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Quantitative analysis of nerve growth factor (NGF) in the atopic dermatitis and psoriasis horny layer and effect of treatment on NGF in atopic dermatitis

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SUMMARY

Background and objective: The expression of nerve growth factor (NGF) is known to increase in the skin of patients with atopic dermatitis (AD) and is related to disease aggravation. In the present study, we measured skin NGF levels in AD patients and determined whether they correlate to AD severity as well as treatment effects.

Methods: NGF in the horny layer (horn NGF) of skin lesions found on the cubital fossa of AD patients was collected via tape stripping and measured using ELISA before and after 2 and 4 weeks following initiation of treatments. Itching and eruptions on the lesions were also evaluated. Peripheral blood eosinophil count, serum LDH level and total serum IgE level were also examined.

Results: The level of NGF was significantly higher in AD patients than in healthy controls, and correlated with the severity of itch, erythema, scale/xerosis, eosinophil count, and LDH level. The NGF level decreased significantly at 2 and 4 weeks of treatment with olopatadine, a histamine H₁ receptor antagonist, and/or topical steroid. The reduction in NGF correlated with the decrease in the severity of itching and erythema, papule, scale/xerosis and lichenification of the lesion, eosinophil count, and LDH level. In psoriatic lesional skin with itch, the horn NGF was significantly higher than in non-lesional skin of psoriasis, but the value was lower than NGF in atopic skin.

Conclusions: The level of horn NGF was found to reflect the severity of itching and eruptions in AD. Therefore, quantification of NGF in the samples collected directly from the horny layer appears to be useful in assessing severity and therapeutic effects in AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin disease characterized by highly pruritic, eczematous skin lesions. Various factors contribute to the pathogenesis and development of AD, including allergic and immunological abnormalities, as well as physiological and biochemical barrier dysfunctions of the skin [1].

Recently, neurogenic inflammation, including effects of neuropeptides, e.g. substance P, has provided a new perspective in understanding the pathogenesis of AD [2]. Increasing evidence suggests that neurotrophins may be involved in the neurogenic inflammation of AD and may be one of factors which regulate AD development [3].

Nerve growth factor (NGF), discovered more than 50 years ago [4,5], belongs to the neurotrophin family, together with brain-derived neurotrophic factor and neurotrophins. NGF is essential for the survival, development, differentiation, and function of peripheral sympathetic and sensory neurons, as well as basal forebrain cholinergic neurons in the central nervous system [6]. NGF is one of the major neurotrophins [7] and has diverse activity in the skin. It is mainly produced by keratinocytes [6,8]. In human skin, NGF is released in increasing amounts by the basal proliferative cell compartment of keratinocytes [9–11]. Immunohistochemistry of human skin shows that NGF is expressed in suprabasal keratinocytes and in single cells of the basal epidermal layer [10].

It is known that expression of NGF is increased in the skin of patients with AD and is related to aggravation of the disease [12]. NGF levels of AD patients have been measured in plasma [8], urine and saliva [13]. A correlation of NGF level and disease severity has been reported in the peripheral blood sample. However, recently Schulte-Herbruggen et al. have reported that they did not find a significant correlation between NGF and total IgE, or severity of

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disease assessed by SCORAD [14]. The problem seems that NGF level in blood sample does not accurately express the NGF level in the skin. However, thus far, there had been no useful method, in terms of non-invasiveness and repeated use, to measure the NGF level in the skin.

To quantify the NGF level in the epidermis, we have established a method using tape stripping and ELISA, which can be performed repeatedly in the same patient. In preliminary experiments, we measured the NGF levels directly from the horny layer (i.e., horn NGF) using this method and showed that NGF in AD lesions was significantly elevated as compared to levels in healthy skin (data not shown). In the present study, we measured the horn NGF in AD lesions using this method and assessed for correlation with the severity of itch and AD, as well as the effects of treatment. We also measured horn NGF in psoriasis with or without itch to compare the NGF in AD.

