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The mast cell stabilizing activity of Chaga mushroom critical for its therapeutic effect on food allergy is derived from inotodiol

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ABSTRACT

While an anti-allergic effect of Chaga mushroom (*Inonotus obliquus*) has been indicated, its therapeutic effect on allergy and immunoregulatory mechanisms and chemical constituents directly responsible for that are hardly known. We examined the effect of 70% ethanol extract of Chaga mushroom (EE) and its dichloromethane (DF) and aqueous (AF) fractions using a mouse model of chicken ovalbumin (cOVA)-induced food allergy, and found that only EE and DF ameliorated allergy symptoms to a significant extent. The in vivo mast cell-stabilizing activity was also found only in EE and DF whereas the activities to suppress Th2 and Th17 immune responses and cOVA-specific IgE production in the small intestine were observed in all three treatment regimens, implying that inhibition of the mast cell function by lipophilic compounds was vital for the therapeutic effect. Results also indicated that inotodiol, a triterpenoid predominantly present in DF, played an active role as a mast cell stabilizer.

1. Introduction

Food allergy is defined as a series of unfavorable immune reactions against an ingested food protein. While innocuous food proteins taken up through the intestinal mucosa normally induce immune tolerance called oral tolerance, such an immune tolerance mechanism does not operate properly in certain circumstances to cause food allergy [1,2]. Symptoms of food allergy vary from mild itching, skin rash, breathing problem, vomiting and diarrhea to life-threatening anaphylaxis which claims thousands of death each year worldwide [3]. The population to suffer from food allergy has been increasing in an alarming rate during the last decades particularly in developed countries [4].

Food allergy is an IgE-mediated (type-1) hypersensitivity reaction. When a food allergen absorbed through the digestive track interacts with IgE decorating the surface of mast cells via the interaction with FcεRI, mast cells undergo a rapid activation process to release granules containing a variety of effector molecules such as histamine, leukotriene and mast cell proteases [5–7]. In general, mast cell activation and degranulation lead to dilation of blood vessels to cause redness and edema and the recruitment of eosinophil to the gut to exacerbate the allergic inflammation [8]. Besides mast cell and eosinophil, $CD4^+$ T lymphocytes also play a role. Production of Th2 cytokines by $CD4^+$ T cells is critical for class switching of IgM or IgG to IgE in B lymphocytes [9–11]. In addition, Th2 cytokines produced by $CD4^+$ T cells activated in the draining lymph node (dLN) promote recruitment of mast cell and eosinophil to the inflicted tissue as well as their development [12].

Epinephrine, a α-1 agonist to cause vasoconstriction, is routinely used for treating severe anaphylactic reactions in the clinic along with other medications such as anti-histamines, glucocorticoids and β-agonist [13,14]. Still, as dangerous anaphylactic response tends to arise acutely and intensely within a few minutes to an hour after contact with a food allergen, development of preventative therapeutics for food allergy is highly desirable. Special traditional herbal formulas (THFs), hot water extract of assorted plant species, are known to have reliable therapeutic effects on food allergy [15]. While THFs have been used for treatments of various diseases and medical conditions in oriental medicine for generations, their preparation is often cumbersome as a group of selected medicinal herbs have to be collected and decocted in right ratio. In addition, reports that the use of THFs may cause renal impairment and other health complications raise concerns over long-term uses [16–18]. Single herbal preparations, pure substances or mixtures from aqueous or organic solvent extracts of special medicinal

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herbs, are also used for improving various medical conditions [39,40]. Single herbal preparations, which are often used as food (dietary) supplements, are considered as more cost-effective and safer alternatives to THFs.

Chaga mushroom (*Inonotus obliquus*) is a parasitic fungus to grow on birch and other trees belonging to the family *Hymenochaetaceae*. This fungus naturally inhabits the forests of Russia, Korea and Northern Europe. Chaga mushroom is well-known for its therapeutic effects on various diseases including mental, immunological and metabolic disorders and cancer, and has been used as a folk medicine in East Asian countries and Russia for centuries [19,20]. An anti-allergic effect of Chaga mushroom has been also inferred in a study by others [21]. Yet, its therapeutic effect has never been investigated in an animal allergy model. Studies on chemical components and their immunoregulatory activities directly responsible for the anti-allergic effect of the mushroom have not been conducted, either. In this study, we examined the therapeutic effect of 70% ethanol (EtOH) extract of Chaga mushroom and its dichloromethane (CH_2Cl_2) and aqueous fractions on food allergy using a mouse model of chicken ovalbumin (cOVA)-induced food allergy. We also analyzed immunoregulatory activities of the extract and the fractions to better understand the mechanism(s) directly linked to the therapeutic effect. Further, a role of inotodiol, a major tetracyclic triterpenoid in the organic fraction of the mushroom extract, with respect to the anti-allergic effect of the mushroom was investigated and characterized.

