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Inhibitory effect of 5,6-dihydroergosterol-glucoside on atopic dermatitis-like skin lesions via suppression of NF- κ B and STAT activation.

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ABSTRACT

Background: Atopic dermatitis (AD) is a Th2-type disease. Keratinocytes, a major type in the skin, produce Th2 chemokines such as thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22, which play pivotal roles in the development of Th2-dominant inflammatory skin diseases. Recently, it was reported that 5,6-dihydroergosterol-glucoside (DHE-Glc) was synthesized and exhibited strong anti-inflammatory activity.

Objective: We aimed to investigate the effects of DHE-Glc, a synthetic molecule derived from ergosterol, on AD-like skin lesions induced by 2,4-dinitrochlorobenzene (DNCB) in mice and to elucidate the effects of DHE-Glc on TNF- α /IFN- γ -induced production of CCL17 and CCL22 in human keratinocytes (HaCaTs) and DNCB induced skin inflammation mice model.

Method: Mice were sensitized and challenged on the skin of their backs with DNCB. At 30–60 days after sensitization, mice were treated with cutaneous administration of DHE-Glc by skin smear. HaCaT cells were used to evaluate the effects of DHE-Glc on production of CCL17 and CCL22 and investigate mechanisms of action by RT-PCR, ELISA, Western blot, and reporter assays.

Result: Topical administration of DHE-Glc attenuated AD-like skin inflammatory symptoms. DHE-Glc decreased infiltration of epidermal eosinophils and mast cells, and reduced levels of IgE, histamine, and mRNA expression and protein levels of CCL17/CCL22 in the plasma of DNCB-treated animals. In addition, DHE-Glc suppressed TNF- α /IFN- γ -induced expression of the Th2 chemokines CCL17 and CCL22 by inhibiting NF- κ B and STAT activation in TNF- α /IFN- γ -induced HaCaT cells.

Conclusion: DHE-Glc improved AD-like skin inflammatory symptoms on the backs of DNCB-induced mice, partly by suppressing production of Th2 chemokines, CCL17 and CCL22 in inflamed skin. Therefore, DHE-Glc is a potential therapeutic agent for skin inflammatory diseases such as AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by erythema, pruritic and eczematous skin lesions, edema, and thickening of the skin [1,2]. The pathogenesis of AD has been attributed to complex interactions between multiple factors such as genetic and environmental factors, altered skin barrier function, and the immune system [3,4]. Extrinsic AD caused by environmental factors or allergens induces Th2 cells to produce

Th2 cytokines such as IL-4 and IL-13 and high serum IgE levels [5,6]. Th2 cell numbers are elevated during the acute and chronic stages of AD. Th2 cytokines have direct effects on skin cells such as epidermal keratinocytes, which produce pro-inflammatory cytokines and chemokines that induce infiltration of immune cells including T cells, monocytes, eosinophil and mast cells into inflammatory skin lesions [5].

Thymus and activation regulated chemokine (TARC/CCL17) is a CC chemokine that binds to the CC chemokine receptor 4 (CCR4), which is expressed selectively on Th2 cells [7–9]. CCL17 is constitutively expressed in the thymus and produced by various cells including monocyte-derived dendritic cells, endothelial cells and keratinocytes [10–12]. Macrophage-derived chemokine (MDC/

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CCL22) is also a CC chemokine that binds to CCR4. CCL22 is constitutively produced by dendritic cells, macrophages, and keratinocytes [13,14]. The interaction between CCR4 and its ligands is important for the migration of Th2 cells into inflamed tissue [15]. CCL17 and CCL22 are significantly elevated in AD patients and correlate with the severity in patients with AD [16–18]. Thus, the reduction of CCL17 and CCL22 in keratinocytes can be effective for treatment of the skin diseases.

A number of pharmacological controls for AD involve topical or systemic administration of steroids, antihistamines or specific inhibitors of NF- κ B [19]. Steroids reduce all Th cell functions and are widely used as immunosuppressants [20]. However, prolonged use of steroid has side effects such as thinning of the skin, leading to cracking and bleeding [21]. Natural compounds from herbs and plants are potential sources of therapeutic agents for preventing and treating inflammatory skin diseases [22–24]. The phytosterol 3-O- β -D-glucopyranosylspinasterol (spinasterol-Glc) from *Stewartia koreana* leaf extracts is strongly anti-inflammatory and inhibits production of CCL17 in keratinocytes [25,26]. However, spinasterol-Glc is not readily available by isolation from plants. A derivative of spinasterol-Glc, 5,6-dihydroergosteol-glucoside (DHE-Glc), has been synthesized recently, which exhibited a strong anti-inflammatory activity [27]. In this study, we evaluated the effects of DHE-Glc on 2,4-dinitrofluorobenzene (DNFB)-induced AD mouse models and on production of CCL17 and CCL22 in HaCaT keratinocytes. We found that topical administration of DHE-Glc inhibited production of IgE and histamine and reduced epidermal thickness, infiltration of eosinophils, and lymph node and spleen size. In addition, we observed inhibitory effects by DHE-Glc on production of CCL17 and CCL22 in TNF- α /IFN- γ induced HaCaT keratinocytes.

2. Materials and methods

2.1. Cell culture

Immortal human keratinocyte (HaCaT) cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin at 37 °C in a humidified 95% and 5% (v/v) mixture of air and CO₂, respectively.

2.2. Cell viability assay

Cells were seeded in 96-well plates at 5×10^4 cells/well and allowed to attach for 18 h. After discarding the growth medium, HaCaT cells were treated with indicated concentrations of DHE-Glc in serum-free medium for 24 h. After incubation, cells were treated with 100 μ g/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for 1 h. Formazan precipitates were dissolved in 200 μ l of DMSO and absorbance at 560 nm was determined spectrophotometrically. Analyses were repeated three times, and the results were expressed as means of three independent experiments.

