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Morus bombycis extract suppresses mast cell activation and IgE-mediated allergic reaction in mice

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ABSTRACT

Ethnopharmacological relevance: Morus bombycis Koidzumi (MB) is widely distributed throughout Korea, where it is used as a traditional folk remedy for the treatment of allergic diseases including asthma. However, the pharmacological effect and the mechanistic study of MB have not been investigated. We aimed to investigate the anti-allergic activity of MB in vitro and in vivo and the mechanism of its action on mast cells.

Materials and methods: The anti-allergic activity of MB extract (MBE) was assessed using passive cutaneous anaphylaxis (PCA) in mice and mouse bone marrow-derived mast cells (BMMCs) in vitro. The effects of MBE on mast cell activation were evaluated by using the β-hexosaminidase release assay, reverse transcriptase-polymerase chain reaction, enzyme-linked immunosorbent assay, and western blotting analysis.

Results: MBE reversibly inhibited degranulation and generation of cytokines (TNF-α and IL-4) in antigen-stimulated mast cells. With regard to its mechanism of action, MBE inhibited the activation of Lyn and Syk, which have essential roles in degranulation and the production of various inflammatory cytokines. MBE also inhibited the activating phosphorylation of mitogen-activated protein (MAP) kinases, Erk1/2, p38, JNK, and Akt. In agreement with its in vitro effect, MBE significantly inhibited mast cell-mediated PCA reactions in IgE-sensitized mice.

Conclusions: The present results strongly suggest that MBE exerts an anti-allergic effect, both in vitro and in vivo by inhibiting the Lyn and Syk pathways in mast cells. Therefore, MBE may be useful for the treatment of allergic diseases, including atopic dermatitis and allergic asthma.

1. Introduction

Allergic diseases are common diseases caused by hypersensitive immune disorders. It is observed in 10–20% of the world's population, and the number of allergy patients has steadily increased in recent years (Flohr et al., 2009; Erb, 2009). Most patients with allergies are genetically predisposed to produce IgE in the body. Under allergic conditions, mast cells are the major effector cells sensitized by IgE through the IgE high-affinity receptor (FcεRI) on their membrane (Metzger, 2004).

The aggregation of FcεRIs by antigens activates a variety of signaling molecules, which result in the release of mediators such as histamine, products of arachidonic acid metabolism, and an array of inflammatory cytokines (Gilfillan and Beaven, 2011). The antigen stimulation of mast cells activates Src family kinases (SFKs) such as Lyn, Fyn, and Fgr (Gilfillan and Beaven, 2011; Lee et al., 2011). Subsequently, the association of Syk with the tyrosine-phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of FcεRIγ results in a conformational change that increases its enzymatic activity (Siraganian et al., 2002; Mócsai. et al., 2010). This leads to the downstream propagation of signals, such as tyrosine phosphorylation of Linker for activation of T cells (LAT), phospholipase Cγ, and Ca^{2+} influx (Gilfillan and Rivera, 2009). These pathways are essential for the secretion of allergic mediators (Beaven, 2009; Gilfillan and Beaven, 2011).

Morus bombycis Koidzumi (MB), which belongs to the Moraceae family, is a species of forest tree. It is largely distributed throughout eastern Asia, including Korea. The bark of this tree is appreciably grayish brown, and the branches appear as thorns with stinging hairs. MB extract (MBE) has been used as an

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Oriental folk medicine for allergic diseases including asthma, tuberculosis, arthritis, stroke, and asthenia (Mun, 2011). Although anti-arthritis (Kim et al., 2011), anti-obesity (Kim et al., 2010), and anti-oxidant effects (Jin et al., 2005) of MBE have been reported, its effect on allergic responses remained to be investigated.

In this study, we demonstrate for the first time that a traditional folk medicine, MBE, suppresses antigen-stimulated mast cell activation and mast cell-mediated allergic responses. The current study suggests that MBE can be a new herbal medicine for treating various allergic diseases, including asthma and atopic dermatitis.

2. Materials and methods

2.1. Reagents

Dinitrophenol (DNP)-specific IgE, DNP-conjugated bovine serum albumin (BSA, antigen), Evans blue, and cetirizine were obtained from Sigma-Aldrich (St. Louis, MO). (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-d]pyrimidine (PP2) and thapsigargin were obtained from Calbiochem (La Jolla, CA). Antibodies against Syk and actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against the phosphorylated forms of Akt, Erk1/2, p38, JNK, Syk, linker for activation of T cells (LAT), and phosphotyrosine (pY) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Enzyme-linked immunosorbent assay (ELISA) kits for assessing TNF-α and IL-4 in media were obtained from Invitrogen to Biosource Cytokine & Signaling (Camarillo, CA). Cell-culture media and other components were obtained from GIBCO/Life Technologies Inc. (Rockville, MD).

