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Topical application of *Rehmannia glutinosa* extract inhibits mite allergen-induced atopic dermatitis in NC/Nga mice

Yoon-Young Sung[a], Taesook Yoon[a,*], Ja Young Jang[a], Sang-Joon Park[b], Ho Kyoung Kim[a]

[a] Center of Herbal Resources Research, Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea
[b] Department of Veterinary Histology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

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ABSTRACT

Aim of the study: Rehmannia glutinosa is known in Asia as a traditional herbal medicine with anti-inflammatory properties. Atopic dermatitis (AD) is an inflammatory skin disease associated with enhanced T-helper 2 (Th2) lymphocyte responses to allergens that results in elevated serum IgE levels and leukocyte infiltration. Although some studies have shown that *Rehmannia glutinosa* extract (RGE) has anti-inflammatory and anti-allergic activities, these properties have not been demonstrated in AD. This study investigated the effectiveness of RGE as a therapeutic candidate in an AD model as well as its underlying mechanism of action.

Materials and methods: The effects of RGE on mite allergen (*Dermatophagoides farinae*)-treated NC/Nga mice were evaluated by skin symptom severity, ear thickness, production of serum IgE and histamine, and expression of cytokines, chemokines, and adhesion molecules in the ear lesions. In addition, the levels of thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and regulated on activation, normal T cell expressed and secreted (RANTES) produced in both TNF-α- and IFN-γ-stimulated human keratinocytes were investigated by enzyme-linked immunosorbent assay (ELISA).

Results: RGE treatment of NC/Nga mice significantly reduced dermatitis scores, ear thicknesses, and serum histamine levels. Histological analyses demonstrated decreased thickening of the epidermis/dermis as well as dermal infiltration by inflammatory cells. In the ear lesions, mRNA expression levels of IL-4, TNF-α, VCAM-1, and ICAM-1 were inhibited by RGE treatment. RGE also suppressed the production of TARC, MDC, and RANTES in both the ear lesions and keratinocytes.

Conclusions: RGE inhibits the development of AD in NC/Nga mice by suppressing the expression of cytokines, chemokines, and adhesion molecules.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is occurring in infants and children with an increasing incidence (Spergel and Paller, 2003). In AD, the clinical symptoms are characterized by pruritic and relapsing eczematous skin lesions, which are distinguished by the infiltration of inflammatory cells, such as lymphocytes, macrophage, eosinophils, and granulated mast cells (Soter, 1989; Leung and Bieber, 2003). T-helper 2 (Th2) cells, producing IL-4, IL-5, and IL-10, play major roles in the onset and development of AD (Leung et al., 2004; Homey et al., 2006). Although Th2 cells are dominant during the acute phase of AD, IFN-γ-producing Th1 cells are highly expressed and contribute to pathogenesis during the chronic phase (Vestergaard et al., 1999).

Topical glucocorticoids are very effective remedies for the treatment of AD. However, it is well known that long-term use of glucocorticoids frequently causes significant adverse effects (Barnetson and White, 1992; Leung and Barber, 2003). Tacrolimus, an immunosuppressant, has been evaluated for AD treatment in ointment form (Nakagawa et al., 1994; Bieber, 1998; Inagaki et al., 2006; Rustin, 2007). The effect of tacrolimus ointment in a dust mite allergen-induced AD model was investigated in NC/Nga mice (Sasakawa et al., 2004; Oshio et al., 2009). Recently, results from several studies indicated that AD patients might benefit from herbal Oriental medicine therapy (Gao et al., 2005; Lee et al., 2006; Park et al., 2007; Kim et al., 2008; Makino et al., 2008).

Rehmannia glutinosa, which belongs to the family of Scrophulariaceae, is a traditional, medicinal herb. It is believed that *Rehmannia glutinosa* possesses the effects of hemostasis, activation of blood circulation, and improvement of kidney function (Zhang et al., 2008).

* Corresponding author at: Center of Herbal Resources Research, Korea Institute of Oriental Medicine, 483 Expo-ro, Yuseong-gu, Daejeon 305-811, Republic of Korea. Tel.: +82 42 868 9529.
E-mail address: tsyoon@hotmail.com (T. Yoon).