2. Materials and methods

2.1. Subjects and treatments

An ethical approval for our study was obtained from the ethics committee of the Yokohama City University. A full explanation of the procedure was given to all participants, and their written consent was obtained before testing. Thirty-three AD patients (20 males and 13 females; average age, 27.9 years) and 9 healthy non-AD volunteers (4 males and 5 females; average age, 34.7 years) were included in the study. Diagnosis of AD was made according to the criteria of Hanifin and Rajka [15]. Of these patients, 26 were continuously treated at our clinic. To assess the effects of treatments on the level of horn NGF, these 26 AD patients were treated with olopatadine (10 mg/day) and/or mild class topical corticosteroids (i.e. alclometasone dipropionate), with a moisturizer (e.g., petrolatum). Olopatadine hydrochloride ((Z)-11-(3-dimethylaminopropylidene)-6,11-dihydrodibenz[b,e]oxepin-2-acetic acid monohydrochloride, Allelock[®], Kyowa Hakko Kogyo Co., Ltd., Japan) is a selective and potent histamine H₁ receptor antagonist [16]. The patients had a wash-out phase for at least 2 weeks before initiation of the study. To define the significance of NGF in AD, horn NGF in lesional and non-lesional skin was also measured in 41 patients with psoriasis.

2.2. Assessment of the clinical conditions

Patients were evaluated for clinical conditions (i.e., itch, eruption, and laboratory findings). Itch was evaluated using the Visual Analogue Scale (VAS) [17] and pruritus scores on a five-point scale (0 = none; 4 = very severe) on morning and night [18]. Severity of eruptions on the lesion used for NGF measuring was evaluated by total skin score, defined as the sum of individual scores, graded as: 0 (none), 1 (slight), 2 (moderate), 3 (mild) and 4 (severe) for the symptoms of erythema, papule, scale/xerosis, erosion/crust, and lichenification. These clinical conditions were evaluated before and at 2 and 4 weeks following initiation of treatment. Peripheral blood eosinophil count, serum LDH level, and total serum IgE levels were measured before and after 4 weeks of treatment. Serum-specific IgE levels (house dust mite, cedar, soy bean, *Malassezia*, wheat, rice, and *Candida*) were also examined by CAP-FEIA before treatment.

2.3. Measurement of the NGF level in the horny layer of atopic and psoriatic skin

The horny layer was collected via the tape stripping method from skin lesions found on the cubital fossa of AD patients and

normal skin from the cubital fossa of healthy controls. With respect to psoriasis, the horny layer was collected from skin lesions on forearm with or without itch.

The patients had stopped to apply topical treatment about 12 h before tape stripping, and taken a bath or shower on the previous day. The tape adherence to the skin was performed without wiping the skin surface and identical before and after treatment in patients. The identical tape adherence was also performed in psoriatic patients and healthy controls. The tape (17 mm × 45 mm, Serotape[®], NICHIBAN, Tokyo) was applied 3 times to the skin, removed gently, cut finely, and immersed in 600 μ l PBS (–). After ultrasound sonication (Sonifier[®], Branson Ultrasonics Corporation, Connecticut) for 1 min at 4 °C, the supernatant was collected by centrifugation for 15 min (11755 G) and used as horny layer extract samples. NGF levels in the extracts were measured via sandwich ELISA (NGF E_{MAX}[®] ImmunoAssay System, Promega, Wisconsin), with a minimum detection level of 7.8 pg ml^{–1} of NGF, as per the manufacturer's instructions. Total protein levels of the samples were measured via Bradford's method (Bio-rad protein assay[®], Bio-rad, California), according the manufacturer's instructions, and horn NGF contents were corrected by total protein contents in the samples (NGF protein^{–1} value, pg μ g^{–1}).

2.4. Immunohistochemistry

Formalin-fixed, paraffin-embedded skin sections were deparaffinized with xylene and graded ethanol. Endogenous peroxidases were blocked using peroxide. After washing with PBS, sections were incubated in normal goat serum. Sections were then incubated at 4 °C overnight with polyclonal rabbit antibodies against NGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections were washed with PBS and then incubated with biotinylated goat anti-rabbit IgG antibody. Slides were washed with PBS and then incubated with avidin-biotinylated peroxidase complex. Peroxidase activity was visualized using stable diaminobenzidine. Staining was monitored under a bright-field microscope, and sections were washed with distilled water to stop the reaction. Sections were then counterstained with methyl green, washed, and mounted.

2.5. Data analysis

Results were expressed as the mean $\pm$ S.D. of the indicated number of independent experiments. Statistical analysis was performed using the Mann-Whitney *U*-test, steel test or Tukey's test, as indicated. The correlations between horn NGF levels and clinical conditions before and after each treatment were analyzed via the Spearman rank-order correlation test. A difference with a *p*-value <0.05 was considered statistically significant. Distributions of scores were visualized using box-whisker-plots of the indicated number of independent experiments. Medians were indicated using horizontal bars. Vertical bars were used to indicate the range, with box horizontal boundaries representing the first and third quartiles.

