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Oral administration of ginsenoside Rh1 inhibits the development of atopic dermatitis-like skin lesions induced by oxazolone in hairless mice

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ABSTRACT

In the present study, we examined the inhibitive effect of ginsenoside Rh1 on oxazolone-induced atopic dermatitis-like skin lesions in hairless mice. Oral administration of ginsenoside Rh1 improved clinical symptoms, and it was confirmed by histopathological analysis. In ginsenoside Rh1 (20 mg/kg) group, ear swellings and ear weights were significantly lower than the control group. Moreover, elevation of IL-6 and total IgE levels in serum were suppressed by ginsenoside Rh1 (20 mg/kg). In addition, ginsenoside Rh1 (20 mg/kg) significantly increased mRNA expression of IFN γ and Foxp3, and slightly decreased IL-4 expression in draining lymph nodes. The results suggest that ginsenoside Rh1 can alleviate inflammatory symptoms in atopic dermatitis (AD) by reduction of IgE and IL-6 levels in peripheral blood, increase of Foxp3 expression in draining lymph nodes and suppression of inflammation in skin regions. Indeed, ginsenoside Rh1 exhibited therapeutic possibility in immune disorders.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritus and relapsing eczematous lesions [1,2]. As in other allergic diseases, environmental factors play an important role in the development and manifestations of AD in genetically predisposed individuals [3,4]. Although the prevalence of early childhood infections has decreased in recent years due to declined family size, improved basic amenities, and enhanced personal cleanliness [5], the occurrence of allergic diseases has increased in the industrialized regions such as Europe and North America [6].

Patients with AD tend to have peripheral blood eosinophilia and increased levels of serum IgE, which are due to the elevated production of IL-4, IL-5, IL-13 and suppressed production of IFN γ by T_H2 cells [7,8]. IL-4 and IL-13 promote class switching to IgE in B cells [9,10], while IL-5 is related to the development, activation and survival of eosinophils [11]. However, IFN γ favors T_H1 development and suppresses production of IgE and proliferation of T_H2 cells [2]. It is considered that controlling the balance of T_H1/T_H2 lineages, which is mainly regulated by IL-4 and IFN γ , is critical in the treatment of AD [12]. On the other hand, the reduced immune suppression by

regulatory T cells (T_{reg}s) is regarded as another important mechanism for immune disorders [13]. The T_{reg}s suppress autoimmune responses and also improve allergic disorders [14,15]. Indeed, decreased proportion of T_{reg}s in lymphoid organ is correlated with the symptoms of AD as well as inflammatory bowel disease (IBD) [15].

Various murine models has been used to research mechanisms or treatments of AD: spontaneous model using Nc/Nga mouse in conventional environment [16,17]; transgenic models that express abnormal cytokine levels in mice [18,19]; and induced AD-like models by repeated challenges with allergens (mite extracts [20–22] or haptens [23–26]). Of these models, hapten-induced mice models are advantageous with convenience, reproducibility and low cost [25], but are not characterized by immunologic, epidermal structural and biochemical abnormalities. Nevertheless, oxazolone-challenged skin lesions in hairless mouse induced structural, immunologic and biochemical similarities with human AD [25].

Ginsenoside Rh1 (Fig. 1), a bioactive component of ginseng, is one of the widely used traditional herbs in Asia [27]. Ginsenoside Rh1 has been reported to decrease the mortality due to anaphylaxis in mice [28]; suppress iNOS expression in IFN γ stimulated microglia [29]; inhibit histamine release from rat peritoneal mast cells [27]; and decrease NO production in murine peritoneal macrophages [30]. However, the therapeutic effect of ginsenoside Rh1 in AD murine model has not been reported.

In this study, oxazolone challenged hairless mice were used to evaluate if ginsenoside Rh1 can alleviate AD as determined by clinical signs, histology, cytokine (IL-4 and IFN γ) and Foxp3 expressions in draining lymph nodes (DLN), IgE and proinflammatory cytokine (IL-6) levels in serum.

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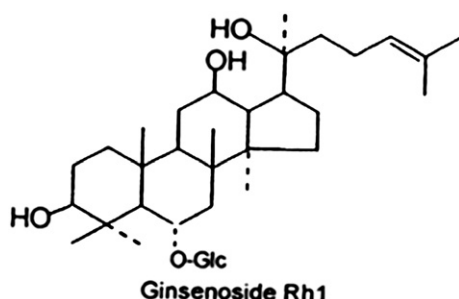


Fig. 1. Structure of ginsenoside Rh1.

2. Materials and methods

2.1. Animals

Female hairless mice, 5 weeks old, were purchased from Orient Co. (Korea) and maintained under SPF (specific pathogen free) conditions at $23 \pm 3^\circ\text{C}$ with $50 \pm 10\%$ humidity. The mice were fed a laboratory rodent diet and water ad libitum with a 12 h/12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University.

