



The (2'S,7'S)-O-(2-methylbutanoyl)-columbianetin as a novel allergic rhinitis-control agent

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ABSTRACT

Aims: The (2'S,7'S)-O-(2-methylbutanoyl)-columbianetin (OMC) is a novel secondary metabolite extracted from *Corydalis heterocarpa*, which has long been used as a folk medicine for various inflammatory diseases in Korea. We examined the effect of OMC on allergic rhinitis (AR).

Main methods: We assessed the therapeutic effects and regulatory mechanisms of OMC on the phorbol 12-myristate 13-acetate plus A23187-stimulated mast cell line, HMC-1 cells and ovalbumin (OVA)-induced AR models.

Key findings: OMC significantly decreased the releases of histamine and tryptase from stimulated HMC-1 cells. The degranulation process, characterized by morphological extension of the filopodia on the surface and membrane ruffling, was strongly induced in the stimulated-HMC-1 cell, however OMC suppressed the morphological changes in stimulated-HMC-1 cells. OMC reduced the production and mRNA expression of inflammatory cytokines. These inhibitory actions by OMC were dependent on the regulation of mitogen-activated protein kinases, nuclear factor- κ B, and caspase-1 signaling pathways. In the AR animal model, the increased rub scores and AR biomarkers (histamine and IgE) in ovalbumin (OVA)-sensitized mice were significantly reduced by the administration of OMC. Furthermore, eosinophils and mast cell infiltrations in nasal mucosa tissue were also blocked through the regulation of macrophage-inflammatory protein and intercellular adhesion molecule-1 levels.

Significance: OMC showed the possibility to regulate AR in activated mast cells and OVA-induced AR models. Hence, we suggest that OMC is a powerful and feasible new agent to suppress AR.

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Introduction

Allergic rhinitis (AR), referred to as hay fever, is the most common cause of all atopic diseases afflicting roughly 500 million people worldwide and defined clinically as an inflammatory disorder of the nasal mucous membrane (Barnes, 1999; Bousquet et al., 2008; Gravit, 2011). AR is a type 1 immune response mediated by T helper 2 (T_H2)-cells and innate immune cells such as eosinophils, mast cells, basophils, and macrophages (Palm et al., 2012). The AR reaction includes a hypersensitivity phase, which induces IgE synthesis and humoral responses, and a clinical phase divided into early, late and chronic phases, which

are induced by re-exposure of allergen (Barnes, 1999; Ramírez-Jiménez et al., 2012). In the hypersensitivity phase, dendritic cells (DCs), the antigen presenting cells, in the mucosal surface present the processed allergen peptides to cognate naive T cells using major histocompatibility complex (MHC) class II molecules. Then, naive T cells acquire a T_H2 cell phenotype, which induces B cells to undergo the class-switch recombination to produce IgE antibodies through secreted interleukin (IL)-4 and IL-13 (Min, 2010; Galli and Tsai, 2012). The IgEs bind to FcεRI receptors on mast cells and the subsequent exposure to the allergen induces the cross-linking of IgE on mast cells.

Mast cells play a key part in the early response in the clinical phase because the cross-linking of IgE triggers mast cell de-granulation and the synthesis of a variety of mediators including biogenic amines, serglycin proteoglycans, serine proteases, cytokines, and growth factors. Histamine is an important biogenic amine that leads to clinical symptoms of congestion, rhinorrhea, and bronchoconstriction by increasing vascular permeability and constricting smooth muscle (Perlman,

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1999; Pawankar et al., 2011). Furthermore inflammatory mediators such as IL-3, IL-5, IL-9, IL-6, tumor necrosis factor (TNF)- α , macrophage inflammatory protein-2 (MIP-2), and intercellular adhesion molecule-1 (ICAM-1) induce the recruitment of inflammatory cells leading to the late allergic response manifested by nasal congestion, nasobronchial hyper-reactivity, and chronic wheeze (Mandhane et al., 2011; Xu et al., 1995; Perlman, 1999). Importantly, intracellular signaling pathways such as mitogen-activated kinases (MAPKs), nuclear factor- κ B (NF- κ B), and caspase-1 regulate the production of these pro-inflammatory cytokines (Oh et al., 2012).

(2'S,7'S)-O-(2-methylbutanoyl)-columbianetin (OMC, Fig. 1) is a novel secondary metabolite extracted from *Corydalis heterocarpa*, which has long been used as a folk medicine for various inflammatory diseases in Korea. Libanoridin and columbianetin extracted from *C. heterocarpa* were very effective on allergic inflammatory reaction (Jeong et al., 2009; Kim et al., 2010a). Kim et al. identified OMC for the first time in 2010 and reported its anti-proliferative effects against U-937 human leukemia cells (Kim et al., 2010b). However the effect of OMC on AR has not been determined. In this study, we focused on the possibility of OMC for the regulation of AR and considered a possible mechanism in the phorbol 12-myristate 13-acetate plus A23187 (PMACI)-stimulated mast cells and ovalbumin (OVA)-induced AR models.

Materials and methods

Reagents

We purchased OVA, phorbol 12-myristate 13-acetate (PMA), calcium ionophore (A23187), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), bovine serum albumin (BSA), bicinchoninic acid (BCA), and avidin–peroxidase from Sigma Chemical Co. (St Louis, MO, USA); Isocove's Modified Dulbecco's Medium (IMDM), penicillin, streptomycin, and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY, USA). Anti-mouse IgE/IL-1 β [mature form detection antibody (Ab)]/TNF- α /thymic stromal lymphopoietin (TSLP)/interferon (IFN)- γ /MIP-2/ICAM-1 Ab, biotinylated anti-mouse IgE/IL-1 β /TNF- α /TSLP/IFN- γ /MIP-2/ICAM-1 Ab, recombinant mouse

IgE/IL-1 β /TNF- α /TSLP/IFN- γ /MIP-2/ICAM-1, anti-human IL-1 β /TNF- α /IL-6/IL-8 Ab, biotinylated anti-human IL-1 β /TNF- α /IL-6/IL-8 Ab, and recombinant human IL-1 β /TNF- α /IL-6/IL-8 Ab were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Ab for extracellular signal-regulated protein kinase (ERK), phosphorylated-ERK (pERK), c-jun amino N-terminal kinase (JNK), pJNK, p38, pp38, I κ B- α , pI κ B- α , NF- κ B, histone, procaspase-1, caspase-1, actin, and α -tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse OVA-specific IgE kit was purchased from DS Pharma Biomedical Co. Ltd. (Osaka, Japan). The caspase-1 assay kit was supplied by R&D Systems, Inc. (Minneapolis, MN, USA). The tryptase assay kit was supplied by Millipore Co. (Billerica, MA, USA).

Cells culture and stimulation

Human leukemic cell line, HMC-1 cells were grown in an IMDM medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 μ M monothioglycerol, and 10% heat-inactivated FBS at 37 °C, in 5% CO₂ and 95% humidity. Cells were treated with OMC for 30 min prior to stimulation with PMACI (20 nM of PMA plus 1 μ M of A23187) and incubated at 37 °C. The time points by PMACI stimulation were selected according to a previous report (Oh et al., 2012).

Preparation of OMC

The specimens of OMC were collected along the shores of Cheju Island and Busan, South Sea, Korea. The voucher specimens of the plant were identified by an algal taxonomist, Jong-Su Yoo, in the Research Institute of Marine Science and Technology, Korea Maritime University, Busan, Republic of Korea, and deposited in the Herbarium of the Division of Ocean Science, Korea Maritime University, Busan, Republic of Korea, under the curatorship of Jong-Su Yoo (voucher no. 02JP-13). The dried samples (30 g) were extracted twice with 300 ml of methanol for 24 h at room temperature (Lin et al., 1997). The methanol extract (600 ml) was concentrated under reduced pressure. The yield of extraction was about 6.7% (w/w). The extract was stored in a freezer at –80 °C until tests were performed.

