Virchows Arch (1998) 432:363–370 © Springer-Verlag 1998

ORIGINAL ARTICLE

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Cutaneous nerves in atopic dermatitis

A histological, immunohistochemical and electron microscopic study

Received: 21 April 1997 / Accepted: 27 October 1997

Abstract Although pruritus is the cardinal symptom of atopic dermatitis, its mechanism is not well understood. Free nerve endings in the skin are involved in pruritus as itching receptors. We studied the cutaneous nerve fibres in lichenified lesions of 16 patients with adult atopic dermatitis. On immunohistochemistry, fibres immunoreactive for neurofilament, neuron-specific enolase, and protein gene product 9.5 were observed in the papillary dermis and dermoepidermal junctions as well as in the epidermis. In these areas, no fibres stained positively for substance P, neuropeptide Y, vasoactive intestinal peptide, beta endorphin, somatostatin or serotonin. On electron microscopy, the ultrastructure of subepidermal and intraepidermal free nerve endings appeared to be essentially normal. However, the distribution density of the cutaneous nerve fibres was much higher than in normal controls, and the diameter of these fibres was much larger, because of the large number of axons in each nerve fibre. Degranulation of mast cells was not seen. These findings suggest that pruritus in lichenified atopic skin is probably not caused by damage to the cutaneous free nerve endings. In such lesions, the number of the cutaneous free nerve endings is greatly increased, but they may have a normal function.

Key words Atopic dermatitis · Pruritus · Cutaneous nerve · Immunohistochemistry · Electron microscopy

Introduction

Atopic dermatitis (AD) is typically accompanied by severe pruritus that elicits vigorous scratching leading to the aggravation of the lesions. A vicious circle of itch–scratch results. Generally speaking, the neural activity that is related to each type of sensation travels along a defined neural pathway from the receptor in the skin, along spinal pathways, to the higher centres in the brain [18]. The receptors of pruritic sensation are regarded as being free nerve endings in the dermoepidermal junction [19, 20].

Previous studies have revealed that the number of cutaneous nerve fibres is increased in atopic skin lesions [12, 17]. Although such studies were immunohistochemical studies with light microscopy, few papers on electron microscopy have been published [14,15]. The present investigation was undertaken to elucidate the morphological features of the nerve fibres, particularly free nerve endings, in cutaneous lesions of adults with AD, by means of histology, immunohistochemistry and electron microscopy.

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Materials and methods

Samples were obtained from 16 Japanese patients, 8 men and 8 women aged 15–59 years (mean 20.3 years), who fulfilled the diagnostic criteria of AD recently proposed by the working group for AD of the Japanese Dermatological Association [16]. None had received topical or systemic treatments in the past month. Under local anaesthesia with 1% lidocaine hydrochloride, 3- or 4-mm punches were made to obtain biopsy specimens from the lichenified lesions. The biopsy specimens came from the antecubital fossa in 13 cases, the upper back in 2 cases, and the lateral chest in 1 case. Informed consent was obtained from each subject. As controls, specimens of normal skin of adults (5 men and 1 woman, age range 31–65, mean 50.3 years) from around benign skin tumours such as seborrhoeic keratosis were obtained from the antecubital fossa in 4 cases, and the upper back and lateral chest in 1 case each. All the specimens were cut in half. One half of each was fixed in 3% buffered formalin and embedded in paraffin; these specimens were used for histological and immunohistochemical studies. The other half was cut into smaller pieces for examination by conventional transmission electron microscopy (TEM). These specimens were fixed in 2.5% glutaraldehyde solution buffered by 0.1 M sodium cacodylate at pH 7.4. They were postfixed with 1% osmium tetroxide diluted by the same buffer, dehydrated in a graded series of ethanol, and in propylene oxide, and embedded in Epon 812.

For histological examination, all paraffin-embedded sections were stained with haematoxylin and eosin.

The deparaffinized sections were immunostained with the following primary antibodies: monoclonal anti-neurofilament (NF) (70 kDa, 200 kDa) (Medac, Germany; dilution 1:10), polyclonal

anti-human neuron-specific enolase (NSE; Dako Japan, Kyoto; 1:1000), monoclonal anti-human protein gene product 9.5 (PGP 9.5; Ultraclone, UK; 1:100), polyclonal anti-calcitonin gene-related peptide (CGRP) (Affiniti Research Products, UK; 1:1000), polyclonal anti-substance P (SP; Biomeda, USA; 1:50), polyclonal anti-neuropeptide Y (NPY; Peninsura, USA; 1:400), polyclonal anti-vasoactive intestinal peptide (VIP; Biomeda; 1:20), polyclonal anti-beta endorphin (End; Biomeda; 1:100), polyclonal anti-somatostatin (SOM; Chemicon, USA; 1:1000), and polyclonal anti-serotonin (Ser; Biomeda; 1:1000). The specimens were then analysed by the streptavidin–biotin-peroxidase complex method, using the Histofine SAB-PO (M) Kit (Nichirei, Tokyo). The reaction products were visualized by the diaminobenzidine (DAB) or cobalt-enhanced DAB reaction. The details of the positive controls are described elsewhere [7].