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A steamed root of *Rehmannia glutinosa*, known as 'Sook-Ji-Whang' in Korean, has been used in traditional Oriental medicine for treating the allergic inflammatory disease (Huang, 1993). An aqueous extract from a steamed root of *Rehmannia glutinosa* (RGAE) inhibits the secretion of both TNF-α and IL-1 from mouse astrocytes, indicating that RGAE has anti-inflammatory effects on the central nervous system (Kim et al., 1999). In addition, RGAE has been shown to inhibit both compound 48/80-induced systemic allergic and passive cutaneous anaphylaxis (PCA) reactions, two typical models of immediate-type allergic reaction (Kim et al., 1998). In a veterinary study, a mixture of plant extracts (*Rehmannia glutinosa*, *Paeonia lactiflora* and *Glycyrrhiza uralensis*) reduced erythema and pruritus in canine AD (Ferguson et al., 2006). Chemically, *Rehmannia glutinosa* was found to contain iridoid glycosides such as catalpol, aucubin and ajugol (Oshio and Inouye, 1982). Aucubin inhibits 12-O-tetradecanoylphorbol- 13-acetate (TPA)-induced mouse ear edema and production of TNF-α and IL-6 in mast cells (Recio et al., 1994; Jeong et al., 2002). Recently, it has been reported that beta-sitosterol, one of the major constituents of *Rehmannia glutinosa* (Ni et al., 1992), inhibits ovalbumin-induced airway inflammation in a guinea pig asthma model (Mahajan and Mehta, 2011). These results suggest that *Rehmannia glutinosa* might be beneficial in the treatment of human allergic disorders.

Therefore, this study investigated the effects of *Rehmannia glutinosa* extract (RGE) on AD using NC/Nga mice exposed to dust mite allergens as an AD model. We also determined the effects of RGE on chemokine production in the human keratinocyte cell line, HaCaT, to examine a possible mechanism for the anti-allergic effects of RGE.

2. Materials and methods

2.1. Preparation of Rehmannia glutinosa extract (RGE)

The steamed root of *Rehmannia glutinosa* as a dried herb was purchased from Omniherb Co. (Yeoungcheon, Korea) and authenticated based on its macroscopic characteristics by the Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM). The committee was composed of nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. A voucher specimen (KIOM 79081) was deposited in the herbarium of the Department of Herbal Resources Research at KIOM. The dried herb (50 g) was extracted twice with 70% ethanol (with a 2-h reflux), and the extract was then concentrated under reduced pressure. The decoction was filtered, lyophilized, and serially stored at 4 °C. The yield of dried extract from starting crude materials was approximately 26.82% (w/w). The extract was dissolved in either saline or phosphate-buffered saline (PBS) for experiments.

2.2. Experimental animals

Male 8-week-old NC/Nga mice were purchased from SLC, Inc. (Hamamatsu, Japan) and maintained for 1 week prior to experiments. Animals were housed in an air-conditioned animal room with a 12-h light/12-h dark cycle at a temperature of 22 ± 1 °C and a humidity of 50 ± 10%. Mice were provided with a laboratory diet and water *ad libitum*. All experimental protocols involving the use of animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication No. 85-23, revised 1996), and approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Institute of Oriental Medicine (KIOM).

2.3. Induction of dermatitis

Mice were anesthetized with ether, and the hair on the upper back of each animal was shaved with a clipper and a shaver 1 day before the experiments. The exposed dorsal region was treated with ointment (Biostir-AD, Biostir, Kobe, Japan) prepared from a crude extract of house dust mite *Dermatophagoides farinae* (DfE) as described previously (Yamamoto et al., 2007). The DfE (mite extract, lyophilized) was mixed in ointment base (hydrophilic petrolatum) to make a 0.5% solution, referred to as DfE ointment. The ointment base (hydrophilic petrolatum) was applied to normal mice instead of DfE ointment. For topical application, barrier disruption was achieved using 150 μl of a 4% sodium dodecyl sulfate (SDS) treatment on the shaved dorsal skin and both surfaces of each ear 3 h before DfE ointment application. One hundred micrograms of DfE ointment were then applied on the shaved dorsal skin twice a week for 3 weeks. SDS treatment was repeatedly performed prior to each DfE ointment application. Protopic ointment containing 0.1% tacrolimus in ointment base (Astellas Pharma Inc., Deerfield, IL, USA) was used as a positive control for treating AD. Mice were randomly divided into four groups: (1) normal group without DfE application (8 mice); (2) control group with DfE application (100 mg/mouse) (8 mice); (3) RGE-treated group (400 μg/mouse) with DfE application (7 mice); and (4) protopic-treated group (100 μg/mouse) with DfE application (7 mice). The lyophilized RGE powder was dissolved in an acetone/olive oil (4:1) mixture for topical application. The same volume of acetone/olive oil vehicle was applied to normal and control group instead of RGE solution. On the day of fourth DfE application (day 11), RGE treatment was started. RGE was treated daily for 23 days. Following the last application of RGE (day 33), the mice were killed to investigate immunological and histological changes (day 34). Ear thickness was measured twice a week using a micrometer (Mitutoyo Corporation, Kanagawa, Japan).

