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Evaluation of epidermal nerve density and opioid receptor levels in psoriatic itch

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Summary

Background Psoriasis is a complex, multifactorial inflammatory skin disease with genetic and environmental interactions. Patients with psoriasis exhibit erythematous plaques with itch, but the mechanisms of psoriatic itch are poorly understood.

Objectives This study was performed to investigate epidermal nerve density and opioid receptor levels in psoriatic skin with or without itch.

Methods Twenty-four patients with psoriasis aged between 39 and 82 years were included in this study. The number of epidermal nerve fibres, the levels of semaphorin 3A (Sema3A) and the expression patterns of μ- and κ-opioid systems were examined immunohistologically in skin biopsies from psoriatic patients with or without itch and healthy volunteers as controls.

Results The number of epidermal nerve fibres tended to increase in approximately 40% of psoriatic patients with itch compared with healthy controls, while such intraepidermal nerves were not observed in other itchy patients. In comparison with healthy controls, Sema3A levels also tended to decrease in the epidermis of psoriatic patients with itch. However, no relationship was found between nerve density and Sema3A levels in the epidermis of psoriatic patients with itch. The levels of μ-opioid receptor and β-endorphin in the epidermis were the same in healthy controls and psoriatic patients with or without itch. The levels of κ-opioid receptor and dynorphin A were significantly decreased in the epidermis of psoriatic patients with itch compared with healthy controls.

Conclusions Based on Sema3A levels in the epidermis, epidermal opioid systems, rather than hyperinnervation, may be involved in the pathogenesis of psoriatic itch.

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Conflicts of interest
None declared.

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Psoriasis is a common chronic inflammatory skin disease. Patients with psoriasis show erythematous plaques with or without itch. Previous studies have indicated that approximately two-thirds of patients with psoriasis have associated itch; however, intense itch (as in atopic dermatitis) is found only rarely in these patients.[1–3]

Histamine, one of the major pruritogenic mediators, does not seem to be involved in the development of itch in psoriasis. There was no correlation between pruritus intensity and plasma histamine level in psoriasis, and there was no difference in plasma histamine levels between pruritic and non-pruritic patients with psoriasis.[3] In addition, oral H_1-receptor blockers often lack efficacy in psoriatic patients with itch,[2] suggesting that histamine blockade does not prevent pruritus in psoriasis. In these patients, itch also induces scratching, and brings about aggravation of exanthema by the Köbner phenomenon.[1–3] Therefore, it is clinically important to control itch in patients with psoriasis.

Many possible mediators have been suggested to transmit or modulate itch sensation in psoriasis, but none has been clearly demonstrated to be a causative agent of itching.[3] The most commonly discussed theory is the importance of altered innervation in psoriatic skin. One study indicated increased numbers of protein gene product 9.5 (PGP9.5)-immunoreactive nerve fibres in the epidermis and in the upper dermal areas in psoriatic patients with itch.[4] An increase in the number of nerve fibres containing substance P (SP), which is related to neurogenic inflammation and/or pruritus, was also observed in the perivascular areas.[4] The hyperinnervation is probably caused by nerve growth factor produced by keratinocytes, mast cells,

eosinophils and fibroblasts.[4–7] However, other studies have indicated negative correlations between pruritus severity and nerve density in patients with psoriasis.[8,9] Therefore, the pathogenic mechanisms of itch related to skin nerve density in psoriasis remain controversial.

Our recent study indicated that the level of expression of semaphorin 3A (Sema3A), a nerve repulsion factor, is decreased in the epidermis of patients with atopic dermatitis.[10] Several semaphorins are also produced by fibroblasts and immune cells.[11,12] These findings imply that cutaneous innervation is regulated by Sema3A levels in atopic skin, and not only by neurotrophin levels, but the relationship between Sema3A levels and nerve density in psoriatic skin is still unclear.

