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Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema

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Abstract The association between mast cells and sensory nerves and the distribution of the neuropeptides substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide (CGRP) were studied immunohistochemically in lesional and nonlesional skin of 26 atopic dermatitis (AD) and 23 nonatopic nummular eczema (NE) patients. Mast cell-nerve contacts were counted morphometrically and confirmed by confocal laser scanning microscopy. Neuropeptide positivity was assessed semiquantitatively. Dermal contacts between mast cells and nerves were increased in number in both lesional and nonlesional samples of AD and NE when compared to those in normal controls, although only the values in lesional AD reached statistical significance ($P<0.05$). Nerve-mast cell contacts in the basement membrane zone were seen practically only in lesional NE. SP and CGRP fibres were prominently increased in lesional samples when compared to their nonlesional controls both in AD and NE in the epidermis and in the papillary dermis. In both AD and NE, only small differences were found regarding VIP positivity in lesional and nonlesional biopsies. The epidermis was devoid of VIP positivity. In conclusion, SP and CGRP but not VIP fibres were more frequent in lesional than in nonlesional papillary dermis of both AD and NE. Since mast cells are also increased in number in lesions of AD and NE, they are able to maintain neurogenic inflammation through activation by SP and CGRP. The increased SP/CGRP nerves in the epidermis of AD and NE lesions may stimulate keratinocytes to release cytokines which affect various cell types enhancing inflammation.

Keywords Mast cells · Nerves · Neuropeptides · Atopic dermatitis · Nummular eczema

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Introduction

Mast cells are increased in number in inflammatory dermatoses such as psoriasis [1] and lichen planus [2]. We have previously studied the distribution of mast cells in atopic dermatitis (AD) and in nonatopic nummular eczema (NE) patients [3]. Mast cells are increased in number in lesional versus nonlesional samples in both dermatoses. Reports are available suggesting that inflammation in AD could be neurogenic [4, 5]. In NE, this aspect has not been previously studied. Close contact between mast cells and sensory nerves has been shown in psoriasis [6, 7] and in AD [8]. It is known that neuropeptides such as substance P (SP), vasoactive intestinal polypeptide (VIP) and somatostatin can release histamine and other inflammatory mediators from mast cells in the skin [9].

Patients with AD often complain of itch. A subpopulation of the receptive endings of small unmyelinated nerve fibres, the C-fibres, transduce itch [4]. Liberation of histamine and other inflammatory mediators from mast cells may be an initiating factor in producing this sensation. In this study, we investigated the association between mast cells and sensory nerves, and the distribution of different neuropeptides in the epidermis and upper dermis of AD and nonatopic NE. The nonatopic NE patients also served as controls for the atopic AD patients in this study.

Materials and methods

Patients

Three groups of patients were included. The first group comprised 26 AD patients (15 females and 11 males; ages 16–60 years, average 32 years). The second group comprised 23 nonatopic NE patients (7 females and 16 males; ages 28–75 years, average 53 years). The control group comprised 8 healthy subjects (6 females and 2 males; ages 30–84 years, average 49 years).

The AD patients were selected according to the criteria of Hanifin and Rajka [10]. The NE patients were selected according to the definition of Burton [11] who defines NE as characterized by circular or oval plaques of eczema with a clearly demarcated edge. In most cases the cause is unknown [11]. Patients without any sys-

Table 1 The number of mast cells (MC) in morphological contact with neurofilament-positive nerves in the basement membrane, the papillary dermis and the upper dermis (0.4 mm down from the papillary dermis) in atopic dermatitis(AD), nummular eczema (NE) and control patients. Values are means±SEM

*P<0.05 vs healthy normal skin, **P<0.05 vs nonlesional skin

	Basement membrane (/mm)		Papillary dermis (/mm^2)		Upper dermis (/mm^2)	
	Total MC	MC-nerve contacts	Total MC	MC-nerve contacts	Total MC	MC-nerve contacts
AD (n=26)						
Nonlesional	0.44±0.17	0.02±0.01	150±35.1	17±6.03	112±9.55	25.3±4.94
Lesional	0.7±0.19	0.02±0.01	162±23.6	13.8±4.68	115±8.27	28.3±4.86*
NE (n=23)						
Nonlesional	0.33±0.1	0±0	86.9±17.2	13±5.45	95.7±11.1	15.8±4.17
Lesional	0.76±0.23	0.1±0.04**	128±24.9	17.9±5.41	93.8±7.66	17.8±3.93
Control (n=8)	0.18±0.07	0±0	89.4±34.3	2.31±2.16	108±22.9	9.06±2.12

temic or effective local treatments for at least 1 month prior to the biopsy were included. Only 1% hydrocortisone cream was allowed to be used during the 2 weeks before the study. The clinical diagnoses were verified by a pathologist.

