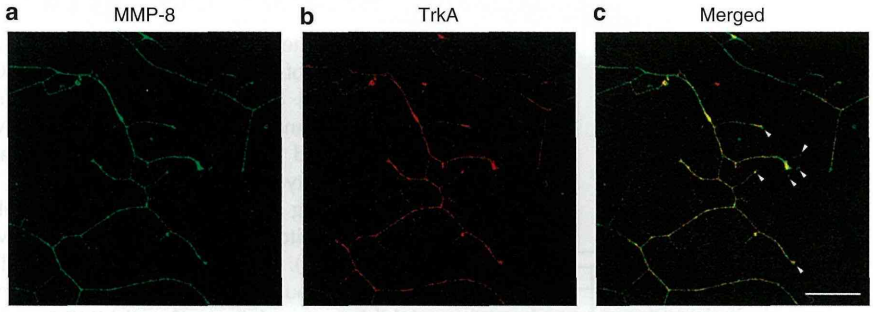

Figure 4. Immunocytochemical distribution of matrix metalloproteinase-8 (MMP-8) in cultured dorsal root ganglion neurons. (**a**) Neurons cultured for 48 hours in serum-free medium supplemented with 10 ng ml^{-1} nerve growth factor were immunostained with anti-MMP-8 (green) antibody. (**b**) The cells were also immunostained with anti-tropomyosin receptor kinase (TrkA; red) antibody. (**c**) MMP-8 immunoreactivities were observed in the growth cones of TrkA^{+} fibers. Yellow parts show double labeling. White arrowheads in **c** show the tips of nerve fibers. Bar = 47.62 μm.

as several MMPs, such as MMP-3, -7, -10, and -14, and even several bacterial proteases (Van Lint and Libert, 2006; Page-McCaw *et al.*, 2007). These observations indicate that MMP-8 activation is indeed strongly regulated and mostly limited to sites of inflammation. Our expression analyses using western blotting showed that tissue plasminogen activator proteins, not cathepsin G, were present in the conditioned medium of DRG neuronal cultures without induction by NGF stimulation (Supplementary Figure S3 online). Therefore, neuronal tissue plasminogen activator may constitutively convert pro-MMP-8 into the active form in our culture system. This was supported by the results of axonal outgrowth assay with aprotinin, which does not inhibit plasminogen activators (Sprengers and Kluft, 1987). In addition, pro-MMP-8 may be activated by other serine proteases as described above from non-neuronal cells in the skin.

MMP-8 expression in the cultured neurons was modulated by other factors in addition to NGF, such as Sema3A, which induces growth cone collapse and axonal repulsion (Fujisawa, 2004). This molecule inhibited nerve fiber penetration because of the NGF concentration gradient, concomitant with downregulation of *MMP-8*, suggesting reciprocal nerve fiber penetration mechanisms between these two molecules. Such effects of Sema3A were also found in our previous study using the BCC system (Tominaga *et al.*, 2009a). Increases in nerve density are observed in the dermis of patients with AD or psoriasis (Urashima and Mihara, 1998; Nakamura *et al.*, 2003). Similar findings have been observed in animal models, such as NC/Nga mice (Tominaga *et al.*, 2009b). A recent study showed that spontaneous scratching is enhanced by intradermal injection of pruritogens in a mouse model of chronic dry skin (Akiyama *et al.*, 2010). This hyperknesis may be because of sensitization of itch-signaling neurons. Indeed, anti-NGF and NGF receptor inhibitor approaches or recombinant Sema3A replacement approaches suppressed pruritus in atopic NC/Nga mice (Takano *et al.*, 2005, 2007; Yamaguchi *et al.*, 2008). Therefore, the participation of *MMP-8* under the control of the axonal guidance molecules is suggested in these clinical backgrounds.

Additional factors are components of the ECM, especially substrates for MMP-8. A role of integrins in growth cone movement during neural development and repair has been suggested by *in vitro* studies of neurite outgrowth on different ECM components (Reichardt and Tomaselli, 1991). In addition, neurotrophins and ECM together induce robust axon outgrowth (Goldberg *et al.*, 2002; Liu *et al.*, 2002), suggesting that coordinated activation of neurotrophin and ECM-integrin signaling is necessary for efficient and long-distance axon extension (Rossino *et al.*, 1990; Lefcort *et al.*, 1992; Grabham and Goldberg, 1997; Werner *et al.*, 2000; Danker *et al.*, 2001). Thus, when the elongation of nerve fibers is initiated by NGF stimulation in our BCC system or *in vivo*, integrins will be accumulated at the growth cone to interact with a variety of ECM components (Grabham and Goldberg, 1997). During this process, MMPs are required for the growth cone to abrogate the three-dimensional ECM barriers. Appropriate MMPs corresponding to surrounding ECM components of the growing nerve fibers are probably selected and upregulated for efficient nerve fiber penetration. This may also affect the gene expression of molecules involved in pro-MMP activation. This is supported by our previous observation that MMP-2 expression is enhanced by its substrates in cultured DRG neurons (Tominaga *et al.*, 2009a). Meanwhile, Sema3A stimulation on the growing nerve fiber may provide a reverse signaling pathway in these events because class 3 semaphorin signaling inhibits integrin-mediated adhesion signaling (Zhou *et al.*, 2008). Therefore, although the integrin-mediated regulatory system remains unclear from the results of this study, such a mechanism may be applicable to pruritic skin diseases involving hyperinnervation.

MATERIALS AND METHODS

Antibodies and reagents

The antibodies and reagents used in this study are described in the Supplementary text online.

DRG neuron cultures

Neonatal rat DRG neurons were purchased from Lonza Walkersville (Walkersville, MD), and maintained in serum-free medium

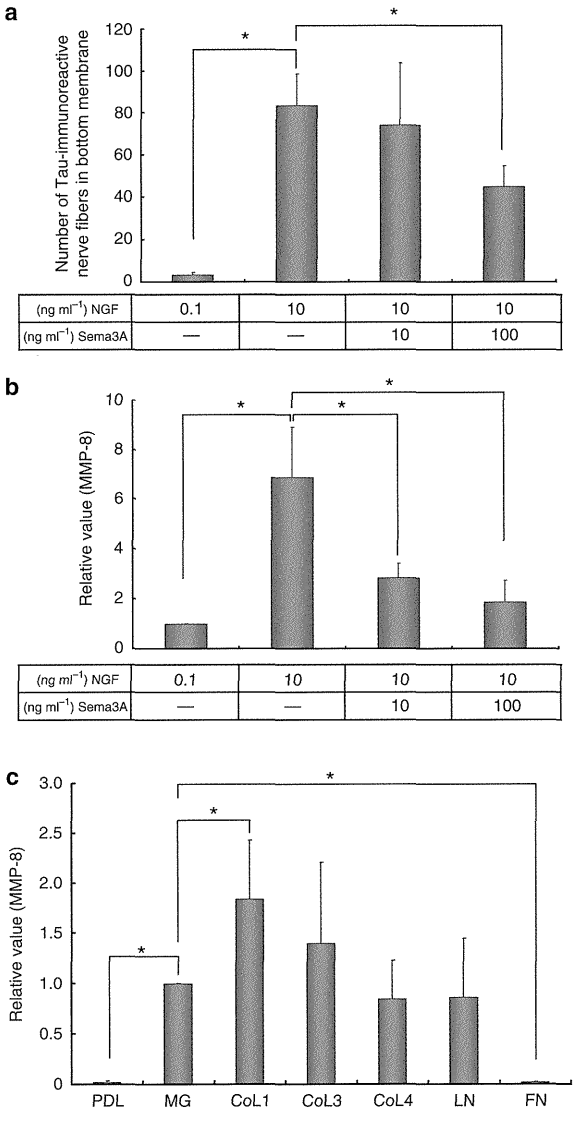

Figure 5. Modulation of matrix metalloproteinase-8 (*MMP-8*) expression by semaphorin 3A (Sema3A) or extracellular matrix components. (**a**) Axonal outgrowth assays in the Boyden chamber culture system with concentration gradients of both nerve growth factor (NGF) and Sema3A. Sema3A dose dependently inhibited NGF-induced nerve penetration. (**b**) Quantitative reverse transcriptase-PCR analysis of *MMP-8* expression in the cultured neurons. Sema3A inhibited NGF-induced *MMP-8* expression. (**c**) Quantitative reverse transcriptase-PCR analysis of *MMP-8* expression in the neurons cultured on various extracellular matrices in the presence of NGF concentration gradient. Results are shown as values compared with the levels of gene expression in neurons cultured on the MG-coated membrane. The values in **a** and **c** represent the means ± SD from three independent experiments. *$P<0.05$. CoL, collagen; FN, fibronectin; MG, Matrigel; LN, laminin; PDL, poly-D-lysine.

