

The Inhibitory Effect of *Duchesnea chrysantha* Extract on the Development of Atopic Dermatitis-like Lesions by Regulating IgE and Cytokine Production in Nc/Nga Mice

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Duchesnea chrysantha belongs to the Rosaceae family and has been used traditionally for the treatment of various diseases in Korea and other parts of East Asia. This study examined the antiinflammatory effect of *Duchesnea chrysantha* extract (DcE) on atopic dermatitis *in vitro* and *in vivo*. DcE inhibited the production of IL-6, IL-8 and MCP-1 in THP-1 cells and the release of IL-6 and MCP-1 in EoL-1 cells after treatment with house dust mite extract. In the *in vivo* experiment, Nc/Nga mice were sensitized to DNCB and then orally and dorsally administered DcE (50 mg/kg in PBS) for 3 weeks. The DcE administration significantly reduced the skin severity score when compared with the control group and inhibited the thickening of the epidermis and infiltration of inflammatory cells into the dermis. In addition, the serum IgE levels decreased markedly in the DcE-treated mice when compared with the control group. The synthesis of IL-5, IL-13, MCP-1 and eotaxin was also decreased in splenocytes of the DcE-treated group, while IFN- γ was increased in the Dc-administered group. These results may indicate that DcE attenuates the development of atopic dermatitis-like lesions by lowering the IgE and inflammatory cytokine levels, and that it is useful in drug development for the treatment of atopic dermatitis. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: *Duchesnea chrysantha*; atopic dermatitis; antiinflammatory effect; IgE.

INTRODUCTION

Atopic dermatitis (AD) is a common pruritic and inflammatory skin disease. It primarily occurs in children and is associated with hypersensitivity to allergens, high levels of serum immunoglobulin E (IgE) and genetic abnormality (Bieber, 2008; De Benedetto *et al.*, 2009; Barnes, 2010). Allergens such as house dust mites play an essential role in AD (Gavino *et al.*, 2008). The level of serum IgE increases in patients with AD and includes antibody to a variety of food and inhaled allergens (Gould and Sutton, 2008). Previous studies have reported an association between increased IgE production and T helper (Th) cytokines such as Th1 and Th2 in AD (Horan *et al.*, 1992; Habu *et al.*, 2001; Jin *et al.*, 2009). Because the exact pathogenic mechanism of atopic dermatitis in patients remains to be elucidated, general therapy for AD depends on antiinflammatory or immunosuppressive drugs; however, some drugs can elicit toxic side effects.

An animal model of AD has been developed for investigation of the pathogenesis of AD, as well as for drug development for its treatment. The mouse models of AD are primarily classified into three groups; one in

which AD is induced using an epicutaneous sensitizer, a transgenic model associated with the depletion of specific molecules, and a model representing spontaneous AD-like skin lesions (Jujo *et al.*, 1992). NC/Nga mice have been used widely in AD studies. When Nc/Nga mice have been kept in a conventional environment they exhibit clinical features similar to human AD such as edema, hemorrhage and erosion on the skin and up-regulation of the total IgE level in serum.

Duchesnea chrysantha is a perennial plant that belongs to the Rosaceae family. *Duchesnea chrysantha* has been used traditionally as a herb in East Asia for the prevention and treatment of a variety of diseases including asthma, congenital fever, toothache and cancer, and its water extract is known to be edible and non-toxic (Kim *et al.*, 2002; Kang *et al.*, 2008; Kim *et al.*, 2008). *Duchesnea chrysantha* extract (DcE) was shown previously to have antiinflammatory properties with no toxicity and to be effective in preventing asthma (Kim *et al.*, 2007). This study first examined the antiinflammatory effect of DcE *in vitro* and then investigated the effect of DcE on attenuation of the development of AD in Nc/Nga mice.

MATERIALS AND METHODS

DcE preparation. Whole plants of *Duchesnea chrysantha* Miq (Rosaceae) were collected in Okchon, Chungbuk,

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Korea, in October 2004 and identified by one of our co-authors, Professor J.-H. Kim. A voucher specimen and a standard extract have been deposited at the Herbarium of Department of the Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Daejeon, Korea and Division of Life Science, Daejeon University (TUT), Korea. The whole plants of *D. chrysanth* (36 g) were dried, powdered and then extracted with 80% ethanol (3 × 0.5 L) for 2 days at room temperature. The combined liquid extracts (4.84 g) were concentrated under reduced pressure.

