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Mechanisms Regulating Epidermal Innervation in Pruritus of Atopic Dermatitis

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1. Introduction

Histamine, the best-known pruritogen in humans, is also regarded as an experimental itch-causing substance. Clinically, antihistamines, *i.e.*, H_1-receptor blockers, are used to treat all types of itch resulting from renal and liver diseases, as well as from serious skin diseases such as atopic dermatitis. Antihistamines, however, often lack efficacy in patients with chronic itch involving other agonists, including proteases, neuropeptides, cytokines, and opioids, as well as their cognate receptors, including thermoreceptors, PAR-2, and opioid receptors. Release of these pruritogenic mediators and modulators into the periphery may directly activate itch-sensitive C-fibers by binding to specific receptors on the nerve terminals (Ikoma et al., 2006; Paus et al., 2006). Nerve fibers can also be activated by exogenous mechanical, chemical, or biological stimuli, resulting in itch responses (Tominaga and Takamori, 2010). Histological examination has shown increased epidermal nerve densities in patients with atopic dermatitis (AD), suggesting that this higher density may be at least partly responsible for the intense itching in the skin. Such hyperinnervation is probably caused by an imbalance of nerve elongation factors (*e.g.* nerve growth factor, amphiregulin, and gelatinase) and nerve repulsion factors (*e.g.* semaphorin 3A and anosmin-1) produced by keratinocytes (Tominaga and Takamori, 2010; Tengara et al., 2010). Using a unique system of culturing rat dorsal root ganglion (DRG) neurons, consisting of Boyden chambers and extracellular matrix (ECM), we recently demonstrated that neuronal matrix metalloproteinase-2 (MMP-2) is involved in the penetration of sensory nerve fibers into basement membrane through modulation by axonal guidance molecules and/or ECM (Tominaga et al., 2009a). Clinically, psoralen-UVA (PUVA) therapy may reduce epidermal hyperinnervation in patients with AD by normalizing abnormal Sema3A and NGF expression in the epidermis, decreasing in visual analog scale (VAS) scores of pruritus severity (Tominaga et al., 2009c). Such anti-nerve growth effects have been observed in the dry skin of acetone-treated mice following exposure to narrowband-UVB and excimer lamps (Kamo et al., 2011a). These findings may help understand the mechanisms by which UV-based therapy modulate epidermal innervation. This chapter presents recent knowledge regarding the relationship between pruritus and epidermal nerve density, especially in AD.

2. Itch involving epidermal nerve fibers

Many pruritogenic mediators and modulators released into the periphery may directly activate itch-sensitive C-fibers by binding to specific receptors on the nerve terminal. Alternatively, these molecules may act indirectly by inducing other cells to release pruritogenic mediators and modulators. Nerve fibers are activated by exogenous mechanical, chemical, and biological stimuli, resulting in itch responses (Ikoma et al., 2006; Paus et al., 2006; Tominaga and Takamori, 2010).

Sensory nerve fibers are acceptors of itch and pain sensations in the skin. The neuronal mechanisms underlying intractable pruritus have been partially identified to date. Histological examination has shown that the density of epidermal nerve fibers is higher in the skin of patients with AD, contact dermatitis and xerosis than in control individuals (Figure 1) (Ikoma et al., 2006; Tominaga and Takamori, 2010), although the nerve density in patients with pruigo nodularis and psoriasis remain unclear (Stander et al., 2011; Taneda et al., 2011). Similar findings have been observed in animal models such as AD NC/Nga (Tominaga et al., 2007a) and dry skin (Tominaga et al., 2007b) mice, indicating increases in sensory receptors responsive to exogenous triggering factors and endogenous pruritogens from immune cells and keratinocytes and suggesting that hyperinnervation is at least partly responsible for intense itch sensations (Ikoma et al., 2006).

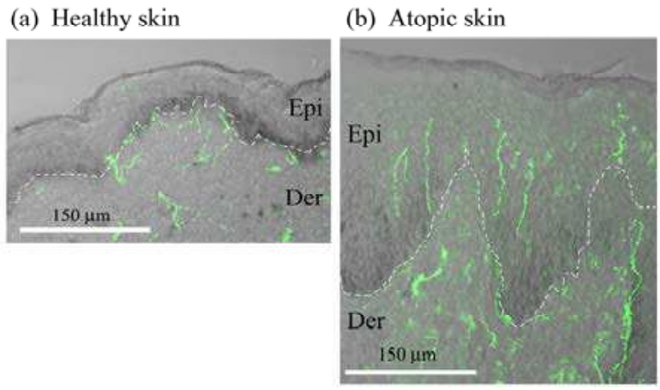

Fig. 1. Distribution of epidermal nerve fibers in healthy and atopic skin.
Staining of the skin of healthy volunteers and AD patients with antibody to protein gene product 9.5 (anti-PGP9.5). Nerve fibers images were overlapped with differential interference microscopic images. (a) PGP9.5-immunoreactive nerve fibers (green) were occasionally present in the epidermis of healthy volunteers. (b) Epidermal nerve fibers were observed at higher densities in AD patients. Scale bars = 150 μm.

In patients with lichen amyloidosis, itch has been associated with low densities of nerve fibers in the epidermis and dermoepidermal junctions (Maddison et al., 2008). Recently, a missense mutation in the OSMR gene, which encodes oncostatin M-specific receptor beta (OSMRb), was found in three families affected by familial primary localized cutaneous amyloidosis, an autosomal dominant disorder (Tanaka et al., 2009). OSMRb is a component of the interleukin (IL)-31 receptor, and IL-31 is an inducer of itch (Sonkoly et al., 2006). In addition, IL-31 receptor and OSMRb are expressed in afferent fibers in the spinal cord and the dermis of the skin (Bando et al., 2006). Therefore, cross-talk between cutaneous nerve fibers and IL-31 may induce itch in lichen amyloidosis, although further studies are required to determine the correlation between IL-31 receptor function and nerve degeneration in lichen amyloidosis.

In addition, diminished skin innervation has been observed in the skin of patients with neuropathic itch (Wallengren et al., 2002). This spontaneous itching may emanate from a central nervous system disorder, such as stroke, and continue in partly denervated skin. However, its mechanisms have not yet been elucidated.

3. Regulation of epidermal nerve fiber density by axonal guidance molecules

3.1 Nerve elongation factors

Nerve growth factor (NGF) is a neurotrophin that affects neurite outgrowth and neuronal survival (Lewin et al., 1993). Keratinocyte-derived NGF is a major mediator of skin innervation density, with higher local NGF concentrations in the lesional skin of patients with prurigo nodularis, AD, psoriasis, contact dermatitis and xerosis than in normal skin (Ikoma et al., 2006). In adult rat primary sensory neurons, NGF has been shown to upregulate neuropeptides, especially substance P (SP) and calcitonin-gene-related peptide (CGRP) (Verge et al., 1995), both of which are involved in the hypersensitivity of itch sensation and neurogenic inflammation (Steinhoff et al., 2003). Several studies using NC/Nga mice have demonstrated that anti-NGF approaches significantly inhibited both epidermal nerve growth and scratching behavior, but did not ameliorate scratching that had already developed (Takano et al., 2005; Takano et al., 2007). These anti-NGF approaches, however, did not completely inhibit itch responses, indicating that other mechanisms may also regulate epidermal innervation.

Amphiregulin (AR), a protein belonging to the epidermal growth factor (EGF) family, has been found to affect nerve fiber elongation (Kimura et al., 1992; Nilsson and Kanje, 2005). AR expression was also shown to be upregulated in the epidermis of NC/Nga mice with AD (Tominaga et al., 2007a), suggesting that AR is a regulator of epidermal nerve density in the skin.

Matrix metalloproteinases (MMPs) have been reported to catalyze the release of AR from transmembrane precursors, a release blocked by GM6001, a broad-spectrum MMP inhibitor, and by MMP-2/MMP-9 (i.e. gelatinase A/B) inhibitors (Kansra et al., 2004). Gelatinase activities were found to be higher in the suprabasal layer of atopic NC/Nga mice than in controls (Tominaga et al., 2007a). In addition, transmembrane-type AR was found to localize on the cell surface of basal cells, whereas AR was diffused in the suprabasal layer. Thus, gelatinase in suprabasal cells may be involved in AR elaboration into the intercellular space between keratinocytes.