All patients volunteered for this study, and the protocol was approved by the Ethics Committee of the Kuopio University Hospital.

Skin samples

Punch biopsies (4 mm) were taken under local anaesthesia (1% lidocaine with adrenaline) from acute or subacute lesional skin, mostly from the medial side of the forearm, and also from healthy-looking skin at least 2 cm away from lesions. After removal, the specimens were embedded in OCT (Miles Scientific, Naperville, Ill.) and frozen in isopentane cooled with a mixture of dry ice and absolute ethanol. The frozen biopsies were kept at –70°C before processing.

Double staining for mast cells and sensory nerves

Sensory nerves were visualized by staining neurofilament proteins with a mixture of monoclonal antibodies against 68, 160, and 200 kDa subunits (Amersham, Little Chalfont, UK; dilution 1:50). Mast cells were stained enzyme-histochemically for tryptase. This double stain was applied on 16-µm thick acetone-fixed cryosections as described in detail previously [12].

Immunohistochemical staining of neuropeptides

Cryosections of 12 µm thickness were cut on poly-L-lysine-coated slides. The sections were fixed in 4% paraformaldehyde and cryoprotected in 15% sucrose containing 0.01% sodium azide, then air-dried and stored in –20°C before staining. The neuropeptides were visualized with polyclonal antibodies against SP (Amersham; diluted 1:50), VIP (Milab, Malmö, Sweden; 1:500) and CGRP (Peninsula Laboratories, Belmont, Calif.; 1:200) combined with the avidin-biotin-peroxidase method (Vectastain Elite ABC kit, PK-6101 Vector Laboratories, Burlingame, Calif.). The stainings were carried out and controlled as described in detail previously [12].

Technique for confocal laser scanning microscopy

Cryosections of 12 µm thickness were cut from three AD and seven NE samples for staining of sensory nerves and tryptase-positive mast cells. Both components were stained using the indirect immunofluorescence method. The staining protocol and the confocal laser scanning microscopy procedure are described in detail in our previous work [7].

Quantitative analysis of mast cell-nerve contacts

Contacts between mast cells and sensory nerves were counted under light microscopy (×40 objective) as described previously [6]. The papillary dermis, and five adjacent grid fields (each 0.04 mm^2) of 0.4 mm depth of the upper dermis were analysed in each slide (mast cell-nerve contacts/mm^2). Mast cells in contact with both a nerve and the basement membrane were also counted (mast cell-nerve contacts/mm of the basement membrane). The area of the papillary dermis and the length of the basement membrane were measured using an automated image analysis system (Quantimet, Leica, Nussloch, Germany). The paired Student's t-test was used to compare the means of lesional and nonlesional samples of the same patient. An unpaired t-test was applied when comparing patient data to that of healthy skin. The data were analysed by a professional statistician.

Semiquantitative analysis of neuropeptides

Neuropeptide-positive nerves were estimated in the epidermis, in the papillary dermis and in a 0.4-mm zone of the upper dermis beneath the papillary dermis. Neuropeptide positivity was assessed semiquantitatively: – (negative), + (scanty positivity, mainly near the basal epidermis), ++ (positivity both in the papillary dermis and around capillaries). Special attention was paid to the epidermis, where positivity was marked separately [12]. The investigators were blinded to which slides were under study. Two representative sections out of three per sample were examined.