2.3. Animals and maintenance

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Kyung Hee University (KHUASP (SU) 11-012). Male BALB/c mice were from Orient Bio. Inc. (Gyeonggi-do, Korea) and were 6 weeks old at initiation of experiments. All mice were maintained at the animal facility of Kyung Hee University (Yoinin-si, Korea) and were housed in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to water. Room temperature was maintained at 22 ± 1 °C with a relative humidity of $50 \pm 10\%$. Animals were fed with a sterilized pelleted diet (Orient Bio. Inc.). DNFB (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in vehicle (3:1 acetone:

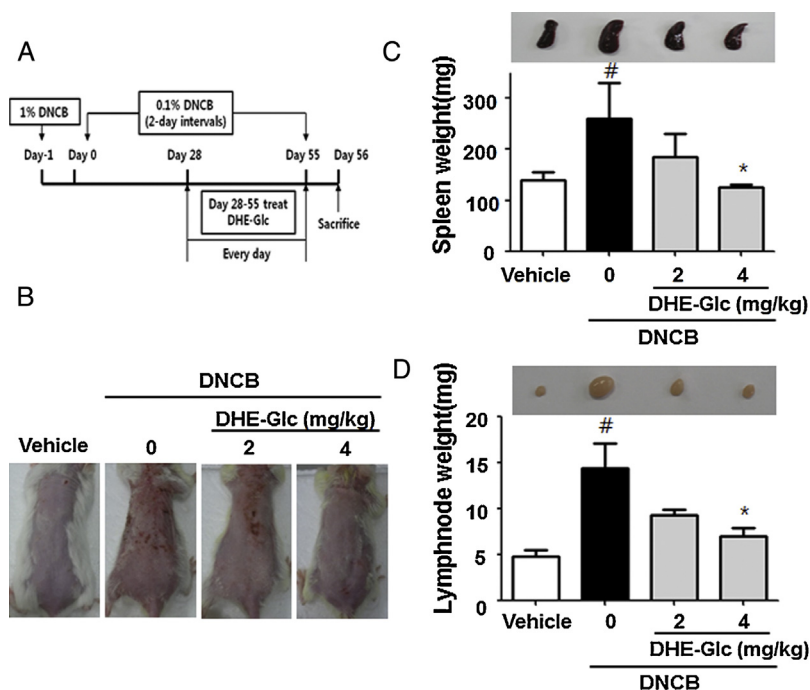


Fig. 1. Effects of DHE-Glc on DNFB-induced AD skin severity. (A) Sensitization of DNFB mice ($n = 5$): stimulation was with DNFB for 1 month, and after 1 month, with cream containing 2 mg/kg and 4 mg/kg of DHE-Glc for 1 month. Mice were sacrificed on day 60. (B) Clinical severity of inflammatory skin lesions. Photographs were taken on the day before mice were sacrificed. (C, D) Organ sizes were compared by photographic images. Organs and whole-body weights of five mice per group were measured. $\#p < 0.05$ vs. negative control, $*p < 0.05$ vs. positive control.

olive oil) and used as a sensitizer for inducing AD-like skin lesions in mice. Backs of mice were shaved with a clipper and depilatory cream, washed with sterilized PBS and gauzed a day before sensitization. Mice were divided into 4 groups with 5 mice per group: vehicle, DNCB, and DNCB plus topical treatment of 50 μ g of DHE-Glc or 100 μ g of DHE-Glc. Sensitization was as shown in Fig. 1. Exposed skin was treated with vehicle alone or with 0.1 ml of 1% of DNCB in vehicle. On day 4 after sensitization, skin was re-sensitized with 0.1% of DNCB solution daily for about two months. On day 30 after initiation of sensitization, a cream containing 50 μ g or 100 μ g of DHE-Glc was applied to skin lesions with DNCB daily for a month.

2.4. Reverse transcriptase-polymerase chain reaction

For reverse transcriptase-polymerase chain reaction (RT-PCR), HaCaT cells (1×10^6 cells/well) were cultured in 6-well plates and treated with TNF- α /IFN- γ (10 ng/ml) in the presence or absence of DHE-Glc for 18 h. Total RNA from HaCaT cells was isolated using TRIzol reagent kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription of total RNA (2 μ g) was performed with oligo dT primers and M-MuLV reverse transcriptase (Fermentas Life Science, Burlington, Canada). The cDNA was used for amplification of chemokine mRNAs with gene-specific primers using Taq PCR master mix kits (Qiagen, Hilden, Germany). Primers used in this study were as follows: CCL17 (forward) 5'-ACT GCT CCA GGG ATG CCA TCG TTT TT-3', (reverse) 5'-ACA AGG GGA TGG GAT CTC CCT CACTG-3', CCL22 (forward) 5'-AGG ACA GAG CAT GGC TCG CCT ACA GA-3', and (reverse) 5'-TAA

TGG CAG GGA GGT AGG GCT CCT GA-3'. GAPDH mRNA was used as an internal control.

2.5. Enzyme-linked immunosorbent assay

HaCaT cells were pre-incubated in 6-well plates and treated with TNF- α /IFN- γ (10 ng/ml) in the presence or absence of DHE-Glc for 18 h. CCL17 and CCL22 in culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. We collected blood from mice after sacrifice. Serum samples were obtained after centrifugation. Using mouse CCL17 and CCL22 ELISA kits, CCL17 and CCL22 levels were measured according to the manufacturer's instructions.

2.6. Western blots

HaCaT cells were pretreated with DHE-Glc for 1 h and exposed to TNF- α /IFN- γ (10 ng/ml) for 30 min to 1 h. Cells were then lysed in RIPA buffer (Pierce Biotechnology, Rockford, IL, USA). Total protein was extracted from skin inflammatory lesion using RIPA lysis buffer according to the manufacturer's protocol. Lysates were resolved using SDS polyacrylamide gel electrophoresis and western blots were carried out as previously described [26]. Membranes were developed with an enhanced chemiluminescence system (GE healthcare, Amersham, UK) and exposed to X-ray film (Fuji Photo Film Co., Ltd., Japan).