TNF-α is a pivotal proinflammatory cytokine in the innate immune response and a key molecule for skin inflammation. Mast cells have been identified as important sources of TNF-α (Steinhoff et al., 2003). Plasma TNF-α concentration is increased in AD (Sumimoto et al., 1992), and both TNF-α and its receptors are upregulated in dermal blood vessels from patients with psoriasis (Kristensen et al., 1993). A study using mast cell- and TNF-deficient mice demonstrated that TNF produced by mast cells promotes the elongation of epidermal and dermal nerve fibers in a mouse model of contact dermatitis (Kakurai et al., 2006). Partly because of their close anatomical association, it has been suggested that cutaneous sensory nerves and mast cells may represent a functional unit, whereby stimulated nerve fibers may activate local mast cells, which in turn can control local nerve function (Steinhoff et al., 2003). Thus, mast cell-derived TNF may act as a nerve elongation factor in inflamed skin. TNF receptors are also expressed on peripheral nerves (Shubayev et al., 2004). TNF may also directly affect sensory nerves, but the details are still uncertain. More recently, TNF-α was

reported to enhance NGF production in human keratinocytes (Takaoka A., 2009), suggesting a close relationship between mast cells and keratinocytes in nerve fiber elongation.

3.2 Nerve repulsion factors

During neural development, nerve fibers are regulated by both attraction and repulsion factors to reach its targets (e.g. skin and muscle). Semaphorin 3A (Sema3A) is a diffusible molecule that induces growth cone collapse and axonal repulsion of several neuronal populations through its interaction with a neuropilin-1 (Nrp-1)/plexin-A receptor complex (Fujisawa, 2004). Sema3A acts by selectively repelling axons from a subset of embryonic dorsal root ganglion (DRG) neurons, which are small in diameter and responsive to NGF (Messersmith et al., 1995; Shepherd et al., 1997). Sema3A has been found to induce the retraction of NGF-responsive sensory afferents in adult mammalian spinal cord (Dontchev and Letourneau, 2002).

Sema3A transcripts are also expressed in cultured normal human epidermal keratinocytes, and Sema3A proteins are mainly distributed in the suprabasal layer of normal human skin (Tominaga et al., 2008) (Figure 2).

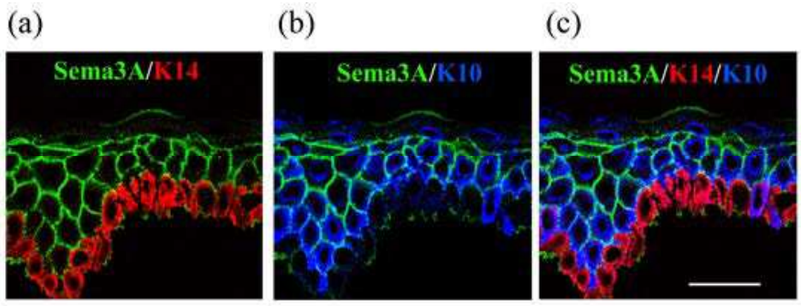

Fig. 2. Distribution of Sema3A in human healthy skin.
Normal human skin was triply stained for Sema3A (green), keratin 14 (K14; red) and K10 (blue). (a) A merged image of Sema3A (green) and K14 (red). (b) A merged image of Sema3A (green) and K10 (blue). (c) A merged image of Sema3A (green), K14 (red) and K10 (blue). Immunoreactivity for Sema3A was slight in the K14-positive cell layer but stronger in the K10-positive cell layer. Scale bars = 30 μm.

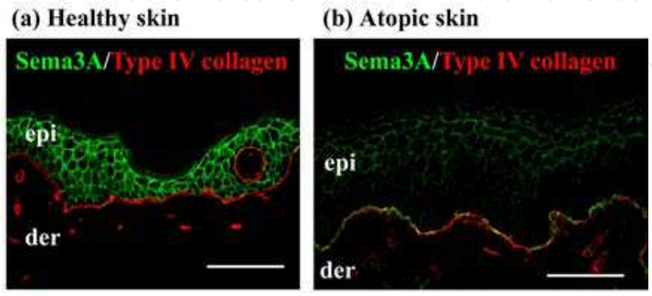

Fig. 3. Decreased production of Sema3A in the epidermis of AD patients.
Skins of healthy volunteers (a) and AD patients (b) were doubly stained for Sema3A (green) and type IV collagen (red). Sema3A expression was lower in the epidermis of AD patients than in healthy volunteers. Scale bars = 75 μm. epi: epidermis, der: dermis.

Recently, epidermal Sema3A levels were reported to be lower in patients with AD than in healthy volunteers, concomitant with an increase in epidermal nerve density (Tominaga et al., 2008), indicating a good correlation between epidermal innervation and Sema3A levels (Figure 3). Moreover, Sema3A has been found to inhibit NGF-induced sprouting of sensory afferents in adult rat spinal cord (Tang et al., 2004), whereas elevated levels of NGF reduced the Sema3A-induced collapse of sensory growth cones (Dontchev and Letourneau, 2002).
These findings suggest that decreasing the expression of Sema3A can accelerate epidermal nerve growth in individuals with AD. Thus, epidermal innervation may be regulated by a fine balance between nerve elongation and repulsion factors (Figure 4). These findings may also provide new potential therapeutic targets for ameliorating pruritus associated with epidermal nerve density, including AD. The role of Sema3A in abnormal itch perception has been confirmed by recombinant Sema3A replacement approaches in atopic NC/Nga mice (Yamaguchi et al., 2008).

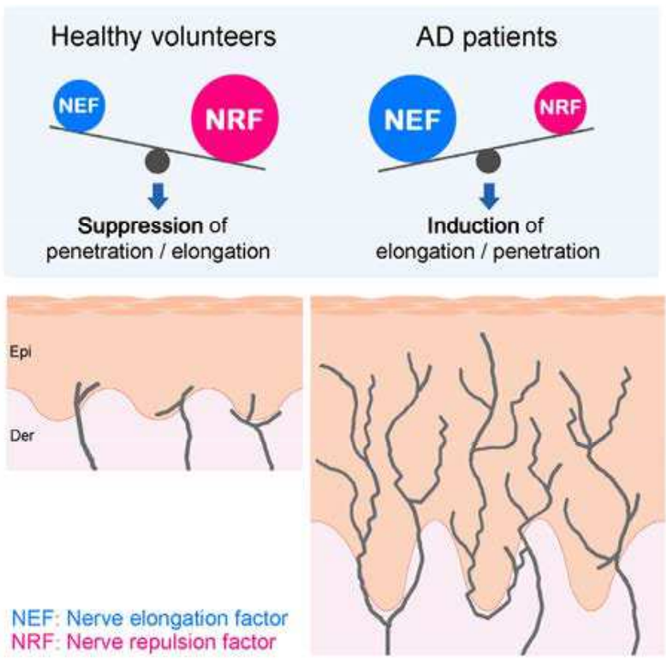

Fig. 4. A regulatory model of sensory nerve fiber penetration into the epidermis by a balance of nerve elongation and repulsion factors.
Epidermal NEF levels were lower and epidermal NRF levels were higher in healthy than in atopic skin, suggesting the suppression of penetration and/or elongation into the normal epidermis. In contrast, epidermal NEF levels were higher and epidermal NRF levels were lower in atopic than in healthy skin. Epidermal nerve density may be regulated by a fine balance between NEF and NRF. Epi, epidermis; Der, dermis; NEF, nerve elongation factors; NRF, nerve repulsion factors.

Anosmin-1, an extracellular matrix glycoprotein anosmin-1 encoded by *KAL1* (Kallmann syndrome 1 sequence), the gene responsible for the X chromosome-linked recessive form of Kallmann syndrome (Soussi-Yanicostas et al., 1996; Kim et al., 2008), was recently shown to be involved in epidermal innervations in AD (Tengara et al., 2010). Anosmin-1 has been shown to play several roles during neural development. For example, it was found to

promote the migration of gonadotropin-releasing hormone-producing neurons, to guide the navigation of axons from mitral cells and to participate in the formation of their collaterals, and to stimulate the outgrowth and branching of Purkinje axons *in vitro* (Soussi-Yanicostas et al., 1998; Kim et al., 2008). Interestingly, coculturing of cerebellar granular neurons with anosmin-1-overexpressing CHO cells showed that anosmin-1 also has an inhibitory effect on neurite outgrowth (Soussi-Yanicostas et al., 1998) and further indicates the importance of anosmin-1 in regulating neurons.

