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Keratinocyte-derived anosmin-1, an extracellular glycoprotein encoded by the X-linked Kallmann syndrome gene, is involved in modulation of epidermal nerve density in atopic dermatitis

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ABSTRACT

Background: Epidermal nerve density is increased in atopic dermatitis (AD), suggesting that the hyperinnervation is partly responsible for abnormal itch perception. It is probably controlled by axonal guidance molecules produced by keratinocytes. An extracellular matrix glycoprotein anosmin-1 encoded by *KAL1* has chemoattractive or chemorepulsive effects on different neuronal types.
Objective: This study was performed to investigate the roles of anosmin-1 in skin innervation.
Methods: Rat dorsal root ganglion (DRG) neurones were cultured in conditioned medium from control or *KAL1*-overexpressing cells for neurite outgrowth assay. *KAL1* expression in cultured epidermal keratinocytes or human skin was examined by quantitative RT-PCR (qRT-PCR). Anosmin-1 distribution in normal and atopic skin was examined immunohistochemically. The effects of calcium concentrations and cytokines on *KAL1* expression in cultured normal human epidermal keratinocytes (NHEK) were analysed by qRT-PCR.
Results: Neurite outgrowth in cultured DRG neurones was inhibited by conditioned medium from *KAL1*-overexpressing cells, while it was rescued by addition of recombinant fibroblast growth factor receptor 1 for capturing anosmin-1. *KAL1* transcripts were expressed in cultured keratinocytes or in normal skin. Anosmin-1 was strongly expressed in the basal cell layer of normal skin, but decreased in atopic skin, concomitant with increases of epidermal nerve fibres. *KAL1* expression was downregulated during keratinocyte differentiation. The expression was also upregulated by interleukin-4 (IL-4), IL-13 or transforming growth factor (TGF)-β1. TGF-β1 acted synergistically with IL-13 to enhance *KAL1* expression, while interferon-γ inhibited its expression.
Conclusion: Anosmin-1 produced by epidermal keratinocytes in response to calcium concentrations or cytokines may modulate epidermal nerve density in AD.

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with pruritus as one of the most important symptoms. Histamine is the best-known pruritogen and has been regarded as a target of antipruritic therapy. However, there remain some patients whose pruritus is not satisfactorily improved by antihistamines [1–3].

Histological observations have indicated that cutaneous unmyelinated nerve fibres are present at higher densities in the epidermis of AD patients with intractable pruritus, suggesting that the hyperinnervation is partly responsible for abnormal itch perception [2–4]. Hyperinnervation in AD is probably caused by nerve growth factor (NGF) released from keratinocytes. Moreover, recent studies have demonstrated that amphiregulin, gelatinase

Abbreviations: AD, atopic dermatitis; DRG, dorsal root ganglion; FGFR1, fibroblast growth factor receptor 1; GnRH, gonadotropin-releasing hormone; IFN-γ, interferon-γ; IL, interleukin; K10, keratin 10; K14, keratin 14; *KAL1*, Kallmann syndrome 1 sequence; NHEK, normal human epidermal keratinocytes; NGF, nerve growth factor; PBS, phosphate buffered saline; PGP9.5, protein gene product 9.5; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Sema3A, semaphorin 3A; STAT6, signal transducer and activator of transcription 6; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α.

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and tumour necrosis factor, which are nerve elongation factors, and semaphorin 3A (Sema3A), which is a nerve repulsion factor, are involved in modulation of nerve density in atopic skin [5–8]. Thus, various nerve elongation and repulsion factors are related to the pathogenesis of AD.

The *KAL1* gene (Kallmann syndrome 1 sequence), encoding the extracellular matrix glycoprotein anosmin-1, is responsible for the X chromosome-linked recessive form of Kallmann syndrome [9,10]. This molecule has been shown to play a number of roles in different events during neural development. It promotes the migration of gonadotropin-releasing hormone (GnRH)-producing neurones, guides the navigation of axons from mitral cells, as well as participating in the formation of their collaterals and stimulates outgrowth and branching of Purkinje axons *in vitro* [11–14]. Interestingly, anosmin-1 also has an inhibitory effect on neurite outgrowth in cerebellar granular neurones co-cultured with anosmin-1-overexpressing CHO cells [11]. These findings indicate that anosmin-1 plays various roles in a neurone type-specific manner. One study also indicated that *KAL1* transcripts are overexpressed in dermatomyositis, suggesting a fibrogenic effect of chronic inflammation [15]. However, the expression patterns and roles of anosmin-1 in inflammatory skin diseases such AD are unknown.

In the present study, we examined the expression patterns in human skin and the roles of anosmin-1 in skin innervation of patients with AD using skin biopsies, cultured epidermal keratinocytes and rat DRG neurones. Here, we report a possible role of anosmin-1 in modulation of epidermal nerve density in AD.

2. Materials and methods

2.1. Antibodies and reagents

Primary antibodies used in this study were as follows: mouse anti-anosmin-1 antibody (1:100 dilution, Abnova, Taipei, Taiwan), mouse anti-neuronal class III β-tubulin (Tuj1, 1:500 dilution; Convance, Berkeley, CA, USA), mouse anti-type IV collagen (1:20 dilution; Progen Biotechnik GmbH, Heidelberg, Germany), guinea pig anti-keratin-14 (K14, 1:200 dilution; Progen Biotechnik GmbH, Heidelberg, Germany), rabbit anti-protein gene product 9.5 (PGP9.5, 1:4000 dilution; BIOMOL International L.P., PA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA). For Western blotting, horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from DAKO (Glostrup, Denmark).

All recombinant human cytokines and recombinant fibroblast growth factor receptor 1 (rhFGFR1) used in this study were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human anosmin-1 fragment (residues 548–658) was purchased from Abnova. Dulbecco's Modified Eagle's Medium (DMEM), bovine serum albumin, poly-D-lysine, recombinant human nerve growth factor (rhNGF), 5-fluoro-2′-deoxyuridine, uridine and Ponceau solution were purchased from Sigma (St. Louis, MO, USA). Minimal Essential Medium Alpha (MEM-α), DMEM/F12, N-2 supplement and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). Trypsin/ethylenediaminetetraacetic acid (EDTA) solution, Keratinocyte Basal Medium (KBM-2) and KGM-2 SingleQuots were purchased from Clonetics (Walkersville, MD, USA). Trypsin solution was purchased from Life Technologies (Karlsruhe, Germany). Normal donkey serum was purchased from Chemicon (Temecula, CA, USA). VECTASHIELD mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA). An enhanced chemiluminescence detection kit was purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Skin biopsies

Three-millimetre punch biopsies were taken with informed consent from the lesional skin of 10 patients (8 men and 2 women, average age 31.3 years) with AD diagnosed according to Hanifin and Rajka criteria [16], and 8 healthy male volunteers (average age 29.8 years) with no history of skin disease. The degree of pruritus was evaluated by visual analogue scale score, and its mean in the patients with AD was 82.7%. None of the patients took oral corticosteroid or cyclosporine, and topical corticosteroids were not applied for more than 1 week before the collection of skin samples.

All of the protocols used to obtain skin biopsies were approved by the Medical Ethical Committee of Juntendo University Urayasu Hospital for the Protection of Human Subjects. The clinical investigation was conducted according to the Principles of the Declaration of Helsinki.

2.3. Animals

Male ICR mice (8–12 weeks; SLC Japan, Shizuoka, Japan) were maintained in Juntendo University Graduate School of Medicine's experimental animal facility. They were kept under a 12-h light:12-h dark cycle at 22–24 °C. Food and tap water were provided ad libitum. Mice care and handling conformed to the NIH guidelines for animal research. All animal procedures were approved by the Institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine.

2.4. Cell cultures

HaCaT cells were cultured in DMEM containing 10% heat-inactivated foetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin.

Normal human epidermal keratinocytes (NHEK) were purchased from Cambrex (Walkersville, MD, USA). They were cultured in KBM-2 supplemented with KGM-2 SingleQuots (0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone and 0.1% gentamicin). Cells at passages 1–3 were used for *in vitro* experiments. Trypsin/EDTA solution was used for detaching the cells during passages. NHEK were grown until 80–90% confluency. For keratinocyte differentiation, the cells were cultured in the presence of 1.4 mM $CaCl_2$ for 5 or 14 days.

