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Effect of (E)-2-(3,4-dimethoxyphenyl)-4-oxo-4H-chromen-7-yl-3-(3,4-dimethoxyphenyl) acrylate on the development of atopic dermatitis-like lesions

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ABSTRACT

Aims: In this study, we synthesized a novel chemical, (E)-2-(3,4-dimethoxyphenyl)-4-oxo-4H-chromen-7-yl-3-(3,4-dimethoxyphenyl) acrylate (CSH) and investigated the effect of CSH on atopic dermatitis (AD) by evaluating the anti-inflammatory effect of CSH on immune cells *in vitro* and on atopic dermatitis-like lesions *in vivo*.

Main methods: Human monocytic THP-1 cells and human eosinophilic EoL-1 cells were treated with house dust mite extract in the absence and presence of CSH. Nc/Nga mice were sensitized to 2,4-dinitrochlorobenzene (DNCB) for 5 weeks and then orally and dorsally administered with CSH or dexamethasone for 3 weeks.

Key findings: CSH inhibited the secretion of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6 and IL-8 due to house dust mite extract in THP-1 cells. CSH also suppressed the secretion of MCP-1 and IL-8 in EoL-1 cells. *In vivo*, the skin severity score decreased after CSH treatment as compared to the control group. CSH suppressed the inflammatory cell infiltration into the dermis and thickened the epidermis. CSH reduced serum IgE level as compared to the control group. The levels of IL-4, IL-5, IL-13 and eotaxin in mouse splenocytes increased after treatment with concanavalin A and the increase of the cytokines was decreased by pre-treatment with CSH. The inhibitory effects of CSH on atopic lesions of DNCB-treated Nc/Nga mice were similar to those of dexamethasone, despite differing degrees depending on results evaluated in this study.

Significance: These results may contribute to the development of a therapeutic drug for the treatment of AD.

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Introduction

Atopic dermatitis (AD) is characterized by chronically pruritic and inflammatory dermatitis and is increases annually in the world (Leung, 2000). AD is caused by a variety of pathogenic factors, which include environmental components such as allergen and genetic factors. House dust mite is one of the most common allergens and induces the production of immunoglobulin E (IgE), which is very important in the pathogenesis of allergic diseases such as AD (Bieber, 2008; Cookson and Moffatt, 2002; Marsella and Samuelson, 2009). The increase of total IgE or house dust mite-specific IgE in serum is an important factor

in the classification of AD such as extrinsic and intrinsic types of AD (Tokura, 2010). T helper 2 (Th2) lymphocytes are the main cells associated with the pathogenesis of AD (Romagnani, 1994). The cells excessively secrete Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13, which disrupt the Th1/Th2 balance and increase the production of IgE.

For the development of a drug for treatment of AD, the Nc/Nga mouse has been used as an animal model of AD (Shiohara et al., 2004; Han et al., 2012). The mouse produces AD-like skin lesions in non-specific pathogen-free (SPF) conditions. Chemicals such as 1-chloro-2,4-dinitrobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) enhance the clinical features of AD. We previously reported our results associated with an *in vitro* screening system to identify drug candidates for treatment of allergic diseases including AD (Lee et al., 2008, 2009).

Despite efforts to develop pharmacological drugs for the treatment of AD, the effective drug for AD remains elusive. Therefore, as part of our ongoing anti-atopic drug discovery project, we have synthesized a novel series of flavone compounds. From the structure–activity relationships, we found out a novel chemical, (E)-2-(3,4-dimethoxyphenyl)-4-oxo-4H-chromen-7-yl-3-(3,4-dimethoxyphenyl)acrylate (CSH), and examined the therapeutic effect of CSH in DNCB-sensitized Nc/Nga

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mouse as well as the inhibitory effect of CSH on cytokine production in immune cells.