Five Epon-embedded blocks were randomly sampled for TEM in each case. Each grid had three ultrathin sections, and six to eight such grids were made for each block. In two randomly sampled grids, the ultrathin sections were double-stained with uranyl acetate and lead citrate. A total of 30 ultrathin sections were observed with an electron microscope (Hitachi H 500, Ibaragi, Japan) at 75 kV.

In addition, seven to eight blocks of the antecubital lesions were sampled in all 13 cases. In these blocks, we counted all basal cells, all intraepidermal nerve fibres or Schwann cell–axon complexes (SAs), all subepidermal SAs within 24 μm of the epidermal basal lamina, and all mast cells with nuclei. The total number of SAs (number of subepidermal SAs plus intraepidermal SAs) was calculated per 100 basal cells. Finally, 100 subepidermal SAs were randomly sampled. Their shortest diameters were measured, and the number of the axons in each SA were counted. The shortest diameters of all the axons were also measured. Mean values were compared with those of 30 SAs obtained from the antecubital skins of 4 normal controls. All statistical comparisons were analysed by the Mann-Whitney U test.

Results

In the lesion-bearing skin specimens there were parakeratosis, moderate acanthosis with elongation of rete ridges, and spotty spongiosis with an exocytosis whose component cells were largely lymphocytes. From the papillary dermis to the upper reticular dermis there was mild to moderate perivascular lymphohistiocytic infiltration, intermingled with eosinophils. A number of melanophages were also seen. Thick cutaneous nerve fibres were occasionally encountered below the mid-dermis through the subcutaneous fatty tissues both in the lesion-bearing skin and in the normal controls. Their distribution density seemed not to differ much between lesion-bearing skin and the normal controls. The cutaneous nerve fibres showed no pathologic changes in the lesion-bearing skin.

In the deep dermis, thick fibres stained positive for NF in the lesion-bearing skin specimens and the normal controls (see Table 1). In the papillary dermis of the lesion-bearing skin, thin NF-positive fibres were seen in only 1 case, and NF-positive fibres were also present in the epidermis in this patient, while no NF-positive fibres were observed in and above the papillary dermis in the normal controls. Both thin fibres in the papillary dermis and thick fibres in the deep dermis stained extensively positive for NSE and PGP 9.5 in lesion-bearing skin. These fibres also were seen in the dermoepidermal junctions in many patients (Fig. 1A). Some fibres reached the