Cell culture. The human monocytic cell line THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). The human eosinophilic leukemia cell line EoL-1 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). They were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL).

MTT assay. The MTT assay was performed to determine cell viability using the cell proliferation kit (Roche, Penzberg, Germany). Both THP-1 cells and EoL-1 cells in 100 µL of the culture medium were plated into a 96-well plate. DcE was added to each well at a concentration of 1, 10 or 100 µg/mL. After incubation for 24 h at 37 °C, 10 µL of MTT solution was added and incubated for 4 h. Solubilization solution (100 µL) was added to each well. After 24 h incubation, the absorbance was measured at 550 nm by using an ELISA reader (Bio-Tek Instruments, Winooski, VT).

Enzyme-linked immunosorbent assay (ELISA). Cytokine release of THP-1 cells and EoL-1 cells was measured using the method described by our reports (Lee and Yang, 1994; Lee *et al.*, 2008). After pretreatment with DcE for 30 min, the THP-1 cells and EoL-1 cells were treated with the extract of house dust mite, *D. pteronissinus*, supplied by Dr Tai-Soon Yong (Yonsei University College of Medicine, Seoul, Korea). The concentrations of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in the supernatant were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using an OptEIA Set (BD Biosciences, San Diego, CA), according to the manufacturer's instructions using an ELISA reader, Bio-Tek ELx808. For detection of the cytokines released from mouse splenocytes, the cells were treated with concanavalin A for 24 h and the supernatants of the cells were collected. The concentrations of IL-4, IL-5, IFN-γ and MCP-1 in the supernatant were measured using an 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.

Induction of allergic dermatitis in Nc/Nga mice. Five-week-old female Nc/Nga mice (25 ± 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 ± 2 °C and a humidity of

50 ± 10%. Mice were provided with solid feed (CRF-1, Oriental Yeast, Tokyo, Japan). Induction of AD was developed by 2,4-dinitrochlorobenzene (DNCB, Sigma, USA) in Nc/Nga mice. Briefly, 1% 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 there was no sign of skin damage such as chafed skin or hemorrhage. The 1% DNCB solution (0.15 mL) was applied to the same area of the dorsal skin. After the first sensitization, the mice were dorsally treated with 0.3% DNCB at 1 week intervals for 4 weeks.

Drug administration and evaluation of skin severity. The Nc/Nga mice were divided into four groups ($n = 7$ per group); they include the untreated group, the control group, the DcE group and the dexamethasone group. The control group, the DcE group and the dexamethasone group were dorsally administered with 1% DNCB and thereafter were dorsally treated with 0.3% DNCB for 4 weeks. The control group, the DcE group and the dexamethasone group were treated with PBS, DcE (50 mg/kg) and dexamethasone (5 mg/kg) by gastric inoculation with an animal-feeding needle and applied to the same area of 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 symptom), 1 (mild), 2 (moderate) and 3 (severe) (Lee *et al.*, 2009).

Measurement of serum IgE level. Blood was collected from the retro-orbital plexus of the mice on the day of killing. Serum was obtained by centrifugation and stored at −70 °C until required. Total IgE levels in the serum were measured using a sandwich enzyme-linked immunosorbent assay kit (R&D systems Inc.) following the manufacturer's instructions.

Splenocyte preparation. Mice were killed at 3 weeks after DcE treatment, and their spleens were removed under aseptic conditions. Splenocytes were prepared from the spleens according to a previous report (Leung, 1995), and the red blood cells were removed using RBC lysis solution (Sigma). Splenocytes were seeded in a 24-well plate at a concentration of 5×10^6 cells/mL.

Histological analysis. After the mice were killed, the dorsal skin was removed, fixed in carnoy solution, embedded in paraffin and sectioned. The 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).

Statistical analysis. The results were expressed as the mean ± SD. Statistical differences were analysed using a one-way ANOVA. SPSS statistical software, Version 10.0 (SPSS, Inc., Chicago, IL) was used for statistical analysis. A value of $p < 0.05$ was considered significant.

