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Inhibitory Effects of Ginsenoside Rb_1 on Atopic Dermatitis-Like Skin Lesions in Mice

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Allergies are immediate hypersensitive responses to antigens and interleukin (IL)-4 is involved in the initiation and development of allergic responses. Rb_1 has been known to have a variety of biological activities including anti-inflammatory activity, but the effect of Rb_1 on allergic responses is not known yet. The present study was undertaken to examine whether Rb_1 has an inhibitory effect on allergic response in mouse model. In allergic mouse model, our results showed that topical application of Rb_1 on atopic dermatitis (AD)-like skin lesions improved skin condition and inhibited starching behaviors. In addition, Rb_1 application not only suppressed mRNA expression of IL-4 and IL-10, but also prevented the nuclear factor of activated T cells 1 transcription. Moreover, Rb_1 application suppressed IL-4's secretion. Taken together, these results suggest that Rb_1 has a potent inhibitory effect in AD-related T cell cytokine production and may be a candidate for therapeutic agent in allergy.

Keywords: Ginsenoside Rb_1, Atopic dermatitis, Scratching behaviors, Interleukin-4

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied by several common symptoms including erythema, eczema, and pruritus [1]. In AD, skin becomes extremely itchy followed by severe scratching behavior that induces the production of proinflammatory cytokines. This in turn activates immune cells and initiates inflammatory cycle of AD that accompanied by erythema, keratosis and its scaling. All these symptoms are the consequence of an imbalanced immune response to various allergens [2]. Another defining characteristic of the atopic immune system is the capacity to generate elevated IgE antibodies and type 2 helper T cells (T_H2) are critical for IgE synthesis. T_H2 cells predominantly produce interleukin (IL)-4, IL-5, IL-10, and IL-13, and these cytokines are associated with specific function of immune cells in AD. For example, they act as excellent helpers for B cell activation, particularly antibody responses [3-5]. IL-4, mainly produced by activated T cell, plays a prominent role in immune responses. It also works as the most important determinant of IgE isotype switching in B cell, which in turn elicits allergic immune response, and induces differentiation of naïve CD4 T cells into T_H2 cells [6,7]. Expression of IL-4 appears to be regulated at the transcriptional level and the nuclear factor of activated T cells (NFAT) has been suggested as a transcriptional activator of cytokine genes [8,9].

Red ginseng (*Panax ginseng* C.A. Meyer Radix Rubra) has been most widely used for a long time to enhance immune function as well as stamina, and it has been reported that ginseng has pharmacological effects on the cardiovascular, endocrine, and central nervous systems [10]. Among the components of ginseng,

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ginsenosides appear to be responsible for most of its pharmacological activities. More than 40 ginsenosides have been identified and each ginsenoside may have different biological effect for structural difference. Rb_1, Rb_2, Rg_1, Rg_3, and Rh_2 are the most commonly studied ginsenosides in research and hundreds papers have been reported that ginsenosides show various biological activities including anti-inflammatory, anti-allergic, and anti-tumor activities [11-13]. However, anti-atopic effect of Rb_1 *in vivo* has not been thoroughly studied. The aim of the present study therefore was to determine whether topical application of Rb_1 has inhibitory effects on AD-like skin lesions in animal model.

MATERIALS AND METHODS

Animals and reagents

Six-week-old male BALB/c mice were obtained from the Daehan Biolink Co. (Eumseong, Korea) and housed with free access to food as well as water ad libitum. During the experimental period, All animals were maintained at a temperature of 23±1°C, a humidity of 55±5% with 10 to 18 circulation/hour under 12 hours light/dark cycle. The animals were randomly divided into five groups with 5 mice. Group 1, normal control group; group 2, vehicle-treated group; group 3, dexamethasone-treated group; and group 4 and 5, Rb_1-treated groups. All reagents including Rb_1 were purchased from the Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated. The chemical structure of Rb_1 was shown in Fig. 1.

Induction and treatment of atopic dermatitis-like skin lesions in mice

1-chloro-2,4-dinitrobenzene (DNCB) was used as an inducer of AD. Induction of AD-like skin lesions was performed according to the method of Kim *et al*. [14] with modification. Briefly, 1% of DNCB was freshly dissolved in aceton:olive oil (4:1) and topically applied on the shaved area of dorsal surface twice for a week, and 0.2% of DNCB was then applied in a same way (four times in total). After inducing AD-like skin lesions, skin lesions were treated with Dulbecco's phosphate buffered saline vehicle, dexamethasone (positive control), or Rb_1 (50 and 100 μg/mL) every other day (four times in total). The concentrations of Rb_1 were selected based on previous reports [15]. To compare the improvement of skin condition by treatment with each compound, pictures were taken before and after the treatment.