consisting of Dulbecco's minimal essential medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 0.1 or 10 ng ml^{-1} NGF, 0.5% N-2 supplement, 87.5 ng ml^{-1} 5-fluoro-2′-deoxyuridine, 37.5 ng ml^{-1} uridine, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin at 37 °C, 5% CO_2 according to the manufacturer's recommendations. CoL1 was used in the DRG neuron culture system for axonal outgrowth assays using Boyden chambers. For immunocytochemistry, DRG neurons (5×10^3 cells) were plated in the wells of Biocoat CultureSlides (BD Falcon, Bedford, MA) coated with CoL1 in 1 ml of culture medium as described above.

Axonal outgrowth assays using a BCC system

Ice-cold CoL1 solution, 10 × minimal essential medium, and reconstitution buffer containing 0.05 N NaOH, 2.2% $NaHCO_3$, and 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were mixed at a ratio 8:1:1, and the mixture was kept on ice to prevent gel formation. The ice-chilled reconstituted CoL solution (40 μl per chamber) was added to the upper surface of the 0.4 μm pore size polyester insert of a 24-well Boyden chamber (Millipore, Beverly, MA), and allowed to gel at 37 °C for 30 minutes. DRG neurons (1×10^4 cells) were placed on the CoL gel in 200 μl of culture medium with 0.1 ng ml^{-1} NGF. Then, 1 ml of culture medium with 10 ng ml^{-1} NGF was also added to the lower chamber of the BCC system. In some experiments, MMP-8 inhibitor I (5 or 50 nM), MMP-8 inhibitor I negative control (50 nM), aprotinin (0.2 or 20 μg ml^{-1}), anti-MMP-8 (10 μg ml^{-1}), and anti-MMP-2 (10 μg ml^{-1}) were added to both upper and lower chambers of the BCC system, and recombinant Sema3A protein (10 or 100 ng ml^{-1}) was added to the lower chamber only.

After culture for 48 hours in this system, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The CoL1 gel-coated membrane was removed from the Boyden chamber, and then stained with anti-Tau antibody. Immunofluorescence staining of the underside of the membrane was observed by confocal laser scanning microscopy (DMIRE2; Leica, Wetzlar, Germany), which revealed nerve fibers that had crossed the CoL1 gel-coated membrane. For measurement of the number of crossed nerve fibers, three membranes in each group were stained with anti-Tau antibody. The number of Tau$^+$ fibers per membrane was hand counted by two researchers (ST and AK) in a blinded manner.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes. They were washed three times with phosphate buffered saline (phosphate-buffered saline (PBS), pH 7.4) for 5 minutes and blocked in PBS with 2% bovine serum albumin and 5% normal donkey serum for 1 hour at room temperature. Cells were overlaid with primary antibodies at working dilutions and incubated for 16 hours at 4 °C. After washing with PBS, the cells were incubated with secondary antibodies for 1 hour at room temperature. After washing with PBS, they were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK). Immunoreactivity was visualized by confocal laser scanning microscopy.

Neurite outgrowth assays in two-dimensional cultures of DRG neurons

Wells of 24-well tissue culture plates were coated with a thin layer of CoL1, and then DRG neurons (5×10^3 cells) were plated on each well for neurite outgrowth assays. Cells were maintained in

serum-free medium as described above. In some experiments, MMP-8 inhibitor I (5, 25, or 50 nM) was added to the medium. After culture for 48 hours, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes. The fixed cells in each well were observed by phase-contrast microscopy (DMIL; Leica). For quantification of neurite outgrowth, photographs were taken of nine random fields per well, and the length of the longest process of DRG neurons was measured with BZ-H2A software (Keyence, Osaka, Japan). At least 100 cells from two replicate wells per group were analyzed in each experiment. The data from three independent experiments (at least 300 cells per group) were used for statistical analysis.

Methylthiazole tetrazolium assay

Wells of 96-well tissue culture plates were coated with a thin layer of CoL1 and then DRG neurons (5×10^3 cells) were cultured in serum-free medium with NGF (0.1 or 10 ng ml^{-1}). In some experiments, Sema3A (10 or 100 ng ml^{-1}), MMP-8 inhibitor I (5, 25, or 50 nM) or vehicle (PBS or dimethyl sulfoxide) was added to the medium, concomitant with 10 ng ml^{-1} NGF. After culture for 48 hours, cell viability was determined by methylthiazole tetrazolium Cell Growth Assay (Millipore) according to the manufacturer's instructions. The data from three independent experiments were used for statistical analysis.

Western blotting analysis

Conditioned media were collected from DRG neurons for 48 hours in the BCC system, and then concentrated with Amicon Ultra-4 (Millipore) according to the manufacturer's instructions. Human neutrophil lysates were prepared according to the protocol described previously (Nakayama *et al.*, 2008). Aliquots of 15 μg of total protein were loaded per lane. Samples, rat recombinant MMP-8 standard (30 ng per lane) and molecular weight markers were subjected to 10% SDS-PAGE. After electrophoresis, western blotting was performed as described previously (Tengara *et al.*, 2010) using goat anti-MMP-8 antibody (1:1,000 dilution; R&D Systems, Minneapolis, MN). After western blotting, the membranes were stained with Ponceau solution. Densitometry was also performed with a Scion Image software (Scion, Frederick, MD) for semiquantitative analysis.

Total RNA preparation

DRG neurons were cultured in the BCC system for 48 hours. To prepare total RNA from the cultured cells, the membranes coated with various ECM proteins were removed from the Boyden chamber, and then total RNA was isolated with an RNeasy Fibrous Tissue Mini kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. Universal Rat Reference RNA (Stratagene, La Jolla, CA) was used as a positive control qRT-PCR.

qRT-PCR analysis

The protocols for qRT-PCR analysis were described previously (Tominaga *et al.*, 2007b). The primers used in this study are listed in Supplementary Table S1 online. They were designed to meet specific criteria and were synthesized by Perfect Real Time support system (TaKaRa, Kyoto, Japan). The PCR specificity was confirmed by dissociation curve analysis and gel electrophoresis. The levels of gene expression were calculated relative to expression of ribosomal protein S18 (*RPS18*).

Statistical analysis

Statistical analyses were performed by two-tailed Student's *t*-test and one-way analysis of variance with Bonferroni's multiple comparison test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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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.

K.T. and M.T. contributed equally to this work.

