CCR7 in DCs is not sufficient to guarantee its functionality (Sanchez-Sanchez *et al.*, 2006); therefore, it is possible that iNOS alters certain downstream functions of LCs without affecting CCR7 expression.

iNOS inhibitor reduces LPS-induced apoptosis of LC

We then evaluated the effect of endogenous iNOS activity on the viability of LCs. EC suspensions from the earlobes of B6 mice were cultured for 9 hours with or without LPS in the presence or absence of L-NIL, an iNOS inhibitor. LPS stimulation reduced the number of LCs, but this reduction was reversed by the addition of the iNOS inhibitor (Figure 6a). It has been reported that epidermal LCs are unable to proliferate *in vitro* when they are incubated as an EC suspension (Schuler and Steinman, 1985), suggesting that the observed effects of the iNOS inhibitor stem from a survival change.

To examine whether the iNOS inhibitor promotes the survival of LCs, cellular viability was assessed through flow cytometry after Annexin V/propidium iodide staining and 9 hours of culture (Figure 6b). This flow cytometry experiment used anti-MHC class II and anti-CD11c mAbs. The percentage of Annexin V and propidium iodide double-positive cells in samples that had been treated with 100 μM iNOS inhibitor and those that had not was as follows: 100 μM, 1.6 ± 0.4%; no addition, 2.2 ± 1.0% (mean ± SD, $n = 3$). These results suggest that the reduction of apoptotic cells that occurs through iNOS inhibitor treatment is not due to the increment of necrotic cells. We found that LPS-induced apoptosis of LCs was reduced by the addition of the iNOS inhibitor (Figure 6c), suggesting that the iNOS inhibitor promotes DC survival.

DISCUSSION

The results of this study on the effects of an iNOS inhibitor include several major findings about the involvement of NO in the sensitization phase of CHS. First, CHS as a model of acquired skin immune response was enhanced by treatment with the iNOS inhibitor. Second, the iNOS inhibitor markedly increased the number of migrating cutaneous DCs. Accordingly, the chemotactic response of LCs to CCL21 was enhanced by *in vitro* incubation with the iNOS inhibitor. Finally, the iNOS inhibitor was capable of reducing LPS-induced apoptosis of LCs.

It has generally been believed that iNOS is involved in CHS as a producer of NO and a trigger of inflammatory responses (Cals-Grierson and Ormerod, 2004). It has been

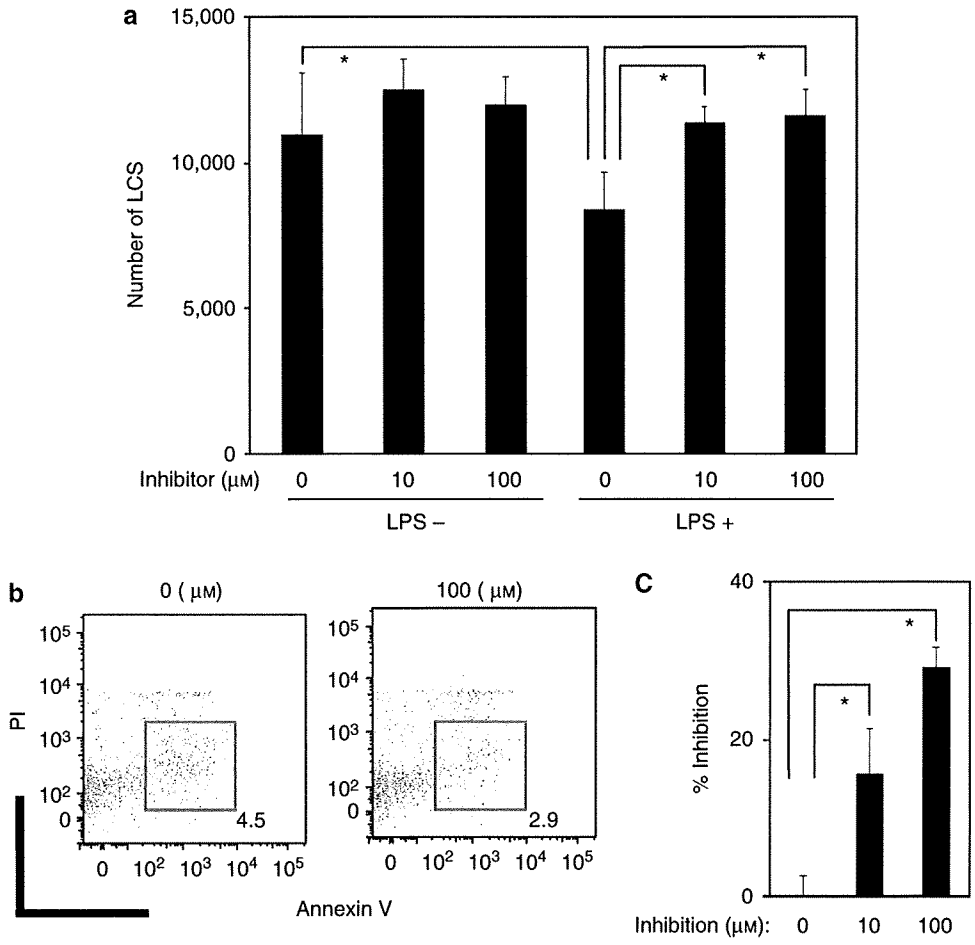

Figure 6. The effect of LPS on LC survival. (**a**) EC suspensions were cultured with or without LPS and iNOS inhibitor for 9–12 hours. iNOS inhibitor dose dependently increased the number of LCs. Columns show the mean ± SD. *$P<0.05$. Data are representative of three independent experiments. (**b**) Apoptosis was determined using annexin V/propidium iodide double staining. (**c**) The percentage inhibition is calculated. Data represent the mean ± SD. *$P<0.05$.

reported that iNOS and NO were produced in human skin subjected to positive patch tests to contact allergens (Cruz *et al.*, 2007; Ormerod *et al.*, 1997) and to the irritant sodium lauryl sulfate; nevertheless, it remains controversial whether iNOS is inhibitory or augmentative in CHS (Ross and Reske-Kunz, 2001). It has also been reported that an iNOS inhibitor exerted a suppressive effect on the CHS response to 2,4,6-trinitrochlorobenzene (TNCB) (Musoh *et al.*, 1998), although this effect was limited to the first few hours of the response, and neither NO production, NO-expressing cells, nor NOS isoenzymes were identified. Thus, the mode of action of iNOS in CHS remains a matter of debate.

It is possible that iNOS first modulates keratinocytes so that they produce cytokines, thereby subsequently modifying LC function. Yet, we found that the production levels of GM-CSF and tumor necrosis factor-α in the culture supernatant of primary keratinocytes of B6 mice cultured for 72 hours were not significantly affected by the presence of the iNOS inhibitor (Supplementary Figure S2). With regard to the effect of iNOS on T cells, we cultured immune $CD4^+$ T cells for 72 hours with varying concentrations of the iNOS inhibitor in the presence of anti-CD3 mAb and found that the iNOS inhibitor was incapable of stimulating T cells *per se* (Supplementary Figure S3).

LCs have traditionally been believed to have a role in the induction of CHS, but three research groups have reported three contradictory findings after applying haptens to transgenic mice deficient in LCs: a diminished reaction (Bennett *et al.*, 2005), an enhanced reaction (Kaplan *et al.*, 2005), and an unchanged response (Kissenpfennig *et al.*, 2005). Moreover, recent findings suggest that dDCs has a critical role in initiating CHS (Fukunaga *et al.*, 2008). In our study, dDCs augmented iNOS expression in response to hapten application more than LCs did. Our findings suggest that iNOS can suppress cutaneous DC migration and survival. Given that, in CHS, dDCs and LCs have positive and regulatory capacities, respectively, our findings on cutaneous DCs seem to be consistent with the observation that iNOS inhibitor induces an enhancement of CHS.

