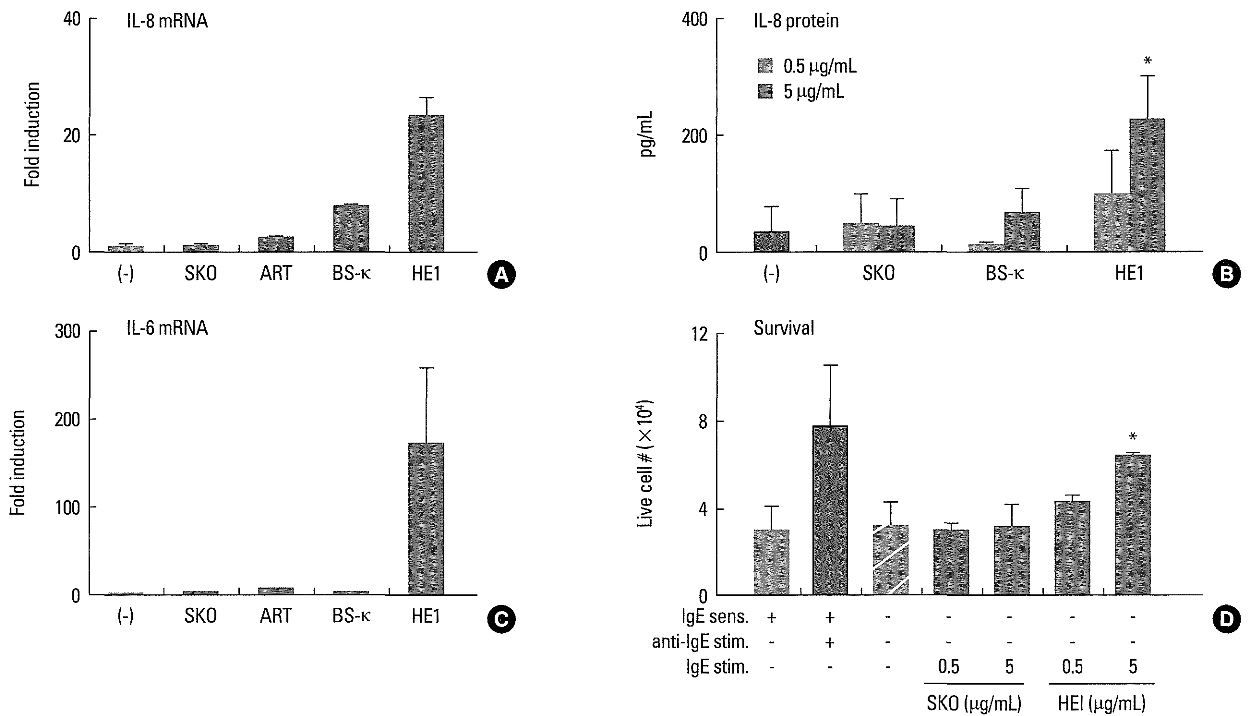

Fig. 2. A human IgE preparation can induce IL-8 and IL-6 production and promote survival in human mast cells. (A-C) Human CBMCs were incubated with 0 (PBS, [-]), 0.5 (white bar), or 5 (gray or black bar) μg/mL of the indicated human IgEs for 2 hr. (A,C) IL-8, IL-6, and GAPDH mRNA levels were measured by quantitative RT-PCR. The amount of cytokine mRNA was normalized against GAPDH mRNA and the relative value in PBS-treated cells was set at 1. (B) IL-8 protein was measured by ELISA. *$P<0.05$ vs PBS control by Student's *t*-test. (D) Human CBMCs (1×10^5 cells) were incubated with 0 (PBS), 0.5, or 5 μg/mL of the indicated human IgEs in the absence of SCF and IL-6 for 72 hr. As controls, CBMCs were pre-incubated with 1 μg/mL of PS IgE in the presence of IL-4 for 24 hr, and washed cells were incubated with 0 or 1 μg/mL anti-IgE for 72 hr. Trypan blue excluding live cell numbers were counted. *$P<0.05$ vs control (no IgE sensitization/no IgE stimulation) by Student's *t*-test.

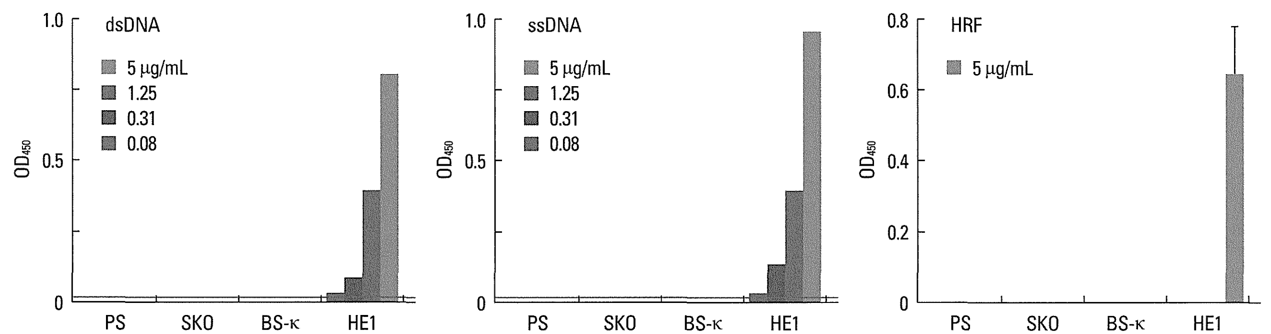

Fig. 3. A human HC IgE HE1 reacts with dsDNA, ssDNA and HRF. ELISAs were performed on the indicated human IgE samples, as shown in Fig. 1 except that anti-human IgE was used in place of anti-mouse IgE.

pathogenesis of AD, asthma and other allergic diseases[30-33] and preferential IgE reactivity with autoantigens in AD,[34] we measured levels of IgE that reacted with dsDNA, ssDNA, β-galactosidase, and LPS in sera of AD patients. AD patients had significantly higher serum levels of ssDNA-reactive and β-galactosidase-reactive IgE than healthy controls (Fig. 4A and 4C). Levels of dsDNA-reactive IgE also tended to be higher than healthy controls, although the difference did not reach statistical significance (Fig. 4B). AD patients also had higher serum levels of HRF than healthy controls (Fig. 4D). Four of 34 AD patients had measurable levels of IgE that reacted with HRF (Fig. 4E). Two of these patients had extremely high IgE levels of 48.7 and 384 μg/mL, whereas another patient had 0.17 μg/mL and IgE levels were not known for the other patient. By contrast, none of 25

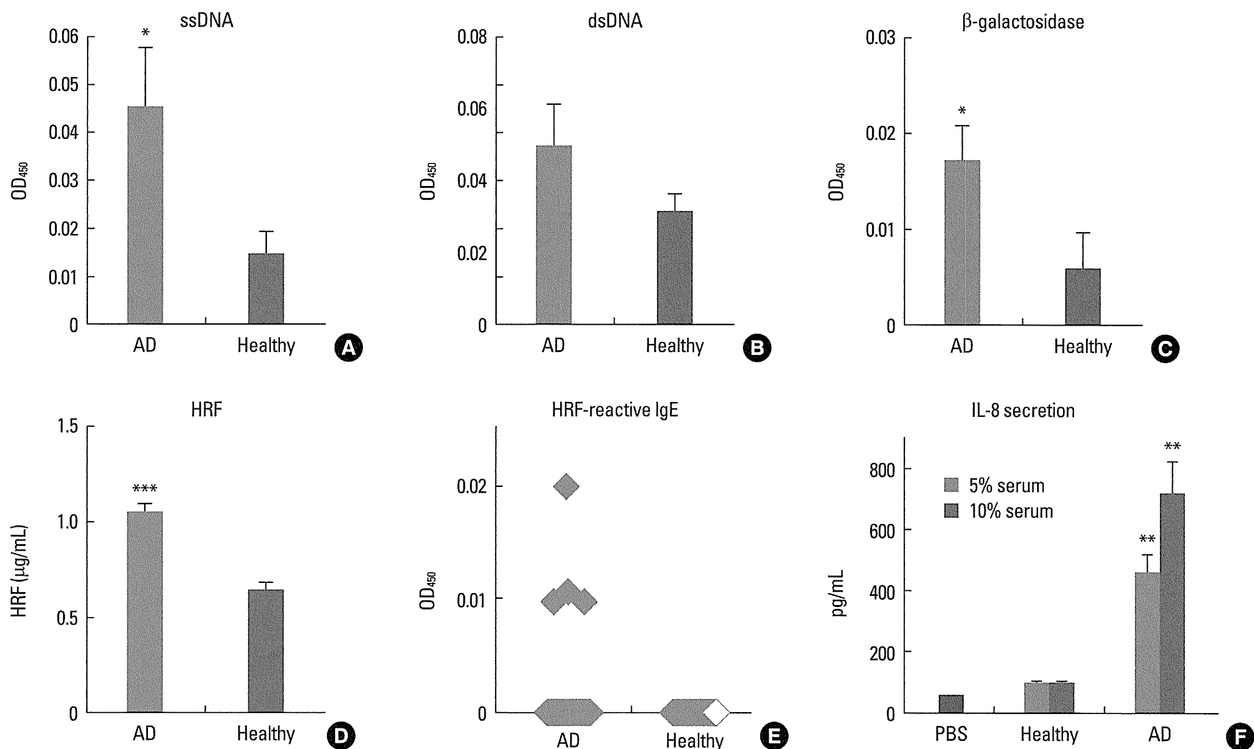

Fig. 4. AD patients have higher serum levels of ssDNA- or β-galactosidase-reactive IgE and HRF. ELISAs were performed on human sera using wells coated with ssDNA (A), dsDNA (B), β-galactosidase (C) and HRF (E). Sera were taken from cohorts of AD patients and healthy individuals. Note that low OD_{450} values, compared to those in Fig. 3, were obtained with sera diluted tenfold. (D) Serum HRF levels were quantified by immunoblotting. (A-D) n=27, AD patients; n=23, healthy subjects; (E) n=34, AD patients; n=25, healthy subjects. (F) Human CBMCs were pretreated with 10 ng/mL IL-4 for 24 hr and stimulated with sera (5% or 10%) from normal subjects (n=2) or AD patients (n=8) for 24 hr. Supernatants were collected and IL-8 was quantified by ELISA.

healthy individuals had detectable levels of anti-HRF IgE. However, we found no increase in anti-LPS IgE titers ($OD_{450} < 0.01$) in AD or healthy individuals, ruling out the possibility that the measured high O.D. values reflected antigen-non-specific bindings due to high concentrations of total IgE.