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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

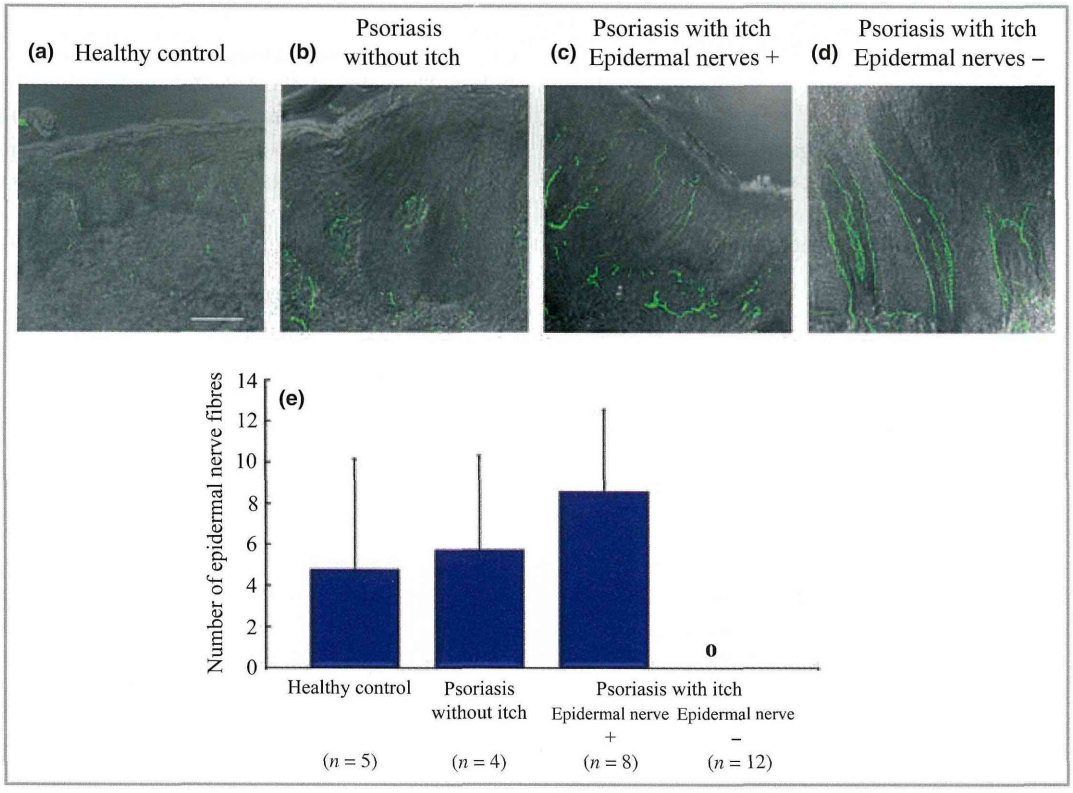

Fig 1. Nerve densities in the epidermis of patients with psoriasis. (a–d) Skin specimens from healthy volunteers and patients with psoriasis were stained with antiprotein gene product 9.5 (PGP9.5) antibody. The images of PGP9.5-immunoreactive nerve fibres (green) were superposed with differential interference 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.

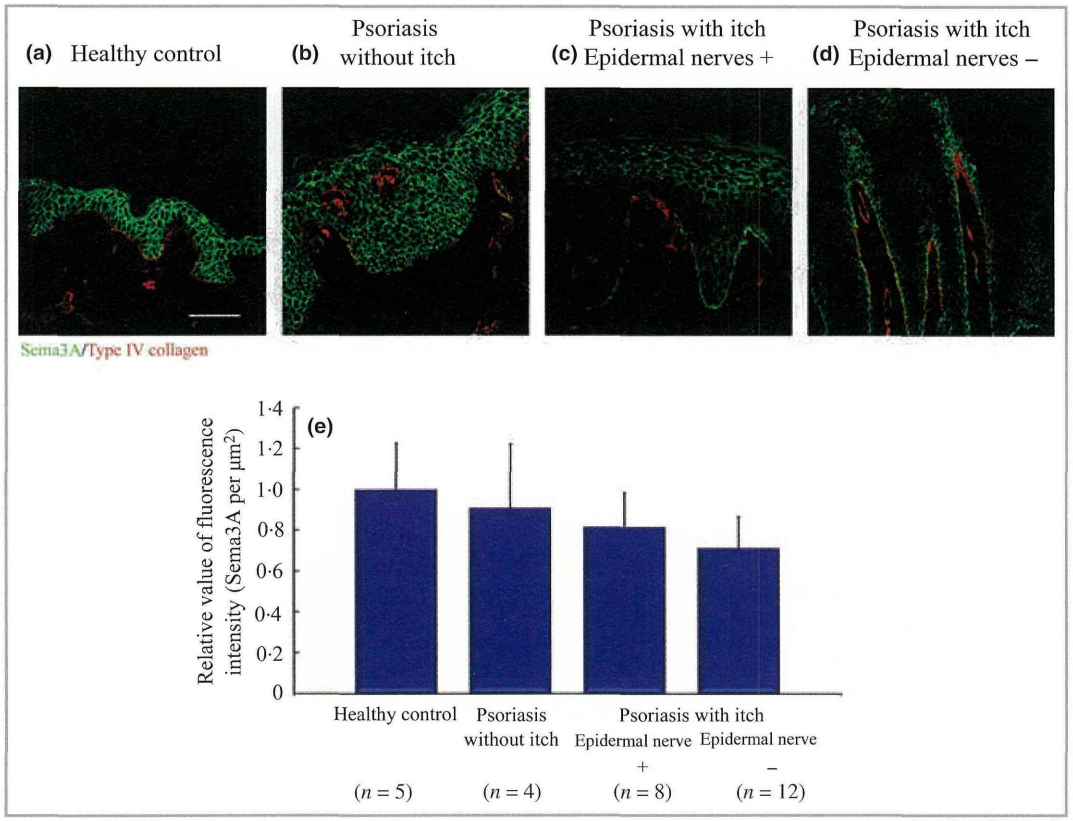

Fig 2. Expression levels of semaphorin 3A (Sema3A) in the epidermis of patients with psoriasis. (a–d) Double labelling for Sema3A (green) and type IV collagen (red) was performed on skin of healthy volunteers and psoriatic patients with or without itch. Scale bar: 75 μm. (e) The fluorescence intensity 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. 2e).