The opioid system in the skin is another possible cause of pruritus in psoriasis. It is generally believed that activation of μ-opioid receptors induces pruritus, while activation of κ-opioid receptors has a suppressive effect.[13] Our recent findings indicated significant alterations in μ- and κ-opioid receptor expression in the epidermis of patients with atopic dermatitis, showing mainly downregulation of the κ-opioid system.[14] Opioids may also induce pruritus acting in the central nervous system. Intrathecal administration of morphine elicits pruritus and both naloxone and naltrexone, μ-opioid receptor antagonists, reduce histamine-induced pruritus in subjects with atopic dermatitis to a greater extent than H_1-receptor blockers.[15,16] Meanwhile, nalfurafine, a κ-opioid receptor agonist, significantly reduces uraemic or cholestatic pruritus.[17,18] Therefore, μ- and κ-opioid systems are important in several pruritic conditions, but have yet to be fully investigated in psoriasis.

In the present study, we investigated nerve density and expression of Sema3A and μ- and κ-opioid receptors in the epidermis of psoriatic patients with and without itch. Here, we describe the possible mechanism of pruritus in psoriasis.

Materials and methods

Skin biopsies

Punch biopsies 3 mm in diameter were taken with informed consent from normal abdominal skin of five healthy male volunteers (age range 27–33 years, mean 31). Spindle-shaped biopsies were taken with informed consent from lesional skin of 24 patients (18 men and six women, age range 39–82 years, mean 60) with the clinical appearance of psoriasis. Biopsies were taken from the site of itching in the lesional skin of 15 men and five women who had psoriasis with itch and from the lesional skin of three men and one woman who had psoriasis without itch. Psoriatic severity was evaluated by Psoriasis Area and Severity Index (PASI) score.[19] A standardized visual analogue scale (VAS) was also used to measure the intensity of pruritus. The mean ± SD PASI and VAS scores in the psoriatic patients with itch were 9·41 ± 4·72 and 62·0 ± 24·1%, respectively, and the mean ± SD PASI score in psoriatic patients without itch was 9·11 ± 3·31. This study was approved by the Medical Ethical Committee of the Juntendo University Urayasu Hospital, and was conducted in accordance with the Principles of the Declaration of Helsinki.

Antibodies

The primary antibodies used in this study were as follows: rabbit anti-PGP9.5 (1 : 4000 dilution; BIOMOL International LP, Plymouth Meeting, PA, U.S.A.), rabbit anti-Sema3A (1 : 200 dilution; Abcam Ltd, Cambridge, U.K.), rabbit anti-μ-opioid receptor (1 : 200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), goat anti-κ-opioid receptor (1 : 100 dilution; Santa Cruz Biotechnology), rabbit anti-β-endorphin (1 : 200 dilution; Chemicon, Temecula, CA, U.S.A.), rabbit antidynorphin A (1–17) (1 : 200 dilution; Bachem, Bubendorf, Switzerland) and mouse antitype IV collagen (1 : 40 dilution; Progen Biotechnik GmbH, Heidelberg, Germany). Secondary antibodies conjugated with Alexa 488 or Alexa 594 used in this study were obtained from Molecular Probes (1 : 300 dilution; Eugene, OR, U.S.A.).

Immunohistochemistry

Half-sized skin biopsies cleaved with a knife were fixed with 4% paraformaldehyde in 0·1 mol mL^{-1} phosphate buffer for 4 h. After washing with phosphate-buffered saline (PBS, pH 7.4), they were immersed successively in PBS solution containing 10%, 15% and 20% sucrose. The skin specimens were embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan) and frozen in liquid nitrogen, and then cut into cryosections (20 μm thick for PGP9.5 staining or 7 μm thick for opioid receptor staining) using a CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were mounted on silane-coated glass slides. After blocking in PBS with 5% normal donkey serum (NDS) and 2% bovine serum albumin (BSA), the sections were incubated with antibodies against PGP9.5 and opioid receptors for 16 h at room temperature.

