



Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

DA-9601 suppresses 2, 4-dinitrochlorobenzene and dust mite extract-induced atopic dermatitis-like skin lesions

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ARTICLE INFO

Article history:

Received 16 February 2011

Received in revised form 22 March 2011

Accepted 5 April 2011

Available online xxxx

Keywords:

Atopic dermatitis

DA-9601

IgE

Skin inflammation

ABSTRACT

DA-9601 (Stillen™) is a novel anti-peptic formulation prepared from the ethanol extracts of *Artemisia asiatica* possessing anti-oxidative, anti-allergic and anti-inflammatory activities. However, their effect on atopic dermatitis (AD) has not been studied yet. In this study, we report that topical application of DA-9601 suppressed house dust mite extract (*Dermatophagoides farinae* extract, DFE) and 2, 4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in BALB/c mice model. We established atopic dermatitis model in BALB/c mice by repeated local exposure of DFE/DNCB to the ears. Repeated alternative treatment of DFE/DNCB caused AD-like lesions. DA-9601 reduced AD-like skin lesions based on ear thickness and histopathological analysis, and serum IgE levels. DA-9601 inhibited mast cell infiltration into the ear and elevation of serum histamine in AD model. In addition, DA-9601 suppressed DFE/DNCB-induced expression of IL-4, IL-13, IL-31, and TNF- α in the ears. Taken together, our results showed that topical application of DA-9601 exerts beneficial effects in animal model of AD, suggesting that DA-9601 might be a candidate for the treatment of AD.

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1. Introduction

Atopic dermatitis (AD), characterized by chronic and relapsing inflammatory dermatitis with immunologic disturbances and pruritic and eczematous skin lesions is one of the most common skin diseases. AD is a biphasic inflammatory skin disease, provoked by unbalance between Th1 and Th2 immune response [1]. In particular, AD has been associated with the Th2 phenotype, showing the dominance of IL-4 and IL-13 secretion, and Th2 type cytokine-mediated IgE production [2,3]. Recently, association of IL-31 with AD-induced skin inflammation and pruritus was also reported [4]. For human, house dust mites, such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are the most common environmental allergens associated with AD [5].

Many therapeutic trials have been performed to modulate AD with limited success. These include prevention of Th2 responses, enhancement of Th1 responses and decrease of IgE concentration. Topical glucocorticoids are important and effective remedies for treatment of AD. It is well known, however, that prolonged use of high dose of glucocorticoids causes a variety of side effect. Despite of several side effects, steroid has been used as potent anti-AD treatment [6,7]. Recently, natural herbs come into spotlight as an alternative therapeutics in immune disorders due to

their proved safety with potent immunomodulatory effects. There are many reports about the use of natural herbs as a drug for treatment of AD and psoriasis [8–10].

DA-9601 (Stillen™), a formulated ethanol extract of *Artemisia asiatica*, is developed to treat patients with gastric mucosal ulcers and is now used in South Korea and other Asian countries. *Artemisia asiatica* is widely used in traditional Asian medicine for the treatment of inflammation, cancer and microbial infection [11]. DA-9601 has been reported as having anti-oxidative, anti-allergic, and anti-inflammatory effects [11–14]. From the chemical analysis, DA-9601 contains flavonoids and essential oils such as eupatilin, 1,8-cineole, and terpinen-4-ol.

In this study, we examined the effect of DA-9601 on AD-like lesions using BALB/c model. Assessment was made by measuring ear thickness, histopathological changes including mast cell count, cytokine expression in ear tissue, and serum IgE and histamine in each group.

2. Materials and methods

2.1. Animals

Six-week-old female BALB/c J mice were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed 5–10 per cage in a laminar air flow room, maintained at a temperature of $22 \pm 2^\circ\text{C}$, with a relative humidity of $55 \pm 5\%$ throughout the study. The care and treatment of the mice were in accordance with the guidelines

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established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Drugs and chemicals

DA-9601 was provided from Dong-A Pharmaceutical (Kyunggi-do, Korea). A freeze-dried crude *Dermatophagoides farinae* extract (DFE) (Greer Laboratories, Lenoir, NC) was used as an antigen. All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. DFE was dissolved in phosphate buffered saline (PBS) containing 0.5% Tween 20. 2,4-Dinitrochlorobenzene (DNCB, 1%) was dissolved in acetone/olive oil (1:3) solution.