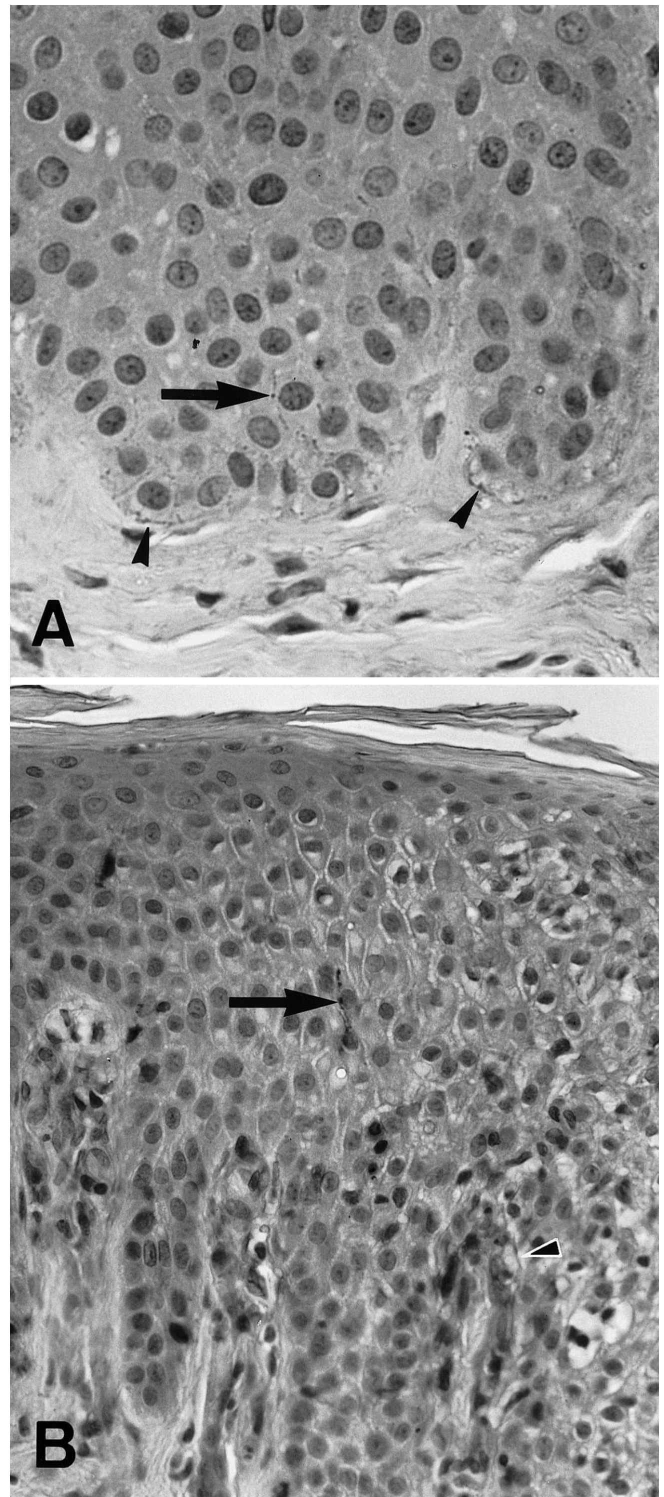

Fig. 1A There are NSE-immunoreactive fibres in the dermoepidermal junction (*arrowheads*) and the lower spinous layer (*arrow*). DAB-cobalt, × 340. **B** There are NSE-immunoreactive fibres in the suprabasal area (*arrowhead*) and the spinous layer (*arrow*). The latter nerve fibre has a beaded appearance. DAB-cobalt, × 225

Table 1 Immunohistochemistry (*AD* atopic dermatitis, *NF* neurofilament, *NSE* neuron-specific enolase, *PGP* protein gene product, *CGRP* calcitonin gene-related product, *SP* substance P, *NPY* neuropeptide Y, *VIP* vasoactive intestinal peptide, *End* beta endorphin, *SOM* somatostatin, *Ser* serotonin)

	Deep dermis		Papillary dermis		DE junction		Epidermis	
	AD	Control	AD	Control	AD	Control	AD	Control
NF	+	+	+ ~ –	–	+ ~ –	–	+ ~ –	–
NSE	+	+	+	+ ~ –	+	–	+ ~ –	–
PGP9.5	+	+	+	+ ~ –	+	–	+ ~ –	–
CGRP	+	+	+ ~ –	–	–	–	–	–
SP	+	+	–	–	–	–	–	–
NPY	+	+	–	–	–	–	–	–
VIP	+	+	–	–	–	–	–	–
End	+	+	–	–	–	–	–	–
SOM	+	+ ~ –	–	–	–	–	–	–
Ser	+	+	–	–	–	–	–	–