For immunofluorescence staining of Sema3A and opioid peptides, half-sized skin specimens were embedded in OCT compound without fixation, and then cryosections 7 μm thick were fixed with ice-cold acetone for 10 min at −20 °C. Sections were rehydrated in PBS, blocked in PBS with 5% NDS and 2% BSA, and then incubated with antibodies against Sema3A and opioid peptides for 16 h at 4 °C. After washing with PBS, secondary antibodies were added to the sections for 1 h at room temperature followed by mounting in Vectashield mounting medium (Vector Laboratories Ltd, Peterborough, U.K.). Immunoreactivity was viewed with a confocal laser-scanning microscope (DMIRE2; Leica Microsystems).

Semiquantitative measurements

For semiquantitative determination of the fluorescence intensities of epidermal Sema3A, opioid peptides and opioid receptors, at least five confocal images were analysed per skin biopsy in

each group. Exposure and acquisition settings were fixed and were such that no signal saturation occurred. The total fluorescence intensity in the epidermis of each skin biopsy specimen was measured using Leica Confocal Software (Leica Microsystems), and fluorescence intensity per unit area was calculated.

For semiquantitative determination of the number of epidermal nerve fibres, the skin biopsy specimens from all 29 subjects were stained with anti-PGP9.5 antibody. In confocal microscopic analysis, optical sections 0·9 μm thick were obtained by scanning through the z-plane of the stained specimens (thickness 20 μm). Three-dimensional reconstruction of the images was performed with Leica Confocal Software (Leica Microsystems). For measurement of the number of epidermal nerve fibres, at least 25 confocal images were analysed per skin biopsy in each group. The number of epidermal nerve fibres per $1{\cdot}6 \times 10^5$ μm^2 in the images was counted by hand by two researchers (M.T. and O.N.) in a blinded manner, although intra- and interobserver reliability was untested and variability was not calculated. All values are presented as mean ± SD of each group.

Statistical analyses

One-way analysis of variance with Bonferroni's multiple comparison test was used for statistical analyses.

Results

Epidermal nerve densities in patients with psoriasis

Nerve fibres were present at low density in the epidermis of healthy volunteers (Fig. 1a) and psoriatic patients without itch (Fig. 1b), and their densities were higher in approximately 40% of psoriatic patients with itch than in healthy controls (Fig. 1c). Semiquantitative analyses indicated that the number of epidermal nerve fibres showed a tendency to increase in the 40% of psoriatic patients with itch compared with healthy volunteers, although the differences were not statistically significant (Fig. 1c,e). The penetration of nerve fibres into the epidermis was not observed in approximately 60% of psoriatic patients with itch (Fig. 1d,e).

Expression of semaphorin 3A in the epidermis of patients with psoriasis

The epidermis of psoriatic patients with itch tended to show decreased Sema3A expression in comparison with healthy volunteers and psoriatic patients without itch (Fig. 2a–d). Fluorescence intensity per unit area of epidermal Sema3A was calculated in each group, and statistical analysis was performed. Expression levels of epidermal Sema3A tended to