CHO cells were cultured in MEM-α containing heat-inactivated foetal bovine serum at 10% supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Cells at passages 1–3 were used for *in vitro* experiments. Trypsin solution was used for detaching the cells during passages. All cells were cultured at 37 °C, 5% CO_2.

2.5. Immunohistochemistry

Skin samples were embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and cryosections 8 μm thick were cut using a CM1850 cryostat (Leica, Wetzlar, Germany). The sections were mounted on silane-coated glass slides and then were fixed with ice-cold acetone for 10 min at −20 °C. They were washed three times with phosphate buffered saline (PBS, pH 7.4) and blocked in PBS with 5% normal donkey serum and 2% bovine serum albumin. The sections were incubated with primary antibodies overnight at 4 °C. After washing with PBS, secondary antibodies were added to the sections for 1 h at room temperature. The sections were mounted in VECTASHIELD mounting medium. As controls, the primary antibodies were either omitted or replaced with normal IgG. Immunoreactivity was visualized with a confocal laser-scanning microscope (DMIRE2; Leica). Immunostanning for cutananeous nerve fibres was performed as described previously [5].

2.6. Total RNA preparation

Total RNA from cultured cells was isolated by using an RNeasy Mini Kit (Qiagen KK, Tokyo, Japan). Total RNA from skins of human or mouse was isolated using an RNeasy Fibrous Mini Kit (Qiagen KK) according to the manufacturer's guidelines. Universal Human Reference RNA (UHR RNA; Stratagene, La Jolla, CA, USA) and mouse skin RNA were used as positive and negative controls for quantitative reverse transcription-polymerase chain reaction (qRT-PCR), respectively.

2.7. QRT-PCR

The protocols for qRT-PCR analysis were described previously [5]. The primers used in this study are listed in Table 1. They were designed to meet specific criteria and were synthesised using Perfect Real Time support system (TaKaRa Bio Inc., Kyoto, Japan). The specificity of PCR was confirmed by dissociation curve analysis and gel electrophoresis. QRT-PCR was performed in triplicate, and the measured mRNA levels are expressed relative to the internal reference ribosomal protein S18 (*RPS18*) mRNA level and further adjusted to the level in the control group.

2.8. Transfection of plasmid into CHO cells

KAL1 cDNA (accession no. NM_000216.1) in the mammalian expression vector pCMV6-XL4 was purchased from OriGene (Rockville, MD, USA). For transfection of plasmid into CHO cells, the cells were grown in culture plates in DMEM/F12 for 24 h and incubated with pCMV6-XL4-KAL1 or mock vector in the presence of the Lipofectamine™ 2000 reagent according to the manufacturer's instructions. After 24 h, conditioned media were collected from cultures of transfected cells for neurite outgrowth assay. The levels of anosmin-1 in the conditioned media were confirmed by Western blot analysis.

2.9. Western blot analysis

Conditioned media were collected from CHO cells transfected with pCMV6-XL4-KAL1 or with vector alone, and then concentrated with Amicon Ultra-4 (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Equal amounts of total protein (50 μg per lane) were applied to 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto Immobilon-P Transfer Membranes (Millipore) by Electroblotting Trans Blot (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, the membranes were incubated with anti-anosmin-1 antibody (1:1000 dilution; Abnova, Taipei, Taiwan) overnight at 4 °C. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was used as the secondary antibody. The membrane was developed with an enhanced chemiluminescence detection kit. Autoradiographs were prepared by exposing the membranes to X-ray film (Kodak Japan, Tokyo, Japan). After Western blotting, the membranes were stained with Ponceau solution.

Table 1
Sequences of primer pairs used for quantitative RT-PCR analyses.

Gene	Product (bp)	Primer	Sequence (5′–3′)
KAL1	108	Forward	CAACCGGATCAGAGGCATCA
		Reverse	TTGGCCGTATTGGTTGGACA
K14	136	Forward	CAAGACCATTGAGGACCTGAGGA
		Reverse	GCGCAGGTTCAACTCTGTCTCATA
K10	111	Forward	AGGCTGGCAGCTGATGACTTC
		Reverse	CAGGGTCAGCTCATCCAGCA
Human RPS18	123	Forward	ACTCAACACGGGAAACCTCA
		Reverse	AACCAGACAAATCGCTCCAC
Mouse RPS18	128	Forward	AGGATGTGAAGGATGGGAAG
		Reverse	ACGAAGGCCCCAAAAGTG

KAL1, Kallmann syndrome 1 sequence; K14, keratin 14; K10, keratin 10; RPS18, ribosomal protein S18.

2.10. Neurite outgrowth assay

Bio Falcon™ CultureSlides (BD Falcon, Bedford, MA, USA) were coated with poly-D-lysine, and then neonatal rat DRG neurones (4×10^3; Cambrex) were cultured in conditioned medium from cells transfected with pCMV6-XL4-KAL1 or vector alone. Neuronal survival factors (0.1 ng/ml rhNGF and 0.5% N-2 supplement) and cell growth inhibitors (87.5 ng/ml 5-fluoro-2′-deoxyuridine and 37.5 ng/ml uridine) were added to the conditioned media. In some experiments, rhFGFR1 (50 ng/ml) was added to the culture system to capture free anosmin-1 for 24 and 48 h. Immunocytochemistry was performed using anti-neuronal class III β-tubulin antibody (Tuj1) to visualize neurites as described previously [17]. Immunoreactivity was observed by fluorescence microscopy (BIOREVO BZ-9000; Keyence, Osaka, Japan). Neurite lengths in whole culture area (0.69 cm^2) were measured using BZ Analyzer II software (Keyence) and then mean total neurite length per neurone was determined.

2.11. Treatment of cultured cells with cytokines

NHEK were cultured in triplicate in 6-well plates in 2 ml KBM-2 supplemented with KGM-2 SingleQuots for 24 h to allow adherence. The cells were washed and incubated with the indicated concentrations of cytokines. After incubation for 24 h, total RNA was isolated from the cultured cells as described above.

2.12. Statistical analysis

Statistical analyses were performed by two-tailed Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison test.

3. Results

3.1. Anosmin-1 inhibited neurite outgrowth in cultured DRG neurones

The effects of anosmin-1 on neurite outgrowth of sensory neurones were examined in rat DRG neurones cultured with conditioned media from *KAL1*-overexpressing cells. After 24 h in culture, no differences were observed between neurones cultured in conditioned media from control cells and *KAL1*-overexpressing cells with or without rhFGFR1 (data not shown). After 48 h, the neurones cultured in conditioned medium from control cells displayed long and ramified neurites (Fig. 1a), whereas the neurite length of the neurones cultured in conditioned medium from *KAL1*-overexpressing cells was reduced relative to the controls (Fig. 1b and d). The neurones cultured in the conditioned medium with rhFGFR1 showed longer neurites compared to that in controls (Fig. 1c and d). We also confirmed by Western blot analysis whether anosmin-1 was secreted into the conditioned media. Anosmin-1 was detected in at approximately 100 kDa (full-length) and 40 kDa (C-terminal part) in conditioned media from *KAL1*-overexpressing cells, but was consistently absent in the conditioned media from control cells (Fig. 1e).