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

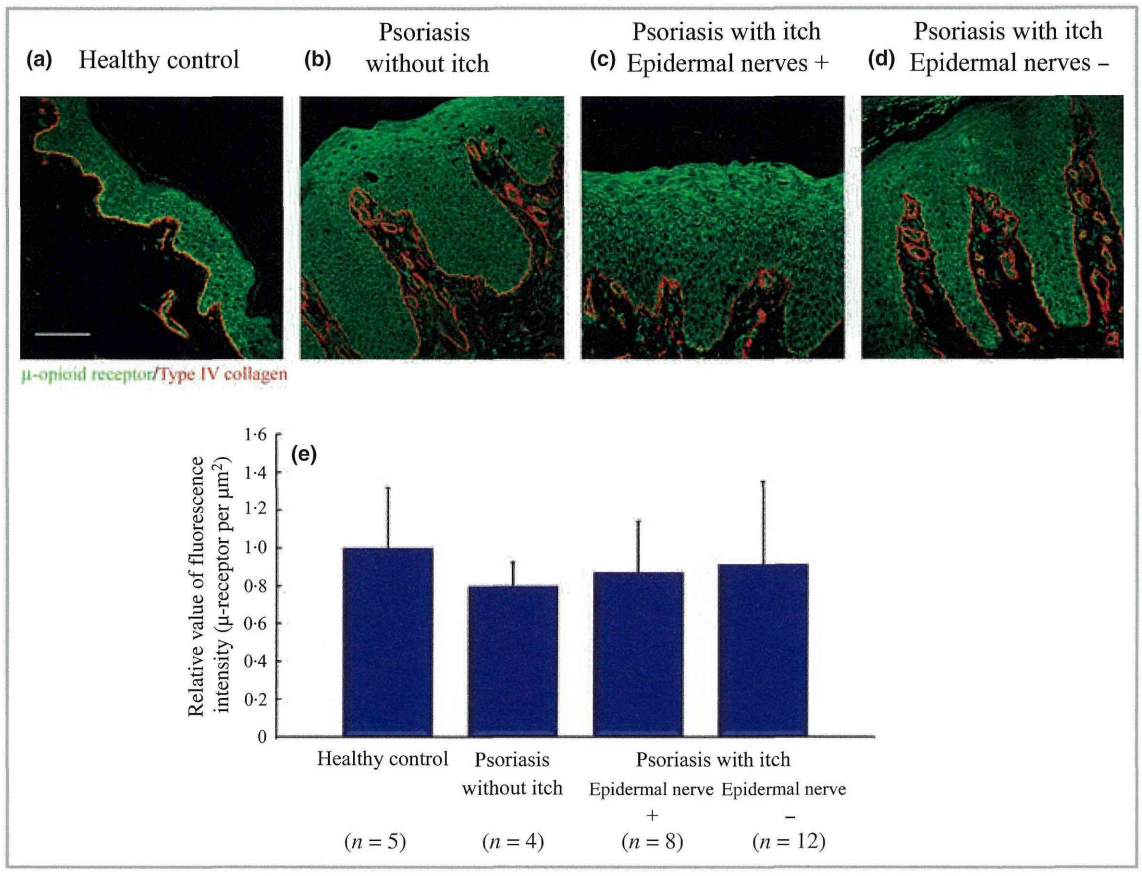

Fig 3. Expression levels of μ-opioid receptor in the epidermis of patients with psoriasis. (a–d) Double labelling for μ-opioid receptor (green) and type IV collagen (red) was performed on skin of healthy volunteers and psoriatic patients with or without itch. Scale bar: 75 μm. (e) Fluorescence intensity 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

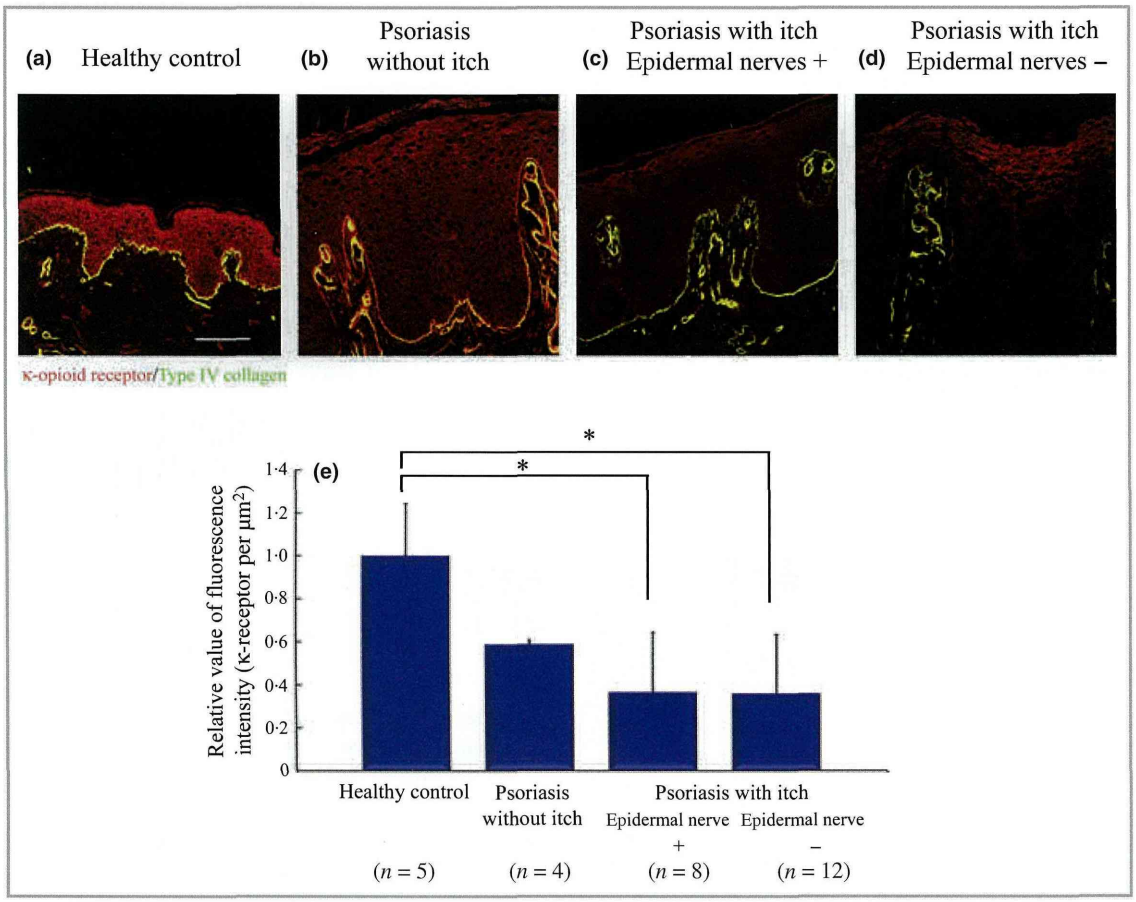

Fig 4. Expression levels of κ-opioid receptor in the epidermis of patients with psoriasis. (a–d) Double labelling for κ-opioid receptor (red) and type IV collagen (green) was performed on skin of healthy controls and psoriatic patients with or without itch. Scale bar: 75 μm. (e) Fluorescence intensity 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

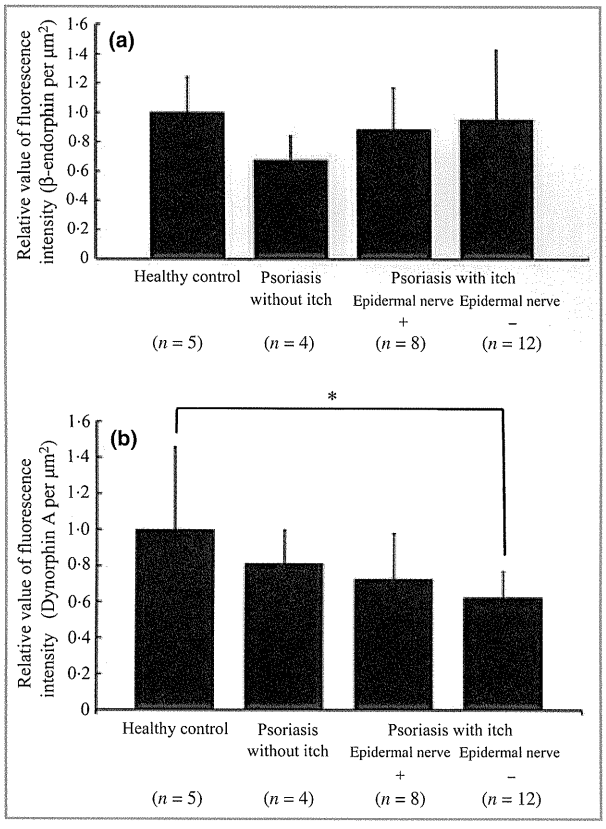

Fig 5. Expression levels of β-endorphin and dynorphin A in the epidermis of patients with psoriasis. (a) Fluorescence intensity per unit area of epidermal β-endorphin was calculated in each group, and statistical analysis was performed. Expression levels of β-endorphin were unchanged among healthy controls, 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 (*$P < 0.05$). 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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Inhibitory effects of UV-based therapy on dry skin-inducible nerve growth in acetone-treated mice

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ABSTRACT

Background: UV-based therapy has anti-pruritic effects in inflammatory skin diseases, such as atopic dermatitis and psoriasis. These anti-pruritic effects may be partly due to inhibition of intraepidermal nerve growth, but they have not been fully characterized.
Objective: This study was performed to characterize the anti-nerve growth effects of UV-based therapies in acetone-treated mice as an acute dry skin model.
Methods: Nerve fibers penetrate into the epidermis 24 h after acetone treatment in mice, and nerve growth peaks 48 h after acetone treatment. To investigate the effects of UV-based therapies on the epidermal nerve fibers, including combination treatment with corticosteroid ointment, the mice were treated with psoralen ultraviolet A (PUVA), PUVA and betamethasone valerate ointment (PUVA + BV), narrowband ultraviolet B (NB-UVB), or an excimer lamp. Each therapy was provided 24 h after acetone treatment, and skin samples were taken 48 h later. Nerve fiber densities and expression levels of nerve growth factor (NGF) and semaphorin 3A (Sema3A) in the epidermis were examined by immunohistochemistry.
Results: Penetration of nerve fibers into the epidermis was observed in the acetone-treated mice, concomitant with increased NGF and decreased Sema3A levels in the epidermis. The acetone-induced intraepidermal nerve growth was significantly decreased by PUVA, PUVA + BV, NB-UVB, and excimer lamp treatments compared with controls. In addition, PUVA + BV and NB-UVB normalized the abnormal expression of NGF and Sema3A in the epidermis, but no such normalization was observed with excimer lamp treatment.
Conclusion: UV-based therapies, especially NB-UVB and excimer lamp treatments, may be effective therapeutic methods for pruritus involving epidermal hyperinnervation.