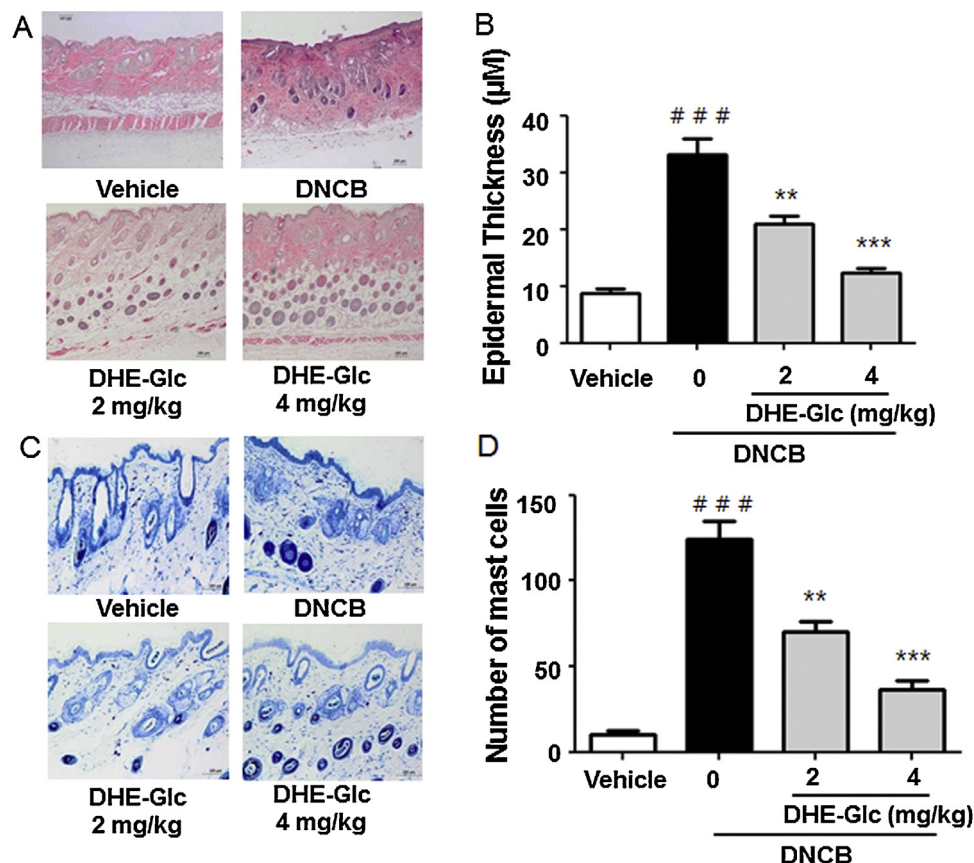


Fig. 2. Effect of DHE-Glc on mast cell infiltration in DNCB-induced AD-like skin. Mouse back skin lesions were fixed in 3.7% formaldehyde, sectioned at 4.5 μ m and stained with hematoxylin and eosin (A) and toluidine blue O (C). Epidermal thickness was measured with a micrometer (B). The number of cells were presented as means of cell numbers at five random sites per each animal (D). Five mice were used per group. ### $p < 0.0001$ vs. negative control, ** $p < 0.01$, *** $p < 0.001$ vs. positive control.

2.7. Luciferase reporter assays

HaCaT cells were plated in 24-well plates and transiently transfected with pGAS/Luc or pNF- κ B/Luc with pCis-CK using lipofectamine according to the manufacturer's instructions (Stratagene, Santa Clara, CA, USA). Transfected cells were recovered for 24 h, pretreated with DHE-Glc for 1 h and stimulated with TNF- α /IFN- γ for 8 h. Cells were lysed with reporter lysis buffer (Stratagene) and NF- κ B and GAS luciferase reporter gene activity was analyzed using a luciferase reporter assay system (Promega, Madison, WI, USA).

2.8. Measurement of serum IgE and histamine level

We collected blood from mice after sacrifice. Serum samples were obtained after centrifugation. Using mouse IgE ELISA kits (Shibayagi, Gunma, Japan), total IgE serum levels were measured. For serum histamine levels, mouse histamine ELISA kits (Oxford Biomedical Research Inc., Oxford, MI, USA) were used.

2.9. Histological analysis

Skin samples of mice were shaved and fixed in 3.7% formalin, embedded in paraffin and serially sectioned at 4.5 μ m. Sectioned samples were stained with a hematoxylin/eosin solution or toluidine blue O, anti-CCL17 and anti-CCL22 antibody solution according to the manufacturer's instructions (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for detecting epidermal thickness or infiltration of various inflammatory cells, mast cells and expression of CCL17/CCL22 on AD-like skin lesions induced by 2,4-dinitrochlorobenzene (DNCB) in mice. Histopathological changes were examined by light microscopy. The number of mast

cells were counted in four random fields ($\times 400$) and presented as means of five independent numbers of cell counts.

2.10. Statistical analysis

Unless stated otherwise, all experiments were performed with triplicate samples and repeated at least three times. Data are presented as mean $\pm$ SD and statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t*-test.

3. Results

3.1. DHE-Glc attenuated DNCB-induced skin inflammation

To investigate the effects of DHE-Glc on AD-like skin lesions *in vivo*, we sensitized BALB/c mice with DNCB allergen to induce AD-like contact skin inflammation. Mice were challenged with multiple applications of the indicated doses of DNCB to back skin (Fig. 1A). Edema, erythema excoriation and scarring were apparent on the skin of DNCB-sensitized mice compared with untreated control mice. Severity of lesions was reduced in DNCB-sensitized mice treated with topical DHE-Glc (Fig. 1B).

The weight of local lymph node increases with topical application of materials that are allergenic or have sensitizing potential [28,29]. Local lymph node weight was validated as a measure of atopic symptoms in a murine local lymph node assay. The spleen also proliferates during immune system activation through cell-mediated reaction, especially through activation of T and B cells. Therefore, we investigated morphological changes in spleens. DNCB-sensitized mice had enlarged spleens that were reduced to almost normal size in mice treated with DHE-Glc