2.2. Plant material

The *Morus bombycis* Koidzumi (MB) was collected from Jeju Island in Korea, and the sample was authenticated by the Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). The methanol extract of MB was prepared using the dried stem of the plant, according to the institute's standard protocol (Kim et al., 2011). The extraction yield was ~15% relative to the dry starting material. A voucher specimen (0 0 0 4–0 4 0) was deposited at the Plant Extract Bank and Konkuk University. The extract was dissolved in dimethyl sulfoxide (DMSO) for the in vitro assay and suspended in 5% gum arabic for the in vivo animal study.

2.3. Culture of bone-marrow-derived mast cells (BMMCs) and rat basophilic leukemia (RBL)-2H3 mast cells.

BMMCs were prepared with bone marrow cells from Balb/c mice, according to a previous report with minor modification (Jensen et al., 2006). The BMMCs were cultured for 4 weeks in a medium (RPMI 1640, containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal bovine serum (FBS) containing 10 ng/mL IL-3. RBL-2H3 cells were grown in minimum essential medium (MEM) with Earle's salts, supplemented with glutamine, antibiotics, and 15% FBS.

2.4. Measurement of degranulation in mast cells

The degree of degranulation of mast cells was determined by β-hexosaminidase (granule marker protein) release assay. Briefly, the mast cells on 24-well cluster plates (2×10^5 cells/0.5 mL/well) were sensitized overnight with 50 ng/mL of DNP-specific IgE. The cells were washed twice, and then buffered solution was added (0.2 mL/well), as required. Experiments were performed in 1,4-piperazinediethanesulfonic acid (PIPES)-buffered medium [25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum] for RBL-2H3 cells or in Tyrode buffer [20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% bovine serum albumin (BSA)] for BMMCs. The cells were incubated for 30 min with or without MBE before treatment with 25 ng/mL of antigen for 10 min or for the indicated times. The release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-*b*-D-glucosaminide was measured for β-hexosaminidase activity. Degranulation of mast cells was determined by calculating the ratio of β-hexosaminidase activity in culture media to the total activity in the media and cell lysate (Ozawa et al., 1993).

2.5. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from RBL-2H3 cells by using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was performed at 94 °C for 45 s, at 55 °C for 45 s, and at 72 °C for 60 s for 30 cycles. The specific primers used were as follows: rat TNF-α forward 5′-CACCACGCTCTT CTGTCTACTGAAC-3′; rat TNF-α reverse: 5′-CCGGACTCCGTGATGTC-TAAGT ACT-3′; rat IL-4 forward 5′-ACCTTGCTGTCACCCTGTTC-3′; rat IL-4 reverse 5′-T TGTGAGCGTGGACTCATTC-3′; rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-GTGGAGTCTACTGGC-GTCTTC-3′; rat GAPDH reverse: 5′-CCAAGGCTGTGGGCAAGGTCA-3′.

2.6. ELISA of TNF-α or IL-4

IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL DNP-BSA for 6 h, with or without MBE. The level of TNF-α and IL-4 secreted from the cells was measured in the media by using an ELISA kit (Invitrogen, Carlsbad, CA).

2.7. Generation of passive cutaneous anaphylaxis (PCA) in mice

A total of 0.5 μg of DNP-specific IgE was injected subcutaneously into the right ears of BALB/c mice (male, 4 weeks old) 24 h before antigen challenge. After IgE-sensitization, either MBE (0, 100, 300, and 1000 mg/kg) or cetirizine (20 mg/kg) was orally administered to the mice. After 60 min, the mice were intravenously injected with 250 μg of antigen in 250 μL of 1 × phosphate-buffered saline (PBS) containing Evans blue (5 mg/mL). The ear tissues were removed after mice were euthanized 1 h later. Dye extravasated into the ear tissue was extracted overnight in 700 μL of formamide at 63 °C. The absorbance of the formamide solution containing Evans blue was measured at 620 nm, and the amount of dye was calculated by using a standard curve (Kim et al., 2009). The animal study was performed using a protocol pre-approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University.

2.8. Immunoprecipitation and immunoblotting analysis

For preparation of the cell lysate, RBL-2H3 cells were harvested, washed twice with 1 × PBS, and lysed with lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 mM nitrophenylphosphate, 0.7 mg/mL pepstatin, and a protease-inhibitor cocktail tablet). After lysis, cell lysates were kept on ice for 30 min and then centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant protein solution was "precleared" by the addition of 50 μL of protein G-agarose, and the supernatant solution of equal protein content was used for immunoprecipitation.