2.3. Induction of AD-like skin lesions in the ear

Induction of AD using DNCB and DFE was performed as previously described [15]. The schematic experimental procedure was described in Fig. 1. For the induction of AD, mice were divided into four groups and surface of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μ l of DNCB (1%) was painted on each ear and then 20 μ l of DFE (10 mg/ml) 4 days later. Treatment of DFE/DNCB was repeated once a week alternatively for 4 weeks. After 2 weeks later of 1st induction, tail bleeding was performed to check the serum IgE level. After confirming an atopic condition by IgE level, ears were treated with DA-9601 by painting brush until the end of 4 weeks of induction. Ear thickness was measured 24 h after DNCB or DFE application with a dial thickness gauge (Kori Seiki MFG, Co., Japan).

At days 14 and 28, blood samples were collected by orbital puncture. Plasmas were prepared from blood samples and stored at -70°C for further analysis. After blood collection, ears were removed and used for histopathological analysis. The serum IgE levels were measured using IgE ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX) according to manufacturer's instruction.

2.4. Histological observation

Excised ears are fixed in 4% paraformaldehyde for 16 h and embedded in paraffin. Thin, 6 μ m sections were stained with hematoxylin and eosin. Infiltrated lymphocytes, thickening of the epidermis and fibrosis in the dermis were observed by microscope. For measurement of mast cell infiltration, skin sections were stained with toluidine blue and the number of mast cells in five sites chosen at random was counted.

2.5. Real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan) according to the manufacturer's protocol. Total RNA was isolated from superficial cervicel, axillary of each group. The conditions for PCR were similar to ones previously described [16]. Briefly, 2 μ l of cDNA (100 ng), 1 μ l of sense and antisense primer solution (0.4 μ M), 12.5 μ l of SYBR Premix Ex Taq (Takarabio Inc.), and 9.5 μ l of dH₂O were mixed together to obtain a final 25 μ l reaction mixture in each reaction tube. The primers used for PCR were (IL-4 F 5'-

ACAGGAGAAGGGACGCCAT, R 5'-GAAGCCCTACAGACGAGCTCA; IL-13 F 5'-GCAACATCAACAGGACCAGA, R 5'-GTCAGGGAATCCAGGGCTAC; IL-31 F 5'-TCGGTCATCATAGCACATCTGGAG, R 5'-GCACAGTCCCTTTGGAGT-TAAGTC; TNF- α F 5'-CCTGTAGCCACGTCGTAGC, R 5'-TTGACCT-CAGCGCTGAGTTG). The amplification conditions were 10 s at 95°C , 40 cycles of 5 s at 95°C and 30 s at 60°C , 15 s at 95°C , 30 s at 60°C , and 15 s at 95°C . The normalization and quantification of mRNA expression was performed using the TP850 software supplied by the manufacturer.

2.6. Histamine assay

The histamine content was measured by the o-phthalaldehyde spectrofluorometric procedure as previously described [17]. The serum was separated from the released histamine by centrifugation at $400\times g$ for 5 min at 4°C . The fluorescent intensity was measured at an excitation 355 nm and emission 450 nm using a Perkin-Elmer Fluorescence Spectrometer LS-50B (Norwalk, CT).

2.7. Statistical analysis

Statistical analyses were performed using SAS statistical software (SAS institute, Cary, NC). Treatment effects were analyzed using ANOVA followed by Dunnett's multiple range tests. Significance was set at $P<0.05$.

3. Results

3.1. Clinical symptoms at the inflamed site

To investigate the effect of DA-9601 on AD, a BALB/c AD-like lesion model was established by alternate painting of DFE and DNCB for 4 weeks on both ear lobes [15]. As shown in Fig. 2, repeated topical application of DFE/DNCB significantly increased ear thickness in mice. However, treatment of DA-9601 (1 and 5 μ g/ear) significantly suppressed DFE/DNCB-induced ear thickness. Different from the control group, no remarkable AD-like skin lesions, such as hemorrhage, edema, excoriation and scaling, were observed in DA-9601 (5 μ g/ear) treated groups.

3.2. Histopathological observation and serum IgE

To analyze the effect of DA-9601 on the skin hypertrophy and granulocyte infiltration, sections of ear were stained and observed under an optical microscope. Repeated DFE/DNCB exposure caused

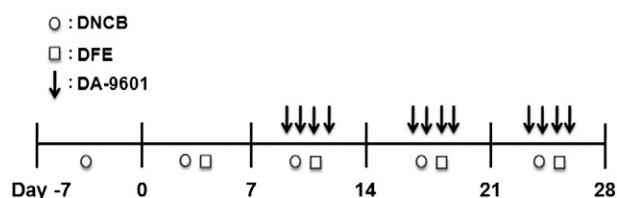


Fig. 1. Experimental schedule for the induction of AD-like lesion. After 7 days of first boosting with DNCB, DNCB and DFE were treated to the both ears once a week alternatively for 4 weeks. After 1 week of first induction, DA-9601 was painted to the ears with painting brush every day for 3 weeks.