3. Results

3.1. Histological distribution of NGF in the horny layer of atopic skin

Previous histological examinations have demonstrated that NGF is mainly expressed in basal and suprabasal cells in human epidermis, and that its expression levels is increased in the epidermis of AD patients compared with healthy controls [9–12].

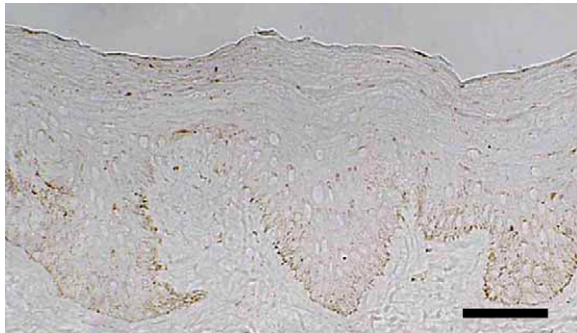


Fig. 1. Histological distribution of NGF in the horny layer of atopic skin. Immunohistochemical staining of NGF was performed as described in Section 2.4. In addition to basal cells and keratinocytes, horny layer express NGF proteins. Original magnification 100×; Bar = 100 μm .

To define the histological distribution of NGF in the horny layer, immunohistochemical staining was performed. In addition to basal cells and keratinocytes, horny layer express NGF proteins (Fig. 1).

3.2. Characteristics of the AD patients

For the 33 initially enrolled patients, total skin score was 12.7 ± 4.2 , peripheral blood eosinophil count was $1004.3 \pm 1207.0 \mu\text{L}^{-1}$, LDH level was $300.7 \pm 94.4 \text{ IU L}^{-1}$, and total serum IgE level was $6754.8 \pm 9963.8 \text{ U mL}^{-1}$ (Table 1).

3.3. Correlations of the horn NGF level and the clinical conditions

The horn NGF level was significantly higher in AD lesions than in the normal skin of healthy controls ($p < 0.01$, Student's *t*-test) (Fig. 2). Horn NGF level correlated with itch severity [(a) pruritus scores ($R = 0.390$, $p = 0.040$)], eruptions on measured lesional skin [(b) erythema ($R = 0.436$, $p = 0.023$), (c) scale/xerosis ($R = 0.466$, $p = 0.014$) and (d) total skin score ($R = 0.479$, $p = 0.015$)], (e) eosinophil count ($R = 0.628$, $p = 0.001$), and (f) serum LDH level ($R = 0.432$, $p = 0.028$) (Fig. 3, Spearman rank-order correlation test). However, positive correlations were not observed between horn NGF level and skin scores of papule, erosion/crust, and lichenification on measured skin and serum IgE levels (data not shown).

Table 1
AD patient characteristics

	AD patients
Patient number	33
Age (years)	27.9 ± 9.6
Total skin score (score)	12.7 ± 4.2
Peripheral blood eosinophil count (number μL^{-1})	1004.3 ± 1207.0
LDH level (IU L^{-1})	300.7 ± 94.4
Total serum IgE level (U mL^{-1})	6754.8 ± 9963.8
Specific IgE (UA mL^{-1})	
House dust mite	312.8 ± 478.6
Cedar	58.6 ± 106.0
Soy bean	0.4 ± 0.7
Malassezia	10.9 ± 13.1
Wheat	1.00 ± 1.4
Rice	0.6 ± 0.7
Candida	7.2 ± 12.8

Thirty-three well-informed AD patients were subjected to the study. Patient clinical conditions were evaluated, as shown above. Values represent mean $\pm$ S.D.