3.2. Expression and distribution of anosmin-1 in skin from healthy controls and patients with AD

In RT-PCR analyses, *KAL1* transcripts were expressed in cultured keratinocytes and normal skin (Fig. 2a). To further determine the

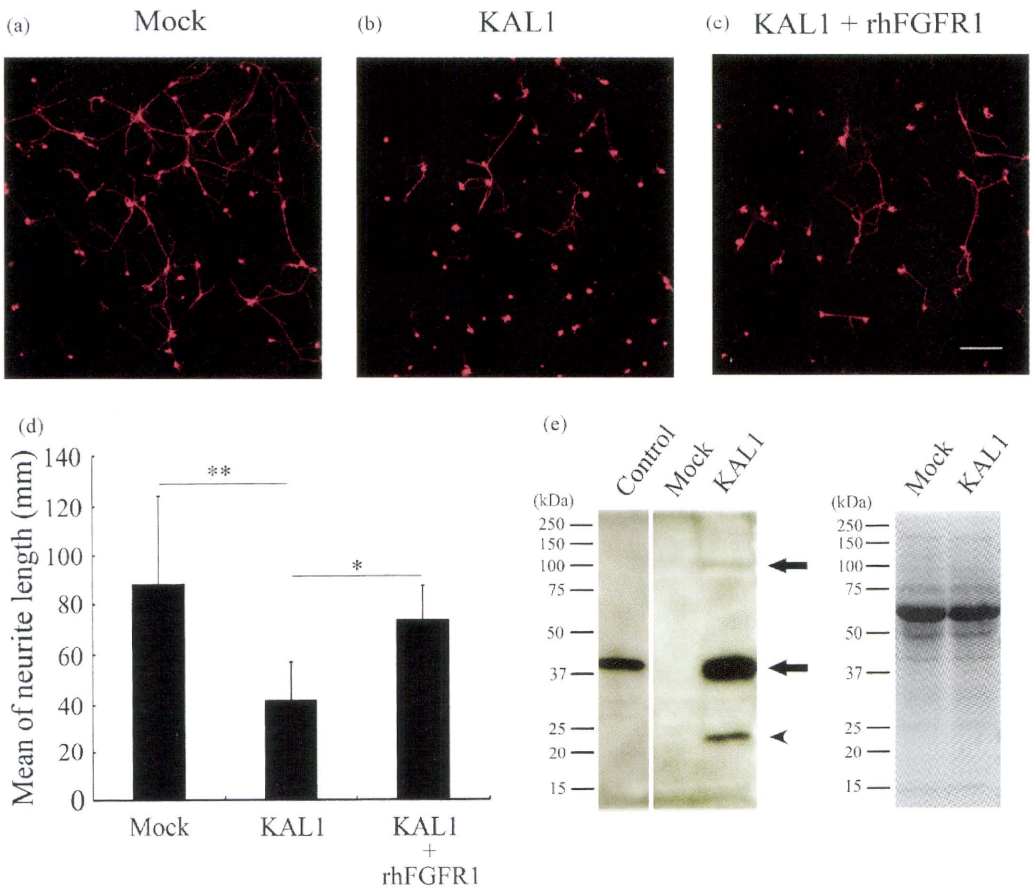

Fig. 1. Effects of anosmin-1 on neurite outgrowth of cultured DRG neurones. (a–c) DRG neurones were cultured for 48 h in conditioned medium from mock vector-transfected cells (a) or pCMV6-XL4-KAL1-transfected cells (b) and pCMV6-XL4-KAL1-transfected cells with 50 ng/ml rhFGFR1 (c). The cultured neurones were immunostained with Tuj1 antibody (red). Scale bar: 200 μm. (d) Mean total neurite length per neurone at 48 h in culture was determined over the whole culture area (0.69 cm^2). The neurite length of the neurones cultured in conditioned medium from *KAL1*-overexpressing cells was significantly reduced compared with the controls, while its inhibitory effect was blocked by addition of rhFGFR1. Values are means ± SD from four independent experiments (*$P < 0.05$; **$P < 0.01$). (e) Western blot analysis of anosmin-1 secreted into the conditioned media from CHO cells transfected with vector alone or with pCMV6-XL4-KAL1. Immunoreactive bands of anosmin-1 were detected at approximately 100 and 40 kDa (arrows in left panel). A degradation product was also detected (arrowhead). Recombinant anosmin-1 fragment was used as a positive control (0.15 μg per lane). After Western blotting, the membranes were stained with Ponceau solution (right panel).

distribution of anomin-1 in human skin, skin specimens from healthy controls and patients with AD were subjected to immunolabelling with anti-anosmin-1 antibody. In the normal skin, anosmin-1 was strongly expressed in the basal layer (Fig. 2b). Anosmin-1 was also expressed in some dermal cells, such as vascular endothelial cells and fibroblasts, as described previously [15]. Weak signals were detected in the suprabasal cell layer and stratum corneum with normal IgG (data not shown).

In comparison with healthy controls, the expression of anosmin-1 in the basal layer of atopic skin was reduced, and the distribution was diffuse in the epidermis (Fig. 3a and b). On qRT-PCR analysis, *KAL1* expression levels tended to be lower in patients with AD compared with healthy controls although the differences were not statistically significant (Fig. 3c). We also found the increasing of epidermal nerve fibres in patients with AD compare with healthy controls (Fig. 3d–f).

3.3. Expression of *KAL1* is downregulated by keratinocyte differentiation

KAL1 expression during keratinocyte differentiation was examined in NHEK treated with 1.4 mM $CaCl_2$ for 5 or 14 days. In qRT-PCR analysis, *KAL1* expression was significantly downregulated in the differentiated cells (Fig. 4a). The differentiation status was confirmed by examining expression of the proliferation-specific marker keratin 14 (Fig. 4b) and the differentiation marker keratin 10 (Fig. 4c).

3.4. Regulation of *KAL1* expression by cytokines in cultured NHEK

To further examine the effects of cytokines on *KAL1* expression, qRT-PCR analyses were performed on NHEK cultured in low-calcium serum-free medium with several cytokines for 24 h. The analyses showed that *KAL1* expression was significantly upregulated in the cultured NHEK stimulated by IL-13, IL-4, or TGF-β1 but not with the others, in comparison with controls (Fig. 5a). These cytokines also dose-dependently induced *KAL1* expression (Fig. 5b, c, and d).

The cross-regulation in the effects of cytokines on *KAL1* expression was examined in NHEK treated with IL-13 and in combination with the other cytokines mentioned above for 24 h. QRT-PCR analyses showed that IL-13 and TGF-β1 acted synergistically to enhance *KAL1* expression in the cultured NHEK, while IFN-γ inhibited gene expression induced by IL-13 (Fig. 5e). These effects were also dose-dependent (Fig. 5f and g).

4. Discussion

KAL1 encodes anosmin-1 and underlies the X-linked form of Kallmann syndrome, a neurological disorder that impairs development of the olfactory and GnRH systems [9,10]. Anosmin-1 has

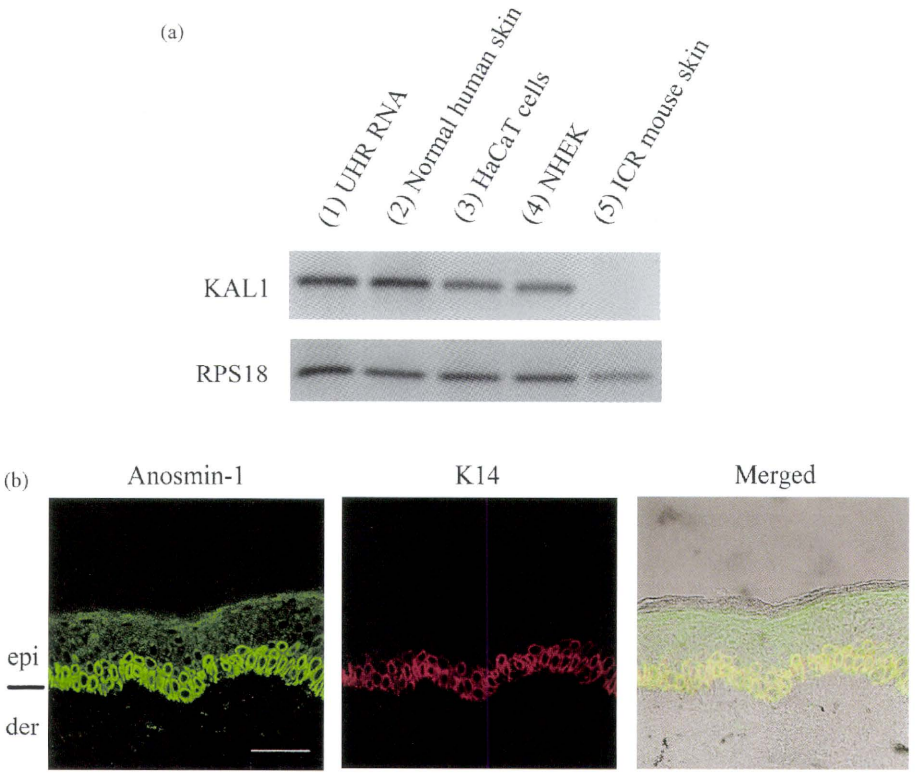

Fig. 2. Expression analyses of *KAL1* in cultured keratinocytes and normal human skin. (a) Total RNA was isolated from cultured HaCaT cells and NHEK, and RT-PCR was performed with specific primers for *KAL1*. Lane 1, Universal Human Reference (UHR) RNA (positive control); lane 2, normal human skin; lane 3, HaCaT cells; lane 4, NHEK; lane 5, ICR mouse skin (negative control). *RPS18* was used as an internal control for mRNA intensity and quality. (b) Double-labelling for anosmin-1 (green) and keratin-14 (K14, red) was performed on cryosections of normal human skin. Strong anosmin-1 immunoreactivity was detected in K14-positive cells and in some dermal cells. These images of anosmin-1 and K14 were superposed. Yellow areas in the merged image are double-labelled. Scale bar: 50 μm. epi, epidermis; der, dermis.

