Chamaecyparis obtusa Essential Oil Inhibits House Dust Mite Induced Nasal Epithelial Cell Activation and Immune Responses

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Abstract: Essential oils extracted from plants contain protective volatile compounds and are known to processes antibacterial, antifungal, anti-oxidative, and anti-inflammatory effects. This study was conducted to explore the immunomodulatory effects of essential oil extracted from *Chamaecyparis obtusa* (EOCO) on house dust mite-induced mucosal inflammation. Cultured primary nasal epithelial cells were stimulated with *Dermatophagoides pteronyssinus* (DP), and *Dermatophagoides farina* (DF) for 48 h. The production of interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) was measured by enzyme-linked immunosorbent assay, and the expression levels of nuclear factor (NF)- κ B, activator protein (AP)-1, and mitogen-activated protein kinase (MAPK) were determined by western blot analysis. To examine the effect of EOCO on the production of chemical mediators and the expression of transcription factors, epithelial cells were pretreated with EOCO for 1 h before stimulation. Peripheral blood mononuclear cells (PBMCs) were cultured in nasal epithelial cell conditioned media (NECM) for 72 h, after which the levels of IL-5, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α were measured. DP and DF enhanced the production of IL-25, IL-33, and TSLP, and EOCO pretreatment inhibited their production from nasal epithelial cells. EOCO pretreatment also significantly suppressed the expression of NF- κ B and AP-1. NECM induced the production of IL-5, IFN- γ , and TNF- α from PBMCs, and only TNF- α production was significantly inhibited by EOCO pretreatment. EOCO pretreatment inhibited the DP and DF induced nasal epithelial cell derived cytokine production and TNF- α production from PBMCs. These results indicate the potential value of EOCO in the treatment of airway inflammatory or immunological diseases.

Key words: house dust mite, nasal epithelial cell, essential oil, *Chamaecyparis obtusa*, cytokine, transcription factor

1 Introduction

Allergic rhinitis (AR) is one of the most common airway diseases associated with high social and economic burden. AR is characterized by the upregulation of Th2 cells and an increased production of their immune mediators^{1,2}. Nasal epithelial cell derived cytokines, such as interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP), are critical regulators of Th2 immune responses. These cytokines are involved in the activation of innate lymphoid cells to produce effector type 2 cytokines^{3,4}. IL-25 belongs to the IL-17 cytokine family, which initiates eosinophil infiltration or Th2 cytokine production, and is associated with the level of extracellular matrix and tissue fibrosis⁵. IL-33 is a member of the IL-1 cytokine family, which play a vital role in the development of eosinophilia, airway hyperresponsiveness, and tissue remodeling⁶. TSLP belongs to the IL-7

cytokine family, which plays a crucial role in the maturation of antigen-presenting cells, skewing the Th cells toward the Th2 phenotype, and in the development of various immune-mediated diseases⁷.

Aeroallergens comprise an important causative factor of AR through direct or indirect contact with the nasal mucosa. House dust mites (HDM) are one of the most common causes of AR in South Korea⁸. *Dermatophagoides pteronyssinus* (DP) and *Dermatophagoides farina* (DF) are the major causes of allergic diseases and are strongly implicated in the increasing prevalence of allergies. Proteases from HDM disrupt the physical barrier of respiratory epithelial cells and induce the production of damage-associated molecular patterns that induce airway inflammation⁹. HDM also trigger the activation of epithelial cells through pattern-recognition receptors such as, prote-

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ase-activated receptors (PARs) and toll-like receptors (TLRs) and induce the production of inflammatory mediators and airway inflammation through interaction with inflammatory cells¹⁰.

Essential oils are volatile hydrophobic compounds that protect plants from harmful insects and microorganisms. *Chamaecyparis obtusa* is a species of cypress found in the southern part of Korea and central Japan. The essential oil extracted from *C. obtusa* (EOCO) contains monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and several other substances that are known to process antibacterial, antiatopic, and anti-inflammatory properties^{11, 12}. Although the biological activities of EOCO are not completely understood, it is known to exert immunomodulatory activities that influence Th immune responses¹³. HDM are a major causative allergen for the development of airway allergic inflammation. In the present study, we evaluated the effects of HDM on the production of the epithelial cell derived cytokines IL-25, IL-33, and TSLP from nasal epithelial cells and their effect on Th2 immune responses. We also attempted to determine the effect of EOCO on the production of epithelial cell derived cytokines and Th immune responses.