2.2. Induction of AD-like skin lesions

After 1-week adaption, mice were randomly assigned to five groups (group 1—naïve, group 2—control, group 3—prednisolone, group 4—Rh1 (10 mg/kg), and group 5—Rh1 (20 mg/kg)). Group 2–5 sensitized with $20\ \mu\text{l}$ of 0.5% oxazolone (Sigma, USA) dissolved in 100% ethanol, and the group 1 (naïve group) received 100% ethanol vehicle only that were painted to right ear and dorsal. For the subsequent seven weeks, the mice were challenged weekly with $60\ \mu\text{l}$ of 0.1% oxazolone painted to right ear ($20\ \mu\text{l}$) and dorsal ($40\ \mu\text{l}$), while the naïve group painted with 100% ethanol only. The experiment schedule is summarized in Fig. 2.

2.3. Administration of prednisolone and ginsenoside Rh1

Ginsenoside Rh1 (98%, Cogon, China) was dissolved in vehicle (3% Tween 80 (Sigma, USA) in PBS) and orally administrated at 10 mg/kg or 20 mg/kg every day from third week to seventh week, while the naïve group and the control group were given vehicle only. Prednisolone (99%, Sigma, USA), as a positive control, was also dissolved in vehicle and administrated at 3 mg/kg daily from third week to seventh week.

2.4. Evaluation of dermatitis score

Macroscopic assessment of the clinical conditions was evaluated according to the method described by Matsuoka [20]. Briefly, severity of clinical signs was determined by the sum of two signs: dryness/scaling and erosion/excoriation/hemorrhage which were graded by 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe).

2.5. Measurement of ear thickness

Four hours after each application of oxazolone, ear thickness of each mouse was measured with a digital thickness gauge (Mitutoyo Corp., Japan). Ear thickness was measured three times: at the beginning of experiment (0 week); before oral administration (3 week); at the end of oral administration (7 week).

2.6. Histological evaluation

Dorsal skin of each mouse was removed and fixed in 10% formalin. Fixed tissues were embedded in paraffin and the sections were stained with H&E and Toluidine blue (Xpath, Korea). Degranulated mast cells were counted in Toluidine blue staining slides.

2.7. Organ weights

Right ear and total spleen of each mouse were removed and weighed after sacrifice.

2.8. Total serum IgE levels

Blood samples were collected every week, and the levels of total serum IgE were measured by IgE ELISA kit (BD Biosciences, USA), following the manufacturer's instructions.

2.9. Serum IL-6 levels

Blood samples were collected at sacrifice, and the serum was used to measure the concentration of IL-6. Serum IL-6 was measured by IL-6 ELISA kit (BD Biosciences, USA) according to the manufacturer's instructions.

2.10. Quantitative analysis of mRNAs in DLN

Total RNA was extracted from the draining lymph nodes of each mouse using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized with reverse transcriptase primed with oligo(dT) primers (PrimeScript 1st strand cDNA Synthesis Kit, Takara, Japan). Quantitative real-time PCR (SYBR green, Takara, Japan) was performed by ABI Setpone system (USA), and all the PCR procedures using the following conditions: 95°C for 10 s, 95°C for 5 s and 60°C for 30 s up to 40 cycles.

The following primers were used: murine IL-4 (forward) 5'-ACA GGA GAA GGG ACG CCA T-3' and (reverse) 5'-GAA GCC CTA CAG ACG AGCTCA-3', IFN γ (forward) 5'-TCA AGT GGC ATA GAT GTG GAA GAA-3' and (reverse) 5'-TGG CTC TGC AGG ATT TTC ATG-3', Foxp3 (forward) 5'-CCC ATC CCC AGG AGT CTT G-3' and (reverse) 5'-CCA TGA CTA GGG GCA CTG TA-3', and HPRT (forward) 5'-TTA TGG ACA GGA CTG AAA GAC-3' and (reverse) 5'-GCT TTA ATG TAA TCC AGC AGG T-3'. HPRT was used as an endogenous control [15].

2.11. Statistical analysis

Results are presented as mean $\pm$ SEM. Statistical significance was determined by Student's t tests with $p < 0.05$ as a significance level.

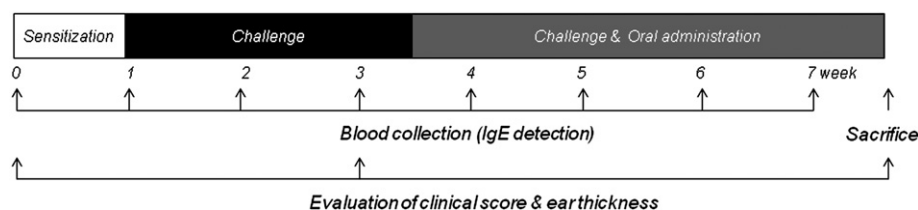


Fig. 2. Schedule for whole experiment.