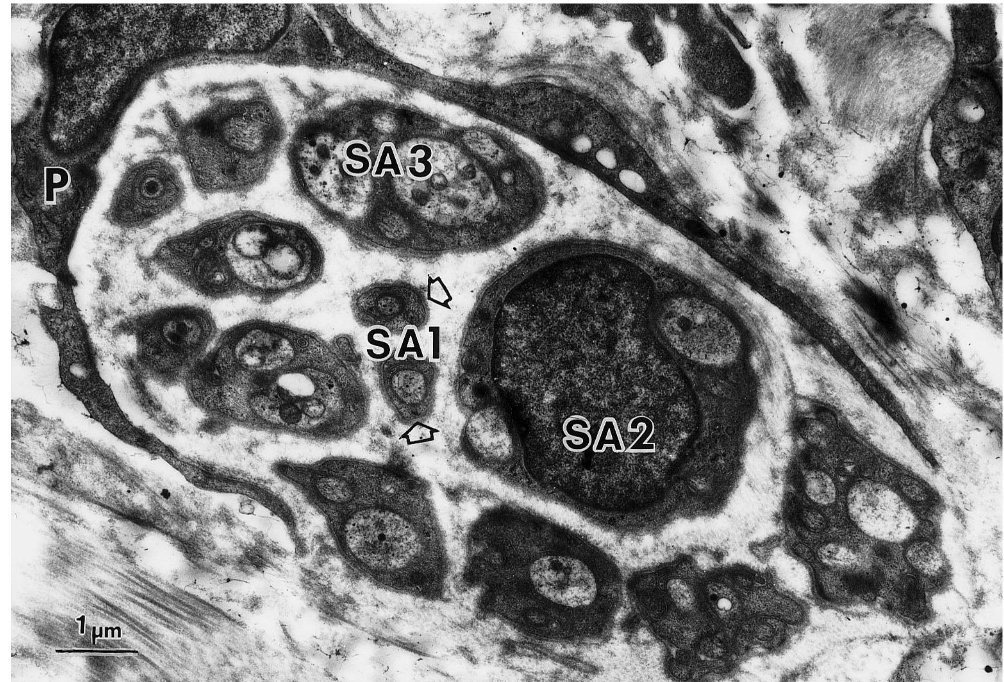

Fig. 2 A nerve fascicle partially ensheathed by a perineurial cell (*P*) is seen. The nerve facicle has many Schwann cell–axon complexes (SAs), in which there are mono- (*SA1* and *hollow arrows*), oligo- (*SA2*), and polyaxonal (*SA3*) nerve fibres. × 14,000

granular layer. Other fibres 1–2 µm in diameter had a beaded appearance and ran up to 7 or 8 prickle cell layers above the basal layers (Fig. 1B). In contrast, there were no NSE- or PGP 9.5-positive fibres in or above the dermoepidermal junctions in normal controls. In the lesion-bearing skin CGRP-positive fibres were seen up to the papillary dermis, but not in the dermoepidermal junctions or the epidermis, while in the normal controls CGRP-positive fibres were not seen in or above the papillary dermis. No fibres were positive for the other neuropeptides from the papillary dermis to the epidermis either in the lesion-bearing skin specimens or in the normal controls.

Nearly all the cutaneous nerve fibres observed on TEM were unmyelinated. In the dermis deep to the epidermis, each nerve fascicle with many SAs was ensheathed completely by the perineum and endoneurium in both the lesion-bearing skin and control. As the nerve fascicles came to lie near the epidermis, they were partially ensheathed by the perineurium and endoneurium in lesions (Fig. 2) as well as in controls. However, the number of the SAs in the nerve fascicles appeared to be much greater in the lesion-bearing skin than in normal controls. In the subepidermal portions the nerve fibres lost their perineurium and endoneurium both in the lesion-bearing skin and in the normal controls. In contrast to the normal controls, in which there were only occasionally SAs in the dermal micropapillae, in the lesion-bearing skin specimens every dermal micropapilla had one SA in the same areas (Fig. 3A), and SAs were grouped in the remaining areas (Fig. 3B). These axons were completely or partially ensheathed by Schwann sheaths (Fig. 3C). Unlike the normal controls, the lesion-bearing specimens had the nucleated and non-nucleated SAs coexisting next to each other immediately beneath the epidermis (Fig. 3D). In the epidermis of lesion-bearing skin the SAs were found in the intercellular spaces between the basal keratinocytes or in the spaces between the basal