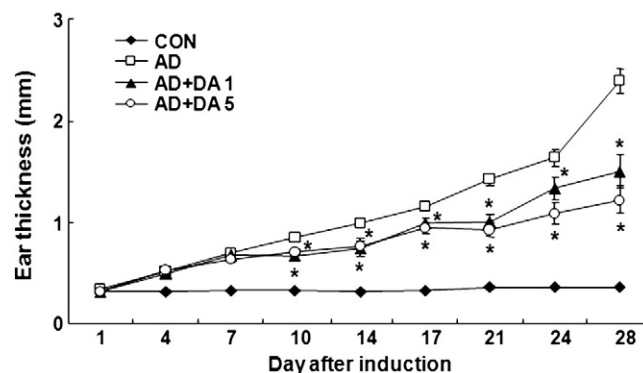


Fig. 2. Effect of DA-9601 on ear thickness and macroscopic ear features. Detailed experimental procedure is described in Materials and methods. (A) Vehicle (acetone/olive oil) and DA-9601 (1 or 5 μ g/ear) were pretreated for 2 h before 1% DNCB (20 μ l/ear) or DFE treatment (10 mg/ml, 20 μ l/ear). Ear thickness was measured 24 h after DNCB or DFE application with a dial thickness gauge. Data are presented as mean $\pm$ SD of six determinations. *Significant difference from the DFE/DNCB-treated value at $P<0.05$. DA: DA-9601 treatment; AD: DFE and DNCB treatment.

potent inflammatory changes, such as thickening of the epidermis, fibrosis in the dermis and accumulation of inflammatory cells such as lymphocytes, eosinophils and neutrophils in the ear tissue of the atopic dermatitis (Fig. 3). Hyper-production of IgE is a major characteristic of AD. We investigated the effect of DA-9601 on IgE production. Repeated application of DFE/DNCB caused an apparent elevation of serum total IgE (Fig. 4A) and DFE mite specific IgE (Fig. 4B). DA-9601 significantly reduced DFE/DNCB-induced serum total and DFE mite specific IgE levels.

3.3. Effect of DA-9601 on the histamine release and mast cell infiltration

Next, to understand the inhibitory mechanism of DA-9601, we measured a representative symptom-inducing substance such as histamine [18] to test whether DA-9601 suppresses the release of histamine. Interestingly, DA-9601 almost completely inhibited the enhanced level of histamine induced by DFE/DNCB (Fig. 5A). To further investigate, we assessed the effects of DA-9601 on the infiltration of mast cells, an important effector cell involved in AD, into the ears. As shown in Fig. 5B, the infiltration of mast cells induced by DFE/DNCB was suppressed by DA-9601 treatment.

3.4. Effect of DA-9601 on expression of cytokines in the ear

We examined the expression levels of cytokines in the ear by real-time PCR. It is known that Th2 cytokine such as IL-4 and IL-13 and IL-31 are highly expressed in acute stage of AD. Repeated application of DFE/DNCB increased the expression of IL-4 and IL-13 and IL-31 (Fig. 6A–C). DA-9601 significantly reduced the expression of these Th2 cytokines. In addition, one of the important pro-inflammatory cytokines, TNF- α , was markedly increased by DFE/DNCB. The elevated TNF- α was also reduced by DA-9601 (Fig. 6D).

4. Discussion

In this present study, we investigated the effect of DA-9601 on DFE/DNCB-induced AD-like skin lesion in BALB/c mice model. Among the several AD models, we used DFE/DNCB-induced AD model based