MTT assay

Cell viability was assessed by an MTT assay. HMC-1 cells suspension was cultured in 24-well plates for 24 h after treatment by each concentration of OMC. 20 μ l of MTT solution (5 mg/ml) was added and the cells were incubated at 37 °C for 4 h. The insoluble formazan product was dissolved in DMSO after washing the supernatant. Then, the optical densities of 96-well culture plates were measured using an ELISA reader at 540 nm.

Histamine assay

HMC-1 cells were preincubated for 30 min at 37 °C before the addition of PMACI for stabilization. The cells were preincubated with OMC for 1 h and then incubated for 6 h with PMACI. The cells were separated from the released histamine by centrifugation at 400 rpm for 5 min at 4 °C. The levels of histamine in the supernatant were measured by the OPA spectrofluorometric procedure (Jeong et al., 2006). The fluorescent intensity was measured at 460 nm (excitation at 355 nm) using a spectrofluorometer.

Tryptase assay

HMC-1 cells were preincubated with OMC for 1 h and then incubated for 6 h with PMACI. Tryptase from culture supernatants was assayed by using a mast cell degranulation assay kit (Millipore Co., Billerica, MA, USA).

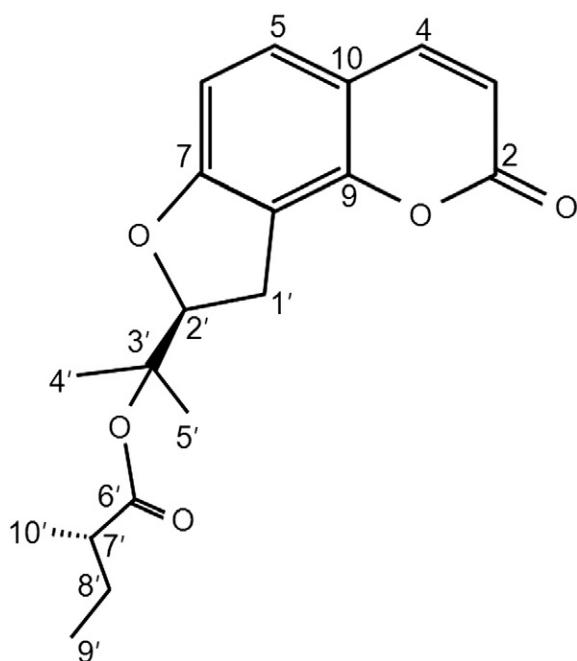


Fig. 1. Chemical structure of OMC.

Electron microscopy

Briefly, for the scanning electron microscopy (SEM), cells were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer for 4 h at 4 °C. Then the cells were postfixed in 1% OsO₄ for 2 h at 4 °C and rinsed in distilled water. The tissue was dehydrated in ethanol, dried in hexamethyldisilazane, and coated with gold. Samples were examined with a JEOL 5200 scanning electron microscope. For the transmission electron microscopy (TEM), cells were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer for 4 h at 4 °C. Cells were postfixed in 1% OsO₄ for 2 h at 4 °C, rinsed in distilled water, dehydrated through a graded series of ethanols, rinsed in acetone, and embedded in Enbed 812. Thin sections were cut with an ultramicrotome (MT-X, RMC, Tucson, AZ, USA) and stained for 10 min each in Reynolds' lead citrate and uranyl acetate. Sections were examined in a Libral 120 Electron Microscope (Carl Zeiss, Germany).

ELISA assay

Cytokines of serum, mucosa, and spleen tissue and supernatant were measured by an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed by coating 96-well plates with 1 µg/well capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with 1 × PBS containing 0.05% Tween 20. All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from the known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the biotin-conjugated secondary antibodies and an avidin peroxidase and 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablet substrate solution containing 30% H₂O₂. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. The OVA-specific IgE was measured from the serum according to the manufacturer's specifications using an OVA-specific IgE kit. Cytokine levels in the spleen and nasal mucosa were divided according to the total protein. The protein was estimated using the BCA method with the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

$$\% \text{ Inhibition} = (a - b) \times 100/a$$

where 'a' is a cytokine secretion without OMC and 'b' is a cytokine secretion with OMC.

Reverse transcription-polymerase chain reaction (RT-PCR)

HMC-1 cells were treated with OMC (0.1, 1, and 10 µg/ml) for 1 h before stimulation with PMACI incubated for 6 h. The total RNA was isolated from the cells and nasal mucosa according to the manufacturer's specification using an easy-BLUE RNA extraction kit (iNtRON Biotechnology, Kyunggi-do, Korea). The concentrations of total RNA in the final elutes were determined by spectrophotometry. Total RNA (2.5 µg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The polymerase chain reaction (PCR) was performed with the following primers for the human IL-1β (5'-CCG GAT CCA TGG CAC CTG TAC GAT CA-3'; 5'-GGG GTA CCT TAG GAA GAC ACA AAT TG-3'); IL-6 (5'-GAT GGATGC TTC CAATCT GGAT-3'; 5'-AGT TCT CCATAG AGA ACA ACA TA-3'); IL-8 (5'-CGA TGT CAG TGC ATA AAG ACA-3'; 5'-TGA ATT CTC AGC CCT CTT CAA AAA-3'); TNF-α (5'-CAC CAG CTG GTT ATC TCT CAG CTC-3'; 5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'); GAPDH (5'-CAA AAG GGT CAT CAT CTC TG-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3'). GAPDH was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. Saturation curves for the PCR were

obtained from various experimental conditions (RNA concentrations, annealing temperatures, and PCR cycle numbers). We determined the optimal amplification conditions (annealing temperature and PCR cycle number) of primers for the PCR. The annealing temperature was 50 °C for human IL-1β, 56 °C for IL-6, and 60 °C for IL-8, TNF-α, and GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Western blot analysis

HMC-1 cells were treated with OMC (0.1, 1, and 10 µg/ml) for 1 h before stimulation with PMACI which had been incubated for 2 h. Western blot analysis was used for nasal mucosa tissue extracts, and cell extracts were prepared by a detergent lysis procedure. Samples were heated at 95 °C for 5 min and briefly cooled on ice. After the centrifugation, 50 µg aliquots were resolved by 10% SDS-polyacrylamide gel electrophoresis. The resolved proteins were electro-transferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, and 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 × phosphate-buffered saline (PBS) containing 0.05% Tween 20 containing 5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions.

Caspase-1 assay

HMC-1 cells were treated with OMC (0.1, 1, and 10 µg/ml) for 1 h before stimulation with PMACI incubated for 2 h. Equal amounts of the total protein were quantified by a BCA protein quantification kit (Sigma-Aldrich) in each lysate. Caspase-1 activity was measured according to the manufacturer's specifications using a caspase assay kit (R&D Systems, Inc.).

OVA-induced AR animal model

We maintained 6-week-old female BALB/c (Charles River Laboratories, Inc., Wilmington, MA, USA) mice under pathogen-free condition. Mouse care and experimental procedures were performed under the approval from the animal care committee of Kyung Hee University [Approval no. KHUASP (SE)-10-016]. The mice were sensitized on days 1, 5, and 14 by intraperitoneal injection of 100 µg of OVA emulsified and 20 mg of aluminum hydroxide (Sigma-Aldrich) in a 100 µl of PBS and challenged intranasally with 2 µl of 1.5 mg of OVA or PBS. The mice were challenged intranasally with PBS in a similar manner for the negative control. OMC (1 and 10 mg/kg) and a control vehicle (distilled water.) were administered orally for 10 days before the intranasal OVA challenge. Nasal symptoms were only evaluated by counting the number of nasal rubs that occurred in 10 min after OVA intranasal provocation at the 10-day mark after the challenge. We measured OVA-specific IgE as the relevant endpoint for AR. The number of mice in each group was 5.