The findings of our study are clinically relevant in two respects. First, iNOS and NO exert immunosuppressive effects on cutaneous inflammation. In this context, the *in vivo* immunosuppressive effect of NO has also been shown in human studies (Kuchel *et al.*, 2003). Second, iNOS reduces cutaneous DC function and survival in the sensitization phase of CHS. The observation that NO directly reduces the number of LCs in the human epidermis supports our conclusion (Mowbray *et al.*, 2008).

MATERIALS AND METHODS

Animals and reagents

Female B6 mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were conducted on 8-week-old mice. The mice were maintained on a 12-hour light/dark cycle under a specific pathogen-free condition. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health. L-NIL (a highly selective inhibitor of iNOS enzymatic activity) and LPS were obtained from Sigma-Aldrich (St Louis, MO). CCL21 was purchased from R&D Systems (Minneapolis, MN).

DNFB-induced CHS model

B6 mice were sensitized through the application of 25 μl of 0.5% (v/v) DNFB in 4:1 acetone/olive oil to their shaved abdomens on day 0. They were then challenged on both sides of each ear with 20 μl of 0.3% (v/v) DNFB. Ear thickness change was calculated as follows: (ear thickness 24 or 48 hours after challenge)−(ear thickness before challenge). iNOS was inhibited with L-NIL as described previously (Diefenbach *et al.*, 1998). Briefly, L-NIL was applied by intraperitoneal injection (2.5 mg in 0.5 ml PBS twice daily) for 6 consecutive days starting 1 day before sensitization. We chose his protocol because treatment with L-NIL at this concentration and frequency for 4-13 days is one of the most common methods of blocking *in vivo* activity of iNOS (Diefenbach *et al.*, 1998; Stallmeyer *et al.*, 1999).

EC preparation and culture

EC suspensions were prepared as described previously (Tokura *et al.*, 1994). Ears of naive mice were split along the plane of the cartilage, which was then removed together with the subcutaneous tissue. These specimens were incubated for 1 hour at 37 °C in a 0.2% solution of trypsin in PBS. After incubation, the epidermis was separated from the dermis and the separated epidermal sheets were rubbed to disperse the ECs in PBS supplemented with 10% fetal calf serum. The cells were filtered and washed twice in PBS. As a culture medium, RPMI-1640 (Sigma-Aldrich) was supplemented with 10% heat-inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

Preparation of dermal cell suspensions

Dermal cells were obtained from normal murine skin from which the epidermis had been removed. Samples were minced and incubated for 2 hours at 37 °C in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with collagenase (2 mg ml^{-1}; Sigma-Aldrich), hyaluronidase (260 U ml^{-1}; Sigma-Aldrich), DNase (0.1 mg ml^{-1}; ICN, Costa Mesa, CA), and 10 mM HEPES (Sigma-Aldrich). The obtained cells were filtered through a 40-μm filter.

Flow cytometry

For flow cytometry, cells were plated at a density of 1×10^6 cells per well in 96-well U-bottomed plates (Falcon, BD Biosciences, San Jose, CA). Cells were then stained for 20 minutes on ice with mAbs in 25 μl of PBS containing 2% fetal calf serum, 1 mM EDTA, and 0.1% NaN_3, and washed twice with 200 μl of this buffer. Data were collected on a FACSCanto system (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, CA). The mAbs used were as follows: FITC-conjugated anti-CD86 and Annexin V mAbs, PE-conjugated anti-CD80 and CD40 mAbs, PE-Cy5-conjugated anti-MHC class II mAb, APC-conjugated anti-CD11c mAb (all from BD Biosciences), and PE-Cy7-conjugated anti-CCR7 mAb (eBioscience, San Diego, CA). For detection of Langerin and iNOS, anti-Langerin Ab (eBioscience), PE-conjugated anti-iNOS Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and PE-Cy5-conjugated streptavidin were

used after fixation and permeabilization of cells using a Cytofix/Cytoperm Kit (BD Biosciences).

Histology

At 48 hours after the challenge with hapten, the ears of B6 mice were excised and fixed in 10% formaldehyde. Sections of 5-μm thickness were prepared and stained with hematoxylin and eosin.

FITC-induced cutaneous DC migration

The shaved abdomens of the mice were painted with 200 μl of 2% FITC (Sigma-Aldrich) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma-Aldrich) mixture, and the iNOS inhibitor was applied through intraperitoneal injection (2.5 mg in 0.5 ml PBS) twice daily for 4 days. Cutaneous DCs migrating into the draining inguinal and axillary lymph nodes were then counted by means of flow cytometry (Kabashima *et al.*, 2007) using Flow-Count Fluorospheres (Beckman Coulter, Fullerton, CA). The principle of Flow-Count Fluorospheres is based on the precise mixing of microparticles whose concentration and volume are known. Before flow cytometric analysis, 10 μl of Flow-Count Fluorospheres were added to each specimen. The percentages of fluorospheres and migrating DCs within each node were then determined using the FACSCanto system (BD Biosciences). To find the number of migrating DCs, the ratio of DCs to fluorospheres was counted using the following formula, based on Reimann *et al.* (2000), with some modifications: number of migrating DCs = (percentage of migrating DCs/percentage of fluorospheres) × number of fluorospheres.

Chemotaxis assay

EC suspensions were incubated for 9 hours with or without the iNOS inhibitor, and then tested for transmigration across uncoated 5-μm transwell filters (Corning Costar, Corning, NY) to CCL21 or medium in the lower chamber for 3 hours. Migrating cells were enumerated by means of flow cytometry (Ngo *et al.*, 1998). The medium used in this assay was RPMI-1640 with 0.5% fatty acid-free bovine serum albumin (Calbiochem, San Diego, CA).

Apoptosis analysis

The EC suspensions from B6 mice were stained with PE-Cy5-conjugated anti-MHC class II mAb for 20 minutes on ice, then stained with FITC-conjugated Annexin V and propidium iodide (BD Pharmingen, Franklin Lakes, NJ), according to the manufacturer's protocol. The number of LCs was assessed by means of flow cytometry with anti-MHC class II and APC-conjugated anti-CD11c mAbs. Apoptosis in LCs was analyzed using a FACSCanto system with FlowJo software.

Statistical analysis

Data were analyzed using an unpaired two-tailed *t*-test. $P<0.05$ was considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Comparison of skin barrier function and sensory nerve electric current perception threshold between IgE-high extrinsic and IgE-normal intrinsic types of atopic dermatitis

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Summary

Background Two types of atopic dermatitis (AD) have been proposed with different pathophysiological mechanisms underlying this seemingly heterogeneous disorder. The extrinsic type shows high IgE levels presumably as a consequence of skin barrier damage and feasible allergen permeation, whereas the intrinsic type exhibits normal IgE levels and is not mediated by allergen-specific IgE.

Objectives To investigate the relationship between pruritus perception threshold and skin barrier function of patients with AD in a comparison between the extrinsic and intrinsic types.

Methods Enrolled in this study were 32 patients with extrinsic AD, 17 with intrinsic AD and 24 healthy individuals. The barrier function of the stratum corneum was assessed by skin surface hydration and transepidermal water loss (TEWL), and pruritus perception was evaluated by the electric current perception threshold (CPT) of sensory nerves upon neuroselective transcutaneous electric stimulation.

Results Skin surface hydration was significantly lower and TEWL was significantly higher in extrinsic AD than intrinsic AD or normal controls. Although there was no statistically significant difference in CPT among extrinsic AD, intrinsic AD and normal controls, CPT was significantly correlated with skin surface hydration and inversely with TEWL in intrinsic AD and normal controls, but not extrinsic AD. Finally, CPT was correlated with the visual analogue scale of itch in the non-lesional skin of patients with extrinsic but not intrinsic AD.