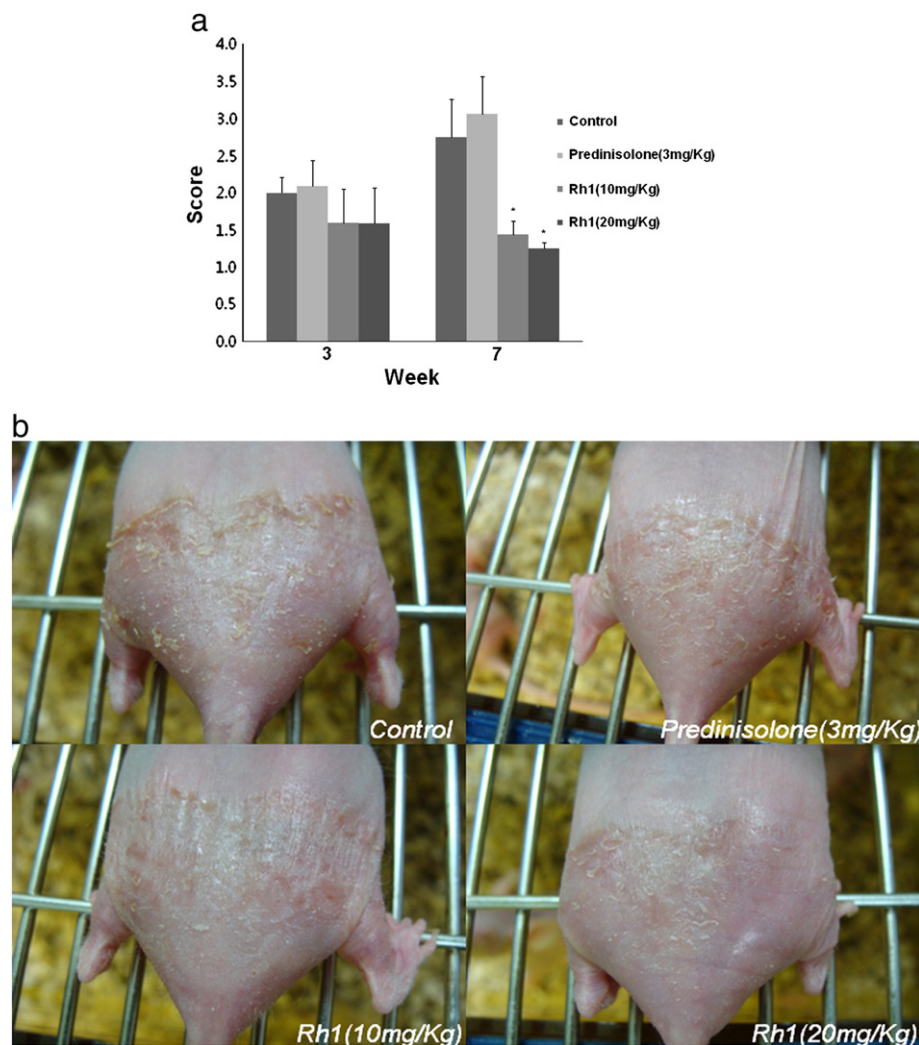


Fig. 3. Clinical severity of AD-like skin lesions. a) Clinical scores were determined by the sum of the dryness/scaling and erosion/excoriation/hemorrhage, and graded as 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe). The score was evaluated before (3 week) and after (7 week) administration of vehicle, prednisolone (3 mg/kg) and Rh1 (10 mg/kg or 20 mg/kg). The values are the mean $\pm$ SEM of six mice. * $p < 0.05$: significantly different from the control group (Student's *t*-test). b) Skin features of mice at the end of experiment (7 week). Images are representative of six mice.

3. Results

3.1. AD-like skin lesions

Dermatitis scores evaluated at the beginning (3-week) and at the end (7-week) of oral administration revealed that prednisolone (3 mg/kg) did not inhibit the development of AD-like skin lesions compared with the control group (Fig. 3a). In comparison, ginsenoside Rh1 significantly improved skin symptoms both at the dose of 10 mg/kg and 20 mg/kg. Dryness/scaling and erosion/excoriation/hemorrhage were alleviated after Rh1 administration (Fig. 3b).

3.2. Ear swelling and ear weight

Before oral administration at 3 week, no significant differences were observed in ear swelling among challenged groups. However, after 4-week's administration, both prednisolone and ginsenoside Rh1 (10 mg/kg, 20 mg/kg) suppressed ear swellings significantly ($p < 0.05$, Fig. 4a). Ear weight of ginsenoside Rh1 (20 mg/kg) group was significantly lower than that of the control group, and it was lowest in prednisolone group (Fig. 4b).