1. Introduction

Itching, or pruritus, an unpleasant sensation associated with the desire to scratch, frequently accompanies a variety of inflammatory skin conditions and systemic diseases. Histamine is the best-known pruritogen in humans and is also used as an experimental itch-causing substance. Clinically, antihistamines, *i.e.*, histamine H_1 antagonists, are commonly used to treat all types of itch resulting from renal and liver diseases, as well as from serious skin diseases, such as atopic dermatitis (AD). However, antihistamines often lack efficacy in patients with chronic itch [1–3].

Increased epidermal nerve density is considered as one cause of antihistamine-resistant itch, suggesting that the lesioned skin is susceptible to stimulation and sensitive to itching [4]. The sprouting of epidermal nerve fibers associated with pruritus is found in AD [5,6], xerosis [7], allergic contact eczema [8], and prurigo nodularis [9], and in experimental animal models [10,11]. Nerve growth factor (NGF) produced by keratinocytes is an important growth factor that determines skin innervation [12], and increased plasma concentrations of NGF in AD patients are correlated with disease severity [13]. We recently demonstrated that levels of semaphorin 3A (Sema3A), which induces retraction of NGF-responsive sensory afferents, are decreased in the epidermis of AD patients [14], suggesting that epidermal innervation is regulated by the fine balance between nerve elongation factors

Abbreviations: AD, atopic dermatitis; AP-1, activator protein-1; BSA, bovine serum albumin; BV, betamethasone valerate; IL, interleukin; 8-MOP, 8-methoxypsoralen; NB-UVB, narrowband ultraviolet B; NDS, normal donkey serum; NGF, nerve growth factor; PBS, phosphate-buffered saline; PGP9.5, protein gene products 9.5; PUVA, psoralen ultraviolet A; Sema3A, semaphorin 3A; TEWL, transepidermal water loss; TNF, tumor necrosis factor.

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(*e.g.*, NGF, amphiregulin, gelatinase) [6,10] and nerve repulsion factors (*e.g.*, Sema3A, anosmin-1) [14,15].

UV-based therapies, such as psoralen ultraviolet A (PUVA) and narrowband-ultraviolet B (NB-UVB), are efficacious in the treatment of chronic pruritus in patients with AD [16], psoriasis [17], and lichen amyloidosus [18]. Recently, excimer laser has been shown to ameliorate dermatitis in psoriasis patients and also pruritus in AD patients [19]. Our previous study showed that PUVA therapy reduces epidermal hyperinnervation in AD patients by normalizing abnormal Sema3A and NGF expression in their epidermis [6]. However, the anti-nerve growth effects of other UV-based therapies, such as NB-UVB and excimer lamps, have not been fully characterized.

In this study, we examined the anti-nerve growth effects of UV-based therapies, including combination therapy with corticosteroid ointment, in acetone-treated mice, which show epidermal hyperinnervation. Here, we describe an effective UV-based therapeutic strategy for epidermal hyperinnervation that partly involves the regulation of itch.

2. Materials and methods

2.1. Animals

Male ICR mice (10 weeks old; SLC Japan, Shizuoka, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under a 12 h light:12 h dark cycle at a regulated temperature of 22–24 °C, with food and tap water provided *ad libitum*. Care and handling of all animals conformed to the NIH guidelines for animal research, and all animal procedures were approved by the Institutional Animal Care and Use Committee of the Juntendo University Graduate School of Medicine.

2.2. Antibodies and reagents

The primary antibodies used in this study were anti-protein gene product 9.5 (PGP9.5, 1:4000 dilution; BIOMOL International Corporation, Plymouth Meeting, PA, USA), rabbit anti-NGF (1:500 dilution; Millipore Corporation, Billerica, MA, USA) and rabbit anti-Sema3A (1:200 dilution; Abcam Inc., Cambridge, MA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA).

Bovine serum albumin (BSA) and 8-methoxypsoralen (8-MOP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Normal donkey serum (NDS) was purchased from Chemicon (Temecula, CA, USA). Vectashield mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA).

2.3. Treatment of cutaneous barrier disruption

The protocol for cutaneous barrier disruption has been described previously [11]. Briefly, the hair over the rostral part of the back was shaved at least 3 days before acetone treatment, and each shaved area was treated with acetone-soaked cotton balls for 5 min. In the control group, the shaved area was treated with sterile water.

Following anesthesia with sevoflurane (Abbott Japan, Osaka, Japan), transepidermal water loss (TEWL) was measured using a Tewameter® TM210 (Courage & Khazawa, Cologne, Germany) for 30 s.

2.4. UV-based therapy

The skin of acetone-treated mice was treated with UV-based therapies 24 h after acetone treatment ($n = 6$, each group). An untreated group was also used as a control.

2.4.1. Psoralen and ultraviolet A (PUVA) therapy or combination of PUVA and betamethasone valerate (BV) ointment

Following intraperitoneal injection of 4 mg/kg body weight of 8-MOP, mice were irradiated with 4 J/cm^2 of UVA (320–375 nm) using a 6 W UVL-56 Handheld UV Lamp (UVP Inc., Upland, CA, USA) [20]. 8-MOP was dissolved in absolute ethanol to 5 g/L and diluted 1:10 with sterile water immediately prior to injection as described [21]. In the PUVA + BV group, mice were treated with 100 mg per site of BV ointment (Rinderon®V; Shionogi & Co., Ltd., Osaka, Japan) after PUVA therapy. Control groups were treated with 8-MOP or BV alone.

2.4.2. Narrow-band ultraviolet B (NB-UVB) therapy

Mice were irradiated with 1 J/cm^2 of NB-UVB (311 nm) [22], using an 18 W 120UVB-NB model (Solark Systems, Inc., Ontario, Canada).

2.4.3. Excimer lamp therapy

A xenon-chloride gas 308-nm excimer lamp (E500-308P model; Excimer, Inc., Kanagawa, Japan) was used to irradiate mice at a dose of 250 mJ/cm^2 [23].

Each UV irradiance was measured using a radiometer (Sato Shouji, Inc., Kanagawa, Japan).

2.5. Immunohistochemistry

Skin samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h. After washing with phosphate buffered saline (PBS, pH 7.4), the samples were immersed successively in PBS solutions containing 10%, 15%, and 20% sucrose. The skin samples were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and cut into cryosections (20 μm thick for PGP9.5 staining or 8 μm thick for NGF staining) using a CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were mounted on silane-coated glass slides. After blocking in PBS with 5% NDS and 2% BSA, the sections were incubated with antibodies against PGP9.5 and NGF overnight at 4 °C.

