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Cultivated ginseng inhibits 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice and TNF-α/IFN-γ-induced TARC activation in HaCaT cells

Jae Ho Choi [a,1], Sun Woo Jin [a,1], Bong Hwan Park [a], Hyung Gyun Kim [a], Tilak Khanal [a], Hwa Jeong Han [a], Yong Pil Hwang [a,b], Jun Min Choi [c], Young Chul Chung [c], Sang Kyu Hwang [d], Tae Cheon Jeong [e,*], Hye Gwang Jeong [a,*]

[a] Department of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Department of Pharmaceutical Engineering, International University of Korea, Jinju, Republic of Korea
[c] Department of Food and Medicine, International University of Korea, Jinju, Republic of Korea
[d] Department of Management, International University of Korea, Jinju, Republic of Korea
[e] College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea

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ABSTRACT

Ginseng contains many bioactive constituents, including various ginsenosides that are believed to have anti-allergic, anti-oxidant, and immunostimulatory activities; however, its effects on atopic dermatitis (AD) remain unclear. In the current study, we hypothesized that cultivated ginseng (CG) would inhibit 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in NC/Nga mice by regulating the T helper (Th)1/Th2 balance. Also, CG inhibits TNF-α/IFN-γ-induced thymus- and activation-regulated chemokine (TARC) expression through nuclear factor-kappa B (NF-κB)-dependent signaling in HaCaT cells. CG ameliorated DNCB-induced dermatitis severity, serum levels of IgE and TARC, and mRNA expression of TARC, TNF-α, IFN-γ, IL-4, IL-5, and IL-13 in mice. Histopathological examination showed reduced thickness of the epidermis/dermis and dermal infiltration of inflammatory cells in the ears. Furthermore, CG suppressed the TNF-α/IFN-γ-induced mRNA expression of TARC in HaCaT cells. CG inhibited TNF-α/IFN-γ-induced NF-κB activation. These results suggest that CG inhibited the development of the AD-like skin symptoms by modulating Th1 and Th2 responses in the skin lesions in mice and TARC expression by suppressing TNF-α/IFN-γ-induced NF-κB activation in keratinocytes, and so may be a useful tool in the therapy of AD-like skin symptoms.

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with a clinical presentation of erythematous skin, lichenification with cutaneous hypersensitivity, and intense pruritus (Leung, 2000). AD is a multifactorial skin disease, with complex interactions of innate and adaptive immune responses based on a strong genetic, environmental, pharmacological, and psychological predisposition and triggered by environmental factors (Udompataikul and Limpa-o-vart, 2012). Various factors, including immunological abnormalities contribute to the pathogenesis and development of AD. The early onset of AD in infancy results in it being the most prevalent chronic skin disorder in childhood and can affect individuals throughout their lifetimes (Choi et al., 2012a,b).

The NC/Nga strain originated from Japanese mice and is the most extensively studied animal model of AD. Skin changes develop spontaneously in NC/Nga mice exposed to various allergens and closely mimic human AD. NC/Nga mice have been reported to develop AD-like eczematous skin lesions when kept in conventional conditions but not when maintained under specific pathogen-free (SPF) conditions (Hiramoto et al., 2010). To reduce experimental error and to induce a stable clinical AD-like skin disease, many researchers have used the chemical haptens, DNCB, 2,4-dinitrofluorobenzene (DNFB), and 2,4,6-trinitro-1-chrolobenzene (TNCB). Application of the haptens DNCB, DNFB, or TNCB produces an AD-like skin disease in NC/Nga mice (Park et al., 2012; Kim et al., 2011). DNCB was used on the basis of evidence of its efficacy in creating AD-like skin lesions and IgE hyperproduction in NC/Nga mice (Jung et al., 2010; Yang et al., 2011; Fujii et al., 2009). The pathogenesis of DNCB-induced contact hypersensitivity is

* Corresponding authors. Tel.: +82 53 810 2819 (T.C. Jeong), tel.: +82 42 821 5936 (H.G. Jeong).
E-mail addresses: hgjeong@cnu.ac.kr (T.C. Jeong), taecheon@ynu.ac.kr (H.G. Jeong).
[1] These authors are contributed equally to this work.