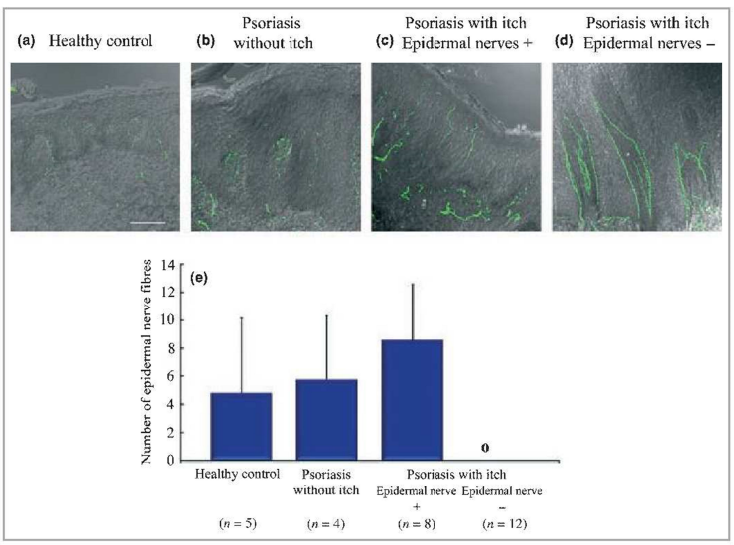

Fig 1. Nerve densities in the epidermis of patients with psoriasis. (a–d) Skin specimens from healthy volunteers and patients with psoriasis were stained with antiprotein gene product 9.5 (PGP9.5) antibody. The images of PGP9.5-immunoreactive nerve fibres (green) were superposed with differential interference microscopic images. Scale bar: 75 μm. (e) Semiquantitative analyses of the number of PGP9.5-immunoreactive nerve fibres in the epidermis of healthy volunteers and psoriatic patients with or without itch. Each value represents the mean ± SD of each group.

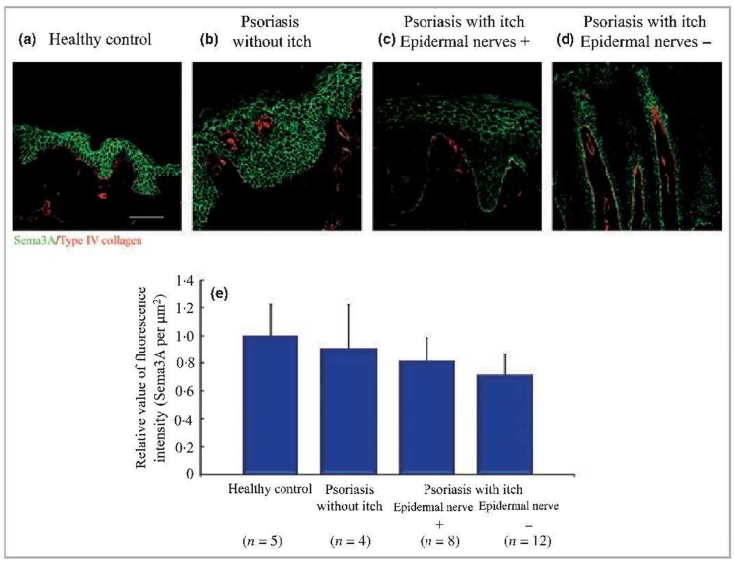

Fig 2. Expression levels of semaphorin 3A (Sema3A) in the epidermis of patients with psoriasis. (a–d) Double labelling for Sema3A (green) and type IV collagen (red) was performed on skin of healthy volunteers and psoriatic patients with or without itch. Scale bar: 75 μm. (e) The fluorescence intensity per unit area of epidermal Sema3A was calculated in each group, and statistical analysis was performed. Results are shown as values compared with the levels of fluorescence intensity in healthy volunteers. Each value represents the mean ± SD of each group.

decrease in pruritic psoriatic patients with and without penetration of nerve fibres into the epidermis, although the differences were not statistically significant (Fig. 2c).

Expression levels of μ- and κ-opioid receptors in the epidermis of patients with psoriasis

The level of μ-opioid receptor expression in the epidermis was the same in healthy volunteers and psoriatic patients with or without itch (Fig. 3a–d). Fluorescence intensity per unit area of epidermal μ-opioid receptors was unchanged between healthy controls and patients with psoriasis (Fig. 3e). However, the levels of κ-opioid receptor expression were decreased in the epidermis of patients with psoriasis, especially in those with itch, compared with healthy controls (Fig. 4a–d). Fluorescence intensity per unit area of epidermal κ-opioid receptors was significantly decreased in psoriatic patients with itch compared with healthy controls (Fig. 4e).

Expression levels of β-endorphin and dynorphin A in the epidermis of patients with psoriasis

There were no differences in expression levels of β-endorphin in the epidermis of psoriatic patients with or without itch in comparison with healthy volunteers (Fig. 5a). In contrast, dynorphin A levels were significantly decreased in the epidermis of pruritic psoriatic patients without penetration of intraepidermal nerve fibres compared with healthy controls (Fig. 5b).