We recently reported that conditioned medium from *KAL1*-overexpressing cells inhibited neurite outgrowth in cultured DRG neurons (Tengara et al., 2010). *KAL1* transcripts are expressed in cultured keratinocytes and in normal human skin. Anosmin-1 is strongly expressed in the basal cell layer of normal skin, but its expression is lower in atopic skin, concomitant with increases of epidermal nerve fibers (Figure 5). Moreover, *KAL1* expression is downregulated during keratinocyte differentiation in a high-calcium medium but is upregulated by IL-4, IL-13 or transforming growth factor (TGF)-β1. TGF-β1 was found to act synergistically with IL-13 to enhance *KAL1* expression, whereas IFN-γ inhibited its expression. Thus, anosmin-1 produced by epidermal keratinocytes in response to calcium concentrations or cytokines may modulate epidermal nerve density in individuals with AD.

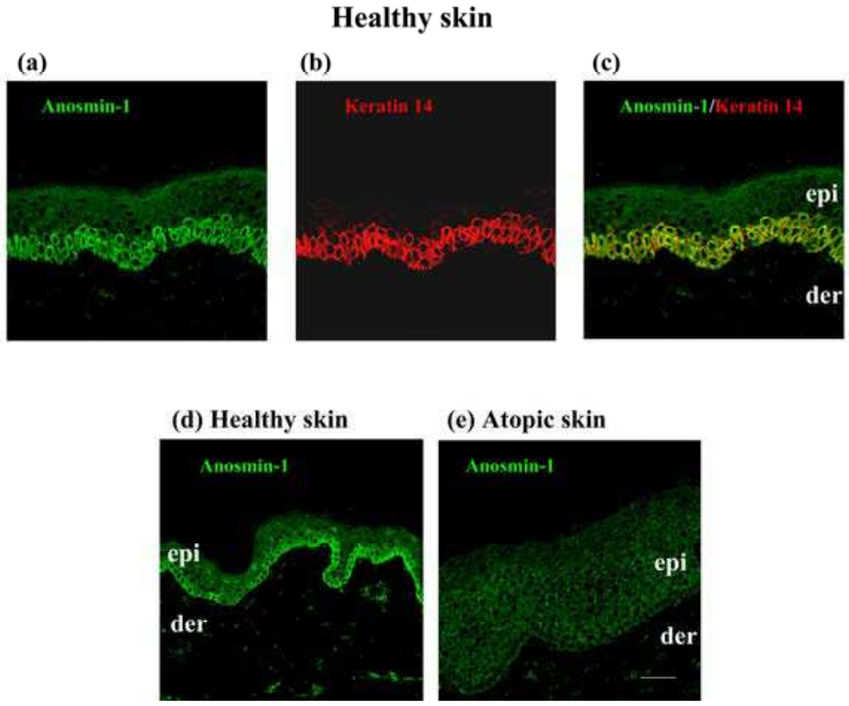

Fig. 5. Patterns of anosmin-1 expression in healthy and atopic skin.
(a,b) Cryosections of normal human skin were doubly labelled for anosmin-1 (a; green) and keratin-14 (b; red). Strong anosmin-1 immunoreactivity was detected in keratin-14-positive cells and in some dermal cells. (c) Superimposition of (a) and (b); the yellow areas were those doubly labelled. (d,e) Immunolabelling with anti-anosmin-1 antibody (green) of healthy (d) and atopic (e) skin. Anosmin-1 was strongly expressed in the basal cell layer of normal skin, but its expression was decreased in the basal cell layer of atopic skin. Scale bar: 50 μm. epi, epidermis; der, dermis.

Epidermal innervation in atopic skin is probably regulated by skin concentrations of both nerve elongation and nerve repulsion factors. A more recent study in psoriasis patients with pruritus reported no close relationship between the number of epidermal nerve fibers and Sema3A levels (Taneda et al., 2011). Although patients with Kallmann syndrome do not express anosmin-1 due to the lack of the *KAL1* gene (Soussi-Yanicostas et al., 1996; Kim et al., 2008), there have been no reports of itchy skin in these patients (Sato et al., 2004). Thus, in many individuals who have skin diseases with pruritus, epidermal innervation may be regulated by combinations of axonal guidance molecules. Further research should involve the altered balance of expression of these molecules in skin diseases with pruritus.

4. Skin barrier disruption and epidermal nerve fibers

Seasonal changes affect the condition of normal skin and trigger various cutaneous disorders. In common dermatoses, such as xerosis, AD and psoriasis, a decline in skin barrier function often parallels an increased severity of clinical symptomatology, including pruritus. These conditions all tend to worsen during the winter season, when humidity is lower (Yosipovitch et al., 2004; Loden and Maibach, 2006). Other indirect evidence suggests that decreased humidity precipitates these disorders (Rycroft and Smith, 1980), whereas increased skin hydration appears to ameliorate these conditions (Chernosky, 1976; Rawlings et al., 1994). Moreover, histological studies have shown that xerotic and AD patients have a higher density of nerve fibers and higher levels of NGF expression than normal individuals (Tominaga and Takamori, 2010). Basal transepidermal water loss (TEWL) is also higher in individuals with AD, including in clinically uninvolved skin, than in normal individuals (Yosipovitch et al., 2004).

Skin barrier disruption causes changes in epidermal innervation, making the skin more susceptible to any stimulation and more sensitive to itching. This has been demonstrated in studies using acetone and acetone/ether/water (AEW)-treated mice, models of acute and chronic dry skin, respectively (Grubauer et al., 1989; Miyamoto et al., 2002; Tominaga et al., 2007b). In acetone-treated mice, the number of epidermal nerve fibers is increased (Tominaga et al., 2007b), suggesting that barrier disruption causes nerve fibers located at the epidermal-dermal border to penetrate into the epidermis. Moreover, acetone treatment led to immediate increases in epidermal NGF and AR mRNA levels, followed by increased expression of the respective proteins (Grubauer et al., 1989; Tominaga et al., 2007b), as well as decreased levels of Sema3A in the epidermis (Kamo et al., 2011a). All of these changes occurred before the nerve fibers penetrated into the epidermis. Artificial restoration of the barrier by latex occlusion immediately after acetone-induced barrier disruption inhibited the increases in epidermal NGF and AR mRNAs (Grubauer et al., 1989; Liou et al., 1997). Thus, alterations in cutaneous barrier permeability induced the abnormal expression of nerve elongation and repulsion factors (Figure 4), suggesting that topically applied emollient may work by normalizing the expression of these genes.

Recently, application of petrolatum or heparinoid cream was found to attenuate dry skin-inducible intraepidermal nerve growth (Kamo et al., 2011b). Immediate application of these emollients after acetone treatment significantly inhibited the acetone-induced increase in epidermal nerve density. Both emollients also attenuated the acetone-induced increase in epidermal NGF levels, but had no effects on epidermal Sema3A levels. These anti-nerve growth effects were also observed when petrolatum or heparinoid cream, especially the latter, was applied 24 hours after acetone treatment, although immediate-type application seemed to

be more effective. Therefore, prompt application of emollients after skin barrier disruption may be therapeutically effective for pruritus involving epidermal hyperinnervation.

A close relationship between skin barrier disruption and itch sensation has been demonstrated using AEW-treated mice (Miyamoto et al., 2002). AEW treatment elicited spontaneous scratching, concomitant with an increase in TEWL and a reduction in stratum corneum (SC) hydration. Treatment also induced spontaneous scratching in mast cell-deficient mice, indicating that mast cells may not be involved in the AEW-inducible scratching behavior. Although the mechanisms are unclear, scratching behaviors in mast cell-deficient mice may be caused, at least in part, by increases in epidermal nerve fibers or pruritogens from other dermal cells and keratinocytes. This idea is partly supported by a recent study using this model (Akiyama et al., 2010).