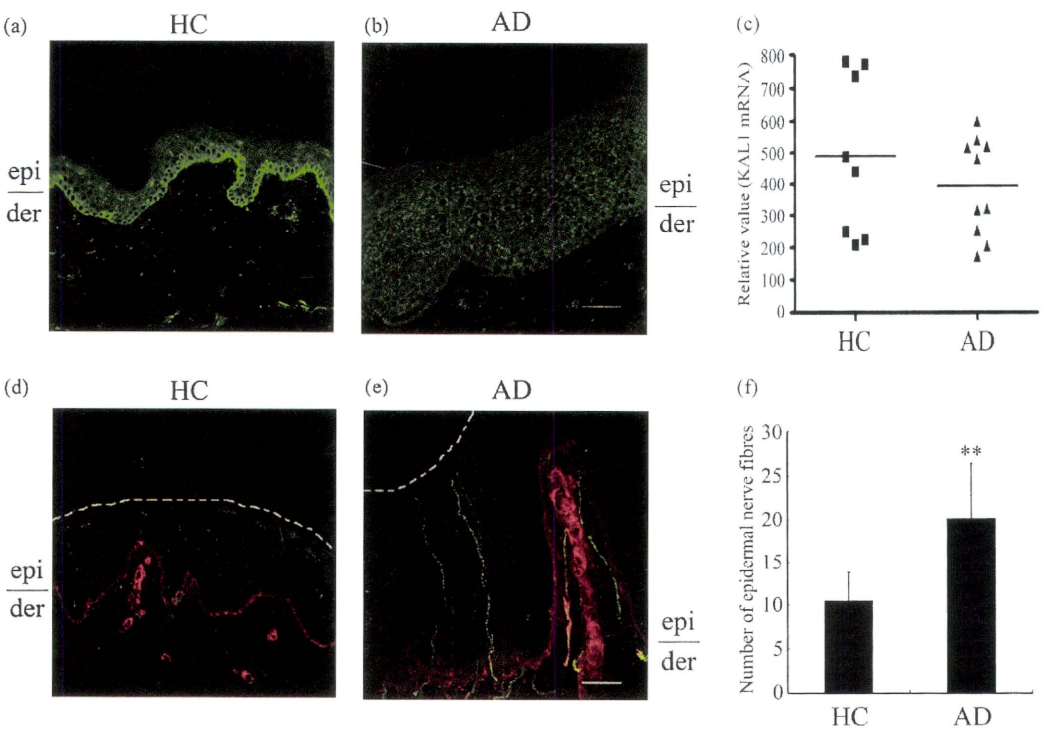

Fig. 3. Decreased expression of anosmin-1 in the lesional skin of patients with AD. Immunolabelling with anti-anosmin-1 antibody (green) in the skin of healthy control (a) and AD patient (b). Anosmin-1 was strongly expressed in the basal cell layer of normal skin, while the expression was decreased in the basal cell layer of atopic skin. (c) QRT-PCR analysis for *KAL1* mRNA expression was performed in the skin of healthy controls (HC) ($n = 8$) and patients with AD ($n = 10$). The levels of *KAL1* mRNA expression were calculated by the comparative C_t-method compared to *RPS18*. (d and e) Double immunostanning with anti-PGP9.5 (green) and type IV collagen (red) in skins from healthy controls (d) and AD patients (e). PGP9.5 positive fibres were occasionally present in the normal epidermis. In contrast, the epidermal nerve fibres were observed at higher densities in AD patients. White broken lines in panels (d) and (e) indicate the skin surface. Scale bars = 50 μm. (f) The number of epidermal nerve fibres was significantly increased in AD patients compared with that in healthy controls. Values are mean ± SD from three experiments ($^{**}P < 0.01$).

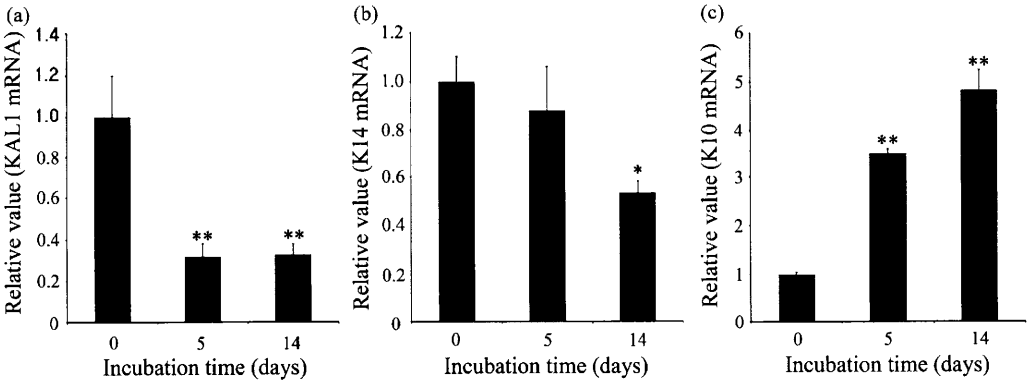

Fig. 4. *KAL1* expression is regulated by calcium concentration. NHEK treated with 1.4 mM $CaCl_2$ for 5 or 14 days, and qRT-PCR was performed using *KAL1* (a), *K14* (b) and *K10* (c) primers. *KAL1* expression was significantly downregulated in the differentiated cells. *K14* and *K10* genes were used as markers of proliferation and differentiation, respectively. The levels of mRNA expression were calculated by the comparative Ct-method in comparison to *RPS18*. Results are shown relative to the levels of gene expression in cells cultured with low-calcium serum-free medium (0 day). Values are means ± SD from three independent experiments (*$P < 0.05$; **$P < 0.01$).