Discussion

Numerous pruritogenic mediators and modulators released in the periphery can directly activate the itch-sensitive C-fibres by specific receptors on the nerve terminals or they can act indirectly by inducing the release of pruritogenic mediators and modulators from other cells. The nerve fibres are also activated by mechanical and chemical stimuli from the external environment, and thereby may elicit itch responses.[5,6,20]

Our immunohistochemical data showed that epidermal nerve fibre densities were higher in approximately 40% of pruritic psoriasis patients than in healthy controls. However, epidermal hyperinnervation was not found in all psoriatic patients with itch. Other researchers have reported a significant decrease in number of epidermal PGP9.5-immunoreactive nerve fibres in uninvolved psoriatic skin, and a further decrease was observed in mature lesions with almost complete

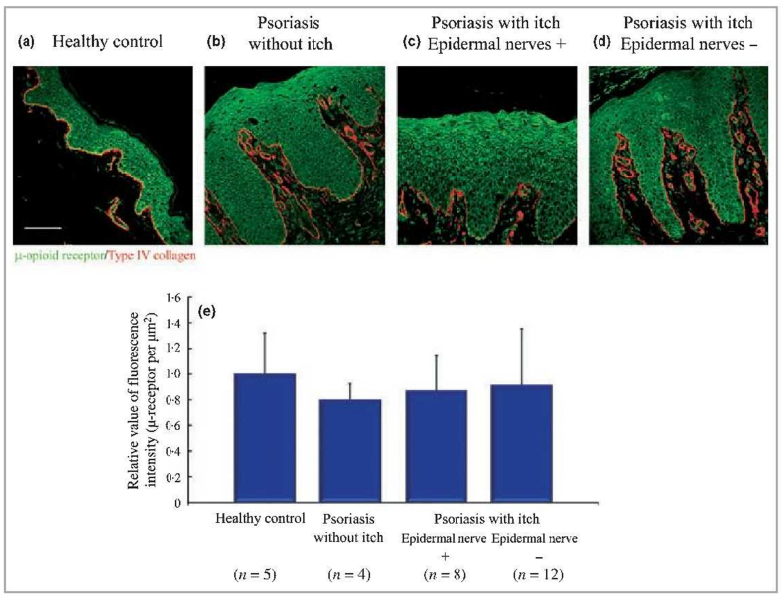

Fig 3. Expression levels of μ opioid receptor in the epidermis of patients with psoriasis. (a–d) Double labelling for μ opioid receptor (green) and type IV collagen (red) was performed on skin of healthy volunteers and psoriatic patients with or without itch. Scale bar: 75 μm. (e) Fluorescence intensity per unit area of epidermal μ-opioid receptor was calculated in each group, and statistical analysis was performed. Expression levels of μ-opioid receptor were unchanged among healthy volunteers, nonpruritic and pruritic patients with psoriasis. Results are shown as values compared with the levels of fluorescence intensity in healthy volunteers. Each value represents the mean ± SD of each group.

lack of epidermal nerve fibres in long-established psoriatic lesions.[8] Moreover, other groups did not find any relationship between SP-immunoreactive nerve fibres in the epidermis and the intensity of pruritus.[9] However, negative correlations between pruritus severity and plasma SP levels were also reported in patients with psoriasis.[21] Therefore, increased nerve fibres in the epidermis may not be an essential factor for the pathogenesis of itch in psoriasis.

Unlike patients with atopic dermatitis,[10,22] no significant relationship was found between nerve density and Sema3A levels in the epidermis in psoriasis patients with itch, although expression levels of epidermal Sema3A tended to be lower in patients with psoriasis compared with the healthy controls. More recently, it has been suggested that keratinocyte-derived anosmin-1, an extracellular glycoprotein, inhibits the penetration of sensory nerve fibres into the epidermis of patients with atopic dermatitis.[23] This may suggest the existence of other neuronal repulsion factors in the skin of patients with psoriasis.