3.3. Infiltration of inflammatory cells

H&E staining of dorsal skin showed that repeated application of oxazolone induced infiltration of inflammatory cells. Ginsenoside Rh1 inhibited the infiltration of inflammatory cells significantly at the dose of 20 mg/kg but less pronounced at 10 mg/kg. Prednisolone did not exhibit any considerable inhibitory effect (Fig. 5a).

3.4. Degranulation of mast cells

Infiltration and degranulation of mast cells in dorsal skin were detected in Toluidine blue staining (Fig. 5b). The number of degranulated mast cells in ginsenoside Rh1 and prednisolone groups was significantly lower than the control group. Comparing three treatment groups, high dose (20 mg/kg) of ginsenoside Rh1 was more effective than the others (Fig. 5c).

3.5. Spleen weight

Prednisolone treatment significantly decreased spleen weight ($p < 0.05$) compared with naïve group, while the other groups markedly increased spleen weights (Fig. 6).

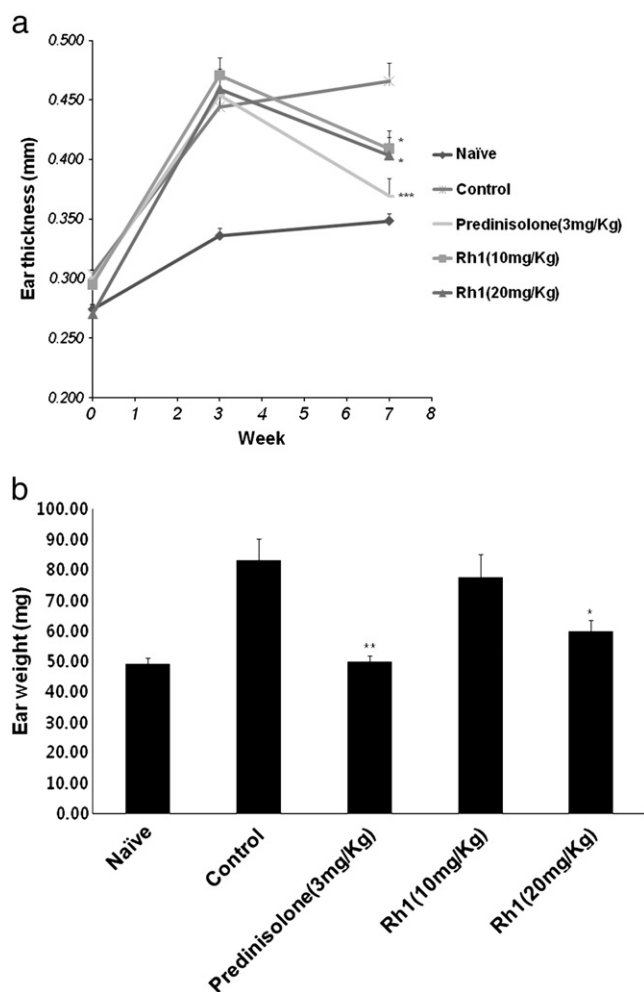


Fig. 4. Ear thickness and ear weight. a) Ear thicknesses were measured 4 h after application of oxazolone in 0, 3 and 7 week. b) Ear weight of each mouse was measured after sacrifice. Each point represents the mean $\pm$ SEM. Data are from six mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different from the control group (Student's *t*-test).

3.6. IgE levels in serum

Both ginsenoside Rh1 and prednisolone prevented the elevation of IgE levels from fifth week (Fig. 7a). Compared with the control group, IgE levels were significantly lower in ginsenoside Rh1 groups in fifth week. Apparently ginsenoside Rh1 was more effective at the dose of 20 mg/kg than at 10 mg/kg even though the difference was not significant after fifth week. On the other hand, prednisolone significantly decreased IgE concentrations from fifth week to seventh week.

3.7. Serum IL-6 levels

The elevation of IL-6 concentration in serum was slightly decreased by 10 mg/kg but inhibited significantly by 20 mg/kg of ginsenoside Rh1. Prednisolone group exhibited significantly lower concentration of IL-6 compared with the control group (Fig. 7b).

3.8. Relative mRNA expression of IL-4, IFN γ and Foxp3 in DLN

Ginsenoside Rh1 did not decrease mRNA expression of IL-4, but increased the expression of IFN γ and Foxp3 ($p < 0.05$) at the dose of

20 mg/kg. On the contrary, prednisolone decreased IL-4 expression and increased Foxp3 expression ($p < 0.05$), while IFN γ expression in prednisolone group did not differ from that in the control group (Fig. 8).