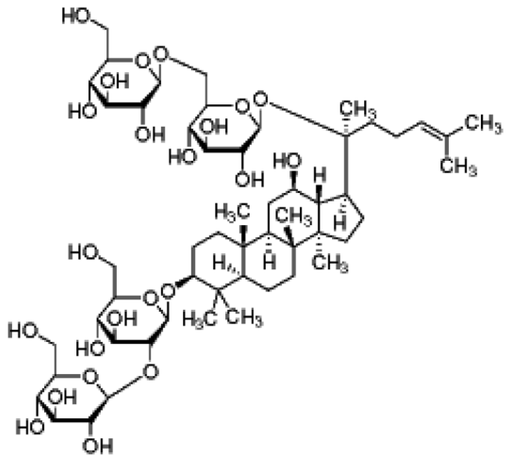

Fig. 1. Chemical structure of ginsenoside Rb_1.

Measurement of scratching behavior frequency

Scratching behavior was observed after completion of all treatments. In brief, the mice were placed into cages and the number of scratching behaviors was counted for 10 minutes. Measurement was repeated for five times (50 minutes in total). The scratching behavior was defined as the movement with hind paws in this experiment.

Determination of interleukin-4 secretion

The spleens were aseptically removed and total splenocytes were prepared. Splenocytes (5×10^5 cells/well) were plated at 96-well plates and incubated for 24 hours in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% of FBS (Hyclone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 humidified incubator. Culture supernatants were collected and the amount of secreted IL-4 was measured using enzyme-linked immunoassay kit purchased from Biolegend, Inc. (San Diego, CA, USA).

Measurement of serum IgE level

Blood samples were collected from the inferior vena cava after finishing treatment and serum was separated. The serum concentration of IgE was measured by enzyme-linked immunoassay kit purchased from Biolegend, Inc.

RT-PCR analysis of gene expression

The expression of mRNA transcripts of IL-4, IL-10 and NFAT was determined by reverse transcriptase-polymerase chain reaction. Total RNA was extracted from splenocytes using the Trizol (Invitrogen, Carlsbad, CA, USA) reagent and processed to cDNA with RevertAid (Fermentas, Vilnius, Lithuania). Solgent™ EF-Taq DNA Polymerase kit (Solgent Co., Daejeon, Korea) was

Table 1. Primers for reverse transcriptase PCR

Genes	Sequence of forward and reverse primers
Interleukin -4	Forward: 5'-ATGGGTCTCAACCCCCAGCTAGT-3 Reverse: 5'-GCTCTTTACGCTTTCCAGGAAGTC-3
Interleukin-10	Forward: 5'-CTGCTCTTACTGACTGGCATGAG-3 Reverse: 5'-GACTCAATACACACTGCAGGTGT-3
NFATc2(NFAT1)	Forward: 5'-TGGCCCGCGACATCTACCCT-3 Reverse: 5'-TGGTAGAAGGCGTGCGGCTT-3
GAPDH	Forward: 5'-CCATGGAGAAGGCTGGGG-3 Reverse: 5'-CCAAGTTGTCATGGATGACC-3

NFAT, nuclear factor of activated T cells.

used for PCR analysis and cDNA was amplified using primers as shown in Table 1. PCR conditions were as follows: 94°C 30 seconds, 58°C 45 seconds, and 72°C 30 seconds repeated 32 times for IL-4; 94°C 1 minute, 65°C 2 minutes, and 72°C 1 minute repeated 40 times for IL-10; 95°C 25 seconds, 54°C 30 seconds, and 68°C 45 seconds repeated 31 times for NFAT1; 92°C 30 seconds, 58°C 30 seconds, and 72°C 1 minute repeated 25 times for GAPDH.

RESULTS

Effect of Rb_1 on atopic dermatitis-like skin lesions

AD-like skin lesions were induced in BALB/c mice by topical application of DNCB compared to untreated group (Fig. 2A). After the first application of DNCB, erythema began to appear on the back followed by eczema and keratosis with final application (Fig. 2B-E). In contrast, treatment of AD-like skin lesions with Rb_1 resulted in improved skin condition (Fig. 2I and 2J) as compared to the DNCB-treated group (Fig. 2G). We also examined the inhibitory effect of dexamethasone, a well-known anti-inflammatory and immunosuppressant. As shown in Fig. 2B, dexamethasone significantly reduced DNCB-induced AD-like skin lesions. Based on these findings, Rb_1 may have activities similar to dexamethasone.