Alternatively, spontaneous scratching may be induced by water treatment following AE, but not by organic solvents alone. Water can remove natural moisturizing factors important for skin hydration, impairing SC hydration and flexibility (Yosipovitch et al., 2004). Water may also induce transient swelling of the SC followed by a drying out of the surface layers. Physical swelling and shrinking may act as a mechanical stimulus of C-fibers in the upper epidermis, where it is perceived as itch. This hypothesis is supported by findings showing that mechanical stimuli were associated with enhanced neurogenic inflammation (Yamaoka et al., 2007).

5. Relationship between epidermal nerve fibers and abnormal expression of cell-cell junction molecules

Adherens junctions and tight junctions are critical for skin barrier function and have been shown to be altered in individuals with psoriasis (Pummi et al., 2001; Perez-Moreno et al., 2003; Zhou et al., 2003; Harhaj et al., 2004) and AD (Tominaga et al., 2007a). Epidermally targeted amphiregulin (AR)-transgenic mouse strains develop many features of psoriasis spontaneously (Cook et al., 1997; Cook et al., 2004). The levels of expression of the adherins junction protein E-cadherin (Chung et al., 2005) and the tight junction proteins zona occludens 1 (ZO-1) and ZO-2, are decreased in the epidermis of these transgenic mice. In addition, the levels of expression of E-cadherin and ZO-1 are decreased in the epidermis of atopic NC/Nga mice, while the expression of AR is increased (Tominaga et al., 2007a). These findings suggest that AR downregulates epithelial junctional molecules in atopic and psoriatic skin and that AR affects the integrity of cell-cell junctions. Moreover, skin barrier function against external mechanical, chemical, and biological stimuli may be attenuated or abrogated in inflammatory skin diseases.

In cocultures of human corneal fibroblasts and epithelial cells, overexpression of Sema3A by corneal fibroblasts increased the expression of E- and N-cadherin mRNA and protein by corneal epithelial cells (Ko et al., 2010), suggesting that Sema3A may modulate the expression of cell-cell junctional molecules in epidermal keratinocytes.

Desmosomes are complex intercellular junctions that link the keratin filaments of adjacent cells, providing mechanical strength to epithelial tissues such as the epidermis. Desmoglein 3 (Dsg3) is a desmosomal cadherin highly expressed in the basal layer of mammalian skin (Wheelock and Johnson, 2003). Following differentiation, however, the expression of Dsg3 decreases (Wheelock and Johnson, 2003). Electron microscopic analysis has shown that a keratin 1 promoter increases intercellular spaces in the basal and spinous layers of Dsg3-transgenic mice (Merritt et al., 2002). Dsg3 is also aberrantly expressed in the epidermis of atopic NC/Nga mice (Tominaga et al., 2007a).

Taken together, these findings suggest that widening of intercellular spaces in the epidermis is required for the penetration and/or elongation of nerve fibers into the epidermis (Figure 6), as well as for inflammatory cell infiltration into the dermatitis (Wittmann and Werfel, 2006). Thus, epidermal hyperinnervation is enhanced by the abnormal expression of cell-cell junctional molecules, and thereby may induce and/or enhance itch in skin diseases associated with barrier disruption.

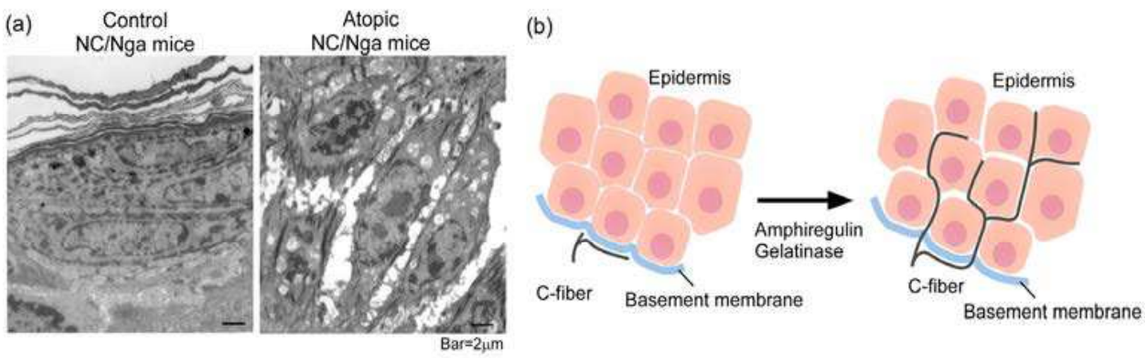

Fig. 6. Relationship between widening of intercellular spaces in the epidermis and nerve fiber density.
(a) Electron micrographs of the skins of NC/Nga mice. Intercellular spaces between keratinocytes were tight in the skin of control, specific pathogen-free (SPF)-NC/Nga mice, but were wider in the skins of conventional (Cnv)-NC/Nga mice, which developed AD-like symptoms. (b) Increased AR downregulates epithelial junctional molecules in atopic skin, suggesting that AR affects the integrity of cell-cell junctions. Gelatinase activities were also high in atopic skin, suggesting that gelatinase may be involved in the activation of transmembrane-type AR. Moreover, desmoglein 3 (Dsg3) is aberrantly expressed in the epidermis of atopic NC/Nga mice, suggesting that Dsg3 may be involved in widening intercellular spaces in the epidermis. Increased spaces may be required for the penetration and/or elongation of nerve fibers into the epidermis.

6. Mechanism of penetration of nerve fibers into basement membrane

Although epidermal innervation was found closely related to itch in AD, the mechanisms by which dermal nerve fibers pass through the basement membrane (BM) at the epidermal-dermal border remain unclear.
Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of degrading extracellular matrix (ECM) components, including BM proteins. The MMP family is divided into two major groups according to their cellular localization, secreted and membrane-type (MT) MMPs. Breakdown of ECM by MMPs is important in cell migration, tissue remodelling, inflammatory diseases and tumor cell invasion and metastasis (Page-McCaw et al., 2007). Interestingly, studies using DRG neurons showed that MMPs promote neurite extension (Muir et al., 1994; Hayashita-Kinoh et al., 2001), suggesting that MMPs may be involved in the penetration of nerve fibres into the BM and that axonal guidance molecules modulate the expression and enzymatic activity of MMPs that degrade BM components.

We have developed an *in vitro* model of BM, in which DRG neurons are cultured in a unique system, consisting of Boyden chambers and Matrigel (MG) (Figure 7). This system mimics the pathological skin condition of intractable pruritus because nerve fiber penetration into the MG was induced by the NGF concentration gradient (Tominaga et al., 2009a). We found that MMP-2 is localized on the growth cone in the penetration mechanism and that it may be involved in intractable pruritus (Tominaga et al., 2009a).

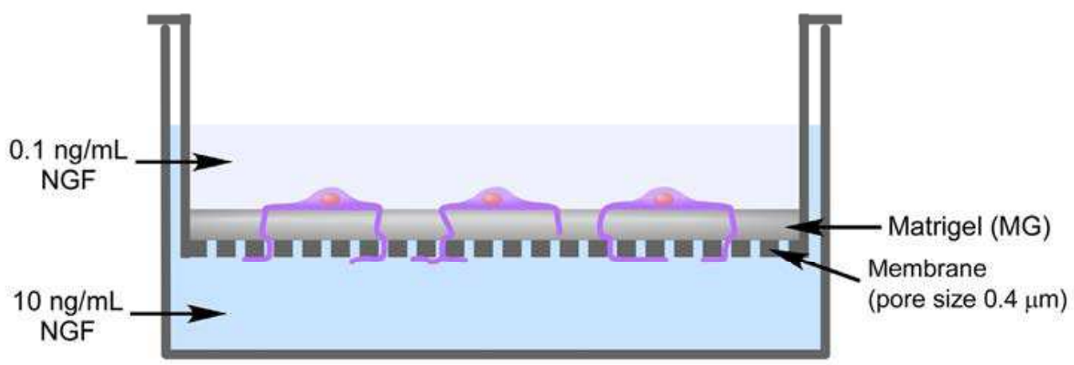

Fig. 7. Schematic representation of the Boyden chamber culture system.
The chamber used to assess the mechanism by which nerve fibers penetrate into the BM is shown schematically. The upper surface of the 0.4-μm pore size polyester insert of a 24-well Boyden chamber was coated with Matrigel (MG). DRG neurons, in 200 μL culture medium containing 0.1 ng/mL NGF, were placed on the MG, and 1 mL of culture medium containing 10 ng/mL NGF was added to the lower chamber. After culture for 24 hours, the cells were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The MG-coated membrane was removed from the Boyden chamber and stained with an antibody against Tau, a protein enriched in axons. Immunofluorescence staining of the underside of the membrane was assessed using a confocal laser-scanning microscope, which revealed nerve fibers that had crossed the MG-coated membrane.