RESULTS

DcE suppresses the cytokine production of THP-1 cells and EoL-1 cells

The study first investigated the DcE to determine if it exerted a cytotoxic effect against THP-1 and EoL-1 cells. The DcE had no cytotoxic effect on these cells because cell viability was not altered after treatment for 24 h with DcE at concentrations in the range 1–100 µg/mL (Fig. 1). Next the inhibitory effect of DcE on cytokine expression that had been increased by treatment with mite extract was evaluated. As shown in Fig. 2, DcE increased the protein release of IL-6, IL-8 and MCP-1 from THP-1 cells and DcE strongly inhibited the protein release in a dose-dependent manner. The DcE even suppressed the upregulation of cytokines at a low concentration (0.1 µg/mL). In EoL-1 cells, DcE inhibited the upregulation

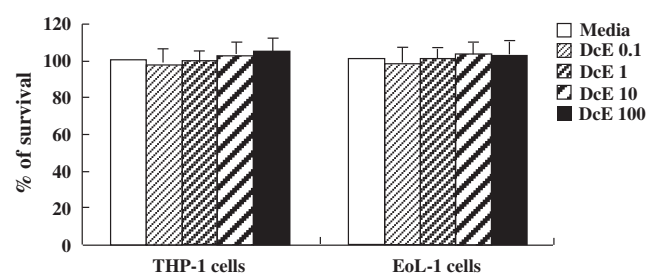


Figure 1. DcE exerts no cytotoxicity against THP-1 cells or EoL-1 cells. THP-1 cells and EoL-1 cells were incubated in the absence (media) or the presence of DcE at the indicated concentration for 24 h. The survival rate was measured by performing a MTT assay as described in the Materials and Methods section. 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.

of IL-6 and MCP-1 in response to mite extract in a fashion similar to the effect of DcE observed in THP-1 cells (Fig. 3). However, DcE increased IL-8 production in EoL-1 cells in a dose-dependent manner.

DcE decreases the aggravation of atopic-like skin lesion and diminishes the serum IgE level in DNCB-induced dermatitis mice

The therapeutic effect of DcE in DNCB-induced dermatitis mice was examined by use of a histological analysis and evaluation of the skin severity score. Histological evaluation showed hypertrophy, hyperkeratosis of the epidermis and infiltration of inflammatory cells in the untreated group (Fig. 4A). However, oral and dorsal administration of DcE ameliorated the histopathological alteration in a fashion comparable to the dexamethasone group. The development of symptoms of dermatitis such as mild eczema was observed in the control group, but treatment with DcE led to a significant decrease in the skin severity score when compared with the control group ($p < 0.01$) (Fig. 4B). Because IgE plays a central role as an inducer of allergic diseases such as AD, the suppressive effect of DcE on AD-like lesions was evaluated to determine if it is associated with regulation of IgE expression. In DNCB-induced mice, the serum IgE level was strongly upregulated when compared with the untreated group (Fig. 4C). DcE decreased the elevated IgE level in serum (approximately 43%), and the inhibitory effect of DcE was comparable to that of dexamethasone. Moreover, the body weight was not altered in any of the experimental groups (Fig. 4D). Taken together, these results indicate that oral and dorsal administration of DcE inhibits the pathogenic development of AD in DNCB-induced NC/Nga mice.

