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Full paper

Red ginseng extracts attenuate skin inflammation in atopic dermatitis through p70 ribosomal protein S6 kinase activation

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ABSTRACT

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease with increased immunoglobulin E (IgE) levels. Activation of the mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70S6K) signaling is known to occur in the inflammatory regions of AD skin. We previously demonstrated that red ginseng extract (RGE), as an anti-inflammatory agent, had potential for treating AD. However, it is still unclear whether RGE inhibits mTOR/p70S6K signaling. Thus, we examined the anti-inflammatory effects of RGE on IgE or interferon- γ (IFN- γ) induced signaling pathways. In KU812 human basophils, activation of Fc ϵ receptor type 1 α (Fc ϵ RI), also known as the high affinity IgE receptor, induced phosphorylation of both mTOR and p70S6K. Moreover, levels of phosphorylated p70S6K (p-p70S6K), but not p-mTOR, were decreased by RGE. RGE also decreased p-p70S6K levels in IFN- γ -stimulated human keratinocytes, suppressing the IFN- γ induced increase in levels of C-C chemokine ligand 2 mRNA. Interestingly, the increased p70S6K phosphorylation in skin lesions of AD model mice was attenuated by RGE treatment. In conclusion, RGE is a potential therapy against inflammatory responses involving the p70S6K signaling pathway.

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1. Introduction

Atopic dermatitis (AD) is an important chronic or relapsing inflammatory skin disease characterized by itching. Blood basophils and mast cells mediate chronic allergic inflammation, such as that in AD, that is dependent on the high affinity immunoglobulin E (IgE) receptor type I (Fc ϵ RI). In the response to IgE, these cells rapidly release intracellularly located mediators, including interleukins, leukotrienes and histamine. Major Fc ϵ RI downstream pathways include the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway and the phosphoinositide 3-kinase (PI3-kinase)/Akt pathway.^{1–3} Furthermore,

PI3-kinase was recently identified as a major signaling molecule responsible for growth of neoplastic mast cells.⁴ Therefore, PI3-kinase downstream signaling molecules, such as the mammalian targets of rapamycin (mTOR) and p70 ribosomal protein S6 kinase (p70S6K), are considered potential therapeutic targets for diseases associated with activated basophils and mast cells or abnormal mast cell growth.^{5,6} More recently, the mTOR inhibitor, rapamycin, applied topically, alleviated dermatitis induced by Dermatophagoides farina body antigen in NC/Nga mice.⁷ Therefore, the mTOR/p70S6K signal pathway is considered a promising therapeutic target for AD.

A recent report showed that impaired tight junctional protein expression contributed to barrier dysfunction in AD.⁸ Interferon- γ (IFN- γ) and tumor necrosis factor- α were shown to increase cellular permeability through downregulating tight junctional proteins.⁹ In specially epidermal keratinocytes and basophils have primary roles

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Abbreviations

AD	atopic dermatitis
RGE	red ginseng extracts
mTOR	mammalian target of rapamycin
p-mTOR	phosphorylated mTOR
p70S6K	p70 ribosomal protein S6 kinase
p-p70S6K	phosphorylated p70S6K
FcεRIα	Fcε receptor type Iα
IFN-γ	interferon-γ
IgE	immunoglobulin E
DNFB	2,4-dinitrofluorobenzene
D-MEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
DNP-ascaris	2,4-dinitrophenylated ascaris extract
HRP	horseradish peroxidase
KU812	human basophils
NHEK (NB)	human keratinocytes
CCL2	C-C chemokine ligand 2

in amplification of skin inflammation,¹⁰ IFN-γ is known to be the most potent activator of the proinflammatory functions of keratinocytes.^{11,12} In fact, IFN-γ-activated keratinocytes express a broad array of chemokines, cytokines and membrane molecules, which can direct the recruitment, activation and retention of specific leukocyte subpopulations in the skin.^{11,13}

Recently, we examined effects of red ginseng extract (RGE) on histamine-induced vascular permeability in rats and on experimental dermatitis in mice, both caused by repeated applications of 2,4-dinitrofluorobenzene (DNFB) solutions.¹⁴ RGE significantly reduced DNFB-induced increases in scratching behavior and ear swelling, suggesting that it is potentially beneficial as an anti-allergic agent. However, the anti-allergic mechanism of RGE is unclear. Because the mTOR/p70S6K signal pathway plays a key role in various immune responses and inflammatory processes, in this study we assessed whether RGE inhibited activation of mTOR/p70S6K signaling in human basophils and keratinocytes to explore the molecular mechanisms of the beneficial effects of RGE on AD. Moreover, we examined the relationship between the anti-allergic mechanisms of RGE and the mTOR/p70S6K signal pathway in AD mice.

2. Material and methods

2.1. Animals

All procedures involving animals were performed in compliance with the Osaka City University animal care guideline. The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male BALB/c mice, 8–10 weeks of age (Japan SLC, Inc., Hamamatsu) were used in the present study.