4. Discussion

Atopic dermatitis (AD) is a complex inflammatory skin disease that significantly affects the quality of life of patients whose peripheral bloods often contain elevated levels of IgE [31,32]. Various murine models have been used to study the mechanisms of human AD and investigate preventive measures [20,23,24,26,33–35]. Since oxazolone-induced skin lesions in hairless mouse exhibited structural, immunologic and biochemical similarities with human AD [25], we induced AD-like skin lesions in SKH1 mice by repeated challenges of oxazolone. Consequently, AD-like skin lesions were observed in company with the progressive increase of total IgE levels in serum during the period of experiment (Fig. 7a). IgE is known to induce the degranulation of mast cells and basophils, and participate in the activation of macrophage/monocytes [36]. Therefore, elevated levels of IgE render AD individuals susceptible to the manifestation of inflammatory reactions [12]. Accordingly, reduction of IgE levels may be a feasible way to treat AD. In this study, the elevation of IgE concentrations (Fig. 7a) and the symptoms of skin regions (Fig. 3) were considerably improved by ginsenoside Rh1, suggesting that the reduction of IgE contributes to the alleviation of skin lesions to a certain extent.

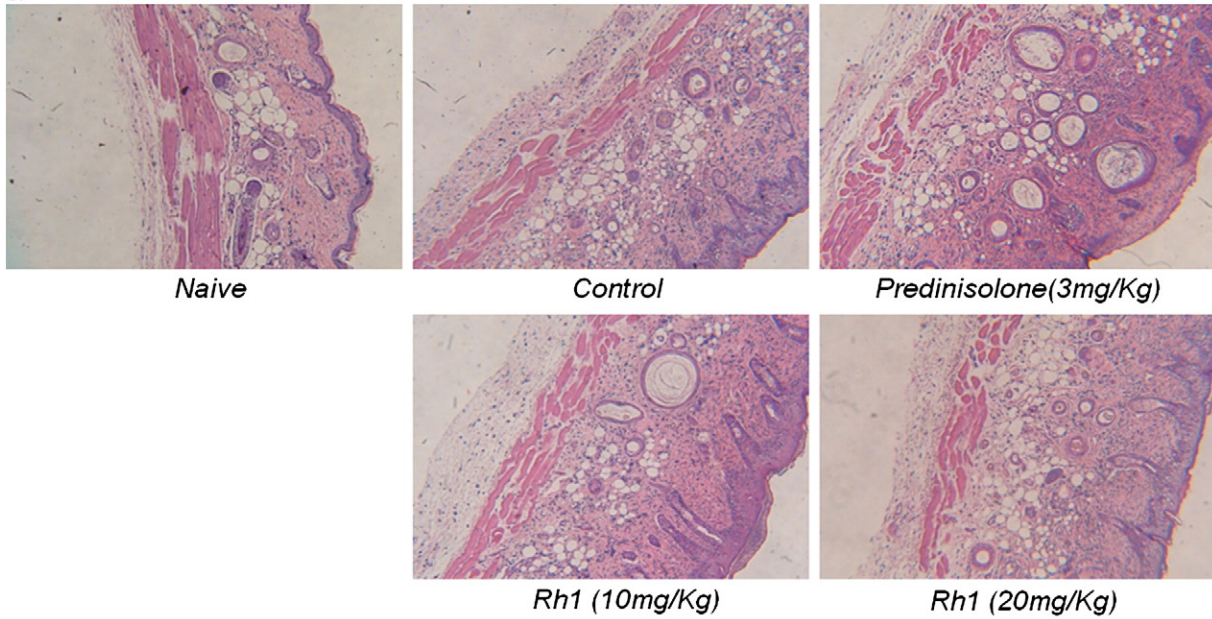
The regulation of IL-4 and IFN γ is the key point of T_H1/T_H2 balance and, crucial for the maintenance of T_H cell homeostasis [12]. IFN γ is a signature cytokine of T_H1 differentiation, suppresses the activity of IL-4 which promotes proliferation of T_H2 lineages and isotype switching to IgE [12,36]. PBMCs from AD patients tend to produce increased level of IL-4 and decreased level of IFN γ [37,38]. Likewise, repeated challenges of oxazolone in hairless mice induced elevated expression of IL-4 and reduced expression of IFN γ in DLN (Fig. 8a and b), both of which are thought to be related to the increased IgE levels in serum [38]. In the present study, ginsenoside Rh1 did not decrease IL-4 expression markedly, but promoted the expression of IFN γ in DLN ($p < 0.05$, Fig. 8a and b). The results demonstrated that inhibition of IgE elevation by ginsenoside Rh1 might be due to the up-regulation of IFN γ expression.