Conclusions Patients with extrinsic AD have an impaired barrier, which increases the pre-existing pruritus but rather decreases sensitivity to external stimuli. In contrast, patients with intrinsic AD retain a normal barrier function and sensory reactivity to external pruritic stimuli.

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electric current perception threshold, extrinsic atopic dermatitis, intrinsic atopic dermatitis, skin barrier function

Conflicts of interest
None declared.

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Atopic dermatitis (AD) is a chronic inflammatory skin disease with complicated pathophysiological mechanisms and causative agents. Two subtypes of AD have been proposed: extrinsic AD and intrinsic AD. The extrinsic type is the IgE-mediated common form of AD and is associated with respiratory allergies, such as rhinitis and asthma, and high serum levels of IgE.[1–3] In contrast, intrinsic AD is characterized by the absence of allergen-specific IgE and thus shows normal total IgE levels, although this newly introduced concept is still controversial among academic dermatologists.[1–3] Approximately 20%[4] or fewer[5] patients are estimated as having intrinsic AD. Its characteristics include female predominance, absence of atopic diseases, later onset of disease, and milder disease severity.[3–6] A history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, and hand and/or foot eczema are significantly less present in the intrinsic type, but Dennie-Morgan fold is positively associated with intrinsic AD.[3]

Several studies have suggested differences in various aspects of pathophysiology between extrinsic and intrinsic AD.

Increased transepidermal water loss (TEWL) and reduced skin surface hydration are hallmarks of atopic skin, and there are some differences in these values between the two types of AD.[4] Immunologically, surface expression of the high- and low-affinity receptor for IgE and of the interleukin (IL)-4Rα chain is elevated in monocytes from patients with extrinsic AD, but serum levels of IL-13 are significantly increased in patients with intrinsic AD.[7] Skin lesions of extrinsic AD show high levels of chemokines such as CCL18.[8] Expression of neurotrophins is increased comparably in both types.[6]

The stratum corneum of the epidermis, consisting of more than 10 layers of corneocytes and intercellular lipids, serves as the skin barrier.[9] In extrinsic AD, impairment of the skin barrier may be the primary condition which facilitates permeation of environmental allergens and leads to immunological responses such as elevation of allergen-specific IgE.[1] A recent finding of filaggrin gene mutations in a high percentage of patients with AD,[10,11] together with an older finding of ceramide reduction in the stratum corneum,[12,13] have further suggested the presence of skin barrier damage in extrinsic AD. On the other hand, intrinsic AD shows normal or mildly elevated serum IgE, in striking contrast to extrinsic AD.[14] The mechanisms underlying intrinsic AD remain unclear and more speculative than those underlying extrinsic AD.[2,6,8,15]

Patients with AD are well known to be sensitive to irritation from the environment due to the impaired skin barrier function. Given that the extrinsic and intrinsic types are different from each other in the skin barrier condition, each type might respond to external stimuli in a different manner. However, little is known regarding the difference in sensitivity to irritants and in elicitability of pruritus between the two types. It appears that most previous studies on sensitivity were performed in patients with extrinsic AD because of its higher incidence.

There are several reported methods to assess the threshold for the itch sensation to various environmental stimuli.[16–18] Local administration of histamine, either by needle injection or by iontophoresis, is one of the most common procedures for this purpose.[19] Electrically evoked itch is another useful method with the use of a neuroselective transcutaneous electrical stimulator, Neurometer™ CPT/C (Neurotron Inc., Baltimore, MD, U.S.A.), in a noninvasive fashion. Evaluation of electric current perception threshold (CPT) quantifies the sensory threshold to electric stimulation of the sensory nerves.[20,21] This device has been used mainly by neurologists to demonstrate abnormalities in a variety of neuropathic conditions. It does not measure the sensation only to histamine, but the device directly excites large- and small-diameter sensory nerve fibres in a differentiating fashion, independent of local factors such as skin thickness, temperature and substances involved in the induction of pruritus.[20,22] The CPT for 250- and 5-Hz frequency current emitted by the Neurometer CPT/C has been reported to enable quantification of the sensory threshold of Aδ- and C-fibres, respectively, that are thought to transmit the itch sensation from the skin. Therefore, this instrument allows us to investigate the elicitability of pruritus in patients with AD. Kobayashi *et al.*[23] have reported that patients with AD showed lower CPT than healthy controls, and CPT was inversely correlated with TEWL after tape stripping in normal subjects.

To address the differences in the mechanisms between extrinsic and intrinsic AD, we measured CPT in patients with AD, together with measurements of stratum corneum function. Our results show that there are prominent differences in the relationship of CPT with the skin barrier function between the two types.

Materials and methods

Participants

Patients over 18 years of age from our department were included in this study. Forty-nine patients with AD (25 men and 24 women), diagnosed in accordance with the Hanifin and Rajka classification,[24] and 24 healthy controls were enrolled in this study. The distribution of skin symptoms in all patients was characterized for adult AD. The hands, shoulders, neck, flexures and face were the predilection sites, while the extremities were less involved. Patients who had total serum IgE levels < 220 U mL^{-1} (normal range for Japanese subjects) were classified as having intrinsic AD, and those with levels > 400 U mL^{-1} were classified as having extrinsic AD. There was no patient with IgE levels between 220 and 400 U mL^{-1}. IgE RAST for *Dermatophagoides pteronyssinus* was measured in 20 patients. The disease activity was assessed by SCORAD (severity scoring of AD). Patient details are listed in (Table 1). All participants provided written informed consent, and the institutional review board approved this study.

Electric current perception threshold and stratum corneum function

C-fibres are sensory nerves conducting itch and pain. Transcutaneous electric current with 5-Hz sine wave stimulates C-fibres, as assessed by the active action potentials of rat dorsal root ganglia.[21,25] Depending on the body surface site, 5-Hz electric current induces itch and/or pain. In addition, transcutaneous 250-Hz current has also been known to stimulate Aδ-fibres, thus inducing itch.[25] In this direct stimulation of nerve fibres with transcutaneous electric current, the condition of the stratum corneum possibly modifies the perception by affecting the current or other factors.

We measured CPT, skin surface hydration and TEWL at the nonlesional flexor forearm, the nonlesional lower leg, and at lesional skin on the trunk or extremities. When the patients had skin lesions on the flexor forearms or lower legs we avoided these regions and chose clinically normal areas on the volar skin as the sites to perform measurements on nonlesional skin. The patients did not apply any ointment or cream to the examined sites for at least 2 days before the measurements. Concerning the inflammatory state at the clinically normal sites tested, we have previously demonstrated

Table 1 Patients and healthy individuals enrolled in this study

	Extrinsic AD	Intrinsic AD	Healthy controls
Number of subjects	32 (21 men and 11 women)	17 (four men and 13 women)	24 (nine men and 15 women)
Age (years), mean ± SD (range)	30·0 ± 8·1 (19–51)	33·0 ± 10·4 (18–57)	28·9 ± 3·8 (23–37)
IgE (U mL^{-1}), mean ± SD (range)	5034·8 ± 7538·0 (436–30 000)	110·5 ± 66·8 (11–219)	–
SCORAD, mean ± SD (range)	41·8 ± 19·0 (4·6–84·5)	27·1 ± 20·6 (3·5–73)	–
VAS (nonlesional forearm), mean ± SD	30·9 ± 20·6	15·8 ± 22·1	–
VAS (nonlesional lower leg), mean ± SD	36·0 ± 24·9	20·4 ± 28·5	–
VAS (lesional skin), mean ± SD	55·3 ± 28·3	47·3 ± 36·0	–

AD, atopic dermatitis; VAS, visual analogue scale.

that clinically normal-appearing skin of patients with AD has no histological evidence of inflammation.[26] As control, we measured CPT, skin surface hydration and TEWL on the mid-flexor forearm and lower leg in healthy individuals. CPT was measured by using the Neurometer CPT/C as described previously.[23] Skin surface hydration was evaluated by capacitance using the Corneometer CM825 (Courage & Khazaka Electronic GmbH, Cologne, Germany) and was expressed as arbitrary units.[27] TEWL was measured by detecting the evaporated water using the VapoMeter SWL-2 (Delfin Technologies Ltd, Kuopio, Finland).

