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Inhibition of MDM2 expression by rosmarinic acid in TSLP-stimulated mast cell

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ABSTRACT

Rosmarinic acid (RA) has an anti-inflammatory property while thymic stromal lymphopoietin (TSLP) has an important role in mast cell-mediated inflammatory responses. Thus, the aim of this study was to determine the regulatory effect of RA in TSLP-stimulated human mast cell line, HMC-1 cells, and short ragweed pollen-induced allergic conjunctivitis mouse model. As a result, we found that RA significantly decreased the TSLP-induced mast cell proliferation and murine double minute (MDM) 2 expression. RA significantly decreased the levels of interleukin (IL)-13 and phosphorylated the signal transducer and activation of transcription 6 in the TSLP-stimulated HMC-1 cells. RA induced the increment of p53 levels, caspase-3 activation, and poly-ADP-ribose polymerase cleavage and the reduction of the procaspase-3 and Bcl2. RA significantly reduced the production of tumor necrosis factor-α, IL-1β, and IL-6 on the TSLP-stimulated HMC-1 cells. In addition, RA significantly reduced the levels of IgE, IL-4, and TSLP in the short ragweed pollen-induced allergic conjunctivitis mouse model. In conclusion, the results of the study suggest that RA has a significant anti-inflammatory effect on TSLP-induced inflammatory reactions. These effects of RA are likely to be mediated through inhibiting the MDM2 increased by TSLP.

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1. Introduction

Allergic responses happen in rodents, avian species, humans, non-human primates, and all of the domestic animals. These responses are arbitrated by IgE antibody that bind to FcεRI (mast cells surface marker) of mast cells and induce release or synthesis of potent inflammatory mediators (Gershwin, 2015). Mast cells are originated from hematopoietic progenitor cells and mature in local tissues, such as skin, mucosal surfaces, connective tissues, and vascularized tissues (Metz et al., 2007). Mast cells act important roles in allergic responses because they release histamine, prostaglandins, leukotrienes, and inflammatory cytokines (Jeong et al., 2002). Mast cells degranulation was required for the enhanced expression and production of thymic stromal lymphopoietin (TSLP), which are required for the optimal orchestration and priming of type 2 immunity (Hepworth et al., 2012). TSLP is known to have broad-ranging effects on immune cells including basophils, dendritic cells, mast cells, B cells, epithelial cells, and T cells (Lo Kuan and Ziegler, 2014). TSLP promotes mast cell proliferation *via* the regulation of apoptotic and anti-apoptotic factors by murine double minute 2 (MDM2) (Han et al., 2014). MDM2 works as a specific inhibitor of p53 during embryonic development (Toledo and Wahl, 2007). Inhibition of MDM2 blocked cellular proliferation and migration. In addition, inflammatory responses were attenuated by inhibition of MDM2 levels (Hashimoto et al., 2011; Han et al., 2014).

Rosmarinic acid (RA, an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid) is broadly distributed in diverse plants. RA has various pharmacological and biological activities including anti-inflammatory effect, slowing the development of Alzheimer's disease, cognitive-enhancing effects, and cancer chemoprotection (Khojasteh et al., 2014). However, the effect of RA on inflammatory reactions by TSLP has not yet been clarified. Therefore, we investigated the effect and regulatory mechanism of RA on TSLP-stimulated human mast cell line, HMC-1 cells and short ragweed (SRW) pollen-induced experimental allergic conjunctivitis (EAC) mouse model.

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2. Material and methods

2.1. Reagents

We purchased Isocove's modified Dulbecco's medium (IMDM) from Gibco BRL (Grand Island, NY, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-phospho- signal transducer and activator of transcription 6 (pSTAT6), lipopolysaccharide (LPS), dexamethasone (DEX), and RA (purity:97%) from Sigma Chemical Co (St. Louis, MO, USA); VGX-1027 from Abcam (Cambridge, UK); bromodeoxyuridine (BrdU) from Roche Diagnostics (Mannheim, Germany); Recombinant TSLP, caspase-3 assay kit, interleukin (IL)-13, tumor necrosis factor (TNF)-α, IL-6, and IL-1β antibodies from R&D Systems, Inc. (Minneapolis, MN, USA); Bcl2, procaspase-3, Poly-ADP-ribose polymerase (PARP), MDM2, and actin from Santa Cruz Biotechnology (Dallas, TX, USA). RA was dissolved in distilled water and diluted with IMDM.

2.2. HMC-1 cells culture

HMC-1 cells were incubated in IMDM supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum at 37 °C in 5% CO_2 with 95% humidity.