2.4. Evaluation of skin severity

Severity of dermatitis on the ear and back regions was evaluated twice a week. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores comprised the dermatitis score (Matsuda et al., 1997).

2.5. Measurement of serum total IgE and histamine levels

After blood was collected from the mice after sacrifice, serum samples were obtained by centrifugation (1700 × *g*, 10 min) and were stored at −70 °C until use. Total serum IgE levels were measured using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions. Histamine levels were measured using a mouse histamine ELISA kit (Oxford Biomedical Research Inc., Oxford, MI, USA).

2.6. Histological analysis

Ear samples of NC/Nga mice were removed, fixed in 10% formalin, embedded in paraffin, and serially sectioned at 4 μm. Sections were stained with a hematoxylin/eosin solution. Histological changes were examined by light microscopy.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression levels of mRNA transcripts of cytokines and chemokines in the ear lesion were determined by RT-PCR. Tis-

Table 1
Sequences of primers used RT-PCR analysis.

Genes	Forward	Reverse	Accession number	Length (bp)
IL-4	TCAACCCCCAGCTAGTTGTCA	CATCGAAAAGCCCGAAAGAG	NM_021283	313
TNF-α	CCTGTAGCCCACGTCGTAGC	TTGACCTCAGCGCTGAGTTG	NM_013693	373
TARC	CAGGAAGTTGGTGAGCTGGTATA	TTGTGTTCGCCTGTAGTGCATA	NM_011332	300
RANTES	GCTCCAATCTTGCAGTCGTGTT	ATTTCTTGGGTTTCGTGGTCG	NM_013653	283
MDC	TCTGATGCAGGTCCCTATGGT	TTATGGAGTAGCTTCTTCAC	NM_009137	207
ICAM-1	CCTCTGCTCCTGGCCCTGGT	CGGACTGCTGTCCTCCCCGA	NM_010492	237
VCAM-1	TCGCGGTCTTGGGAGCCTCA	TCGCGGTCTTGGGAGCCTCA	MM_011693	213
GAPDH	AAGCTGTGGCGTGATGGCCG	TGGGCCCTCAGATGCCTGCT	NM_008084	228

sues were homogenized, and total RNA was isolated using the easy-BLUE total RNA extraction kit (Intron, Seoul, Korea) according to the manufacturer's instructions. For cDNA synthesis, 1 μg total RNA was mixed with Maxime RT premix (Intron) containing oligo-dT primers and DEPC-treated water to a final volume of 20 μl and incubated at 45 °C for 60 min. The reaction was terminated by heat inactivation at 95 °C for 5 min. cDNAs were then amplified with gene-specific primers using a Taq PCR master mix kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The 20-μl amplification mixture was composed of 1 μl cDNA and 10 μl of a 2× *Taq* PCR master mix containing 1.5 mM $MgCl_2$, 0.1 μM each primer, and water. After a 15-min preincubation at 94 °C, PCR amplification was performed for 35 cycles as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. Primers were designed using primer3 software, and the sequences are currently available in the GenBank database. Table 1 shows the sequences of the gene-specific PCR primers. The relative expression levels of target genes were normalized using GAPDH as an internal control.

2.8. Reagents and cell culture

Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin, PBS (pH 7.4), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). Human recombinant IFN-γ and human recombinant TNF-α were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Human keratinocyte cell line HaCaT was kindly provided from Dr. H. K. Shin (Korea Institute of Oriental Medicine, Daejeon, Korea). HaCaT cells were maintained in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 μg/ml penicillin. HaCaT cells were cultured in 96-well plates and stimulated with 10 ng/ml human recombinant IFN-γ and 10 ng/ml human recombinant TNF-α in the presence or absence of several doses (0–200 μg/ml) of RGE for 24 h as previously described (Vestergaard et al., 2000). Culture supernatants were collected and stored at −70 °C for ELISA.

2.9. Measurement of chemokine production by ELISA

The levels of TARC, MDC, and RANTES in the culture supernatant of HaCaT cells were determined using a human ELISA kit (R&D Systems Inc.). Briefly, capture antibody was added to wells of a 96-well plate, which was then blocked with 1% BSA in PBS. Supernatants were then added, and the plate incubated for 2 h at room temperature (RT). After washing, detection antibody was added, and the plate was then incubated for 2 h at RT. After washing, streptavidin-HRP conjugate was added to each well, and the plate was then incubated for 20 min at RT, followed by the addition of substrate solution and incubation for 20 min at RT. Finally, stop solution was added, and the optical density of each well was measured at 450 nm using a Benchmark plus microplate spectrophotometer (BioRad, Hercules, CA, USA).