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predominantly the result of a T cell mediated immune response (Zhang et al., 2009). The scratching behavior is the first symptom of the AD-like skin changes and is followed by rapidly developing hemorrhages, edema, scarring, and erosion of the face, ears, neck, and back. Histological examination shows dermal infiltration with mast cells, mononuclear cells, and eosinophils prior to the appearance of clinical skin symptoms.

The pathogenesis of AD is not fully understood, but specific immune and inflammatory mechanisms culminate in a complex series of cellular interactions, leading to the signs and symptoms of AD (Liu et al., 2011). Along with the AD-like skin changes, NC/Nga mice show preferential Th differentiation toward Th2 cells in the spleen, accumulation of eosinophils and mast cells in the skin lesions, and increased IgE levels (Kohara et al., 2001; Vestergaard et al., 1999). Acute AD skin lesions reveal the important role of the Th2 type immune response, characterized by the infiltration of $CD4^+$ T cells and the secretion of interleukin (IL)-4, IL-5, and IL-13, whereas in chronic AD skin lesions, Th1 type immune responses, characterized by the production of tumor necrosis factor (TNF)-α and interferon (IFN)-γ by Th1 cells, and delayed-type hypersensitivity reactions are responsible (Jin et al., 2009; Leung and Bieber, 2003; Werfel et al., 1996). Recent reports have shown that typical AD symptoms involve increased levels of Th2-mediated cytokines and a deficiency in Th1-mediated cytokines (Sawada et al., 2012). Immunoglobulin E (IgE) production is also closely related to AD; indeed, elevated IgE levels are a hallmark of AD and the expression of IL-4 contributes to this elevation. IL-4 stimulates IgE production in B cells. IgE is released from B cells and then binds to mast cells. Mast cells degranulate and release various biological mediators in IgE-mediated AD (Amin, 2012; Kishimoto and Hirano, 1988; Poulsen and Hummelshoj, 2007). AD is dependent on the secretion of the cytokines IL-4, IL-5, and IL-13 by Th2 cells that are generated from precursors. Furthermore, recent studies have shown that T cells constitutively expressing CD25, T regulatory cells (Tregs), regulate AD (Stassen et al., 2004; Szegedi et al., 2009). It has been suggested that IL-10 production by Tregs suppresses both Th1- and Th2-mediated immune responses (Wilson et al., 2007). Thus, it is important to consider the balance among Th1, Th2, and Treg cells in AD patients.

Cultivated ginseng (CG, *Panax ginseng* C.A. Meyer) is one of the most widely used medicinal herbs in traditional and oriental medicine (de Andrade et al., 2007). It has many biological and pharmacological effects, including anti-inflammatory, anti-oxidative, anti-tumor, and anti-aging activities (Jung et al., 2012; Kim et al., 2012; Wong et al., 2010; Ho et al., 2010). We previously reported that CG suppresses ultraviolet B-induced collagenase activation via mitogen-activated protein kinases and nuclear factor-kappa B (NF-κB)/activator protein-1 (AP-1)-dependent signaling in human dermal fibroblasts (Hwang et al., 2012). Although CG has been reported to have activity in various assays, the effects of CG as an anti-atopic agent for AD remain unknown. In this study, we examined the inhibitory effects of CG on the development of AD in *in vivo* and *in vitro*.

2. Materials and methods

2.1. Chemicals

DNCB (2,4-dinitrochlorobenzene) was obtained from Sigma–Aldrich (Milwaukee, WI, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IgE and TARC were obtained from BD Biosciences (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA). Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Daejeon, Korea). All chemicals and solvents were of the highest commercially available grade.