2 Experimental Procedures

2.1 Preparation of microencapsulated EOCO

Microencapsulated EOCO was kindly provided by Qwell Inc. (Seoul, South Korea). Essential oil was isolated from the leaves of *C. obtusa* collected in Masan, Kyunsangnam-do, South Korea. EOCO was produced through steam distillation of leaves according to previously described methods¹⁴. The collected essential oil was stored in nitrogen tank for 1 year at room temperature to prevent the evaporation and composition change of EOCO. The composition of EOCO was determined using a gas chromatography mass spectrometry analysis (Agilent Tech., Santa Clara, CA, USA) and **Table 1** shows the main components of EOCO used in this study. For microencapsulation, EOCO was mixed with a water-soluble styrene maleic anhydride polymer, and melanin precondensate was added to the mixture, which resulted in the formation of microcapsules consisting of a spherical inner core and an outer shell.

2.2 Isolation and activation of primary nasal epithelial cells

Primary nasal epithelial cells were isolated from the inferior turbinates of 10 patients with septal deviation (5 men and 5 women; aged 49.5 ± 13.7 years) during the septal surgery. Subjects were excluded if they had an active inflammation, allergy, and used antibiotics, antihistamines, or other medications for at least 4 weeks preoperatively. Allergy status was evaluated using the skin prick test and

Table 1 Main components of essential oil from *Chamaecyparis obtusa*.

Compound	Retention time (min)	Peak area (%)
α -Pinene	8.75	5.96
Sabinene	10.96	16.72
Myrcene	11.36	19.45
α -Terpinene	12.69	3.05
γ -Terpinene	13.54	4.75
Limonene	13.76	1.82
Terepineol	15.21	1.26
Terpinene-4-ol	16.23	2.82
Bornylacetate	27.53	9.46
α -Terpinylacetate	28.64	15.69

the multiple allergen simultaneous test. This study was approved by the Institutional Review Board of Daegu Catholic University Medical Center, and all subjects signed a consent form that outlined the objectives of the research and experiments.

Specimens were placed in Ham's F-12 medium supplemented with 100 IU penicillin, 100 μ g/mL streptomycin, and 2 μ g/mL amphotericin B. The nasal mucosa was rinsed with Ham's F-12 medium supplemented with antibiotics and incubated with 0.1% dispase (Roche Diagnostics, Mannheim, Germany) for 16 h at 4°C. The epithelial cells were isolated by gentle agitation and the cell suspensions were filtered through a No. 60 mesh cell dissociation sieve. The cells were suspended in Ham's F-12 medium supplemented with antibiotics, 150 μ g/mL glutamine, 5 μ g/mL transferrin, 25 ng/mL epithelial growth factor, 15 μ g/mL endothelial cell growth supplement, 5 IU/mL insulin, 200 pM triiodothyronine, 100 nM hydrocortisone, and 15% fetal calf serum. Cell suspensions (10^6 cells/mL) were placed in culture plates and incubated at 37°C with 5% CO₂. The epithelial cell cultures that reached 80%–90% confluence were treated with 100 and 50 μ g/mL of crushed DP and DF (Greer Lab, Lenoir, NC, USA). After 48 h of incubation, the supernatants and epithelial cells were harvested and stored at -70°C until assayed. The levels of IL-25, IL-33, and TSLP were quantified using the enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

To examine the effects of EOCO on the production of chemical mediators from nasal epithelial cells, the cells were pretreated with 0.01%, 0.05%, and 0.1% EOCO for 1 h before stimulation with DP and DF.

2.3 Cytotoxic effect of EOCO on nasal epithelial cells

The cytotoxic effect of EOCO was evaluated using a CellTiter-96® aqueous cell proliferation assay kit (Promega,

Madison, WI, USA). In a 96-well microstate plate, the nasal epithelial cells were cultured in the presence of EOCO at various concentrations (0.01%, 0.05%, 0.1%, and 0.5%) for 48 h at 37°C with 5% CO₂. A tetrazolium compound and Owen's reagent were added to each well. The reduced tetrazolium compound produced a colored formazan product due to the mitochondrial activity of the cell. The amount of formazan was directly proportional to the number of viable cells. Color intensities were evaluated using a fluorescence microplate reader at wavelength 490 nm.

2.4 Western blot analysis of nasal epithelial cells for transcription factors

After 1 h of treatment with DP and DF, the nasal epithelial cells were harvested and lysed in an ice-cold lysis buffer (Thermo Scientific, Rockford, IL USA). The collected nasal epithelial cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Berkeley, CA, USA). The membranes were blocked with 5% skim milk solution and incubated with antibodies against nuclear factor (NF)- κ B, phosphorylated NF- κ B, C-Jun, phosphorylated C-Jun, p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK, and GAPDH (Santa Cruz Biotechnology, CA, USA). After 1 h incubation, the membranes were washed and treated with peroxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). Bands were visualized using horseradish peroxidase-conjugated secondary antibodies and an ECL system (Pierce, Rockford, IL, USA). Band densities were measured using the Multi Gauge v.2.02 software (Fujifilm, Tokyo, Japan) and expressed as the percentage of treated versus untreated cells.