2.10. Statistics

The differences between groups were examined using an unpaired Student's *t*-test. All data were presented as means ± S.E.M. Significant differences were accepted when the *p* value was less than 0.05.

3. Results

3.1. Effects of RGE on development of dermatitis in NC/Nga mice exposed to mite allergen

To establish a mite allergen-induced AD model, DfE was repeatedly applied twice a week to the dorsal skin of NC/Nga mice. The skin conditions were evaluated twice a week for 34 days using the dermatitis severity score. Fig. 1A and B shows the dermatitis scores and clinical features of NC/Nga mice treated with mite allergen. The dermatitis score of the control mice increased rapidly and significantly after 7 days compared with that of normal mice ($p < 0.001$). However, in RGE-treated mice, the DfE-induced increase in the dermatitis score was significantly decreased after 21 days with respect to that in control mice ($p < 0.05$) (Fig. 1A). As shown in Fig. 1B, repeated application of DfE ointment in control mice first induced skin dryness, followed by erythema, hemorrhage, edema, scarring, erosion, and excoriation. However, the application of RGE as

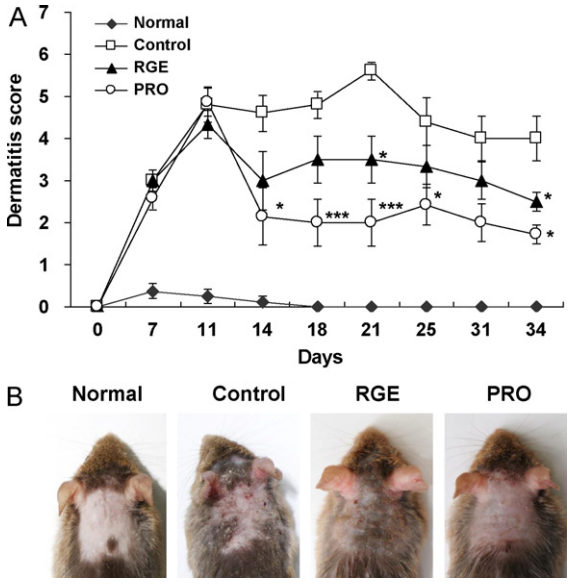

Fig. 1. Effects of RGE on the development of DfE-induced AD in NC/Nga mice. The DfE-induced dermatitis mouse model was divided into four groups: untreated (normal), DfE treatment (100 mg/mouse; control), Protopic treatment (100 μg/mouse; PRO), or RGE treatment (400 μg/mouse; RGE). (A) Dermatitis scores and (B) clinical features were assessed using the criteria described in Section 2. The photograph was taken on day 34. Values are expressed as the means ± S.E.M. ($n = 7–8$). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control.

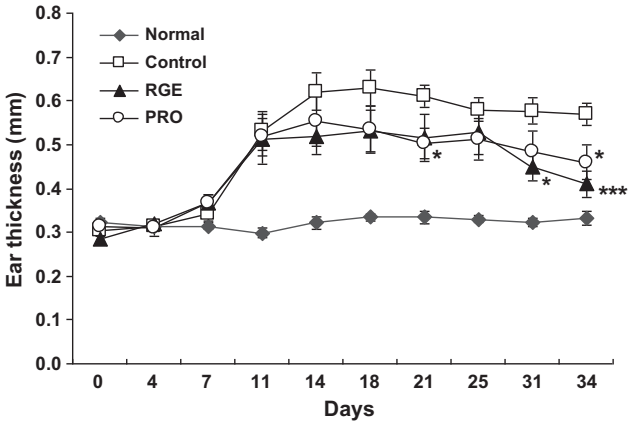

Fig. 2. Effects of RGE on DfE-induced increase in ear thickness in NC/Nga mice. Mice were pretreated with RGE for 30 min before DfE treatment (100 mg/mouse). DfE was administered twice a week for 3 weeks. Ear thickness was measured twice a week for 34 days. Data are presented as mean ± S.E.M. (n = 7–8). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control.

tacrolimus ointment inhibited these symptoms of AD. These results indicate that RGE suppresses spontaneously induced AD in NC/Nga mice.

3.2. Effects of RGE on ear swelling after DfE application

After the third application of DfE to the ears, ear swelling gradually increased. As shown in Fig. 2, ear thickness significantly increased after 11 days of DfE application ($p < 0.001$). However, RGE treatment moderately suppressed DfE-induced ear swelling. No significant difference was observed between RGE-treated mice and Protopic-treated mice.