2.2. Materials

RPMI-1640 medium, Dulbecco's modified Eagle's medium (D-MEM), penicillin and streptomycin, IFN-γ were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Fetal bovine serum (FBS) was from Equitech-Bio, Inc. (Kerrville, TX, USA). Red ginseng produced from 6-year-old ginseng was obtained from Ohki Pharmaceutical Co., Ltd. (Tokyo, Japan). Five-hundred grams of red

ginseng was crushed and refluxed for 2 h twice in 5 L of 70% methanol. The filtrate was evaporated to dryness under reduced pressure to give a brownish extract. This extract was chromatographed over a Lichroprep Rp-18 column with a Water-Methanol system to obtain the saponin fraction (RGE).¹⁴ Ginsenosides (Rb1, Rg1, Rg3, and Rh1) were gift from Dr. Samukawa. Anti-FcεRIα antibody (rabbit antiserum) was from Upstate (#06-725), and antibodies against mTOR, phosphorylated mTOR (p-mTOR), p70S6K, and phosphorylated-p70S6K (p-p70S6K) were from Cell signaling technology (Tokyo, Japan). HuMedia-KG2 and HuMedia-KB2 were from Kurabo (Osaka, Japan). Protease inhibitor cocktail tablet and SYBR Green were from Roche (Tokyo, Japan). Anti-β-actin antibody was from Sigma (St. Louis, MO, USA). Isogen was from Nippongene (Toyama, Japan). ReverTra Ace[®] qPCR RT Kit was from Toyobo (Osaka, Japan). 2,4-dinitrophenylated ascaris extract (DNP-Ascaris) (LSL-LG0009) was from Cosmo Bio, Ltd. (Tokyo, Japan). 2,4-dinitrofluorobenzene (DNFB) was from Nacalai Tesque (Kyoto, Japan).

2.3. Cell culture of human basophils

Human basophil KU812 cells obtained from Health Science Research Resources Bank (Osaka, Japan) were maintained in RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin, under 5% CO₂ at 37 °C. After pre-cultured KU812 cells with RGE or ginsenosides (Rb1, Rg1, Rg3, and Rh1) for 12 h, cells were changed all of free fresh media and were stimulated with an anti-FcεRIα antibody (0.02% serum antibody against human FcεRIα) in the absence or presence of RGE, compared with control serum antibody from non-immune rabbit. KU812 cells were washed with phosphate-buffered saline and collected by centrifugation in ice-cold phosphate-buffered saline.

2.4. Cell culture of human keratinocytes

Human epidermal keratinocyte NHEK (NB) cells obtained from Kurabo were maintained in HuMedia-KG2 containing in 10 µg/mL insulin, 0.1 ng/mL human epidermal growth factor, 0.67 µg/mL hydrocortisone hemisuccinate, 0.4% v/v bovine pituitary extract, 50 µg/mL gentamycin, and 50 ng/mL amphotericin B, under 5% CO₂ at 37 °C. After pre-cultured NHEK (NB) cells with RGE or ginsenosides (Rb1, Rg1, Rg3, and Rh1) for 12 h, cells were changed to HuMedia-KB2 containing 1% HuMedia-KG2, and were stimulated by IFN-γ (50 ng/mL) with or without RGE.

2.5. Western blot analysis

Whole cell lysates were prepared with RIPA buffer containing protease inhibitor. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5 or 10% gel. Immunoblot analysis was performed with antibodies against mTOR (1:5000), p-mTOR (1:5000), p70S6K (1:1000), p-p70S6K (1:1000), and β-actin (1:5000) at 4 °C for 12 h. Blotted proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG and Immobilon Western Chemiluminescent horseradish peroxidase substrate (Merck Millipore; Darmstadt, Germany), and were measured using a computed image analysis system (Image quant LAS-4000 mini, GE Healthcare UK; Buckinghamshire, UK).

2.6. Quantitative real-time PCR

Total RNA (0.1 µg) extracted from cells using Isogen, according to the manufacturer's instructions, was transcribed into cDNA with the ReverTra Ace[®] qPCR RT Kit in a total volume of 10 µL, according

to the manufacturer's instructions, as follows: incubation for 15 min at 37 °C, followed by heating for 5 min at 98 °C to stop the reaction. cDNAs were then used as templates for PCR amplification using SYBR Green. For human C-C chemokine ligand 2 (CCL2), forward; 5'-CAG CCA GAT GCA ATC AAT GCC-3' and reverse 5'-TGG AAT CCT GAA CCC ACT TCT-3' primers were used. For β -defensin-3, forward; 5'-TCC AGG TCA TGG AGG AAT CAT-3' and reverse 5'-CGA GCA CTT GCC GAT CTG T-3' primers were used. For filaggrin, forward; 5'-GGA CTC TGA GAG GCG ATC TG-3' and reverse 5'-TGC TCC CGA GAA GAT CCA T-3' primers were used. For cathepsin H-3, forward; 5'-AGT GGG GAATGA ACG GGT A-3' and reverse 5'-CCA GAG GGA TGG GGT AGG-3' primers were used. For 18S rRNA, forward; 5'-GCA ATT ATT CCC CAT GAA CG-3' and reverse 5'-AGG GCC TCA CTA AAC CAT CC-3' primers were used. The interpolated values for each sample were divided by the corresponding values for 18S rRNA, as the housekeeping gene, and the results are expressed as the ratio relative intensity for specific gene/18S rRNA. Real-time PCR reactions were performed for 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, 10 s at 72 °C in Light Cycler 96 (Roche).

2.7. AD model mice

Two weeks after sensitization with DNP-ascaris and alum, a total volume of 100 μ L of DNFB dissolved in acetone at a concentration of 0.15% was topically applied every 48 h to both sides of each ear lobe, 5 times in total (day 15, 17, 19, 21, and 23). Two weeks after the fifth application (day 37), the mice received a similar final challenge. RGE (1%) or ginsenosides (Rb1, Rg1, Rg3, and Rh1; 0.01%) were dissolved in ethanol and given topically to mice for 8 days before the final DNFB challenge. Control mice were treated with ethanol after fifth dosage of DNFB and no final DNFB challenge. At 6 h after the DNFB challenge, the ears were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

2.8. Statistical analysis

For quantitative PCR and analysis of normalized data from western blot, one-way ANOVA followed by Tukey test or two-way ANOVA followed by Bonferroni test were used. Statistical analysis was performed with Graph Pad PRISM (Version 5.0).

