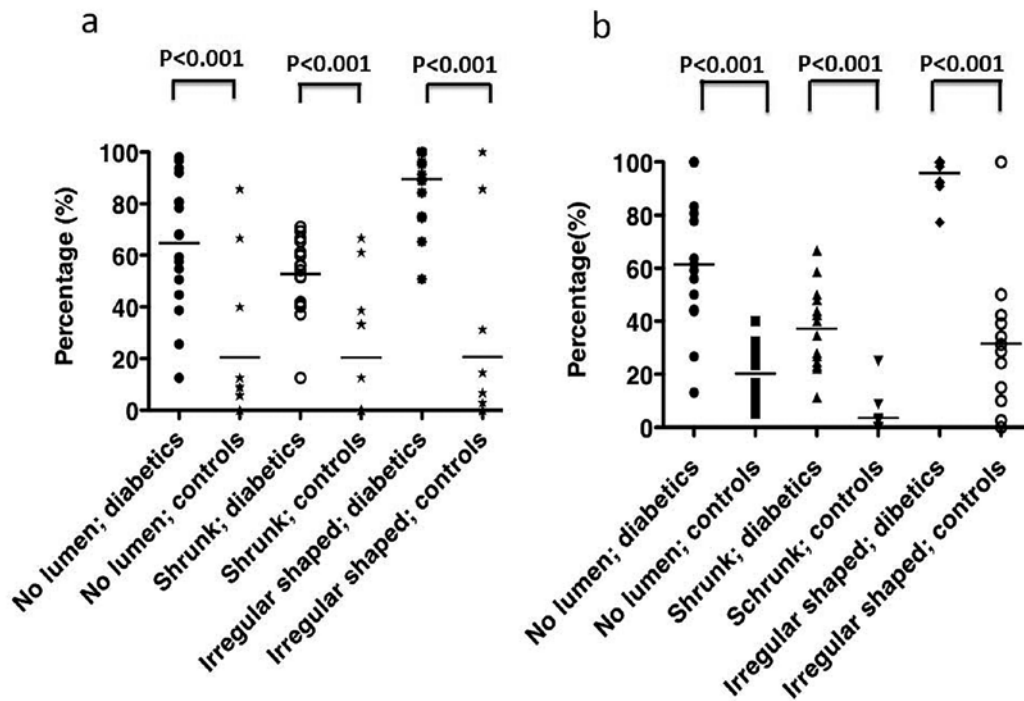


Fig. 2. Quantitative analysis of structural anomalies in the eccrine sweat apparatus. a. Secretory portion. b. Ductal portion. Disappearance of the lumen, incidence of shrunken morphology, and irregular outlines in both glands and ducts were significantly more frequent in diabetes patients than in controls.

Conventional ultrastructure

The basement membrane zone of the eccrine sweat gland in diabetic patients was markedly thickened, resulting in irregular outlines. This zone consisted of homogeneous hyaline-like matter with some multilayered fibrous structures at the periphery (Fig. 3). In addition, most of the luminal spaces in the secretory portion were narrowed, and some myoepithelial and epithelial cells had undergone cytolysis, leaving cell debris throughout the thickened basement membrane. Dark secretory cells containing large mucoid granules were aggregated in the center of the zone, and the central lumen was obscured. No epithelial cells undergoing necrosis or apoptosis were found, and neither nerve fibers nor Schwann cells were identified around the secretory glands (Fig. 3).

Duct components in the secretory portion were also surrounded by a homogeneous, thickened basement membrane zone, with admixed cell debris and vacuoles. Cytolysis of the epithelial cells at the basal side resulted in irregular outlines (Fig. 4).

The basement membranes of microvessels consisted of multilayered structures with less homogeneous zones and admixed cell debris, while those of eccrine sweat glands and ducts showed a homogeneous ultrastructure (Fig. 5). Necrotic changes, such as swollen mitochondria and sparse chromatin, were not appreciable in the eccrine sweat apparatus of diabetic patients.