Visual analogue scale

All patients rated current itching on a 100-mm visual analogue scale (VAS)[28] at the following sites: nonlesional forearm, nonlesional lower leg and lesional skin.

Statistical analyses

Data were expressed as mean ± SD and assessed for statistical significance. We used Student's t-test to compare skin surface hydration, TEWL and CPT. A linear regression analysis was performed for correlations between the skin surface hydration or VAS and CPT, using Peason's correlation coefficient. For all tests, $P < 0{\cdot}05$ was considered statistically significant.

Results

Impaired skin barrier function in extrinsic but not intrinsic atopic dermatitis

Patients were classified as having extrinsic or intrinsic AD by means of IgE level (> 400 and < 220 U mL^{-1}, respectively). IgE RAST was scored by index values 0–6 according to the manufacturer's criteria (BML, Tokyo, Japan). An index value > 3 to *D. pteronyssinus* was obtained in 11 of 12 (92%) patients with extrinsic AD, but in only one of eight (12·5%) patients with intrinsic AD. Moreover, 67% of the patients with extrinsic AD showed a RAST score index value of 6, and none of the patients with intrinsic AD showed this highest score. As summarized in (Table 1), more patients had extrinsic AD than intrinsic AD, and women predominated in the intrinsic type, as previously reported.[3–5] No significant difference was noted in age between the two types. There was a tendency that SCORAD and VAS at the three test sites were higher in extrinsic than intrinsic AD, as reported previously.[3–5]

As extrinsic AD is caused by external allergens invading through the damaged skin barrier, we initially examined the skin surface hydration (capacitance) and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between extrinsic and intrinsic AD. Skin surface hydration was significantly lower in extrinsic AD than in normal control subjects (Fig. 1). There was no significant difference in the hydration level between intrinsic AD and healthy controls. Extrinsic AD tended to show lower values than intrinsic AD at both sites. TEWL, another assessment of the barrier function, was statistically higher in extrinsic AD than intrinsic AD and normal controls at the nonlesional forearm (Fig. 1). Thus, the skin barrier function was impaired in extrinsic AD and preserved in intrinsic AD, validating this clinical dichotomy.

Correlation between disease severity and pruritus in both extrinsic and intrinsic atopic dermatitis

In advance of analysing CPT, we also examined the correlation between the itch levels and SCORAD in the two types of AD. In both types, VAS scores on the lesional skin were correlated with SCORAD (Fig. 2), suggesting that both types of AD are associated with disease severity-dependent pruritus.

Significant correlation between electric current perception threshold (CPT) and skin surface hydration and between CPT and transepidermal water loss in intrinsic atopic dermatitis as well as in normal individuals

CPT for 5- and 250-Hz current stimuli was measured in patients with AD and normal volunteers. In all experiments, the results from 5- and 250-Hz current stimuli were virtually the same. Figure 3 shows the mean ± SD CPT in each group, and there was no significant difference between the groups in the nonlesional or lesional skin.

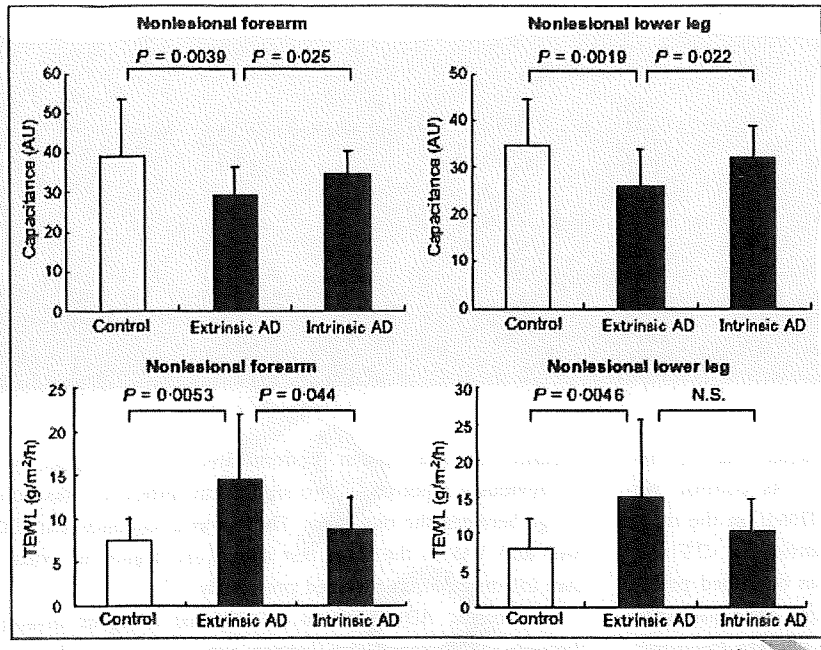

Fig 1. Skin surface hydration on nonlesional forearm and lower leg in extrinsic and intrinsic atopic dermatitis (AD) and healthy controls. Skin surface hydration is represented by capacitance in arbitrary units (AU). Results are shown as mean ± SD. N.S., not significant.

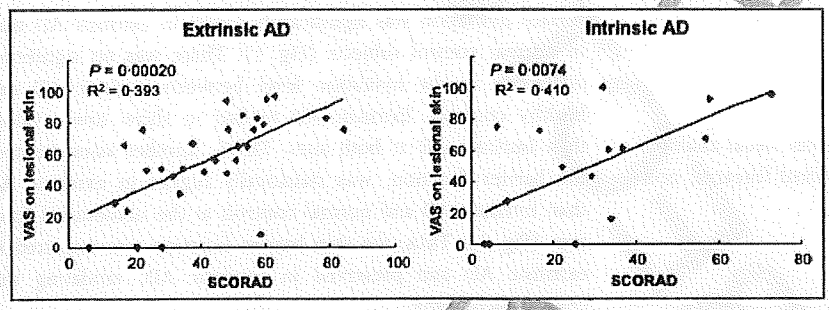

Fig 2. Relationship between SCORAD and visual analogue scale (VAS) score in extrinsic and intrinsic atopic dermatitis (AD).

When CPT was analysed in relation to skin surface hydration, an interesting finding was obtained. In normal subjects (control), CPT was significantly correlated with skin surface hydration (Fig. 4), suggesting that the water-poor cornified layer has a property to evoke pruritus in response to external stimuli. Similarly, the lesional skin of patients with intrinsic AD showed such a significant correlation between CPT and skin surface hydration. However, there was no correlation in extrinsic AD, as large individual variations of CPT were seen in the patients with extrinsic AD and low levels of skin surface hydration.

As to the relation of CPT to TEWL, there was no significant correlation between these two parameters in the lesional skin of patients with AD. However, CPT of nonlesional forearm, as assessed by 250-Hz sensitivity, was inversely correlated with TEWL in intrinsic AD as well as in controls (Fig. 5). The results suggest that intrinsic AD is associated with a normal stratum corneum and no excess elicitability of externally stimulated pruritus, while extrinsic AD does not show such a regular, surface hydration-related irritant perception.

Different electric current perception threshold levels in relation to pre-existing pruritus between extrinsic and intrinsic atopic dermatitis

It is possible that CPT is affected by the itch state in patients with AD. We therefore investigated the relationship between CPT and the pre-existing pruritus assessed by VAS. In the lesional skin of both types of AD there was no correlation between CPT and VAS (data not shown). In the nonlesional lower leg, however, CPT was significantly correlated with VAS in extrinsic but not intrinsic AD (Fig. 6), suggesting that the pre-existing pruritus rather downmodulates the sensitivity to external stimuli in extrinsic AD. The nonlesional forearm exhibited the same tendency but without statistical significance. Thus, the pruritic normal-appearing skin seems to be insensitive to further itchy stimuli in extrinsic AD.