For immunofluorescence staining of Sema3A, skin samples were embedded in OCT compound without fixation, and cryosections 8 μm thick were fixed with ice-cold acetone for 10 min at −20 °C. The sections were rehydrated in PBS, blocked in PBS with 5% NDS and 2% BSA, and incubated with antibodies against Sema3A overnight at 4 °C. After washing with PBS, the sections were incubated with secondary antibodies for 1 h at room temperature and mounted in Vectashield mounting medium. Immunoreactivity was viewed with a confocal laser-scanning microscope (DMIRE2; Leica Microsystems).

2.6. Semi-quantification of epidermal nerve fibers

Three skin specimens from each mouse were stained with anti-PGP9.5 antibody. Using a confocal microscope, optical sections 0.9 μm thick were scanned through the *z*-plane of the stained specimens (thickness 20 μm), and the images were reconstructed in three dimensions using Leica Confocal Software (Leica Microsystems). The numbers of epidermal nerves were determined by analyzing at least 27 confocal images from each group. The numbers of epidermal nerve fibers in areas of 1.6×10^5 μm^2 were hand-counted in the images by two researchers (M.T. and S.T.) blinded to treatment. All values are reported as the means ± standard deviation (SD) of three experiments.

2.7. Semi-quantitative measurements of immunohistochemical fluorescence intensity

Three skin specimens from each mouse were stained with anti-NGF or anti-Sema3A antibody, and the fluorescence intensity of

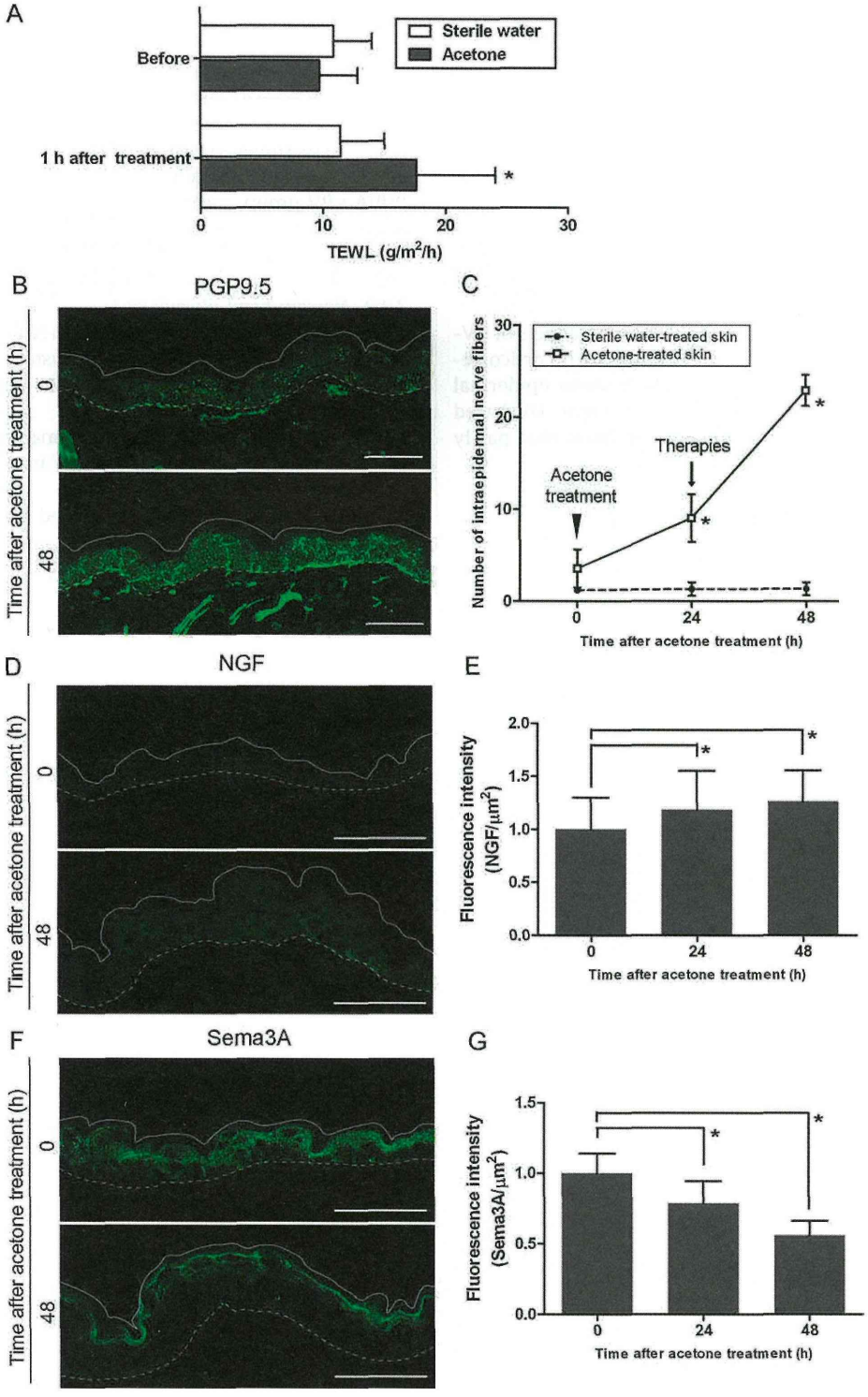

Fig. 1. Distribution of nerve fibers and NGF and Sema3A levels in the epidermis of acetone-treated mice. (A) TEWL was significantly increased 1 h after acetone treatment. $^*P < 0.01$ *vs.* sterile water-treated control mice. (B) Acetone treatment significantly increased the penetration of nerve fibers into the epidermis. (C) Increased numbers of nerve fibers were observed after acetone treatment, but not in skin treated with sterile water. $^*P < 0.001$ *vs.* sterile water-treated control mice at each time point. Arrowheads and arrows show the time points of acetone treatment and therapies, respectively. (D) Expression pattern of epidermal NGF in sterile water- and acetone-treated mice. (E) The fluorescence intensity of epidermal NGF was significantly increased after acetone treatment ($^*P < 0.05$). (F) Expression pattern of epidermal Sema3A in sterile water- and acetone-treated mice. (G) Significant decreases in the levels of epidermal Sema3A were observed after acetone treatment ($^*P < 0.05$). Scale bars, 50 μm. White and broken lines indicate the skin surface and the border between the epidermis and dermis, respectively.

each mouse was evaluated using 15 confocal images. Exposure and acquisition settings were fixed such that no signal saturation occurred. The total fluorescence intensity in the epidermis of each mouse was measured using Leica Confocal Software (Leica Microsystems), and fluorescence intensity per unit area was calculated. All values are reported as the means ± SD of three experiments.

2.8. Statistical analysis

Data were analyzed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The differences between means were analyzed by Tukey's multiple comparison tests.

3. Results

3.1. Distribution of nerve fibers and NGF and Sema3A levels in the epidermis of acetone-treated mice

We confirmed the skin dryness and cutaneous barrier disruption by TEWL measurement. TEWL was increased and peaked at 1 h after acetone treatment (Fig. 1A). The distribution of intraepidermal nerve fibers was examined immunohistochemically in the skin of acetone-treated mice. Intraepidermal nerve fibers were occasionally observed in control mice treated with sterile water (data not shown). Penetration of nerve fibers into the epidermis was observed in the acetone-treated skin, and nerve growth was increased 48 h after acetone treatment (Fig. 1B and C).

Immunohistochemical measurements of the levels of expression of NGF and Sema3A in the epidermis of acetone-treated mice showed that epidermal NGF levels were significantly increased (Fig. 1D and E), while epidermal Sema3A levels were significantly decreased (Fig. 1F and G), 24 and 48 h after acetone treatment.

3.2. Anti-nerve growth effects of UV-based therapies on acetone-treated mice

Acetone-induced epidermal hyperinnervation was significantly decreased in PUVA, PUVA + BV, NB-UVB, and excimer lamp treated groups compared with that in the nontreated group (Fig. 2A and B). Both NB-UVB and excimer lamp treatments had a particularly strong effect on intraepidermal nerve growth (Fig. 2B). No signs of redness or burning of the skin of the mice within the treated site were observed in all groups (data not shown).