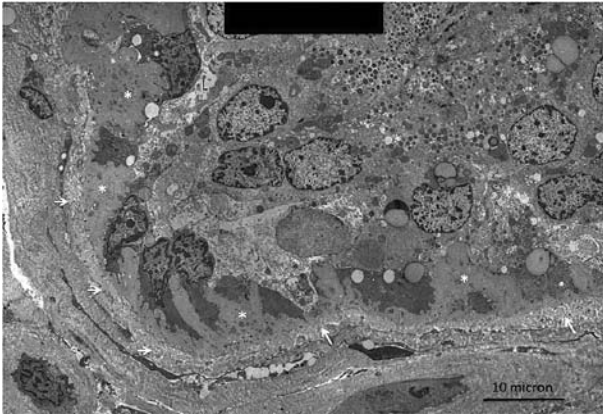


Fig. 3. Electron micrograph of the secretory portion of an eccrine sweat gland in a diabetes patient

The thickened basement membrane zone consisted of homogeneous hyaline-like material (asterisks) with multilayered fibrous structures at the zone peripheries (arrows). L: narrowed luminal space. Bar = 10 μ m.

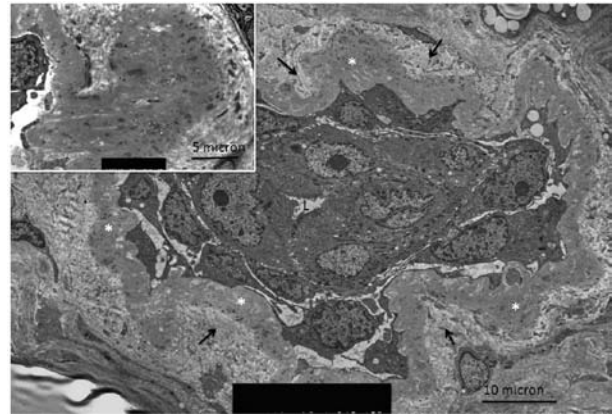


Fig. 4. Electron micrograph of the ductal portion of an eccrine sweat gland in a diabetes patient

The ductal portion was surrounded by a homogeneously thickened basement membrane zone (asterisks) with multilayered fibrous structures at the zone periphery (arrows). The homogeneously thickened basement membrane zone contained admixed cell debris and vacuoles (inset). Cytolysis of the epithelial cells at the basal side resulted in the irregular outlines.

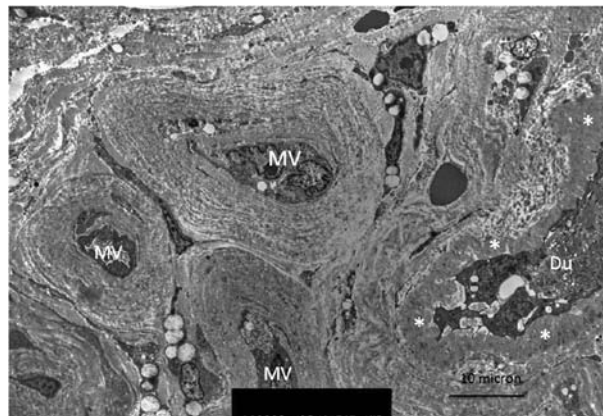


Fig. 5. Electron micrograph of the ductal portion of an eccrine sweat gland and microvessels in a diabetes patient

The basement membranes of microvessels (MV) consisted of multilayered structures with less homogeneous zones, while those of the eccrine sweat duct (Du) showed a moderately electron-dense homogeneous ultrastructure (asterisks). Bar = 10 μ m.

Post-embedding immunoelectron microscopy

In diabetes patients, immunogold labeling for type IV collagen was distributed diffusely in the thickened basement membrane zone of the secretory glands and ducts, and was especially prominent on the amorphous hyaline material. Type IV collagen was also detected in the multilayered

basal membranes of dermal microvessels. In control subjects, type IV collagen labeling was visible in a linear pattern along the basal lamina of the secretory gland (Fig. 6).

Discussion

Our ultrastructural study demonstrated for the first time that significantly thickened basement membrane zones expressing type IV collagen may reflect the shrunken morphology, disappearance of luminal spaces, and irregular outlines typically observed by light microscopy in the eccrine sweat apparatus of diabetic patients. Moreover, neural components were not found around the secretory glands. These morphological alterations could be caused by diabetic and/or uremic neuropathy, with a contribution from angiopathy.