2.3. BrdU assay

Cell (1×10^4) proliferation was determined using a colorimetric immunoassay based on the measurement of BrdU incorporated by DNA synthesis (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Western blot analysis

The stimulated cells were lysed and separated through 10% SDS-PAGE. After electrophoresis, the protein was transferred to nitrocellulose membranes and then the membranes were blocked and incubated with primary (1:500 dilution in PBST) and secondary (1:3000 dilution in PBST) antibodies. Finally, the protein bands were visualized by an enhanced chemiluminesence assay (Amersham Co. Newark, NJ, USA) according to manufacturer's instructions.

2.5. MTT assay

Cell viability was measured by a MTT assay. Briefly, 500 μl of HMC-1 cell (3×10^5) were pretreated with diverse concentrations of RA for 1 h and stimulated with TSLP for 48 h. The cell suspension containing MTT solution (5 mg/ml) was incubated at 37 °C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in dimethyl sulfoxide. Then, the optical density of 96-well culture plate was determined using an ELISA method reader at 540 nm.

2.6. Cytokines assay

The levels of IL-13, TNF-α, IL-6, and IL-1β were determined using a sandwich ELISA method according to the manufacturer's instructions (R&D Systems).

2.7. RNA isolation and Quantitative Real-Time PCR

Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea), we isolated the total RNA from HMC-1 cells in accordance with the manufacturer's specifications. The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 μg) was heated at 75 °C for 5 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 60 min at 42 °C using a cDNA synthesis kit (iNtRON Biotech, Sungnam, Korea). Quantitative real-Time PCR was performed using a SYBR Green master mix and the detection of mRNA was analyzed using an ABI StepOne real-time PCR System (Applied Biosystems, Foster City, CA, USA). We performed real-time with the following primers: IL-13 (5′ GCCCTGGAATCCCT-GATCA 3′; 5′ GCTCAGCAT CCTCTGGGTCTT 3′; GAPDH (5′ TCGA-CAGTCAGCCGCATCTTCTTT 3′; 5′ ACCAAA TCCGTTGACTCCGACCTT 3′). The level of the target mRNA was normalized to the level of the GAPDH and compared with the control. All data were analyzed using the ΔΔCT method.

2.8. Caspase-3 assay

The enzymatic activity of caspase-3 was assayed using a colorimetric assay kit (R&D Systems) according to the manufacturer's protocol.

2.9. Murine model of EAC induced by SRW pollen

Eight-week-old female BALB/c mice from the Dae-Han Experimental Animal Center (Eumsung, Republic of Korea) were maintained under pathogen-free conditions. The mouse care and experimental procedures were performed with the approval of the Animal Care Committee of Kyung Hee University (KHUASP [SE]-12–019). The EAC model was induced according to previous report (Li et al., 2011). In brief, the mice were immunized with 50 μg of SRW pollen (Cosmo Bio, Japan) in 5 mg of Imject Alum (Pierce Biotechnology, Rockford, USA) by means of footpad injection on day 0. Allergic conjunctivitis was induced by means of topically applying 0.15 mg of SRW pollen suspended in 10 μl of PBS into each eye once a day from days 10 to 12. PBS eye drop-treated SRW-sensitized mice were used as control animals. RA was prepared at a dose of 4 mg/kg, which is similar to a previous report (Oh et al., 2011). DEX and VGX-1024 were used as positive control drugs. RA (4 mg/kg), DEX (5 mg/kg), or VGX-1027 (20 mg/kg) was administrated by intraperitoneal (i.p.) injection 1 h before the SRW pollen challenge. On day 13, 24 h after the last SRW challenge, the serum and whole eyes were harvested for gene expression assays.

2.10. Statistics

All results are representative of three independent experiments with duplicate and expressed as the mean ± SEM. The statistical evaluation of the results was performed by an independent *t*-test and an ANOVA with a Tukey posthoc test using SPSS statistical software (IBM Corporation, Armonk, NY, USA). Results with a *P*-value of < 0.05 were considered significant.

3. Results

3.1. Inhibitory effect of RA on the mast cell proliferation and the MDM2 levels in the TSLP-stimulated HMC-1

The mast cell proliferation amplifies the inflammatory allergic response and was increased by TSLP (Han et al., 2014). Thus, we investigated the effect of RA in the TSLP-induced mast cell proliferation. To determine the regulatory effect of RA on the proliferation of HMC-1 cells, a BrdU assay was performed. As shown in Fig. 1A, RA significantly attenuated the TSLP-induced proliferation of HMC-1 cells. We also evaluated the inhibitory effect of RA on the level of MDM2 in the TSLP-stimulated HMC-1 cells. As a result, the levels of MDM2 increased by TSLP were significantly decreased by RA (Fig. 1B and C, $P < 0.05$). Cytotoxicity did not observe at doses of 0.1, 1, and 10 μM of RA (Fig. 1D).

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