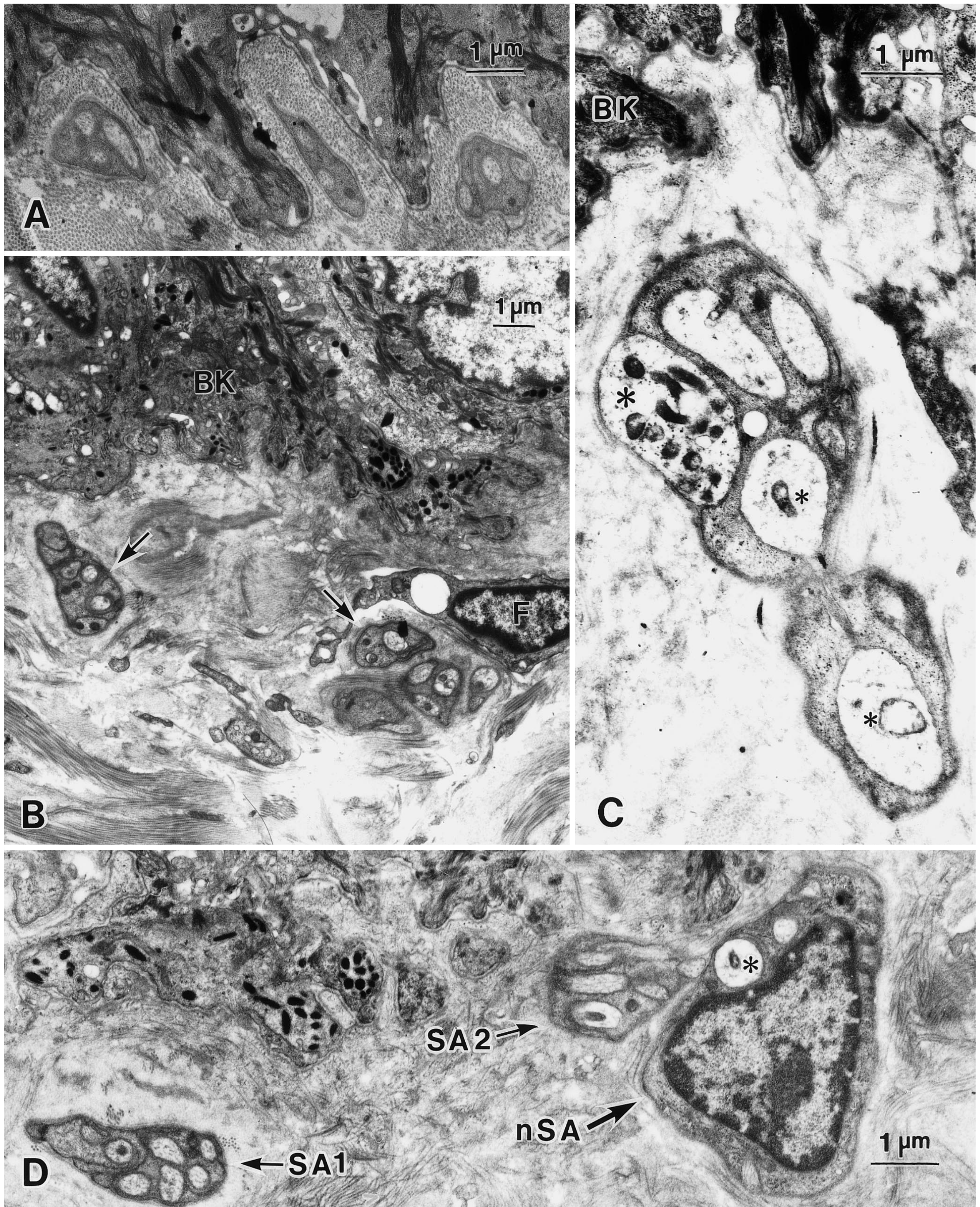

Fig. 3A Every dermal micropapilla has one subepidermal SA. × 9,900. **B** The SAs (*right arrow*) remain grouped. *Left arrow* indicates subepidermal SA (*BK* basal keratinocyte, *F* fibroblast) × 6,600. **C** The axons (*asterisks*) completely or partially ensheathed by Schwann sheath. × 14,000. **D** The nucleated (*nSA*) and non-nucleated (*SA1, SA2*) SAs coexist next to each other immediately beneath the epidermis. *Asterisk* indicates an axon in the nucleated SA. × 11,000