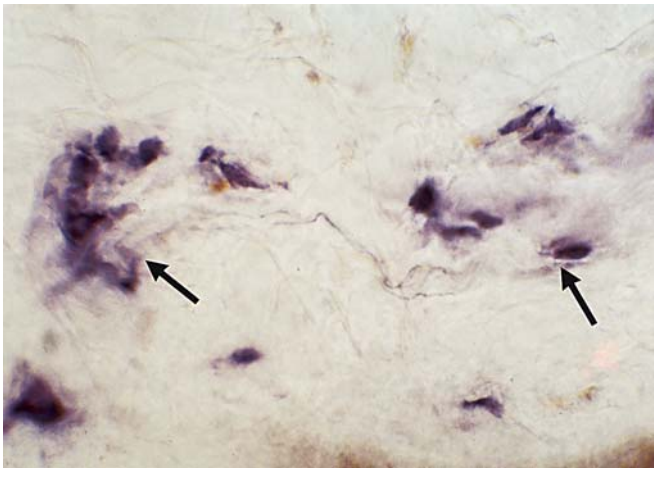

Fig. 1 Double staining of mast cells and sensory nerves demonstrates their morphological association (*arrows*) in the upper dermis of nonlesional skin of AD (×450)

Table 2 Distribution of neuropeptides SP, CGRP and VIP in the epidermis, papillary dermis and upper dermis (a zone of 0.4 mm beneath the papillary dermis) of nonlesional and lesional skin of AD and NE and control patients (– no positivity or only a few nerve fibres, + scanty positivity, ++ clear nerve fibres)

	SP			CGRP			VIP		
	Epidermis	Papillary dermis	Upper dermis	Epidermis	Papillary dermis	Upper dermis	Epidermis	Papillary dermis	Upper dermis
AD nonlesional (*n*=26)									
–	22	14	2	22	16	0	26	6	11
+	4	12	23	4	10	26	0	18	15
++	0	0	1	0	0	0	0	2	0
AD lesional (*n*=26)									
–	13	2	0	11	1	0	26	10	15
+	13	23	16	15	22	18	0	14	11
++	0	1	10	0	3	8	0	2	0
NE nonlesional (*n*=23)									
–	23	13	1	21	17	1	23	9	15
+	0	10	21	2	6	21	0	14	8
++	0	0	1	0	0	1	0	0	0
NE lesional (*n*=23)									
–	13	4	0	11	2	0	23	11	16
+	10	19	16	12	20	16	0	12	7
++	0	0	7	0	1	7	0	0	0
Control patients (*n*=8)									
–	6	2	0	8	7	0	8	5	0
+	2	6	8	0	1	8	0	3	8
++	0	0	0	0	0	0	0	0	0

Results

Mast cell-nerve contacts are shown in Table 1. The number of contacts (Fig. 1) was higher in both lesional and nonlesional samples of AD and NE when compared to those of normal controls, although due to great variation only the values in the lesional upper dermis (0.4 mm beneath the papillary dermis) of AD reached statistical significance ($P<0.05$). In the upper dermis, contacts were somewhat more frequent in AD than in NE. Mast cell-nerve contacts at the basement membrane zone were zero/very few, but significantly more frequent in lesional than nonlesional NE ($P<0.05$).

As shown in Table 2, SP and CGRP fibres were prominently increased in lesional samples when compared to their nonlesional controls or to samples from healthy subjects in the epidermis and papillary dermis of both AD and NE (Fig. 2). The epidermis of both AD and NE was devoid of VIP positivity. More VIP-positive fibres were seen in nonlesional than in lesional samples of AD, whereas there was hardly any difference in VIP positivity between lesional and nonlesional samples of NE.

The nature of the contacts between mast cells and nerve fibres was examined by confocal laser scanning microscopy. As shown in Fig. 3, nerve fibres were in very close morphological contact with mast cells.

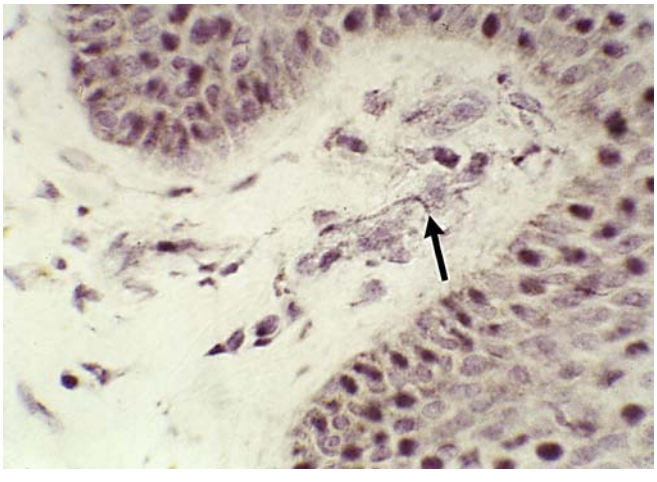

Fig. 2 A prominent nerve fibre (*arrow*) positive for SP is seen in the papillary dermis of lesional skin of AD (×450)

Discussion

In inflammatory skin conditions, histamine increases the permeability of capillaries causing plasma extravasation, which is seen as a wheal, and affects unmyelinated afferent nerve fibres leading to liberation of neuropeptides. Due to this reaction vasodilation occurs appearing as a flare [4]. SP, when injected intradermally into the human forearm, causes a dose-dependent wheal and flare response [13].