3.3. Effects of UV-based therapies on epidermal NGF and Sema3A levels in acetone-treated mice

Epidermal NGF levels were significantly decreased in the skin treated with BV, PUVA, PUVA + BV and NB-UVB, while no alteration was observed in excimer lamp treated group (Fig. 3A). The significant increase of the levels of epidermal Sema3A was observed in the PUVA + BV and NB-UVB treated groups (Fig. 3B).

4. Discussion

Epidermal hyperinnervation is found in the skin of patients with AD, psoriasis, and xerosis, and is associated with intractable itch [4]. In clinical settings, PUVA therapy is often used together with corticosteroid ointment as adjunctive treatment in patients with inflammatory skin diseases [24], although the effects of each therapy on nerve growth in the skin remain unclear. In the present study, the rank order of anti-nerve growth effect was: BV < PUVA < PUVA + BV. Our data also showed that the epidermal NGF and Sema3A levels were significantly normalized in the PUVA + BV-treated group. Previous studies have shown that UV-based therapies reduce the number of epidermal nerve fibers [6,25] and concomitantly normalize the levels of Sema3A and NGF in the epidermis of AD patients [6]. These observations suggest that epidermal hyperinnervation is regulated by the fine balance between nerve elongation and repulsion factors. These findings may explain the differences in the degree of anti-nerve growth effects among the treatments used in this study.

The expression of axonal guidance molecules controlled by PUVA treatment was examined in this study. Psoralen acts by interfering with activator protein-1 (AP-1) in murine keratinocytes, thereby inhibiting the ability of AP-1 to bind to DNA [26]. Although the Sema3A promoter has not yet been investigated, the NGF promoter contains an AP-1 element important for transcriptional activity [27,28]. PUVA has been reported to affect chromatin structure in human epithelial cells [29,30], and these changes in chromatin structure may influence the DNA binding activity of transcription factors [31]. These findings may explain the mechanism of PUVA-regulated gene expression in epidermal keratinocytes.

Another possibility is the gene regulation of axonal guidance molecules by inflammatory cytokines produced by cutaneous cells, such as keratinocytes and immune cells. A recent study indicated that tumor necrosis factor (TNF)-α enhances NGF production *via* the Raf-1/MEK/ERK pathway in cultured normal human epidermal keratinocytes [32]. Although UV irradiation induces cytokine secretion from cultured keratinocytes, successful UV-based therapy of atopic dermatitis is known to be associated with downregulation of cytokine production in the inflamed skin [33]. Our preliminary experiments also showed that Sema3A expression in cultured normal human epidermal keratinocytes is controlled by some inflammatory cytokines (Tengara et al., unpublished observations). Therefore, these findings raise the possibility that PUVA regulates the expression of axonal guidance molecules by reducing the cytokine levels in the skin.

Glucocorticoids have also been reported to reduce the expression of proinflammatory genes by repression of key inflammatory transcription factors, including AP-1 and NF-κB [34]. Thus, BV may also inhibit NGF expression in epidermal keratinocytes through repression of AP-1 transcription factor expression. A more recent study indicated that TNF-α and interleukin (IL)-1β were upregulated in UVA-irradiated keratinocytes and its effect was inhibited by Jun-N-terminal kinase (JNK) inhibitor [35]. JNKs can induce the expression of AP-1 proteins and also increase the transduction activity of AP-1 complexes by phosphorylation [36]. These data suggest that BV application after PUVA therapy suppresses the UVA-induced inflammation response. Therefore, the anti-nerve growth activity of combination treatment may be greater than either treatment alone.

Interestingly, we also found that the anti-nerve growth effects of NB-UVB and excimer lamp treatments were more effective than PUVA + BV treatment. It is generally accepted that UVA penetrates into the dermis, while UVB is limited almost exclusively to the epidermis [37]. This suggests that the high efficacy of UVB irradiation is restricted to the epidermal region alone, and may explain the different anti-nerve growth effects among the UV-based therapies used in this study. This is supported by clinical studies using PUVA, NB-UVB, or excimer lamp therapies [38,39].

Photobiologically, the wavelengths of NB-UVB and excimer lamp are very close to each other, and therefore their therapeutic effects seem to be similar [40]. Although our study showed a strong inhibitory effect of NB-UVB and excimer lamp on epidermal nerve growth, these UV-based therapies had different effects on the expression of axonal guidance molecules in the epidermis of acetone-treated mice.

Short-wave radiation, such as UVB, excites DNA directly and generates photoproducts, such as cyclobutane pyrimidine dimers

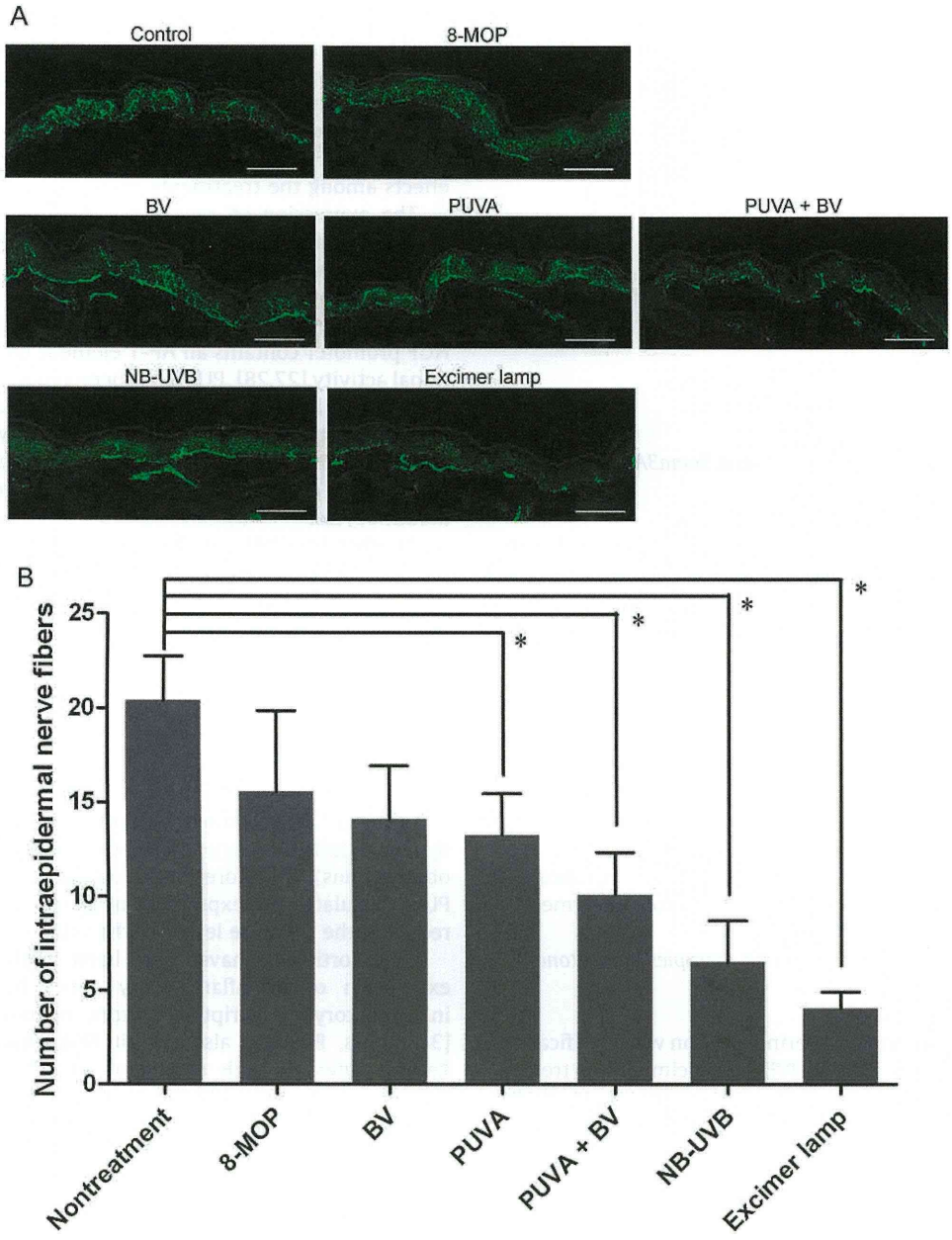

Fig. 2. Effects of UV-based therapy on intraepidermal nerve fibers in acetone-treated mice. (A) Distributions of intraepidermal nerve fibers after a single topical application of UV-based therapy including combination treatment with BV ointment in acetone-treated mice were examined immunohistochemically with anti-PGP9.5 antibody. White and broken lines indicate the skin surface and the border between the epidermis and dermis, respectively. Scale bars, 50 μm. (B) A marked decrease in the number of intraepidermal nerve fibers was observed in the group of mice treated with PUVA, PUVA + BV, NB-UVB and excimer lamp (*$P < 0.001$). All values represent the means ± SD of 6 animals.