Thickening of the basement membrane is also a key finding in diabetic microangiopathy of the skin⁹⁾. The basement membranes of tissue and blood vessel endothelia are composed of type IV collagen, laminin, heparin sulfate proteoglycans, and fibronectin¹⁰⁾. With respect to collagen, the secretory portion of the eccrine sweat gland is rich in $[\alpha 1 (IV)]_2 \alpha 2 (IV)$ and has less $[\alpha 5 (IV)]_2 \alpha 6 (IV)$, while $[\alpha 5 (IV)]_2 \alpha 6 (IV)$ is abundant in the ductal portion¹¹⁾. A dot blot hybridization analysis of diabetic skin demonstrated significantly reduced mRNA levels for pro $\alpha 1 (IV)$ collagen, γ -actin, and fibronectin, while those for $\alpha 1 (I)$ collagen and laminin were not changed¹²⁾. These data suggest that basement membrane thickening develops more as a consequence of reduced basement membrane degradation than elevated synthesis of basement

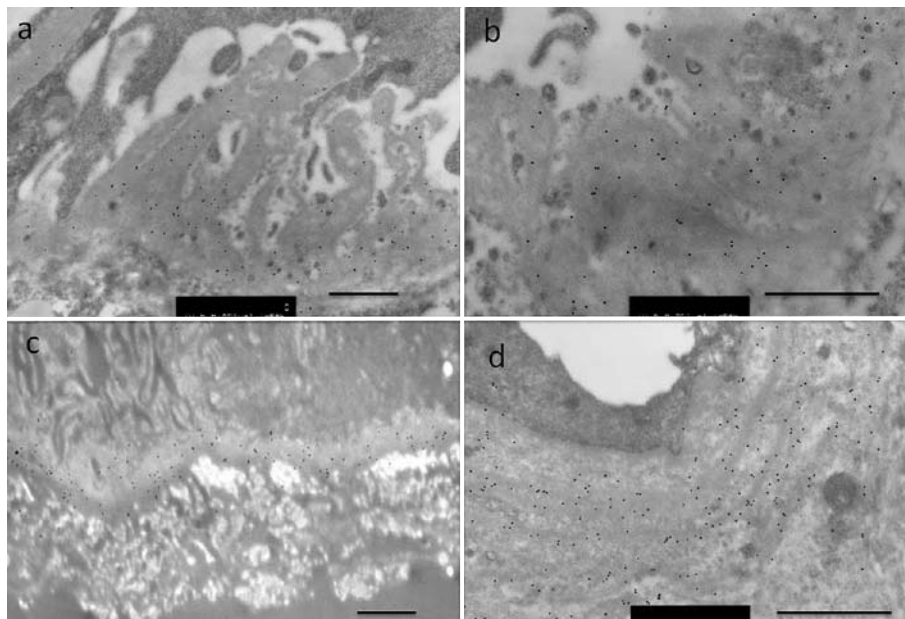


Fig. 6. Post-embedding immunoelectron microscopy of type IV collagen
 a, b. Immunogold-labeling of the homogeneously thickened basement membrane zone of a secretory gland in a diabetes patient. Bar = $1\mu\text{m}$
 c. Linearly arranged immunogold particles labeled the basal lamina of a secretory gland in a control subject. Bar = $1\mu\text{m}$.
 d. Immunogold particles specific for type IV collagen also labeled the multilayered basal membranes of dermal microvessels. Bar = $1\mu\text{m}$.

membrane components. A potential explanation for the reduced degradation is glycation of type IV collagen and other structural components^{13, 14)} because glycation accelerates the crosslinking of type IV collagen and makes it less susceptible to proteinase digestion¹⁴⁾.

Based on our ultrastructural observation, the thickened basement lamina of the eccrine sweat apparatus comprised electron-dense, homogeneous hyaline-like material with small areas of fibrous structures at the periphery. By contrast, the basement membrane of microvessels consisted of multilayered structures with less homogeneous zones. Although the ultrastructural properties were different, immunoelectron microscopy of type IV collagen labeled both the homogeneous zones in the eccrine sweat apparatus and the multilayered zones in microvessels. Such multiple basal lamina layers adjacent to microvessels are not a specific finding in diabetes mellitus, and are found in the lower extremities of normal subjects¹⁵⁾. The multilayered thickening of basal membranes is due to repeated episodes of endothelial cell death and cell replacement on the inside, with each basal lamina layer representing residual evidence of cell generation^{15, 16)}. Indeed, cell debris found in the multilayered basal lamina supports this idea, and since more basal lamina layers were observed in diabetes patients than in non-diabetic controls in this study, the cycle of cell death and replacement must be more rapid during diabetes¹⁷⁾. The vascular luminal space narrowed as this cycle was repeated, although the pathogenesis of narrowing or absence of luminal spaces and shrunken morphology in the eccrine sweat apparatus may be different from that in microvessels. Cytolysis of the basal epithelial and/or myoepithelial cells may occur, leaving cell debris and causing thickening of the basement membrane zone. However, no cell replacement was found in this study. Thus, the thickened basement membrane zone bands could tighten the epithelial cells, resulting in the narrowing or absence of lumen seen by light microscopy.