3.3. Histological analysis

Fig. 3 shows the histopathological features of the ear lesions in DfE-treated NC/Nga mice. On day 34 after the first application of DfE ointment, it was found that lesioned skin showed a thickening

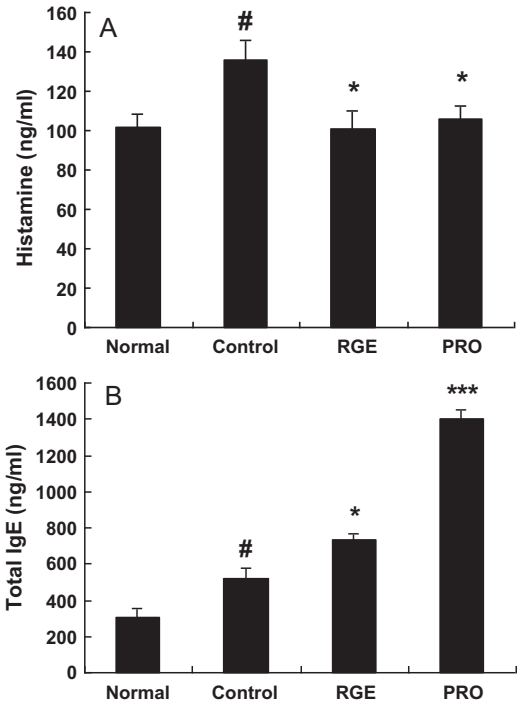

Fig. 4. Effects of RGE on serum histamine and total IgE levels. Serum was collected 24 h after the last RGE treatment. (A) Serum histamine and (B) total IgE levels were analyzed by ELISA. Values are expressed as the means ± S.E.M. (n = 7–8). $^{\#}p < 0.05$ vs. normal; $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control.

of the dermis and epidermis and an accumulation of inflammatory cells in DfE-treated control mice compared with the skin of normal mice. However, RGE- and Protopic-treated mice showed decreased thickening of the epidermis/dermis and dermal infiltration by inflammatory cells.

3.4. Effects of RGE on serum histamine and IgE levels

Histamine, the main content of mast cell granules, exerts many effects related to the immediate phase of allergic inflam-

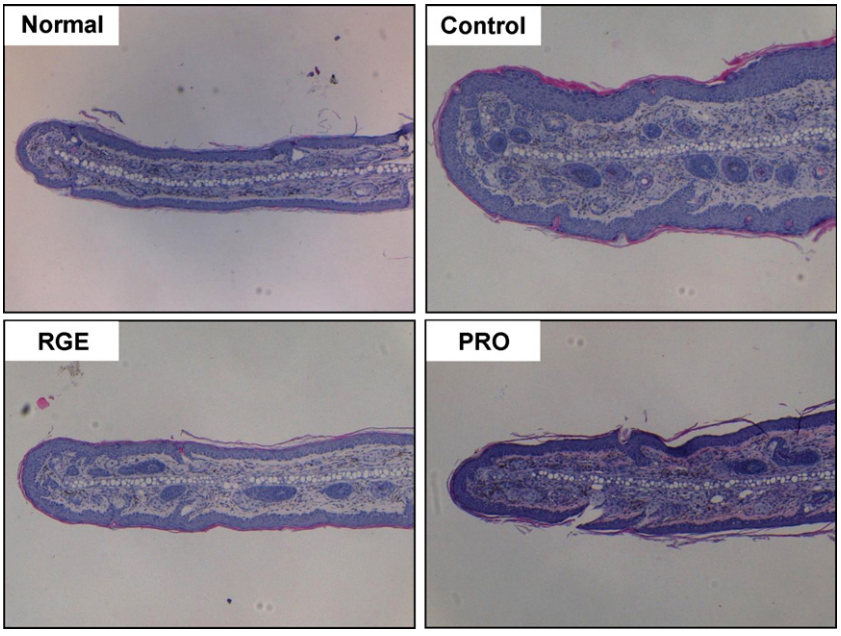

Fig. 3. Histological features of the ears of NC/Nga mice. The ears were excised, fixed with 10% formaldehyde, embedded in paraffin, and sectioned. The sections were stained with H&E (magnification 100×).