2. Materials and methods

2.1. Chemicals and other reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin-glutamine solution, and dinitrophenol (DNP)-bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (Carlsbad, USA). cOVA (grade VI), alum [$KAl(SO_4)_2$-$12H_2O$], eosin, hematoxylin and BSA were purchased from Sigma-Aldrich (St. Louis, USA). Toluidine blue was purchased from Samchun Chemical (Pyungtack, Korea). IL-4, IL-17 and IFN-γ ELISA kits were purchased from Biolegend (San Diego, USA). IL-5 and IL-10 ELISA kits were purchased from BD Biosciences (San Diego, USA). Biotinylated anti-mouse total IgG and IgG2a were purchased from Jackson ImmunoResearch (West Grove, USA). Biotinylated anti-IgG1 and IgE were purchased from Biolegend and BD Biosciences, respectively. Horse radish peroxidase (HRP)-conjugated streptavidin was purchased from Biolegend. Mouse mast cell protease-1 (MCPT-1), and IL-13 ELISA kits were purchased from eBioscience (San Diego, USA). Anti-DNP IgE was purchased from Sigma-Aldrich (St. Louis, USA). IFN-γ intracellular staining kit was purchased from Biolegend (San Diego, USA).

2.2. Animals

Five week-old female BALB/C mice were purchased from SAMTAKO Co. (Osan, Korea) and housed in Core Animal Facility in Chungnam National University. The animal study protocol used in this study was approved by Animal Ethics Committee in Chungnam National University (Approval Number: CNU-00570), and animal experiments were carried out in accordance with the approved protocol.

2.3. Preparation of the 70% EtOH Chaga extract and the fractions and purification of inotodiol

Chaga mushroom (*Inonotus obliquus*) used in this study was purchased from a herbal market in Kumsan, Korea, and identified by Professor Y. H. Kim, College of Pharmacy, Chungnam National University (Daejeon, Korea). A voucher specimen (CNU 15001) was deposited at the herbarium of the College of Pharmacy, Chungnam National University.

The Chaga mushroom (630.0 g) was ground into fine particles and extracted with 70% aqueous EtOH (3 × 3.0 L) under ultrasonic agitation at 90 Hz (40 °C). The ethanol solution was evaporated in vacuo to produce a dried brown extract (58.0 g). The EtOH extract was suspended in distilled water and successively partitioned with CH_2Cl_2 and EtOAc to prepare CH_2Cl_2 (6.0 g), EtOAc (3.2 g), and water fractions (48.5 g).

The CH_2Cl_2 fraction was separated via silica gel column chromatography and eluted repeatedly with *n*-hexane-EtOAc (20:1, 9:1, 4:1, 2:1 and 1:2) to yield thirteen smaller subfractions (C-1 to C-13). Subfractions C-6 and C-7 were combined and were subjected to a silica gel column with a solvent mixture of *n*-hexane-EtOAc (20:1) to afford inotodiol (210.0 mg) (Supp. Fig. 1).

For animal experiments, the dried powders obtained from the EtOH extract and the fractions were pulverized and mixed with the Tween 20-saline solution to the final concentration 10 mg/mL or 40 mg/mL [22].

2.4. High performance liquid chromatography (HPLC) analysis

The solid EtOH extract and the fractions were dissolved in methanol (MeOH) at concentrations of 0.2% (w/v). The standards were also dissolved in MeOH at concentrations of 0.2 mg/mL. All solutions were filtered through a 0.2 μm syringe filter before analysis. HPLC analysis was performed using a LC-10AD series HPLC system (Shimadzu, Kyoto, Japan) coupled with UV/VIS detector at ambient temperature. A HECTOR-M C_{18} column (250 × 4.6 mm, 5 μm, RStech, Korea) was used stationary phase for separation. The mobile phase consisted of water (A) and 100% acetonitrile (B) was operated under gradient program following conditions; 0 min at 60% B, 30 min at 95% B and 60 min at 95% B. The flow rate of 1 mL/min and the detection wavelength of 210 nm were used.

Inotodiol and 3β-hydroxylanosta-8,24-dien-21-al purified from the CH_2Cl_2 fraction as described above were used as standards.

2.5. Protocol for the mouse model of cOVA-induced food allergy

Mice were immunized i.p. with cOVA (20 μg) plus alum (2 mg) twice with an interval of two weeks. One week after the second immunization, the blood was drawn from the immunized mice and the level of cOVA-specific IgG in the serum was measured with ELISA. The mice producing cOVA-specific IgG at a level in the range of mean ± SD were chosen and randomly divided into 7 groups (6 mice per group) (Fig. 1A). Two weeks after the second immunization, the mice were challenged p.o. with cOVA (50 mg) five times once every three days. During the challenge period, mice were either left untreated or treated daily with EE, DF, AF or Dexamethasone (p.o.). One hour after the fifth challenge, mice were examined for allergy symptoms (change in rectal temperature, diarrhea, and anaphylaxis) and the severities of allergy symptoms were rated and scored as previously reported [23]. The rectal temperature was measured using a rectal probe thermometer (Physitemp, Clifton, USA).

2.6. Histology

Histology experiments were performed as described by others [24]. Briefly, the small intestine (duodenum) was collected 1 day after the fifth challenge and washed with PBS and fixed with 10% formalin. The tissue was embedded in paraffin, and the paraffin-embedded tissue sample was sliced into sections of 5-μm thickness with microtome. The thin sections were then stained either with Toluidine Blue or H&E staining solution. The stained samples were observed under the microscope and the numbers of mast cells and eosinophil in high power field (HPF) were enumerated as described [25,26].