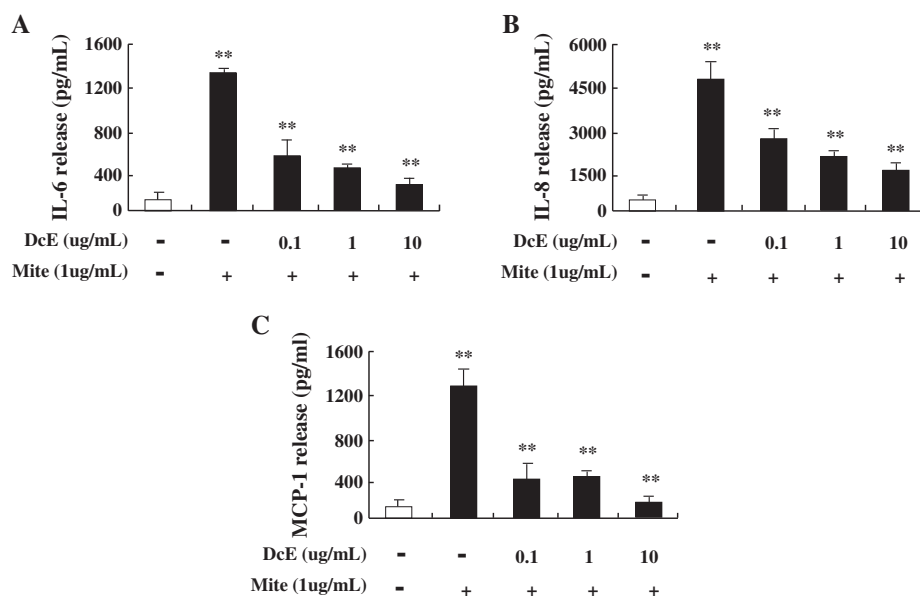


Figure 2. DcE inhibits the increased production of IL-6, IL-8 and MCP-1 after treatment with house dust mite extract in THP-1 cells. The THP-1 cells were seeded into 24-well plates and cultured in RPMI 1640 containing 0.5% FBS at 37 °C for 24 h. The cells were pretreated in the absence or presence of DcE at the indicated concentration for 24 h. Next, the cells were treated with 1 µg/mL of the mite extract for 24 h. The supernatant was collected and analysed by ELISA as described in the Materials and Methods section. 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 mite only treated group or between the mite only treated group and the DcE treated group.

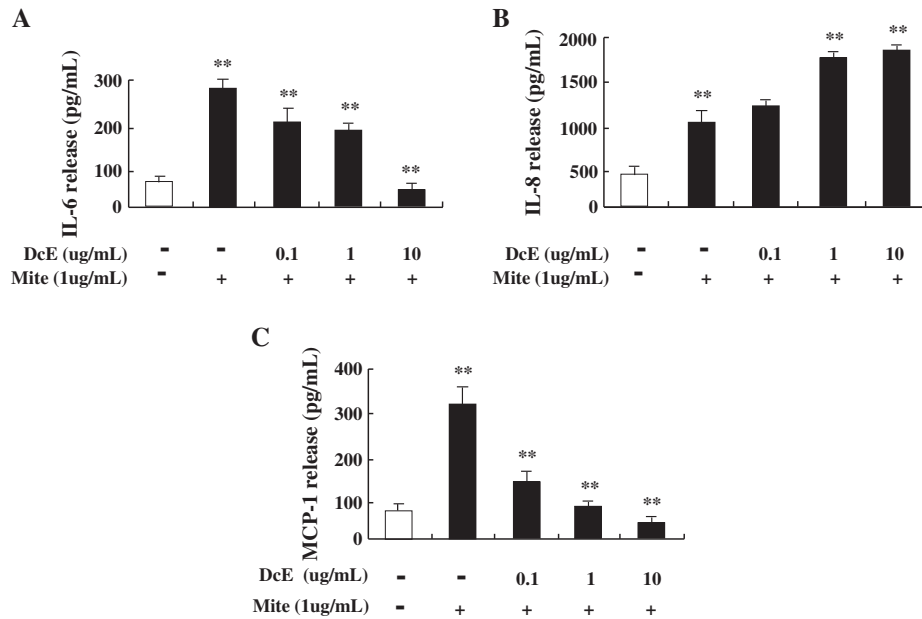


Figure 3. DcE inhibits the increased production of IL-6 and MCP-1 after treatment with the mite extract in THP-1 cells. EoL-1 cells were seeded into 24-well plates and cultured in RPMI 1640 containing 0.5% FBS at 37 °C for 24 h. The cells were pretreated in the absence or the presence of DcE at the indicated concentration 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 analysed by ELISA as described in the Materials and Methods section. The data are presented as the mean ± SD of three independent experiments. ** $p < 0.01$ was 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 DcE treated group.