2.2. Preparation of cultivated ginseng (CG)

Mountain CG (*Panax ginseng* C.A. Meyer; Jangnoisam), naturally grown in forested areas, was collected and identified by the Ham Yang Agricultural Association (Ham Yang, Korea). Cultivated ginseng roots used in this experiment were 5 years old. The CG roots (585 g) were cut into small pieces and extracted twice at 90 °C in distilled water (DW) for 5 h. The solutions were combined, filtered through Whatman No. 1 filter paper, concentrated using a rotary vacuum evaporator (EYELA, Tokyo Rikakikai Co., Tokyo, Japan) under reduced pressure, refrigerated in a recirculating chiller (EYELA CCA-1110) to obtain concentrated extracts, and then lyophilized (EYELA FD-800). The concentration of ginsenosides, the major active ingredients, was measured by high-performance liquid chromatography (HPLC) (Lee et al., 2007). The composition of CG was described previously (Choi et al., 2007; Lee et al., 2007). CG was dissolved with distilled water and then mixed with emollient cream at 5 or 20 mg/kg concentration.

2.3. Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT (gift from Fusenig, German Cancer Research, Germany) (Fusenig and Boukamp, 1998) was cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells (2.5×10^5) were plated in 60 mm plates and cultured until confluent. Cells were treated with CG for 1 h, and then stimulated with TNF-α and IFN-γ (each 20 ng/mL) for 24 h in serum-free culture medium. Control cells were treated with DW alone.

2.4. Measurement of cytotoxicity

Cytotoxicity was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and a lactate dehydrogenase (LDH) release assay kit (Roche, Indianapolis, IN, USA), both according to the manufacturer's instructions. Briefly, the cells (5×10^4/500 μL) were seeded in 48-well plates in 10% FBS-containing medium. After cells had been subjected to the MTT reduction assay, the supernatants were used in the LDH release assay. After incubation, the cells were treated with MTT solution (final concentration, 1 mg/mL) for 1 h. The dark blue formazan crystals that formed in intact cells were solubilized with dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured with a spectrophotometer (Varioskan, Thermo Electron Co., Finland). LDH levels in the supernatants were quantified using a colorimetric test based on the NADH concentration. CG did not interfere with the absorbance measurements at the wavelengths used in these assays.

2.5. Animals and treatment

Specific pathogen free male 6-week-old NC/Nga mice were obtained from SLC, Inc. (Shizuoka, Japan). Animals were acclimatized to the temperature (22 ± 2 °C) and humidity (55 ± 5%) in controlled rooms with a 12/12-h light/dark cycle for at least 2 weeks prior to experiments. Animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water. All experimental protocols for animal care were performed according to the rules and regulations of the Animal Ethics Committee, Chungnam National University. The mice were divided into four groups ($n = 5$ per group). To induce AD-like immunological and skin lesions, DNCB was applied to the dorsal skin and ears. After complete removal of dorsal hairs within an area of approximately 8 cm^2, 200 μL of 0.2% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) was challenged for three times per week for 9 weeks. After inducing AD, emulsion containing CG was applied topically to the dorsal skin and ears of the mice six times per week for 4 weeks. In the CG-treated mice, emollient cream containing CG (5 or 20 mg/kg mouse body weight), 100 μL, was applied topically 1 h before DNCB application each time. Control and DNCB-treated mice received topically applied 100 μL emulsions without CG on the dorsal skin and ears at the same time. Animals were sacrificed 64 days after the first application of DNCB (Fig. 1). Blood was collected from the vena cava, and the right ear was removed and subjected to histopathological examination.

2.6. Measurement of ear thickness

Ear thickness was measured with a micrometer (Mitutoyo, Kawasaki, Japan) on the day of sacrifice. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges, and the thickness was recorded in micrometers. To minimize technique variation, a single investigator performed the measurements throughout each experiment.

2.7. Histopathological studies

The right ear lesions were sliced and tissue slices were fixed in 10% buffered-neutral formalin for 24 h. The fixed ear tissue slices were embedded in paraffin wax, sectioned, deparaffinized, and rehydrated using standard techniques. Sections 5 μm thick were subjected to hematoxylin and eosin (H&E) staining for the detection of various inflammatory cells. Histopathological changes were examined by light microscopy. An arbitrary scope was given to each microscopic field viewed at a magnification of 100×.