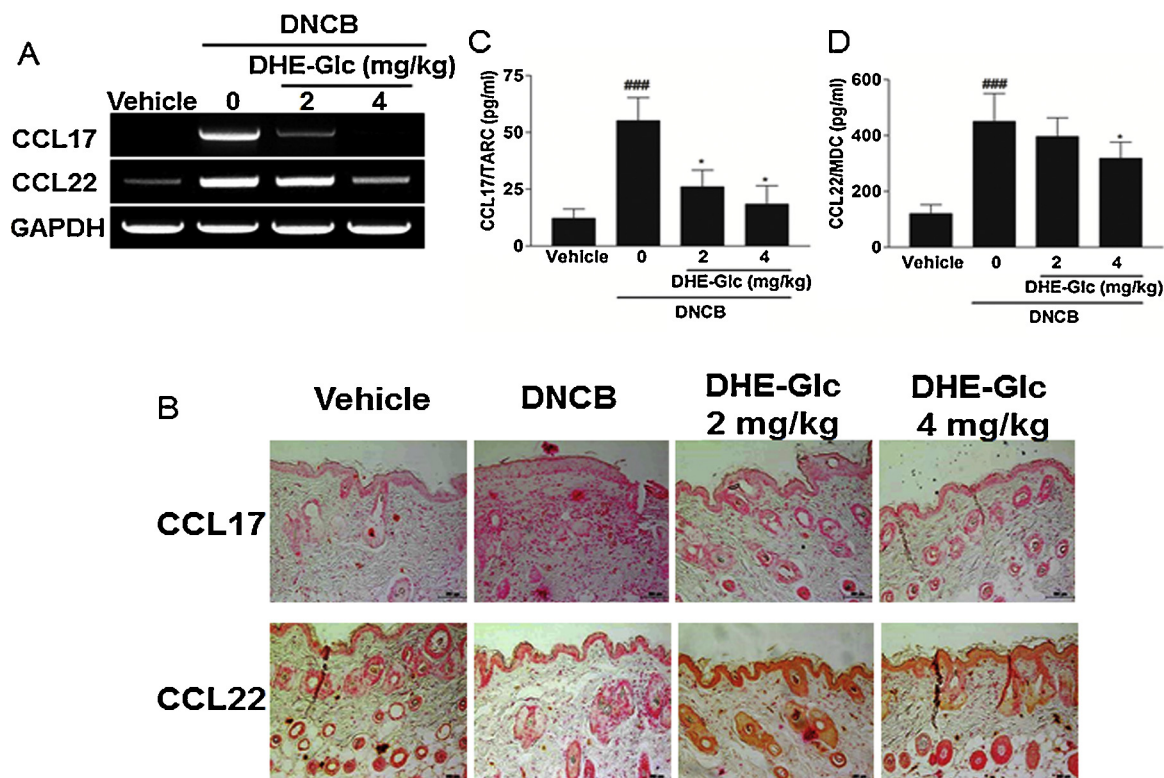


Fig. 3. Effect of DHE-Glc on DNCB-induced mRNA and protein expression levels of CCL17 and CCL22 in mice. (A) Total RNA was extracted from mouse back skin inflammatory lesion tissue and mRNA levels of CCL17 and CCL22 were determined by RT-PCR. (B) Mouse back skin lesions were fixed in 10% formaldehyde, sectioned at 4.5 μ m and stained with anti-CCL17 and anti-CCL22. (C and D) Serum was collected 24 h after final DHE-Glc and/or DNCB treatment. Serum CCL17 (C) and CCL22 (D) were analyzed by ELISA. Data shown are the average of five samples per group and are shown as the mean values $\pm$ SD. ^{###}*p* < 0.001 vs. negative control, ^{*}*p* < 0.05, vs. positive control.

(Fig. 1C). We demonstrated an increase in lymph node weight in DNCB-sensitized mice, which was markedly reduced by topical application of DHE-Glc (Fig. 1D).

3.2. DHE-Glc reduces infiltration of eosinophils and mast cells in skin

DNCB induced hyperkeratosis in mice. Increased epidermal thickness was probably due to increased epidermal proliferation caused by altered differentiation at the inflamed skin tissues. Our results showed that the thickness of the epidermal layer was substantially reduced by treatment of DHE-Glc (Fig. 2A and B). Skin sections were stained with H&E to examine eosinophil infiltration. Eosinophil infiltration decreased significantly in the skin of AD-like mice treated with DHE-Glc compared with DNCB-sensitized mice (Fig. 2A). Skin sections were stained with toluidine blue O to examine mast cell infiltration. Mast cell infiltration was higher in the skin of AD-like mice. Topical administration of DHE-Glc suppressed mast cell infiltration in the skin. Detectable basal levels of infiltrated mast cells were 10.5 ± 3.318 . Upon stimulation with DNCB, infiltrated mast cells increased up to 124.6 ± 23.36 (about 12-fold). DHE-Glc markedly inhibited the DNCB-induced infiltration of mast cells. The numbers of mast cells were 69.9 ± 14.04 and 36.63 ± 13.86 in the AD-like skin of mice treated with DHE-Glc after days 2 and 4, respectively (Fig. 2C and D).

3.3. DHE-Glc suppressed DNCB-increased mRNA and protein expression levels of CCL17 and CCL22 in mice

Keratinocytes at inflammatory sites of epidermis induces abnormal expression of cytokines and chemokines including Th2 chemokines such as CCL17 and CCL22, leading to infiltration of T cells or leukocytes [5]. Therefore, we examined the effects of DHE-Glc on chemokine expression in the DNCB-treated AD-like mice model. We measured the mRNA and protein expression of CCL17 and CCL22 in the skin lesion by RT-PCR, immunohistochemistry and ELISA. As shown in Fig 3A, topical application of DNCB increased mRNA expression of CCL17 and CCL22 in the DNCB-treated group vs. the negative group. DHE-Glc treated group significantly decreased the CCL17 and CCL22 mRNA expression. Next, we examined the expression pattern of CCL17 and CCL22 in skin lesion by IHC. There was specific staining by an anti-CCL17 and anti-CCL22 antibody. High increases in CCL17 and CCL22 protein expression was observed in DNCB-treated group by IHC, showing that DHE-Glc treated group significantly decreased the CCL17 and CCL22 protein expression (Fig. 3B). We analyzed the effectiveness of DHE-Glc on the serum CCL17 and CCL22 levels. As shown in Fig. 3C and D, DHE-Glc significantly reduced DNCB-induced production of CCL17 and CCL22 in a dose-dependent manner.

3.4. DHE-Glc reduced serum levels of histamine and IgE in DNCB-sensitized mice

Mast cells are sources of histamine, which is a symptom-inducing substance in AD [30]. Thus, the serum levels of histamine were examined. DHE-Glc inhibited the serum levels of histamine in DNCB-sensitized mice (Fig. 4A). IgE antibody production is associated with Th2 immune response [1]. To examine the effect of DHE-Glc on Th2 response, we measured serum levels of IgE. Compared with AD-induced control mice, total IgE was significantly reduced by topical administration of DHE-Glc (Fig. 4B).

3.5. DHE-Glc suppresses TNF- α /IFN- γ induced CCL17 and CCL22 in HaCaT keratinocytes

HaCaT cells were pretreated with various doses of DHE-Glc for 1 h and stimulated with TNF- α /IFN- γ for 18 h. Production of

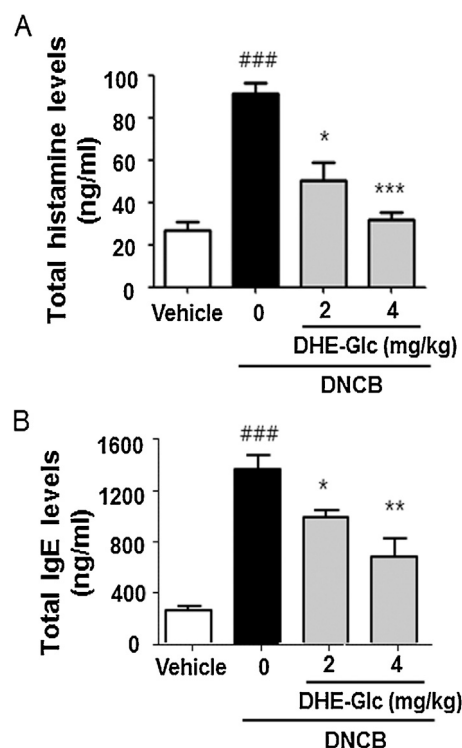


Fig. 4. Effects of DHE-Glc on IgE and serum histamine levels and immune cell recruitment. Serum was collected 24 h after final DHE-Glc and/or DNCB treatment. Serum histamine (A) and IgE (B) were analyzed by ELISA. Data shown are the average of five samples per group and are shown as the mean values $\pm$ SD. ### p < 0.001 vs. negative control, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. positive control.