Ginsenoside Rh1 has been reported by Park et al. and Kim et al. [27,28] to inhibit anaphylaxis in mice and decrease histamine release from rat mast cells. Consistent with these results, ginsenoside Rh1 suppressed the degranulation of mast cells in inflammatory regions in the present study (Fig. 5c). Moreover, ginsenoside Rh1 inhibited the infiltration of inflammatory cells in skin lesions (Fig. 5a). These results could be explained by the reduction of cell adhesion molecules in monocytes by ginsenoside Rh1 [39]. Besides, inflammation of ear tissues was remarkably improved by treatment of ginsenoside Rh1 (Fig. 4), similarly to the result of Yong-Wook Shin et al. [40]. Taken together, it is conceivable that ginsenoside Rh1 performs anti-inflammatory effect in local regions by the inhibition of histamine release from mast cells and cell adhesion of monocytes to endothelium to some degree.

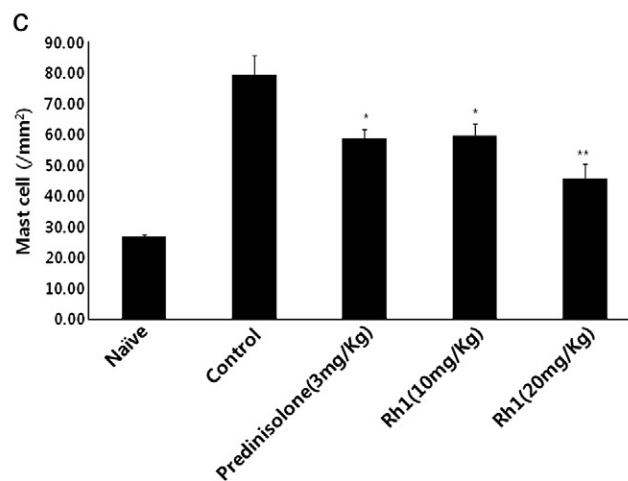
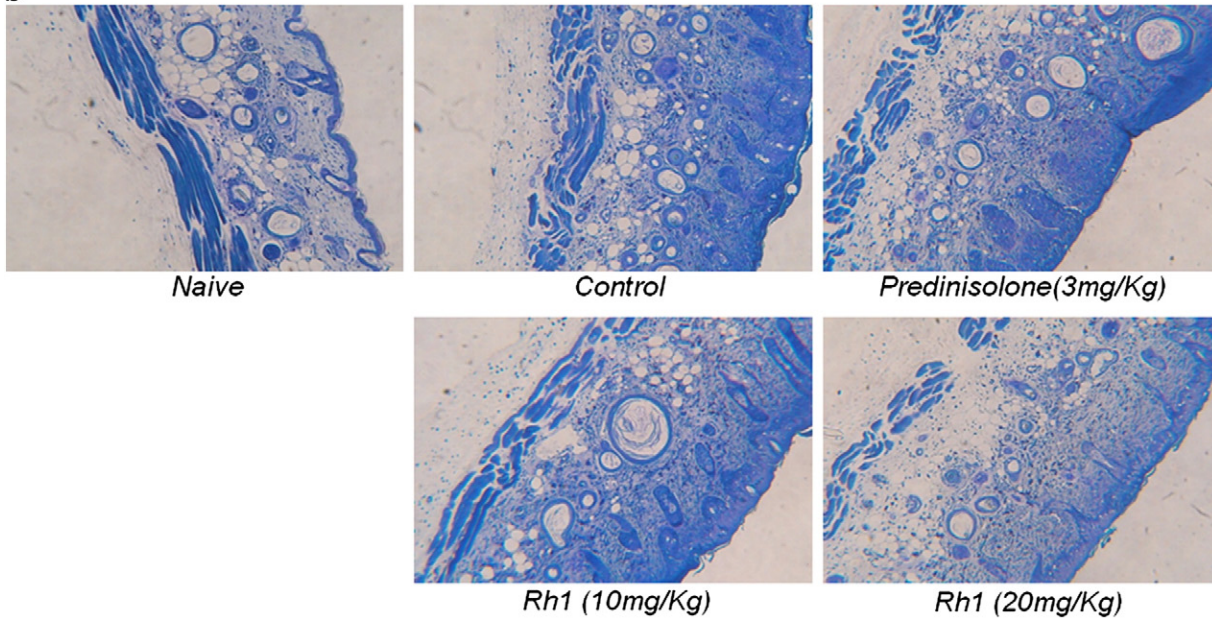
Interestingly prednisolone did not alleviate the symptoms and inflammation of AD-like skin lesions despite of IgE and IL-4 down regulation (Figs. 3, 7a, 8a). Prednisolone group resulted in significantly lower spleen weight than naïve group (Fig. 6), which evidenced that prednisolone decreased the number of spleenocytes even in the case of hypersensitivity. Prednisolone, a synthetic corticosteroid drug, suppresses the immune response, but comes with many side effects including elevated susceptibility to infections in patients [41]. In our