Materials and methods

Preparation of CSH

The synthesis of CSH is depicted in Scheme 1. A solution of 2-(3,4-dimethoxyphenyl)-7-hydroxy-flavone (1) dissolved in dry methylene chloride was added to 3,4-dimethoxycinnamic acid (2), EDC and 4-dimethylaminopyridine (4-DMAP). The reaction mixture was stirred at room temperature for 1 day. Then, the solvent was removed in a rotary evaporator under reduced pressure and the resulting residue was purified by column chromatography using n-hexane:ethyl acetate in a ratio of 3:1 to produce yield CSH as a white solid. The characteristics of CSH are described as follow:

Yield: 74.5%, White solid, mp 121.5–122 °C, R_f = 0.35 (n-hexane:ethyl acetate = 1:2), ^1H NMR(CDCl_3) δ ppm 8.27 (d, J = 8.8 Hz, 1H), 7.88 (d, J = 16 Hz, 1H), 7.56 (dd, J = 2.0, 8.4 Hz, 1H), 7.53 (d, J = 2.0 Hz, 1H), 7.39 (d, J = 2.4 Hz, 1H), 7.25–7.20 (m, 2H), 7.14 (d, J = 2.0 Hz, 1H), 6.97 (d, J = 8.4 Hz, 1H), 6.92 (d, J = 8.4 Hz, 1H), 6.76 (s, 1H), 6.52 (d, J = 16 Hz, 1H), 3.99 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 3.95 (s, 3H); ^{13}C (CDCl_3) δ ppm 177.60(C-3), 164.82(C-1'), 163.58(C-1), 156.58(C-7), 154.73(C-9), 152.05(C-12), 151.77(C-6'), 149.27(C-13), 149.22(C-7'), 147.69(C-3'), 126.96(C-4'), 126.77(C-5), 123.94(C-9'), 123.34(C-10), 121.55(C-15), 119.96(C-4), 119.31(C-6), 113.83(C-2'), 111.08(C-14), 111.03(C-8'), 111.01(C-5'), 109.67(C-8), 108.63(C-11), 106.44(C-2), 56.05(C-OCH₃), 56.02(C-OCH₃), 56.00(C-OCH₃), 55.90(C-OCH₃); ESI-MS m/z 489.0842(M + H).

Cell culture

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA). The human eosinophilic leukemia cell line EoL-1 was obtained from the Riken Cell Bank (Tsukuba, Japan). Both cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$).

Viability assay

Viability was assayed based on the conversion of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using a cell proliferation kit (Roche, Penzberg, Germany). Both THP-1 cells and EoL-1 cells in 100 μl of culture medium were seeded into wells of a 96-well plate. CSH was added to each well as a concentration of 0.1 $\mu\text{g}/\text{ml}$ (0.2 μM), 1 $\mu\text{g}/\text{ml}$ (2 μM), or 10 $\mu\text{g}/\text{ml}$ (20 μM). After incubation for 24 h at 37 °C, 10 μl of MTT solution was added and incubated for 4 h. One hundred microliters of solubilization solution was added to each well. After 24 h incubation, the absorbance was measured at 550 nm using a BIO-TEK ELx808 enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, Winooski, VT).

ELISA

Cytokine release of THP-1 and EoL-1 cells was measured as we have previously detailed (Lee et al., 2008, 2009). After pre-treatment with

CSH for 30 min, THP-1 and EoL-1 cells were treated with the extract of house dust mite, *Dermatophagoides pteronissinus* supplied by the Korea National Arthropods of Medical Importance Resource Bank (Seoul, Korea). The concentrations of monocyte chemotactic protein-1 (MCP-1), IL-6, and IL-8 in the supernatant were measured by a sandwich ELISA using OptEIA Set (BD Biosciences, San Diego, CA), according to the manufacturer's instructions. For detection of cytokines released from mouse splenocytes, the cells were treated with concanavalin A (Sigma Korea, Seoul, Korea) for 24 h and the supernatants of the cells were collected. The concentrations of IL-4 and IL-5 in the supernatant were measured using OptEIA Set (BD Biosciences) and the concentrations of IL-13 and eotaxin were measured using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN). All assays were performed in triplicate. The cytokine concentration was calculated using a linear-regression equation obtained from the standard absorbance values.