We previously showed that polyclonal IgE in AD sera can induce IL-8 production, a signature of HC IgEs, in human CBMCs.[20] Therefore, we tested this ability of AD sera (n=8) with high titers (≥ 0.01 OD_{450}) of dsDNA- and ssDNA-reacitve IgEs, compared to sera from healthy subjects. As shown in Fig. 4F, the AD sera exhibited 4.7- and 7.2-fold higher IL-8-producing activity at 5% and 10% serum concentrations. Therefore, these results strongly suggest that AD patients have autoantigen-reactive IgEs and these IgEs may be HC IgEs.

DISCUSSION

This study demonstrates that most of mouse HC IgEs react with multiple autoantigens. Since some of the autoantigens, e.g., HRF and dsDNA and ssDNA, have multivalency in terms of the IgE-binding ability, it seems reasonable to assume that such an autoantigen can trigger cross-linking of IgE-bound receptors, thus activation of mast cells and basophils, eventually leading to allergic reactions. Indeed, HRF was shown to amplify allergic inflammation by this mechanism in passive cutaneous anaphylaxis and airway inflammation in mice.[24] Similar to HRF, dsDNA and ssDNA released from damaged tissues during allergic reactions may contribute to exacerbate allergic inflammation by interacting with HC IgE bound to FcεRI on mast cells and basophils. One of the eight mouse IgE mAbs tested failed to react with autoantigens. This negative result might be due to the fact that the number of autoantigens tested was not exhaustive enough or appropriate autoantigens were not tested.

As encountered by western blotting or other immunoassays on a daily basis, immunologists have long known that an antibody recognizes more than one antigen.[35] Particularly, monoclonal antibodies were shown to have multispecificity.[36] Conformational diversity of antigen recognition, whereby one antibody (e.g., IgE) sequence adopts multiple structures, can increase the effective size of the antibody repertoire and may lead to autoimmunity.[28] Autoimmunity underlies allergic diseases.[34] Epidemiological data indicate that the prevalence of allergies and autoimmune diseases have increased in parallel.[37,38] Recent studies support the concept that IgE autoreactivity may

play a pathogenic role in severe and chronic forms of atopy.[34,39] While acute allergic reactions following exposure to exogenous allergens are understood as immediate type inflammation triggered by degranulation of mast cells via allergen-IgE-FcεRI interactions, chronic allergic inflammation with Th1 characteristics can occur and persist in the absence of exogenous allergens. IgE-mediated presentation of autoallergens may activate autoreactive Th1 cells to release proinflammatory cytokines.[40,41] Several environmental allergens have striking structural and immunologic similarities with human proteins.[42-46] A broad spectrum (≥140 proteins) of IgE-reactive autoantigens were found in AD patients and some of them were shown to have the ability to activate basophils.[47] As shown in this study, some AD patients who had high levels of ssDNA- (or dsDNA-) and/or β-galactosidase-reactive IgEs also had high levels of HRF-reactive IgEs, the autoimmune mechanism could be an important part of the disease pathogenesis in this subset of patients. This observation also suggests the heterogeneity of AD pathogenesis, as not all AD patients have increased IgE reactivity to autoantigens. Interestingly, NC/Nga mice spontaneously develop AD-like skin lesions[48] and IgE autoantibodies including anti-histone H3 IgE;[49] this age-dependent increase in the serum anti-histone H3 IgE was in parallel with the onset of dermatitis, and correlated well with dermatitis severity at 12-16 weeks of age. As global gene expression patterns are similar in lesional skin between NC/Nga mice and human AD patients (Ando et al., in preparation), these data and our present study support the notion that IgE autoreactivity may play a pathogenic role in AD. Thus, further characterization of the role of IgE autoantibodies in the NC/Nga model will be interesting.

This study also demonstrates that human IgE molecules exhibit heterogeneity in the ability to induce cytokine production and survival promotion in human mast cells in a similar way that mouse HC versus PC IgE molecules exhibit differences in inducing various activation events in mouse mast cells.[19,50] Thus, a human HC IgE induces cytokine/chemokine production more strongly than do human PC IgEs. However, there are some differences between human and mouse systems. For example, significant survival promotion was seen only with a human HC IgE, in contrast with mouse IgE effects showing that even PC IgEs can promote mast cell survival albeit with weak potency.[7,10] Degranulation could not be induced by either type of human IgE. These results could be due to the type of human mast cells used in this study. Indeed, even IgE+anti-IgE-stimulated human CBMCs degranulate only 10%-20% of the granule content (data not shown). This low responsiveness may be related to low expression levels of FcεRI in CBMCs, which can be enhanced by IL-4 stimulation.[29] Unlike CBMCs pretreated or not with IL-4, human monomeric myeloma IgE induced dose-dependent histamine release, leukotriene C4 production, and IL-8 synthesis in cultured human lung mast cells.[22] That study suggests that cultured human lung mast cells are more responsive to IgE than human CBMCs, although this and our studies were performed using different IgE molecules. Despite the above-mentioned minor differences in monomeric IgE effects between mouse and human, a human HC IgE bound autoantigens such as dsDNA, ssDNA and HRF, like mouse HC IgEs.

In conclusion, HC, but not PC, IgEs exhibit reactivity to various autoantigens. Some of the autoantigens are multivalent, thus potentially capable of activating HC IgE-bound mast cells and basophils. Therefore, these results indicate an autoimmune component in the pathogenic mechanisms of allergic diseases.

ACKNOWLEDGMENTS

The authors thank Drs. Shane Crotty and Rachel Soloff for providing normal sera. This study was funded in part by an NIH grant R01 AI050209 (T.K.). RPS was supported by the Intramural Research Program of the National Institutes of Health, NIDCR. This is Publication No. 1465 from the La Jolla Institute for Allergy and Immunology.

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CED
Clinical and Experimental Dermatology

Neurotropin inhibits the increase in intraepidermal nerve density in the acetone-treated dry-skin mouse model

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doi:10.1111/ced.12100

Summary

Epidermal hyperinnervation is considered one cause of sensitization to itch, and is thought to regulated by keratinocyte-derived axonal guidance molecules, including nerve growth factor (NGF) and semaphorin (Sema)3A. Neurotropin (NTP) shows antipruritic effects in allergic disease and is also used for pain relief. Using cultured rat dorsal root ganglion neurones, we previously found that NTP inhibited NGF-induced neurite outgrowth. However, no such inhibitory effect has been shown *in vivo*. We therefore assessed the effects of intraperitoneal administration of NTP on nerve density and expression of NGF and Sema3A mRNAs in the epidermis of acetone-treated mice showing epidermal hyperinnervation. We found that NTP significantly reduced intraepidermal nerve growth in these acetone-treated mice. NTP significantly upregulated epidermal Sema3A mRNA, but had no effect on expression of epidermal NGF mRNA. These findings indicate that NTP may reduce intraepidermal nerve density by inducing expression of Sema3A in the epidermis.

Itch (pruritus) is a sensation of discomfort that leads to scratching. It often accompanies a variety of dermatological and systemic diseases. One itch mediator, histamine, acts as a pruritogen in humans, and is used as an experimental itch-causing substance. Histamine type 1 receptor (H1R) antagonists (antihistamines) are therefore used to treat many types of itch, including those resulting from renal and liver disease, and from several skin diseases, such as atopic dermatitis (AD) and xerosis. However, antihistamines are often ineffective in patients with chronic itch.[1]

Epidermal hyperinnervation is considered one cause of intractable itch, suggesting that this area of the skin is susceptible to stimulation and is sensitive to itching.[1] The sprouting of epidermal nerve fibres associated with pruritus is seen in patients with AD, xerosis and allergic contact eczema, and also in experimental animal models.[1,2] Epidermal innervation is regulated by a fine balance between nerve elongation factors, such as nerve growth factor (NGF), amphiregulin and gelatinase, and nerve repulsion factors, such as semaphorin (Sema)3A and anosmin-1, produced by epidermal keratinocytes.[1] Therefore, NGF and Sema3A have been regarded as therapeutic targets in patients with pruritus. We previously showed that commonly used clinical therapies, such as emollients and ultraviolet light-based therapies, have inhibitory effects on intraepidermal nerve fibres, and that these effects may be due to imbalances between NGF and Sema3A.[3]

Neurotropin (NTP), a nonprotein extract isolated from the inflamed skin of rabbits inoculated with vaccinia virus, is widely used in Japan and China to treat various chronic pain conditions.[4] In clinical studies in Japan, NTP has been shown to have antipruritic effects in patients with eczema, dermatitis and urticaria,[5] and in those undergoing haemodialysis.[6] We also found that NTP inhibits NGF-induced neurite outgrowth of dorsal root ganglion (DRG) neurones *in vitro*.[7]

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Conflict of interest: none declared.