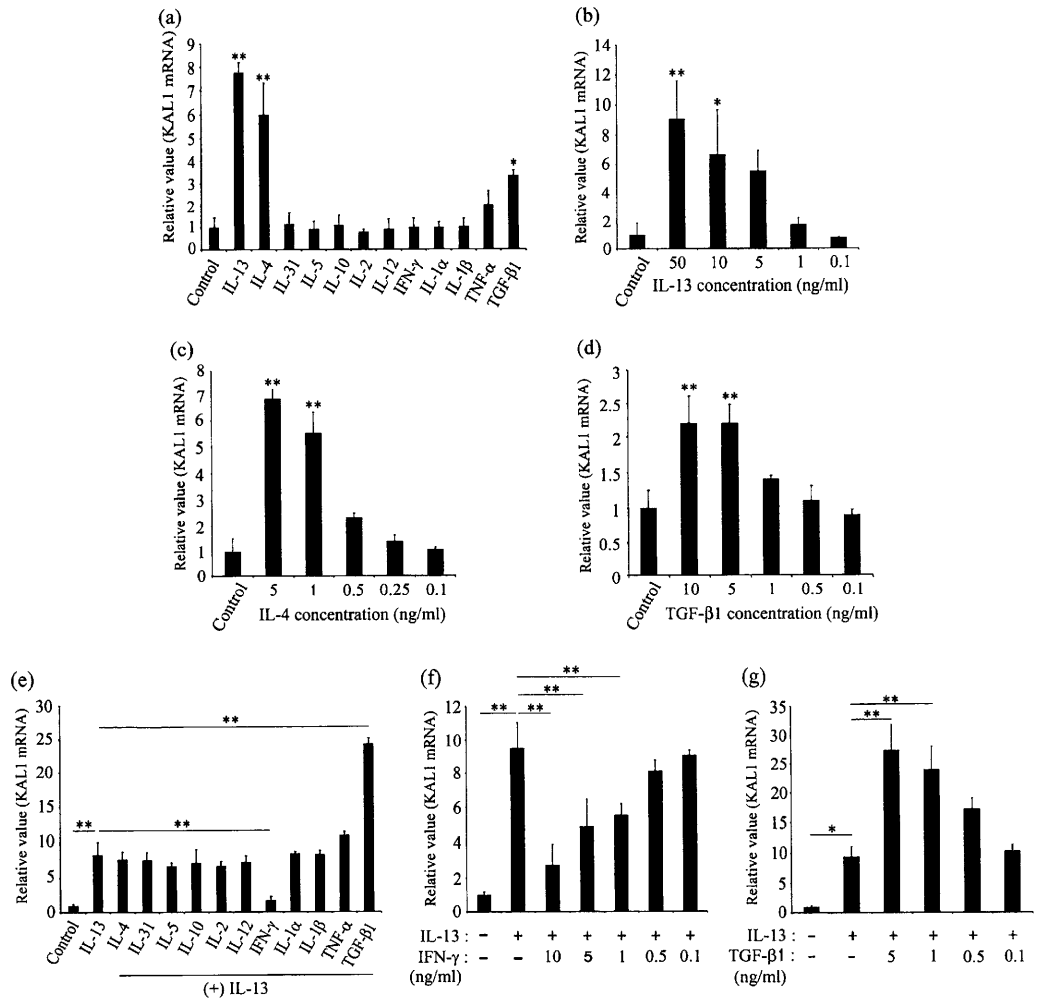

Fig. 5. Regulation of *KAL1* expression by cytokines in cultured NHEK. (a) Total RNA was isolated from NHEK stimulated with IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-31, IFN-γ, TNF-α and TGF-β1 at 50 ng/ml, or IL-1β and IL-1α at 50 pg/ml for 24 h, and then the expression level of *KAL1* was determined by qRT-PCR analysis. (b–d) *KAL1* expression was significantly upregulated in the cultured NHEK stimulated by IL-13, IL-4, or TGF-β1. IL-13 (b), IL-4 (c) and TGF-β1 (d) dose-dependently increased the expression levels of *KAL1* in cultured NHEK. (e) QRT-PCR analyses were performed on NHEK treated with IL-13 (50 ng/ml) and in combination with the other cytokines and concentrations mentioned above for 24 h. IFN-γ inhibited IL-13-induced *KAL1* expression, while TGF-β1 enhanced its expression in the presence of IL-13. (f and g) IFN-γ (f) and TGF-β1 (g) showed dose-dependent effects on IL-13-induced *KAL1* expression. The levels of mRNA expression were calculated by the comparative Ct-method in comparison to *RPS18*. Results are shown relative to the levels of gene expression in non-treated cells (control). Values are means ± SD from three independent experiments (*$P < 0.05$; **$P < 0.01$).

been shown to play roles in cell adhesion, cell migration, axonal guidance and branching activities [11–14,18].

The present study demonstrated the inhibitory effect of anosmin-1 on neurite outgrowth in cultured DRG neurones. Such inhibitory effects have been demonstrated in cerebellar neurones co-cultured with anosmin-1-producing cells. Thus, anosmin-1 may act as a nerve repulsion factor in human skin. Meanwhile, the neurite outgrowth was promoted in the cerebellar neurones cultured on anosmin-1-coated plates [11]. This reciprocal result suggests an allosteric mode of action of anosmin-1 on neurite outgrowth.

A recent study has indicated that anosmin-1 binding to fibroblast growth factor receptor 1 (FGFR1) inhibits FGF2–FGFR1–heparan sulfate complex formation [19]. In our DRG neuronal culture system, the neurite outgrowth was also rescued by addition of rhFGFR1. Activation of FGFR1 on adult sensory neurones is involved in promotion of neurite outgrowth [20]. However, we did not use FGF2 in the DRG neuronal culture system. Therefore, although we could not exclude FGF2 produced by CHO cells, this might imply the existence of other receptors or targets for anosmin-1 related to neurite outgrowth.

Unlike our observations, previous studies also indicated that anosmin-1 had no effect on axonal outgrowth of rodent DRG neurones [11,12]. This discrepancy may be explained by different responses to anosmin-1 in cultured neurones derived from different developmental stages.

Sensory nerve fibres are acceptors of itch (or pain) sensations in the skin. A number of studies have demonstrated that epidermal nerve fibres are present at higher densities in the skin of patients with AD than in control individuals [2]. Similar findings have been observed in animal models, such as NC/Nga mice [5]. Our recent studies also demonstrated that the epidermal nerve density is regulated by nerve elongation factors (e.g., NGF, amphiregulin, gelatinase) and repulsion factors (e.g., Sema3A) [5–7]. 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 sensations [2]. This was also supported by recent studies indicating that anti-NGF and recombinant Sema3A replacement approaches suppressed pruritus in NC/Nga mice [21,22].

In all patients with AD examined in this study, immunoreactivity of anosmin-1 reduced in the basal cell layer compared with healthy controls. Our immunohistological data showed increases of epidermal nerve fibres in the all patients. Therefore, these imply a correlation between anosmin-1 levels in the basal cell layer and epidermal nerve number. Unlikely anosmin-1 levels, the levels of *KAL1* mRNA were slightly decreased in the skin of AD patients. This might suggest the existence of post-transcriptional regulation in anosmin-1 expression. Another possibility is a complex regulation of *KAL1* expression by several Th1 and Th2 cytokines *in vivo*. This idea is supported by our data using cultured human keratinocytes.

Epidermal innervation is probably regulated by nerve elongation factor levels in the atopic skin, and not only by nerve repulsion factor levels [7]. In our preliminary data, no close relationship between number of epidermal nerve fibres and Sema3A levels was observed in psoriasis patients with pruritus (Taneda et al., unpublished observations). Although patients with Kallmann syndrome do not express anosmin-1 due to the lack of the *KAL1* gene [9,10], there have been no reports of itchy skin in Kallmann syndrome [23]. Taken together, epidermal innervation could be regulated by combination of several axonal guidance molecules in many skin diseases with pruritus. Therefore, altered balance of expression of these molecules will be needed to investigate in the skin diseases with pruritus.

It has been shown that Sema3A is mainly distributed in upper layers of epidermis [7]. Meanwhile, we showed that anosmin-1 was mainly distributed in the basal cell layer. Additionally, anosmin-1 may function in neurite outgrowth by different mechanisms of Sema3A [19]. Therefore, the different axon guidance regulators may be involved in the topography formation of sensory nerves in the human skin [24].

The mammalian epidermis shows a characteristic calcium gradient, and normal keratinocyte differentiation can be reproduced *in vitro* by culturing cells in media containing high-calcium concentrations [25,26]. In our experiments, *KAL1* expression was downregulated in NHEK cultured in a high-calcium environment in comparison with a low-calcium environment. This was also consistent with our immunohistochemical observation that basal cells mainly express anosmin-1. Thus, *KAL1* expression may be regulated by calcium concentration in normal skin during keratinocyte differentiation. Therefore, further studies are required to elucidate the function of *KAL1* in regulation of keratinocyte proliferation and/or differentiation.

Moreover, we found that *KAL1* expression was upregulated by IL-13 or IL-4 in cultured NHEK. These biological effects may be mediated by a shared receptor composed of the IL-4Rα and the IL-13Rα1 receptor chains [27]. Intracellular signal transduction in response to IL-4/IL-13 occurs through activation of signal transducer and activator of transcription 6 (STAT6) [28]. Therefore, although the *KAL1* promoter is not completely understood, these findings raise the possibility that *KAL1* expression is controlled by the STAT6 signalling pathway. Increased TGF-β1 expression is found in chronic lesions compared with acute lesions in AD, suggesting a role in the development of tissue remodelling [29]. Our data showed that TGF-β1 slightly induced *KAL1* expression in cultured NHEK as compared with IL-4/IL-13 stimulation. This growth factor also acts as an inducer of *KAL1* expression in human muscle cells and fibroblasts [15]. However, in combination with IL-13, it acted synergistically to enhance *KAL1* expression in the present study. The synergistic effect of IL-13 and TGF-β1 has been validated by a recent study on *CCL5* expression using cultured keratinocytes [30]. Therefore, these findings suggest that TGF-β1 acts as an enhancer rather than an inducer of *KAL1* expression in the presence of Th2 cytokines. This may be explained by a previous report that the TGF-β signalling pathway can modify the cellular responses to several cytokines and stress signals [31].