AD induction in Nc/Nga mice

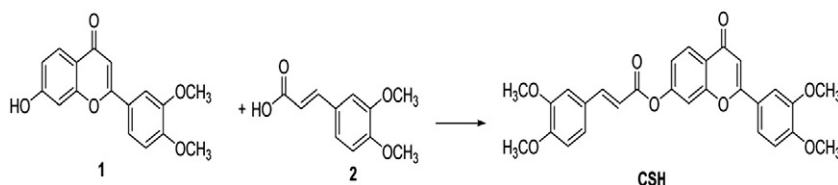
Five-week-old female NC/Nga mice (25 $\pm$ 2 g) were purchased from SLC Japan (Shizuoka, Japan) and were maintained for 1 week before the start of the experiments. They were housed in an air-conditioned animal room with a temperature of 23 $\pm$ 2 °C and a humidity of 50 $\pm$ 10%. Mice were provided with solid feed (CRF-1; Oriental Yeast, Tokyo, Japan). AD induction was developed by DNCB (Sigma Korea) in Nc/Nga mice. Briefly, one percentage of the DNCB was dissolved in acetone–olive oil mixture (acetone:olive oil = 3:1). The dorsal hair of the mice was shaved off with an electric razor, and skin damage such as chafed skin and hemorrhage was invisible. DNCB solution (1%, 0.15 ml) was applied at the same area of the dorsal skin. After the first sensitization, mice were dorsally treated with 0.3% DNCB at 1-week intervals for 4 weeks.

Drug administration and evaluation of skin severity

Nc/Nga mice were divided into four groups (n = 7 per group), which include the untreated group, control group, CSH group, and the dexamethasone (Dex) group. The control group, CSH group, and Dex group were dorsally administered with 1% DNCB and thereafter were dorsally treated with 0.3% DNCB for 4 weeks. The control group, CSH group, and Dex group were treated with phosphate buffered saline (PBS), CSH (8 mg/kg), and Dex (5 mg/kg) by gastric inoculation with an animal-feeding needle and applied at the same area of the dorsal skin for 3 weeks after sensitization with 0.3% DNCB, respectively. The untreated group was treated with PBS without drug administration of DNCB and the drugs. The severity of dermatitis was assessed macroscopically in a blinded fashion. Five signs of skin lesions: (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema, (4) excoriation/erosion, and (5) scaling/dryness were expressed as follows: 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe) (Leung, 1995).

Histological analysis

The dorsal skin of the mice was removed after they were sacrificed and fixed in Carnoy's solution, embedded into paraffin and sectioned. The paraffin sections were stained with hematoxylin–eosin solution, and examined by light microscopy for histological evaluation, depending on the epidermis (hypertrophy and infiltration by inflammatory cells) and on the corium (infiltration by inflammatory cells).



Scheme 1. Reagents and conditions: EDC, 4-DMAP, anhydrous methylene chloride, rt, 1 day.

Measurement of serum IgE level

Blood was collected from the retro-orbital plexus of the mice on the day of sacrifice. Serum obtained by centrifugation was stored at -70°C until required. Total IgE level in the serum was measured using sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Splenocyte preparation

Mice were sacrificed at 10 weeks after the drug treatment and their spleens were removed under aseptic conditions. The red blood cells (RBCs) were removed using RBC lysis solution (Sigma Korea) and splenocytes were collected after centrifugation. Isolated splenocytes were seeded in wells of a 24 well plate at a concentration of 5×10^6 cells/ml.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Statistical differences were analyzed using a one-way ANOVA. SPSS statistical software, Version 10.0 (SPSS, Chicago, IL) was used for statistical analysis. A significant value was considered as $p < 0.05$.

Results

CSH inhibits the secretion of MCP-1, IL-6, and IL-8 in THP-1 cells and the production of MCP-1 and IL-8 in EoL-1 cells

For evaluating the effect of CSH on immune cells *in vitro*, MTT assays were performed to determine the optimal concentration of CSH in THP-1 and EoL-1 cells. A maximal concentration (10 $\mu\text{g/ml}$) as well as low concentrations (0.1 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$) of CSH were not cytotoxic to THP-1 and EoL-1 cells during 24-h exposures (Fig. 1). The secretion of MCP-1, IL-6, and IL-8 increased after treatment with the extract of house dust mite, *D. pteronissinus* (Fig. 2A). In THP-1 cells, CSH significantly suppressed the increased production of MCP-1, IL-6, and IL-8 in a dose-dependent manner, despite different inhibitions for the particular cytokine ($p < 0.05$). In EoL-1 cells, the mite extract enhanced the expression of MCP-1, IL-6, and IL-8. MCP-1 and IL-8 increased after treatment with 0.1 $\mu\text{g/ml}$ CSH (Fig. 2B). However, CSH at a high concentration of 10 $\mu\text{g/ml}$ diminished MCP-1 and IL-8 as compared to the mite extract-treated cells. CSH upregulated the production of IL-6 in EoL-1 cells. Although CSH inhibited cytokine secretion of THP-1 and EoL-1 cells, the results indicated that CSH is a possible candidate chemical for treatment of AD.

