INVESTIGATIVE REPORT

Decreased Expression of Semaphorin-3A, a Neurite-collapsing Factor, is Associated With Itch in Psoriatic Skin

Kenzen KOU1, Fumio NAKAMURA2, Michiko AIHARA1, Huichin CHEN1, Katsuya SETO1, Junko KOMURI-YAMAGUCHI1, Takeshi KAMBARA4, Yoji NAGASHIMA3, Yoshiro GOSHIMA2 and Zenro IKEZAWA1

Departments of 1Environmental Immuno-Dermatology, 2Molecular Pharmacology and Neurobiology and 3Molecular Pathology, Yokohama City University Graduate School of Medicine, and 4Department of Dermatology, Yokohama City University Medical Center, Yokohama, Japan

Psoriasis is a common chronic inflammatory skin disease, with intractable itch as one of its main symptoms. The histopathological features of psoriasis are characterized by thickening of the epidermis, parakeratosis and elongated rete ridges (1). Patients with psoriasis present with erythematous plaques, and a previous study found concomitant pruritus in 84% of patients (2). Pruritus is a daily symptom for most patients, which significantly affects quality of life, including the ability to sleep (3). Scratching, caused by the itch sensation, can aggravate exanthema, as seen in the Koebner phenomenon (2, 4–6). Itch intensity has been shown to correlate with the severity of psoriasis (4).

The itch sensation is mediated by afferent C-fibres (7), which are unmyelinated nerve fibres of the dorsal root ganglia (8–10). The outgrowth of C-fibres is facilitated by nerve growth factor (NGF) (11–13). NGF binds to the Trk-A receptor, which is expressed on C-fibre endings (14–17). In healthy skin, most C-fibres terminate at the epidermal–dermal junction, and few invade the epidermis (18, 19). However, C-fibres abnormally innervate in psoriatic epidermis, which could lead to hypersensitivity (18). The number of C-fibres mediating the dermal itch sensation is increased in psoriatic skin lesions, and this increased innervation may be induced by increased expression of NGF in the epidermis (20, 21) and/or by an increased level of Trk-A in C-fibres (22, 23).

In addition, neurite outgrowth of C-fibres is negatively regulated by semaphorin-3A (Sema3A) (9, 24–26). Sema3A is an axon-guidance molecule that inhibits neurite outgrowth of sensory C-fibres (27, 28). Studies have demonstrated that Sema3A is expressed in keratinocytes (19–29). Sema3A may restrict C-fibre outgrowth and invasion in the epidermis of healthy skin (18, 19). The results of our previous study indicated that Sema3A alleviates skin lesions and scratching behaviour in an atopic dermatitis mouse model (30). Sema3A binds to the primary receptor neuropilin-1 (NRP1), which is expressed in sensory C-fibres. NRPI forms functional receptor complexes with plexin-A and mediates the Sema3A-induced repulsive response of outgrowing neu-rones (31–37). It has been shown that the epidermis also expresses NRPI and that this expression may regulate the migration of keratinocytes (38, 39).

Based on this knowledge, we hypothesized that decreased Sema3A expression in psoriatic keratinocytes might be involved in the pathogenesis of psoriasis. The present study compared Sema3A expression in psoriatic skin and healthy skin and examined its correlation with the clinical score of psoriasis. The study also assessed the number of nerve fibres in the epidermis and NGF and NRPI expression.

MATERIALS AND METHODS

Patients and diagnosis of psoriasis

Experimental protocols for this study were approved by the ethics committee of Yokohama City University Graduate School of...
Medicine, Yokohama, Japan (notice of approval IRB protocol number B080911013). Written informed consent was obtained from all participants. A total of 61 patients with psoriasis and 65 healthy volunteers were recruited for the study from Yokohama City University Hospital and Yokohama City University Medical Center. Patients and healthy control subjects were selected to be as similar as possible in terms of age, sex, and biopsy region (Table I). In preparation for the study, patients with strong pruritus were left untreated to the greatest extent possible. Clinical data were obtained from archival records of Yokohama City University Hospital. Diagnosis of psoriasis was based on clinical and histological examinations, and clinical severity was evaluated with the use of the Psoriasis Area and Severity Index (PASI) (40). The degree of pruritus was evaluated with the use of a visual analogue scale (VAS) (41). The VAS was scored by presenting a 100-mm horizontal line to each patient and asking them to mark the point that represented their current state of itchiness. The scale ranged from 0 (no itch) to 100 (maximal itch).