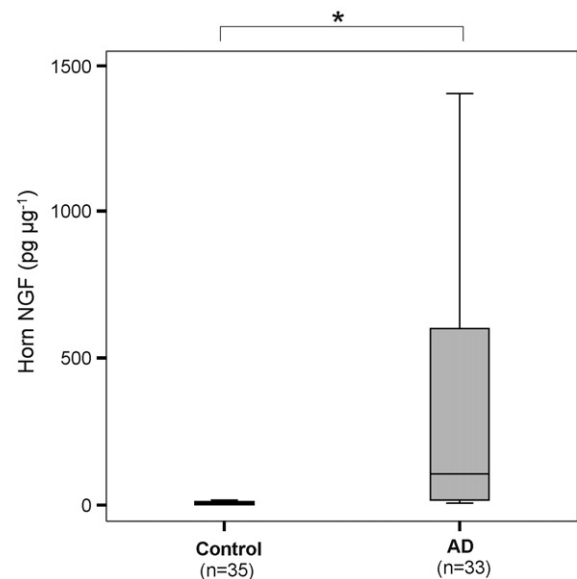


Fig. 2. Horn NGF level in the AD lesions and in the normal skin of healthy control subjects. Before treatments, the level of horn NGF in the AD lesion was significantly higher than that in the normal skin of healthy controls ($p < 0.01$, Student *t*-test). The horny layer was collected via the tape stripping method from skin lesions found on the cubital fossa of AD patients ($n = 33$) and normal skin from the cubital fossa of healthy controls ($n = 35$). NGF and total protein levels of the samples were measured as described in Section 2.3. Horn NGF contents were corrected by total protein contents in the samples. Before treatments, the level of horn NGF in the AD lesion was significantly higher than that in the normal skin of healthy controls ($p < 0.01$, Student *t*-test).

3.4. Effects of treatment on clinical conditions and horn NGF in AD patients

Olopatadine and/or mild class topical corticosteroids were given to continuously treated 26 AD patients as mentioned in Section 2. Olopatadine was administered the 21 patients.

At 2 and 4 weeks of treatment, the itch severity [(b) pruritus scores], eruptions on the lesion [(c) erythema, (d) papule, (e) scale/xerosis, (f) erosion/crust, (g) lichenification and (h) total skin score] and (j) serum LDH level decreased significantly compared to values before treatment. The (i) eosinophil count also decreased after 4 weeks of treatment, although this was not significant. The (k) total serum IgE level remained constant before and after treatments (Fig. 4).

The horn NGF level in these patients decreased significantly at both 2 and 4 weeks of treatment, as compared to levels before treatment ($p < 0.01$, steel test) (Fig. 5). There was no significant difference in horn NGF levels, between the 2 and 4 weeks treatment timepoint.

Correlations between the decrease in horn NGF level and the improvement in clinical conditions were assessed at 4 weeks of treatment. The decrease in horn NGF correlated with the decrease in itch severity [(a) VAS, (b) pruritus scores], skin scores on the lesion from the cubital fossa [(c) erythema, (d) papule, (e) scale/xerosis, (f) lichenification and (g) total skin score], (h) eosinophil count, and (i) serum LDH level (Fig. 6, Spearman rank-order correlation test). Positive correlations were not observed at 4 weeks of treatment between the decrease in horn NGF and erosion/crust and total serum IgE level (data not shown).

3.5. Horn NGF level in psoriatic lesions

Horn NGF was measured in psoriatic patients with itch ($n = 33$) and without itch ($n = 34$). The horn NGF was significantly higher in