CCL17 and CCL22 increased up to 140.1 ± 14.35 pg/ml and 1495 ± 51.97 pg/ml in response to TNF- α /IFN- γ , compared to the basal levels of 25.016 ± 4.263 pg/ml and 15.67 ± 4.041 pg/ml, respectively (Fig. 5A and B). DHE-Glc dose-dependently inhibited TNF- α /IFN- γ -induced production of CCL17 and CCL22. To conform that the inhibitory effect of DHE-Glc on the chemokine production was not due to cytotoxicity of DHE-Glc, we examined the effects on cell viability of HaCaT cells. The cells were incubated at 37 °C for 24 h with or without various concentrations of DHE-Glc. MTT assays showed that DHE-Glc did not affect cell viabilities of keratinocyte HaCaT cells in these conditions (Fig. 5C).

To determine whether DHE-Glc inhibited production of CCL17 and CCL22 at the gene expression level, we investigated the effects of DHE-Glc on CCL17 and CCL22 mRNA in TNF- α /IFN- γ -stimulated HaCaT cells. CCL17 and CCL22 mRNA levels were induced by TNF- α /IFN- γ by 5.51 ± 1.87 and 2.57 ± 0.53 folds, respectively, which were reduced by DHE-Glc in concentration-dependent manners (Fig. 6). Our results indicated that DHE-Glc inhibited TNF- α /IFN- γ -induced expression of CCL17 and CCL22 mRNA, which resulted in reduction of the chemokine levels in HaCaT cells.

3.6. JAK2 and p38 MAPK signaling pathways are involved in TNF- α /IFN- γ -induced expression of CCL17 and CCL22 in HaCaT keratinocytes

Expression of CCL17 and CCL22 genes is mainly induced by NF- κ B and STAT1 in TNF- α /IFN- γ -stimulated keratinocytes [31]. Since the transcription factors are activated by upstream signaling molecules such as MAP kinases and JAK2 [32,33], we examined the effects of signaling molecule inhibitors on production of CCL17 and CCL22 in TNF- α /IFN- γ -stimulated HaCaT cells. Cells were

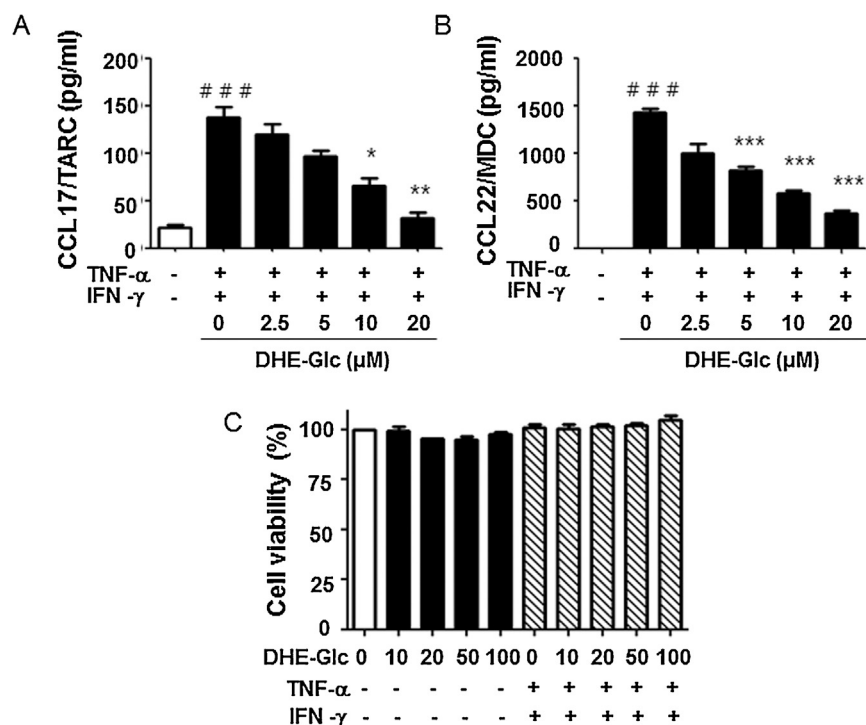


Fig. 5. Inhibition of TNF-α/IFN-γ-induced CCL17 and CCL22 production by DHE-Glc. HaCaT cells were pretreated with indicated concentrations of DHE-Glc for 1 h and stimulated with IFN-γ/TNF-α for 18 h. ELISA was used to measure protein levels of CCL17 and CCL22 (A, B). Data shown are the average of triplicate samples of three independent experiments and are shown as the mean values ± SD. ^{###}*p* < 0.001 vs. negative control, ^{*}*p* < 0.05, ^{**}*p* < 0.01, ^{***}*p* < 0.001 vs. positive control. (C) Cells were incubated with DHE-Glc at the indicated concentrations for 24 h. Cell viability was measured by MTT assays.

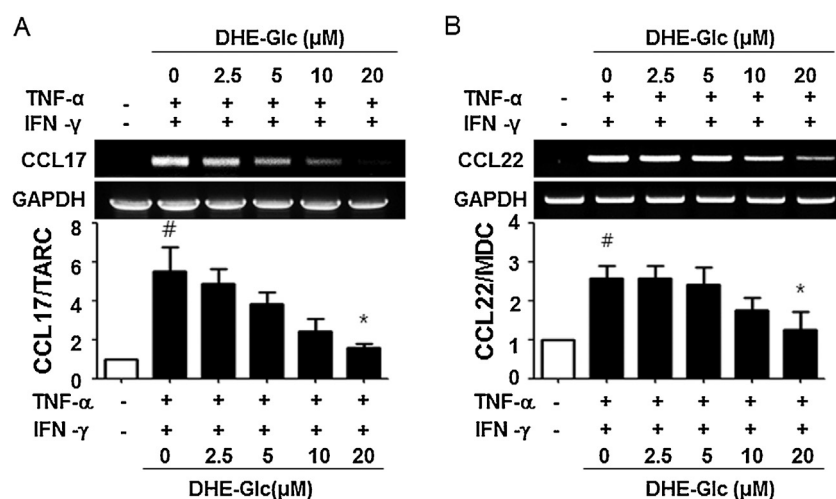


Fig. 6. Inhibition of TNF-α/IFN-γ-induced CCL17 and CCL22 mRNA expression by DHE-Glc. HaCaT cells were pretreated with indicated concentrations of DHE-Glc for 1 h and stimulated with IFN-γ/TNF-α for 18 h. Total RNA was extracted, and CCL17 and CCL22 mRNA were analyzed by RT-PCR and quantified by densitometry. GAPDH was used as the internal control. Data shown are the average of triplicate samples of three independent experiments and are shown as the mean values ± SD. (A, B). [#]*p* < 0.05 vs. negative control; ^{*}*p* < 0.05 vs. positive control.

incubated with inhibitors of JAK2 (AG490), ERK MAPK (PD98059), p38 MAPK (SB203580), JNK (SP600125) or NF-κB (Bay11-7082) for 1 h and stimulated with 10 ng/ml TNF-α/IFN-γ for 18 h. CCL17 and CCL22 in cell supernatants were measured by ELISA. Inhibitors of p38 MAPK, JAK2, and significantly reduced TNF-α/IFN-γ-induced production of CCL17 and CCL22. JNK and ERK MAPK inhibitors only weakly inhibited the production of CCL17 and CCL22 (Fig. 7A and B). Analysis of mRNA levels showed that the inhibitors suppressed expression of CCL17 and CCL22, similar to suppression of CCL17 and CCL22 protein (Fig. 7C and D). These results suggested that JAK2 and p38 MAPK were mainly involved in induction of

CCL17 and CCL22 gene expression in the activation of TNF-α/IFN-γ-stimulated keratinocytes.