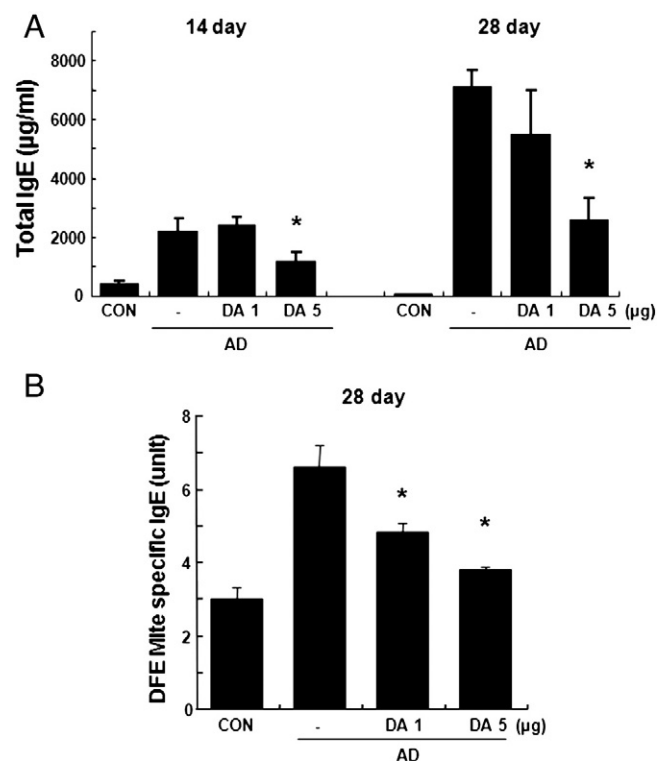


Fig. 4. Effect of DA-9601 on serum total and mite specific IgE. (A) The blood samples of groups of vehicle, DFE/DNCB plus vehicle and DFE/DNCB plus DA-9601 (1 or 5 µg/ear) were collected by orbital puncture at 14 and 28 day, and plasma levels of total IgE was quantified by ELISA. (B) The DFE mite specific IgE level was measured at 28 day. Data are presented as mean $\pm$ SD of triplicate determinations. *Significant difference from the DFE/DNCB-treated value at $P < 0.05$. DA: DA-9601 treatment; AD: DFE and DNCB treatment.

on the reproducibility and repeatability from our previous study [15]. In this report, we clearly showed that typical AD symptoms were observed by the repeated alternative application of DFE/DNCB. Ear

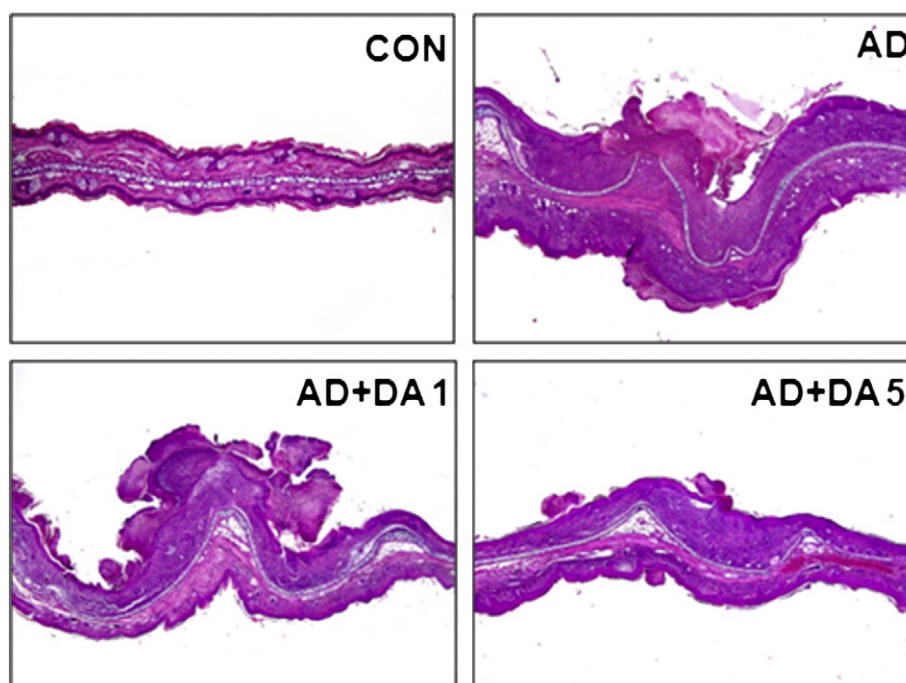


Fig. 3. Histopathological analysis. The ears of groups of vehicle, DFE/DNCB plus vehicle and DFE/DNCB plus DA-9601 (1 or 5 µg/ear) were excised, fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. The skin sections were stained with hematoxylin and eosin. DA: DA-9601 treatment; AD: DFE and DNCB treatment.