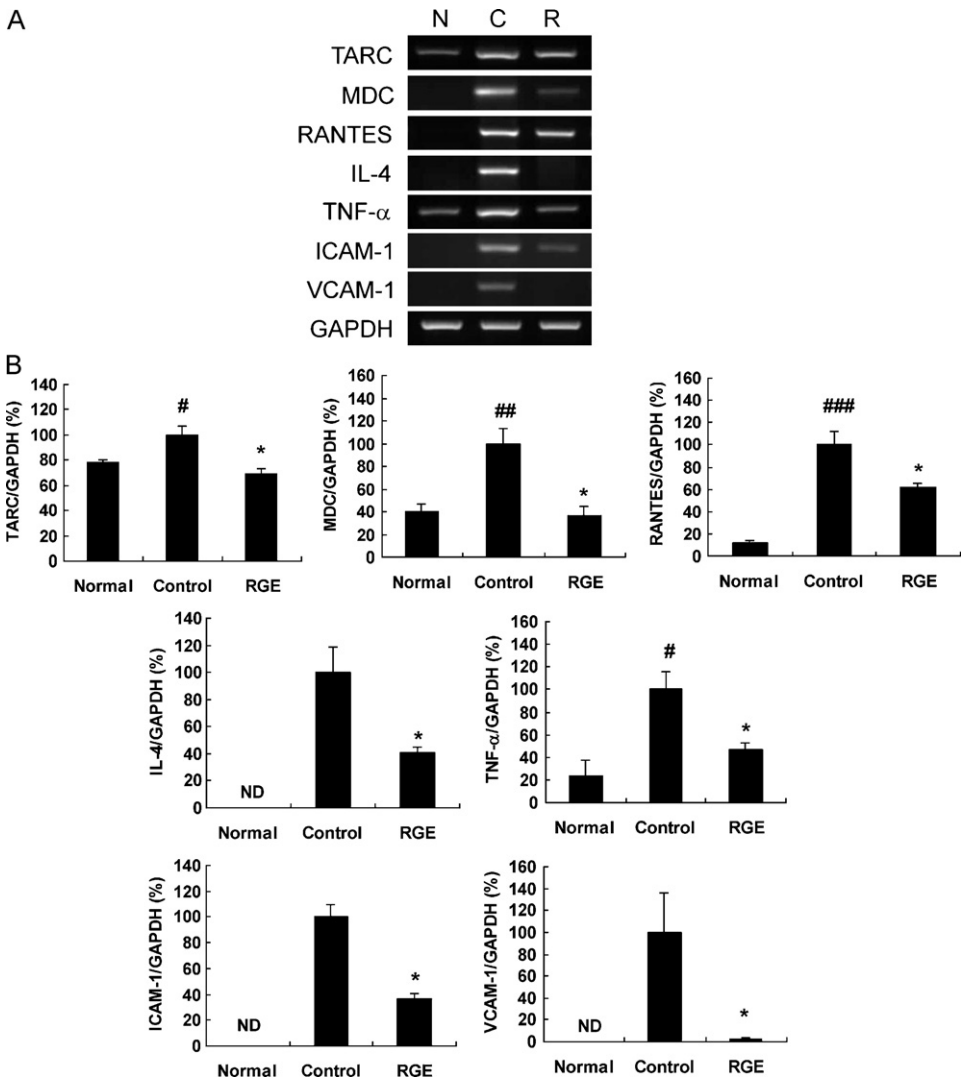

Fig. 5. Effects of RGE on DfE-induced mRNA expression levels of cytokines, chemokines, and adhesion molecules in the ear lesions of NC/Nga mice. (A) The mRNA expression levels of TARC, MDC, RANTES, IL-4, TNF-α, ICAM-1, and VCAM-1 were determined. Total RNA was prepared from the ears of mice treated with DfE or RGE for 34 days, and RT-PCR was performed. Typical results are shown from five independent analyses. (B) The intensity of PCR bands was measured using the NIH ImageJ program. Values are expressed as the means ± S.E.M. ($n = 5$). ND, not detected. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. normal; $^{*}p < 0.05$ vs. control.

mation, including vasodilation, increase vascular permeability, tissue erythema, bronchial and gastrointestinal contraction, and increased mucus production (Guo et al., 1997; White, 1999). As shown in Fig. 4A, application of mite allergen for 34 days in control mice enhanced serum histamine levels compared with normal mice, whereas RGE treatment as tacrolimus ointment significantly reduced histamine levels compared with control mice ($p < 0.05$).

An increase in serum IgE levels is an important component of AD. Previous studies have reported that serum IgE levels are elevated in DfE-treated NC/Nga mice with AD-like skin lesions (Matsuda et al., 1997; Matsuoka et al., 2003; Gao et al., 2004). Thus, we investigated whether RGE application decreased serum IgE levels after DfE treatment in NC/Nga mice. A significant increase in serum IgE levels was observed in the DfE-treated group. Unexpectedly, RGE application did not reduce serum IgE levels but instead increased IgE levels, similar to the tacrolimus ointment (Fig. 4B). These results indicate that the RGE-mediated suppression of DfE-induced AD-like skin lesions is independent of DfE-induced serum IgE levels.