3. Results

3.1. IgE-dependent activation of the mTOR/p70S6K signaling pathway in human basophils

To examine whether IgE activated the mTOR/p70S6K signaling pathway in human basophils, KU812 cells were incubated with an anti-Fc ϵ R1 α antibody (0.02% serum antibody against human Fc ϵ R1 α) for 45 min. Levels of p-mTOR were increased at 15 min after stimulation of Fc ϵ R1 α with the antibody and returned to control levels after 45 min (Fig. 1A and B). We next examined whether p70S6K acted downstream of the mTOR signaling pathway. Similar to the levels of p-mTOR, p-p70S6K levels were increased by the anti-Fc ϵ R1 α antibody with a peak at 15 min (Fig. 1A and B). In contrast, the control antibody did not affect mTOR or p70S6K levels (data not shown). Moreover, we confirmed the effects of RGE on mTOR/p70S6K pathway activation through Fc ϵ R1 α in KU812 cells. RGE (100 μ g/mL) significantly inhibited p70S6K phosphorylation, but it did not affect mTOR phosphorylation (Fig. 2A and B). Interestingly, ginsenosides Rg3 and Rh1 each suppressed the increase in phosphorylated p70S6K in a dose-dependent manner (Fig. 2C). However, the ginsenosides did not affect mTOR phosphorylation.

3.2. IFN- γ -dependent mTOR/p70S6K signaling in human keratinocytes

We next examined whether IFN- γ activated the mTOR/p70S6K signaling pathway in a human keratinocyte line, NHEK (NB). p-mTOR levels were increased by IFN- γ (50 ng/mL) over 24 h in a time-dependent manner. Levels of p-p70S6K peaked at 6–9 h and returned to control levels by 24 h (Fig. 3A and B). In contrast, total mTOR and total p70S6K levels were unchanged by IFN- γ treatment. Furthermore, RGE (100 μ g/mL) suppressed the increase in p-p70S6K levels, but had no effects on p-mTOR levels at 12 h after IFN- γ addition (Fig. 4A and B). Similar to IgE-dependent p70S6K activation in basophils, Rg3 and Rh1 strongly suppressed p70S6K phosphorylation in IFN- γ -stimulated human keratinocytes (Fig. 4C).

3.3. Expression of chemokines in human keratinocytes

Because IFN- γ induced expression of pro-inflammatory genes through the p70S6K signaling pathway, we confirmed CCL2 mRNA expression in NHEK (NB) cells. RGE suppressed the increase in CCL2 mRNA levels by IFN- γ (Fig. 5A). In contrast, mRNA expression of antimicrobial/host defense peptide human β -defensin-3 was not decreased by RGE (Fig. 5B). In addition, neither IFN- γ nor RGE affected mRNA expression of filaggrin, an important component of the skin barrier to the external environment (Fig. 5C). In contrast, mRNA expression of the protease cathepsin H was decreased by IFN- γ (Fig. 5D). These results suggested that RGE suppressed the increase in CCL2 mRNA levels by IFN- γ via inhibition of p70S6K phosphorylation.

3.4. The p70S6K signaling pathway in AD mice

We previously demonstrated that RGE attenuates histamine-induced vascular permeability and scratching behavior in rat and mouse AD models. We next examined whether mTOR and p70S6K were phosphorylated in AD mice treated with or without RGE (AD + RGE and AD groups, respectively). Levels of p-p70S6K and total p70S6K were elevated in AD mice compared with control mice (Fig. 6A and B). p-p70S6K levels in the AD + RGE group were lower than those in the AD group, while levels of total p70S6K were the same in both groups. In contrast, p-mTOR and total mTOR levels were similar in both groups. These results demonstrated that RGE may be an effective drug through suppression of p70S6K activation in the inflammatory skin of AD.

4. Discussion

Red ginseng is one of the most popular medicinal plants in traditional medicine of East Asian countries, including Korea, China, and Japan, which is prepared by a process of repeated steaming and drying of fresh ginseng until it has less than 15% moisture content. It has been reported that the steaming process increases red ginseng bioactivity.¹⁵ Red ginseng contains various pharmacologically active substances such as ginsenosides, polysaccharides, polyacetylenes, phytosterols, and essential oils. Notably, ginsenosides are not usually found in raw ginseng.¹⁶ Many previous studies have shown that red ginseng inhibits PI3-kinase, Akt, and MAP kinase signaling pathways, causing apoptosis in neuroblastoma cells.¹⁷ However, the effects of red ginseng on p70S6K as a downstream PI3-kinase signaling molecule were previously unknown. In this study, our results revealed that a methanol extract of red ginseng, RGE, inhibited p70S6K phosphorylation induced by Fc ϵ R1 α in basophils and IFN- γ in keratinocytes (Figs. 2A, B and 4A, B). Moreover, we confirmed in AD model mice that p70S6K was indeed