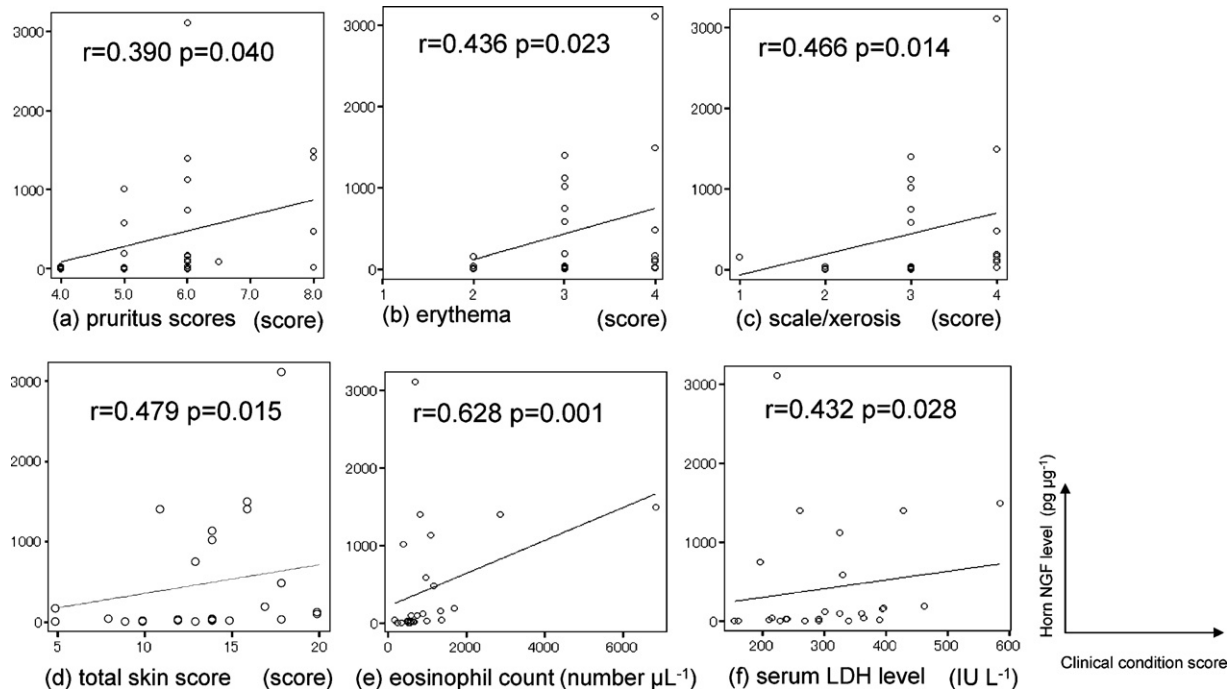


Fig. 3. Correlations of the horn NGF level and the clinical conditions. Patients with AD ($n = 33$) were evaluated for clinical conditions before treatments. The horn NGF levels were correlated with the severity of itch (the pruritus scores), eruptions of the measured lesion (erythema, scale/xerosis and total skin score), blood eosinophil count and serum LDH level (Spearman rank-order correlation test).

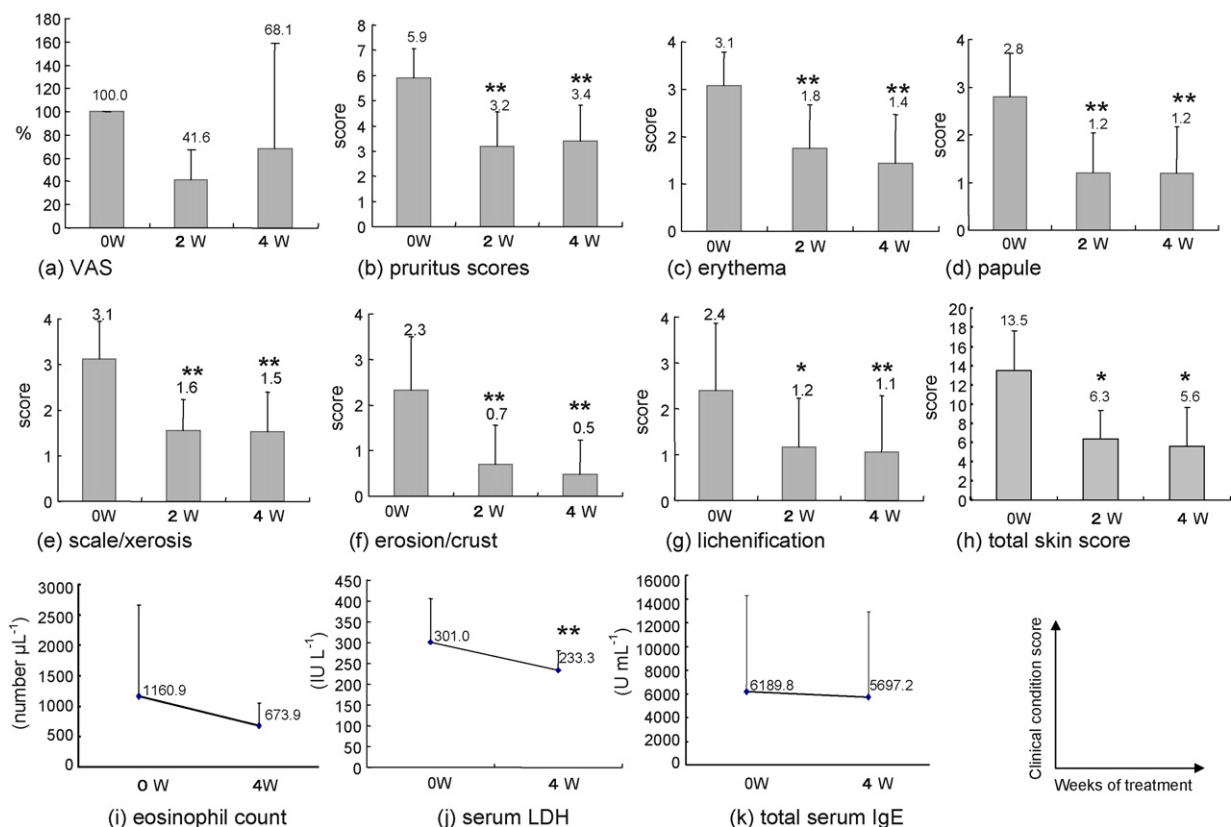


Fig. 4. Changes of clinical conditions with AD treatments. The changes of clinical conditions were evaluated in patients with AD ($n = 26$). At 2 and 4 weeks of treatment, the severity of itch (the pruritus scores), the severity of eruptions on the lesion from cubital fossa (erythema, papule, scale/xerosis, erosion/crust, lichenification and total skin score) and the serum LDH level significantly decreased compared to those before the treatments ($p < 0.05$, $**p < 0.01$, steel test).