Accepted for publication 9 October 2012

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To expand these observations, we examined whether NTP affects intraepidermal nerve density and the epidermal expression of NGF and Sema3A mRNA in a mouse dry-skin model. We describe the possible inhibitory effects of NTP on the pruritus accompanying epidermal hyperinnervation.

Report

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Juntendo University Graduate School of Medicine. Male 10-week-old ICR mice (SLC Japan, Shizuoka, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine.

The protocol for cutaneous barrier disruption has been described previously.[3] Briefly, the hair over the rostral back of the mice was shaved at least 3 days before acetone treatment, and each shaved area was treated with acetone-soaked cotton balls for 5 min. Each mouse was injected intraperitoneally with 200 NTP units (NU) per kg of NTP (experimental group) or saline (control group), immediately and 8, 16, 24, 32 and 40 h after acetone treatment.

Because epidermal nerve density in acetone-treated mice peaks at 48 h after acetone treatment,[3] skin samples were collected at that time, and analysed by immunohistochemistry and quantitative reverse transcription (qRT)-PCR (Fig. 1a).

The numbers of epidermal nerve fibres were assessed immunohistochemically using an antibody to protein gene product (PGP)9.5 at 1 : 4000 dilution (Biomol International Corp., Plymouth Meeting, PA, USA), with Alexa 488-conjugated secondary antibodies (Life Technologies Corp., Carlsbad, CA, USA) used at 1 : 300 dilution. The numbers of PGP9.5-positive nerve fibres penetrating into (Fig. 1b, arrow) and within (Fig. 1b, arrowheads) the epidermis in areas of 1.6×10^5 μm in size in the images of nine sections (20 μm thick) from each mouse were counted by hand. In these analyses, each typical line structure with a minimum length of 12.5 μm was counted as one fibre, and counting was based on intraepidermal nerve-fibre counting rules.[8]

Following treatment with trypsin, epidermal sheets were separated from the skin using forceps, and qRT-PCR analysis was performed as described previously.[2] The primers used to assay NGF and Sema3A mRNA (Perfect Real Time Support System, TaKaRa, Kyoto, Japan) were designed to meet specific criteria.

All statistical analyses were performed using Prism software (version 5; GraphPad Software Inc., La Jolla,

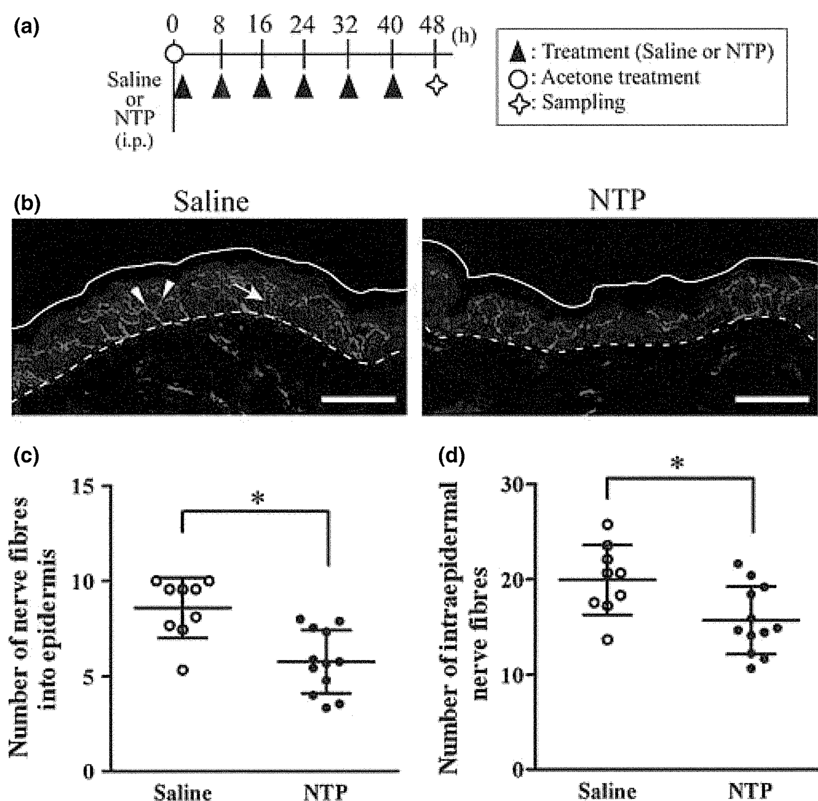

Figure 1 (a) Mice were injected with saline or neurotropin (NTP) immediately and 8, 16, 24, 32 and 40 h after acetone treatment. (b) Distribution of the intraepidermal protein gene product (PGP)9.5-immunoreactive fibres in mice treated with saline or NTP. White and broken lines indicate the skin surface and the border between the epidermis and dermis, respectively. The arrow indicates penetrating nerve fibres and the arrowheads indicate the branching neurones. Scale bars, 50 μm. (c,d) Numbers of PGP9.5 positive nerve fibres (c) penetrating into and (d) lying within the epidermis in NTP-treated and control mice. All values represent the mean ± SD of 9–12 animals. *$P < 0.05$.

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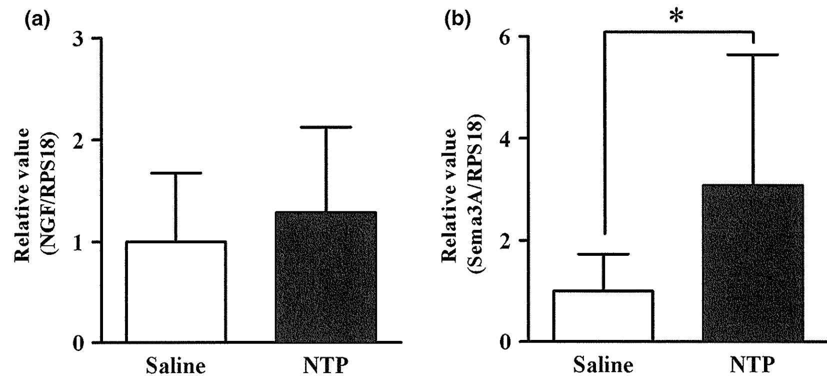

Figure 2 (a,b) Effects of neurotropin (NTP) on epidermal levels of (a) nerve growth factor (NGF) and (b) semaphorin (Sema)3A mRNA levels: NGF mRNA levels were similar in NTP-treated and control mice, whereas Sema3A mRNA levels were significantly higher in NTP-treated than in control mice. All values represent the means ± SD of seven animals. $^*P < 0.05$.

CA, USA). The differences between means were analysed by the Student *t*-test.

NTP administrations after acetone treatment significantly reduced the numbers of nerve fibres penetrating into (Fig. 1c) and within (Fig. 1d) the epidermis, compared with saline-treated mice. When we assessed the expression levels of NGF and Sema3A mRNA in the epidermal sheets from saline-treated and NTP-treated skin samples, we found that the expression of NGF mRNA was similar in the two groups (Fig. 2a), whereas the expression of Sema3A mRNA was significantly higher in the NTP than in the saline group (Fig. 2b).

NTP contains a number of small molecules, including nucleic acids, amino acids and sugars (Nippon Zouki Pharmaceutical Company, unpublished observations). Vaccinia virus-infected cells were recently shown to generate RNA species by activating interferon-β gene transcription, which modulates the expression of numerous cellular microRNAs.[9] Moreover, endogenous microRNA was shown to regulate axonal guidance.[10] Therefore, NTP may contain some effectors that bind certain molecules involved in the induction of Sema3A expression in the epidermis.

In conclusion, we found that NTP inhibited acetone-induced increases in intraepidermal nerve density by inducing expression of Sema3A in the epidermis of acetone-treated mice. Previously, using cultured DRG neurones, we reported that NTP inhibited NGF-induced neurite outgrowth.[7] Thus, in addition to having a direct effect on nerve growth *in vitro*, NTP may have an indirect effect *in vivo*. In this study, although the effect of NTP on scratching behaviour was not measured, our previous studies have shown that epidermal hyperinnervation can be correlated with pruritus.[1] Therefore, NTP may be a possible treatment to relieve pruritus accompanying epidermal hyperinnervation, and there is support for this idea from several clinical studies of NTP treatment for pruritus.[5]

Learning points

- Neurotropin induces Sema3A expression in the epidermis of acetone-treated mice and may inhibit nerve growth.
- Neurotropin may act through both direct and indirect mechanisms to regulate intraepidermal nerve density.
- This inhibitory effect raises the possibility of an effective treatment for pruritus accompanying epidermal hyperinnervation.

Acknowledgements

We thank Nippon Zouki Pharmaceutical Company for providing us with Neurotropin and their valuable scientific supports. This work was partly supported by the High-Tech Research Centre Project for Private Universities, with a matching fund subsidy from MEXT. The funders had no other input into the work.

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CPD questions

Learning objective

To demonstrate an understanding of the up-to-date mechanism about treating intractable itch involving epidermal hyperinnervation.

Question 1

Which of the following is a repulsive factor for intra-epidermal nerve fibres?
a) Histamine.
b) Nerve growth factor.
c) Amphiregulin.
d) Semaphorin 3A.
e) Neurotropin.

Question 2

Which of the following is **not** a pharmacological action of neurotropin (NTP)?
a) Normalization of the expression balance of epidermal axonal guidance molecules.
b) Relief of pain.
c) Inhibition of nerve growth factor-induced neurite outgrowth of dorsal root ganglion neurones.
d) Relief of itch.
e) Increase in epidermal nerve growth factor levels.

Instructions for answering questions

This learning activity is freely available online at http://www.wileyhealthlearning.com/ced.

Users are encouraged to
- Read the article in print or online, paying particular attention to the learning points and any author conflict of interest disclosures
- Reflect on the article
- Register or login online at http://www.wileyhealth-learning.com/ced. com and answer the CPD questions
- Complete the required evaluation component of the activity

Once the test is passed, you will receive a certificate and the learning activity can be added to your RCP CPD diary as a self-certified entry.