Histological examination

The nasal mucosa tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. The sections of the sample were 4 µm thick. Each section was stained with hematoxylin and eosin (for eosinophils) before de-waxing and dehydration. The numbers of eosinophils on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

Confocal laser scanning microscopy

Tissue samples and cells were fixed with 4% formaldehyde and embedded in paraffin. After de-waxing and dehydration, sections were blocked with bovine serum albumin followed by 60 min of incubation with an anti-mouse c-Kit or anti-rabbit p65 (Santacruz, CA, USA) at a concentration of 1 $\mu\text{g}/\text{ml}$. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated anti-rabbit IgG (Invitrogen), was added to the incubation for 30 min. The mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) was used to counterstain DNA. All specimens were examined with a confocal laser-scanning microscope. The numbers of mast cells on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

Statistical analysis

All results are representative of three independent experiments and expressed as the mean $\pm$ S.E.M. The statistical evaluation of the results was performed by an independent *t*-test and an ANOVA with a Tukey post hoc test using SPSS statistical software. The results were significant with a value of $P < 0.05$.

Results

OMC inhibited degranulation of mast cells

Mast cells play an important role in AR, producing a variety of cytokines, chemokines, proteases, and histamine after re-exposure to an

allergen. Histamine and tryptase are major biogenic amine and serine proteases released after the degranulation of mast cells, respectively. We examined the effects of OMC on PMACI-induced histamine and tryptase release. OMC significantly inhibited histamine and tryptase release in PMACI-stimulated mast cells as shown in Fig. 2B and C. OMC (10 $\mu\text{g}/\text{ml}$) had no effect on the histamine and tryptase release by itself (Fig. 2B and C). We also examined the ability of OMC to inhibit the degranulation of PMACI-stimulated HMC-1 cells. To visualize the responses of mast cells to OMC, we undertook microscopy studies. Through SEM, we observed that morphological changes occurred in PMACI-stimulated mast cells. The activated-mast cells displayed irregular shapes, filopodia extension, and membrane ruffling (Fig. 2D). By contrast, cells treated with OMC showed a round shape, a few of the filopodia, and few holes in the surface. Moreover, we observed the cytoplasm of mast cells to analyze granule contents by TEM. The number of granules in cytoplasm and the electron density of the granules were significantly decreased by OMC (Fig. 2D). We examined cell viability using a MTT assay. OMC had no effect on cell viability (Fig. 2A).

OMC inhibited production and mRNA expression of pro-inflammatory cytokines in HMC-1 cells

It is reported that the production of pro-inflammatory cytokines including IL-1 β , IL-6, IL-8, and TNF- α was increased in nasal mucosa (Mandhane et al., 2011; Oh et al., 2012). We examined the regulatory effect of OMC on the production of IL-1 β , IL-6, IL-8, and TNF- α . PMACI significantly increased cytokine production compared with the media control (Fig. 3A–D). IL-1 β , IL-6, IL-8, and TNF- α which were increased by PMACI, were significantly inhibited by OMC (Fig. 3A–D, $P < 0.05$). The inhibition rates for IL-1 β , IL-6, IL-8 and TNF- α at

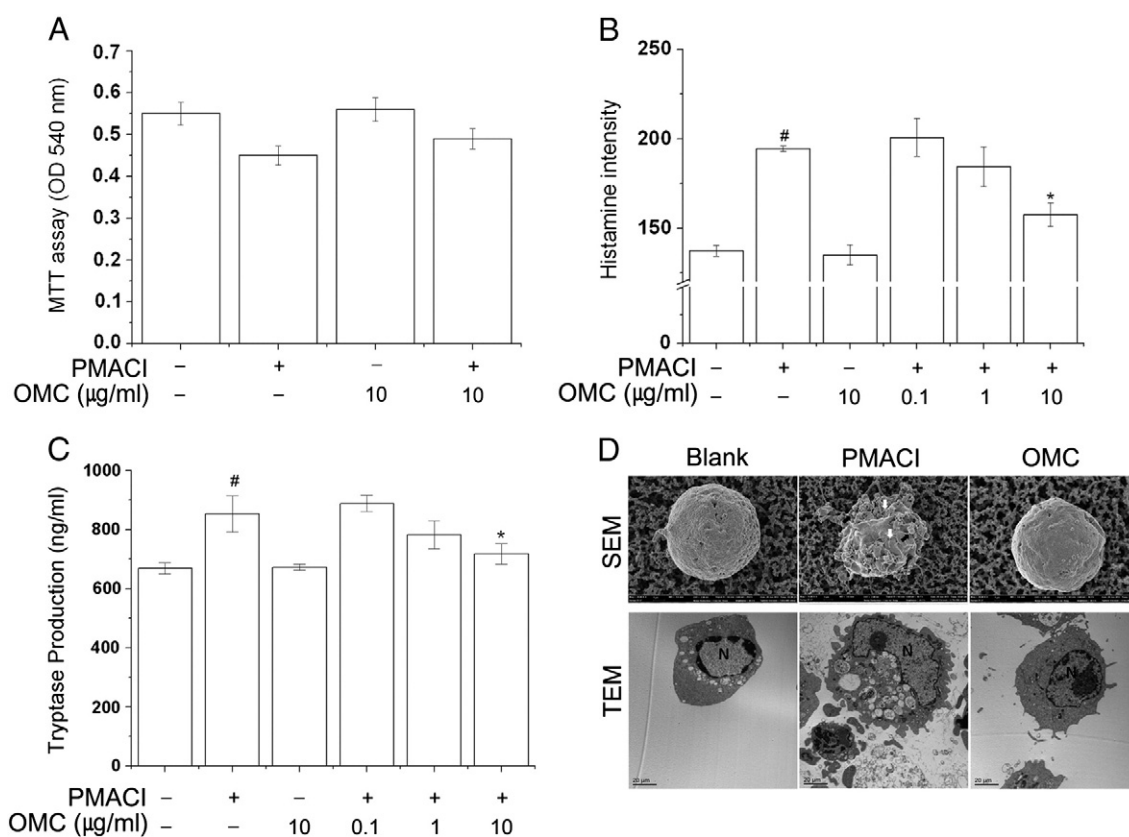


Fig. 2. OMC inhibited the degranulation of mast cells. (A) MTT assay. (B) HMC-1 cells (3×10^6) were treated with OMC (0.1, 1, and 10 $\mu\text{g}/\text{ml}$) and then stimulated by PMACI for 6 h. The level of histamine in the supernatant was measured by a histamine assay method. (C) The level of tryptase in the supernatant was measured by using a tryptase assay kit. (D) The surface morphology and cytoplasm of HMC-1 cells were observed by SEM and TEM. White arrows indicate extended filopodia. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. Blank, unstimulated cells. [#] $P < 0.05$; significantly different from the unstimulated cells. ^{*} $P < 0.05$; significantly different from the PMACI-stimulated cells. Blank, unstimulated cells; PMACI, PMA plus A23187; OMC, PMA plus A23187 plus OMC (10 $\mu\text{g}/\text{ml}$); N, nucleus.