**Sema3A mutant mice**

Sema3A mutant mice were generated as described previously (42). Genotypes of the offspring of all heterozygous and homozygous knockout mice were assessed by PCR, as described previously (43).

### RESULTS

#### Histology and immunohistochemistry

Biopsies of normal and psoriatic skin were taken from the healthy volunteers and patients. Samples were fixed immediately in 10% formalin and embedded in paraffin for histopathology and immunohistochemistry. Tissue sections (3-μm thick) were stained with haematoxylin–eosin for conventional histopathological diagnosis. Primary antibodies used for immunohistochemical staining were as follows: rabbit anti-Sema3A (1:200 dilution; sc-548; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PGP 9.5 (1:800 dilution; UltraClone, Abcam, Cambridge, UK), rabbit anti-NRP1 (1:200 dilution; sc-5541; Santa Cruz Biotechnology), rabbit anti-PGP 9.5 (1:800 dilution; UltraClone, Isle of Wight, UK), and rabbit anti-substance P (1:100 dilution; Biotechnology), rabbit anti-PGP 9.5 (1:100 dilution; UltraClone, Isle of Wight, UK), and rabbit anti-substance P (1:100 dilution; Biotechnology), rabbit anti-PGP 9.5 (1:800 dilution; UltraClone, Osaka, Japan). Tissue sections were deparaffinized, rehydrated and autoclaved (3-μm thickness) were deparaffinized, rehydrated and autoclaved in citrate buffer at 121°C for 15 min for antigen retrieval. The sections were then immersed in 0.3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with 10% normal goat serum at 37°C for 15 min, the sections were incubated at 4°C overnight with primary antibodies diluted as described above. Labelled antigens were visualized with a Histofine SAB (streptavidin–biotin) kit (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine (DABplus; DAKO, Glostrup, Denmark). Mouse skin samples were stained with the same method.

#### Quantification of Sema3A, NGF and NRP1 immunostaining

Tissue sections immunostained with anti-Sema3A, anti-NGF, or anti-NRP1 antibodies were analysed for 3 patients with psoriasis and 3 healthy volunteers for each antibody. The total immunostaining intensity in the epidermis was measured with the use of a BZ-9000 microscope with the Dynamic Cell Count image analysis program (Keyence); immunostaining intensity per unit area was calculated.

#### Quantitative reverse-transcription PCR

Quantitative reverse-transcription (qRT) PCR was used to quantify NGF mRNA expression in 26 psoriasis samples and 26 healthy samples, Sema3A mRNA expression in 61 psoriasis samples and 65 healthy samples, and NRP1 mRNA expression in 39 psoriasis samples and 39 healthy samples. Total RNA was extracted from the skin samples with an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Hertfordshire, UK) according to the manufacturer’s instructions. Total RNA was transcribed with the use of random primers and a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The complementary DNA generated was used in qRT-PCR analysis with an ABI PRISM7500 Sequence Detection System (Applied Biosystems) based on the SYBR Green method, as described previously (45). Sequences of primers and probes used in this study are listed in Table II. Expression of β-actin was used as an internal control. PCR products were electrophoresed on 2% agarose gels, and the relative expression of each gene in healthy skin was set at 1. The expression of each gene in psoriatic skin was calculated relative to expression in healthy skin.

### Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical differences were assessed by Mann-Whitney U test. Correlations between Sema3A/β-actin expression and clinical scores (VAS and PASI) were calculated with the Spearman’s correlation coefficient test. All statistical analysis was performed with SPSS 19 software (SPSS, Chicago, IL, USA). All tests were 2-sided, and p-values of <0.05 were considered statistically significant.