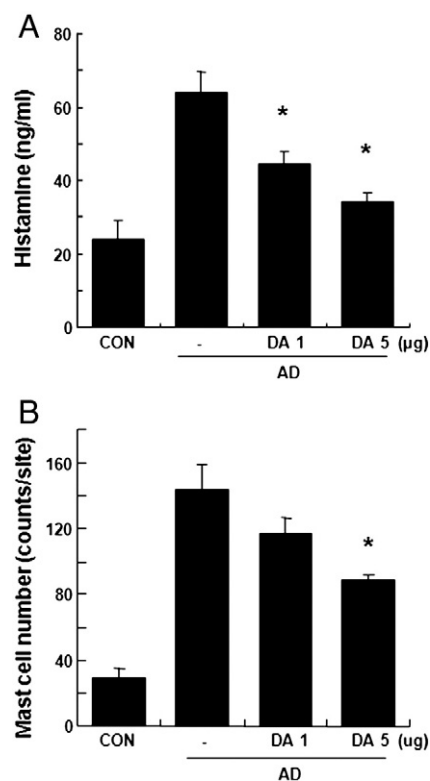


Fig. 5. Effect of DA-9601 on serum histamine and mast cell infiltration. (A) The blood was obtained from the heart of each mouse, and histamine content was measured using the spectrofluorometric procedure. (B) The ear sections were stained with toluidine blue. The number of mast cells in five sites chosen at random was counted. Data are presented as mean $\pm$ SD of triplicate determinations. *Significant difference from the DFE/DNCB-treated value at $P < 0.05$. DA: DA-9601 treatment; AD: DFE and DNCB treatment.

swelling and eczematous skin lesion were observed, and serum total and mite specific IgE were increased. Histopathological analysis also revealed the infiltration of leukocytes, especially mast cells, into the AD region. Topical application of DA-9601 inhibited these AD symptoms. Our results suggest the beneficial effect DA-9601 on AD.

Mast cells are known as key effector cells in IgE-mediated allergic disorders and activated by cross-linking of a high affinity IgE receptor. Upon activation, mast cells undergo degranulation and release a variety of biologically active substances, which play an important role in host defense and allergic reactions including AD. Among the immune mediators released from the mast cells, histamine is one of the best characterized and most potent mediators [19,20]. In patients with AD, increases in histamine levels have been observed [21]. Infiltration of mast cells into the dermis is a necessary factor to define an appropriate animal model for AD [5]. In the present study, we demonstrated that repeated treatment of DFE/DNCB resulted in mast cell infiltration into the dermis in the ear, suggesting that the animal model of AD used in this study satisfy AD conditions. Topical application of DA-9601 significantly suppressed the number of mast cells infiltrating in the skin lesions of the AD mice and the serum histamine release, suggesting that the activation and migration of mast cells may be a target of DA-9601 in AD. Our results demonstrate that treatment with DA-9601 suppresses DFE/DNCB-induced AD-like lesions, suggesting DA-9601, a known anti-peptic and anti-allergic formulation, as a promising agent for AD.

It is known that mast cell activation and histamine release are tightly regulated by IgE from B cells, and serum IgE levels are elevated in proportion to the development of AD [5,8,17]. As shown in the present study, DFE/DNCB-induced serum total and mite specific IgE levels were reduced by DA-9601. From these results, we assume that DA-9601 alleviates AD-like skin lesions through the inhibition of IgE production. IgE synthesis by B cells is regulated by Th2 cytokines, especially IL-4 and IL-13. In human AD, overproduction of IL-4 is a critical factor [22]. Sensitization to an allergen reflects the allergen's