Fig. 5. Histology of dorsal skin lesions. a) H&E staining, and b) Toluidine blue staining. Dorsal skin of each mouse was removed and fixed for staining. Images are representative of four mice. Original magnification $\times 400$. c) The number of degranulated mast cells. Mast cells were counted in the slide of Toluidine blue staining at the magnification of $\times 400$. Each point represents the mean $\pm$ SEM. Data are from four mice. * $p < 0.05$, ** $p < 0.01$: significantly different from the control group (Student's *t*-test).

a



b



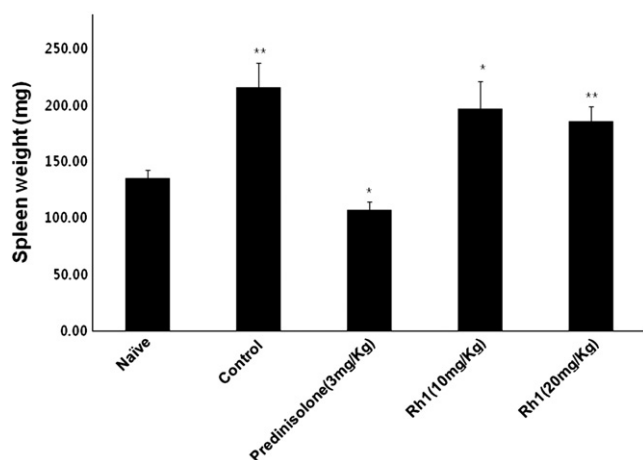


Fig. 6. Spleen weight. Spleen weight of each mouse was measured after sacrifice. Each point represents the mean $\pm$ SEM. Data are from six mice. * p <0.05, ** p <0.01: significantly different from the naïve group (Student's t -test).

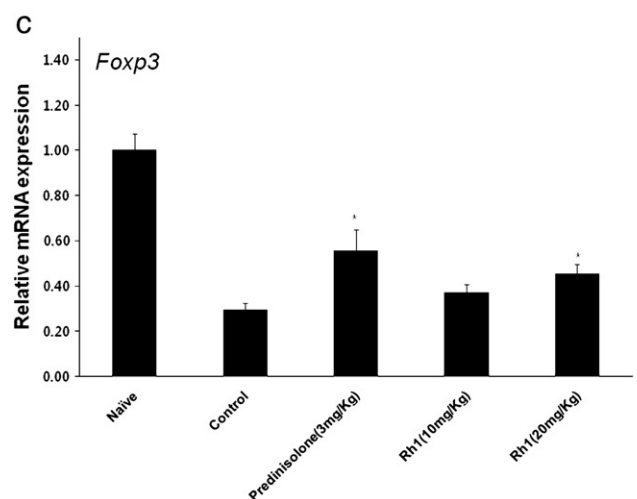
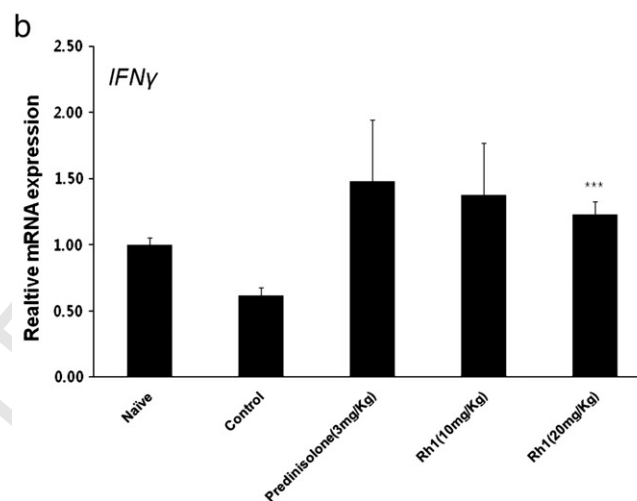
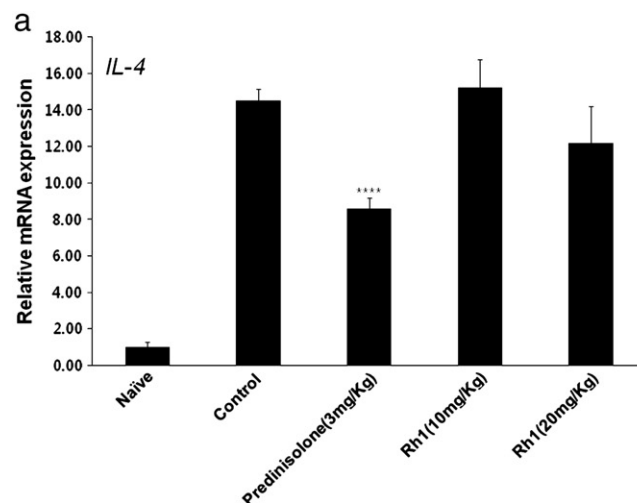


Fig. 8. Relative mRNA expression of IL-4, IFN γ and Foxp3 in DLN. a) IL-4, b) IFN γ , and c) Foxp3. DLN of each mouse was removed then homogenized to isolate mRNAs. Reverse transcription and real-time PCR were performed to evaluate the relative mRNA expression in DLN. HPRT was used as an endogenous control. Each point represents the mean $\pm$ SEM. Data are from three to six mice. * p <0.05, *** p <0.001, **** p <0.0001: significantly different from the control group (Student's t -test).