3.5. Effects of RGE on mRNA expression of cytokines, chemokines, and adhesion molecules in the ear lesions

We investigated the effects of RGE on the mRNA expression levels of cytokines, chemokines, and adhesion molecules, which are important markers involved in the pathogenesis of AD, in the ear lesions of NC/Nga mice. As shown in Fig. 5A and B, the mRNA expression levels of inflammatory cytokines IL-4 and TNF-α were markedly increased after DfE application, but their expression levels were reduced in the RGE-treated group. It was also observed that RGE application inhibited DfE-induced expression of the Th2 chemokines TARC and MDC as well as RANTES in the ears of mice. Moreover, the expression levels of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), were markedly increased by DfE application that was suppressed by RGE treatment. These results reveal that RGE treatment downregulated the levels of cytokines, chemokines, and adhesion molecules, leading to inhibition of skin inflammation caused by the infiltration of inflammatory cells.

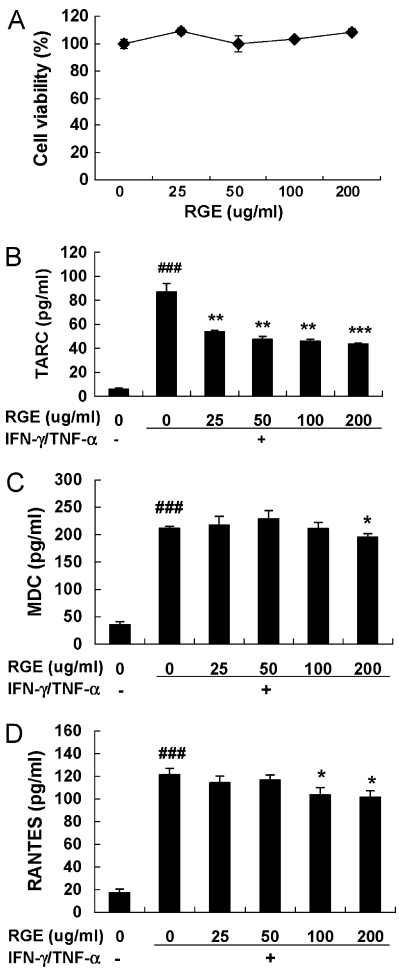

Fig. 6. Effects of RGE on IFN-γ- and TNF-α-induced chemokine production in HaCaT cells. (A) Cell viability. Cells were cultured in 96-well plates and stimulated with or without the extracts (0–200 μg/ml). After 24 h, cell viability was measured using the MTT assay. Production of TARC, MDC, and RANTES in IFN-γ- and TNF-α-stimulated HaCaT cells was measured using an ELISA kit according to the manufacturer's directions (B–D). The cells were cultured in 24-well plates and stimulated with human recombinant IFN-γ (10 ng/ml) and human recombinant TNF-α (10 ng/ml) in the presence or absence of RGE (0–200 μg/ml) for 24 h. Values represent the means ± S.E.M. of three independent experiments. $^{###}p < 0.001$ vs. normal (IFN-γ/TNF-α untreated); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control (IFN-γ/TNF-α alone).

3.6. Effects of RGE on chemokine production in HaCaT cells treated with IFN-γ and TNF-α

Chemokines including TARC, MDC, and RANTES are thought to be involved in the infiltration of inflammatory cells to inflamed sites (Kaburagi et al., 2001; Kakinuma et al., 2002; Shimada et al., 2004). Because RGE suppressed the development of dermatitis in mite antigen-treated NC/Nga mice, we examined whether RGE affected the production of chemokines in human keratinocytes after stimulation with IFN-γ and TNF-α.

To evaluate the cytotoxic effects of RGE, an MTT assay was performed with HaCaT cells. The cells were stimulated in the absence or presence of RGE (0–200 μg/ml) for 24 h. Fig. 6A shows that RGE does not affect cell viability and is not toxic to HaCaT cells.

When HaCaT cells were stimulated with 10 ng/ml IFN-γ and 10 ng/ml TNF-α for 24 h, TARC levels increased 14-fold compared with the vehicle group. However, in RGE-treated HaCaT cells, TARC production was significantly inhibited in a dose-dependent manner ($p < 0.01$) (Fig. 6B). MDC and RANTES production increased 6- and 7-fold, respectively, in IFN-γ/TNF-α-stimulated HaCaT cells, and their levels were significantly reduced after RGE treatment ($p < 0.05$) (Fig. 6C and D). From these results, it appears reasonable to suggest that RGE treatment prevents AD by inhibiting the infiltration of inflammatory cells, such as Th2 cells and eosinophils.

4. Discussion

In this study, we evaluated the effects of RGE on skin inflammation in AD mice. In NC/Nga mice, RGE significantly suppressed the development of AD-like skin lesions induced by dust mite extract. Histological analyses demonstrated that the infiltration of leukocytes into the ear lesions decreased after RGE treatment. These results indicate that RGE exerts therapeutic effects in AD model mice.

