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Oleanolic acid acetate inhibits atopic dermatitis and allergic contact dermatitis in a murine model

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ABSTRACT

Atopic dermatitis (AD) and allergic contact dermatitis (ACD) are common allergic and inflammatory skin diseases caused by a combination of eczema, scratching, pruritus, and cutaneous sensitization with allergens. This paper examines whether oleanolic acid acetate (OAA) modulates AD and ACD symptoms by using an existing AD model based on the repeated local exposure of mite extract (*Dermatophagoides farinae* extract, DFE) and 2,4-dinitrochlorobenzene to the ears of BALB/c mice. In addition, the paper uses a 2,4-dinitrofluorobenzene-sensitized local lymph node assay (LLNA) for the ACD model. The oral administration of OAA over a four-week period attenuated AD symptoms in terms of decreased skin lesions, epidermal thickness, the infiltration of immune cells ($CD4^+$ cells, eosinophils, and mast cells), and serum IgE, IgG2a, and histamine levels. The gene expression of Th1, Th2, Th17, and Th22 cytokines was reduced by OAA in the lymph node and ear tissue, and the LLNA verified that OAA suppressed ACD. The oral administration of OAA over a three-day period attenuated ACD symptoms in terms of ear thickness, lymphocyte proliferation, and serum IgG2a levels. The gene expression of Th1, Th2, and Th17 cytokines was reduced by OAA in the thymus and ear tissue. Finally, to define the underlying mechanism, this paper uses a TNF-α/IFN-γ-activated human keratinocyte (HaCaT) model. OAA inhibited the expression of cytokines and chemokines through the downregulation of NF-κB and MAPKs in HaCaT cells. Taken together, the results indicate that OAA inhibited AD and ACD symptoms, suggesting that OAA may be effective in treating allergic skin disorders.

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Introduction

Atopic dermatitis (AD) is a multifactorial disease arising from complex interactions between genetic and environmental factors as well as between features of immune and skin cells (Boguniewicz and Leung, 2010; Finkelman et al., 2010; Novak et al., 2010). *Dermatophagoides farinae* is a typical environmental allergen, and the application of *D. farinae* extract (DFE) to the uninvolved skin of AD patients through an atopy patch causes eczematous reactions in 30–50% of these patients (Leung et al., 2004). DFE-induced skin lesions display two phases: an initial phase with predominantly IL-4-producing Th2 cells and a subsequent phase after 24–48 h characterized by IFN-γ-producing Th1 cells (Leung and Bieber, 2003; Leung et al., 2004). The important role of Th1 and Th2 cytokines in skin inflammation has been demonstrated through experimental models (Novak, 2009).

Acute AD skin lesions are characterized by epidermal and dermal thickening, dermal infiltration of $CD4^+$ T cells and eosinophils, and local expression of Th2 cytokines (Frohlich et al., 2009; Novak, 2009). Chronic AD skin lesions undergo tissue remodeling caused by chronic inflammation. These skin lesions are associated with epidermal hyperplasia, increased collagen deposition in the dermis. Recently, Th17 and Th22 cells have been detected in skin lesions of AD patients (Nograles et al., 2009). IL-17-producing Th17 cells are present in acute AD skin lesions (Toda et al., 2003). IL-22-producing Th22 cells are increased in skin lesions of patients with chronic AD (Fujita et al., 2011). In addition, the activation of keratinocytes is a hallmark of the pathogenesis of AD (Leung et al., 2004; Oyoshi et al., 2009).

Abbreviations: AD, atopic dermatitis; ACD, allergic contact dermatitis; OAA, oleanolic acid acetate; DFE, *Dermatophagoides farinae* extract; DNCB, 2,4-dinitrochlorobenzene; LLNA, local lymph node assay; DNFB, 2,4-dinitrofluorobenzene.

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Allergic contact dermatitis (ACD) is a chronic disease mediated by T cells caused by repeated skin exposure to contact allergens and is characterized by redness, papule, and vesicles, followed by scaling and dryness (Rose et al., 2012; Vocanson et al., 2005). Many commercial formulations for the treatment of ACD are available in gels, creams, lotions, or ointments but show limited effectiveness (Shah et al., 2012). Many studies have addressed the effects of the topical or systemic administration of corticosteroids and calcineurin inhibitors such as cyclosporine A (Cys A) and tacrolimus (FK506) (Quemeneur et al., 2003), but it is well known that the prolonged use of glucocorticoids and immune suppressors can cause various side effects.

Recently, several effective triterpenoid compounds such as ursolic acid, oleanolic acid, and butulinic acid have been introduced for the treatment of anti-allergic, anti-asthmatic, and anti-inflammatory activities (Giner-Larza et al., 2001; Lee et al., 2010). Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid: OA) is a triterpenoid compound found in many plants. OA inhibits delayed-type hypersensitivity induced by 2,4-dinitrofluorobenzene (DNFB) in the mouse ear skin (Giner-Larza et al., 2001) and has been shown be a remarkable inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase and c-Fos gene expression in the mouse skin (Oguro et al., 1998). Similarly, OA analogs are powerful inhibitors for cellular inflammatory processes such as the induction of NO synthase and cyclooxygenase 2 (Dinkova-Kostova et al., 2005). The inhibitory effect of the extract of *Vigna angularis* on 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in NC/Nga mice has been reported (Collantes et al., 2012). For incremental research, the present paper isolates OA and oleanolic acid acetate (OAA), a derivative of OA, from *V. angularis* through activity-guided fractionation. Despite the similarity in their chemical formulations (Supplementary Fig. S1), little is known about the biological effects of OAA. The paper develops a DFE- and DNCB-induced AD model (Choi et al., 2011b) and modifies the local lymph node assay (LLNA) as a stand-alone method for ACD. The purpose of this paper is to elucidate the effects of OAA on AD and ACD and define the underlying mechanism.