DcE inhibits the synthesis of cytokines and chemokines in mouse splenocytes

To clearly identify the effect of DcE in DNCB-induced Nc/Nga mice, the splenocytes from spleens of mice at 8 weeks after the first DNCB sensitization were isolated and then treated with concanavalin A for 24 h. Following treatment, the splenocytes of the control group showed increased synthesis of the measured cytokines (IL-4, IL-5 and IL-13) and chemokines (MCP-1 and eotaxin) when compared with those of the untreated group (Fig. 5). The synthesis of IL-5, IL-13, MCP-1 and eotaxin was diminished in the splenocytes of the DcE group and IL-4 release was not altered in the splenocytes of the DcE group. The level of the Th1 cytokine, IFN- γ , was lower in the splenocytes of the control group when compared with the untreated group. The splenocytes of the DcE group inhibited IFN- γ production when compared with the control group, and this IFN- γ production was similar to that of the untreated group. Taken together, these results indicate that DcE regulates the expression of cytokines and chemokines in the clinical state of AD.

DISCUSSION

Here, it is reported for the first time that treatment with the DcE alleviates the clinical features of AD in Nc/Nga mice after DNCB induction. Oral and dorsal administration of DcE strongly decreased the serum level of total IgE in DNCB induced Nc/Nga mice. In the *in vitro* study, DcE was found to suppress both IL-6 and MCP-1 expression in THP-1 cells and EoL-1 cells, as well as to decrease the production of IL-5, IL-13 MCP-1 and eotaxin in the splenocytes of mice after concanavalin A treatment.

Duchesnea chrysantha belongs to the Rosaceae family and has been used for the treatment of asthma, congenital fever and cancer in Oriental medicine in Korea. Recently, Yang *et al.* demonstrated that DcE inhibits ovalbumin-induced lung inflammation in an asthmatic mouse model (Kim *et al.*, 2007). Before examining the effect of DcE on AD, the DcE was investigated to determine if it inhibited cytokine production in the human monocytic cell line, THP-1, and the human eosinophilic cell line, EoL-1, to evaluate its possibility for use as a drug candidate for the treatment of AD. The usefulness of the *in vitro* screening system for identifying a drug candidate was reported (Lee and Yang, 1994; Lee *et al.*, 2008). As shown in Figs 2 and 3, DcE inhibited IL-6, IL-8 and MCP-1 in the mite-treated THP-1 cells and suppressed IL-6 and MCP-1 in the mite-treated EoL-1 cells without cytotoxicity, although DcE increased the IL-8 expression in EoL-1 cells.

Nc/Nga mice have been used recently as an animal model of AD and are closely associated with clinical features of human AD (Matsumoto *et al.*, 2001; Novak, 2009). Although some Nc/Nga mice spontaneously develop atopic dermatitis outside of the specific pathogen free (SPF) environment, house dust mites such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, as well as haptens such as picryl chloride and DNCB have been applied to Nc/Nga mice in many studies (Reinhold *et al.*, 1990; Jujo *et al.*, 1992; Robinson *et al.*, 1992). As shown in Figs 4 and 5, the untreated group did not show any clinical features of AD, including histopathological lesions or changes in the clinical skin severity and total IgE level in serum. After induction by treatment with DNCB, the clinical signs and symptoms of AD appeared in conventional Nc/Nga mice. Specifically, the total severity score was increased by DNCB treatment and decreased by treatment with either DcE