3.7. DHE-Glc inhibited phosphorylation of JAK2, p38 MAPK, and IκBα in TNF-α/IFN-γ-activated HaCaT keratinocytes and DNCB-treated mice

To elucidate the molecular mechanism by which DHE-Glc suppressed production of CCL17 and CCL22 in keratinocytes, we next examined which cellular signaling molecules are affected by DHE-Glc. HaCaT cells were pretreated with DHE-Glc for 1 h,

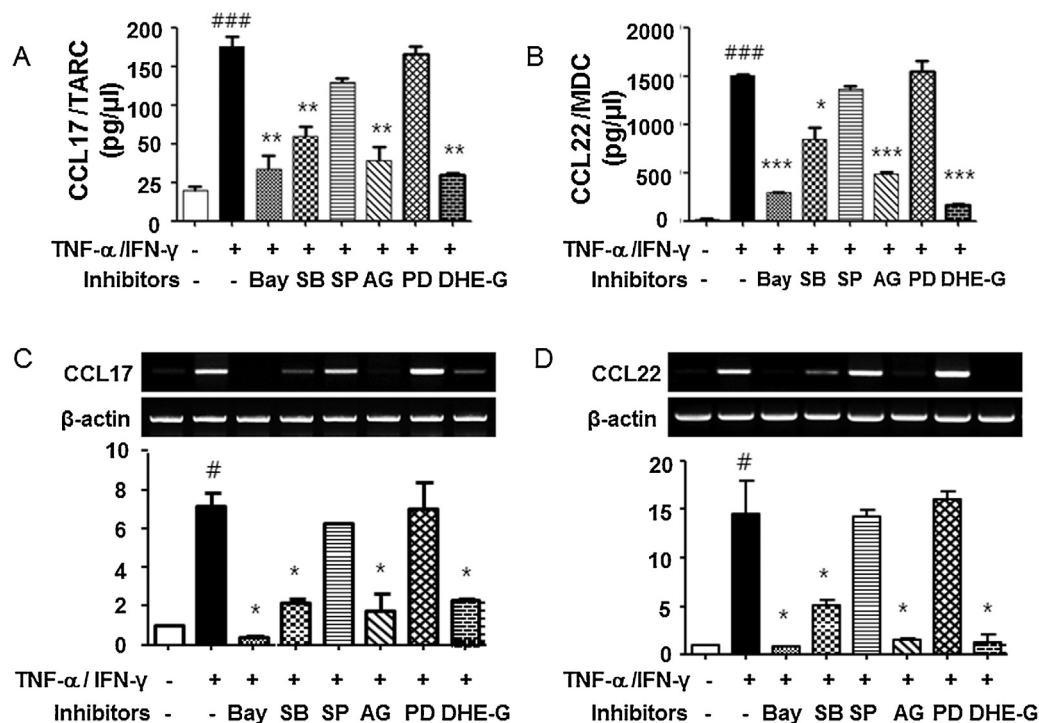


Fig. 7. Effects of signal inhibitors on TNF-α/IFN-γ-induced CCL17 and CCL22 expression. HaCaT cells (1×10^6 cells/well) were cultured in 6-well plate and pretreated with various signal inhibitors, Bay11-7082 (10 μM), SB203580 (10 μM), SP600125 (10 μM), AG490 (100 μM), or PD98059 (20 μM) for 1 h and induced with TNF-α/IFN-γ for 18 h. CCL17 (A) and CCL22 (B) mRNA were analyzed by RT-PCR. Bay, Bay11-7082; SB, SB203580; SP, SP600125; AG, AG490; PD, PD98059; DG, DHE-Glc (20 μM). Data shown are the average of triplicate samples of three independent experiments and are shown as the mean values $\pm$ SD. # $p < 0.05$, ### $p < 0.0001$ vs. negative control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. positive control.

followed by induction by TNF-α/IFN-γ for 10–30 min. We analyzed phosphorylation of MAP kinases ERK, p38 and JNK, and IκBα by Western blot. The results showed that DHE-Glc dose dependently

inhibited phosphorylation of p38 MAPK and IκBα but did not affect phosphorylation of ERK and JNK significantly in TNF-α/IFN-γ-stimulated cells (Fig. 8A).