In the DRG neuron cultures, NGF induced expression of *MMP-2*, whereas MMP-2 blockers inhibited the penetration of nerve fibers across the membrane, suggesting that NGF-inducible MMP-2 is involved in the process of nerve fiber penetration into MG, similar to findings using chick DRG neurons (Muir et al., 1994). Pruritogens and cytokines have been found to upregulate keratinocyte MMP-9 production (Gschwandtner et al., 2008; Purwar et al., 2008), and gelatinase activities were found to be higher in the epidermis of atopic NC/Nga mice than in control mice (Tominaga et al., 2007a). Thus, non-neuronal cell-derived gelatinases may contribute indirectly to nerve fiber penetration *in vivo*.
Growth cones are subject to multiple environmental cues as they navigate (Goodman, 1996). MMP-2 was shown present within the cell bodies, neurites and growth cones of permeabilized DRG neurons (Zuo et al., 1998). In the absence of permeabilization, however, MMP-2 localized to the growth cones of NGF-responsive fibers, and zymographic analyses showed type IV collagenase activity on the cell surface of growth cones, including filopodia, in NGF-responsive fibers (Tominaga et al., 2009a). These results suggest that nerve fiber penetration is caused by activated MMP-2 on the cell surface of growth cones (Figure 8).

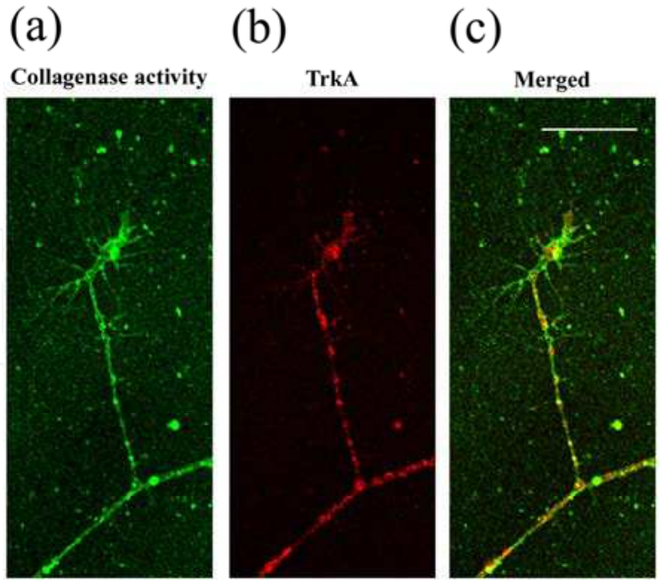

Fig. 8. Type-IV collagenase activities on DRG growth cone.
(a) DRG neurons were cultured for 48 hours in serum-free medium supplemented with 10 ng/mL NGF, followed by *in situ* zymography using FITC-labelled DQ-type IV collagen (green). (b) Following *in situ* zymography, the cells were stained with anti-TrkA (red) antibody. (c) Merged image of (a) FITC-labelled DQ-type IV collagen (green) and (b) TrkA (red). Type IV collagenase activity was detected primarily on the cell membrane of growth cones including the filopodia of $TrkA^+$ fibers. Scale bars, 23.81 μm.

MMP-2 is produced as pro-MMP-2, an inactive zymogen, which is activated by MT-MMPs rather than serine proteases. During the activation process, the MT-MMPs form a complex with pro-MMP-2 through interaction with tissue inhibitor of metalloproteinase-2 (TIMP-2) (Seiki, 1999; Wang et a., 1999). Immunocytochemical analyses of unpermeabilized DRG neurons indicated that MT5-MMP partially colocalized with MMP-2 and/or TIMP-2 in NGF-responsive growth cones. Nerve fiber penetration into the MG was also inhibited by anti-TIMP-2 neutralizing antibody (Tominaga et al., 2009a), suggesting that MT5-MMP also functions as an adaptor when complexed and that MT5-MMP may be involved in MMP-2 activity on the cell surface of growth cones. Moreover, NGF may enhance the ability of MMP-2 to degrade BM components through the upregulation of pro-MMP-2 activation molecules in cultured neurons (Tominaga et al., 2009a). Accordingly, activated MMP-2 on the cell surface may be more effective than its free form in degrading BM components during the nerve fiber penetration process (Figure 9).
In addition to NGF, *MMP-2* expression in cultured neurons is modulated by other factors, including Sema3A, which induces growth cone collapse and axonal repulsion (Fujisawa, 2004). Sema3A molecule inhibits nerve fiber penetration due to the NGF concentration gradient, concomitant with the downregulation of *MMP-2* and *MT5-MMP*, suggesting that these two molecules have reciprocal mechanisms in the regulation of nerve fiber penetration.
MMP-2 is also modulated by its ECM substrates. *In vitro* studies of neurite outgrowth on different ECM components has suggested the involvement of integrins in growth cone movement during neural development and repair (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 signalling is necessary for efficient and long-distance axon extension (Rossino et al., 1990; Lefcort et al., 1992; Grabham et al., 1997; Werner et al., 2000; Danker et al., 2001). Thus, NGF stimulated elongation of nerve fibers, either in experimental culture systems or *in vivo*, will result in the accumulation of integrins at the growth cone, enabling them to interact with a variety of ECM components (Grabham et al., 1997). During this process, MMPs are required for growth cones to abrogate the three-dimensional ECM barriers. This process involves the selection and upregulation of MMPs corresponding to surrounding ECM components of the growing nerve fiber, resulting in efficient nerve fiber penetration. The expression of genes encoding molecules involved in pro-MMP activation may also be affected. In contrast, Sema3A stimulation of growing nerve fibers may constitute a reverse signalling pathway because class 3 semaphorin signalling inhibits integrin-mediated adhesion signalling (Zhou et al., 2008). Therefore, although the integrin-mediated regulatory system remains unclear in our culture system, this mechanism may be applicable to pruritic skin diseases involving epidermal hyperinnervation.