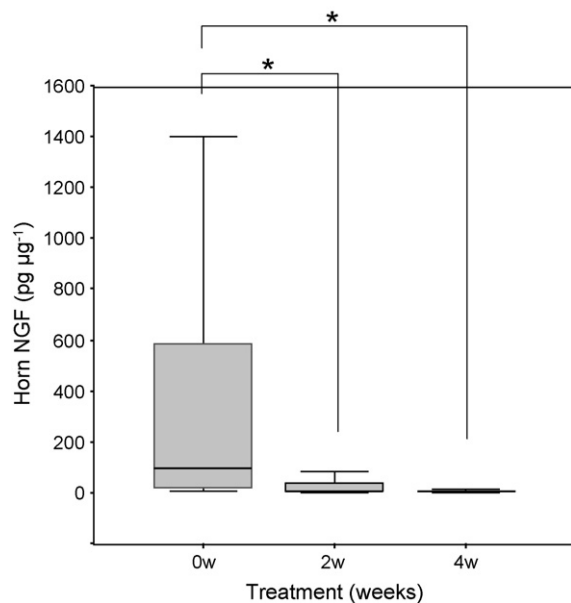


Fig. 5. Effects of treatments on the horn NGF in AD patients. Level of horn NGF was measured in AD patients ($n = 26$) who were treated with olopatadine ($n = 21$) and/or topical steroid for 4 weeks. The level of horn NGF decreased significantly as compared to before treatments ($p < 0.01$, steel test). It showed a significant decrease even after 2 weeks of treatment ($p < 0.01$, steel test). There were 26 patients at the initiation of treatment, 21 patients after 2 weeks of treatment and 18 patients after 4 weeks of treatment.

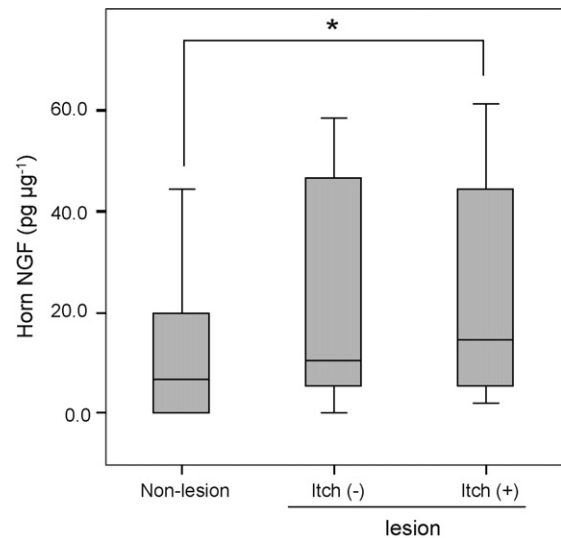


Fig. 7. Horn NGF level in psoriatic lesions. Horn NGF was measured in psoriatic patients with itch ($n = 33$) and without itch ($n = 34$). The horn NGF was significantly higher in lesional skin with itch than in non-lesional skin of psoriasis ($p < 0.05$, Tukey's test).

lesional skin with itch than in non-lesional skin of psoriasis ($p < 0.05$, Tukey's test), but the value was lower than NGF in atopic skin (Fig. 7).

4. Discussion

Neurotrophic factors in human keratinocytes include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3),