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Current Topics

Recent Advances in the Study of Itching

An Update on Peripheral Mechanisms and Treatments of Itch

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Received April 25, 2013

Histamine H_1-receptor blockers are used to treat all types of itch resulting from serious skin diseases such as atopic dermatitis, as well as from renal and liver diseases. However, they often lack efficacy in chronic itch, a profound clinical problem that decreases quality of life. The development of effective treatments requires a full understanding of the fundamental mechanisms of itch. Recent studies have indicated that the pathogenic mechanisms of itch also involve agonists other than histamine, including proteases, neuropeptides, cytokines, and opioids, as well as their cognate receptors. Release of these pruritogenic mediators and modulators into the periphery may directly activate itch-mediating C-fibers *via* specific receptors on the nerve terminals. Histological observations have shown increased epidermal nerve densities in patients with atopic dermatitis, suggesting that the higher density is at least partly responsible for itch sensitization. This hyperinnervation is likely induced by an imbalance between nerve elongation and repulsion factors produced by keratinocytes. Neuronal matrix metalloproteinases are also involved in the penetration of nerve fibers into the extracellular matrix. Moreover, itch-mediating fibers such as gastrin-releasing peptide$^+$ (GRP$^+$) and Mas-related G-protein coupled receptor A3$^+$ (MrgprA3$^+$) fibers are present in the skin. Clinically, emollients or UV-based therapies can partly control epidermal nerve density, but new substances and classes of antipruritic drugs are needed. This review highlights recent knowledge regarding epidermal nerve fibers that are partly involved in itch sensitization, and discuss peripheral mechanisms and treatments of itch, especially in atopic dermatitis.

Key words atopic dermatitis; epidermal keratinocyte; semaphorin 3A; sensory nerve fiber; UV-based therapy

1. INTRODUCTION

Itch (or pruritus) has been defined as an unpleasant sensation that provokes the desire to scratch. Itch is also believed to signal danger from various environmental factors or physiological abnormalities. Clinically, chronic itch is a burdensome clinical problem that decreases quality of life,[1] and it frequently accompanies a variety of inflammatory skin conditions and systemic diseases. Recent studies have indicated that chronic itch is associated with increases in insomnia[2] and suicide[3] and reductions in patient productivity at work and in the classroom.[4] The development of anti-pruritic treatments therefore requires an understanding of the fundamental mechanisms of itch.

Itch and pain are two basic modalities that are initiated and mediated by primary sensory neurons with cell bodies in the dorsal root ganglia (DRG) and trigeminal ganglia. These neurons are highly diverse in somal sizes, expression of ion channels and receptors, innervation territories, and electrophysiological properties.[5] Small-diameter DRG neurons with unmyelinated axons (C-fibers) are the major neuronal types that mediate itch and pain.[5,6] The sensations of itch and pain are distinct, and each can elicit different behavioral responses, such as scratching to remove irritants and withdrawal to avoid tissue injury, respectively.

Recent studies have implicated histamine-dependent and histamine-independent pathways in transmitting itch. Other systems, including proteases, neuropeptides, cytokines, and opioids, and their cognate receptors, such as thermoreceptors, proteinase-activated receptors (PARs), Mas-related G-protein coupled receptor (Mrgprs) and opioid receptors, are involved in the histamine-independent itch pathway. These pruritogenic mediators and modulators, released in the periphery, may directly activate itch-mediating fibers, especially C-fibers, by binding to specific receptors on the nerve terminals.[5,7] These systems also cross-sensitize each other in the enhancement of itch.[8] In addition, cutaneous nerve fibers are activated by exogenous mechanical, chemical, and biological stimuli, resulting in itch responses.[7,9] Therefore, they are regarded as anti-pruritic targets in patients with chronic itch, including those with atopic dermatitis.

2. HISTOLOGY OF CUTANEOUS NERVE FIBERS IN ATOPIC DERMATITIS

Histological investigations have shown that the density of epidermal nerve fibers is higher in the skin of patients with atopic dermatitis (Fig. 1A) and xerosis than in healthy controls[5,7] (Fig. 1B), although the increases of nerve density in patients with pruigo nodularis and psoriasis remain unclear.[10–12] Similar findings have been observed in animal models, such as NC/Nga mice, a model of atopic dermatitis

The authors declare no conflict of interest.

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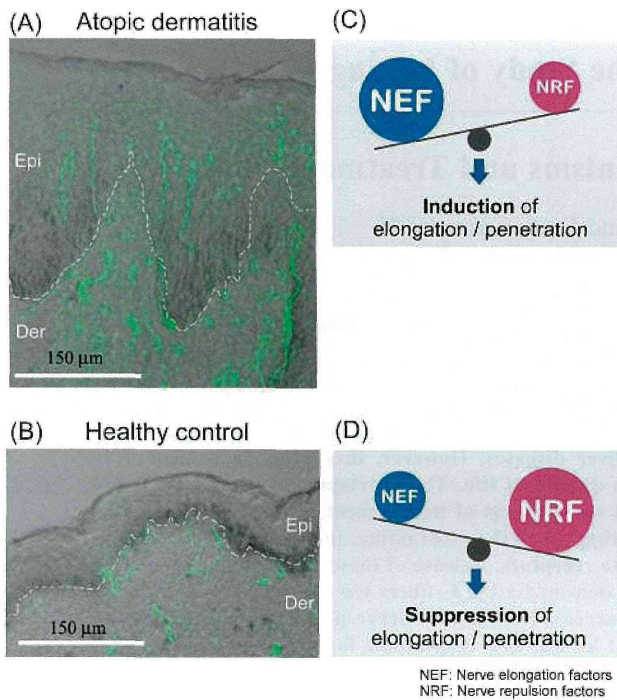

Fig. 1. Distribution of Epidermal Nerve Fibers in Lesional Skin of Atopic Dermatitis and Regulation by Nerve Elongation and Repulsion Factors

A, B; Immunohistochemical images of nerve fibers overlapped with differential interference microscopic images. PGP9.5-immunoreactive fibers (green) are observed at higher densities in the epidermis of atopic dermatitis (A). In contrast, PGP9.5-immunoreactive fibers are mainly distributed in the epidermal–dermal border of healthy controls (B). Scale bars=150 μm. epi, epidermis; der, dermis. C, D; Epidermal NEF levels are higher and epidermal NRF levels are lower in atopic than in healthy skin, suggesting the induction of penetration and/or elongation into the epidermis (C). In contrast, epidermal NEF levels are lower and epidermal NRF levels are higher in healthy than in atopic skin, suggesting the suppression of penetration and/or elongation into the epidermis (D). NEF, nerve elongation factors; NRF, nerve repulsion factors.

model,[13,14] and in dry skin model mice.[15,16] Such increases in nerve density are also found in the dermis of patients with atopic dermatitis and psoriasis.[17,18] These findings are indicative of increases in sensory nerve fibers responsive to exogenous triggering factors and to various endogenous pruritogens from cutaneous cells, such as immune cells and keratinocytes, suggesting that hyperinnervation is partly responsible for itch sensitization.

3. MECHANISMS REGULATING SENSORY NERVE DENSITY IN THE SKIN

Epidermal hyperinnervation is probably caused by an imbalance in nerve elongation factors, such as nerve growth factor (NGF), and nerve repulsion factors, such as semaphorin 3A (Sema3A), produced by keratinocytes[7] (Figs. 1C, D). These axonal guidance molecules may also act on keratinocytes, immune cells and vascular endothelial cells, and may be indirectly involved in the modulation of itching.

3.1. Nerve Elongation Factors

Nerve Growth Factor (NGF) Keratinocyte-derived NGF is a major mediator of cutaneous innervation density, in that local NGF concentrations are higher in the lesional skin of patients with atopic dermatitis, psoriasis, prurigo nodularis, contact dermatitis and xerosis than in normal skin.[5] In adult rat primary sensory neurons, NGF has been shown to upregulate neuropeptides, especially substance P and calcitonin-gene-related peptide (CGRP),[19] both of which are involved in the hypersensitivity of itch sensation and neurogenic inflammation.[20] Using a co-culture model of porcine DRG neurons and human skin cells, human atopic keratinocytes were found to produce elevated levels of NGF and to mediate an increased outgrowth of CGRP-immunoreactive fibers, whereas human atopic fibroblasts did not mediate this outgrowth.[21] These findings indicate that keratinocytes are key factors in hyperinnervation in individuals with atopic dermatitis. Intradermal injection of NGF was also shown to sensitize nociceptors for

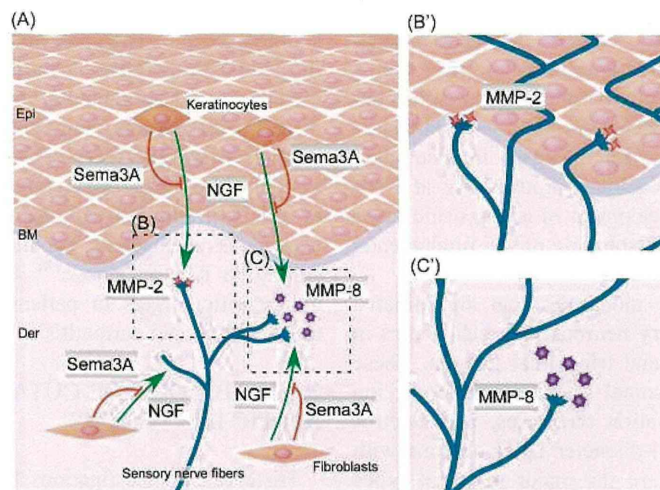

Fig. 2. A Model of Nerve Fiber Penetration into the Extracellular Matrix

(A) NGF and Sema3A, which are produced by skin cells such as epidermal keratinocytes and fibroblasts, modulate the outgrowth of nerve fibers into the extracellular matrix (ECM) through production of neuronal MMPs. (B) NGF promotes MMP-2 production in sensory nerve fibers and activates pro-MMP-2 on the growth cone. Sema3A produced by keratinocytes and fibroblasts may have opposite effects on NGF-dependent events. (B′) Activated MMP-2 on the growth cone may contribute to penetration of nerve fibers into the basement membrane. (C) In the dermis, NGF also promotes MMP-8 production by sensory nerve fibers and its secretion by nerve fibers, probably growth cones. Sema3A may have opposite effects on NGF-dependent events. (C′) Activated MMP-8 may be involved in sensory nerve growth within the interstitial collagen matrix. epi, epidermis; der, dermis; BM, basement membrane.