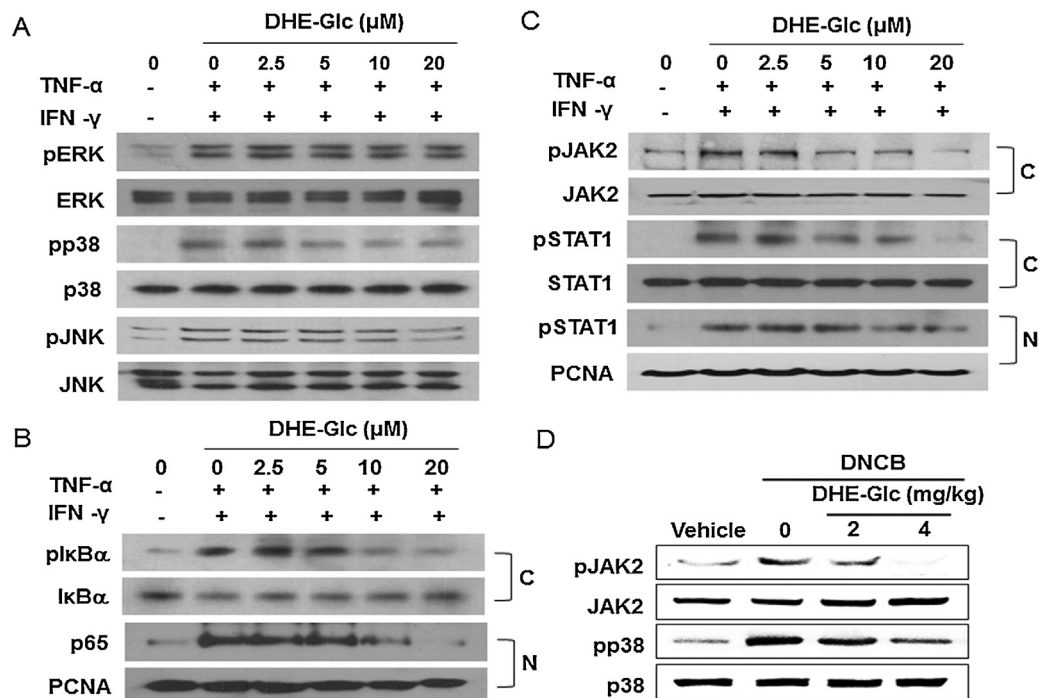


Fig. 8. Effects of DHE-Glc on TNF-α/IFN-γ activation of MAPKs, NF-κB and JAK-STAT. (A) Cells were pretreated with DHE-Glc for 1 h and stimulated with TNF-α/IFN-γ. After 30 min to 1 h, whole cell extracts were subjected to western blot using antibodies against MAPKs or phospho-MAPKs. (B, C) Cells were pretreated with DHE-Glc for 1 h and induced with TNF-α/IFN-γ. After 30 min to 1 h, whole cell or nuclear extracts were subjected to western blot using antibodies against IκB-α, phospho-IκBα or p65(B) or STAT1 or phospho-STAT1 (C).

Activation of NF- κ B plays critical roles in the TNF- α /IFN- γ -induced expression of the chemokines in keratinocytes. Since nuclear translocation of NF- κ B is preceded by I κ B phosphorylation and degradation of I κ B α [34], we analyzed the effects of DHE-Glc on phosphorylation and degradation of I κ B α . The results demonstrated that TNF- α /IFN- γ -induced phosphorylation of I κ B α was inhibited by DHE-Glc. We measured translocation of NF- κ B p65 subunit levels following treatment with TNF- α /IFN- γ in the presence or the absence of DHE-Glc. Basal levels of p65 protein were detected in nuclei of unstimulated cells, but treatment of cells with TNF- α /IFN- γ for 1 h induced the nuclear translocation of p65 dramatically. The amount of p65 in the nucleus of cells was significantly reduced in cells treated with DHE-Glc, comparing to those of cells treated with TNF- α /IFN- γ . I κ B α phosphorylation significantly increased after stimulation of HaCaT cells with TNF- α and IFN- γ . Pretreatment of keratinocytes with DHE-Glc attenuated TNF- α /IFN- γ -induced I κ B α phosphorylation and nuclear translocation of NF- κ B (Fig. 8B). Our results suggested that DHE-Glc suppressed expression of CCL17 and CCL22 by blocking activation of NF- κ B via inhibition of I κ B α phosphorylation.

We next examined phosphorylation of JAK2 and STAT1 in HaCaT after stimulation with TNF- α /IFN- γ . The results demonstrated that TNF- α /IFN- γ -induced phosphorylation of JAK2 was inhibited by DHE-Glc. STAT1 is known to be phosphorylated by JAK2 in response to IFN- γ and translocated into the nucleus. We analyzed phosphorylation of STAT1 in response to DHE-Glc, and observed that TNF- α /IFN- γ -induced phosphorylation of STAT1 was inhibited by DHE-Glc in cytosol and nucleus. Phosphorylation of both JAK2 and STAT1 decreased in a dose-dependent manner (Fig. 8C). We also investigated that the effect of DHE-Glc on phosphorylation of JAK2 and p38 in DNCB-induced mice model. The results showed that DHE-Glc inhibited phosphorylation of JAK2 and p38 in DNCB-stimulated skin lesion (Fig. 8D). Furthermore, JAK2 and p38 MAPK are known to be involved in activation of STAT1. Our results demonstrated that phosphorylation and nuclear translocation of STAT1 were stimulated by TNF- α /IFN- γ in HaCaT cells, which was inhibited by treatment with DHE-Glc. Taken together, our data suggest that DHE-Glc may inhibit the expression of CCL17 and CCL22 via inhibition of NF- κ B and STAT1 activation.

3.8. DHE-Glc suppresses NF- κ B and STAT1 activation by TNF- α /IFN- γ in HaCaT keratinocytes

We examined the effects of DHE-Glc on TNF- α /IFN- γ -induced transcriptional activation. HaCaT cells were transiently transfected with NF- κ B-driven or GAS-driven luciferase constructs and a pCis-CK negative control plasmid. Transfected cells were pretreated with DHE-Glc for 1 h, followed by induction by TNF- α or IFN- γ for 8 h. Stimulation of cells with TNF- α or IFN- γ increased luciferase activity by 4- to 5-fold compared to the negative control. DHE-Glc dose-dependently suppressed TNF- α -induced NF- κ B and IFN- γ -induced STAT1 activation in HaCaT cells at the transcriptional level (Fig. 9). These results suggested that DHE-Glc inhibited production of CCL17 and CCL22 by blocking activation of NF- κ B and STAT1, which are essential activators for expression of CCL17 and CCL22 in HaCaT keratinocytes.

4. Discussion

We previously reported that DHE-Glc, a derivative of spinasterol-Glc inhibited LPS-induced NO production in macrophage cells [27]. In this study, we demonstrated that topical DHE-Glc reduced eosinophil and mast cell infiltration in the skin of DNCB-induced mice and reduced lymph node and spleen weights that were increased by DNCB-sensitization. Mast cells release immune mediators such as histamine, which has potent pro-inflammatory

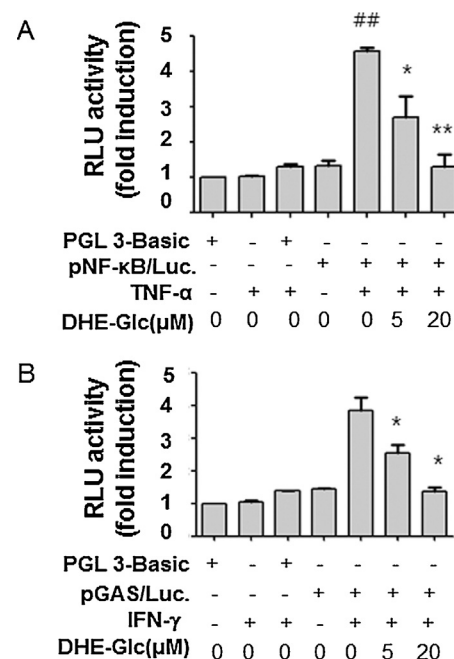


Fig. 9. Effects of DHE-Glc on TNF- α /IFN- γ -induced STAT-1 and NF- κ B promoter activities. (A) For NF- κ B promoter activity, cells were transiently transfected with an NF- κ B-specific reporter gene. After 24 h, transfected cells were pre-incubated with DHE-Glc for 1 h and stimulated with TNF- α for 18 h. (B) Cells were transiently transfected with a GAS-promoter luciferase construct and transfected cells were pretreated with DHE-Glc for 1 h and stimulated with IFN- γ for 18 h. Luciferase activity was determined by the manufacturer's instruction. ^{##} $p < 0.001$ vs. negative control; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. positive control.