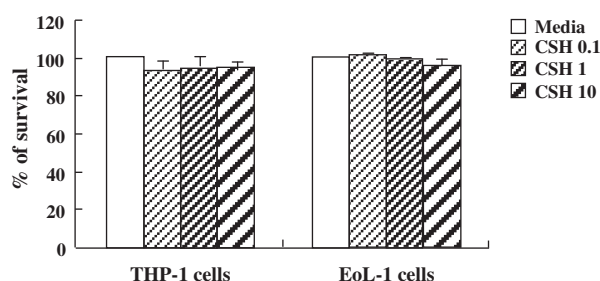


Fig. 1. CSH has no cytotoxicity in THP-1 cells and EoL-1 cells. THP-1 cells and EoL-1 cells were incubated in medium in the absence (media) or presence of CSH at the indicated concentrations for 24 h. The survival rate was measured by performing an MTT assay. The data are expressed as the relative ratio to the absorbance of the untreated cells, which was set at 100%. The data are expressed as the mean $\pm$ SD of three independent experiments.

CSH attenuates the severity of atopic-like skin lesion in DNCB-induced AD mice

We evaluated the anti-inflammatory effect of CSH on Nc/Nga mice as an AD mouse model *in vivo*. Nc/Nga mice were treated with DNCB for 5 weeks and thereafter CSH administered the mice for 3 weeks. As shown in Fig. 3, CSH treatment lowered the skin severity score as compared to the control group at 7–10 weeks and the score of CSH-treated group at 10 weeks was similar to that of the Dex-treated group. The body weight of the CSH-treated group was comparable to that of the control group. The weight decreased after Dex administration, in contrast to CSH administration (Fig. 3C).

CSH decreases histopathological features and serum IgE in DNCB-induced AD mice

For confirming the effect of CSH in AD mice, we performed lung histological analyses using hematoxylin and eosin staining and determination of serum IgE concentration by ELISA. The control group displayed hypertrophy, hyperkeratosis, and infiltration of inflammatory cells into lungs, and CSH treatment relieved the histological features of AD (Fig. 4A). The level of serum IgE increased in the control group as compared to the untreated group, and CSH treatment suppressed the increased IgE concentration in the serum (Fig. 4B). The effect of CSH treatment was comparable to that of Dex administration. These results indicate that CSH treatment relieved the histopathological alteration of AD mice and its effect is associated with the decreased level of serum IgE.

CSH inhibits the release of IL-4, IL-13, and eotaxin of mouse splenocytes increased by DNCB

We evaluated whether CSH regulated a cytokine secretion in DNCB-induced Nc/Nga mice. We separated the spleens from the mice of each group at 10 weeks after the first sensitization of DNCB and splenocytes were isolated from the spleens. After the treatment with concanavalin A for 24 h, the secretion of IL-4, IL-5, IL-13, and eotaxin in the control group is higher than the release in the untreated group (Fig. 5). CSH treatment significantly suppressed the secretion of IL-4, IL-13, and eotaxin ($p < 0.01$). The effect of CSH treatment was comparable to that of Dex administration. These results indicate that the increased production of the cytokine is associated with DNCB treatment and CSH treatment is effective on inhibition of cytokine production.

Discussion

In this study, the possibility of CSH as an anti-AD drug was examined for the first time using a human inflammatory cell line and a DNCB-induced AD mouse model. We demonstrated that (1) CSH inhibits the production of MCP-1, IL-6 and IL-8 in THP-1 cells; (2) CSH suppresses the secretion of MCP-1 and IL-8 in EoL-1 cells; (3) CSH reduces the severity score, histopathological alteration, and serum IgE in DNCB-induced AD mice; and (4) CSH inhibits production of IL-4, IL-13, and eotaxin in mouse splenocytes by CSH treatment.

AD is a chronic skin disease and its characteristics are an inappropriate epidermal-barrier function, a relapsing skin inflammation, IgE-mediated sensitization to environmental allergens such as house dust mite. Although drug development for treatment of AD has actively proceeded, steroid is a main drug for AD therapy until now. Because long-term administration of steroid evokes a variety of side effects, a new drug for AD treatment is needed. With this goal in mind, we examined the effect of CSH on AD *in vitro* and *in vivo*.