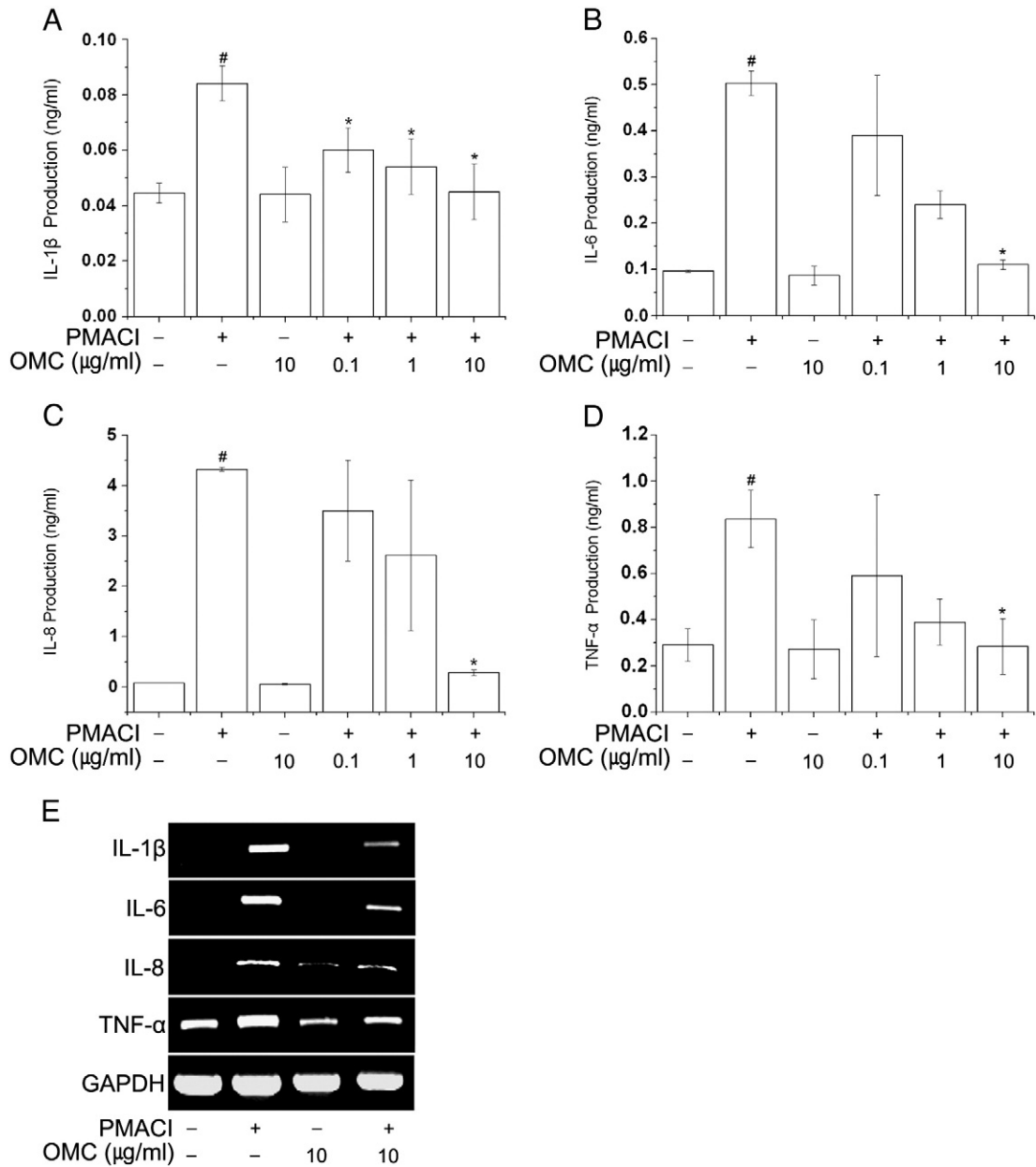


Fig. 3. OMC inhibited the production and mRNA expression of pro-inflammatory cytokines. HMC-1 cells (3×10^6) were treated with OMC (0.1, 1, and 10 μ g/ml) for 1 h and then stimulated by PMACI for 8 h. (A–D) The production of cytokines in the supernatant was measured by the ELISA method. (E) HMC-1 cells (3×10^6) were treated with OMC (10 μ g/ml) for 1 h and then stimulated by PMACI for 6 h. IL-1 β , IL-6, IL-8, and TNF- α mRNA expressions were analyzed by the RT-PCR. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. [#] $P < 0.05$; significantly different from the unstimulated cells. ^{*} $P < 0.05$; significantly different from the PMACI-stimulated cells. PMACI, PMA plus A23187.

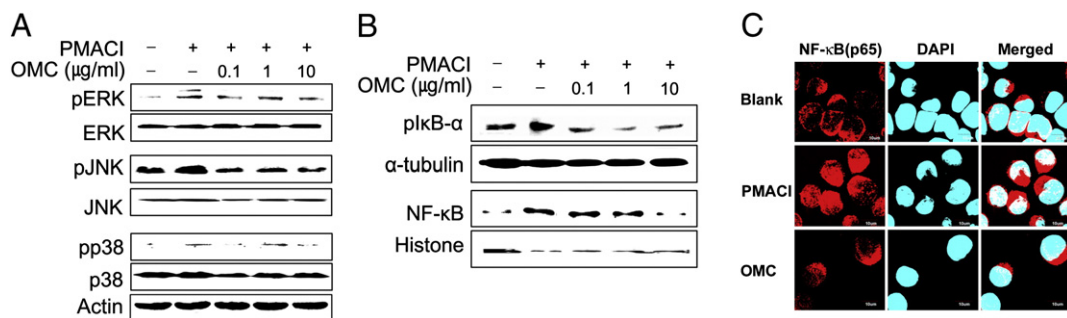


Fig. 4. OMC inhibited the activation of MAPKs and NF- κ B. (A) HMC-1 cells (3×10^6) were treated with OMC (0.1, 1, and 10 μ g/ml) for 1 h and then stimulated by PMACI for 2 h. The phosphorylated-ERK, JNK, and p38 were determined by Western blot analysis. (B) NF- κ B and phosphorylated I κ B- α were determined by Western blot analysis. (C) NF- κ B was stained by a primary anti-p65 for 1 h and then incubated by secondary TRITC-conjugated IgG for 1 h. Blank, unstimulated cells; PMACI, PMA plus A23187; OMC, PMA plus A23187 plus OMC (10 μ g/ml). (Original magnification 400 $\times$, scale bar = 50 μ m).

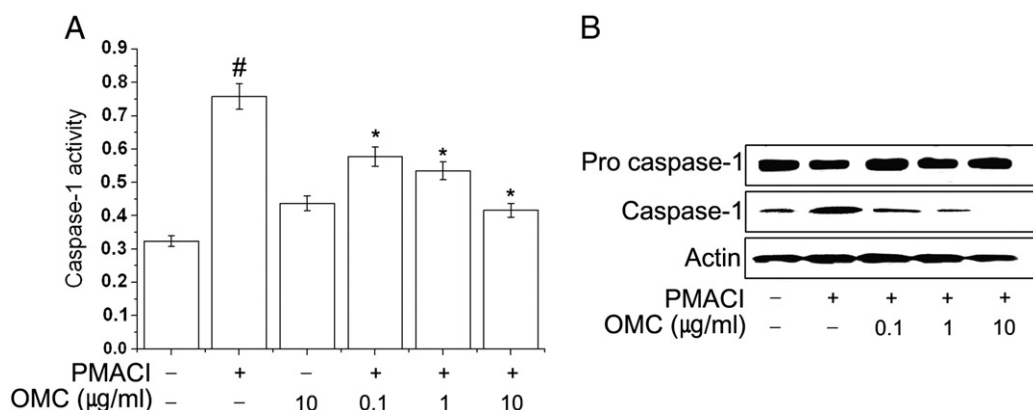


Fig. 5. OMC inhibited the activation of caspase-1. (A) HMC-1 cells (3×10^6) were treated with OMC (0.1, 1, and 10 μg/ml) for 1 h and then stimulated by PMACI for 2 h. Caspase-1 activity was measured by using a caspase-1 assay kit. (B) The expression of caspase-1 was analyzed by Western blot analysis. The data of caspase-1 activity represents the mean $\pm$ S.E.M. of three independent experiments. [#] $P < 0.05$; significantly different from the unstimulated cells. ^{*} $P < 0.05$; significantly different from the PMACI-stimulated cells.