activities. DHE-Glc suppressed serum histamine and IgE. Furthermore, we showed that DHE-Glc inhibited production of TNF- α /IFN- γ -stimulated CCL17 and CCL22 in HaCaT human keratinocytes and DNCB-induced AD-like skin inflammation model. We observed rapid phosphorylation of JAK2 and p38 MAPK in HaCaT keratinocytes in response to stimulation with TNF- α /IFN- γ . DHE-Glc treatment of keratinocytes inhibited TNF- α /IFN- γ -induced activation of the signaling kinases JAK2 and p38 MAPK as assessed by phosphorylation state. Our results suggested that DHE-Glc might attenuate AD-like symptoms by suppressing production of CCL17 and CCL22 chemokines by inhibiting JAK2 and p38 MAPK signaling kinases and downstream transcription factors NF- κ B and STAT1.

AD is a common inflammatory disorder characterized by dry, itchy, eczematous skin lesions with infiltration of immune cells such as lymphocytes, eosinophils and mast cells [2,3]. Topical application of DNCB on the backs of mice induced many of the cutaneous histopathological symptoms of AD within a month. AD-like skin lesions in mice were similar to those in AD patients. We observed a high degree of eosinophil infiltration in the skin tissue of DNCB-sensitized mice. Symptoms were significantly improved after 4 weeks of treatment with DHE-Glc, as observed in AD-like skin lesions on mice. Epidermal thickening, infiltration of eosinophils and an increase in mast cells are the main features of AD lesions. Topical administration of DHE-Glc effectively and dose dependently attenuated epidermal thickening and infiltration of inflammatory eosinophils and mast cells. Clinically, 80% of AD patients have increased serum IgE, which is closely related to disease severity. IgE induced activation of mast cells, which resulted in the release of inflammatory mediators such as histamine. Histamine, a major component of mast cells, is involved in induction of itching and edema [35]. Our data showed that DHE-Glc significantly inhibited serum histamine levels in AD-induced mice. We propose that topical application of DHE-Glc mitigated

AD-like symptoms partly by inhibiting the elevation of serum histamine via reduction in IgE.

Previous data from human and animal studies indicates that AD is a Th2-dominant inflammatory disease at an acute stage followed by Th1 involvement at the chronic stage [2,3,36]. Chemokines play a pivotal role in AD development. CCR4 is a predominant chemokine receptor on Th2 cells. CCL17 and CCL22, ligands for CCR4, attract Th2 cells into inflamed tissues and are responsible for AD initiation. Therefore, targeting chemokine production is considered an important potential treatment for AD. This study demonstrated that DHE-Glc suppressed expression of CCL17 and CCL22 in dose-dependent manner in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. Analysis by RT-PCR and reporter assays indicated that DHE-Glc dose-dependently inhibited CCL17 and CCL22 at the transcriptional level, suggesting that DHE-Glc might attenuate AD-like symptom via down-regulation of CCL17 and CCL22 production by keratinocytes. The chemokine genes contain different combinations of transcription factor-binding elements for NF- κ B and STAT [31]. Pro-inflammatory cytokines such as TNF α and IFN- γ activate NF- κ B and STAT1 in keratinocytes [37]. We demonstrated that the anti-inflammatory activity of DHE-Glc appeared to be mediated by inhibition of NF- κ B and STAT-1 activity. NF- κ B is composed of two subunits, p65 and p50, and binds the inhibitory protein I κ B in the cytoplasm in a resting state. Upon stimulation by inflammatory cytokines, I κ B is phosphorylated and degraded by the 26S proteasome [38]. NF- κ B is released and translocated into nucleus where it activates target genes containing NF- κ B-binding sites. Our data revealed that DHE-Glc inhibited phosphorylation of I κ B α and reduced transcriptional activation of a NF- κ B/Luc reporter gene. STATs are also present in the cytoplasm before phosphorylation and translocate into the nucleus on activation. STAT1 is activated by JAK or p38 MAP kinase in response to cytokines such as IFN- γ [33]. Luciferase reporter assays demonstrated that DHE-Glc dose-dependently suppressed TNF- α -induced NF- κ B and IFN- γ -induced STAT1 activation in HaCaT cells at the transcriptional level.

Previous studies reported that activation of NF- κ B and STAT1 are regulated by cellular signaling pathways, including by MAPKs and JAK. We explored signaling pathways involved in DHE-Glc-mediated inhibition of CCL17 and CCL22 expression in HaCaT cells. Our results showed that DHE-Glc inhibited TNF- α /IFN- γ -induced phosphorylation of JAK2 and p38 MAP kinases and did not inhibit activation of ERK and JNK. Previous studies [24,39,40] and our results showed that p38 MAP kinase and JAK2, but not ERK and JNK kinases, are involved in TNF- α /IFN- γ -induced expression of CCL17 and CCL22 in HaCaT cells. These results indicated that DHE-Glc suppressed production of CCL17 in TNF- α /IFN- γ -stimulated cells by inhibition of p38 MAPK and JAK2 pathways activated by TNF- α and IFN- γ . IFN- γ induces expression of responsive genes by activating JAK and STAT1 transcription factor [32]. ERK and JNK signaling molecules activate inflammatory genes such as for TNF- α , iNOS and ICAM-1 [20], but protein kinases appear not to be essential for activation of CCL17 and CCL22 expression in HaCaT keratinocytes. Our results showed that DHE-Glc inhibited TNF- α /IFN- γ -induced phosphorylation of JAK2 and activation of STAT1 in HaCaT cells. Taken together, our results indicated that DHE-Glc suppressed TNF- α /IFN- γ -induced expression of chemokine genes by inhibiting NF- κ B and STAT1 by suppressing p38 MAPK and JAK2 pathways.

In summary, this study demonstrated that DHE-Glc, a spinasterol-Glc derivative with little cytotoxicity, inhibited production of Th2-type chemokines CCL17 and CCL22 and had anti-inflammatory effects against atopic symptoms in mice induced with DNCB. DHE-Glc suppressed expression of CCL17 and CCL22 genes in TNF- α /IFN- γ -stimulated HaCaT cells by suppressing NF κ B and STAT1 activation by inhibition of p38 MAP kinase and

JAK2. Our findings suggested that DHE-Glc potentially inhibited AD and could be developed as a therapeutic agent for prevention and treatment of AD.

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