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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 Fcε receptor type Iα (FCεRI), 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 (FcεRI). In the response to IgE, these cells rapidly release intracellularly located mediators, including interleukins, leukotrienes and histamine. Major FcεRI 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% CO_2 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% CO_2 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

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