#### Epidermal innervation

Clinical features of the 61 patients with psoriasis and 65 healthy volunteers are listed in Table I. In healthy skin, the majority of the PGP9.5+ fibres were located at the dermo–epidermal junction (Fig. 1a, c). In contrast, numerous PGP9.5-positive signals were present in the epidermis of psoriatic skin (Fig. 1b, d). The penetration of nerve fibres into the epidermis was observed in approximately 67% of...
Decreased semaphorin-3A expression in psoriasis vulgaris psoriatic patients with itch. Substance P immunostaining showed a similar distribution pattern (Fig. 1e, f). The density of PGP9.5-positive nerve fibres in the epidermis in patients (13 ± 12.3 per 1×10^4 μm^2) was 3 times higher than in control subjects (4 ± 3.7 per 1×10^4 μm^2; p=0.038) (Fig. 1g). The density of substance P-immunoreactive fibres was similar (Fig. 1h). The density of substance P-positive nerve fibres in the epidermis was 6 times higher in psoriasis patients (6 ± 5.8 per 1×10^4 μm^2) than in healthy controls (1 ± 1.7 per 1×10^4 μm^2; p=0.043).

NGF was expressed in keratinocytes in skin biopsies from patients with psoriasis and healthy control subjects. However, the staining signal was stronger in psoriatic skin than in healthy skin (Fig. 2a, b). Quantification revealed that epidermal NGF immunostaining in patients was significantly greater than in control subjects (p<0.001) (Fig. 2c). NGF mRNA expression was also significantly greater in psoriatic samples compared with healthy samples (p<0.001) (Fig. 2d).

**Sema3A protein expression in the epidermis**

We first examined anti-Sema3A immunohistochemical staining in *sema3A* mutant mice. Sema3A immunostaining was present in the epidermal keratinocytes of *sema3A* heterozygous mice, but was not detectable in *sema3A* homozygous mice (Fig. 3a, b). Immunohistochemical staining of healthy human skin biopsies revealed that anti-Sema3A-specific antibody labelled epidermal keratinocytes (Fig. 3c). A few dermal fibroblasts showed

**NGF levels in the epidermis**

Table II. Sequences of primers and probes used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product (bp)</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sema3A</td>
<td>142</td>
<td>Forward</td>
<td>GTCGCAGAGAGCGCTGGTCTATTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTAATTGGCTGAACTCCTCTTGGT</td>
</tr>
<tr>
<td>NRP1</td>
<td>168</td>
<td>Forward</td>
<td>ATCACTGAGCCGCTAAGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCATGCAATGGGACAGATTTC</td>
</tr>
<tr>
<td>β-actin</td>
<td>169</td>
<td>Forward</td>
<td>GTGGACATCGGCAAGCCTGTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGCCGATCCACACGGAGTACT</td>
</tr>
<tr>
<td>NGF</td>
<td>TaqMan Gene Expression Assays (Hs00171458_m1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>TaqMan Gene Expression Assays (Hs99999903_m1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Ectopically innervating fibres in psoriatic epidermis (epi). (a–d) Anti-PGP9.5 immunostaining. Skin samples from healthy volunteers (a) and patients with psoriasis (b) were stained with anti-PGP9.5 antibody. Epidermal nerve fibres were observed in greater numbers in patients with psoriasis. Scale bars = 100 μm. (c, d) Enlarged views of the boxes shown in (a) and (b). Scale bars = 20 μm. (e, f) Anti-substance P immunostaining. Specimens from healthy volunteers (e) and patients with psoriasis (f) were stained with anti-substance P antibody. Substance P-positive nerve fibres were observed in greater numbers in patients with psoriasis. Scale bars = 20 μm. (g, h) The number of epidermal nerve fibres was significantly greater in patients with psoriasis than in healthy volunteers. *p=0.038 vs. healthy volunteers. (h) The number of substance P-positive nerve fibres was significantly greater in patients with psoriasis than in healthy volunteers. Values are shown as mean ± SD from 2 experiments. *p=0.043 vs. healthy volunteers.
faint Sema3A immunoreactivity (Fig. 3c, e). This immunoreactivity was diminished by preincubation of the primary anti-Sema3A antibody with the antigen peptide (Fig. 3d), confirming the specificity of the antibody for the antigen. Psoriatic specimens showed less Sema3A immunostaining than in healthy skin (Fig. 3e). Immunostaining intensity per unit area of epidermis was significantly less in psoriatic patients compared with control subjects ($p < 0.001$) (Fig. 3f). In psoriatic skin, the outer layer of the epidermis showed slightly greater Sema3A immunostaining than the inner and basal layers.