Discussion

The precise concept of intrinsic AD in comparison with extrinsic AD has been a matter of controversy. Extrinsic AD seems

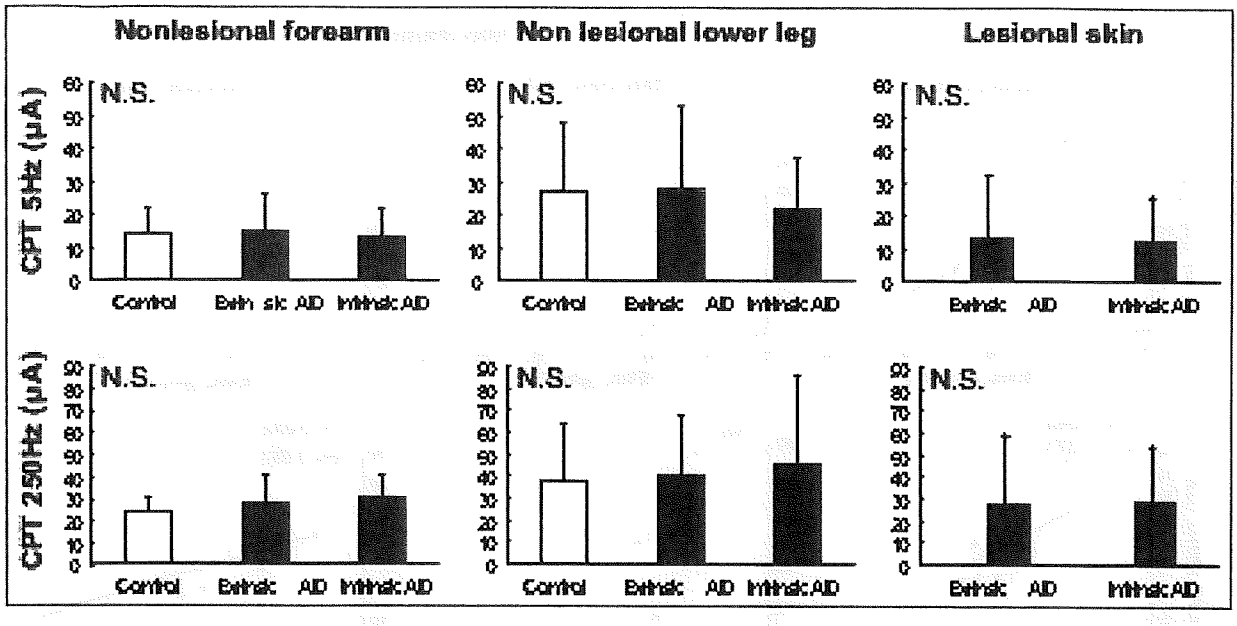

Fig 3. Electric current perception threshold (CPT) on nonlesional forearm or lower leg and lesional skin in extrinsic and intrinsic atopic dermatitis (AD). Results are shown as mean ± SD. N.S., not significant.

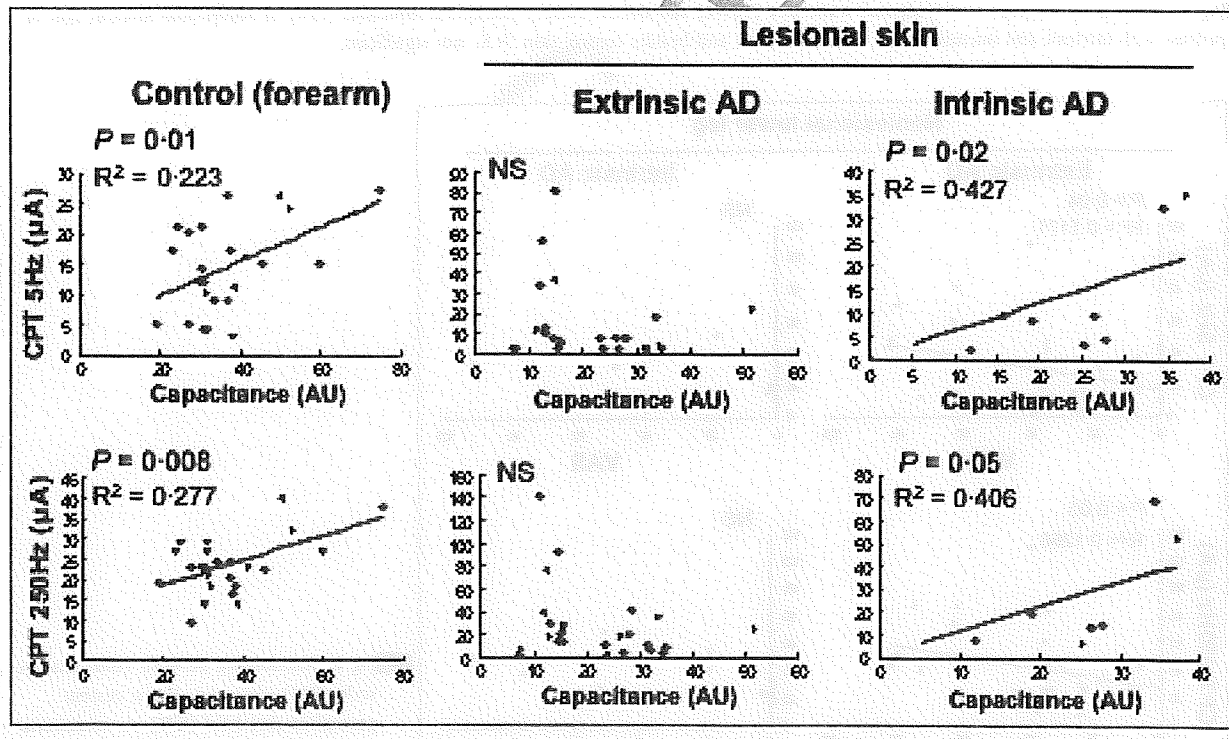

Fig 4. Relationship between skin surface hydration, represented by capacitance in arbitrary units (AU), and electric current perception threshold (CPT) in lesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and in nonlesional forearm skin of healthy controls. N.S., not significant.

to be induced by sequential events, including impairment of stratum corneum, permeation of external substances, exposure of immunocompetent cells to the allergens, and T cell and IgE responses to the antigenic determinants.[1] Growing evidence has supported this mechanism underlying the extrinsic type. The recent finding that filaggrin gene mutations are a predisposing factor for AD has clearly demonstrated the presence of barrier impairment in patients with AD.[10,11] On the other hand, the pathophysiology of intrinsic AD remains obscure, and it may be difficult for clinicians to differentiate the two