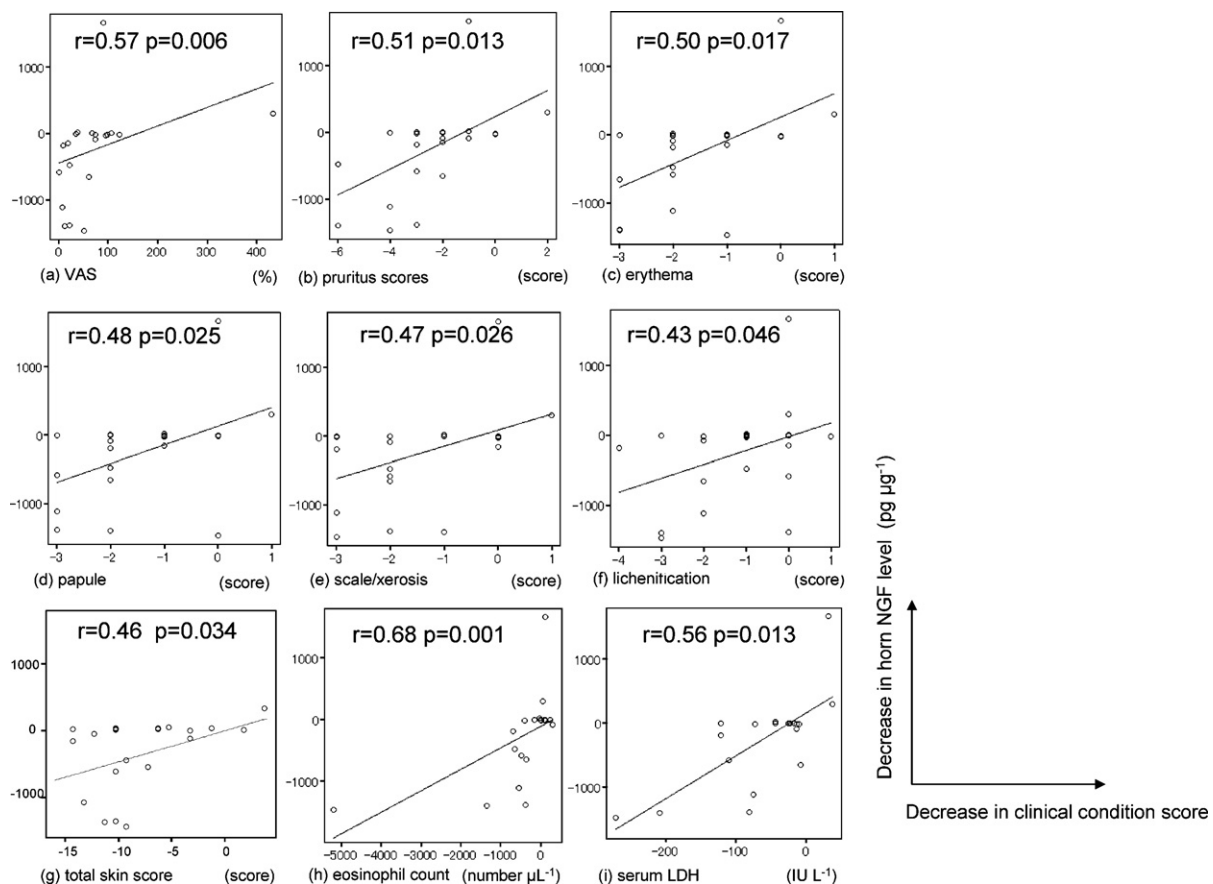


Fig. 6. Correlations between the decrease in horn NGF level and the improvement in clinical conditions. The decrease of horn NGF after 4 weeks treatments with olopatadine and/or topical steroid correlated with the decrease of itch (VAS, the pruritus scores), skin scores (erythema, papule, scale/xerosis, lichenification and total skin score) on the lesion from the cubital fossa, eosinophil count and the serum LDH level (Spearman rank-order correlation test).

and NT-4/5 [7]. Above all, high amounts of biologically active NGF are synthesized and released by human keratinocytes [9]. NGF is important for the growth and preservation of peripheral sensory and autonomic nerves [9]. In addition to keratinocytes, NGF is produced by and secreted from human fibroblasts [19] and Th2 lymphocytes [20], and Merkel's cells in rat [21]. Increased NGF expression in the epidermis, as well as intraepidermal nerve growth, has previously been observed in AD lesions, psoriasis, asteatotic eczema, and allergic contact dermatitis [8,14,22–24].

In addition to its actions on nerve fibers, NGF exerts various biological activities against cells contributing to immune/inflammatory reactions (e.g., mast cells, T cells, B cells, macrophages and basophils), and recently has been recognized as a cytokine [20,25–27].

NGF-transgenic mice displayed hypertrophic sensory ganglia, along with increased skin innervation [28]. Skin-scratching behavior dramatically induced the sprouting of cutaneous nerve fibers, and NGF in the scratched skin increased immediately [29]. Therefore, it is believed that NGF secreted from keratinocytes by stimulation (e.g., scratching) binds with high-affinity NGF receptors (Trk A) on sensory nerve fibers in the epidermis–dermis boundary area, causing the growth of epidermal nerve fibers. It is also believed that persistently elevated secretion of NGF contributes to the exacerbation of AD via epidermal hypertrophy caused by keratinocyte proliferation [11,30], proliferation of human peripheral blood lymphocytes [31,32], and synthesis of neuropeptides [29,33,34]. Furthermore, given the finding that the presence of intraepidermal mast cells is characteristic of AD [35,36], topical NGF produced in AD lesions is expected to induce the activation of mast cells and exacerbate itching.