**Correlation of Sema3A mRNA expression with itch intensity and severity of psoriasis**

Sema3A expression in psoriatic samples was significantly less (mean relative value = 0.3003, $p < 0.001$) than in healthy skin samples (mean relative value = 1.0) (Fig. 4a). Relationships between Sema3A/β-actin expression and extent of pruritus (VAS score) and clinical severity (PASI score) were investigated. VAS score was positively correlated with PASI score in patients with psoriasis (Fig. 4b), with a correlation coefficient ($r$) of 0.41, which was statistically significant ($p = 0.001$). The level of Sema3A/β-actin expression was negatively correlated with VAS score in patients with psoriasis (Fig. 4c), with an $r$ value of $-0.367$ ($p = 0.004$). PASI score was also negatively correlated with Sema3A/β-actin expression (Fig. 4d), with an $r$ value of $-0.383$ ($p = 0.002$).

**Decreased NRP1 expression in psoriatic epidermis**

NRP1 expression in keratinocytes has been reported (38, 39). Therefore, we examined NRP1 immunostaining in skin samples from patients with psoriasis along with those from healthy control subjects. We found NRP1 immunostaining in epidermal keratinocytes in control subjects (Fig. 5a); however, NRP1 staining was relatively decreased in psoriatic skin (Fig. 5b). The intensity of epidermal NRP1 immunostaining was less in patients compared with control subjects ($p = 0.028$) (Fig. 5c). With respect to dermal nerves, NRP1 immunostaining was observed in healthy skin, whereas it was rarely observed in psoriatic skin. Results of qRT-PCR also showed a significantly ($p = 0.007$) decreased NRP1 mRNA expression in psoriatic samples compared with healthy samples, with mean values of 0.52 and 1.0, respectively (Fig. 5d). However, no significant correlation was found between NRP1 expression and VAS or PASI scores (data not shown).

**DISCUSSION**

The results of the present study showed that both immunostaining and mRNA expression levels of Sema3A were lower in psoriatic skin samples compared with healthy skin samples. The results also confirmed that NGF expression was greater in psoriatic lesions. Ectopic innervation of sensory C-fibres in the epidermis was observed in psoriasis but not in healthy subjects. Substance P expression level was also increased in the epidermis of patients with psoriasis and pruritus. Both the itch intensity and the clinical severity of psoriasis were negatively correlated with the level of Sema3A mRNA expression in psoriatic skin samples.

The innervation of C-fibres is regulated, at least to some extent, by NGF-induced nerve elongation and...
Decreased semaphorin-3A expression in psoriasis vulgaris

Sema3A repulsion (29, 46). In healthy skin, sensory C-fibres terminate at the junction of the dermis and the epidermis (Fig. 1a, c). Expression of Sema3A in the epidermis may restrict innervation. When Sema3A expression is decreased, C-fibres may ectopically invade the epidermis. An increase in NGF expression in psoriatic epidermis may also contribute to hyperinnervation. Indeed, an increase in epidermal nerve density has been suggested to be responsible, at least in part, for antihistamine-resistant pruritus in atopic skin (24), and decreased epidermal Sema3A and increased NGF expression levels have been observed in patients with atopic dermatitis (19, 46–48). Therefore, we speculate that downregulation of Sema3A and upregulation of NGF in psoriatic skin may trigger hyperinnervation of C-fibres in the epidermis, leading to increased itch.