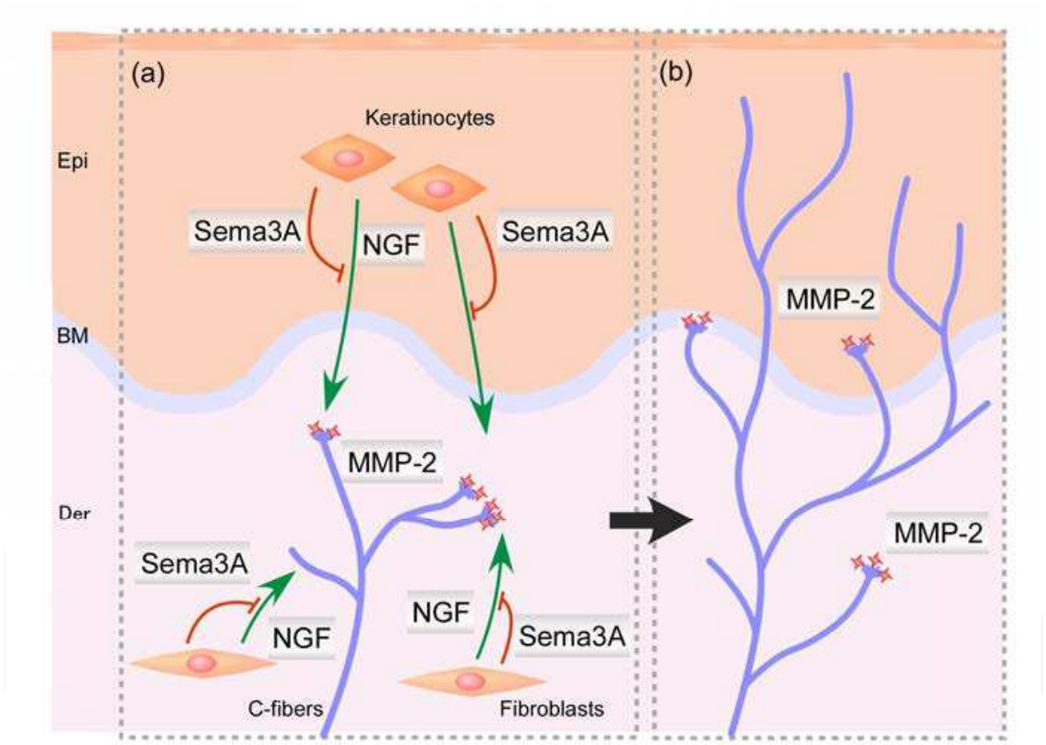

Fig. 9. A model of nerve fiber penetration into the BM.
(a) NGF, which is produced by cutaneous cells such as epidermal keratinocytes, immune cells and fibroblasts, promotes MMP-2 production in sensory nerve fibers and activates pro-MMP-2 on the growth cone. Sema3A produced by keratinocytes and fibroblasts may have opposite effects on these NGF-dependent events. NGF induced expression of *MMP-2* in nerve fibers may be also modulated by the extracellular matrix (ECM) substrates of this enzyme. (b) Activated MMP-2 on the growth cone may contribute to penetration of nerve fibers into the basement membrane.

7. Characterization of nerve fibers containing gastrin-releasing peptide in the skin

It has been difficult to histologically identify itch-specific fibers in the skin because no itch-specific markers have been identified. However, using gastrin-releasing peptide receptor (GRPR)-mutant mice or saporin-conjugating bombesin, the GRP/GRPR system was shown to be involved specifically in itch perception via the spinal cord (Sun and Chen, 2007; Sun et al., 2009). Recently, GRP$^+$ fibers were histologically shown to be present in mouse skin, with the percentage of PGP9.5$^+$ fibers that are GRP$^+$ being exceptionally high only in the epidermis of NC/Nga mice with AD (Figure 10) (Tominaga et al., 2009b). Small- to medium-sized adult DRG neurons expressed GRP, and its receptor was present in the superficial dorsal horn. Intrathecal injection of GRP_{18-27} into wild-type mice induced scratching behavior but did not affect pain sensitivity (Sun and Chen, 2007), suggesting that GRP$^+$ fibers in the skin are itch- but not pain-specific.

Moreover, GRP$^+$ fibers have been found to contain SP or CGRP and to express itch-related molecules such as TRPV1, PAR-2, mu-opioid receptor (MOR) and TrkA, a receptor for NGF (Tominaga et al., 2009b). Although additional research is required to determine whether GRP$^+$ fibers in human and animal skin express histamine receptors or whether different types of itch-mediating fibers coexist in the periphery, GRP$^+$ fiber density may become an objective indicator of itching.

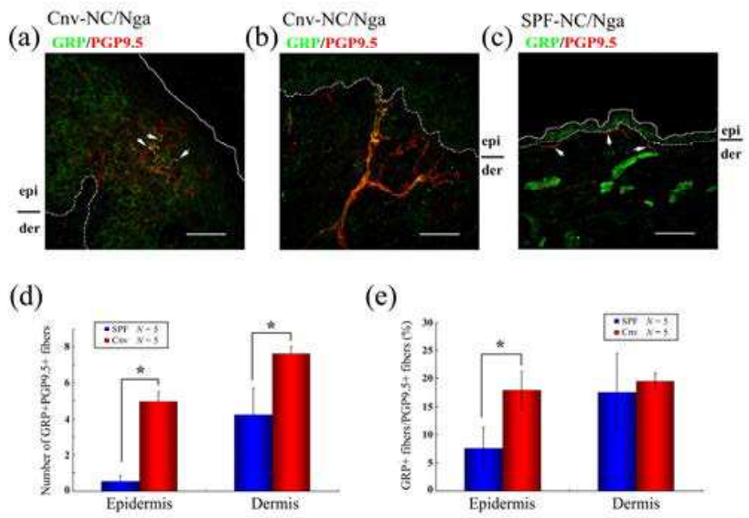

Fig. 10. Distribution of GRP$^+$ fibers in the skin of NC/Nga mice.
a-c, Double-labeling of GRP (green) and PGP9.5 (red) in the skin of NC/Nga mice. A small proportion of PGP9.5$^+$ fibers expressed GRP in the epidermis (arrows in a) and dermis (b) of conventional (Cnv)-NC/Nga mice. GRP$^+$PGP9.5$^+$ fibers were mainly observed in the dermis of specific pathogen-free (SPF)-NC/Nga mice (arrows), but they were occasionally present in the epidermis (c). d, The number of GRP$^+$PGP9.5$^+$ fibers was significantly higher in the epidermis and dermis of Cnv-NC/Nga than of SPF-NC/Nga mice. *$P < 0.05$. e, The percentage of PGP9.5$^+$ fibers that were GRP$^+$ was significantly higher in the epidermis of Cnv-NC/Nga than of SPF-NC/Nga mice, but was similar in the dermis of these mice. Yellow areas are double-labeled, and white and broken lines indicate the skin surface and the border between the epidermis (epi) and dermis (der), respectively. Scale bars = 47.62 μm.

8. UV-based therapy of AD pruritus involving epidermal hyperinnervation

Various types of UV-based therapy, including oral and topical PUVA and narrow-band UVB, are widely used to treat AD (Krutmann, 2000). Interestingly, UV-based therapy was shown to reduce the number of cutaneous nerve fibers, especially in the epidermis, in patients with AD and psoriasis (Wallengren and Sundler, 2004). The intense itch associated with these dermatoses can also be controlled by UV-based therapy. Excimer laser treatment has been shown to ameliorate dermatitis in psoriasis patients and pruritus in AD patients (Baltas et al., 2006). Therefore, these findings suggest a relationship between the antipruritic effects of UV-based therapy and the reduction of epidermal nerve density in atopic skin. The mechanisms underlying UV-induced changes in epidermal nerve density are being assessed.

8.1 Effects of PUVA therapy on epidermal nerve fibers

NGF levels are higher, and Sema3A levels are lower, in the epidermis of patients with AD than in controls, suggesting that abnormal levels of axonal guidance molecules are involved in epidermal hyperinnervation in AD (Tominaga et al., 2008; Tominaga et al., 2009c). We hypothesized that epidermal Sema3A and NGF levels in AD patients are influenced by PUVA therapy, resulting in decreased epidermal nerve density in atopic skin. Using skin biopsies, we recently showed that PUVA therapy reduces epidermal hyperinnervation in AD patients by normalizing abnormal epidermal Sema3A and NGF expression (Tominaga et al., 2009c).

Following PUVA treatment, Sema3A upregulation and NGF downregulation were observed in the epidermis of AD patients (Figure 11). These patients also showed decreases in VAS for itching and clinical severity scores, concomitant with decreases in epidermal nerve densities (Figure 12) (Tominaga et al., 2009c). Sema3A inhibits NGF-induced sprouting of sensory afferents in the adult rat spinal cord (Dontchev and Letourneau, 2002). Although the signaling pathways that mediate the regulation of expression of these axonal guidance molecules remain unknown, these findings suggest that abnormal Sema3A and NGF levels in atopic skin are normalized by PUVA therapy, resulting in decreased epidermal nerve density. These PUVA-induced changes in epidermal innervation also have antipruritic effects, as shown by the use of anti-NGF or recombinant Sema3A replacement approaches against pruritus in atopic NC/Nga mice (Takano et al., 2005; Takano et al., 2007; Yamaguchi et al., 2008).