Effect of Rb_1 on scratching behavior frequency

To investigate the antiatopic effect of Rb_1, we examined the effect Rb_1 on scratching behavior, a typical clinical symptom of AD. DNCB application caused a substantial increase in the frequency of scratching behavior. However, Rb_1 treatment significantly suppressed DNCB-mediated scratching behavior (Fig. 3), although its inhibitory effect was less significant than dexamethasone, a type of steroid drug used to treat many inflammatory diseases.

Effect of Rb_1 on interleukin-4 secretion

Overexpression of IL-4 is associated with allergic response. To further investigate the mechanism of action of Rb_1, we examined the effect Rb_1 on IL-4 secretion. As shown in Fig. 4, DNCB-induced AD mice showed much higher level of secreted IL-4 than that of control mice,

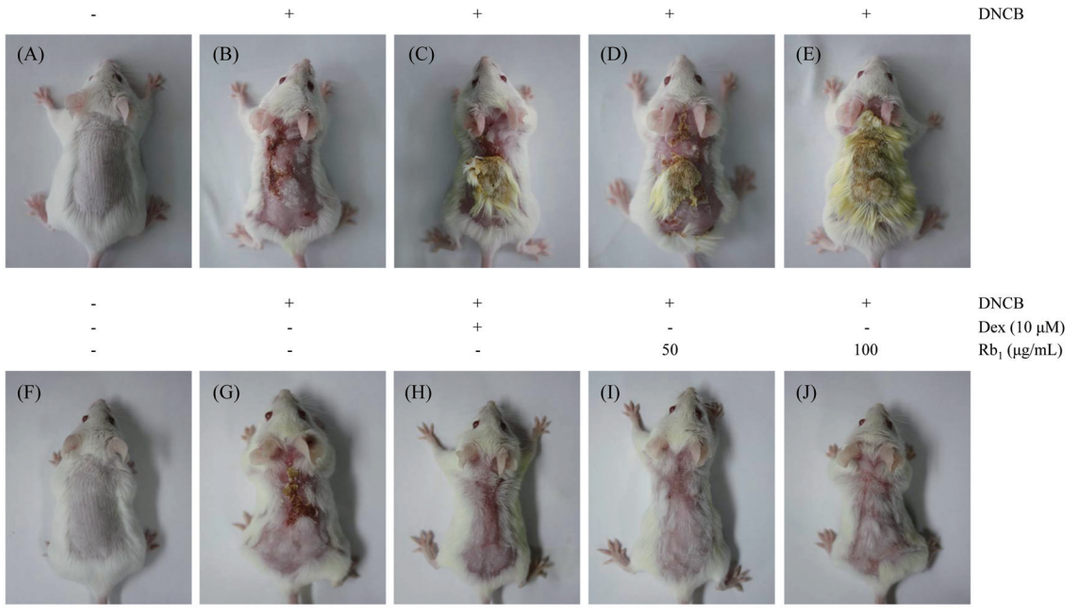

Fig. 2. Atopic dermatitis (AD)-like skin lesions and improvement of skin condition after treatment with Rb_1. AD-like skin lesions were induced by topical application of 1-chloro-2,4-dinitrobenzene (DNCB) for 4 times (B-E), and Rb_1 application on the skin lesions improved the skin condition (G, treatment with PBS; H, treatment with dexamethasone; I, treatment with 50 μg/mL of Rb_1; J, treatment with 100 μg/mL of Rb_1). Dex, dexamethasone.