and (6–4) photoproducts, which induce considerable bending of the DNA [41]. A recent study demonstrated that 311–313 nm UVB radiation (dose: 750 mJ/cm^2) induced AP-1 binding to DNA [42]. These findings suggest that NB-UVB has the potential to modulate the expression of NGF in keratinocytes. Moreover, several groups have proposed that UV irradiation induces ligand-independent activation of cell-surface receptors, such as epidermal growth factor receptor [43,44]. These observations also suggest that NB-UVB may modulate the expression of Sema3A in keratinocytes. This was supported by recent findings that epidermal growth factor increases the expression of Sema3A at both mRNA and protein levels in human corneal epithelial cells [45]. However, as photoproducts are among the factors involved in skin carcinogenesis, further studies are required to determine adequate irradiation doses with low DNA damage potential.

Excimer lamp treatment, the most effective form of therapy on intraepidermal nerve fibers, did not alter the epidermal expression of axonal guidance molecules. Experimentally, keratinocytes were more resistant than lymphocytes to UVB-induced apoptosis [46]. Therefore, although the mechanisms remain obscure, the anti-nerve growth effects may depend on the sensitivity of cutaneous cells to different UV wavelengths. This may also partly explain the greater inhibitory effect of NB-UVB on intraepidermal nerve fibers compared with PUVA + BV, although NB-UVB restored the expression balance of axonal guidance molecules to the same level as PUVA + BV.

In conclusion, the results presented here indicated that NB-UVB and excimer lamp treatment may be an effective therapeutic method for patients with pruritus involving epidermal hyperinnervation. Combination therapy consisting of PUVA + BV may be

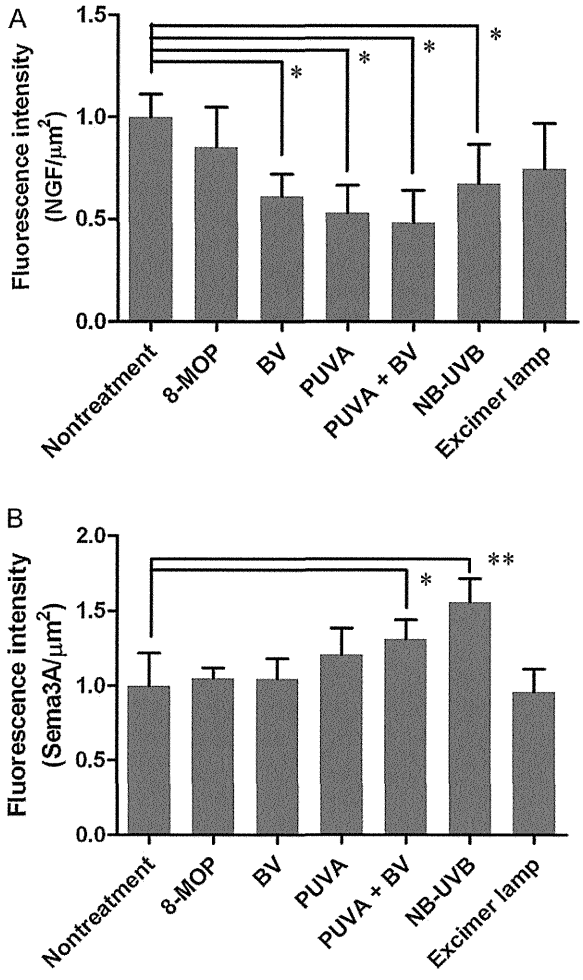

Fig. 3. Effects of UV-based therapy on epidermal NGF and Sema3A levels in acetone-treated mice. (A) The fluorescence intensity of epidermal NGF was significantly decreased in mice treated with BV, PUVA, PUVA + BV and NB-UVB compared with the control mice ($^{*}P < 0.05$). (B) The fluorescence intensity of epidermal Sema3A was significantly increased in PUVA + BV and NB-UVB-treated mice ($^{*}P < 0.05$, $^{**}P < 0.001$). All values represent the means ± SD of 6 animals.

recommended for anti-pruritic therapy rather than either treatment alone. These findings also expand our knowledge regarding effective treatments for pruritic skin diseases.

Acknowledgments

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Experimental dermatology • **Original article**

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Clinical and Experimental Dermatology

Neurotropin inhibits both capsaicin-induced substance P release and nerve growth factor-induced neurite outgrowth in cultured rat dorsal root ganglion neurones

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Summary

Background. Neurotropin (NTP), a biological extract from rabbit skin inoculated with vaccinia virus, is an effective analgesic and anti-allergic agent, and has antipruritic effects in various dermatoses including eczema, dermatitis and urticaria. In patients receiving haemodialysis who have pruritus, NTP appears to exert its antipruritic effect by lowering the plasma levels of substance P (SP), but its underlying mechanisms are not fully understood.

Aim. To investigate the antipruritic mechanisms of NTP.

Methods. The effects of NTP on capsaicin-induced SP release from neonatal rat dorsal root ganglion (DRG) neurones were assessed by measuring SP concentrations in culture media by a competitive ELISA. The effects of NTP on nerve growth factor (NGF)-induced neurite outgrowth were assessed by measuring the length of the longest process of cultured DRG neurones. The neuronal cytotoxicity of NTP was determined using a methylthiazole tetrazolium cytotoxicity assay.

Results. NTP dose-dependently inhibited capsaicin-induced release of SP from cultured DRG neurones, whereas NTP alone had no effect on SP release. Moreover, NTP dose-dependently inhibited NGF-induced neurite outgrowth in cultured DRG neurones. NTP had no observable cytotoxicity.

Conclusions. These results suggest that NTP exerts its antipruritic effects by inhibiting both SP release and neurite outgrowth of cutaneous sensory nerves.

Introduction

Pruritus is an unpleasant sensation inducing a desire to scratch. As a physiological self-protective sensation, pruritus guards the skin against harmful substances including parasites or plants. It is also a major and distressing symptom of many skin and systemic diseases. Histamine, released from mast cells during early phases of inflammation, is the best-known pruritogen in humans and has been regarded as the main target for antipruritic therapies. Chronic pruritus resulting from renal and liver diseases, and from serious skin diseases such as atopic dermatitis (AD), represents a considerable clinical problem. Conventional treatment such as antihistamines often lack efficacy in patients with chronic pruritus, and in such patients, the condition is termed intractable pruritus.[1–3]

To date, the neuronal mechanisms underlying intractable pruritus have only been partly identified. Histological observations indicate that cutaneous nerve fibres are present at higher densities in the skin of patients with AD, psoriasis, contact dermatitis and xerosis than in controls, suggesting that hyperinnervation is partly responsible for the intense itching sensations.[3–9] Keratinocyte-derived nerve growth factor (NGF) is a

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Conflict of interest: none declared.

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