Although the mechanisms by which PUVA influences expression of axonal guidance molecules remain unknown, treatment may affect chromatin remodeling and various transcription factors, such as activator protein-1 (AP-1) and poly(C) binding protein (Borner et al., 2002; Kim et al., 2004; Kim et al., 2005; Park et al., 2005). The NGF promoter contains an AP-1 element important for NGF transcriptional activity (Hengerer et al., 1990; D'Mello et al., 1991). Psoralen functions by interfering with AP-1 in murine keratinocytes, thereby inhibiting DNA binding by AP-1 (Martey et al., 2005). In addition, chromatin structure in human epithelial cells is affected by PUVA (Ree et al., 1981; Gasparro et al., 1997), and changes in chromatin structure influence DNA binding by transcription factors (Park et al., 2005). Although the Sema3A promoter has not yet been investigated, this type of mechanism may occur during the PUVA-induced normalization of Sema3A expression. Therefore, these studies may explain the mechanism of by which PUVA regulates gene expression in epidermal keratinocytes.

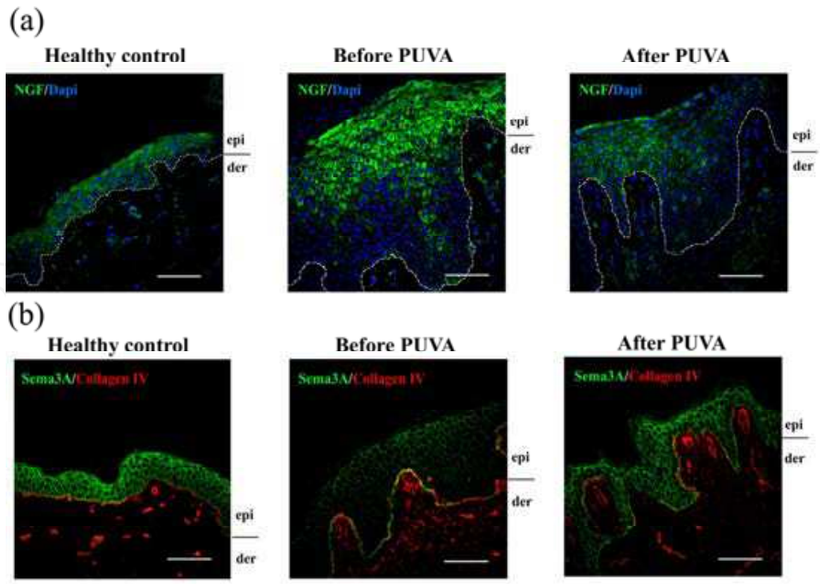

Fig. 11. Epidermal NGF and Sema3A levels in AD patients before and after PUVA therapy. (a) Skin biopsies from healthy volunteers and AD patients before and after PUVA treatment were stained with anti-NGF antibody. Epidermal NGF levels (green) were higher in AD patients than in healthy controls. Nuclei were counterstained with DAPI (blue). NGF expression was reduced in PUVA-treated than in untreated individuals. The white dotted line in each panel indicates the border between the epidermis and dermis (basement membrane). (b) Double labeling for Sema3A (green) and type IV collagen (red) in the skin of AD patients before and after PUVA therapy. Epidermal Sema3A levels were lower in AD patients than in healthy volunteers, but were higher in PUVA-treated than in untreated individuals. Scale bars = 75 μm. epi, epidermis; der, dermis.

Alternatively, genes encoding axonal guidance molecules may be regulated by inflammatory cytokines produced by cutaneous cells, such as keratinocytes and immune cells. TNF-α was recently shown to enhance NGF production *via* the Raf-1/MEK/ERK pathway in cultured normal human epidermal keratinocytes (Takaoka et al., 2009). Although UV irradiation induces cytokine secretion from cultured keratinocytes, successful UV-based therapy of AD has been associated with downregulation of cytokine production in inflamed skin (Krutmann and Morita, 1999). Therefore, PUVA may regulate the expression of axonal guidance molecules by reducing cytokine levels in the skin.
NGF is produced not only by epidermal keratinocytes but by mast cells, eosinophils, and fibroblasts in inflamed skin (Ikoma et al., 2006; Leon et al., 1994). Several semaphorins are also produced by fibroblasts and immune cells (Suzuki et al., 2008; Fukamachi et al., 2011). UV radiation has been shown to affect dermal fibroblasts, dermal dendritic cells, endothelial cells, and skin-infiltrating inflammatory cells, such as T lymphocytes and mast cells (Krutmann and Morita, 1999). UV-based therapy has been shown to affect the production of soluble factors (cytokines, neuropeptides, and prostanoids) and the expression of cell-surface receptors (adhesion molecules, cytokine and growth factor receptors), and to induce apoptosis in these cells (Krutmann and Morita, 1999). Thus, PUVA treatment may modulate the production of axonal guidance molecules in dermal cells and/or inflammatory cells of the atopic skin, as well as in epidermal keratinocytes.

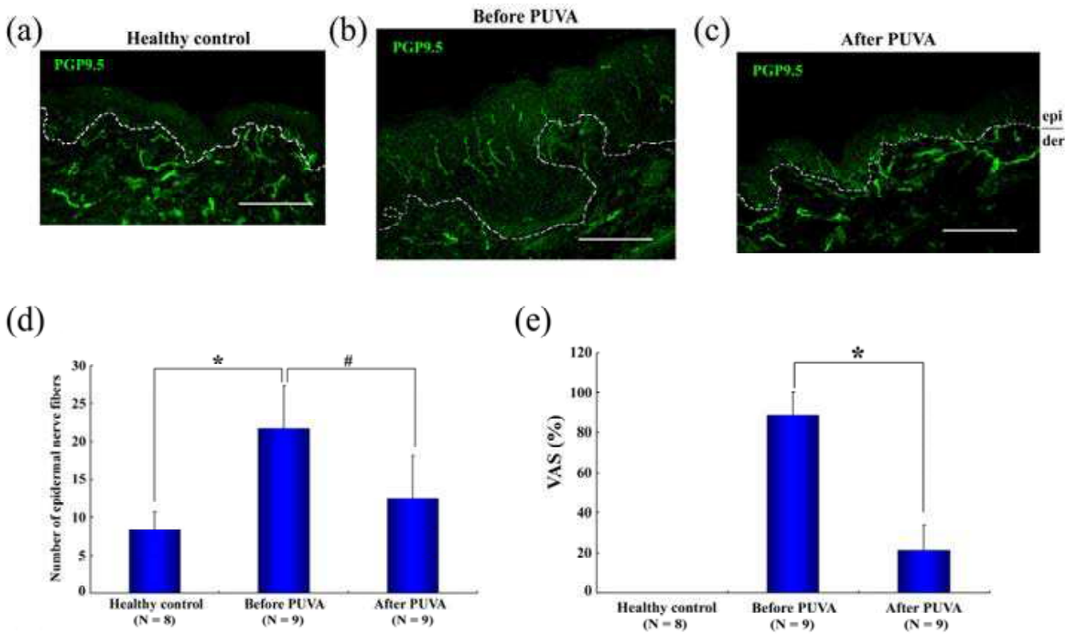

Fig. 12. Epidermal nerve densities in AD patients before and after PUVA therapy.
Skin biopsies from healthy volunteers and AD patients before and after PUVA therapy were stained with anti-PGP9.5 antibody. PGP9.5-immunoreactive fibers (green) were mainly located in the dermis and at the epidermal- dermal border of normal skin, but some nerve fibers penetrated into the epidermis (a). Higher nerve densities were observed in the epidermis of AD patients (b). Reduced nerve densities were observed in the epidermis after PUVA therapy (c). The white dotted line in each panel indicates the border between the epidermis and dermis (basement membrane). Scale bars = 150 μm. epi, epidermis; der, dermis. The number of epidermal nerve fibers was significantly higher in AD patients than in healthy controls, while the number was significantly decreased in AD patients after PUVA treatment (d). Values are the means ± SD ($^{*}P < 0.01$; $^{\#}P < 0.05$). Visual analog scale (VAS) scores were significantly lower after than before PUVA therapy in AD patients ($^{*}P < 0.01$), and there was no itch in healthy controls (e).