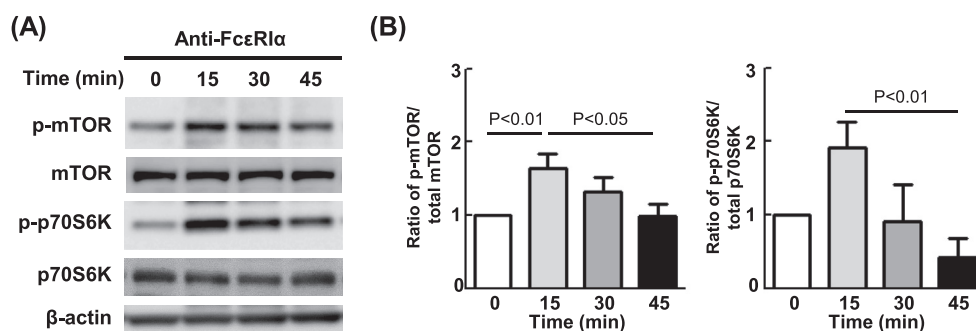


Fig. 1. Time course of mTOR/p70S6K signaling activity in human basophil KU812 cells. (A) Levels of phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K), total mTOR and p70S6K were analyzed by western blotting 15–45 min after treatment of KU812 cells with an anti-FcεRIα antibody (0.02% rabbit serum antibody against human FcεRIα). β-Actin was used as a loading control. (B) Ratios of p-mTOR/total mTOR and p-p70S6K/total p70S6K are shown. The values are means $\pm$ SE ($n = 3-4$). Results of one-way ANOVAs showed a significant increase in p-mTOR/total mTOR ratios after anti-FcεRIα antibody administration and in p-p70S6K/total p70S6K ratios after anti-FcεRIα antibody administration. When one-way ANOVA indicated a significant difference, significance between individual groups were assessed using Tukey's test. The results of one-way ANOVA of p-mTOR/total mTOR were as follows: $F(3,17) = 5.26$, $p = 0.0095$, and p-p70S6K/total p70S6K were as follows: $F(3,11) = 4.17$, $p = 0.0337$.