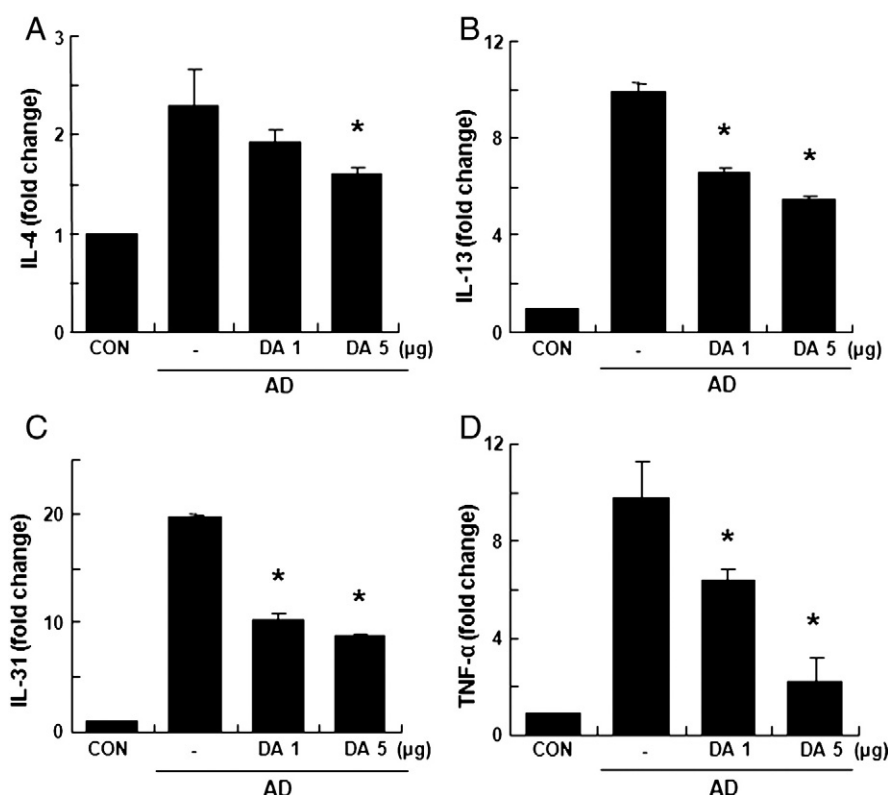


Fig. 6. Effect of DA-9601 on the expression of cytokines in ear. The ears were excised and total RNA was isolated. Quantitative real-time PCR was performed as described in Materials and methods. Data are presented as mean $\pm$ SD of triplicate determinations. *Significant difference from the DFE/DNCB-treated value at $P < 0.05$. DA: DA-9601 treatment; AD: DFE and DNCB treatment.

ability to elicit a Th2 cell response, in which IL-4 and IL-13 drive IgE production by promoting class-switch recombination in B cells [20]. In our results, DA-9601 decreased IL-4 and IL-13 which play important role on isotype switching into IgE. These results imply that DA-9601 can reduce the serum IgE by suppressing the Th2 response especially IL-4 and IL-13 production. IL-31 is a newly characterized Th2 cytokine, which is involved in AD. A major function that has been attributed to IL-31 is the induction of pruritus in the skin and allergic diseases [23]. Our results showing the suppression of DFE/DNCB-induced IL-31 by DA-9601 suggest that IL-31 is also a target of DA-9601 in AD.

The production of pro-inflammatory cytokines, especially TNF- α by epidermal cells is known as one of the key events on the initiation of AD [24]. TNF- α produced at the initiation stage of AD induces the production of various chemokines/adhesion molecules which cause the recruitment and proliferation of leukocytes within the skin. DA-9601 is known to exert anti-inflammatory activity [25,26]. We also previously showed that the expression of TNF- α was inhibited by DA-9601 in activated human mast cells [13]. Therefore, we speculate that the inhibitory effect of DA-9601 on leukocyte infiltration in our AD model might be mediated by blocking TNF- α expression and downstream chemokines/adhesion molecules.

Eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone) is a known active ingredient of DA-9601. Eupatilin is known to possess various biological effect such as anti-oxidant, anti-inflammation, anti-tumor, and anti-gastritis. The molecular target of eupatilin was revealed as NF- κ B [14,27–29]. In the present report, we clearly showed the effect of DA-9601 on the AD; however, the molecular target of this effect is still unknown. We previously reported the anti-allergic inflammatory effect of DA-9601 through the inhibition of immediate-type hypersensitivity, histamine release and serum IgE, and pro-inflammatory cytokines by the attenuation of NF- κ B [13,30]. The intracellular calcium and cAMP are the most important signaling pathways to the degranulation of mast cells and downstream histamine release [17]. The effect of DA-9601 on the calcium, cAMP, and other transcription factors regulating the expression of cytokines in our AD model will be our future study to identify the molecular target of DA-9601.

In conclusion, the present study demonstrates that topical application of DA-9601 inhibits the development of DFE/DNCB-induced AD-like symptoms in BALB/c mice. The inhibitory effect of DA-9601 was mediated by the histamine release, the production of IgE, and the expression of IL-4, IL-13, IL-31 and TNF- α . Our results suggest that DA-9601 could be a potential therapeutic candidate for AD.

Acknowledgements

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A090015), KOSEF grant funded by the Korea government (R01-2008-000-20037-0), and by the Grant of MEST (The Regional Core Research Program/Anti-aging and Well-being Research Center).

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