In the pathogenesis and progression of AD, the regulation of inflammatory cytokine production is an essential step. MCP-1/CCL2 functions as a potent chemoattractant of monocytes, and IL-6 plays

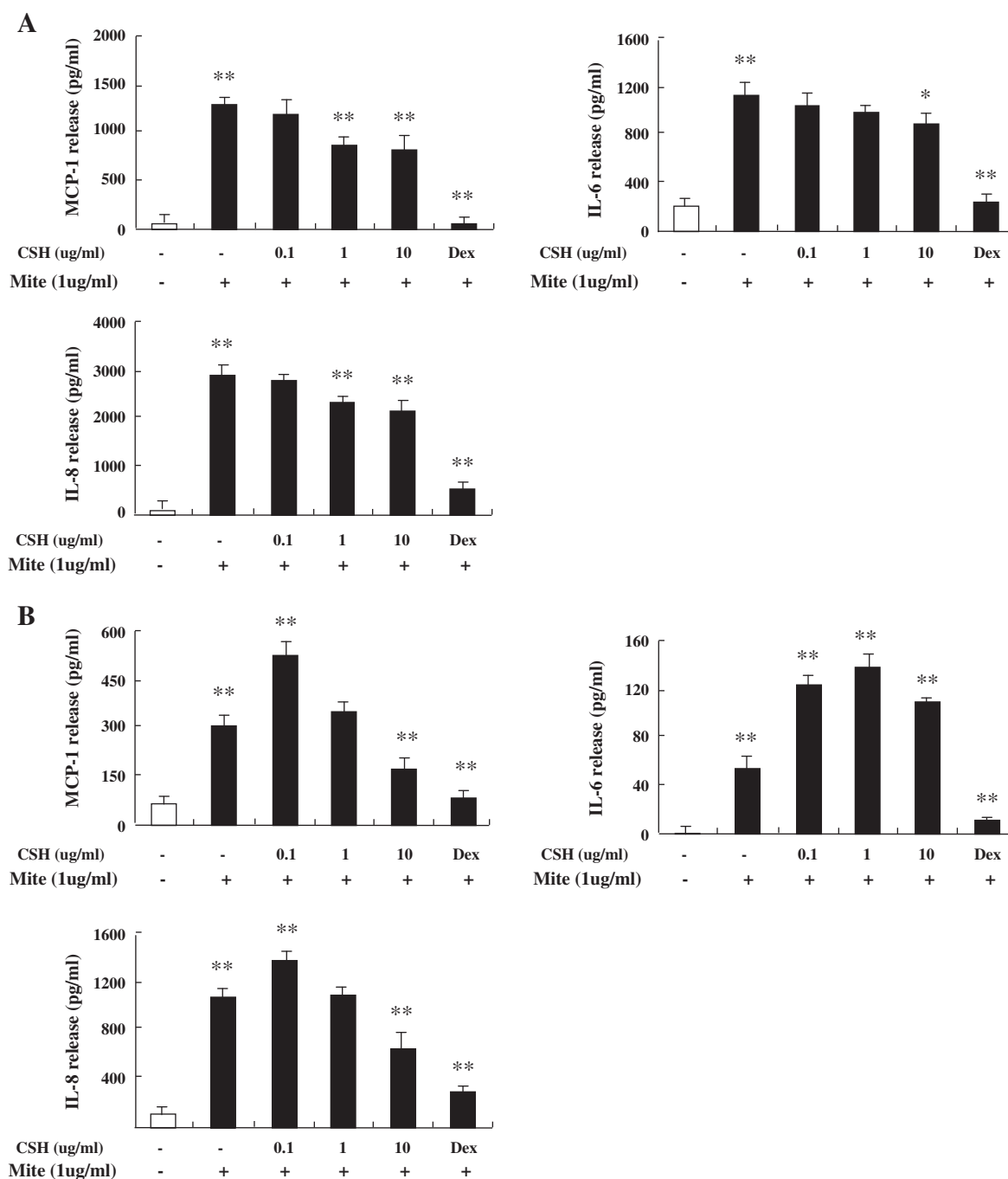


Fig. 2. CSH inhibits the production of MCP-1, IL-6, and IL-8 increased by the mite extract in THP-1 cells. THP-1 cells (A) and EoL-1 cells (B) were seeded in wells of 24 well plates and cultured in RPMI 1640 containing 0.5% FBS at 37 °C for 24 h. The cells were pre-treated in the absence or the presence of CSH at the indicated concentration or dexamethasone (Dex) at a concentration of 1 µg/ml for 24 h. Next, the cells were treated with 1 µg/ml of the mite extract for 24 h. The supernatant was then collected and analyzed by ELISA. The data are presented as the mean ± SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ were considered to indicate a significant difference between the untreated group and the mite only treated group, or between the mite only treated group and the drug-treated group.