10 μg/ml were about 98.7%, 96.6%, 95.2%, and 101.3%, respectively. We examined the effect of OMC on the mRNA expressions of IL-1β, IL-6, IL-8, and TNF-α in PMACI-stimulated mast cells. The mRNA expressions of IL-1β, IL-6, IL-8, and TNF-α were significantly decreased by OMC (Fig. 3E).

OMC inhibited activation of MAPKs and NF-κB in HMC-1 cells

To investigate the regulatory mechanism of OMC on allergic inflammation, we analyzed MAPKs activation. The OMC inhibited the PMACI-

induced ERK, JNK, and p38 MAPKs activation (Fig. 4A). We also examined the effect of OMC on PMACI-induced NF-κB activation. The OMC inhibited the PMACI-induced NF-κB activation (Fig. 4B). As a marker of NF-κB activation, the phosphorylation of IκB-α in cytoplasm lysates was detected. OMC also inhibited PMACI-induced IκB-α phosphorylation (Fig. 4B). Immunocytochemistry was performed to localize NF-κB/p65 protein (Fig. 4C). When HMC-1 cells were stimulated with PMACI, immunoreactive NF-κB was localized to nuclei (Fig. 4C). In contrast, when the cells were pretreated with OMC, immunoreactive NF-κB was located to cytoplasm (Fig. 4C).

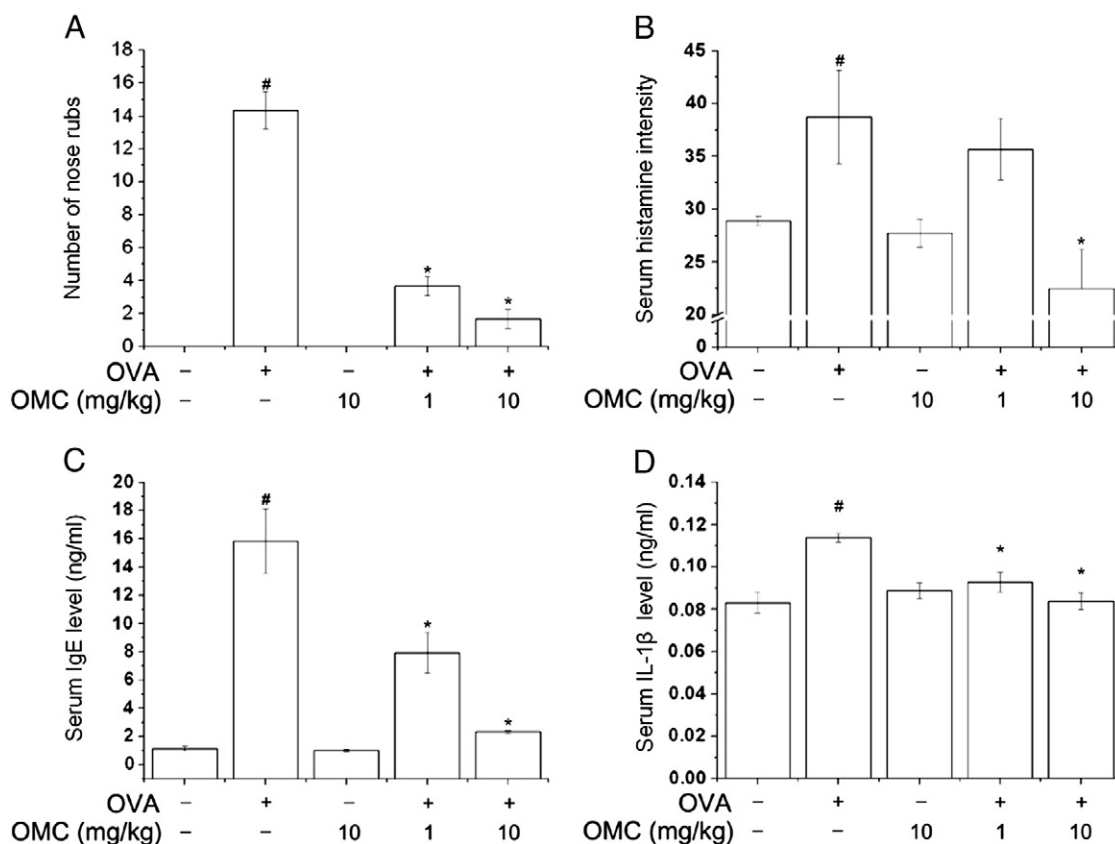


Fig. 6. OMC reduced clinical symptoms in an AR animal model. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μg of OVA emulsified in 20 mg of aluminum hydroxide, and we challenged mice with 1.5 mg of OVA. Mice received OMC before the intranasal OVA challenge for 10 days. (A) The number of the nasal rubs that occurred in 10 min after the OVA intranasal provocation. (B) Serum was isolated from blood to measure histamine. Histamine levels were measured by the histamine assay method. (C) OVA-specific IgE levels were measured by using the ELISA method. (D) The production of IL-1β in serum was measured by using the ELISA method. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. [#] $P < 0.05$; significantly different from the OVA-unsensitized mice. ^{*} $P < 0.05$; significantly different from the OVA-sensitized mice. N = 5.

OMC inhibited activation of caspase-1 in HMC-1 cells

Caspase-1 involves in cleavage and secretion of IL-1 and IL-18 (Stutz et al., 2009). To evaluate whether OMC regulated the caspase-1 activation, we performed a caspase-1 assay and Western blot analysis. Caspase-1 activity increased by PMACI was inhibited by OMC (Fig. 5A). As shown in Fig. 5B, OMC reduced the active form of caspase-1 while it increased the pro caspase-1 form in HMC-1 cells.

OMC reduced clinical symptoms and inflammation-related protein in the AR animal model

To assess the inhibitory effects of OMC in the AR animal model, we sensitized mice on days 1, 5, and 14 by intra-peritoneal injections

of 100 µg of OVA emulsified in 20 mg of aluminum hydroxide and the challenged mice with 1.5 mg of OVA. Mice were administered with various doses of OMC to screen proper dose of OMC. As a preliminary result, we selected 1 and 10 mg/kg of OMC in AR animal model. Increased rub scores were inhibited by the treatment with OMC (Fig. 6A). Histamine, OVA-specific IgE, and IL-1β levels in the serum were reduced by OMC (Fig. 6B–D). The levels of IL-1β, TNF-α, and TSLP in the OVA-induced AR mice were significantly higher than those in the nasal mucosa tissues of the OVA-unsensitized mice (Fig. 7A–C). Increased cytokine levels were reduced by OMC administration (Fig. 7A–C). We also analyzed the MIP-2 and ICAM-1 levels in the OVA-induced AR mice. The levels of MIP-2 and ICAM-1 in the OVA-induced AR mice were significantly higher than those in the nasal mucosa tissues of the OVA-unsensitized mice (Fig. 7D). Increased ICAM-1 levels