IFN-γ inhibited *KAL1* expression induced by IL-13 in cultured NHEK, suggesting that this cytokine functions as a negative regulator of Th2 cytokine-induced *KAL1* expression. This was strongly supported by previous reports that IFN-γ inhibits the development of Th2 cytokine responses [32,33]. AD is an inflammatory skin disorder characterised by local expression of Th2 cytokines, whereas the lesional skin in chronic AD has a mixed Th1 and Th2 pattern [34,35]. Indeed, the expression levels of *KAL1* tended to decrease in the atopic skin. Thus, our observations provide the first evidence regarding cross-regulation of *KAL1* expression by Th1/Th2 cytokine balance with modulation of TGF-β1 in epidermal keratinocytes.

In conclusion, the results of the present study showed that anosmin-1 has an inhibitory effect on neurite outgrowth in cultured DRG neurones. We also demonstrated the distribution and expression patterns of anosmin-1 in human skin. The downregulation of anosmin-1 in the basal cell layer may also cause penetration of nerve fibres into the epidermis of patients with AD. Moreover, the regulation system of *KAL1* by cytokines may be involved in the pathogenesis of AD.

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1 診断基準と重症度分類—海外との比較も踏まえて—

Diagnostic criteria and severity classification
– including comparison with those overseas –

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Key words：アトピー性皮膚炎，診断基準，重症度分類，SCORAD，TARC

Abstract

1994年に策定された日本皮膚科学会「アトピー性皮膚炎の定義・診断基準」(2008年追加改訂)に基づき，1）瘙痒，2）特徴的皮疹と分布，3）慢性・反復性経過の3基本項目を満たすものを，アトピー性皮膚炎と診断する。世界的には1980年に作成されたHanifin & Rajkaの診断基準が頻用されている。2001年に日本皮膚科学会によるアトピー性皮膚炎重症度分類が提唱された。全身を5部位に分け，各部位における皮疹の3要素を最も重症な部分で評価する。世界的はEuropean Task Force on Atopic Dermatitisによる Severity Scoring of Atopic Dermatitis（SCORAD）が頻用されている。また，血清中のTARC（thymus and activation-regulated chemokine）値は短期的な重症度や病勢を鋭敏に反映することが示された。

はじめに

日本皮膚科学会によるアトピー性皮膚炎の診断基準は1994年に[1]，重症度分類は1998年の中間報告を経て，2001年に策定された[2]。日本皮膚科学会アトピー性皮膚炎治療ガイドラインは2000年に初めて策定され，その後2003年，2004年に改訂された[3]。2008年には，アトピー性皮膚炎の診断基準，重症度分類，治療ガイドラインを統合したものとして，アトピー性皮膚炎診療ガイドラインが策定された[4]。また，2009年には新しい治療法の保険適用の追加などに伴い改訂が行われた[5]。本稿では，アトピー性皮膚炎の診断基準と重症度分類について，海外との比較も踏まえて説明し，また重症度の参考となる指標についても概説する。

1. 診断基準

1）日本での診断基準

1994年に策定された日本皮膚科学会「アトピー性皮膚炎の定義・診断基準」(2008年追加改訂)に基づき，1）瘙痒，2）特徴的皮疹と分布，3）慢性・反復性経過の3基本項目を満たすものを，症状の軽重を問わずアトピー性皮膚炎と診断する。疑診例では急性あるいは慢性の湿疹とし，年齢や経過を参考に

表1　アトピー性皮膚炎の定義・診断基準（日本皮膚科学会）（文献5より引用）

＜アトピー性皮膚炎の定義（概念）＞
アトピー性皮膚炎は，増悪・寛解を繰り返す，瘙痒のある湿疹を主病変とする疾患であり，患者の多くはアトピー素因を持つ。
アトピー素因：①家族歴・既往歴（気管支喘息，アレルギー性鼻炎・結膜炎，アトピー性皮膚炎のうちいずれか，あるいは複数の疾患），または②IgE抗体を産生しやすい素因。

＜アトピー性皮膚炎の診断基準＞
1. 瘙痒
2. 特徴的皮疹と分布
　①皮疹は湿疹病変
　　急性病変：紅斑，湿潤性紅斑，丘疹，漿液性丘疹，鱗屑，痂皮
　　慢性病変：浸潤性紅斑・苔癬化病変，痒疹，鱗屑，痂皮
　②分布
　　左右対側性
　　好発部位：前額，眼囲，口囲・口唇，耳介周囲，頸部，四肢関節部，体幹
　　参考となる年齢による特徴
　　乳児期：頭，顔に始まりしばしば体幹，四肢に下降。
　　幼小児期：頚部，四肢屈曲部の病変。
　　思春期・成人期：上半身（顔，頸，胸，背）に皮疹が強い傾向。
3. 慢性・反復性経過（しばしば新旧の皮疹が混在する）
　　乳児では2ヵ月以上，その他では6ヵ月以上を慢性とする。

　上記1, 2, および3の項目を満たすものを，症状の軽重を問わずアトピー性皮膚炎と診断する。そのほかは急性あるいは慢性の湿疹とし，年齢や経過を参考にして診断する。

＜除外すべき診断（合併することはある）＞
接触皮膚炎，脂漏性皮膚炎，単純性痒疹，疥癬，汗疹，魚鱗癬，皮脂欠乏性湿疹，手湿疹（アトピー性皮膚炎以外の手湿疹を除外するため），皮膚リンパ腫，乾癬，免疫不全による疾患，膠原病（SLE，皮膚筋炎），ネザートン症候群

＜診断の参考項目＞
家族歴（気管支喘息，アレルギー性鼻炎・結膜炎，アトピー性皮膚炎），合併症（気管支喘息，アレルギー性鼻炎・結膜炎），毛孔一致性の丘疹による鳥肌様皮膚，血清IgE値の上昇

＜臨床型（幼小児期以降）＞
四肢屈側型，四肢伸側型，小児乾燥型，頭・頸・上胸・背型，痒疹型，全身型，これらが混在する症例も多い

＜重要な合併症＞
眼症状（白内障，網膜剥離など）：とくに顔面の重症例，カポジ水痘様発疹症，伝染性軟属腫，伝染性膿痂疹

して診断する[1]。この診断基準の英訳は1995年に公表されている[6]。なお，2008年の改訂で除外すべき診断として新たに，皮膚リンパ腫，乾癬，免疫不全による疾患，膠原病（SLE，皮膚筋炎），ネザートン症候群が付け加えられた（表1）。除外すべき診断としてあげられた疾患を十分に鑑別でき，重要な合併症としてあげられた疾患について熟知していることが必要である。2008年に改訂された診断基準の英訳は2009年に公表されている[7]。

2）海外での診断基準

世界的には1980年に作成されたHanifin & Rajkaの診断基準が頻用されている（表2）[8]。日本皮膚科学会による診断基準がHanifin & Rajkaの診断基準と異なる点は，アトピー疾患の既往歴・家族歴を基本項目から参考項目にした点であるが，アトピー性皮膚炎の定義（概念）としてアトピー素因が明記されている（表1）。Hanifin & Rajkaの診断基準における23の小項目症状はいずれも本症でしばし

ば観察される特徴的な症状であるが，その発現頻度はさまざまで，抽象的表現を用いた項目もあり，日本皮膚科学会による診断基準では省いてある。その後，“簡易版Hanifin & Rajkaの診断基準”ともいうべきものが2003年に公表されている[9]。この診断基準では，診断に必須な項目として，1）瘙痒，2）湿疹（急性，亜急性，慢性）a）典型的な皮疹の形態と年齢による特徴，b）慢性・再発性経過が挙げられており，日本皮膚科学会の診断基準により近いものとなっている。