Some physiological studies have reported functional abnormalities of eccrine sweat glands in diabetic neuropathy^{18, 19)}. However, there have been no reports regarding morphological changes in eccrine sweat glands due to diabetic neuropathy. In our control patients, at least one nerve component (with components including nerve fibers and Schwann cells) was recognized around each secretory gland section, whereas no such components were observed in the tissues of the diabetes patients. A previous quantitative analysis of PGP 9.5-positive nerve fibers innervating sweat glands demonstrated a significantly reduced nerve fiber density in the distal leg in diabetes patients compared to controls²⁰⁾. Furthermore, sweat gland nerve fiber density in the distal leg of diabetes patients decreased significantly as the neuropathy impairment score in the lower limb worsened in concordance with symptoms of reduced sweat production²⁰⁾. These data suggest that the severely disturbed innervation due to diabetic neuropathy and morphological changes in the eccrine sweat apparatus may be linked. This study revealed no data regarding the significant role of angiopathy in the absence of nerve components around the eccrine sweat apparatus. The time lag between amputation and fixation of the tissue specimen in our study was within 15 minutes, and the observed basement membrane thickening could not occur in this time lag. Moreover, necrotic changes, such as swollen mitochondria and sparse chromatin, were not appreciable by ultrastructural examination in the current diabetic patients.

Electron microscopy in this study revealed the following findings about the eccrine sweat gland apparatus in diabetic patients only: i) thickening of the basement membrane that consisted of homogeneous hyaline-like material expressing type IV collagen; ii) cytolysis of the epithelial cells at the basal side admixed with cellular debris; and, iii) disappearance of neural components around the secretory glands. These first two findings could be contrasted with the shrunken morphology and irregular outlines noted on light microscopy.

Of the 17 diabetes patients we examined, 14 also had peripheral arterial disease, consistent with untreatable ischemia being the principal reason for amputation in diabetes²¹⁾. An older study noted reduced forearm perspiration during tourniquet use²²⁾, while more recently, significantly fewer sweat pores were found on the heel skin of diabetes patients with angiopathy compared to those without angiopathy²³⁾. The authors of the latter study presumed that the insufficient supply of oxygen and nutrients to terminal secretory cells in contact with the vascular plexus in angiopathy causes secretory unit atrophy and a lack of sweat production. However, 60 % of the diabetes patients with angiopathy in that study also had sensory neuropathy, and neuropathy, angiopathy, or both can contribute to the depletion of sweat pores. In this study, we observed no difference in morphological changes under light and electron microscopy between 14 patients with anomalous ABI/SPP and 3 patients with normal ABI/SPP, indirectly suggesting that neuropathy could be a major factor underlying the changes in eccrine apparatus compared to PAD.

Among the 17 diabetic patients described herein, 8 patients were also receiving hemodialysis because of diabetic nephropathy/uremia, and accelerated atherosclerosis in prolonged maintenance hemodialysis has been well documented. Both diabetes mellitus and uremia could be involved in neuropathy; however, we found no association between morphological alteration and the presence of uremia in our patient group. Hence, diabetic neuropathy could be a predominant etiology in these morphological alterations of the eccrine sweat apparatus.

In conclusion, the present study demonstrated that considerable thickening of the basement membrane zone in eccrine sweat glands and ducts, attributed predominantly to type IV collagen, could lead to the shrunken morphology, disappearance of luminal spaces, and irregular outlines typically observed by light microscopy. These morphological alterations to the eccrine sweat apparatus in the amputated feet of diabetes patients may be caused by diabetic and/or uremic neuropathy, with a partial pathogenic contribution from angiopathy.

Conflict of interest disclosure

The authors have declared no conflict of interest.

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