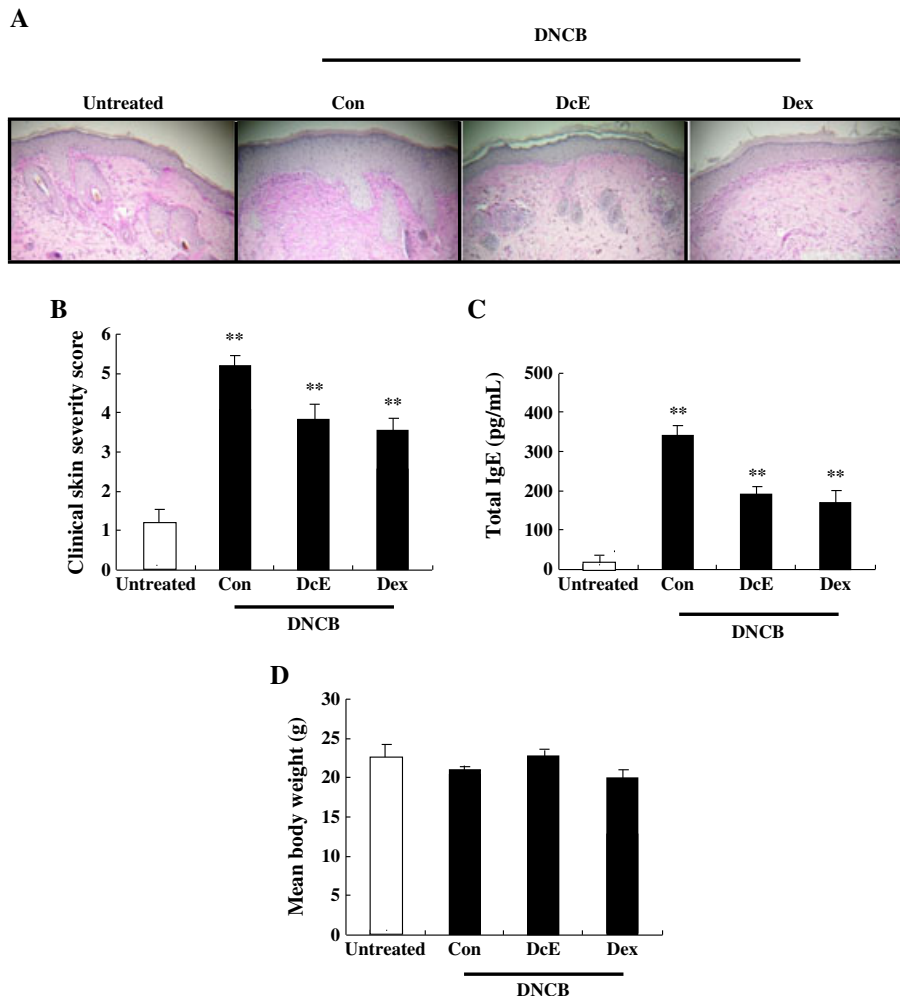


Figure 4. Oral and dorsal administration of DcE inhibits atopic dermatitis-like skin lesions in DNCB-treated NC/Nga mice. The mice were divided into four groups ($n = 7$ per a group): an untreated group, a control group, a DcE group and a dexamethasone group. The control group, the DcE group and the dexamethasone group were dorsally administered 1% DNCB and thereafter were dorsally treated with 0.3% DNCB for 4 weeks. The control group, the DcE group and the dexamethasone group were treated with PBS, DcE (50 mg/kg) and dexamethasone (5 mg/kg) administered via gastric inoculation and applied to the same area of dorsal skin for 4 weeks after sensitization with 0.3% DNCB. The untreated group was treated with PBS alone. (A) For histological analysis, the mice were killed 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) 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 scored as follows: 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe). (C) Serum was collected from the blood of NC/Nga mice on the day of killing. Total IgE levels in the serum were measured using sandwich enzyme-linked immunosorbent assay kits as described in the Materials and Method section. (D) The mean body weight of the mice was measured using an electric scale. 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 DcE-treated group. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

or dexamethasone as a positive control (Fig. 4B). Upon histopathological analysis, DcE strongly inhibited inflammatory features such as epidermis hypertrophy, intracellular edema and infiltration of inflammatory cells, which were observed in the control group. This effect of DcE was comparable to that of dexamethasone (Fig. 4A).

Atopic dermatitis is caused by various factors such as genetic polymorphism, dysregulation of immune cells, innate immune dysfunction and Th1/Th2 cytokine balancing (Romagnani, 1994; Suto *et al.*, 1999; Yang *et al.*, 2008; Sicherer and Leung, 2010). IgE functions as one of the most important molecules leading to the development of atopic allergy. After IgE binds allergens, the Fc ϵ receptor in mast cells and basophils binds the allergen-IgE complex, which induces degranulation

via intracellular signaling. IgE production is upregulated by Th2 cytokines such as IL-4 and IL-13 (Yuk *et al.*, 2007). Both cytokines enhance B cell proliferation and induce immunoglobulin class switching for IgE production. IL-5, as another Th2 cytokine, also increases the proliferation of B cells and eosinophils. IFN- γ is produced in Th1 cells and directly inhibits IgE synthesis of B cells. The results from Fig. 4C led us to investigate whether Th1 and Th2 cytokines are involved in the mechanism by which DcE decreases IgE production. To accomplish this, splenocytes were isolated from the mouse groups and treated with concanavalin A. The release of IL-5 and IL-13 was downregulated in the splenocytes from the DcE-treated group when compared with the control group (Fig. 5). IFN- γ production was increased in the DcE group.