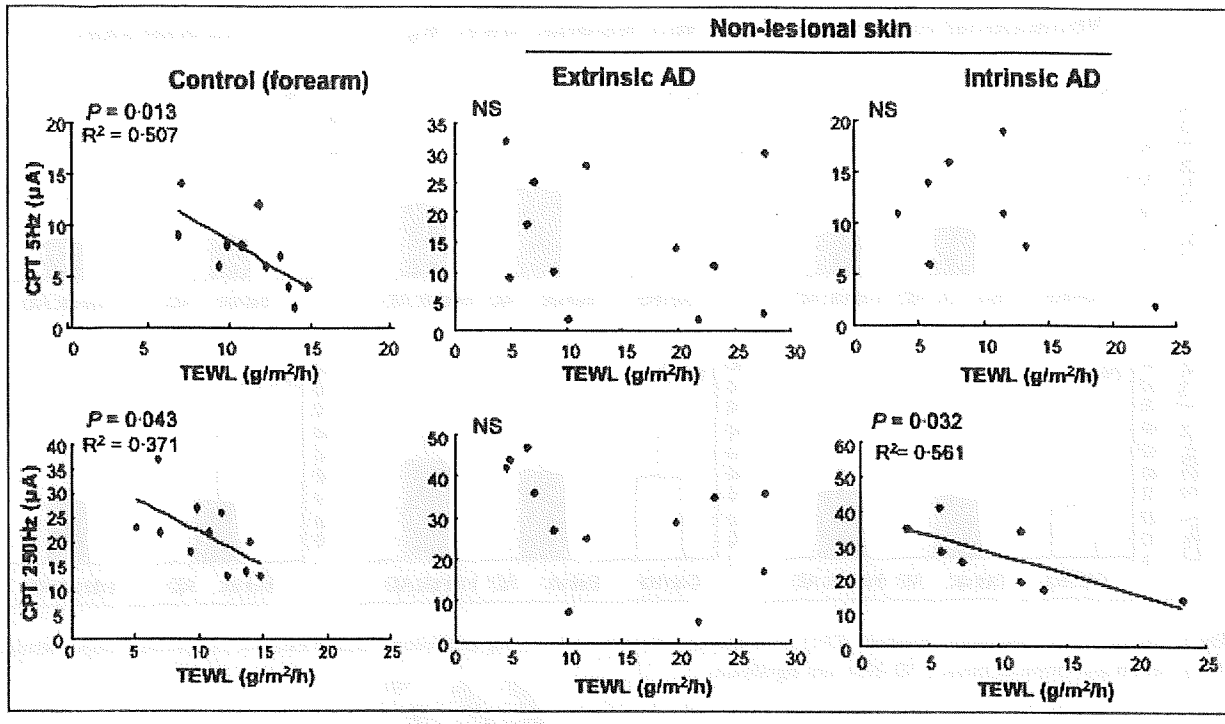

Fig 5. Relationship between transepidermal water loss (TEWL) and electric current perception threshold (CPT) in nonlesional forearm skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and healthy control skin. N.S., not significant.

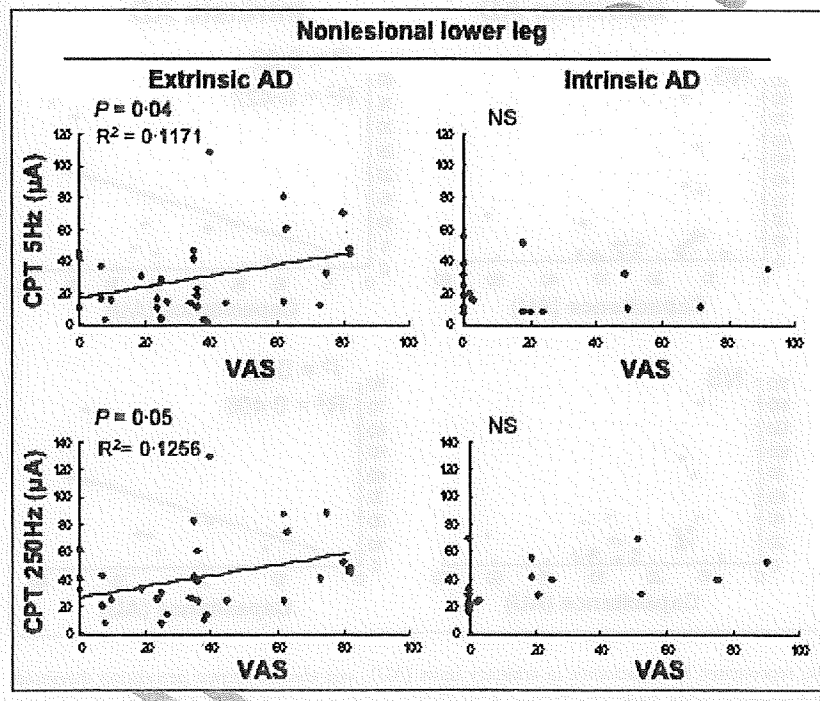

Fig 6. Relationship between visual analogue scale (VAS) score and electric current perception threshold (CPT) on nonlesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD). N.S., not significant.

types accurately. Only one clear way to discriminate the two types is the serum levels of IgE,[29] but its precise cutoff value has not been determined.

In this study, we tentatively divided the patients with AD into two groups by IgE levels of > 400 and < 220 U mL^{-1}, because the normal range in Japanese individuals is < 220 U mL^{-1}. This division was confirmed by a high percentage and high scores of positive RAST to *D. pteronyssinus* in extrinsic AD and a low percentage in intrinsic AD. We found that more of our patients had intrinsic than extrinsic AD, and that women were more likely than men to have intrinsic AD, as already reported in previous studies.[5] In contrast to

extrinsic AD, intrinsic AD is thought to show a normal skin barrier function. We validated this general concept by measuring skin surface hydration and TEWL, and found no significant difference in these values between patients with intrinsic AD and normal individuals, while the patients with extrinsic AD had lower surface hydration levels and higher TEWL levels than the normal subjects.

C-fibres (unmyelinated fibres) are sensory nerves conducting pruritus. A transcutaneous electric current at 5 Hz can stimulate C-fibres.[25] Alternating current stimulus at 250 Hz activates Aδ-fibres (small myelinated fibres), which may also participate in itch. The condition of the stratum corneum may modify the perception by affecting the current or other factors. In our study, low levels of hydration of the stratum corneum reduced CPT. This suggests that the itch perception to external stimuli is promoted in skin with low hydration.

The difference in the barrier function between the extrinsic and intrinsic types raised the possibility that the elicitability of pruritus differs between them. In normal individuals, CPT and skin surface hydration or TEWL were correlated with each other, suggesting that the barrier-damaged skin is sensitive to external irritants. In normal individuals, Kobayashi *et al.*[23] have reported that CPT is inversely correlated with TEWL levels after tape stripping, providing further evidence that barrier damage leads to elicitability of sensation. In our study, the correlation between CPT and skin surface hydration and the inverse correlation between CPT and TEWL were also found in patients with intrinsic AD, suggesting that intrinsic AD shows a normal skin barrier and elicitability of sensation to external stimuli. In contrast, the patients with extrinsic AD showed different elicitability with individual variations presumably due to the low surface hydration. Kobayashi *et al.*[23] have also shown that the skin of patients with AD is not extremely sensitive as compared with that of normal individuals to the electric stimulation of their Aδ- and C-fibres. Their patients seem to include those with both extrinsic and intrinsic AD. Our results suggest that some patients with extrinsic AD have high CPT levels despite the impaired barrier function.

Pre-existing pruritus elevated CPT on the nonlesional skin of patients with extrinsic AD, as CPT and VAS were correlated with each other in the nonlesional sites of the extrinsic type. Accordingly, Ikoma *et al.*[30] found that when histamine prick tests are performed in nonlesional skin of patients with AD, itch rating increases more slowly and is significantly lower than in controls.[30] Our unexpected finding was not observed in intrinsic AD. It is possible that in the already itchy skin of extrinsic AD, Aδ- and C-fibres are in a stimulated state, resulting in the insensitivity to external irritants, while the steady-state interaction between the barrier and sensory fibres might be kept in intrinsic AD. The end of sensory fibres in the skin of extrinsic AD seems to be continuously stimulated by the damaged stratum corneum, leading to the elevated CPT.

Our study suggests that the two types of AD are different from each other in the mode of elicitability of pruritus, because of the different skin barrier states between them. Furthermore, it was recently found that IgE autoantibodies can target keratinocytes in AD; this might promote barrier damage and modify resultant itch elicitability in extrinsic AD.[31] As the response of sensory nerves to irritants appears to be intact in intrinsic AD, the mechanisms of pruritus underlying this type of AD are an important issue to be elucidated.