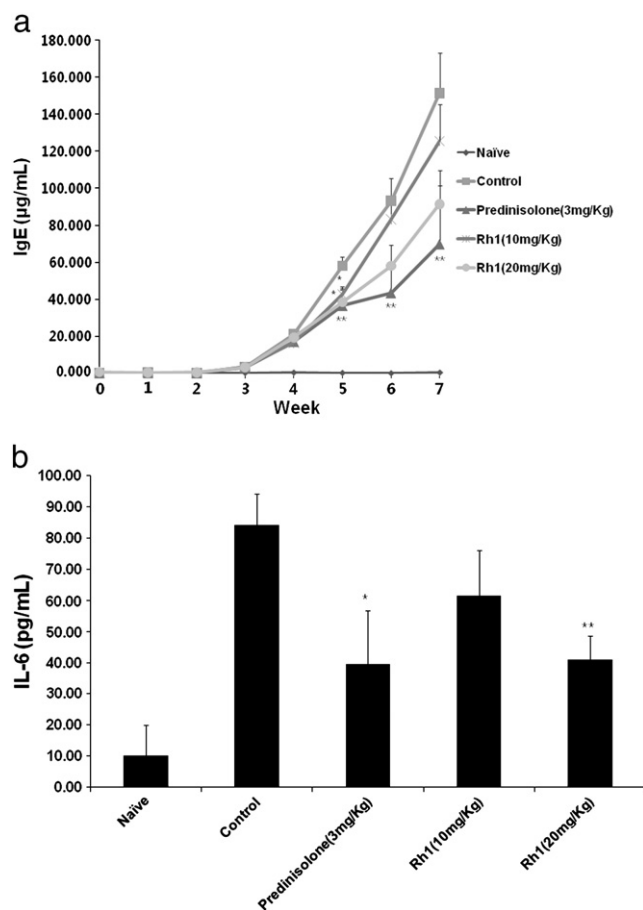


Fig. 7. Total IgE and IL-6 levels in serum. a) IgE concentration of each mouse was measured weekly by ELISA. b) IL-6 concentrations were detected after sacrifice by ELISA. Each point represents the mean $\pm$ SEM. Data are from five to six mice. * p <0.05, ** p <0.01: significantly different from the control group (Student's t -test).

Rh1 may suggest that its mechanism of suppressing immune system differs from that by prednisolone.

IL-6, a proinflammatory cytokine, is originally known as a B-cell differentiation factor [42]. In chronic inflammation, IL-6 attracts

mononuclear cells to the inflammatory site, and then the accumulation of mononuclear cells increases IL-6 levels in blood. Therefore, IL-6 is considered to be an effective mediator in chronic inflammation [43]. It has been reported that IL-6 concentration in peripheral blood was increased in Nc/Nga mice challenged by *Dermatophagoides pteronyssinus* [44], which is consistent with our result that IL-6 levels were elevated after multiple applications of oxazolone (Fig. 7b). Nevertheless, ginsenoside Rh1 significantly suppressed the increase of IL-6 in serum after 4-week's administration (Fig. 7b). Considering IL-6 is an activator of inflammation in chronic stage, we suggest that IL-6 down regulation played a role in the alleviation of skin inflammation.

Recently, T_{reg} s are considered to be an important regulator in immune disorders, such as T_H1 -mediated IBD and T_H2 -mediated AD [15]. T_{reg} s highly express transcription factor Foxp3, which is significant for the development and function of T_{reg} s [45]. In this study, the expression of Foxp3 in DLN was decreased in the control group, but it was up-regulated significantly by ginsenoside Rh1 (Fig. 8c). The expression and function of Foxp3 have been reported to be inhibited by IL-6 [46,47], and thus we may speculate that the reduction of IL-6 levels had affected the elevation of Foxp3 expression. Consequently, T_{reg} s might have played a role in the tolerance of immune disorders and improved the inflammatory status of skin in Rh1 fed mice.

In conclusion, ginsenoside Rh1 alleviated the symptoms of AD-like skin lesions accompanied with decrease of IgE and IL-6 concentrations in serum, improvement of inflammation in skin regions, and up-regulation of Foxp3 expressions in DLN. In addition, our study presented evidence that ginsenoside Rh1 may be therapeutically useful as modulator of immune diseases such as AD.

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