as a key molecule in the acute and chronic phases of inflammation. In acute inflammation, IL-6 enhances the production of acute phase proteins and induces lymphocyte activation in chronic inflammation. IL-8/CXCL8 is a chemoattractant of neutrophils (Rossi and Zlotnik, 2000; Gabay, 2006; Shakoory et al., 2004). The house dust mite, *D. pteronissinus*, is a major allergen that causes the pathogenesis of allergic diseases including AD (Arlan and Platts-Mills, 2001; Friedmann, 1999). In this study, we used the *in vitro* screening system for evaluating the possibility of CSH for treatment of AD. As shown in Fig. 2, CSH significantly decreased the production of MCP-1, IL-6, and IL-8 in THP-1 cells in a dose-dependent

manner ($p < 0.05$) and diminished the secretion of MCP-1 and IL-8 in EoL-1 cells.

Based on the above results *in vitro*, we identified an anti-inflammatory function of CSH and analyzed the effect of CSH for treatment of AD *in vivo*. The Nc/Nga mouse model of AD has proven useful (Novak, 2009; Kim et al., 2011). For inducing AD lesion, Nc/Nga mice were not maintained in SPF conditions and the mice were administered with DNCB, consistent with previous reports (Im et al., 2011; Romagnani, 1994). As shown in Figs. 3 and 4, the untreated group had no clinical manifestation of AD and appeared as the normal skin state. Also, the untreated group showed