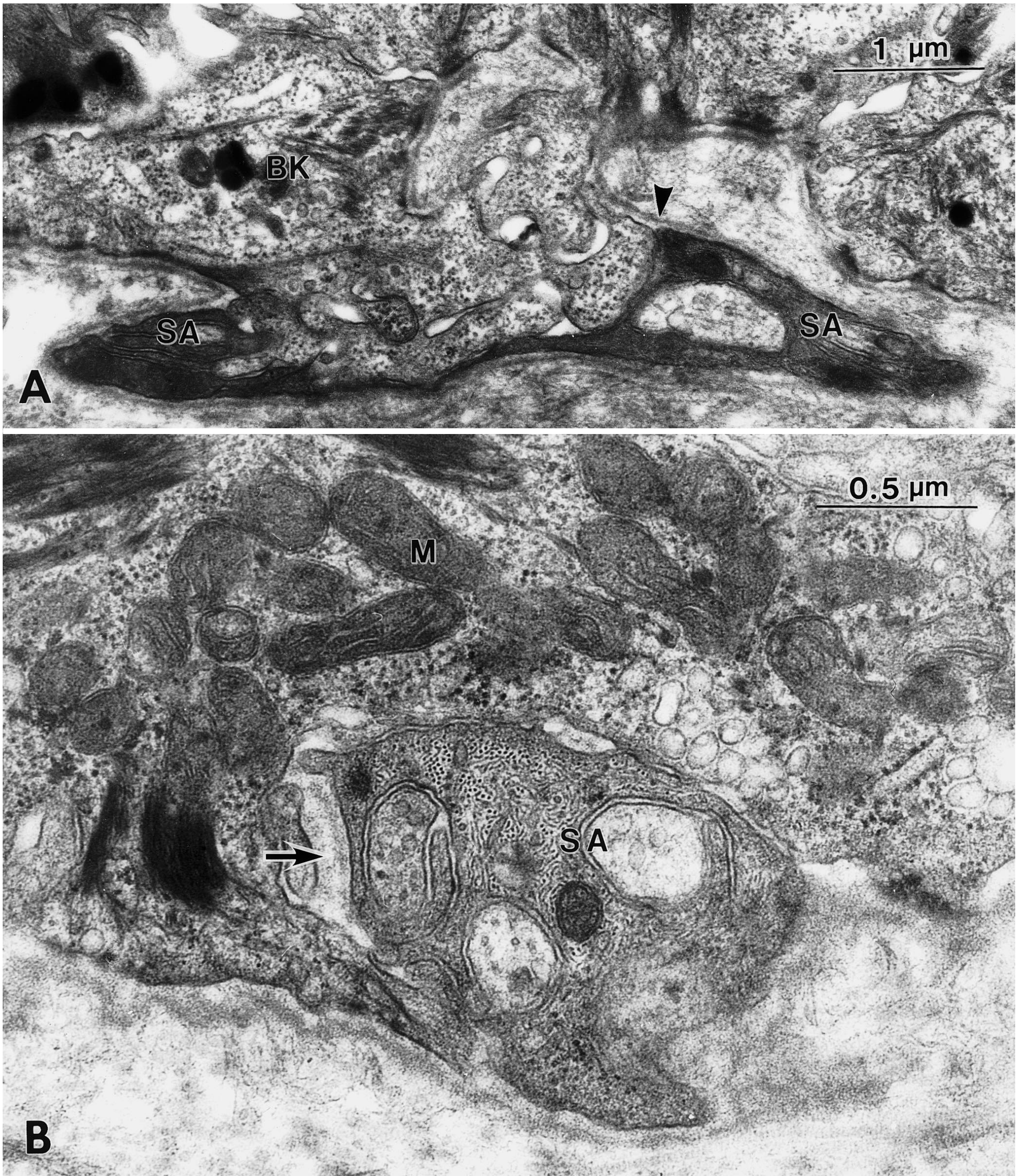

Fig. 4A, B Intraepidermal SA in the space between the basal surface of the cell membrane of the basal keratinocyte and its basal lamina. **A** There are two SAs (*SA*). The basal lamina of the SA fuses with that of the basal keratinocyte (BK; *arrowhead*). × 25,00 **B** The SA has basal lamina (*arrow*) and is surrounded by clustered mitochondria (*M*) in the cytoplasm of the basal keratinocyte. × 52,000

surfaces of the cell membranes of the basal keratinocytes and their basal laminae (Fig. 4). In the last cases, the SAs were in direct contact with tips of the microrete ridges (Fig. 5). Frequently the intraepidermal SAs had many axons (Fig. 5). At their entry into the epidermis, the basal laminae of the SAs fused with those of the basal keratinocytes, and some parts of the Schwann sheaths