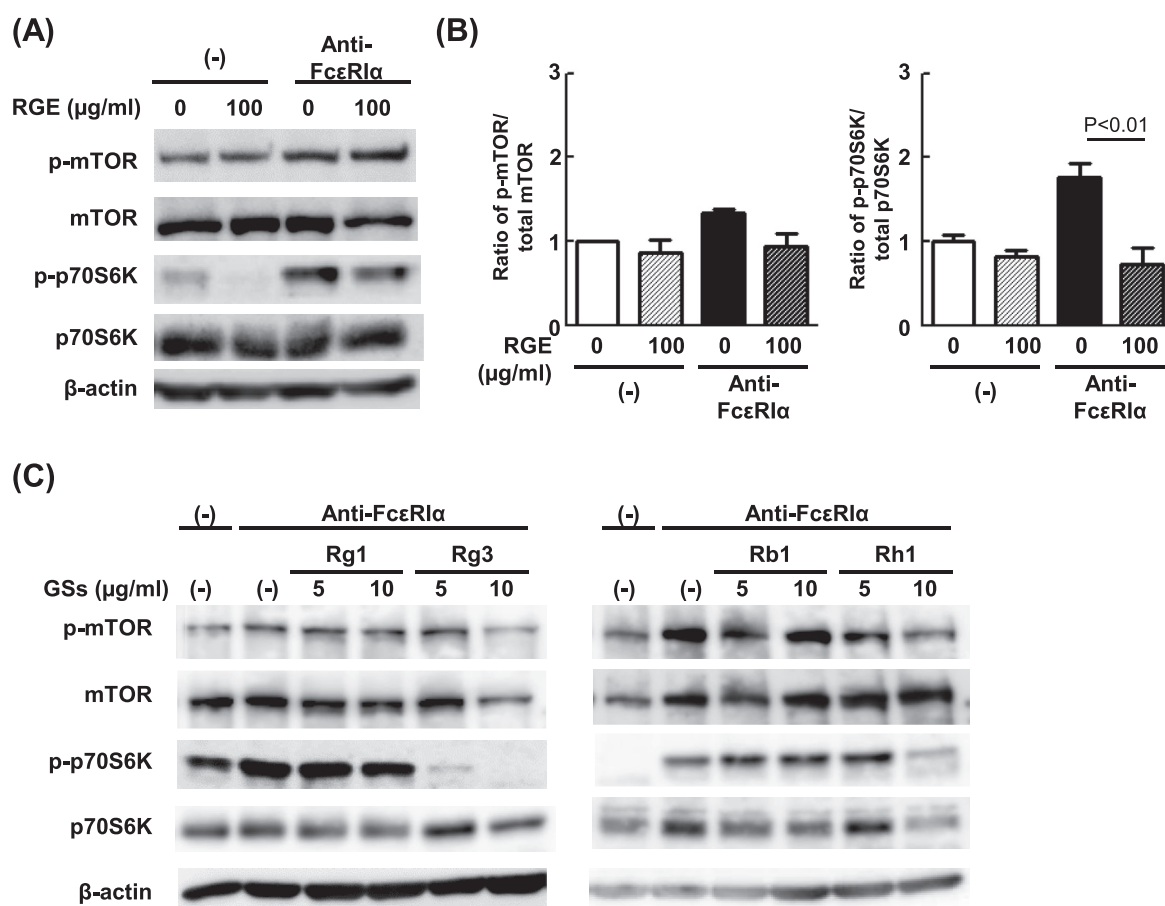


Fig. 2. RGE attenuate p70S6K phosphorylation in human basophil KU812 cells. KU812 cells were pretreated for 12 h with RGE (100 $\mu\text{g}/\text{mL}$) or ginsenosides (GSs; Rg1, Rg3, Rb1, and Rh1) (5 or 10 $\mu\text{g}/\text{mL}$). The anti-FcεRIα antibody (0.02% rabbit serum antibody against human FcεRIα) was added, in the presence or absence of RGE or ginsenosides, with incubation for 15 min. (A) Levels of phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K) and total mTOR and p70S6K, in cells with or without RGE treatment, were analyzed by western blotting. β-Actin was used as a loading control. (B) Ratios of p-mTOR/total mTOR and p-p70S6K/total p70S6K, with or without RGE, are shown. The values are means $\pm$ SE ($n = 3-4$). Statistical differences were determined by two-way ANOVA followed by the Bonferroni test. The results of two-way ANOVA of p-mTOR/total mTOR were as follows: FcεRIα stimulation: $F(1,10) = 5.67$, $p = 0.0386$; RGE inhibition: $F(1,10) = 3.45$, $p = 0.0928$; and FcεRIα $\times$ RGE interaction: $F(1,10) = 1.37$, $p = 0.2694$, and p-p70S6K/total p70S6K were as follows: FcεRIα stimulation: $F(1,11) = 10.70$, $p = 0.0075$; RGE inhibition: $F(1,11) = 4.01$, $p = 0.0705$; and FcεRIα $\times$ RGE interaction: $F(1,11) = 6.07$, $p = 0.0315$. (C) Levels of p-mTOR, p-p70S6K, total mTOR and p70S6K, in cells with or without GEs, were analyzed by western blotting. β-Actin was used as a loading control.

phosphorylated and RGE treatment decreased the p-p70S6K levels in AD mice (Fig. 6). Therefore, we suggest that RGE may be topically effective in the inflamed skin of murine AD. RGE contains certain ginsenosides. Therefore, we examined which of the four kinds of ginsenosides suppressed the p70S6K signaling pathway in cultured

cells. We found that ginsenosides Rg3 and Rh1 inhibited p70S6K phosphorylation through two different signaling pathways (Figs. 2C and 4C). In contrast, FcεRIα- or IFN- γ -activated mTOR phosphorylation was not significantly decreased by RGE. Moreover, p-mTOR levels were similarly increased in AD and AD + RGE mice compared

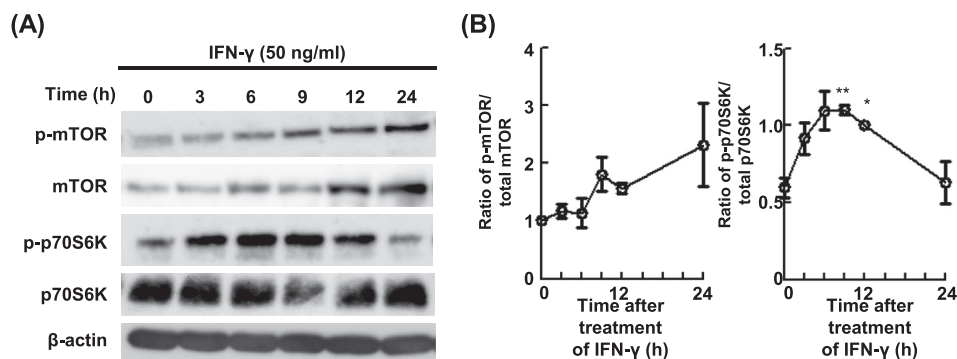


Fig. 3. Interferon- γ caused p70S6K phosphorylation in human keratinocyte NHEK (NB) cells. (A) Levels of phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K), total mTOR and p70S6K were analyzed by western blotting after treatment of NHEK (NB) cells with interferon- γ (IFN- γ) (50 ng/mL) for 24 h. β -Actin was used as a loading control. (B) Ratios of p-mTOR/total mTOR and p-p70S6K/total p70S6K are shown. The values are means $\pm$ SE ($n = 3-4$). One-way ANOVAs did not show a significant increase in the ratio of p-mTOR/total mTOR after IFN- γ administration, whereas there was a significant increase in the ratio of p-p70S6K/total p70S6K after IFN- γ administration. If the one-way ANOVA indicated significance, differences between individual groups were assessed with Tukey's test. Difference with * $p < 0.05$ or ** $p < 0.01$ at 0 h were considered significant. The results of one-way ANOVA of p-mTOR/total mTOR were as follows: $F(5,14) = 2.19$, $p = 0.1138$, and p-p70S6K/total p70S6K were as follows: $F(5,17) = 6.20$, $p = 0.0019$.