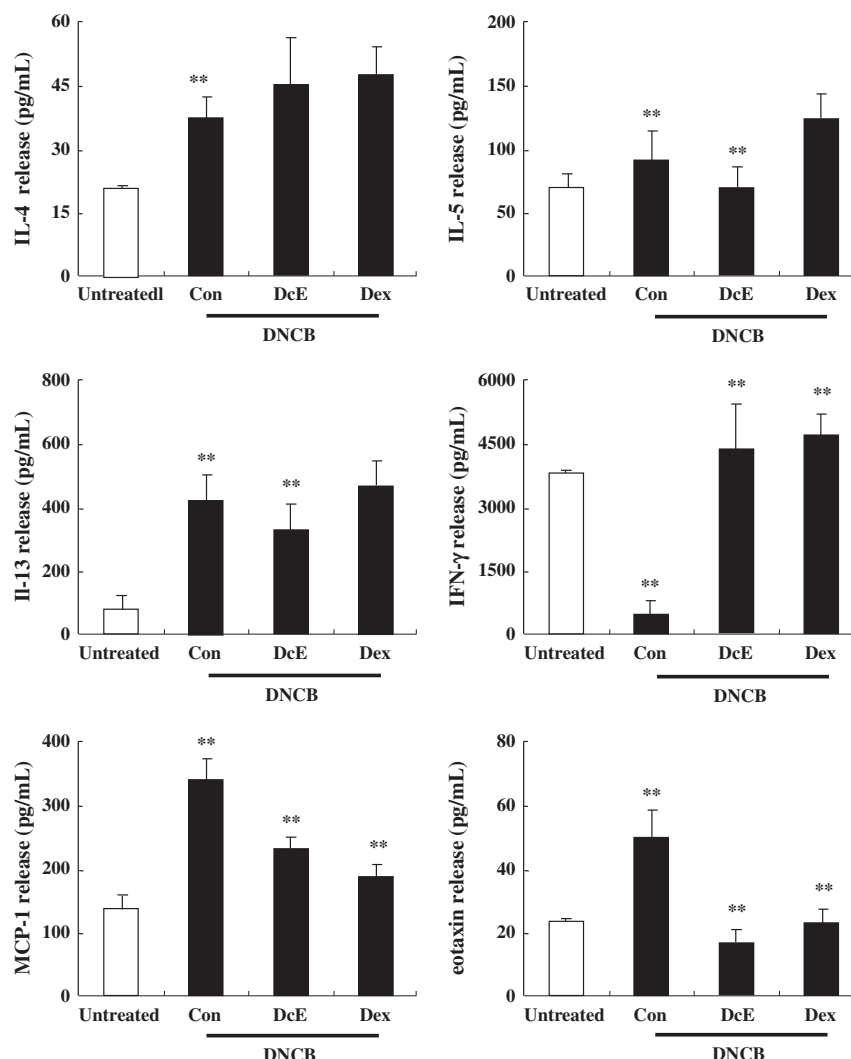


Figure 5. The release of cytokines and chemokines was altered in splenocytes from the DcE-treated group. Splenocytes were isolated from the spleen of each experimental group. The separated cells were then seeded in a 24-well plate at a concentration of 5×10^6 cells/mL, after which they were treated with 1 µg/mL concanavalin A. After the plate was incubated for 24 h, the supernatant was collected and analysed by ELISA as described in the Materials and Methods section. 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 DcE-treated group.

These results indicate that DcE decreases IgE production by regulating Th1 and Th2 cytokines. In addition, the alteration of MCP-1, a potent monocyte chemoattractant, and eotaxin, a powerful eosinophil chemokine, released from the splenocytes was examined. The secretion of both chemokines was found to be downregulated in the DcE-group when compared with the control group.

In conclusion, it was demonstrated that DcE inhibits inflammatory cytokines and chemokines *in vitro* and that it alleviated the pathogenesis of skin damage in a mouse model of AD using Nc/Nga mice. Taken together, these results indicate that DcE has the

potential for use as a therapeutic drug for the treatment of AD.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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