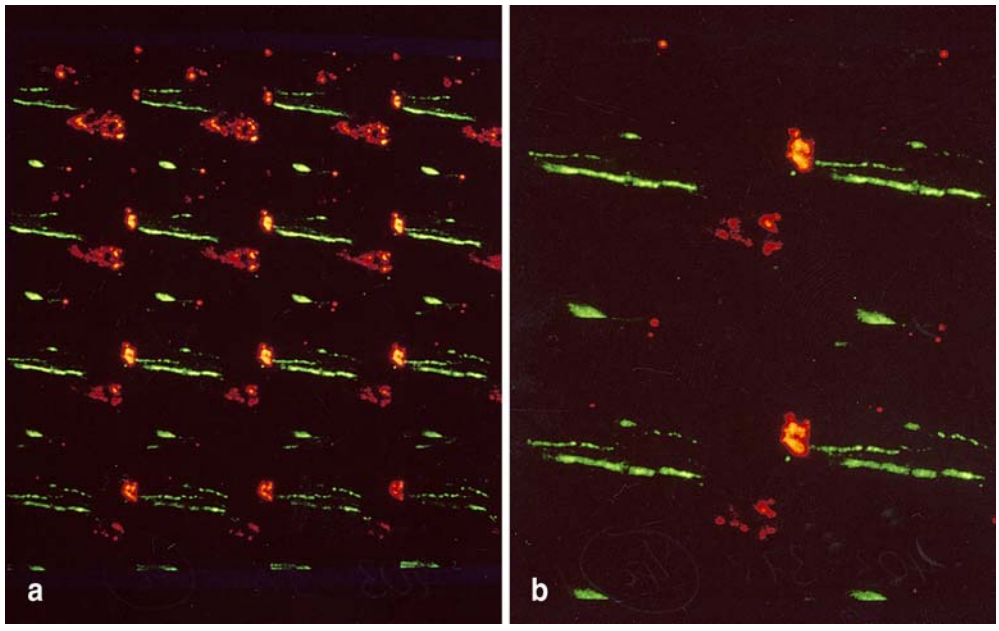

Fig. 3 **a** Sensory nerve fibres (*green*) are in contact with a tryptase-positive mast cell (*red*) in the dermis of lesional skin of NE. The figure shows 16 consecutive confocal laser scanning images with step size of 0.2 µm. **b** Close-up of two contacts shown in **a**. The figure shows 4 consecutive confocal laser scanning images with a step size of 0.2 µm

CGRP coexists in the same sensory nerves with SP, and is also a potent vasodilator [14]. Neuropeptides SP, VIP and somatostatin are capable of inducing degranulation of mast cells in human skin [9], which can lead to the release of more histamine. This stimulus causes an afferent impulse to travel upwards and spread to other nerve branches again inducing liberation of neuropeptides from nerve endings. This is called the axon reflex and is seen as a slowly spreading erythema [4].

Close morphological contact between mast cells and nerve fibres is required for the axon reflex to occur [7, 12]. In the present study, we counted the mast cells that appeared in contact with a nerve. The number of contacts was higher in both lesional and nonlesional skin of AD and NE than in healthy skin (Table 1). The nature of these contacts was further examined by confocal microscopy. Because of the lack of a cell membrane-bound antigen to representatively mark mast cells, we used intracytoplasmic tryptase. Confocal images revealed an intimate association between mast cells and the nerves (Fig. 3). Nerves in AD have been observed at the ultrastructural level in association with mast cells [8], and have been determined to be morphologically normal [15].