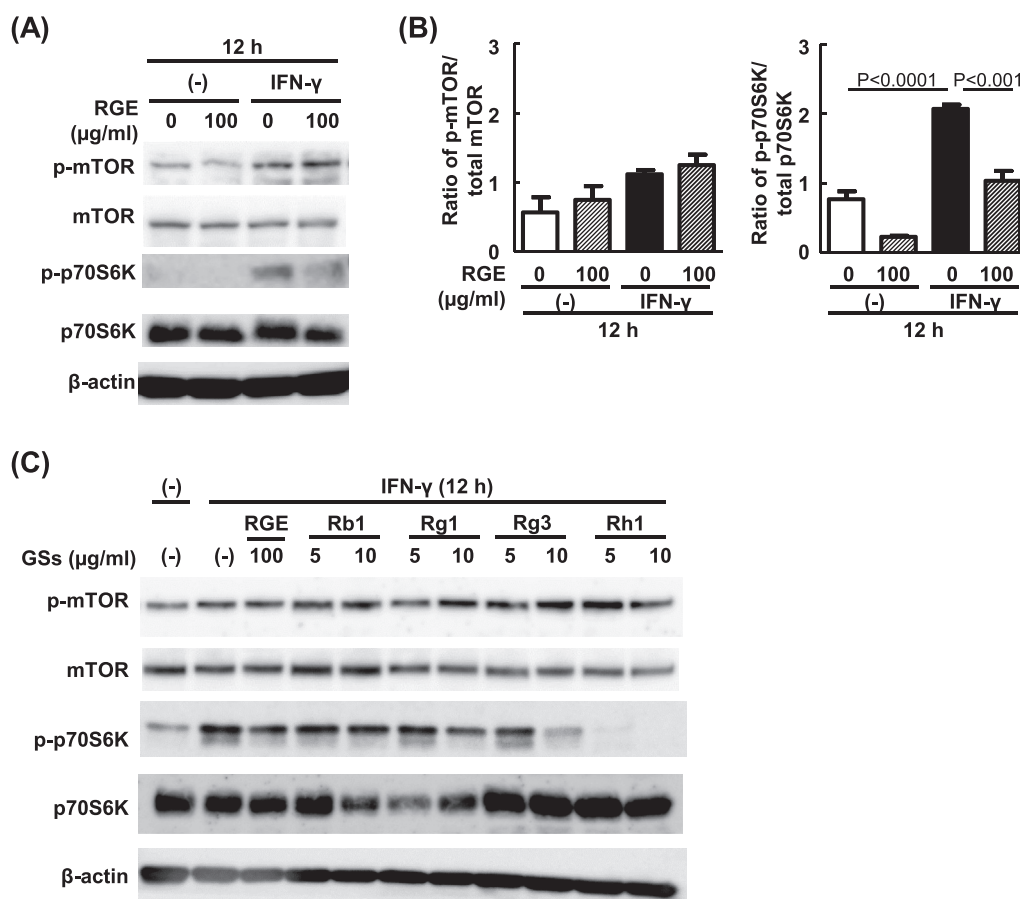


Fig. 4. RGE attenuate p70S6K phosphorylation in human keratinocyte NHEK (NB) cells. Interferon- γ (IFN- γ) (50 ng/mL) was incubated with NHEK (NB) cells for 12 h, in the presence or absence of RGE (100 μ g/mL) or ginsenosides (GSs; Rg1, Rg3, Rb1, and Rh1) (5 or 10 μ g/mL). (A) Levels of phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K) and those of total mTOR and p70S6K were analyzed by western blotting. β -Actin was used as a loading control. (B) Ratios of p-mTOR/total mTOR or p-p70S6K/total p70S6K, in cells with or without RGE, are shown. The values are means $\pm$ SE ($n = 3-4$). Statistical differences were determined by two-way ANOVA followed by the Bonferroni test. The results of two-way ANOVA of p-mTOR/total mTOR were as follows: IFN- γ stimulation: $F(1,10) = 0.79$, $p = 0.3942$; RGE inhibition: $F(1,10) = 0.769$, $p = 0.4248$; and IFN- $\gamma \times$ RGE interaction: $F(1,10) = 0.72$, $p = 0.4166$, and p-p70S6K/total p70S6K were as follows: IFN- γ stimulation: $F(1,16) = 111.27$, $p < 0.0001$; RGE inhibition: $F(1,16) = 62.69$, $p < 0.0001$; and IFN- $\gamma \times$ RGE interaction: $F(1,16) = 6.12$, $p = 0.0249$. (C) Levels of p-mTOR, p-p70S6K, total mTOR and p70S6K, in cells with or without GEs, were analyzed by western blotting. β -Actin was used as a loading control.

with control mice. mTOR forms two multi-protein complexes that regulate different aspects of mTOR signaling. mTOR complex 1 (mTORC1) binds to raptor (regulatory-associated protein of mTOR) and controls 5'-cap-dependent mRNA and protein expression

through two downstream targets, p70S6K and eukaryotic initiation factor 4E-binding protein (4E-BP) 1/2.¹⁸ Our results indicate that RGE retains the ability to inhibit phosphorylation of p70S6K both *in vitro* and *in vivo*. However, the ability of RGE to inhibit 4E-BP1