Acknowledgments

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LETTER TO THE EDITOR

Antihistaminic drug olopatadine downmodulates CCL17/TARC production by keratinocytes and Langerhans cells

Dear Editor,

Olopatadine hydrochloride (OLP; [Z]-11-[3-dimethylaminopropylidene]-6,11- dihydrodinenz [b,e] oxepin-2-acetic acid monohydrochloride) is a histamine H_1-receptor-blocking agent that possesses both acidic and basic residues.[1] This H_1 blocker also suppresses the production by epithelial cells or mast cells of various chemical mediators and cytokines, such as leukotrienes, arachidonic acid, interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α),[2,3] and inhibits intracellular adhesion molecule 1 (CD54) expression on conjunctival cells[4] and activity/migration of eosinophils.[5] Based on these findings, OLP is now widely used for the treatment of allergic rhinitis, urticaria and various itchy skin diseases including eczematous dermatitis.[6]

It has been reported that OLP has a unique anti-allergic property, which may provide implications for the mechanisms underlying its therapeutic actions. Thymus and activation-regulated chemokine (CCL17/TARC) is one of the T-helper (Th)2-associated chemokines, and an important regulator of Th2 cell recruitment into the skin.[7] Serum CCL17 level is proportional to the disease activity of atopic dermatitis (AD), and OLP inhibits CCL17 production by peripheral blood mononuclear cells from AD patients.[7] Serum CCL17 level is also related to the disease activity of bullous pemphigoid, mycosis fungoides, chronic actinic dermatitis and papuloerythroderma.[8,9] In the skin, CCL17 is secreted by keratinocytes (KC) and Langerhans cells (LC). LC are professional antigen-presenting cells in the epidermis, and we have recently shown that they are the main source of CCL17 among epidermal cells.[10] These findings urged us to investigate whether OLP induces inhibition of CCL17 production by KC or LC *in vitro*. To examine the effects of OLP on KC, we used human KC cell line HaCaT cells. LC-enriched epidermal cells (LC-EC) and bone marrow-derived dendritic cells (BMDC) were prepared from BALB/c mice. Our results suggest that OLP exerts its therapeutic effectiveness by inhibiting CCL17 production by both KC and LC.

First, to explore whether olopatadine suppresses CCL17 production by KC, we added olopatadine into the culture medium of KC cell line HaCaT cells. Three-day culture supernatants from HaCaT cells were collected, stored at −80°C and measured for CCL17, CCL22/MDC, monokine induced by γ-interferon (IFN-γ) (CXCL9/Mig) and IFN-γ-inducible protein 10 (CXCL10/IP-10) using enzyme-linked immunosorbent assay (ELISA) kits (Genzyme/Techne, Minneapolis, MN, USA) according to the manufacture's directions. It has been reported that the concentrations of OLP at 10^{-5} to 10^{-7} mol/L suppresses *in vitro* activities of both KC and LC.[11] Therefore, we followed the protocols to examine the inhibitory activity of olopatadine in our experiments. As shown in Figure 1, the IFN-γ/TNF-α-augmented production of CCL17 was suppressed significantly by the addition of olopatadine at a concentration of 10^{-6} or 10^{-5} mol/L. The concentrations of OLP in this *in vitro* study were chosen on the basis of the therapeutic dose of this drug.[12]

To see the effects of olopatadine on CCL17 production by LC-EC, epidermal cell (EC) suspensions freshly isolated from naïve BALB/c mice were subjected to Ficoll gradient separation of LC-EC as described previously.[13] The percentage of LC in LC-EC fraction was 15–20%, as assessed by flow cytometric analysis with anti-I-A^d phycoerythrin (PE)-labeled monoclonal antibody (BD PharMingen, San Diego, CA, USA). OLP

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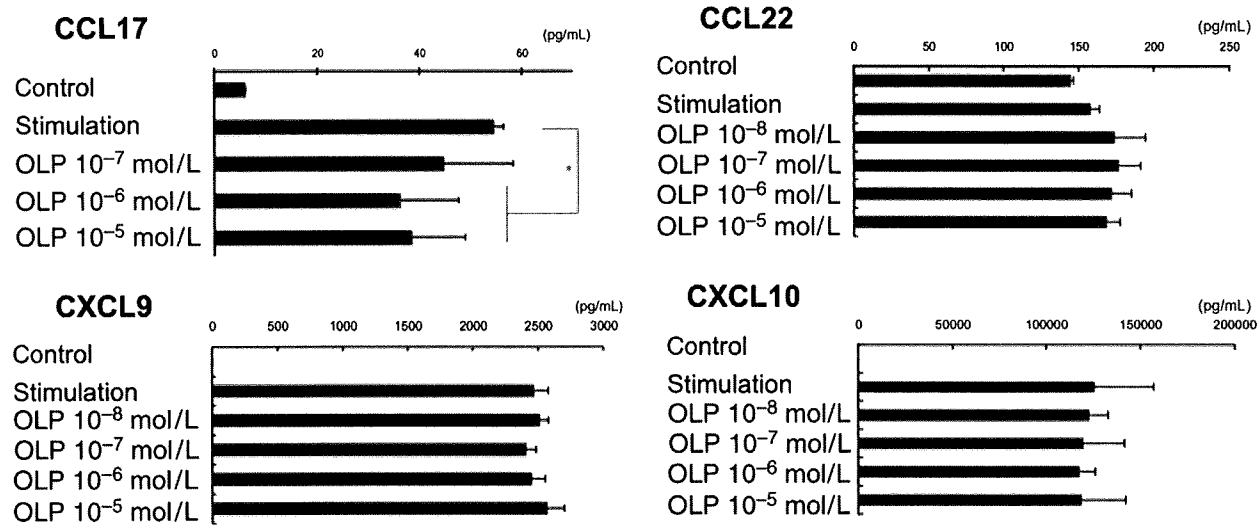

Figure 1. To examine chemokine production, semiconfluent HaCaT cells in 24-well plates were stimulated with 2000 units/mL of recombinant γ-interferon (IFN-γ) (Biogamma; Maruho Pharmaceutical, Osaka, Japan) and 4000 units/mL of tumor necrosis factor-α (TNF-α) (Invitrogen, Carlsbad, CA, USA) for the first 2 h, followed by 200 units/mL IFN-γ and 400 units/mL TNF-α thereafter. Olopatadine (OLP) was added at the starting of culture. Three-day culture supernatants were measured for CCL17, CCL22, CXCL9 and CXCL10 by enzyme-linked immunosorbent assay. Data represent the mean ± standard deviation. $^*P < 0.05$.

downregulated the expression of mRNA for CCL17 but not CCL22 (Fig. 2a). Because KC coexist with LC in LC-EC fraction, we also investigated the production of CCL17 by BMDC, a mimicry of pure LC. Murine immature DC were generated from bone marrow according to standard protocols.[14,15] Minor modification included feed culture medium on day 3 containing granulocyte-macrophage colony-stimulating factor (10 ng/mL). On day 6, BMDC (5×10^6/well) were cultured for 24 h with the two indicated concentrations of OLP. As shown in Figure 2(b), OLP decreased the mRNA expression of CCL17 and CCL22 in mature BMDC. Three independent series of experiments confirmed the result. CCL17 in culture supernatants was quantified by ELISA. OLP significantly suppressed the production of CCL17 by 37%, while the production of CCL22, CXCL9 or CXCL10 was not inhibited (data not shown). The above findings suggested that OLP directly downregulates Th2 chemokine production by DC and LC.

It has been reported that PAM 212 cells, a murine KC cell line, and normal human KC produce CCL17 after stimulation with TNF-α and IFN-γ.[16,17] Consistent with these *in vitro* data, CCL17 is expressed in the lesional KC of AD skin, suggesting that KC is one of the main sources of CCL17.[17] CCL17-transgenic mice showed enhanced Th2 type contact hypersensitivity and reduced Th1 type reactivity.[18] In this study, we demonstrated that OLP downmodulates the production of CCL17 by epidermal KC.