cowhage- but not histamine-induced itch in human skin.[22] Thus, increased NGF in the skin may sensitize primary afferents, thereby contributing to chronic itch such as atopic dermatitis. Interestingly, tumor necrosis factor (TNF)-α has been found to enhance NGF production in human keratinocytes.[23] TNF-α is a pivotal proinflammatory cytokine in the innate immune response and a key molecule in skin inflammation. Mast cells have been identified as important potential sources of TNF-α.[20] In addition, skin barrier disruption up-regulates TNF-α in the epidermis of acetone-treated mice, an acute dry skin model.[24] These findings suggest that TNF-α is a positive regulator of NGF in the skin.

Amphiregulin Amphiregulin (AR), a protein belonging to the epidermal growth factor family, has been found to affect nerve outgrowth.[25,26] AR expression was previously shown to be up-regulated in the epidermis of NC/Nga mice with atopic dermatitis-like symptoms,[13] suggesting that AR may be involved in the modulation of epidermal nerve density. In addition, AR down-regulates expression of epithelial cell–cell junctional molecules such as E-cadherin and ZO-1 to construct adherens junctions and tight junctions, both of which are critical for skin barrier function.[27] Indeed, the levels of expression of E-cadherin and ZO-1 were decreased in the epidermis of atopic NC/Nga mice, together with the increased expression of AR.[13] Since AR affects the integrity of cell–cell junctions, these findings suggest the attenuation or abrogation of protective function against external mechanical, chemical and biological stimuli in inflammatory skin diseases.

Artemin Artemin is a glial cell line-derived neurotrophic factor. Artemin-expressing fibroblasts have been shown to accumulate in skin lesions of patients with atopic dermatitis.[28] Moreover, dermal fibroblasts secrete artemin in response to substance P, released by cutaneous nerve fibers, inducing itch and/or neurogenic inflammation *via* the activation of neurokinin-1 (NK-1) receptor.[29] Intradermal injection of artemin into mice resulted in peripheral nerve sprouting and thermal hyperalgesia.[28] Therefore, artemin may be partly involved in hypersensitivity to warm sensations, mimicking warmth-provoked itch in atopic dermatitis.

3.2. Nerve Repulsion Factors

Semaphorin 3A (Sema3A) Sema3A is the first member of the semaphorin family shown to cause growth cone collapse in neurons, *i.e.*, to function as an axonal repulsion factor, through its interaction with a neuropilin-1/plexin-A receptor complex.[30] In addition, Sema3A inhibits NGF-induced sprouting of sensory afferents in adult rat spinal cord,[31] whereas elevated levels of NGF reduce the Sema3A-induced collapse of sensory growth cones.[32]

Several recent studies showed that *Sema3A* transcripts were expressed in cultured normal human epidermal keratinocytes.[33,34] Sema3A proteins are mainly distributed in the suprabasal layer of normal human skin,[33] consistent with findings showing that Sema3A is expressed in differentiated keratinocyte cultures.[34] Moreover, epidermal Sema3A levels were lower in patients with atopic dermatitis than in healthy controls, while an increase in epidermal nerve density was found in the skin.[33] Increased epidermal nerve density in acute dry skin mice was also associated with decreased levels of Sema3A expression,[35,36] suggesting that decreasing the expression of Sema3A accelerates epidermal nerve growth in patients with dry skin condition such as atopic dermatitis and xerosis. Thus, epidermal innervation may be regulated by a fine balance between NGF and Sema3A.

Anosmin-1 Anosmin-1 is an extracellular matrix glycoprotein encoded by the *KAL1* (Kallmann syndrome 1 sequence) gene, the gene responsible for the X chromosome-linked recessive form of Kallmann syndrome.[37] Anosmin-1 has been found to inhibit neurite outgrowth in cultured rat DRG neurons.[38] *KAL1* transcripts were also expressed in cultured keratinocytes and in normal human skin. Anosmin-1 was strongly expressed in the basal cell layer of normal skin, but its level of expression was lower in atopic skin, concomitant with an increase in epidermal nerve density. Thus, keratinocyte-derived anosmin-1 may be at least partly involved in modulating epidermal nerve density in patients with atopic dermatitis.[38]

3.3. Roles of Matrix Metalloproteinase (MMP)-2 and MMP-8 in Cutaneous Nerve Growth The process of cutaneous nerve growth in pruritic skin such as atopic dermatitis requires several MMPs for growth cones to penetrate the three-dimensional extracellular matrix (ECM) barriers. Using *in vitro* models of ECM, such as Matrigel and type I collagen gel, MMP-2 localized on the growth cone was found to be involved in penetration into the basement membrane[39] (Figs. 2A, B). In addition, MMP-8 secreted by nerve fibers was shown to be involved in nerve growth within the dermis, consisting mostly of types I and III collagens[40] (Figs. 2A, C). The levels of expression of MMP-2 and MMP-8 were up-regulated by NGF and down-regulated by Sema3A, and both were induced by their enzymatic substrates, but not altered by non-substrate molecules. The selection and up-regulation of MMPs corresponding to the ECM components surrounding the growing nerve fibers may be required for efficient nerve fiber penetration, suggesting that the coordinated activation of neurotrophin and ECM-integrin signaling is necessary for efficient and long-distance axon extension.[41,42] Since class 3 semaphorin signaling inhibits integrin-mediated adhesion signaling, Sema3A stimulation of growing nerve fibers may provide a reverse signaling pathway for these events.[43]

4. ITCH-MEDIATING FIBERS IN THE PERIPHERY

4.1. Gastrin-Releasing Peptide (GRP) Since specific markers of itch-mediating fibers have not been identified to date, they could not be histologically identified in the periphery. Recent studies, however, have demonstrated that GRP receptor expressing cells mediate the itch sensation in the spinal cord.[44] In addition, increases in cutaneous nerve fibers containing GRP have been observed in NC/Nga mice with atopic dermatitis-like symptoms.[14] Therefore, GRP secreted from the central terminals of primary afferents may be involved in the transmission of itch signals in the spinal dorsal horn. Intradermal injections of GRP were found to elicit scratching behavior in mice, and the itch-related response was at least partly induced by the release of pruritogens through activation of bombesin receptors in mast cells.[45] A more recent study showed that serum GRP levels correlate with pruritus in patients with atopic dermatitis.[46] Thus, serum GRP level may be useful as a biomarker for itch and disease severity in patients with atopic dermatitis.

4.2. Mas-Related G-Protein Coupled Receptors (Mrgprs) Recent studies have demonstrated that the his-

tamine-independent itch pathway involves members of the family of over 50 Mrgprs, especially MrgprAs, MrgprB4-5, MrgprC11 and MrgprD, which are restricted to small diameter DRG neurons in mice.[47] Chloroquine and bovine adrenal medulla peptide 8-22 (BAM8-22) elicit itch-related scratching through MrgprA3 and MrgprC11, respectively, in mice.[48] Both chloroquine and BAM8-22 also elicited itch in humans.[49,50]

A more recent study using conditional transgenic mice revealed that ablation of MrgprA3$^+$ DRG neurons led to substantial reductions in scratching evoked by multiple pruritogens and occurring spontaneously under chronic itch conditions.[51] However, pain sensitivity remained intact in these mice. Moreover, mice in which transient receptor potential vanilloid 1 (TRPV1) was exclusively expressed in MrgprA3$^+$ DRG neurons exhibited itch, but not pain, behavior in response to capsaicin. Although MrgprA3$^+$ DRG neurons were sensitive to noxious heat, activation of TRPV1 in these neurons by noxious heat did not alter pain behavior. These findings suggest that MrgprA3 defines a specific subpopulation of DRG neurons that mediate itch. In mouse skin, MrgprA3$^+$ fibers exclusively innervated the epidermis and responded to multiple pruritogens, suggesting that they are peripheral itch-specific fibers.

5. ANTI-PRURITIC THERAPIES

5.1. Anti-NGF Antibody and NGF Receptor Antagonists Anti-NGF approaches have been tried to treat itch of atopic dermatitis in NC/Nga mice. Intraperitoneal administration of anti-NGF neutralizing antibody to atopic NC/Nga mice significantly attenuated both the increased number of nerve fibers in the epidermis and scratching behavior, but did not ameliorate scratching that had already developed.[52] Similarly, application of the TrkA antagonists AG879 and K252a to the nape of atopic NC/Nga mice significantly improved established dermatitis and scratching behavior and reduced the numbers of nerve fibers in the epidermis, suggesting the importance of NGF in the pathogenesis of atopic dermatitis-like skin lesions.[53] Thus, NGF and its receptors may be among the antipruritic targets in pruritic skin diseases such as atopic dermatitis.