AD has been described as a Th2-type disease, at least during the initiating phase, as lymphocytes that infiltrate the skin primarily produce IL-4, IL-5, and IL-10 (Vestergaard et al., 1999). The chemokine receptor predominantly expressed on Th2 cells is CC chemokine receptor 4 (CCR4), and the ligands of this receptor are CCL17/TARC and CCL22/MDC (Oshio et al., 2009). TARC and MDC have been shown to be related to the Th2-mediated disease process of AD (Kakinuma et al., 2002; Shimada et al., 2004; Jahnz-Rozyk et al., 2005). It was reported that TARC produced by keratinocytes recruits lymphocytes into AD skin lesions (Vestergaard et al., 2000). In vitro, TARC and MDC are secreted by HaCaT cells after stimulation with IFN-γ and/or TNF-α (Xiao et al., 2003; Kobayashi and Tokura, 2005). In addition, the levels of CC chemokines, such as CCL5/RANTES and eotaxin, are increased in the skin lesions of AD patients and contribute to the migration of eosinophils and mast cells via CC chemokine receptor 3 (CCR3) (Taha et al., 2000). In the present study, RGE treatment significantly inhibited the expression of these chemokines in the ear lesions of NC/Nga mice. In addition, production of TARC in keratinocytes was effectively inhibited in a dose-dependent manner after RGE treatment. Adhesion molecules, such as VCAM-1 and ICAM-1, are also known to be involved in the infiltration of leukocytes into sites of inflammation. It was demonstrated that VCAM-1 and ICAM-1 contribute to the accumulation of mast cells and neutrophils in dust mite extract-induced AD mice (Kang et al., 2007). These adhesion molecules are induced by the proinflammatory cytokine TNF-α at sites of inflammation (Ackermann and Harvima, 1998; de Vries et al., 1998). TNF-α in mast cells has been shown to induce the chemotaxis of neutrophils and T cells, and is an important inflammatory mediator of the allergic response (Männel and Echtenacher, 2000). Therefore, we examined the effect of RGE on the expression of TNF-α, VCAM-1, and ICAM-1; the levels of these molecules were decreased after RGE treatment. These results imply that RGE suppresses the expression of cytokines, chemokines, and adhesion molecules, leading to the inhibition of leukocyte migration to sites of inflammation.

In the present study, repeated DfE treatment resulted in the elevation of serum IgE levels and increased expression of IL-4 mRNA in the ear lesions. Elevated total serum IgE levels are a hallmark of AD, and the expression of IL-4 contributes to this elevation. It is well known that IL-4 (a Th2-type cytokine) is responsible for class switching to the ε heavy chain for IgE antibody production by B cells (Kishimoto and Hirano, 1988). However, neither RGE nor tacrolimus reduced the increased serum IgE levels (Fig. 4B), although they both suppressed the expression of IL-4 mRNA in the ear lesions (Fig. 5). Previously, other studies reported that tacrolimus, an immunosuppressant, increased IgE synthesis in both humans and rodents (Kawamura et al., 1997; Nagai et al., 1997; Inagaki et al., 2006). Furthermore, serum IL-4 levels were below the level of detection (data not shown), suggesting that locally expressed IL-4 in the ear lesions does not contribute to systemic IgE production. Recently, it was reported that neither IgE production

nor Th2 cells are necessary for the development of an AD-like skin disease in NC/Nga mice under conventional conditions (Yagi et al., 2002). It was shown that the IFN-γ-favored skin microenvironment could be a result of AD pathogenesis in NC/Nga mice. Furthermore, it was reported that the repeated application of DfE induces IFN-γ, a Th1-type cytokine (Yamamoto et al., 2007). In addition, IFN-γ is known to play a role in determining the chronicity of AD lesions (Tomimori et al., 2005). Thus, further studies are needed to determine whether the suppression of DfE-induced AD-like skin lesions by RGE treatment is related to IFN-γ, although IFN-γ expression in the ear lesions was not detected (data not shown). Furthermore, it would be interesting to evaluate the effects of long-term RGE treatment. In a short-term study (34 days) with RGE, no effect on serum IgE levels was observed, but prolonged treatment might have different effects.

5. Conclusion

The topical application of RGE prevented the development of dust mite allergen-induced AD in NC/Nga mice. We also demonstrated that the inhibition of the expression of cytokines, chemokines, and adhesion molecules and the subsequent blockade of leukocyte accumulation might be responsible for the inhibitory effects of RGE on AD-like skin lesions. The results from this study suggest that RGE may be used as a therapeutic agent for the treatment of AD.

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