Materials and methods

Animals. Female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan) and housed 5–10 mice per cage in a laminar air flow room maintained at a temperature of 22 ± 2 °C with a relative humidity of 55 ± 5% throughout the experiment. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (approval number KNU-2011-0133).

Preparation of the extract and the purification of compounds from V. angularis. *V. angularis* was purchased from an herbal medicine store in Jeongeup, Korea. The authenticity of the plants was verified (Y. H. Kim, College of Pharmacy at Chungnam National University). *V. angularis* sample (10 kg) was dried, pulverized to a fine powder, and extracted twice with 95% EtOH at 70 °C. The EtOH extract (120 l) was then passed through a 0.45 μm filter and vaporized in a rotary evaporator to yield 100 g of residues. In addition to evaporation, the EtOH extract solution was further extracted to give four fractions: distilled water, hexane, EtOAc, and BuOH extracts. For the isolation of compounds, the EtOAc extract was further chromatographed on a silica gel (230–400 mesh, 1 kg, Merck) by using a step gradient of a hexane–EtOAc solvent system (100:1, 80:1, 60:1, 40:1, 20:1, 10:1, and 1:1; each 1 l, v/v) to yield 5 fractions (H1–H5) by TLC profiles. The recrystallization of H-3 in MeOH yielded compound 1: OAA.

Reagents and cell cultures. All materials and reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. OAA was dissolved in N,N-dimethylacetamide/Cremophor/phosphate-buffered saline (PBS; 1:1:8 solution), and DFE was dissolved in PBS containing 0.5% Tween 20. DNCB (1%) was dissolved in an acetone/olive oil (1:3) solution. A human keratinocyte cell line, namely HaCaT, was maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37 °C in 5% CO_2. DMEM, antibiotics, and trypsin-EDTA were obtained from Invitrogen (Grand Island, NY). Human TNF-α and IFN-γ were purchased from R&D Systems (Minneapolis, MN).

Induction of atopic dermatitis in the mouse ear. The induction of AD by using DFE and DNCB was performed based on previous research (Choi et al., 2011b). Female BALB/c mice (6 weeks) were divided into six groups, and the surfaces of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μl of DNCB (1%) was painted on each ear, followed by 20 μl of DFE (10 mg/ml) after 4 days. The treatment of DFE/DNCB was repeated once a week alternatively for 4 weeks. Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. After confirming the atopic condition by the IgE level, OAA (2, 10, or 50 mg/kg) was orally administered until the end of the 4-week induction period. Ear thickness and clinical scores were measured 24 h after DFE or DNCB application with a dial thickness gauge (Kori Seiki MFG, Co., Japan).

On day 28, blood samples were collected by an orbital puncture. The plasma was stored at −70 °C for further analysis. After blood collection, ears were removed and used for a histopathological analysis. Serum IgE and IgG2a levels were measured using an ELISA kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. The DFE-specific IgE level was indicated by the O.D. value.

Histological and immunohistochemical analysis. The ears were fixed with 10% formaldehyde and embedded in paraffin. Then 5 μm sections were stained with hematoxylin and eosin (H&E). Infiltrated lymphocytes, the thickening of the epidermis, and fibrosis in the dermis were observed using a microscope. For the measurement of mast cell infiltration, skin sections were stained with toluidine blue, and the number of mast cells at five sites chosen at random was counted. Eosinophils and $CD4^+$ T cells were counted blindly in 10 high-power fields at 400× magnification. Dermal thickness was analyzed in H&E-stained sections viewed under 100× magnification and measured in five randomly selected fields for each sample.

Histamine assay. Histamine content was measured through the *o*-phthaldialdehyde spectrofluorometric procedure based on previous research (Bae et al., 2011). The blood from the mice was centrifuged at 400 *g* for 10 min, and the serum was withdrawn to measure histamine content. Fluorescent intensity was measured using 355 nm excitation and 450 nm filters and the fluorescence spectrometer LS-50B (Perkin-Elmer, Norwalk, CT).

FACS sorting. At the end of the experiment, mice were euthanized, and lymph node $CD4^+$ T cells were isolated from each mouse. Separated auricular lymph nodes from each mouse were ground using 70 μm nylon cell strainers (Falcon, Bedford, MA). Cells were washed with PBS, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibodies (Santa Cruz Biotech, Santa Cruz, CA) were added to samples, and incubated at 4 °C for 30 min. The FACS sorting of lymph node single cells was performed using a FACSAria (BD Biosciences, Oxford, UK).

Real-time polymerase chain reaction. A quantitative real-time polymerase chain reaction (PCR) assay was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan) according to the manufacturer's protocol. Briefly, 2 μl of cDNA (100 ng), 1 μl of sense and antisense primer solution (0.4 μM), 12.5 μl of SYBR Premix Ex Taq (Takarabio Inc., Shiga, Japan), and 9.5 μl of dH_2O were mixed together

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