5.2. Sema3A Replacement Therapy Recombinant Sema3A replacement approaches (intradermal injection or ointment application) were found to significantly inhibit scratching behavior and to improve dermatitis in NC/Nga mice with atopic dermatitis-like symptoms compared with controls.[54,55] The therapeutic efficacy of exogenous Sema3A on atopic dermatitis-like symptoms was greater than that of current agents, such as betamethasone and tacrolimus.[55] Moreover, histological analyses showed decreases in (i) the numbers of epidermal nerve fibers; (ii) the numbers of inflammatory infiltrates; (iii) the production of cytokines; (iv) the density of dermal blood vessels; and (v) epidermal thickness in Sema3A-treated lesional skin.[54,55] These findings suggest that exogenous Sema3A not only affects sensory nerve fibers, but other cells that express neuropilin-1, include immune system cells, endothelial cells and keratinocytes.[56] Thus, Sema3A and its receptors are promising therapeutic targets for atopic dermatitis.

5.3. Effectiveness of Conventional Therapies on Axonal Guidance Molecules Several existing therapies normalize abnormal levels of axonal guidance molecules such as NGF and Sema3A in pruritic skin, reducting epidermal nerve density.

5.4. Olopatadine (A Histamine H_1-Receptor Antagonist) Oral administration of olopatadine hydrochloride, a histamine H_1-receptor (H_1R) antagonist, significantly suppressed scratching behavior, improved dermatitis, and inhibited neurite outgrowth in the lesional skin of mice with atopic dermatitis-like symptoms. Notably, olopatadine treatment increased Sema3A expression in the epidermis.[57,58] Although it is unclear whether these effects are caused by specific blocking of H_1R signaling, olopatadine may in part improve imbalances between NGF and Sema3A in the epidermis.

5.5. Neurotropin (NTP) NTP, a non-protein extract isolated from the inflamed skin of rabbits inoculated with vaccinia virus, is widely used in Japan and China to treat various chronic pain conditions.[59] In clinical studies in Japan, NTP has been shown to have anti-pruritic effects in patients with eczema, dermatitis and urticaria,[60] and in those undergoing hemodialysis.[61] NTP was found to inhibit NGF-induced neurite outgrowth of rat DRG neurons *in vitro*[62] and to significantly reduce intraepidermal nerve growth in acetone-treated mice, a model for acute dry skin.[63] In the latter, NTP significantly up-regulated epidermal Sema3A mRNA, but had no effect on expression of epidermal NGF mRNA.[63] Thus, although its mechanisms are as yet unknown, NTP may reduce epidermal nerve density by inducing expression of Sema3A in the epidermis, resulting in suppression of itch.

5.6. Emollient A recent study using dry skin mice showed that application of heparinoid cream resulted in greater improvements in epidermal nerve density and epidermal NGF levels than application of petrolatum, although heparinoid cream had no effect on epidermal Sema3A levels.[35] In addition, the increase of epidermal nerve fibers was more reduced by the immediate than the delayed application of emollients to dry skin, suggesting that the prompt application of emollients is more effective in normalizing epidermal hyperinnervation and expression of axon guidance molecules.

5.7. UV-Based Therapy Various types of UV-based therapy, including psoralen-UVA (PUVA) and narrow-band UVB, have been widely used to treat patients with skin inflammation such as atopic dermatitis and psoriasis.[64] The UV-based therapies were shown to reduce the number of cutaneous nerve fibers, especially in the epidermis, in patients with atopic dermatitis and psoriasis, and to inhibit pruritus.[65,66] Similar effects of UV-based therapy on epidermal nerve fibers were observed in dry skin mice.[36] The imbalance between Sema3A and NGF levels in the epidermis was normalized by PUVA or narrow-band UVB treatment.[36,66] A recent study showed that excimer lamp treatment was the most effective form of UV-based therapy for intraepidermal nerve fibers.[36]

5.8. New Anti-pruritic Drugs

Nalfurafine Hydrochloride (A Selective Kappa-Opioid Receptor Agonist) Several studies have demonstrated that mu- and kappa-opioid systems play pivotal roles in modulation of itch at the central nervous system. It is generally thought that the mu-opioid system induces itch, whereas the kappa-opioid system suppresses itch at the central level.[7,67] Recently, the effectiveness of nalfurafine hydrochloride (REMITCH®), a selective kappa-opioid receptor agonist, on hemodialysis-

related uremic pruritus was validated in a Phase III, randomized double-blind placebo-controlled trial.[68] This drug is also expected to make further contributions to the treatment of patients with intractable itch, such as those with cholestasis and atopic dermatitis.

Peripheral mu- and kappa-opioid systems may also play important roles in pruritus of atopic dermatitis.[69–71] One study revealed that topical application of mu-opioid receptor antagonist (naltrexone) cream to the skin inhibited itch in patients with atopic dermatitis.[70] A peripherally restricted kappa-opioid agonist, ICI 204,448, also antagonized chloroquine-evoked scratching in mice.[69] Moreover, the kappa-opioid system was found to be down-regulated in the epidermis of patients with atopic dermatitis and psoriasis, whereas the mu-opioid system was at normal levels.[11,71] Down-regulation of the mu-opioid system and restoration of the kappa-opioid system by PUVA treatment have been observed in patients with atopic dermatitis, concomitant with a reduction in itch.[71] Thus, these findings suggest that mu-opioid receptor antagonists or kappa-opioid receptor agonists have antipruritic effects at the peripheral level.

5.9. Aprepitant (A Neurokinin-1 (NK-1) Receptor Antagonist) Substance P is a key mediator in pruritus.[72] In the periphery, substance P released from cutaneous nerve fibers induces itch and/or neurogenic inflammation.[29] In addition, a recent study supports a role for NK-1 receptor-expressing spinal neurons in itch.[73] Current evidence suggests that substance P is partially involved in the spinal transmission of itch signals.[74]

Aprepitant is an oral NK-1 receptor antagonist[75] widely used as an antiemetic agent in patients with chemotherapy-induced nausea and vomiting.[76] Aprepitant was also found effective against intractable pruritus associated with Sézary syndrome, a leukemic, cutaneous, epidermotropic T-cell lymphoma.[77] In a recent clinical trial, 16 of 20 patients with chronic itch treated with aprepitant showed a significant reduction in itch.[78] Thus, although some patients did not respond, these findings indicated that aprepitant is effective in patients with chronic itch, including atopic dermatitis.

5.10. Histamine H_4 Receptor (H_4R) Antagonists H_4R have been reported involved in histamine-evoked itch in animal models.[79] While specific H_4R agonists induced itch, pretreatment with the H_4R antagonist JNJ7777120 inhibited this response, and histamine or H_4R agonist-evoked itch was attenuated in H_4R-deficient mice. Moreover, inhibiting both H_1R and H_4R almost completely eliminated histamine-evoked scratching.[80] A more recent study showed that the H_1R antagonist olopatadine and the H_4R antagonist JNJ7777120 reduced scratching behavior and skin inflammation in the NC/Nga mouse model of chronic allergic dermatitis induced by repeated challenges with picryl chloride.[58] Thus, H_4R antagonists may be effective in patients with chronic itch, including those with atopic dermatitis.

6. CONCLUSION

This review presents recent knowledge regarding itch sensitization associated with epidermal nerve density controlled by nerve elongation factors (*e.g.*, NGF) and nerve repulsion factors (*e.g.*, Sema3A) through the regulation of expression of MMPs, especially in atopic dermatitis. In addition, treatment with anti-NGF agents, Sema3A replacement and other treatments such as UV-based therapies may normalize epidermal nerve fiber density. New substances and classes of antipruritic drugs are needed. The research on and development of antipruritic drugs may contribute to improvements in the quality of life of patients who suffer from intractable pruritus such as atopic dermatitis.

Acknowledgments This work was supported by a Health Labor Sciences Research Grant for Research on Allergic Disease and Immunology from the Japanese Ministry of Health, Labour and Welfare, by a KAKENHI (20591354 and 2079081) and a "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT, and by a JSPS Research Fellow.

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ORIGINAL ARTICLE

Matrix Metalloproteinase-8 Is Involved in Dermal Nerve Growth: Implications for Possible Application to Pruritus from *In Vitro* Models

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Cutaneous nerve density is related to abnormal itch perception in dermatoses, such as atopic dermatitis and xerosis. However, the mechanisms underlying the elongation of dermal nerve fibers within the interstitial collagen (CoL) matrix are poorly understood. In this study, a culture system of rat dorsal root ganglion neurons consisting of type I CoL and a Boyden chamber containing a nerve growth factor (NGF) concentration gradient was used. Nerve fibers penetrating into type I CoL gel were observed in the presence of the NGF concentration gradient. Levels of matrix metalloproteinase-8 (MMP-8) mRNA and protein were increased in the cultured neurons and the conditioned medium, respectively. The nerve fiber penetration was dose dependently inhibited by MMP-8 blockers. Moreover, MMP-8 immunoreactivity was partially localized at growth cones in NGF-responsive nerve fibers. Semaphorin 3A stimulation also showed the opposite effects on these NGF-dependent events. Intriguingly, *MMP-8* expression was upregulated by type I and III CoLs, which are substrates for this enzyme. These results suggested that MMP-8 is involved in sensory nerve growth within the interstitial CoL matrix through modulation by the axonal guidance molecules and/or extracellular matrix components. These findings provide insight into the development of pruritus involving skin nerve density.

Journal of Investigative Dermatology (2011) **131**, 2105–2112; doi:10.1038/jid.2011.173; published online 23 June 2011

INTRODUCTION

Pruritus, or itch, is the most common symptom of dermatological diseases in addition to several systemic disorders, such as renal failure. Numerous pruritogenic mediators and modulators released in the periphery may directly activate itch-sensitive C-fibers by binding to specific receptors on the nerve terminals. Alternatively, these molecules may function indirectly by releasing pruritogenic mediators and modulators from other cells. Nerve fibers can be activated by exogenous mechanical and chemical stimulation, resulting in itch responses (Ikoma *et al.*, 2006; Paus *et al.*, 2006).