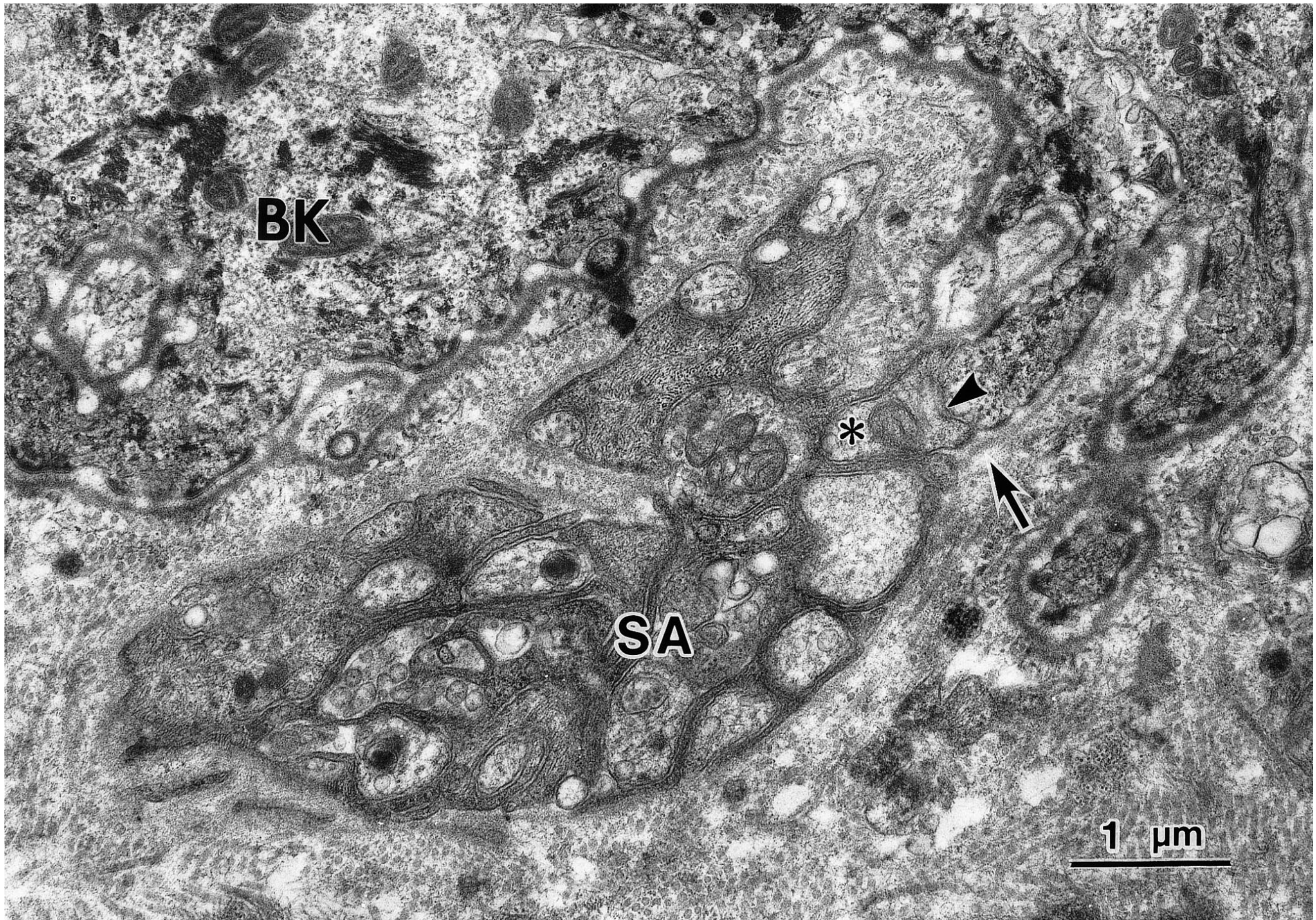

Fig. 5 An intraepidermal SA has many axons, one of which (*asterisk*) contacts directly with the tip of the microrete ridge (*arrowhead*). *Arrow* indicates the fusing portion between the basal lamina of the SA and that of the basal keratinocyte. × 21,000

(Fig. 4) and/or even axons (Fig. 5) made direct contact with the cell membranes of the basal keratinocytes. The SA had basal lamina in a relatively wide space between the SA and the surrounding keratinocyte (Fig. 4B). Intraepidermal nerve fibres were not observed in normal controls. In the cases of AD, the axons and the Schwann cells in the subepidermal and intraepidermal SAs generally had no pathologic changes. In one case with AD the mitochondria were clustered in a small area of the cytoplasm of the basal keratinocyte and surrounded the intraepdermal SA (Fig. 4B).

In the lesion-bearing skin specimens and in normal controls, epidermal keratinocytes had round or oval nuclei with a large amount of euchromatin and a small amount of heterochromatin. Intracytoplasmic organelles, such as mitochondria, lamellar bodies, and melanosomes, showed almost the same distribution and amount in the diseased skins as in the normal controls. Tonofilaments and desmosomes in the lesion-bearing skin samples also did not much differ from those in the normal controls. In contrast with the normal controls, however, the keratinocytes in the horny layer of the lesion-bearing skin still had nuclei and degenerated intracytoplasmic organelles, such as mitochondria and vacuoles. In these skins there were widened intercellular spaces between the keratinocytes, where lymphocytes were scattered. Langerhans cells were often seen in the epidermis in both the lesion-bearing skins and the normal controls, but apparent contacts between Langerhans cells and intraepidermal nerve fibres, described by Hosoi et al. [5], were not found. No Merkel cells could be found in any of the sections observed. Melanocytes had a normal appearance in the diseased skin specimens. There was also mild to moderate oedema in the subepidermal region, where a number of lymphocytes and macrophages were seen, not infrequently intermingled with eosinophils. Mast cells were encountered much more often in lesion-bearing skin than in normal controls. Collagen fibres and elastic fibres in the dermis of the lesion-bearing skin specimens did not differ from those in normal controls. Endothelial cells, smooth muscle cells and fibroblasts in the blood vessels and lymphatic vessels were not altered in comparison with those in the normal controls.

The mean value of the number of the SAs per 100 basal cells was 18.34±7.43 (mean±SD) in the antecubital lesions and 1.11±1.02 in the normal controls. According to the Mann-Whitney U-test, the difference was statistically significant ($U = 0$, $P = 0.0032$). The mean number of mast cells per 100 basal cells was 4.63±4.08 in the

AD skins and 0.07±0.08 in the normal controls. The Mann-Whitney U-test showed that these data were statistically different (U = 0, P = 0.0032).