8.2 Effects of NB-UVB and excimer lamp on epidermal nerve fibers

Narrowband 311-nm ultraviolet B (NB-UVB) is widely recognized as an effective treatment modality for patients with chronic AD (Der-Petrossian et al., 2000). More recently, the 308-nm XeCl excimer laser and lamp was introduced as a new type of UV-based therapy for some dermatoses including AD (Wolkerstorfer and Brenninkmeijer, 2011). Excimer laser treatment has been shown to ameliorate pruritus in AD patients and dermatitis in psoriatic patients (Baltas et al., 2006). The 308-nm excimer lamp and laser has demonstrated similar efficacy in treating vitiligo, although the lamp induced more erythema than the laser (Le Duff et al., 2010). The anti-nerve growth effects of these UV-based therapies have not been fully characterized to date. Using acetone-treated mice as a model of acute dry skin model, we assessed the effects of NB-UVB and excimer lamps on nerve growth (Kamo et al., 2011a). We previously showed that nerve fibers penetrate into the epidermis 24 h after acetone treatment, with nerve growth peaking 48 h after acetone treatment (Tominaga et al., 2007b). We therefore treated the mice with NB-UVB and excimer lamps 24 h after acetone treatment and obtained skin samples 48 h later.

Interestingly, we found that the anti-nerve growth effects of NB-UVB and excimer lamp treatments were more effective than PUVA treatment (Figure 13). UVA penetrates into the dermis, whereas UVB is limited almost exclusively to the epidermis (Meinhardt et al., 2008). Thus, UVB irradiation, which is restricted to the epidermal region, had greater efficacy, and may explain the different anti-nerve growth effects of UV-based therapies. Our findings are supported by clinical studies using PUVA, NB-UVB, and excimer lamp therapies (Van Weelden et al., 1990; Ortel et al., 1993).

Photobiologically, the wavelengths of the NB-UVB and excimer lamp are close to each other, and their therapeutic effects are similar (Asawanonda et al., 2008), with both showing strong inhibition of epidermal nerve growth. Although NB-UVB normalized the abnormal expression of NGF and Sema3A in the epidermis, no such normalization was observed with excimer lamp treatment. Thus, excimer lamp treatment, the most effective form of therapy for intraepidermal nerve fibers, did not alter the epidermal expression of axonal guidance molecules. Experimentally, keratinocytes are more resistant than lymphocytes to UVB-induced apoptosis (Krueger et al., 1995). Therefore, the anti-nerve growth effects may depend on the sensitivity of cutaneous cells to different UV wavelengths.

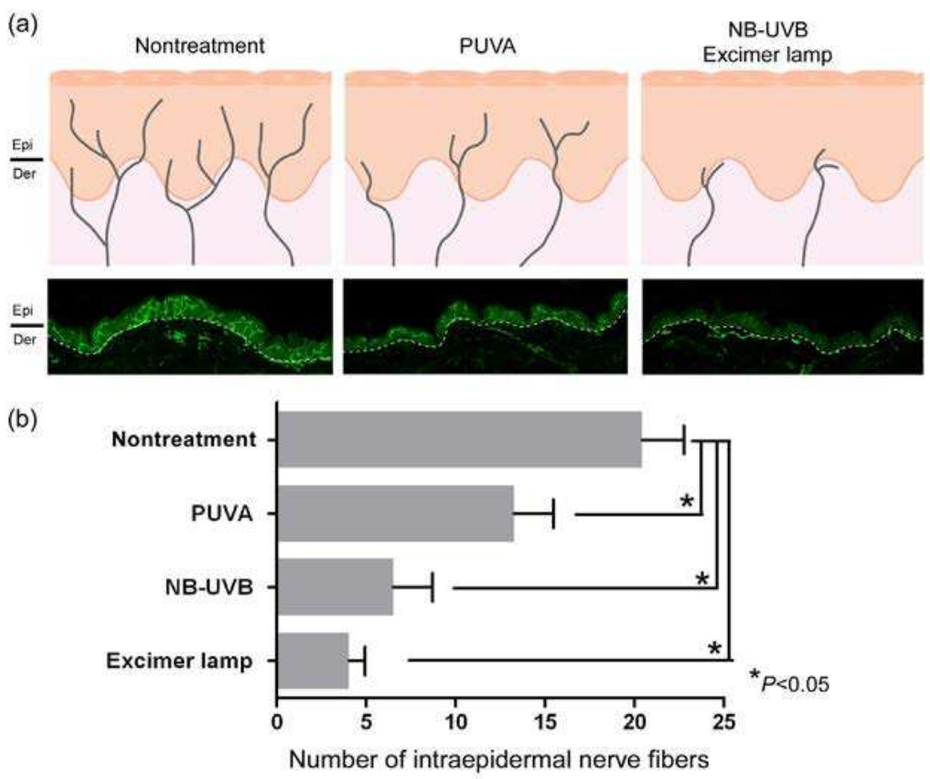

Fig. 13. Effects of UV-based therapy on intraepidermal nerve fibers in acetone-treated mice. (a) Distributions of intraepidermal PGP9.5$^+$ fibers after a single topical application of PUVA, NB-UVB and excimer lamp in acetone-treated mice. White broken lines indicate the border between the epidermis and dermis. Scale bars, 50 μm. (b) A marked decrease in the number of intraepidermal PGP9.5$^+$ fibers was observed in the group of mice treated with PUVA, NB-UVB and excimer lamp (*P < 0.05). All values represent the means ± SD of 6 animals.

Short-wave radiation, such as UVB, also excites DNA directly and generates photoproducts, such as cyclobutane pyrimidine dimers and (6-4) photoproducts, resulting in considerable bending of DNA (Kielbassa et al., 1997). A recent study demonstrated that 311 – 313-nm UVB radiation (dose: 750 mJ/cm^2) induced AP-1 binding to DNA (Hopper et al., 2009), suggesting that NB-UVB can modulate the expression of NGF in keratinocytes. UV irradiation may also induce ligand-independent activation of cell-surface receptors, such as epidermal growth factor receptor (Fisher et al., 1998; Wang et al., 2003), suggesting that NB-UVB may modulate the expression of Sema3A in keratinocytes. Epidermal growth factor was found to increase the expression of Sema3A mRNA and protein in human corneal epithelial cells (Ko et al., 2008). However, as photoproducts are among the factors involved in skin carcinogenesis, further studies are needed to determine therapeutically effective irradiation doses that also have low DNA damage potential.

9. Conclusion

Considerable progress has been made in clarifying the complex pathophysiology of itch. Histamine-independent itch occurs in both humans and animals, with amines, proteases, neuropeptides, cytokines, cannabinoids and opioids, as well as their cognate receptors, acting as mediators and/or modulators of itch. The itch response in the periphery is modulated by interactions among immune cells, keratinocytes and sensory nerve fibers. Epidermal nerve density is partly responsible for abnormal itch perception in several skin diseases, and hyperinnervation is regulated by a fine balance between nerve elongation and repulsion factors. Skin barrier disruption induces the abnormal expression of axonal guidance molecules, thereby increasing epidermal nerve density. There may be a relationship between epidermal nerve fibers and the abnormal expression of cell-cell junctional molecules. Activated MMP-2 on the growth cone may function as a micro-drill to facilitate efficient nerve penetration through the BM, under the control of axonal guidance molecules and/or ECM components. The GRP/GRPR system is specifically involved in itch perception *via* the spinal cord. There is a close relationship between epidermal GRP$^+$ fiber density and pruritus in AD patients. A deeper understanding of these pathways is required for the development of novel antipruritic strategies. Clinically, UV-based therapies such as PUVA, NB-UVB and excimer lamps may be effective for AD patients with pruritus involving epidermal hyperinnervation. These findings will also expand our knowledge regarding effective treatments for pruritic skin diseases.

10. Acknowledgment

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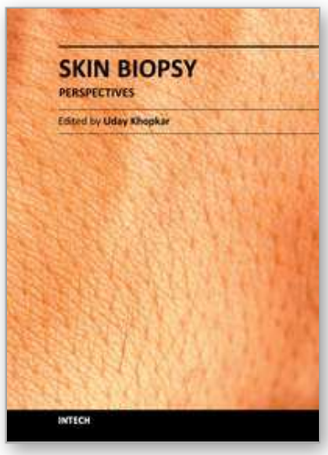

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Skin Biopsy - Perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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