Primary afferent fibers are acceptors of itch and pain sensations in the skin. Histological observations have indicated that epidermal nerve fibers are present at higher densities in the skin of patients with prurigo nodularis, atopic dermatitis (AD), psoriasis, contact dermatitis, and xerosis than in healthy individuals (Ikoma *et al.*, 2006; Tominaga and Takamori, 2010). The hyperinnervation is probably caused by the abnormal expression of axon guidance molecules, such as nerve growth factor (NGF) and semaphorin 3A (Sema3A), produced by keratinocytes (Tominaga *et al.*, 2007a, b, 2008; Tengara *et al.*, 2010). Such increases in nerve density are found in the dermis of patients with AD or psoriasis (Urashima and Mihara, 1998; Nakamura *et al.*, 2003), as well as animal models such as NC/Nga mice (Tominaga *et al.*, 2009b). These findings are indicative of increases in sensory receptors responsive to exogenous trigger factors and to various endogenous pruritogens from immune cells and keratinocytes, suggesting that hyperinnervation is partly responsible for intense itch perception. However, the mechanisms of dermal nerve growth within the interstitial collagen (CoL) matrix remain unknown.

Interstitial CoLs (types I and III) are major structural components of the extracellular matrix (ECM) in the dermis of mammalian skin (Elder *et al.*, 2005). They are composed of three polypeptide chains arranged in a rigid triple helix conformation, rendering them resistant to degradation by proteinases other than the interstitial collagenases. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of degrading ECM components. MMP-8 along with MMP-1 and MMP-13 are the major members of the interstitial collagenase subgroup of the MMP family. The interstitial collagenases mediate the initial and rate-limiting step in interstitial CoL degradation by cleaving the three CoL polypeptide chains (Gross and Nagai, 1965).

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Abbreviations: AD, atopic dermatitis; BCC, Boyden chamber culture; CoL, collagen; DRG, dorsal root ganglion; ECM, extracellular matrix; MMP, matrix metalloproteinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-PCR; Sema3A, semaphorin 3A

Received 19 October 2010; revised 3 March 2011; accepted 23 April 2011; published online 23 June 2011

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Although MMP-8 cleaves all three interstitial CoLs, it differs from MMP-1 in that it cleaves type I CoL (CoL1) at a higher rate than CoL3. MMP-8 also degrades CoL2, the predominant CoL of cartilage, at a higher rate than MMP-1 (Jeffrey, 2001). Our recent study demonstrated that membrane-associated MMP-2 contributes to the penetration of nerve fibers into Matrigel, an *in vitro* model of the basement membrane, through modulation by axonal guidance molecules and/or ECM (Tominaga *et al.*, 2009a). These findings raise the possibility that neuronal interstitial collagenases are associated with sensory nerve growth within the dermis, and that axonal guidance molecules modulate the expression and enzymatic activity of MMPs, which can degrade interstitial CoLs, but the expression and roles of interstitial collagenases in neurons are poorly understood.

In this study, a unique culture system of dorsal root ganglion (DRG) neurons consisting of Boyden chambers and CoL1 was used to investigate the mechanism of nerve fiber elongation within the dermis of skin. Here we report the participation of MMP-8 secreted from the nerve fibers in the elongation mechanism and its possible application to the understanding of pruritus.

RESULTS

Axonal outgrowth assays in the Boyden chamber culture (BCC) system

Axonal outgrowth assays were performed using DRG neurons grown on CoL1 gel-coated membranes in Boyden chambers in the presence of an NGF concentration gradient (Figure 1a). In the BCC system, to assess whether the membranes were coated with CoL1 gel, they were stained with anti-porcine CoL1 antibody. Immunoreactivity for CoL1 was reliably detected in the CoL1 gel-coated membranes but not in the uncoated membranes (Figure 1b).

The CoL1 gel-coated membrane was removed from the Boyden chamber, and then stained with an antibody against Tau, a protein enriched in the axons (Mandell and Banker, 1996). When immunofluorescence staining of the underside of the membrane was observed on confocal laser scanning microscopy, the number of Tau$^+$ fibers that crossed the CoL1 gel-coated membrane was shown to have increased significantly with the NGF concentration gradient compared with controls (Figure 1c). Although DRG neurons were cultured in the presence of axonal guidance molecules or MMP inhibitors in this study, there were no significant differences in neuronal survival among agents in comparison with vehicle controls (Figure 1d and Supplementary Figure S1 online).

NGF-induced MMP-8 is involved in the penetration of nerve fibers into CoL1 gel

To identify NGF-inducible MMPs involved in the process of nerve fiber penetration, quantitative reverse transcription-PCR (qRT-PCR) analyses using primers for interstitial collagenases, such as MMP-1a, -8, and -13, were performed on DRG neurons cultured in the BCC system. In the cultured

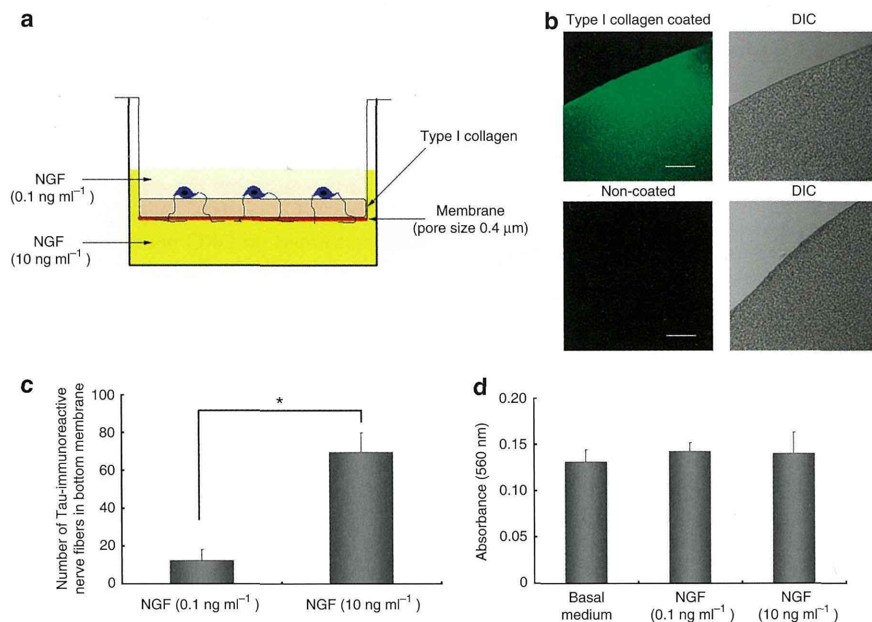

Figure 1. Nerve growth factor (NGF) induced penetration of dorsal root ganglion fibers into collagen 1 gel in the Boyden chamber culture system. (**a**) Schematic representation of the Boyden chamber culture system with collagen 1 gel-coated membranes and NGF concentration gradient. (**b**) Collagen 1 immunoreactivity (green) was detected in the collagen 1 gel-coated membrane but not in the non-coated membrane. They were also observed by differential interference contrast (DIC) microscopy. Bars = 75 μm. (**c**) The number of Tau$^+$ fibers that crossed the collagen 1 gel-coated membrane was significantly increased with the NGF concentration gradient compared with the control (*$P<0.01$). (**d**) Methylthiazole tetrazolium assays for neuronal viability. There were no significant differences among the different culture conditions. The values in **c** and **d** represent the means ± SD from three independent experiments.

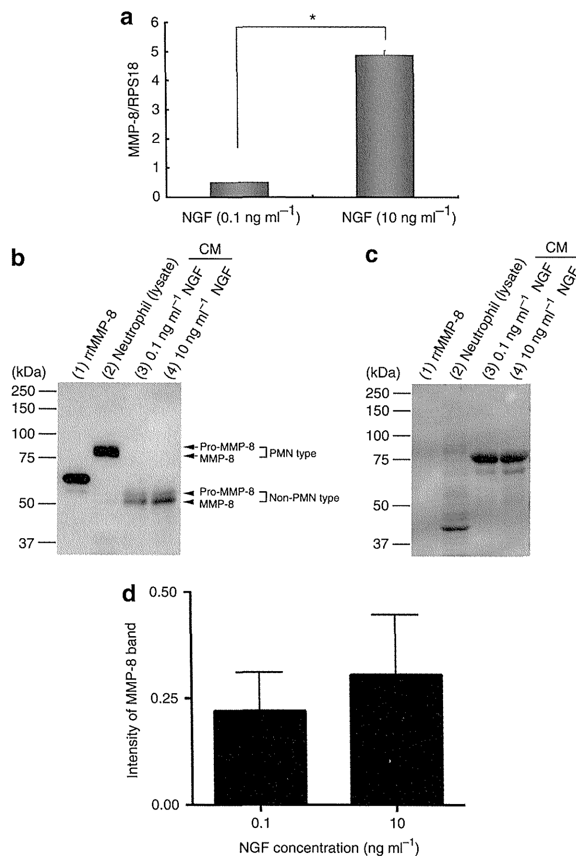

Figure 2. Expression of matrix metalloproteinase-8 (MMP-8) in the cultured dorsal root ganglion (DRG) neurons. (**a**) In quantitative reverse transcriptase-PCR analysis, *MMP-8* expression was increased in DRG neurons cultured for 48 hours in the presence of nerve growth factor (NGF) concentration gradient. (**b**) Western blotting of MMP-8 on conditioned media (CM) from the Boyden chamber culture system. Lane 1, rrMMP-8; lane 2, human neutrophil lysates; lane 3, CM from neuronal cultures without the NGF concentration gradient; lane 4, CM from neuronal cultures with the NGF concentration gradient. PMN, polymorphonuclear leukocyte. (**c**) The membrane stained with Ponceau solution after western blotting. (**d**) In semiquantitative analysis, the MMP-8 levels were modestly increased in the presence of NGF concentration gradient. The values in **a** and **d** represent the means ± SD of three independent experiments (*$P < 0.01$).

neurons, the levels of *MMP-8* transcripts showed the greatest increase with the NGF concentration gradient (Figure 2a and Supplementary Figure S2a, b online). The amount of MMP-8 in the conditioned medium was also examined by western blotting analysis. In culture with the NGF concentration gradient, two species of MMP-8 were detected with molecular weights of approximately 50–60 kDa, representing latent and activated forms of less glycosylated non-polymorphonuclear leukocyte-type MMP-8 (Figure 2b and c), and the amount of MMP-8 was modestly increased compared with the controls (Figure 2d). Two bands of MMP-8 were also detected at approximately 75–80 kDa in total lysate from human neutrophils, representing latent and activated forms of highly glycosylated non-polymorphonuclear leukocyte-type MMP-8 (Prikk *et al.*, 2002).