The mean diameter of the SAs was 1.82±0.63 μm in the antecubital lesions of 13 AD patients, and 1.19±0.42 μm in the skin of normal controls. The Mann-Whitney U-test showed that the difference was statistically significant (U = 7.0, P = 0.0315). The mean number of the axons was 6.38±1.77 in the lesional skins, and 2.57±0.83 in the normal controls, a statistically significant difference according to the Mann-Whitney U-test (U = 0, P = 0.0320). The mean diameter of the axons was 0.39±0.08 μm in the lesion-bearing skin, and 0.41±0.14 μm in normal controls. According to the Mann-Whitney U-test, these values were not statistically significant (U = 22.0, P = 0.6502). When we evaluated the interrelationship between the diameter and the number of axons in the SAs, we obtained a Spearman's rank correlation coefficient of 0.552, indicative of a positive correlation. This indicated that the larger diameter of the SAs in the lesion-bearing skin specimens resulted from an increased number of axons in each SA.

Discussion

Immunohistochemical analysis of the cutaneous nerve fibres in AD has shown that the numbers of NF-, PGP 9.5, CGRP-, and SP-positive fibres are increased in the papillary dermis and the dermoepidermal junctions; they occasionally enter the epidermis [1, 11, 12, 17]. This study confirmed earlier findings regarding NF and PGP 9.5, which are axonal or neuronal markers. Recently interleukin 6-like immunorectivity was found in the nerve fibres in the dermis and even epidermis of normal and inflamed human skin, and these findings suggest that interleukin 6 may have a trophic effect on sensory nerves and that the increasing number of interleukin 6-positive fibres may be associated with pruritus [10].

The present electron microscopic study demonstrated the mode of distribution and the ultrastructures of the cutaneous nerve fibres in the lichenified skin of patients with AD. These cutaneous nerve fibres had far more axons in each SA. In addition, the SAs remained grouped, even when they reached the subepidermal area. These ultrastructural features are uncommon, even in normal guinea pig skin which has many subepidermal and intraepidermal nerve fibres [8]. We also found many nucleated SAs in the subepidermal area, and similar findings have been observed in regenerated epidermis around burn ulcers, where the nucleated SAs even enter the epidermis [6]. The Schwann cells of these nucleated SAs are referred to as terminal Schwann cells and are commonly situated in the dermis at a distance from the epidermis. The terminal Schwann cells stretch their penicillate endings [2, 3], which are composed of the cytoplasmic processes ensheathing the axons, to the targeted tissues and cells. As in the regenerated epidermis of the burn scar, where new formation of cutaneous nerve fibres is actively taking place, the terminal Schwann cells seem to migrate near to and into the epidermis because of the increasing number of axons and stretching [6]. In the lichenified lesions of AD, the terminal Schwann cells may move up to the subepidermal areas because of the formation of numerous axons in the subepidermal and epidermal areas.

Histologically, Mihm et al. [9] described demyelination, vacuolar change, and fibrosis in the cutaneous nerve fibres of AD and the relationship between such derangement of neural structures and pruritus. Similarly, using electron microscopy, Sugiura et al. [14] observed oedematous changes in axons in the cutaneous nerve fibres ensheathed by perineurial cells in patients with pruritic lesions of AD. However, in these studies the terminal portions of the cutaneous nerve fibres, which are the location of the most important itching receptor, were not evaluated. Most cutaneous nerve fibres observed in our electron microscopic study probably belong to the preterminal and terminal portions (free nerve endings, according to Chouchkov's description [4]). Their axons and Schwann cells had an essentially normal ultrastructural appearance, suggesting that cutaneous free nerve endings in atopic lichenified lesions may be functionally intact.

The number of mast cells is greatly increased in the cutaneous lesion of AD [13]. There has been only one previous report of mast cells entering perineurium and degranulating, resulting in oedema of the axons, and this finding was thought to be related to itching in AD [14]. However we could not demonstrate degranulation of mast cells in the lesions of AD. From the clinical viewpoint, in that there is always severe pruritus in lichenified atopic skin lesions, such lesions may be considered as having a continuous pruritogenic stimulus. Thus, there is a possibility that a pruritogenic mechanism not associated with degranulation of the mast cells may be at work in atopic lesion-bearing skin.

Acknowledgements I wish to thank Dr Mikizo Okamoto, Assistant Professor, Department of Hygiene, Faculty of Medicine, Tottori University, for his help for the statistical analysis. The technical help of Mrs Akie Koseki is also highly appreciated.

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