表2　アトピー性皮膚炎診断基準（Hanifin & Rajka）（文献8より引用，改変）

A: 以下の基本項目を3つ以上有すること
1. 瘙痒
2. 典型的な皮疹の形態と分布
成人では屈側部の苔癬化
幼小児では顔面および伸側の皮疹
3. 慢性あるいは慢性再発性皮膚炎
4. アトピー（喘息、アレルギー性鼻炎、アトピー性皮膚炎）の既往または家族歴

B: さらに以下の小項目を3つ以上有すること
1.乾皮症
2.魚鱗癬、手掌の多紋理、毛孔性角化
3.即時型皮膚試験反応陽性
4.高IgE血症
5.年少時発症
6.皮膚感染症を発症する傾向（黄色ブドウ球菌や単純性疱疹）／細胞性免疫低下
7.非特異的手または足の皮膚炎を発症する傾向
8.乳頭湿疹
9.口唇炎
10.再発性結膜炎
11.Dennie-Morgan下眼瞼皺襞
12.円錐角膜
13.前嚢下白内障
14.眼瞼色素沈着
15.顔面蒼白、顔面紅斑
16.白色粃糠疹
17.前頸部皺襞
18.発汗時掻痒
19.羊毛および油脂溶媒に対する不耐性
20.毛嚢周囲顕著化
21.食物不耐性
22.環境、感情因子により影響されやすい
23.白色皮膚描記症、遅発蒼白反応

2. 重症度分類

アトピー性皮膚炎の重症度分類には様々なものが提唱されているが，大別すると全身の重症度を分類したものと，個々の皮疹の重症度を分類したものに分けられる。

1）全身の重症度分類

a）日本での重症度分類

日本皮膚科学会アトピー性皮膚炎重症度分類検討委員会によって，図1に示すような重症度分類が2001年に提唱された[2]。全身を5部位（頭頸部，前体幹，後体幹，上肢，下肢）に分け，各部位における皮疹の3要素（紅斑・急性期の丘疹，湿潤・痂皮，慢性期の丘疹・結節・苔癬化）を最も重症な部分で各々0～3の4段階で評価する（計15回）。別に皮疹の面積も5部位で0～3（0：なし，1：～1/3，2：1/3～2/3，3：2/3～）の4段階で評価する（計5回）。両者を合計（計20回）した点を重症度として評価する（60点満点）。なお，本重症度分類は統計学的信頼性と妥当性が検証されており，臨床試験に用いることが可能である。また，本委員会の検討による簡便法として，全身を頭頸部，前体幹，後体幹，上肢，下肢の5部位に分け，各部位のグローバル評価（0：なし，1：軽症，2：中等症，3：重症，4：最重症）の総和を求める方法も提示されている（最高点数20点）。

さらに簡便な方法として，厚生労働科学研究班により重症度のめやすも提案されている[10]。この方法では，皮疹の面積に関わらず，軽度の皮疹（軽度の紅斑，乾燥，落屑主体の病変）のみみられるものを軽症とする。

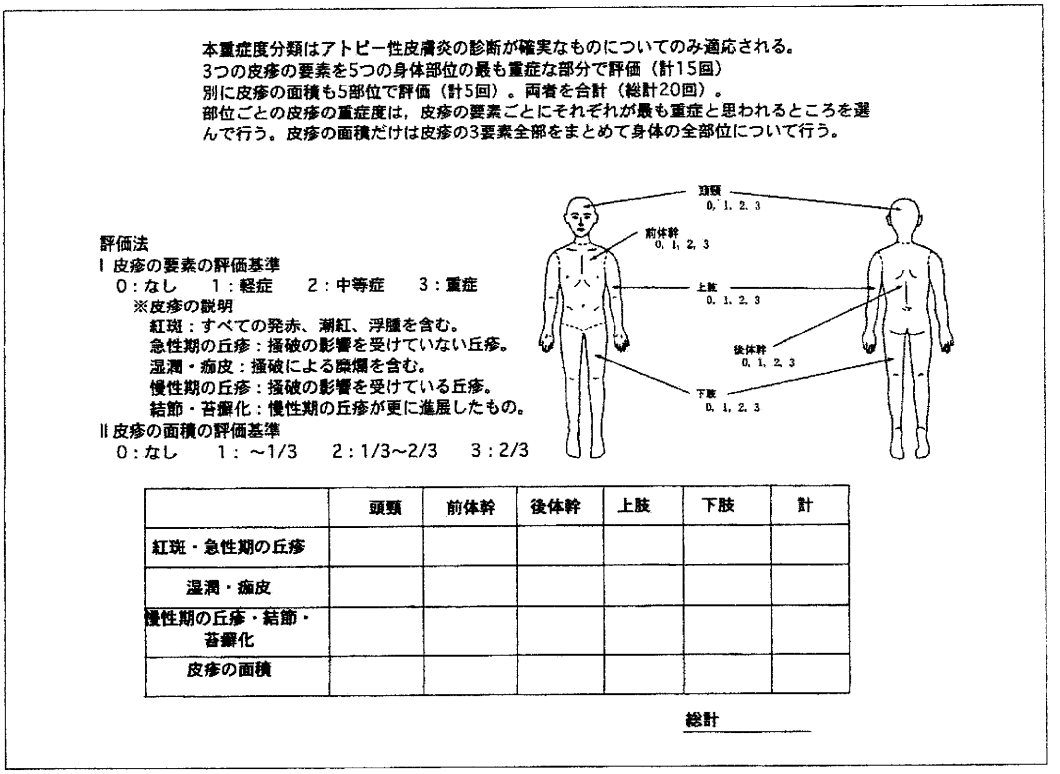

図1　日本皮膚科学会アトピー性皮膚炎重症度分類（文献2より引用，改変）

また，強い炎症を伴う皮疹（紅斑，丘疹，びらん，浸潤，苔癬化などを伴う病変）が体表面積の10％未満にみられるものを中等症，10％以上30％未満にみられるものを重症，30％以上にみられるものを最重症と定めている。

b）海外での重症度分類

世界的にはEuropean Task Force on Atopic Dermatitis によるSeverity Scoring of Atopic Dermatitis (SCORAD)（最高点数103点）あるいは米国のEczema Area and Severity Index (EASI)（最高点数72点）が頻用されている[11][12]。

SCORADによる重症度分類を図2に示す[11]。A. 皮疹の範囲，B. 皮疹の程度，C. 自覚症状の3つの要素で判定する。A. 皮疹の範囲は9の法則に従って計算する。B. 皮疹の程度は，紅斑，浮腫／丘疹，滲出／痂皮，掻破痕，苔癬化，皮膚乾燥の6項目について，平均的な部位で0～3の4段階で評価する。C. 自覚症状は，痒みと睡眠障害に関してvisual analog scale (VAS) を用いて0～10で評価する。SCORADはA／5＋7B／2＋Cの計算式で点数化して表される。103点満点で評価されるが，点数はインターネット上のSCORADのホームページ（http://adserver.sante.univ-nantes.fr/Compute.html）で計算することも可能である。また，C. 自覚症状を除いたA／5＋7B／2をObjective SCORAD（83点満点）として用いる場合もある。

EASIは炎症性角化症である乾癬（psoriasis）の重症度分類PASI (Psoriasis Area and Severity Index)を元にして作られた[12]。全身を4部位（頭頸部，上肢，体幹，下肢）に分け，各部位において皮疹を4つの要素（紅斑，浸潤／丘疹，掻破痕，苔癬化）で各々0～3の4段

階で評価する。また，4部位各々における皮疹の面積を0～6（0=0%，1=1-9%，2=10-29%，3=30-49%，4=50-69%，5=70-89%，6=90-100%）で評価する。（皮疹の4要素の合計点）×（皮疹の面積）を，頭頸部では0.1倍，上肢では0.2倍，体幹では0.3倍，下肢では0.4倍した点を求め，それら全てを総計した点がEASIである（72点満点）。この方法では自覚症状が評価に入っていないため，後にVASを用いて痒みの評価を加えたものがmodified EASI (mEASI)として提唱された。