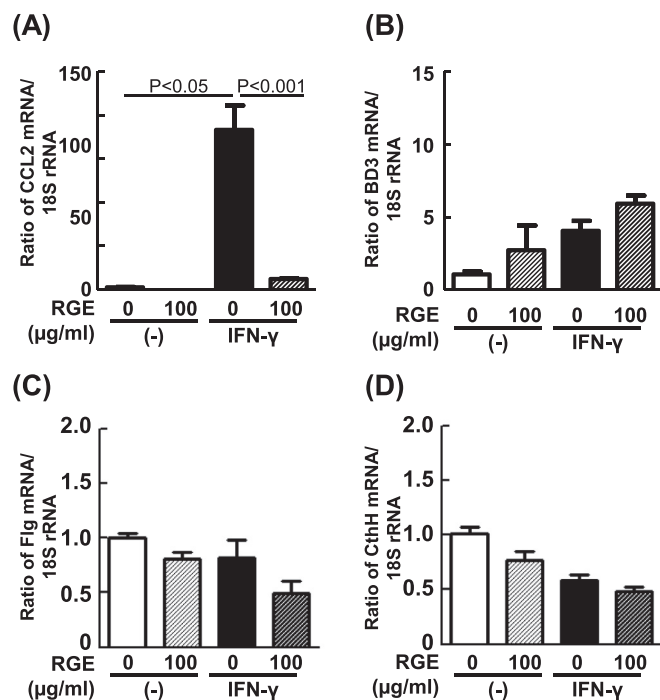


Fig. 5. RGE suppresses the interferon- γ -induced increase in mRNA expression of CCL2 in human keratinocyte NHEK (NB) cells. Interferon- γ (IFN- γ) (50 ng/mL) was incubated with NHEK (NB) cells for 12 h, in the presence or absence of RGE (100 µg/mL). mRNA levels of CCL2, β -defensin-3 (BD3), filaggrin (Flg) and cathepsin H (CthH) were analyzed by real-time PCR in NHEK (NB) cells. Levels of the amplified products were normalized to 18S rRNA levels. Data are means $\pm$ SE ($n = 3-5$) of at least four samples. Statistical differences were determined by two-way ANOVA followed by the Bonferroni test. The results of two-way ANOVA of CCL2 mRNA/18S rRNA were as follows: IFN- γ stimulation: $F(1,13) = 31.09$, $p < 0.0001$; RGE inhibition: $F(1,13) = 25.26$, $p = 0.0002$; and IFN- $\gamma \times$ RGE interaction: $F(1,13) = 23.79$, $p = 0.0003$ (A), BD3 mRNA/18S rRNA: IFN- γ stimulation: $F(1,13) = 15.06$, $p = 0.0019$; RGE inhibition: $F(1,13) = 4.88$, $p = 0.0457$; and IFN- $\gamma \times$ RGE interaction: $F(1,13) = 0.02$, $p = 0.9011$ (B), Flg mRNA/18S rRNA: IFN- γ stimulation: $F(1,21) = 4.73$, $p = 0.0413$; RGE inhibition: $F(1,21) = 4.84$, $p = 0.0391$; and IFN- $\gamma \times$ RGE interaction: $F(1,21) = 0.31$, $p = 0.5827$ (C), and CthH mRNA/18S rRNA: IFN- γ stimulation: $F(1,23) = 35.45$, $p < 0.0001$; RGE inhibition: $F(1,23) = 8.27$, $p = 0.0085$; and IFN- $\gamma \times$ RGE interaction: $F(1,23) = 1.33$, $p = 0.2614$ (D).

phosphorylation is unclear. Rapamycin, which is a candidate mTORC1 inhibitor, is promising as a new drug for AD treatment.⁷ Interestingly, p70S6K is very efficiently inhibited by rapamycin, whereas 4E-BP1 is partially resistant to rapamycin.¹⁹ Therefore, the capability of not only rapamycin but also RGE to inhibit p70S6K may be related to application to AD.

Mukai et al.²⁰ showed that Fc ϵ RI-expressing basophils are essential for development of delayed type allergic inflammation. Moreover, basophils are pivotal for the development of IgE-mediated chronic allergic inflammation as initiators rather than effectors.²¹ Our study showed that IgE activated the mTOR/p70S6K signaling pathway in human basophils, and that p-70S6K levels were decreased by RGE. Therefore, we speculate that RGE might suppress p70S6K signal activation in basophils infiltrating the inflammatory skin of AD mice. In addition, keratinocytes contribute to the inflammatory reactions and immune responses in AD by regulating the release of cytokines, chemokines, proteases, and bioactive lipids, and to initiating crosstalk between adaptive and innate immune responses by activating T cells in AD patients through the release of key molecules.²² Aberrant activation of signal transducer and activator of transcription (STAT) 1 and STAT3 by IFN- γ and IL-22 has been shown in the epidermis of psoriatic lesions, and that they subsequently control production of CCL2, CXCL10, and CXCL8 chemokines and expression of β -defensin-2 and β -defensin-3 in keratinocytes.^{22,23} Several inflammatory chemokines, including CCL2, are associated with the AD phenotype and potentially support leukocyte recruitment.²² Similarly, we confirmed that IFN- γ increased mRNA expression of CCL2 and β -defensin-3 in human keratinocytes (Fig. 5). It is interesting that RGE attenuated the increase in CCL2 mRNA levels. Therefore, we speculated that CCL2 mRNA production was induced through the p70S6K signaling pathway in IFN- γ -activated keratinocytes. Kiat-surayanon et al.²⁴ recently proposed that human β -defensin-3 enhances expression of claudins, which are tight junction membrane proteins, and decreases paracellular permeability in keratinocyte monolayers. Hence, β -defensin-3 is an important factor for innate immunity in keratinocytes during the process of wound closure. We found that the increase in β -defensin-3 mRNA levels was not affected by RGE, enabling induction of β -defensin-3 mRNA independently of the IFN- γ -activated p70S6K signaling pathway.

In our study, we demonstrated that RGE inhibited p70S6K phosphorylation in Fc ϵ RI α -stimulated basophils and IFN- γ -activated keratinocytes. In particular, ginsenosides Rg3 and Rh1 suppressed the p70S6K signaling pathway. Moreover, the levels of phosphorylated p70S6K were elevated in skin lesions of AD model mice, and this effect was attenuated by RGE treatment. However, it still remains unknown whether RGE treatment inhibits basophil infiltration through p70S6K signaling pathways in AD mice. Further research should examine mTOR/p70S6K-dependent inflammatory relationships between keratinocytes and basophils in AD. In conclusion, RGE is an inhibitor of the p70S6K signaling pathway