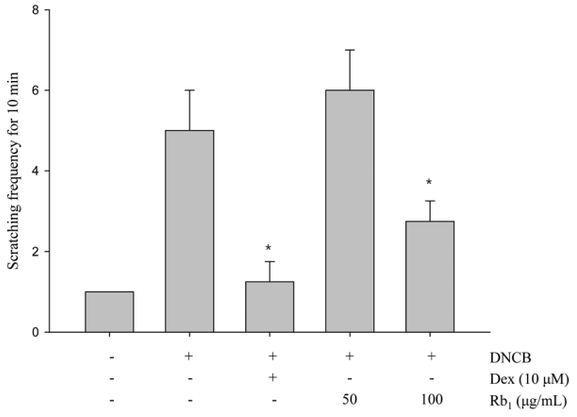

Fig. 3. Inhibitory effect of Rb_1 on 1-chloro-2,4-dinitrobenzene (DNCB)-induced scratching behaviors. Scratching behavior was observed after completion of all treatments. In brief, the number of scratching behaviors was counted for 10 minutes. Measurement was repeated for five times (50 minutes in total). Dex, dexamethasone. *Significantly different from DNCB-treated group (p<0.05).

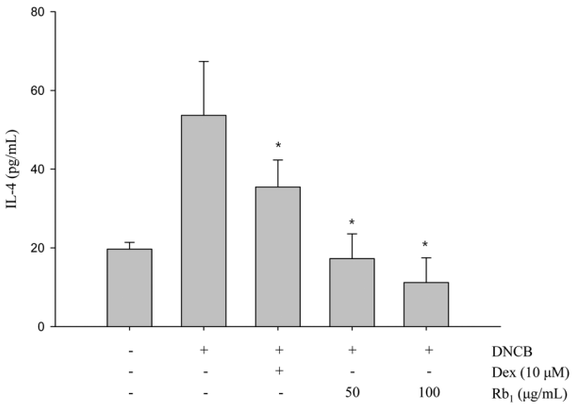

Fig. 4. Inhibitory effect of Rb_1 on 1-chloro-2,4-dinitrobenzene (DNCB)-induced IL-4 secretion in splenocytes. After completion of all treatments, splenocytes (5×10^5 cells/well) were incubated for 24 hours. Culture supernatants were collected and level of secreted IL-4 was determined using ELISA. The data are presented as the mean±SD of a representative experiment of triplicates. Dex, dexamethasone. *Significantly different from DNCB-treated group (p<0.05).

while Rb_1 treatment suppressed secretion of IL-4 and Rb_1 appears to be more effective than dexamethasone on suppression of IL-4 secretion.

Effect of Rb_1 on mRNA levels of cytokines and NFAT1

To determine whether Rb_1 might be involved in cytokine gene regulation, the levels of IL-4 and IL-10 mRNA expression on splenocytes were examined by RT-PCR analysis. DNCB significantly induced mRNA levels of both IL-4 and IL-10. When DNCB-induced AD mice were treated with 100 μg/mL of Rb_1 four times, Rb_1 suppressed DNCB-induced mRNA levels of IL-4 as well as IL-10 (Fig. 5A). The effect of Rb_1 on mRNA level of NFAT1, which is known to induce various cytokine genes including IL-4, was also examined. Consistent with mRNA levels of cytokines, Rb_1 treatment resulted in decrease of NFAT1 expression (Fig. 5 B).

Effect of Rb_1 on serum IgE level

High level of IgE in the blood is a clear indicator of AD. Therefore, we examined whether Rb_1 can suppress serum IgE in AD mice. Although dexamethasone decreased DNCB-induced IgE in serum, Rb_1 treatment did not suppress (Fig. 6).

DISCUSSION

In the present study, we examined the effect of Rb_1 on AD-like skin lesions in mice. Skin sensitivity and inflammation induced by DNCB have been known to be characterized by raised IgE and cytokines such as IL-4 and IL-10 [14,16]. The results of this study demonstrate that topically applied ginsenoside Rb_1 exerts beneficial effects in mouse model of AD.

In atopic dermatitis, skin lesions were characterized by infiltration of IL-4 producing $CD4^+$ T cells, indicating that IL-4 is likely to play a major role in AD since it me-

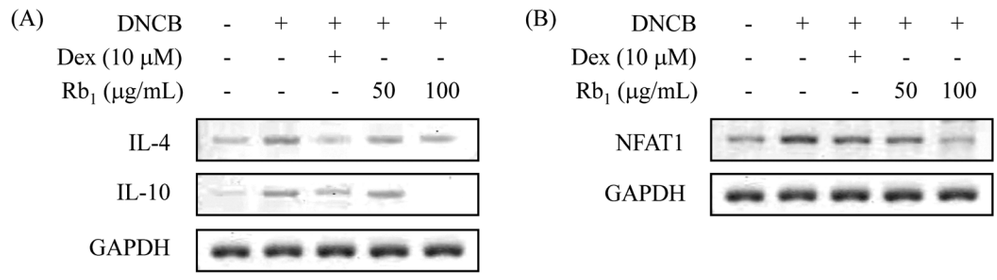

Fig. 5. Suppressive effect of Rb_1 on mRNA expressions of IL-4, IL-10, and the nuclear factor of activated T cells (NFAT) 1. After completion of all treatments, total RNA was extracted from splenocytes. RT-PCR analysis was performed to measure mRNA levels of IL-4, IL-10, and NFAT1, respectively. The results illustrated are from a single experiment, and are representative of three separate experiments. DNCB, 1-chloro-2,4-dinitrobenzene; Dex, dexamethasone.