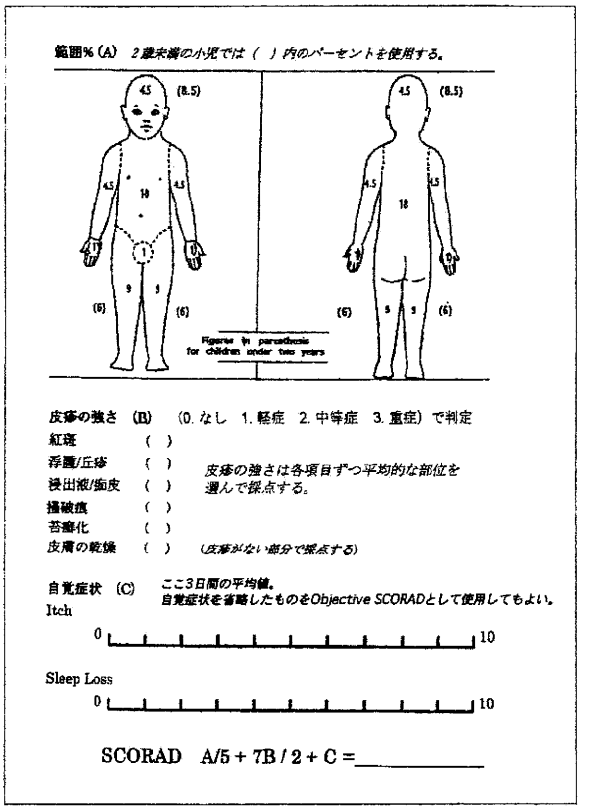

図2 SCORADによる重症度分類
（文献11より引用，改変）

2）個々の皮疹の重症度分類

治療の主体である外用療法の選択は「個々の皮疹の重症度」（表3）により決定される[5]。すなわち，範囲は狭くとも高度な皮疹には十分に強力な外用療法が選択されるが，範囲は広くとも軽度の皮疹には強力な外用療法は必要としない。

重症の皮疹は，高度の腫脹／浮腫／浸潤ないし苔癬化を伴う紅斑，丘疹の多発，高度の鱗屑，痂皮の付着，小水疱，びらん，多数の掻破痕，痒疹結節などを主体とする。中等症の皮疹は，中等症までの紅斑，鱗屑，少数の丘疹，掻破痕などを主体とする。軽症の皮疹は，乾燥および軽度の紅斑，鱗屑などを主体とする。軽微の皮疹は，炎症症状に乏しい乾燥症状を主体とする。

重症の皮疹に対してはベリーストロングないしストロングクラスの，中等症の皮疹に対してはストロングないしミディアムクラス

表3 皮疹の重症度とステロイド外用薬の選択（文献5より引用）

	皮疹の重症度	外用薬の選択
重症	高度の腫脹/浮腫/浸潤ないし苔癬化を伴う紅斑、丘疹の多発、高度の鱗屑、痂皮の付着、小水疱、びらん、多数の掻破痕、痒疹結節などを主体とする	必要かつ十分な効果を有するベリーストロングないしストロングクラスのステロイド外用薬を第一選択とする。痒疹結節でベリーストロングクラスでも十分な効果が得られない場合は、その部位に限定してストロンゲストクラスを選択して使用することもある
中等症	中等度までの紅斑、鱗屑、少数の丘疹、掻破痕などを主体とする	ストロングないしミディアムクラスのステロイド外用薬を第一選択とする
軽症	乾燥および軽度の紅斑、鱗屑などを主体とする	ミディアムクラス以下のステロイド外用薬を第一選択とする
軽微	炎症症状に乏しく乾燥症状主体	ステロイドを含まない外用薬を選択する

の，軽症の皮疹に対してはミディアムクラス以下のステロイド外用薬を第一選択とする。軽微の皮疹に対してはステロイドを含まない保湿剤を主体とした外用薬を選択する[5]。

3. 重症度の参考となる指標

1）痒みの評価

痒みの評価にはVASが有用である[13]。VASは痒みの程度に応じて10cmの線分上の1点に印を付け，左端の「痒みなし」を0，右端の「最もひどい痒み」を100として，左端から印を付けた部位までの距離（mm）を痒みの尺度値として評価する方法である。SCORADでも記載されているように（SCORADでは0～10で評価）VASは睡眠障害に対しても用いうる。また，文章表現による痒みの評価法も提唱されている[14]。

2）Quality of Life (QOL)による評価

Skindex-16ならびにDermatology Life Quality Index (DLQI)が統計学的に詳細に解析され[15][16]，またその邦訳が出版され各種皮膚疾患に応用されている[17][18]。Skindex-16では16項目の質問に，DLQIでは10項目の質問に答えてもらうことにより，過去1週間における皮膚の症状（状態）がQOLに与える影響を評価することができる。

3）病勢の参考となる検査

アトピー性皮膚炎の重症度や病勢の参考となる検査には，末梢血好酸球数，血清総IgE値，LDH (lactate dehydrogenase)値，TARC (thymus and activation-regulated chemokine)値などがある。短期的な病勢のマーカーとしてはLDH，TARCなどが挙げられるが，測定幅からすると，TARCは病勢を鋭敏に反映することが示された[19]。なお，2008年7月よりアトピー性皮膚炎の重症度評価の補助の目的で，血清中のTARC値の測定が保険適用された（月1回に限り算定）。

おわりに

アトピー性皮膚炎の診断基準と重症度分類および重症度の参考となる指標について概説した。アトピー性皮膚炎の診療においては，確実な診断と重症度の評価の後，患者の皮疹の状態に応じて適切な治療（ステロイド外用薬，タクロリムス軟膏，抗ヒスタミン薬・抗アレルギー薬，保湿剤など）をうまく組み合わせて行うことが重要である。

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●総説

免疫異常からみたアトピー性皮膚炎のメカニズムと治療

中村晃一郎*

❶ 要　約

アトピー性皮膚炎の病態は，角質のバリア機能異常による非免疫異常と，サイトカインバランス異常による免疫異常という二つの側面から形成される。免疫異常として皮膚の免疫担当細胞によるサイトカイン産生異常があり，これらにより皮膚炎が形成される。アトピー性皮膚炎の治療の目標は，「症状がない，またはあっても軽微であること，再燃しても遷延化しないこと」であり，このためにステロイド外用薬やタクロリムス軟膏などの免疫調節薬，スキンケア，痒みコントロールなどを組み合わせて行う。寛解導入期に使用するステロイド外用薬などの免疫調節薬は抗炎症作用を有しており，サイトカインバランス異常を是正する。寛解維持期におけるスキンケアは，皮膚バリア機能を改善することによって，バリア機能の低下や痒みを改善する。これらの治療を組み合わせて行うことによって，免疫異常による皮膚炎の増悪を改善することができ，長期的に炎症のない状態を継続することが可能となる。

❷ はじめに

アトピー性皮膚炎，喘息，アレルギー性結膜炎などのアレルギー性疾患は，近年増加傾向を示している。長期に再燃を繰り返すことによって日常生活における QOL や社会活動は著しく阻害される。アトピー性皮膚炎は，多因子遺伝子疾患であり，両親がアレルギー保持者である場合，その児に多く認めるように複数の遺伝因子が関与していると考えられる。同時に悪化因子として，ダニ抗原，細菌感染，接触抗原などの環境因子があり，患者個々において異なるため，これらに対してその患者における悪化因子を積極的に解明し除去することも必要である。本稿では，免疫異常からみたアトピー性皮膚炎の病態について考え，病態からみた治療目標・治療方針について述べたい。

❸ 遺伝因子からみたアレルギー性疾患について

アトピー性皮膚炎は幼少期に発症するアレルギー性疾患で，傾向としては成長とともに自然軽快を示す。アトピー性皮膚炎の発症に遺伝が関与することについては，アトピー性皮膚炎患者で，両親のうちどちらか，もしくは両方がアトピー性皮膚炎に罹患している頻度は 69％と高い値を示すことからも明らかである[1)]。

また，アトピー性皮膚炎と遺伝の関係については，これまでの国内外の遺伝子多型解析の報告から明らかとなってきた。遺伝子多型解析とは，遺伝子領域の，ある特定の塩基の違いの頻度を，比較したもので，これが患者群と健常人群で有意差がみられ，この遺伝子多型が疾患に関与している可能性が考えられる。これまでのこの方法による検証から，アトピー性皮膚炎の遺伝子と相関する多型として，サイトカイン遺伝子，自然免疫に関する遺伝子，細胞接着に関する遺伝子，角層バリ

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