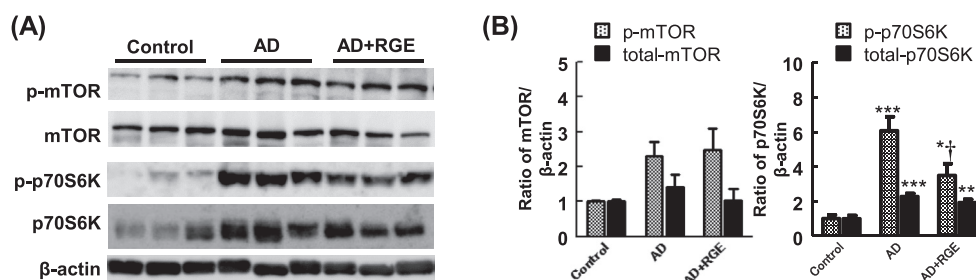


Fig. 6. RGE suppresses p70S6K phosphorylation in AD mice. Mice were treated once with intraperitoneally injected DNP-ascaris and alum and then they were topically applied, along with DNFB, once every 48 h to both sides of each ear lobes, for a total of 5 treatments (on DAY 15, 17, 19, 21 and 23). At 2 weeks after the fifth application (on DAY37), the mice received a similar final challenge. RGE (1%) was dissolved in ethanol and was given topically to mice for 8 d before the final DNFB challenge (AD + RGE). Similar to the treatment of AD mice, Control mice were treated with DNP-ascaris and DNFB, but they did not receive the final DNFB challenges. (A) Levels of phosphorylated mTOR (p-mTOR) and p70S6K (p-p70S6K) and those of total mTOR and p70S6K were analyzed by western blotting. β -Actin was used as a loading control. (B) Each value is shown as a ratio to that of β -actin. The values are means $\pm$ SE ($n = 6-8$) for 9 mice. Statistical differences were determined by one-way ANOVA followed by Tukey's test. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0001$ compared to control group. $\dagger P < 0.05$ compared to AD group. The results of one-way ANOVA of p-mTOR and total mTOR were as follows: $F(2,15) = 3.558$, $p = 0.054$, and $F(2,15) = 0.63$, $p = 0.5454$. p-p70S6K and total p70S6K were as follows: $F(2,20) = 16.23$, $p < 0.0001$, and $F(2,15) = 12.85$, $p = 0.0006$.

and a potential therapeutic agent against inflammatory responses involving activation of p70S6K signaling in AD.

Conflict of interest

None declared.

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References

1. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. *Curr Opin Immunol.* 2003;15:639–646.
2. Kinet J-P. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu Rev Immunol.* 1999;17:931–972.
3. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol.* 2002;2:773–786.
4. Harir N, Friedbichler K, Sonneck K, et al. Oncogenic kit controls neoplastic mast cell growth through a Stat5/PI3-kinase signaling cascade 2008;112: 2463–2473.
5. Lusková P, Dráber P. Modulation of the Fcεpsilon receptor I signaling by tyrosine kinase inhibitors: search for therapeutic targets of inflammatory and allergy diseases. *Curr Pharm Des.* 2004;10:1727–1737.
6. Tedeschi A, Lorini M, Galbiati S, Gibelli S, Miadonna A. Inhibition of basophil histamine release by tyrosine kinase and phosphatidylinositol 3-kinase inhibitors. *Int J Immunopharmacol.* 2000;22:797–808.
7. Yang F, Tanaka M, Wataya-Kaneda M, et al. Topical application of rapamycin ointment ameliorates Dermatophagoides farina body extract-induced atopic dermatitis in NC/Nga mice. *Exp Dermatol.* 2014;23:568–572.
8. Georas SN, Cheadle C, Berger AE. Tight junction defects in atopic dermatitis. *J Allergy Clin Immunol.* 2012;127:773–786.
9. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. *Biochim Biophys Acta.* 2010;1788:864–871.
10. Sugita K, Kabashima K, Atarashi K, Shimauchi T, Kobayashi M, Tokura Y. Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin. *Clin Exp Immunol.* 2007;147: 176–183.
11. Albanesi C, Scarponi C, Sebastiani S, et al. A cytokine-to-chemokine axis between T lymphocytes and keratinocytes can favor Th1 cell accumulation in chronic inflammatory skin diseases. *J Leukoc Biol.* 2001;70:617–623.
12. Federici M, Giustizieri ML, Scarponi C, Girolomoni G, Albanesi C. Impaired IFN-gamma-dependent inflammatory responses in human keratinocytes over-expressing the suppressor of cytokine signaling 1. *J Immunol.* 2002;169: 434–442.
13. Giustizieri ML, Mascia F, Frezzolini A, et al. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. *J Allergy Clin Immunol.* 2001;107: 871–877.
14. Samukawa K, Izumi Y, Shiota M, et al. Red ginseng inhibits scratching behavior associated with atopic dermatitis in experimental animal models. *J Pharmacol Sci.* 2012;118:391–400.
15. Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean Panax ginseng C A Meyer. *Acta Pharmacol Sin.* 2008;29: 1109–1118.
16. Kim WY, Kim JM, Han SB, et al. Steaming of ginseng at high temperature enhances biological activity. *J Nat Prod.* 2000;63:1702–1704.
17. Nguyen CT, Luong TT, Kim GL, Pyo S, Rhee DK. Korean red ginseng inhibits apoptosis in neuroblastoma cells via estrogen receptor β-mediated phosphatidylinositol-3 kinase/Akt signaling. *J Ginseng Res.* 2015;39:69–75.
18. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol.* 2009;10:307–318.
19. Xu L, Brink M. mTOR, cardiomyocytes and inflammation in cardiac hypertrophy. *Biochim Biophys Acta Mol Cell Res.* 2016;1863:1894–1903.
20. Mukai K, Matsuoka K, Taya C, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity.* 2005;23:191–202.
21. Obata K, Mukai K, Tsujimura Y, et al. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood.* 2007;110:913–920.
22. Homey B, Steinhoff M, Ruzicka T, Leung DYM. Cytokines and chemokines orchestrate atopic skin inflammation. *J Allergy Clin Immunol.* 2006;118: 178–189.
23. Lew W, Bowcock AM, Krueger JG. Psoriasis vulgaris: cutaneous lymphoid tissue supports T-cell activation and “Type 1” inflammatory gene expression. *Trends Immunol.* 2004;25:295–305.
24. Kiatsurayanon C, Niyonsaba F, Smithrithee R, et al. Host defense (antimicrobial) peptide, human β-defensin-3, improves the function of the epithelial tight-junction barrier in human keratinocytes. *J Invest Dermatol.* 2014;134: 2163–2173.