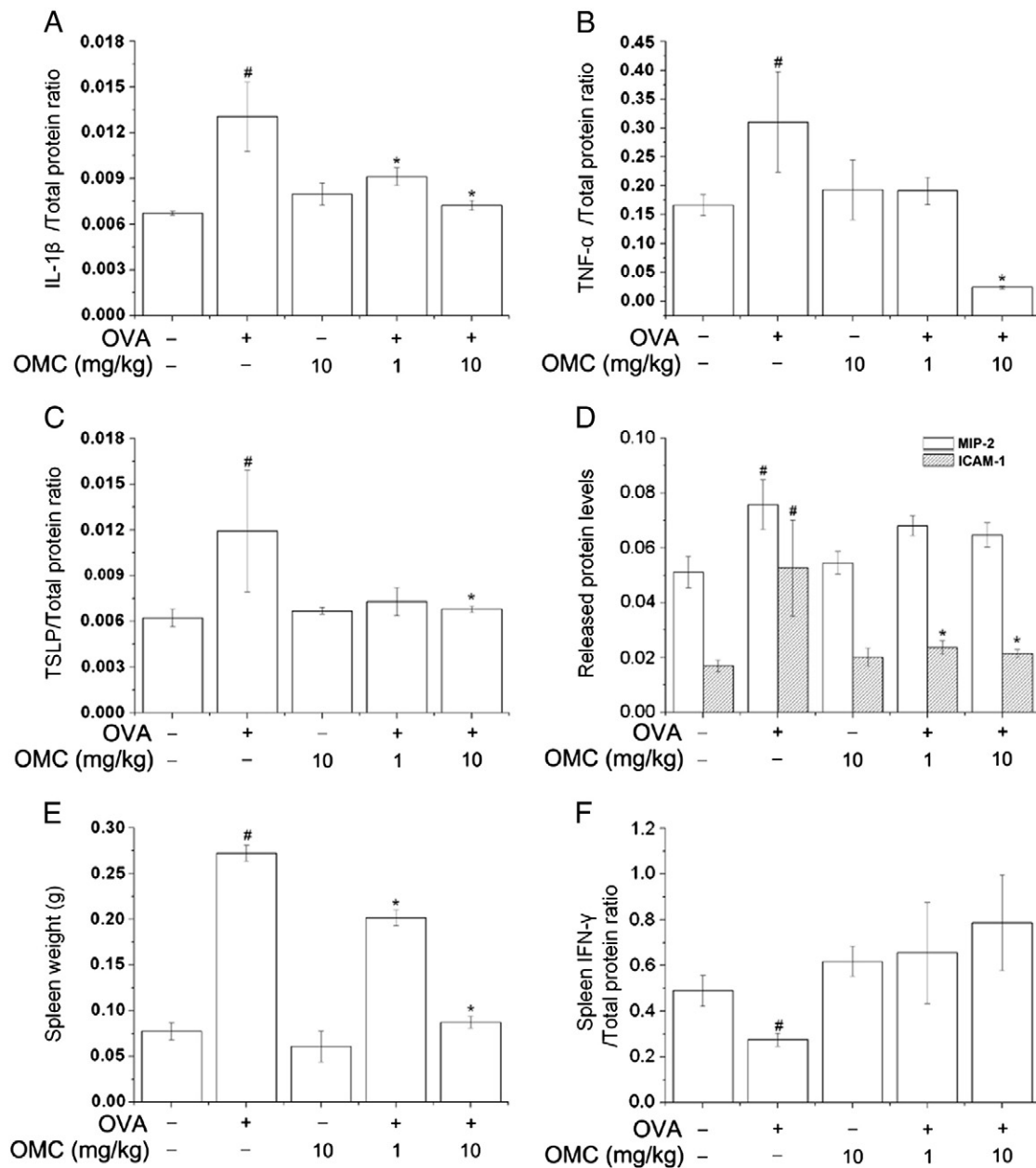


Fig. 7. Effects of OMC on AR biomarkers. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 µg of OVA emulsified in 20 mg of aluminum hydroxide, and we challenged mice with 1.5 mg of OVA. Mice received OMC before the intranasal OVA challenge for 10 days. (A–C) The production of cytokines in the nasal mucosa was measured by the ELISA method. (D) The levels of MIP-2 and ICAM-1 in the nasal mucosa tissue were measured by the ELISA method. (E) Spleen weight. (F) The levels of IFN-γ in the spleen were measured by the ELISA method. Each datum represents the mean ± S.E.M. of three independent experiments. #P < 0.05; significantly different from the OVA-unsensitized mice. *P < 0.05; significantly different from the OVA-sensitized mice. N = 5.

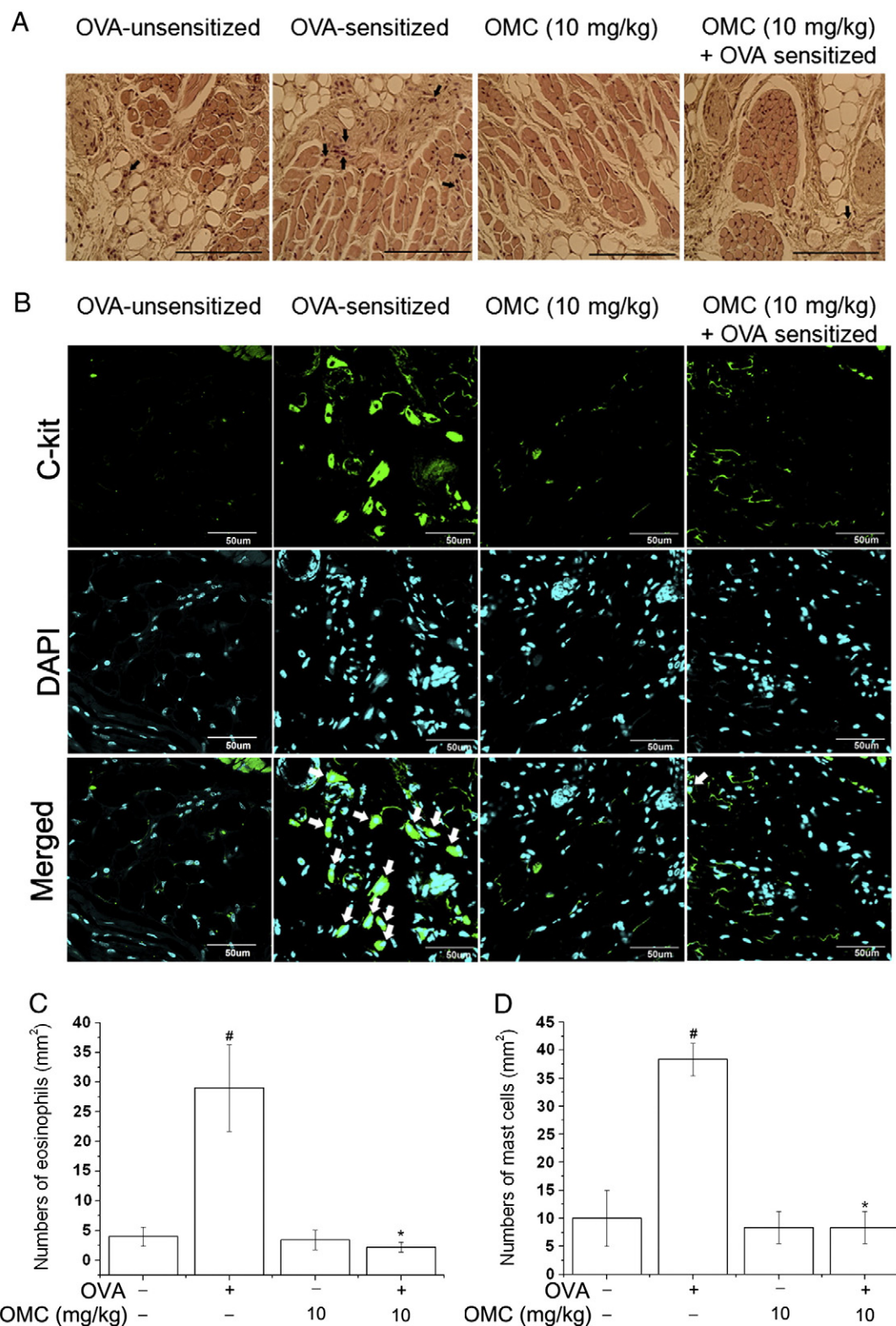


Fig. 8. OMC reduced eosinophils and mast cell infiltration in the AR nasal mucosa tissue. (A) Nasal mucosa tissue was stained with hematoxylin and eosin (H&E) for eosinophils (black arrow). (B) Mast cells were stained by a primary anti-c-Kit for 1 h and then incubated by secondary FITC-conjugated IgG for 1 h (white arrow). (C and D) Eosinophils and mast cells were counted by three individuals. The five randomly selected tissue sections per mouse were counted. The absolute number of cells was counted as the mean $\pm$ S.E.M. [#] $P < 0.05$; significantly different from the OVA-unsensitized mice. ^{*} $P < 0.05$; significantly different from the OVA-sensitized mice. $N = 5$. (Original magnification 400 $\times$, scale bar = 50 μ m).