A recent study reported that anti-NGF antibodies significantly inhibited the development of skin lesions and epidermal innervation, and significantly inhibited an increase in scratching in NC/Nga mice, an atopic mouse model [37,38]. These findings also suggest that NGF plays an important role in the aggravation of AD.

In present study, we measured the horn NGF level in AD patients and assessed the possible relationship with itch severity and eruptions, and blood examination data, as well as the effects of treatment. Due to the use of samples collected directly from the horny layer, this data reflects the topical NGF level in the skin more accurately than previous data, collected using methods that measured NGF levels in the blood, urine, and saliva [8,13]. For reference, NGF levels in AD patients were roughly 2300 pg mL⁻¹ in the plasma [8], 300 pg mL⁻¹ in the saliva [13], and 40 ng mg⁻¹ (NGF creatinine⁻¹) in the urine [13].

Before treatment, the NGF level was significantly higher in AD lesions than in the normal skin of healthy controls (Fig. 2). This is consistent with the previous finding shown by histochemical staining that NGF expression in keratinocytes is increased in AD patients. Horn NGF correlated with itch severity, eruptions (erythema, scale/xerosis and total skin score) on the measured lesional skin, eosinophil count, and serum LDH level (Fig. 3). The NGF level decreased significantly with treatment (Fig. 5), which correlated with the decrease in itch severity, skin scores (erythema, papule, scale/xerosis, lichenification and total skin score) on measured lesional skin, serum LDH level, and especially eosinophil count (Fig. 6). These findings indicate that the horn NGF level reflects to some extent the severity of itch, eruptions, and laboratory data.

The level of NGF had decreased significantly not only at 4 weeks, but also at 2 weeks after beginning treatment (Fig. 5). Considering the fact that the turnover of the horny layer is approximately 14 days, it is suggested that epidermal NGF production is suppressed immediately after beginning treatment. Taken together, the horn NGF level can serve as a marker to evaluate the clinical conditions of AD and the immediate effect of treatment.

Olopatadine hydrochloride is a selective histamine H₁ receptor antagonist possessing inhibitory effects on the release of inflammatory lipid mediators (e.g., leukotriene, thromboxane) from human polymorphonuclear leukocytes and eosinophils. Olopatadine also inhibits tachykinergic contractions in guinea pig bronchi by prejunctional inhibition of peripheral sensory nerves [39]. It has been reported that histamine enhances NGF secretion in human keratinocyte via H₁ receptors *in vivo* [40]. This result suggests that histamine H₁-antagonist may be able to depress the production of NGF. Additionally, we have already reported that olopatadine suppresses increased substance P levels in skin lesions induced by the repeated application of 2,4,6-trinitrochlorobenzene in DS-Nh mouse, an atopic mouse model [41]. Olopatadine also significantly decreased the serum substance P level in patients with AD because of downmodulatory effects on tachykinin release [42]. Results of several *in vivo* and *in vitro* studies have shown that while NGF stimulates the synthesis and release of substance P in the central and peripheral nervous system, substance P induces NGF synthesis via cytokines [34,43]. This suggests that the activations of NGF and substance P are co-dependent. Moreover, in studies of BALB/c mice with chronic contact hypersensitivity induced by oxazolone, it was reported that oral administration of olopatadine suppressed the production of NGF and cytokines such as IL-4, IL-1 β , IL-6, and GM-CSF in affected aural tissues, unlike other histamine H₁ receptor antagonists [16,44,45].

It is well known that topical corticosteroids exert anti-inflammatory, antiproliferative, immunosuppressive, and vasoconstrictive effects for AD [46]. In addition, topical prednisolone significantly inhibited the increased levels of NGF and substance P as well as IL-4, IL-1 β and GM-CSF in the mouse model of chronic inflammatory dermatitis [45]. Taken together with our present results and these previous data, it is suggested that olopatadine and topical steroid exert their effect on AD not only by its anti-inflammatory function but also by suppressing the production of NGF and substance P. Further study is needed to clarify the mechanism of effects on AD.

In conclusion, we show here that the NGF level in the horny layer reflects the severity of itch and eruptions in AD. Therefore, quantification of the NGF in the samples collected directly from the horny layer appears to be useful in assessing severity and therapeutic effects in AD.

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