To further assess the direct involvement of MMP-8 in nerve fiber penetration, its enzyme activities were blocked with inhibitor molecules or neutralizing antibodies in the BCC system. The penetration of nerve fibers into CoL1 gel was dose dependently inhibited by MMP-8 inhibitor I, whereas the negative control of its inhibitor, which is an isomer, or the broad serine proteinase inhibitor aprotinin did not (Figure 3a). Moreover, anti-MMP-8-neutralizing antibody blocked its penetration, whereas control IgG or anti-MMP-2-neutralizing antibody as a specificity control did not (Figure 3b). In two-dimensional cultures using a thin layer of CoL1, MMP-8 inhibitor I showed no inhibitory effect on neurite outgrowth of DRG neurons (Figure 3c).

MMP-8 are localized at growth cones of cultured DRG neurons

The distribution of MMP-8 was examined immunocytochemically in DRG neurons cultured for 48 hours in the presence of 10 ng ml^{-1} NGF. The cultured neurons were also stained with anti-tropomyosin receptor kinase A receptor antibody for detection of NGF-responsive nerve fibers. MMP-8 immunoreactivity was observed in the nerve fibers, including the growth cones of tropomyosin receptor kinase A^{+} fibers (Figure 4).

Modulation of MMP-8 expression by other factors

To examine the possibility that other factors modulate MMP-8 expression, axonal outgrowth assays were performed using the BCC system with concentration gradients of both NGF and Sema3A. The nerve fiber penetration into CoL1 gel induced with the NGF concentration gradient was inhibited by Sema3A in a dose-dependent manner (Figure 5a).

To further examine the effects of Sema3A stimulation on NGF-induced *MMP-8* expression, qRT-PCR analyses were performed on DRG neurons grown in the BCC system. *MMP-8* expression in the cultured neurons was increased in the presence of the NGF concentration gradient, and the increase was significantly inhibited by Sema3A stimulation (Figure 5b).

The effects of ECM components on *MMP-8* expression were also analyzed by qRT-PCR on DRG neurons cultured on Boyden chamber membranes coated with poly-D-lysine or various ECM proteins in the presence of the NGF concentration gradient (Figure 5c). *MMP-8* expression was upregulated by CoL1 and CoL3, which are substrates for the enzyme. The level of gene expression was also increased by basement membrane components, such as CoL4 and laminin or Matrigel, although the abilities of these ECM components to induce gene expression were lower than that of CoL1. Non-substrates, such as fibronectin and poly-D-lysine, had no effect on its expression.

DISCUSSION

This study using the BCC system showed that nerve fiber penetration into the CoL1 gel was induced by the NGF

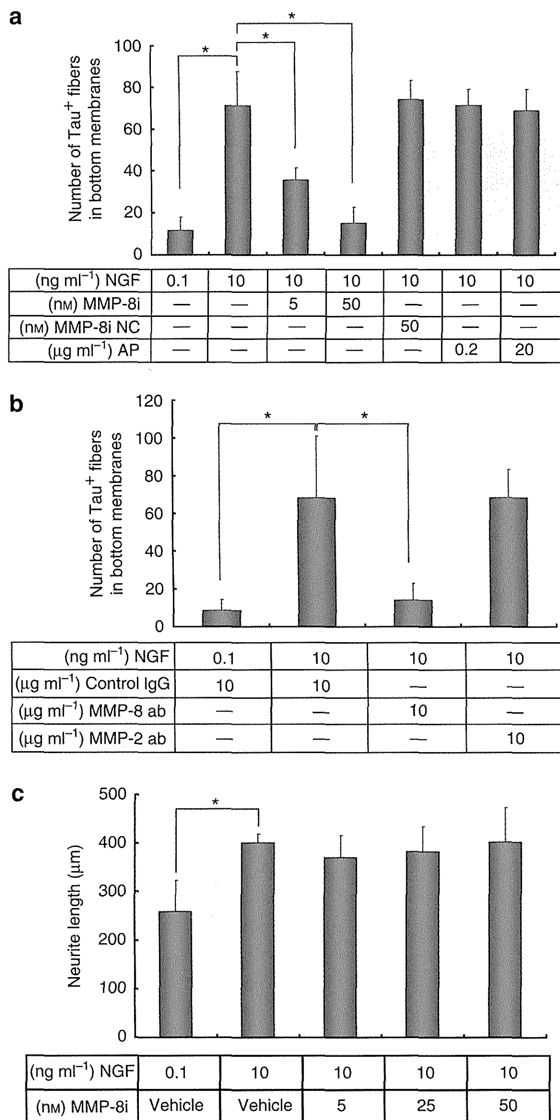

Figure 3. Involvement of matrix metalloproteinase-8 (MMP-8) in nerve fiber penetration into collagen 1 gel. (**a**) In the Boyden chamber culture system, the number of Tau$^+$ fibers that crossed the collagen 1 gel-coated membrane was increased by the nerve growth factor (NGF) concentration gradient, whereas the penetration was inhibited by MMP-8 inhibitor I (MMP-8i) in a dose-dependent manner. MMP-8i negative control (MMP-8i NC) and aprotinin (AP) had no inhibitory effect on nerve fiber penetration. (**b**) Neutralizing antibody (ab) to MMP-8, not MMP-2, blocked the nerve fiber penetration in the Boyden chamber culture system. (**c**) Two-dimensional cultures using a thin-layer collagen 1 showed that neurite outgrowth of DRG neurons grown on collagen 1 gel was not inhibited by MMP-8i. The values in **a** and **c** represent the means ± SD of three independent experiments (*$P<0.01$).

concentration gradient. The axonal outgrowth assays also showed that NGF induced expression of *MMP-8* in the cultured neurons, and an inhibitor and a neutralizing antibody for MMP-8 inhibited penetration of nerve fibers across the membrane. The inhibitor had no effect on neurite outgrowth of DRG neurons grown on CoL1 gel in two-dimensional cultures. The MMP-8 inhibitor was designed previously and investigated by comparative molecular field analysis and X-ray structure analysis (Matter *et al.*, 1999). Its specificity has been demonstrated in previous *in vitro* studies using enzyme assays, and the inhibitory constant 50 of the inhibitor was 4 nM (Matter and Schwab, 1999; Matter *et al.*, 1999). This may also be supported by the data from our axonal outgrowth assays. Moreover, the isomer of MMP-8 inhibitor, aprotinin, and anti-MMP-2-neutralizing antibody did not inhibit the penetration of nerve fibers in this study. MMP-8 cleaves CoL1 at a higher rate than CoL3 (Jeffrey, 2001). Therefore, our findings suggest that NGF-inducible MMP-8 is involved in the process of nerve fiber penetration into CoL1 gel. A recent study indicated that MMP-8 levels in wash samples taken from the lesional skin surface of patients with AD are higher than those of healthy controls and unaffected AD skin (Harper *et al.*, 2010). Thus, non-neuronal cell-derived collagenases may also contribute indirectly to nerve fiber penetration *in vivo*.

MMP-8 is mainly produced by neutrophils, where it is concentrated in secretory granules that are degranulated on neutrophil activation (Lin *et al.*, 2008). However, it has become evident that MMP-8 can be expressed in a wide range of cells, mainly in the course of different inflammatory conditions (Van Lint and Libert, 2006). Our expression analyses showed that the cultured DRG neurons expressed *MMP-8* transcripts. Moreover, the expression of *MMP-8* was induced by the NGF concentration gradient, and the amount of MMP-8 in the conditioned medium was increased. Thus, the neuronal MMP-8 may function in the biological events during the development and disease conditions. Our immunocytochemical study of cultured DRG neurons also showed that MMP-8 is present within nerve fibers including growth cones. Therefore, these data suggest that MMP-8 secreted from nerve fibers cleaves interstitial CoLs, and thereby promotes nerve growth within the CoL gel.

Our data showed that the amount of secreted MMP-8 was modestly increased compared with that at the RNA level. This suggests the existence of a membrane-bound MMP-8 form in the neurons as well as polymorphonuclear cells (Owen *et al.*, 2004). Another possibility is that *MMP-8* transcripts are localized and stored in developing nerve fibers and growth cones. When the nerve fibers newly extend, the growth responses are mediated by rapid protein synthesis and degradation in the nerve fibers and growth cones, but not from cell bodies (Wu *et al.*, 2005), because a great deal of time and energy are necessary to synthesize the nerve growth-related genes in the cell bodies of the DRG, and to transport the proteins to the nerve endings distributed in the skin after they are translated (Campbell and Holt, 2001). These findings may also explain the differences in the MMP-8 mRNA and protein levels.

An inactive form of MMP-8 is activated by serine proteases, such as cathepsin G, chymotrypsin, tissue plasminogen activator, and urinary plasminogen activator, as well