were significantly by OMC administration but not MIP-2 (Fig. 7D). Increased spleen weights by OVA were also significantly reduced by the OMC administration (Fig. 7E). As shown in Fig. 7F, the levels of IFN- γ decreased by OVA were increased in the OMC-administered AR mice.

OMC reduced eosinophils and mast cell infiltration in the AR nasal mucosa tissue

The respective numbers of inflammatory cells (eosinophil and mast cells) in the nasal mucosa in the AR mice were significantly higher than

those in the OVA-unsensitized mice. In the nasal mucosa tissue, the numbers of eosinophils and mast cells increased by OVA sensitization were decreased in the OMC-administered mice (Fig. 8A–D).

Discussion

The treatment of AR consists of major three types: allergic avoidance, drug treatment, and immunotherapy. The antihistamines, anticholinergic agents, corticosteroids, and mast cell stabilizers are widely used to manage AR, although it has adverse side effects including sedation, impaired learning/memory, and cardiac arrhythmias and low clinical efficacy (Mygind, 1993). Recently, natural product-based compounds and herbal formula have reported to be very useful for the treatment of allergic diseases (Thornhimm and Kelly, 2000; Lenon et al., 2007; Fouche et al., 2013). In the present study, we verified that OMC isolated from *Corydalis heterocarpa*, had a therapeutic effect on AR *in vitro* and *in vivo* models.

Mast cells play a pivotal role in the early phase of AR by producing a plethora of mediators, including histamine, proteases, cytokines, and growth factors. Histamine is the most important mediator of mast cells because it causes nasal obstruction, rhinorrhea, and sneezing by the binding histamine H1 receptor (Togias et al., 1987; Takizawa et al., 1995; Wang et al., 2001; Yamauchi et al., 2007). Tryptase, a trypsin-like serine protease, is abundant in mast cell granules and induces the chemoattractants IL-8, ICAM-1, and IL-1 β on epithelial cells in the inflammation process (Payne and Kam, 2004). Therefore, most anti-allergic drugs were designed to inhibit the release of histamine and tryptase from human mast cells (Wang et al., 2009; Kida et al., 2010). Our results also showed that OMC significantly inhibited the release of histamine and tryptase from activated HMC-1 cells. This result indicates that OMC exerts strong anti-allergic effects through the inhibition of mast cell degranulation.

Pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6, and IL-8 are released within the first hours of the allergen challenge. IL-1 β is a strong proinflammatory cytokine, since IL-1 β exerts cytokine and histamine secretions of mast cells as well as the IgE production by stimulating B cells (Artis et al., 1999; Nambu and Nakae, 2010). TNF- α secreted by mast cells and macrophages is associated with T_H2 mediated cytokine production and the homing of T_H2 cells to the site of the allergic inflammation (Cohn et al., 1997; Artis et al., 1999). Recently, it is reported that TNF- α stimulates IgE production by enhancing IL-4 production and eosinophil migration (Bachert et al., 1995; Bradding et al., 1995; Artis et al., 1999). IL-6 is co-related with IgE synthesis and mast cell proliferation and maturation. IL-8 is a chemoattractant leading to eosinophil migration to the site of inflammation (Mukaida, 2000). In our study, OMC markedly inhibited the release of these mediators in activated HMC-1 cells and AR mice. These results demonstrated that OMC exert an anti-inflammatory effect through the regulation of pro-inflammatory cytokines.

The expression of inflammatory cytokines depends on the activation of MAPKs and NF- κ B (Bladh et al., 2009; Jeong et al., 2006). ERK is well documented to regulate cell differentiation by many stimuli. In our previous study, we showed that the blocking of ERK by ERK inhibitor PD98059 decreased the production of IL-6, TNF- α , and IL-8 in stimulated HMC-1 cells (Kang et al., 2011). JNK is the stress-activated protein kinases and activates c-JUN, which then regulates gene expression. The p38 kinases regulate a variety of cytokine production (Johnson and Lapadat, 2002). The blocking of p38 MAPK was correlated with the inhibition of IL-6 and TNF- α (Jeong et al., 2003). Glucocorticoids suppressed the NF- κ B activation and the degradation of I κ B α (Bladh et al., 2009). Our study showed that OMC suppressed the PMACI-induced MAPKs and NF- κ B activation. These results demonstrate that the effect of OMC on mast cell-mediated inflammation, at least in part, might be derived through regulation of the MAPKs and NF- κ B pathways.

Caspase-1 is present as an inactive pro-form in cytoplasm and activated by inflammasome, which are cytoplasmic and multimolecular

protein complexes. Specifically, caspase-1 converts pro-IL-1 β into the bioactive form activating lymphocytes and endothelial cells (Stutz et al., 2009). Metkar et al. (2008) reported that the caspase-1 inhibitor blocked the secretion of IL-1 β , TNF- α , and IL-6. Jeechool-Whan showed the anti-allergic effect through the inhibition of caspase-1 activation in an AR animal model (Oh et al., 2012). In this study, OMC significantly decreased caspase-1 activity in activated HMC-1 cells. These results suggest that OMC reduces AR *via* blocking of the caspase-1 pathway.

Eosinophils play a crucial role in immune response and are linked with allergic inflammatory conditions (Melvin and Ramanathan, 2012). Mast cells contribute to the induction and maintenance of eosinophilic inflammation by IgE-dependent and IgE-independent processes (Pawankar et al., 2007). Patients with AR have shown influx of these cells in the nasal epithelium (Fuentes-Beltrán et al., 2009). ICAM-1 is a chemotactic molecule of eosinophils and expressed in the mucosal epithelial cells of allergic patients. It has been considered as a marker of allergic inflammation (Montoro et al., 2007). We showed that OMC inhibited the levels of inflammation-related protein and infiltration of inflammatory cells. Taken together, OMC provides alternative proof of a possible therapeutic application of this agent in allergic inflammatory diseases.

Conclusion

In conclusion, we have shown that OMC inhibited the histamine and tryptase releases and also regulated the productions of IL-1 β , IL-6, IL-8, and TNF- α in PMACI-stimulated HMC-1 cells through the MAPK and NF- κ B pathways. In an animal model, OMC dramatically inhibited the symptoms of AR by inhibiting the influx of inflammatory cells and pro-inflammatory cytokines production. Given these findings, the regulation of histamine and tryptase levels, which regulated the production of pro-inflammatory cytokines, by OMC may provide an insight into a new strategy for AR.

Conflict of interest statement

The authors declare no conflicts of interest.