In contrast to the epidermis, dermal nerve fibre numbers seem to be increased in almost all psoriatic patients with itch compared with healthy volunteers or patients without itch (Taneda *et al.*, unpublished observations). Therefore, although further studies are needed, dermal nerve density may be at least partly involved in itch perception of patients with psoriasis. This may also be supported by the increased number of PGP9.5-immunoreactive nerve fibres in the upper dermal areas of psoriatic patients with itch, as described previously.[4]

Experimentally, the suppressing activity of κ-opioid receptor agonists such as nalfurafine against different pruritogens has been reported in rodents and monkeys.[24–27] The antiscratch activity of nalfurafine against SP in mice[26] and morphine in monkeys[28] was attenuated by norbinaltorphimine, a κ-opioid receptor antagonist. In addition, nalfurafine was shown to inhibit compulsive scratching in mice elicited by subcutaneous administration of 5′-guanidinonaltrindole, a κ-opioid antagonist.[29] Our results indicate that the κ-opioid, but not the μ-opioid system, is downregulated in the epidermis of psoriatic patients with itch, similar to findings in patients with atopic dermatitis.[14] Moreover, peripheral opioid systems may play a role in pruritus.[13,14,30] For example, topical application to the skin of μ-opioid receptor antagonists inhibited pruritus in patients with atopic dermatitis,[31] and a

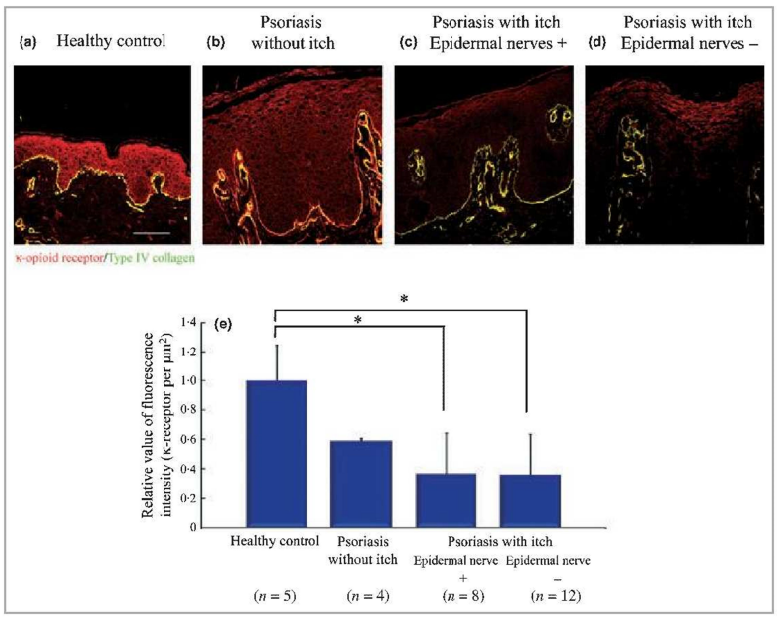

Fig 4. Expression levels of κ-opioid receptor in the epidermis of patients with psoriasis. (a–d) Double labelling for κ-opioid receptor (red) and type IV collagen (green) was performed on skin of healthy controls and psoriatic patients with or without itch. Scale bar: 75 μm. (e) Fluorescence intensity per unit area of epidermal κ-opioid receptor was calculated in each group, and statistical analysis was performed. Expression levels of epidermal κ-opioid receptor were significantly decreased in itchy psoriatic patients with or without epidermal nerves compared with the healthy controls (*$P < 0{\cdot}05$). Results are shown as values compared with the levels of fluorescence intensity in healthy volunteers. Each value represents the mean ± SD of each group.

peripherally restricted κ-opioid receptor agonist was found to antagonize chloroquine-induced scratching in mice.[32] Thus, although the roles of opioid systems in pruritus are unclear and may differ between primates and rodents,[28,33] these findings indicate that the epidermal opioid system is involved, at least in part, in the pathogenesis of psoriatic itch. To our knowledge, it has not yet been determined whether topical application of κ-opioid receptor agonists on to the skin inhibits itch in patients with psoriasis or atopic dermatitis. However, based on this and previous studies, μ-opioid receptor antagonists and/or κ-opioid receptor agonists may hold promise as potentially useful antipruritic agents in human conditions involving itch at the peripheral as well as the central level.