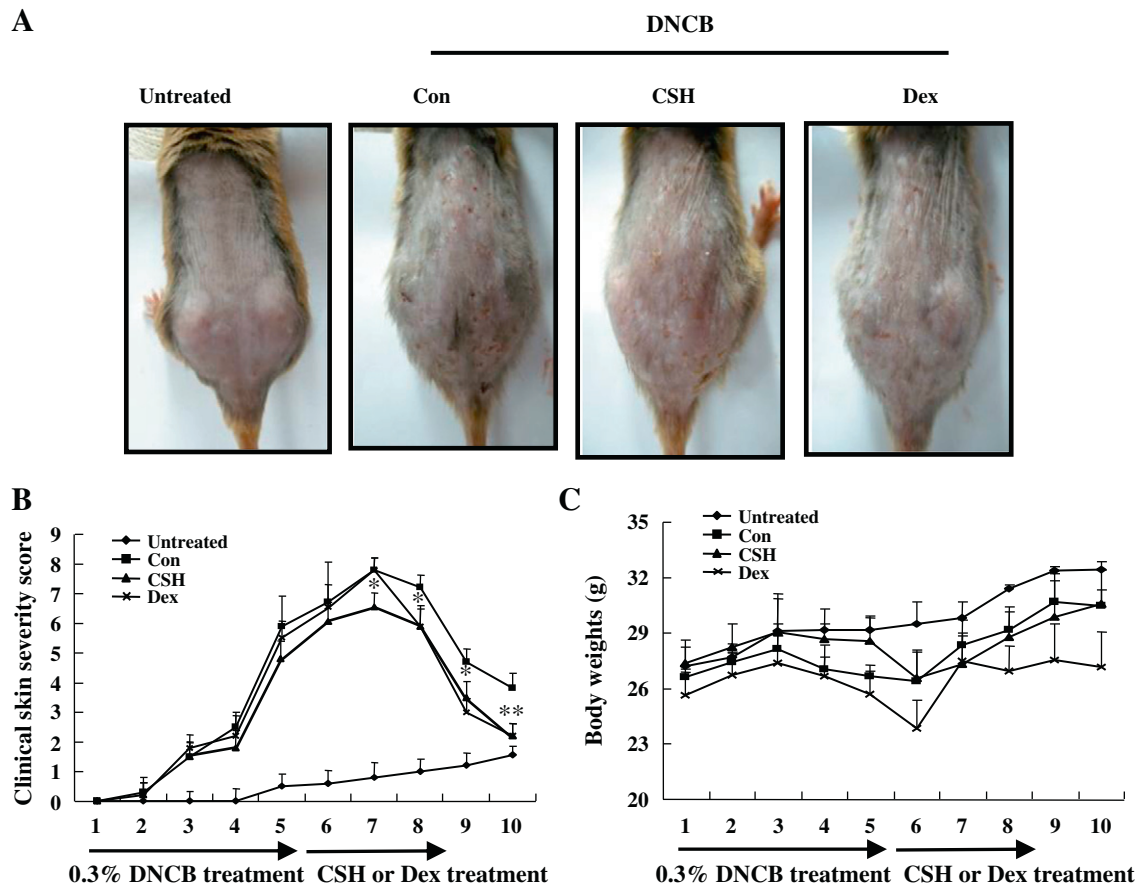


Fig. 3. CSH administration inhibits atopic dermatitis-like skin lesions of Nc/Nga mice after DNCB treatment. (A) The mice were divided into four groups ($n=7$ per group): untreated group (Untreated), control group (Con), CSH-treated group (CSH), and dexamethasone-treated group (Dex). The control group, CSH-treated group, and dexamethasone-treated group were dorsally administered 1% DNCB and thereafter were dorsally treated with 0.3% DNCB for 4 weeks. The control group, CSH-treated group, and dexamethasone group were treated with PBS, CSH (8 mg/kg) or dexamethasone (5 mg/kg) administered via gastric inoculation and applied to the same area of the dorsal skin for 4 weeks after sensitization with 0.3% DNCB. The untreated group was treated with PBS alone. (B) The severity of AD was assessed macroscopically in a blinded fashion. Five signs of skin lesions: (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema, (4) excoriation/erosion, and (5) scaling/dryness were scored as follows: 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe). (C) The mean body weight of the mice was measured using an electric scale.

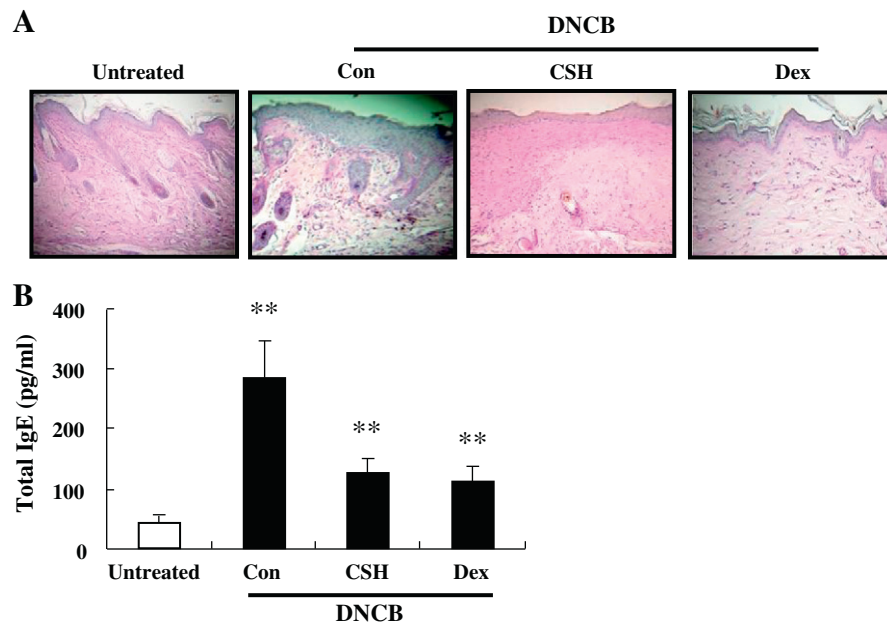


Fig. 4. CSH decreases histopathological features and serum IgE in DNCB-induced AD mice. (A) For histological analysis, the mice were sacrificed and their dorsal skin was removed. The fixed tissue was then cut after embedding in paraffin. The sections were then stained with hematoxylin and eosin solution, after which they were examined by light microscopy. (B) Serum was collected from the blood of Nc/Nga mice on the day of sacrifice. Total IgE level in the serum was measured using sandwich ELISA assay kits. $^{**}p<0.01$ was considered to indicate a significant difference between the untreated group and the control group or between the control group and the drug-treated group.