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References

- Artis D, Humphreys NE, Bancroft AJ, Rothwell NJ, Potten CS, Grecis RK. Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. *J Exp Med* 1999;190:953–62.
- Bachert C, Wagenmann M, Hauser U. Proinflammatory cytokines: measurement in nasal secretion and induction of adhesion receptor expression. *Int Arch Allergy Immunol* 1995;107:106–8.
- Barnes PJ. Therapeutic strategies for allergic diseases. *Nature* 1999;402:B32–8.
- Bladh LG, Johansson-Haque K, Rafter I, Nilsson S, Okret S. Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-kappaB activity by glucocorticoids. *Biochim Biophys Acta* 2009;1793:439–46.
- Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic rhinitis and its impact on asthma (ARIA) 2008 update. *Allergy* 2008;63:8–160.
- Bradding P, Mediawake R, Fealther IH, Madden J, Church MK, Holgate ST, et al. TNF alpha is localized to nasal mucosa mast cells and is released in acute allergic rhinitis. *Clin Exp Allergy* 1995;25:406–15.
- Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997;186:1737–47.
- Fouche G, van Rooyen S, Faleschini T. *Siphonochilus aethiopicus*, a traditional remedy for the treatment of allergic asthma. *TANG* 2013;3:e6.
- Fuentes-Beltrán A, Montes-Vizuet R, Valencia-Maquea E, Negrete-García MC, García-Cruz Mde L, Teran LM. Chemokine CC-ligand 5 production and eosinophil activation into the upper airways of aspirin-sensitive patients. *Clin Exp Allergy* 2009;39:491–9.
- Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012;18:693–704.
- Gravitz L. In search of a booster shot. *Nature* 2011;479:S17–9.
- Jeong HJ, Na HJ, Hong SH, Kim HM. Inhibition of the stem cell factor-induced migration of mast cells by dexamethasone. *Endocrinology* 2003;144:4080–6.

- Jeong HJ, Lee SA, Moon PD, Na HJ, Park RK, Um JY, et al. Alginic acid has anti-anaphylactic effects and inhibits inflammatory cytokine expression via suppression of nuclear factor-kappaB activation. *Clin Exp Allergy* 2006;36:785–94.
- Jeong HJ, Na HJ, Kim SJ, Rim HK, Myung NY, Moon PD, et al. Anti-inflammatory effect of columbianetin on activated human mast cells. *Biol Pharm Bull* 2009;32:1027–31.
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911–2.
- Kang TH, Han NR, Kim HM, Jeong HJ. Blockade of IL-6 secretion pathway by the sesquiterpenoid atractylenolide III. *J Nat Prod* 2011;74:223–7.
- Kida T, Fujii A, Sakai O, Iemura M, Atsumi I, Wada T, et al. Bepotastine besilate, a highly selective histamine H(1) receptor antagonist, suppresses vascular hyperpermeability and eosinophil recruitment in in vitro and in vivo experimental allergic conjunctivitis models. *Exp Eye Res* 2010;91:85–91.
- Kim JJ, Kang TH, Seo JU, Na HJ, Kim SJ, Moon PD, et al. Libanoridin inhibits the mast cell-mediated allergic inflammatory reaction. *Immunopharmacol Immunotoxicol* 2010a;32:258–64.
- Kim YA, Kong CS, Yea SS, Seo Y. Constituents of *Corydalis heterocarpa* and their anti-proliferative effects on human cancer cell. *Food Chem Toxicol* 2010b;48:722–8.
- Lenon GB, Li CG, Xue CC, Thien FC, Story DF. Inhibition of release of vasoactive and inflammatory mediators in airway and vascular tissues and macrophages by a Chinese herbal medicine formula for allergic rhinitis. *Evid Based Complement Altern Med* 2007;4:209–17.
- Lin J, Opoku AR, Geheeb-Keller M, Hutchings AD, Terblanche SE, Jager AK, et al. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. *J Ethnopharmacology* 1997;68:267–74.
- Mandhane SN, Shah JH, Thennati R. Allergic rhinitis: an update on disease, present treatments and future prospects. *Int Immunopharmacol* 2011;11:1646–62.
- Melvin TA, Ramanathan Jr M. Role of innate immunity in the pathogenesis of allergic rhinitis. *Curr Opin Otolaryngol Head Neck Surg* 2012;20:194–8.
- Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, et al. Human and mouse granzyme A induce a proinflammatory cytokine response. *Immunity* 2008;14:720–33.
- Min YG. The pathophysiology, diagnosis and treatment of allergic rhinitis. *Allergy Asthma Immunol Res* 2010;2:65–76.
- Montoro J, Sastre J, Jáuregui I, Bartra J, Dávila I, del Cuvillo A, et al. Allergic rhinitis: continuous or on demand antihistamine therapy? *J Invest Allergol Clin Immunol* 2007;17:21–7.
- Mygind. Glucocorticosteroids and rhinitis. *Allergy* 1993;48:476–90.
- Mukaída N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol*. 2000;72:391–8.
- Nambu A, Nakae S. IL-1 and allergy. *Allergol Int* 2010;59:125–35.
- Oh HA, Ryu JG, Cha WS, Kim HM, Jeong HJ. Therapeutic effects of traditional Korean medicine, Jeechool-Whan in allergic rhinitis model. *TANG* 2012;2:e9.
- Palm NW, Rosenstein RK, Medzhitov R. Allergic host defences. *Nature* 2012;484:465–72.
- Pawankar R, Lee KH, Nonaka M, Takizawa R. Role of mast cells and basophils in chronic rhinosinusitis. *Clin Allergy Immunol* 2007;20:93–101.
- Pawankar R, Mori S, Ozu C, Kimura S. Overview on the pathomechanisms of allergic rhinitis. *Asia Pac Allergy* 2011;1:157–67.
- Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia* 2004;59:695–703.
- Perlman DS. Pathophysiology of the inflammatory response. *J Allergy Clin Immunol* 1999;104:S132–6.
- Ramírez-Jiménez F, Pavón-Romero G, Juárez-Martínez LL, Terán LM. Allergy rhinitis. *J Allergy Ther* 2012:S5.
- Stutz A, Golenbock DT, Latz E. Inflammasomes: too big to miss. *J Clin Invest* 2009;119:3502–11.
- Takizawa H, Ohtoshi T, Kikutani T, Okazaki H, Akiyama N, Sato M, et al. Histamine activates bronchial epithelial cells to release inflammatory cytokines in vitro. *Int Arch Allergy Immunol* 1995;108:260–7.
- Thornhimm SM, Kelly AM. Natural treatment of perennial allergic rhinitis. *Altern Med Rev* 2000;5:448–54.
- Togias A, Proud D, Kagey-Sobotka A, Norman P, Lichtenstein L, Naclerio R. The effect of a topical tricyclic antihistamine on the response of the nasal mucosa to challenge with cold, dry air and histamine. *J Allergy Clin Immunol* 1987;79:599–604.
- Wang DY, Hanotte F, De Vos C, Clement P. Effect of cetirizine, levocetirizine, and dextrocetirizine on histamine induced nasal response in healthy adult volunteers. *Allergy* 2001;56:339–43.
- Wang D, Liu Y, She R, Xu J, Liu L, Xiong J, et al. Reduced mucosal injury of SPF chickens by mast cell stabilization after infection with very virulent infectious bursal disease virus. *Vet Immunol Immunopathol* 2009;131:229–37.
- Xu WB, Haddad EB, Tsukagoshi H, Adcock I, Barnes PJ, Chung KF. Induction of macrophage inflammatory protein 2 gene expression by interleukin 1 β in rat lung. *Thromb* 1995;50:1136–40.
- Yamauchi Y, Fuhikura Y, Shimosawa T. The effect of 1 antagonists carebastine and olopatadine on histamine induced expression of CC chemokines in cultured human asal epithelial cells. *Allergol Int* 2007;56:171–7.