We found increased SP immunoreactivity in pruritic skin compared with healthy skin. This is consistent with a recent report by Haas et al. (49), which showed increased SP-positive nerve fibres in chronic pruritus; however, the strength and maintenance of itch is not related to SP-positive nerve fibres. They suggested that dermal hyperinnervation of sensory SP-positive nerves may be responsible, at least in part, for the intense itch sensation. Amatya et al. (50) in 2011 also reported a similar result, that the numbers of SP-positive nerves in lesional psoriasis skin correlated significantly with pruritus intensity.

Taneda et al. (51) recently reported the absence of a correlation between nerve density and Sema3A level in the epidermis of patients with psoriasis and itch. Their immunohistochemical data showed that the penetration of nerve fibres into the epidermis was observed in approximately 40% of psoriatic patients with itch. In contrast, we found that the penetration of nerve fibres into the epidermis was observed in approximately 67% of psoriatic patients with itch. This discrepancy might be explained by the difference in sampling numbers, different sampling standards for psoriatic patients (VAS value, PASI score and patient age) and/or by different immunostaining methods. We quantified Sema3A mRNA expression in a larger number of psoriatic patients (n = 61) and healthy control subjects (n = 65), whereas Taneda et al. performed a semiquantitative analysis of Sema3A protein expression in relatively small numbers of psoriatic (n = 24) and healthy (n = 5) specimens. For anti-Sema3A

**Fig. 3.** Decreased Sema3A immunostaining in psoriatic skin. (a, b) Sema3A expression in mouse epidermis (epi). Skin sections from sema3A heterozygous (a) and sema3A homozygous (b) knockout mice were immunostained with anti-Sema3A antibody. Epidermal Sema3A staining was absent in sema3A homozygous mice. Scale bars = 20 μm. Skin sections from 3 healthy volunteers (c, d) and 3 patients with psoriasis (e) were immunostained for Sema3A. Sema3A immunostaining was less in the epidermis of patients with psoriasis. Immunoabsorption tests for Sema3A labelling confirmed the specificity of the antibody for the antigen (d). Scale bars = 100 μm. der, dermis. (f) Immunostaining of epidermal Sema3A was significantly decreased in patients compared with healthy control subjects (**p < 0.001). Results are presented as relative values compared with healthy control subjects and are shown as mean ± SD.
immunostaining, we used the SAB method, whereas Taneda et al. used fluorescence-conjugated secondary antibodies. Taneda et al. also reported a significant decrease in epidermal k-opioid receptor in psoriatic skin, in addition to alterations in Sema3A and NGF. Thus, opioid system failure, or some as yet unknown component of pruritus in psoriasis, may be involved and should be investigated in future studies.

Ectopic innervation of the epidermis can trigger a vicious itch-scratch cycle (4, 52). Damage to the skin barrier caused by scratching may induce upregulation of NGF, which could further facilitate hyperinnervation in the epidermis. A correlation has been reported between NGF expression in the epidermis and the clinical severity of psoriasis (41). We speculate that downregulation of Sema3A might also aggravate the itch-scratch cycle.

Fig. 4. Correlation of decreased Sema3A mRNA expression with itch intensity and severity of psoriasis.

(a) Total RNA was isolated from healthy and psoriatic human skin tissues and used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) with primers specific for Sema3A. Lane 1, mRNA from healthy human skin (positive control); lane 2, mRNA from psoriatic skin. The expression level of Sema3A was significantly less in psoriatic samples than in healthy samples. Sema3A mRNA expression was measured by qRT-PCR in 65 healthy volunteers and 61 patients with psoriasis. The level of mRNA expression for the gene was calculated by comparison with β-actin mRNA expression. Results shown are mean ± SD from 3 experiments. ***p<0.001 vs. healthy volunteers.