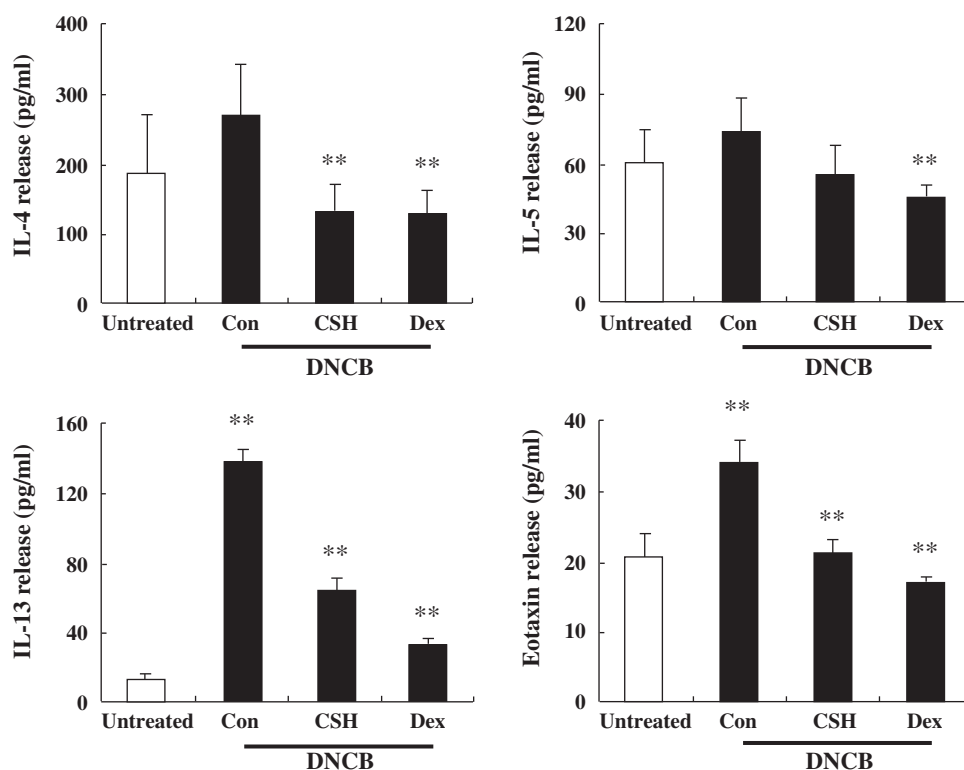


Fig. 5. The release of IL-4, IL-5, IL-13, and eotaxin was diminished in splenocytes from the CSH-treated group. Splenocytes were isolated from the spleen of each experimental group. The separated cells were then seeded in wells of a 24-well plate at a concentration of 5×10^6 cells/ml, after which they were treated with 1 μ g/ml concanavalin A. After the plate was incubated for 24 h, the supernatant was collected and analyzed by ELISA. The data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$ was considered to indicate a significant difference between the untreated group and the control group or between the control group and the drug-treated group.

the normal histological condition and IgE level in serum. The control group displayed increased clinical skin severity and serum IgE level, and histopathological skin lesion by DNCB treatment. The CSH-treated group displayed a low severity score, alleviation of histopathological features such as epidermis hypertrophy, and low serum IgE level as compared to the control group. The effect of CSH treatment was comparable to that of Dex administration, which, as a positive control, showed excellent effects on AD-like lesion. The body weight of CSH is similar to the weight of the control group, although Dex treatment lowered the body weight of the mice. Th1/Th2 cytokine balance is one of the various causes of AD (Romagnani, 1994; Suto et al., 1999). The results in Fig. 5 led us to investigate the effect of CSH on cytokine regulation in Nc/Nga mice. The control group displayed higher cytokine secretion than the untreated group after treatment with concanavalin A (Fig. 5). The CSH-treated group displayed significantly lower production of IL-4, IL-13, and eotaxin as compared to the control group. Since Th2 cytokines such as IL-4, IL-5, and IL-13 are associated with the up-regulation of IgE production, CSH may modulate serum IgE level by regulation of Th2 cytokine production (Yuk et al., 2007).

Conclusion

CSH suppresses the production of inflammatory cytokine of immune cells *in vitro* and alleviates the pathogenesis of skin lesion in an AD mouse model. CSH may be a promising drug candidate for the treatment of AD.

Conflict of interest statement

There is no conflict of interest.

Acknowledgments

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