We have previously demonstrated that the ability of LC to present hapten to prime T cells was reduced by OLP with decreased expression of major histocompatibility complex class II and co-stimulatory molecules.[11] LC are capable of producing a high level of CCL17 constitutively during culture even without exogenous stimuli,[19] and we have recently shown that LC are responsible for the production of CCL17 by epidermal cells.[10] The present study showed that OLP inhibits the production of CCL17 by LC-EC. Thus, OLP is effective for the treatment of Th2-associated skin disorders not only by suppressing antigen-presenting ability but also by inhibiting CCL17 production. In our experiment system using LC-EC, KC coexisted with LC, raising the possibility that OLP alters CCL17 production by LC indirectly by modulating bystander KC. Therefore, another DC population without contamination of KC was tested for the modulatory effect of OLP on the chemokine production. Because Th2 chemokines including CCL17 was

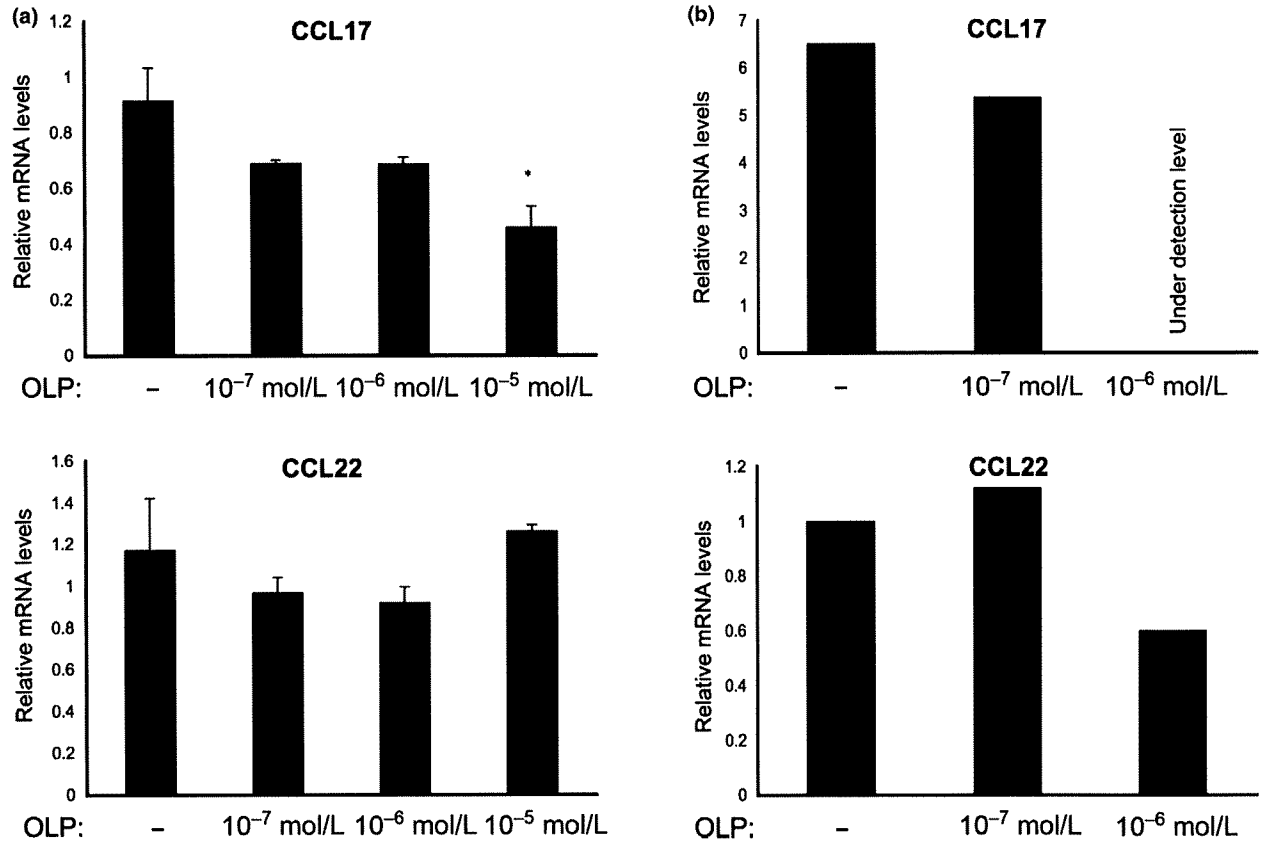

Figure 2. Total cellular RNA was extracted with an RNA extraction kit (Promega, Madison, WI, USA) from cultured Langerhans cell-enriched epidermal cells (LC-EC) and bone marrow-derived dendritic cells (BMDC). RNA was then reverse-transcribed and amplified by random hexamer in single-tube assay using the TaqMan Reverse Transcription Reagants (Applied Biosystems, Foster City, CA, USA) with gene-specific sense and antisense primers and a detection probe labeled on the 5′-end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (accession numbers: CCL17, Mm00516136-m1; CCL22, Mm00436439-m1; β-actin, 4352933E; all for Applied Biosystems). Using an ABI Prism 7000 Sequence Detection Systems (Applied Biosystems), samples were reverse-transcribed and amplified. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse β-actin was performed on all samples to control interspecimen variations in RNA amounts. (a) mRNA expression for chemokines in LC-EC. LC-EC from naïve mice were cultured with or without olopatadine for 24 h. The cultured cells were subjected to real-time polymerase chain reaction analysis for CCL17 and CCL22. Data are expressed as the mean ± standard deviation of triplicate culture. *$P < 0.05$, compared with the olopatadine non-added one. (b) mRNA expression for chemokines in BMDC. BMDC were cultured for 24 h with or without olopatadine. The cultured cells were subjected to real-time polymerase chain reaction analysis for CCL17 and CCL22. The data are from a representative experiment out of three.

expressed in a subset of BMDC, we investigated the effect of OLP on CCL17 production by BMDC. OLP downregulated both CCL17 and CCL22 production by BMDC. Besides the effects of OLP on KC and LC, another study has shown that antihistamines regulate immune responses by affecting the interaction between DC and $CD4^+$ T cells.[20]

In summary, OLP suppresses the production of CCL17 by KC and DC. This suggests that OLP may exerts its therapeutic effect at least partly by down-modulating Th2 chemokine production by epidermal cells.

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CONFLICT OF INTEREST

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Do italicize
- Gene symbols (e.g., *PPARG*)
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Capitalization

Capitalization of gene and protein symbols should be styled according to species:

Species	Gene/protein name[A]	Gene symbol	Protein symbol
Humans/higher mammals			
Humans *(Homo sapiens)*	peroxisome proliferator–activated receptor γ	*PPARG*	PPARγ
Non-human primates	peroxisome proliferator–activated receptor γ	*PPARG*	PPARγ
Rodents			
Mouse (*Mus musculus*), rat (*Rattus norvegicus*)	peroxisome proliferator–activated receptor γ	*Pparg*	Pparγ
Amphibians and fish			
Frog *(Xenopus laevis, X. tropicalis)*	peroxisome proliferator–activated receptor γ	*pparg*	PPARγ
Zebrafish *(Danio rerio)*	engrailed 2b	*eng2b*	Eng2b
Invertebrates			
Fly *(Drosophila melanogaster)*	hedgehog	*hh*	HH
Worm *(Caenorhabditis elegans)*	dumpy-5	*dpy-5*	DPY-5
Unicellular microorganisms			
Bacteria (most genera)	acetylornithine deacetylase	*argA*	ArgA
Budding yeast *(Saccharomyces cerevisiae)*	nicotinate phosphoribosyltransferase	*NPT1*	Npt1

[A]Gene and protein names start with a lower-case letter unless they begin with a person's name (describing a disease/phenotype) or a capitalized abbreviation.