(b) Positive correlation between Psoriasis Area and Severity Index (PASI) and pruritus assessed by visual analogue scale (VAS) score in patients with psoriasis (n=61). Negative correlation between Sema3A mRNA expression and VAS score (c) and PASI score (d) in patients with psoriasis (n=61).

Fig. 5. Decreased neuropilin-1 (NRP1) expression in psoriatic epidermis (epi). (a, b) Skin samples from 3 healthy volunteers (a) and 3 patients with psoriasis (b) were immunostained for NRP1. NRP1 immunostaining was decreased in the epidermis of patients with psoriasis compared with healthy volunteers. Scale bars = 100 μm. der: dermis. (c) Epidermal NRP1 immunostaining was significantly decreased in patients with psoriasis compared with healthy control subjects (*p<0.05). Results are presented as relative values to those of healthy control subjects. (d) Total RNA was isolated from healthy human skin tissues and psoriatic skin tissues and used for quantitative reverse-transcription (qRT) PCR with primers specific for NRP1. Lane 1, mRNA from healthy human skin (positive control); lane 2, mRNA from psoriatic skin. The expression level of NRP1 was significantly less in psoriatic samples compared with healthy human samples. NRP1 mRNA expression was measured by qRT-PCR in 39 healthy volunteers and 39 patients with psoriasis. The level of mRNA expression for the gene was calculated by comparison with β-actin mRNA expression. Results shown are mean ± SD from 3 experiments. **p<0.01 vs. healthy volunteers.
in psoriasis because we found a negative correlation between Sema3A expression and clinical scores (Fig. 4b). This also indicates that monitoring of Sema3A level in the epidermis could become a future tool as a biomarker of pruritus in psoriasis. In addition, Sema3A modulates the immune system by direct action on T cells as a negative-feedback regulator of dendritic cell-induced T-cell proliferation (53). Psoriasis is closely associated with excessive interleukin-4 production as well as inflammatory cell infiltration in the dermis (54). We speculate that decreased Sema3A expression in psoriatic skin may facilitate the infiltration and proliferation of immune cells. Further study is clearly needed to test this hypothesis.

Psoralen-ultraviolet A (PUVA) therapy has been reported to increase Sema3A expression and suppress NGF expression in the epidermis, thereby improving the clinical scores of patients with atopic dermatitis (46). Because a similar imbalance in Sema3A and NGF in psoriatic skin has been demonstrated in the present study, PUVA could be a rational therapy for psoriasis, to restore the Sema3A-NGF balance. In addition, the administration of recombinant Sema3A or anti-NGF antibody has been reported to inhibit itch in an NC/Nga allergic atopic dermatitis model in mice (30, 55). Thus, psoriasis might be treated by the administration of anti-NGF antibody and/or Sema3A to alleviate the hypersensitive itch sensation.

Because Sema3A inhibits the migration of human keratinocytes in culture systems (39), the decreased expression of NRP1 and Sema3A in psoriatic epidermis may contribute to the development of acanthosis, the abnormal proliferation and differentiation of keratinocytes, by facilitating cell migration.

In conclusion, the decrease in Sema3A expression may play a role in pruritus and the pathogenesis of psoriasis; however, more research is needed to confirm the mechanism of its effect. The administration of Sema3A, Sema3A-like agonists, or agents that induce Sema3A expression in the epidermis may play a future role in preventing pruritus and improving the treatment of refractory psoriasis.

ACKNOWLEDGEMENTS

The authors thank Professor Satoshi Morita, and Assistant Professor Masataka Taguri (Department of Biostatistics and Epidemiology, Yokohama City University Medical Centre, Yokohama, Japan) for assistance with the statistical analysis. This work was supported by Grants-in-Aid for Scientific Research numbers 19591320 and 21591471 (to Z. I.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and by Grant no. 07085023 (to Y.G.) from the National Project on Protein Structural and Functional Analyses of MEXT.

The authors declare no conflicts of interest.

REFERENCES

21. Raychaudhuri SP, Jiang WY, Farber EM. Psoriatic kerati-
nocyes express high levels of nerve growth factor. Acta Derm Venereol 1998; 78: 84–86.