Elevated concentrations of histamine have been measured in the skin and plasma of patients with AD [16]. Heyer [4] has suggested that the spontaneous and intense itch suffered by AD patients leads to hyposensitization of histamine and downregulation of histamine receptors on the fine afferent nerve fibres. Because of the intense itch and diminished reactivity to histamine other transmitters, e.g. neuropeptides, might also excite the itch sensation. Skin mast cells have receptors for neuropeptides on their surface, and they are the only mast cells reactive to SP [17]. Increased numbers of nerve fibres containing SP and CGRP have been observed in AD lesions [18, 19]. In contrast, Fantini et al. [5] have reported that SP is decreased and VIP is increased in chronic lesions of AD. In a recent study, SP, CGRP and VIP have been observed in nerve fibres only deeper in the dermis of AD [15]. In the present study with a representative number of patients, SP and CGRP fibres were increased in the epidermis and in the papillary dermis in lesional samples when compared to their nonlesional counterparts both in AD and NE, but VIP was absent from the epidermis and present in somewhat greater amounts, especially in AD, in nonlesional than in lesional dermis (Table 2). In psoriasis, SP and CGRP are increased, but VIP is decreased in lesional skin [7]. Common to psoriasis, AD and NE is the abundance of dermal mast cells, most of which contain enzyme-histochemically detectable tryptase but only a minor proportion of them contain active chymase in lesional skin [3, 20]. Tryptase is known to degrade CGRP and VIP, but it leaves SP intact [21, 22]. Abundant active tryptase as well as less-active chymase in lesional mast cells [3] both have the ability to degrade VIP. This could well explain the immunohistochemically detected distribution of VIP-positive nerves in the present study.

Recently, trypsin and mast cell tryptase have been reported to cleave proteinase-activated receptors [23] thus enhancing inflammation [24]. Primary afferent neurons which express protease-activated receptor 2 also contain CGRP and SP. Trypsin and tryptase directly signal to these neurons and induce the release of CGRP and SP in the spinal cord, but also in peripheral tissues [24]. On the other hand, the study by Weidner et al. [25] suggests that the concentrations of endogenous CGRP or SP are too low to have any acute sensory function in human skin.

An increase in the number of nerve fibres along with the expanding inflammatory reaction has been reported previously [6, 7, 18]. Degradation of VIP in the dermis helps to maintain inflammation, since VIP is able to inhibit T-lymphocyte functions and thus suppress the inflammatory reac-

tion [26]. Support for this idea is given by the findings of a study in which itching and atopic eczema were successfully treated by transcutaneous low-frequency nerve stimulation [27]. During this treatment VIP levels in plasma increased together with the diminishing itch and healing of lesions. However, it has recently been shown that VIP administered intracutaneously to patients with atopic eczema induces pruritus in a dose-dependent manner, although the sensation of itch seemed to be dependent on the activity of the disease [28]. Effective treatment of AD with cyclosporin for 1 month has been shown in a case study to result in the disappearance of the close association between mast cells and cutaneous nerves, suggesting that the mast cell-nerve interaction could be related to disease development and improvement [29].

Existence of epidermal SP and CGRP fibres in human skin has been reported previously [14]. In the present study, scanty epidermal positivity for SP and CGRP was detected in nerve fibres mostly in lesional samples of both AD and NE. The very weak epidermal expression of these neuropeptides could well be due to neutral endopeptidase which effectively degrades neurokinins such as SP. Significant amounts of this proteolytic enzyme have been detected in the epidermis [30]. SP is able to specifically bind to keratinocytes and to stimulate them to release cytokines such as interleukin-1 [30] which in turn can affect various cell types enhancing inflammation.

In conclusion, an increase in the number of mast cell-nerve contacts was observed in lesional and nonlesional dermal skin of AD and NE when compared to those in healthy skin. The results of the present and previous studies indicate that this increase apparently relates to an overall increase of both mast cells and nerves. SP- and CGRP-positive nerve fibres were more frequent in the epidermis and papillary dermis of lesional than nonlesional or healthy skin of both AD and NE. VIP fibres, therefore, were not increased in lesional dermis, and they were absent from the epidermis of both AD and NE. The results again emphasize the role of SP and mast cell tryptase in skin inflammation. The resemblance of the present results with those obtained in psoriatic skin [7] suggest that the changes in mast cells and the substances liberated by them as well as the alterations in nerves and neuropeptides may be linked with the cutaneous inflammatory reaction in general.

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