The pathogenesis of opioid-induced itch is still unclear, although two different mechanisms have been proposed at the peripheral level. The non-neuronal opioid receptors may influence the production of pruritogens or the cytokine pattern in keratinocytes.[34,35] There have also been numerous studies regarding the immune actions of the opioids, and immune cells have been described as targets.[13] Interestingly, morphine directs T cells toward Th2 differentiation.[36] Naloxone induces a shift from Th2 to Th1 cytokine pattern in mice.[37] Activation of κ-opioid receptors decreases the inflammatory response by downregulating several cytokines and chemokines.[13] Meanwhile, activation of μ-opioid receptors may induce a proinflammatory response.[13] Therefore, it will be necessary to carry out comprehensive screening for cytokines and pruritogens controlled by these opioid systems. Sensory neurones also express μ-opioid receptor and/or κ-opioid receptor.[38,39] More recently, we reported that some μ-opioid receptor-immunoreactive nerve fibres expressed gastrin-releasing peptide, which may be a marker for itch-specific nerves, in mouse skin with atopic dermatitis.[40,41] These observations raise the possibility that the opioid receptors on peripheral nerve fibres are directly linked to the modulation of itch.

The present study has the following limitations. Although both observers made observations of intraepidermal nerve fibres in a blinded manner inter- and intraobserver reliability was untested and variability was not calculated. However, as

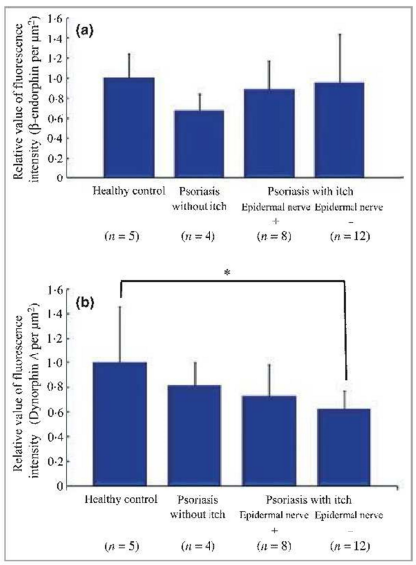

Fig 5. Expression levels of β-endorphin and dynorphin A in the epidermis of patients with psoriasis. (a) Fluorescence intensity per unit area of epidermal β-endorphin was calculated in each group, and statistical analysis was performed. Expression levels of β-endorphin were unchanged among healthy controls, nonpruritic and pruritic patients with psoriasis. (b) Fluorescence intensity per unit area of epidermal dynorphin A was calculated in each group, and statistical analysis was performed. Expression levels of epidermal dynorphin A were significantly decreased in epidermal nerve-negative psoriatic patients with itch compared with the healthy controls (*$P < 0{\cdot}05$). Results are shown as values compared with the levels of fluorescence intensity in healthy volunteers. Each value represents the mean ± SD of each group.

both observers have much experience we do not anticipate this to be a problem. The psoriatic group without itch contained few subjects compared with the psoriatic group with itch. A larger study may be useful in the future. The healthy controls were all male and all younger than the other subjects. Moreover, skin biopsies in the healthy controls were all taken from the abdomen as opposed to parts of the body involved in the psoriatic groups. Although this is not expected to pose a problem it should be mentioned.

In conclusion, our findings on Sema3A levels in the epidermis suggest that epidermal opioid systems, rather than hyperinnervation, may be involved in the pathogenesis of psoriatic itch, although the increase in nerve density may be partly responsible for modulation of itch perception. These findings may help us to understand the control mechanism of psoriatic itch at the peripheral level.

What's already known about this topic?

- Epidermal nerve fibres and opioid systems are partly involved in abnormal itch perception in atopic dermatitis.

What does this study add?

- Our observations indicate that, based on semaphorin 3A levels in the epidermis, epidermal opioid systems, rather than hyperinnervation, may be involved in the pathogenesis of psoriatic itch.

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