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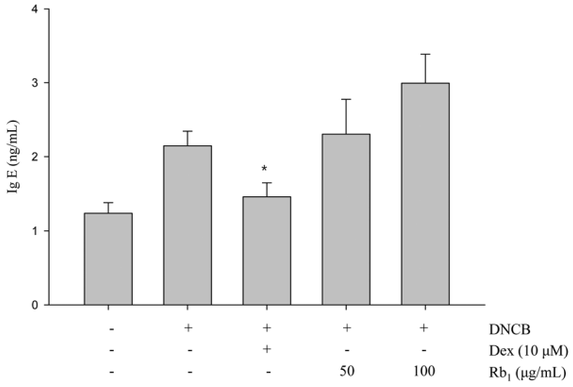

Fig. 6. Effect of Rb_1 on level of IgE in serum. After completion of all treatments, blood samples were collected from the inferior vena cava and serum was separated. Level of serum IgE was measured by ELISA. The data are presented as the mean±SD of a representative experiment of triplicates. *Significantly different from DNCB-treated group (p<0.05).

diates Ig isotype switching from IgM to IgE [6,7]. In addition, IL-10 plays an important role in the T_H2 response in a murine model of allergic dermatitis [17]. It has been also known that the upregulation of total serum IgE is a hallmark of AD [18]. Our data showed that the expression of IL-4 and IL-10 increased in the splenocytes from the control mice developing skin lesions was suppressed by Rb_1 treatment. However, although the application of Rb_1 significantly reduced the severities of AD-like skin lesions induced by DNCB, it did not inhibit total serum IgE levels. For many years there has been an argument for the contribution of IgE-mediated hypersensitivity reactions in the pathogenesis of AD [19], because in some chronic AD skin lesions the development of AD appears to be independent of plasma IgE levels. Moreover, it has recently been suggested that increased IgE production and subsequent IgE-mediated hypersensitivity reactions may not contribute to the development of the AD-like skin lesions observed in NC/Nga mouse model [20]. Therefore, the results of this study do not support the notion that IgE plays a role in modulating AD-like lesions induced by DNCB and might provide an extended scope to the prevailing concept of IgE–independent mechanism underlying the pathobiology of AD.

T_H2 cells produce IL-4, IL-5, and IL-10, and are involved in humoral immunity and the allergic response. Our results demonstrated that Rb_1 inhibited DNCB-induced transcript levels for the cytokines. This suggests that Rb_1 may inhibit the expression of cytokines by interfering with the transcription of their respective genes. It may inhibit either the initiation of transcription or the stability of the mRNAs encoding these molecules. It has been also shown that activation of transcription factor NFAT is required for the transcriptional activation of cytokines [8,9], suggesting that NFAT appears to play a pivotal role in IL-4 and IL-10 gene activation. Therefore, the decreased expression of IL-4 and IL-10 prompted us to examine the effect of Rb_1 on NFAT implicated in the regulation of a variety of genes participating in immune responses, including genes encoding IL-4 and IL-10. The present data showed that Rb_1 inhibited DNCB-induced NFAT activation. However, our results do not eliminate the possible involvement of alternative transcription factors, because STAT6 and c-Maf are known to be involved in gene expression of these cytokines [21-23]. It is, therefore, possible that inhibitory effect of Rb_1 on cytokine expression may be at least partially mediated through suppression of the NFAT transcription factor.

In summary, the application of ginsenoside Rb_1 was found to inhibit the development of AD-like skin lesions induced by DNCB. In addition, the results of the present study demonstrate that Rb_1 is capable of inhibiting the expression of cytokines, which results from the suppression of NFAT activation in splenocytes. Thus, Rb_1 is proposed as an effective new anti-inflammatory agent that may have a potential therapeutic use for preventing the advancement of atopic dermatitis.

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