2.5 Culture of peripheral blood mononuclear cells (PBMCs) with nasal epithelial cell conditioned media (NECM)

PBMCs were isolated from healthy volunteers after obtaining their informed consent, which was approved by the Institutional Review Board of Daegu Catholic University Medical Center. The PBMCs were isolated from heparinized blood by the density gradient centrifugation method using Histopaque (Sigma, St Louis, MO, USA). In total, 2×10^6 cells were cultured in NECM for 72 h, after which the production of IL-5, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α was measured using ELISA kit (R&D Systems).

2.6 Statistical analysis

All experiments were conducted at least five times and produced comparable results. All the measured parameters are expressed as mean $\pm$ standard deviation. One-way analysis of variance followed by Tukey's test was performed for normally distributed data and the Kruskal-Wallis test with post-hoc Bonferroni-Dunn's test was conducted for non-normally distributed data. The analysis was conducted using the Statistical Package for the Social Sciences software version 21 (SPSS Inc., Chicago IL, USA). Results with $p < 0.05$ were considered to be statistically significant.

3 Results

3.1 Effect of EOCO on the proliferation of nasal epithelial cells

The effect of EOCO on cell survival was determined using Mossman's tetrazole test. When the nasal epithelial cells were treated with various concentrations of EOCO for 12, 24, and 48 h, the viability was found to be significantly

Fig. 1 Cytotoxic effect of essential oil extracted from *Chamaecyparis obtusa* (EOCO) on primary nasal epithelial cells at various concentrations and time points analyzed by the CellTiter-96[®] aqueous cell proliferation assay. Cell survival was significantly decreased by treatment with EOCO at 0.5% concentration. Cell survival was not influenced at EOCO concentration less than 0.1% with or without stimulation by *Dermatophagoides pteronyssinus* (DP) and *Dermatophagoides farina* (DF). NC; negative control, H; hours, *, $p < 0.05$ compared with negative control, $n = 5$.

suppressed to 19%–39% by EOCO at the concentration of 0.5%. Next, the nasal epithelial cells were treated with 100 µg/mL DP and DF with or without the various concentrations of EOCO for 48 h. It was observed that the viability of nasal epithelial cells was not influenced by the addition of EOCO to DP or DF (Fig. 1).

3.2 Effect of EOCO on the production of chemical mediators from nasal epithelial cells

The levels of IL-25, IL-33, and TSLP in the supernatants were measured after 48 h stimulation with DP and DF. Results showed that the levels of IL-25, IL-33, and TSLP were significantly increased by treatment with 50 and 100 µg/mL of DF (IL-25: 16.8 ± 4.7 and 17.8 ± 7.2 pg/mL; IL-33: 11.8 ± 5.7 and 12.0 ± 4.2 pg/mL; and TSLP: 35.3 ± 12.4 and 39.7 ± 17.2 pg/mL, respectively) compared with levels in the nontreated group (IL-25: 8.1 ± 3.2 pg/mL; IL-33: 6.2 ± 3.5 pg/mL; and TSLP: 16.6 ± 7.3 pg/mL). DP significantly induced the production of IL-33 (15.3 ± 6.2 and 14.1 ± 3.6 pg/mL, respectively) and TSLP (49.2 ± 13.7 and 47.3 ± 17.7 pg/mL, respectively) from the nasal epithelial cells. The production of IL-25, IL-33, and TSLP induced by DP and DF were significantly inhibited by EOCO in a dose dependent manner (Fig. 2).

3.3 Effect of EOCO on the expression of transcription factors from nasal epithelial cells

We investigated the expression of NF-κB, activator protein (AP)-1, and mitogen-activated protein kinase (MAPK) transcription factor by DP, and DF, which enhanced the production of IL-25, IL-33 and TSLP. Treatment with DP and DF enhanced the expression of NF-κB, phosphorylated NF-κB, phosphorylated C-Jun, and p38 from the nasal epithelial cells. Pretreatment of nasal epithelial cells with EOCO significantly inhibited the expression of NF-κB, phosphorylated NF-κB and phosphorylated C-Jun but not p38 (Fig. 3).

3.4 Effect of EOCO on the production of chemical mediators from PBMCs cultured with NECM

NECM is the cultural supernatants of nasal epithelial cells treated DP and DF with or without EOCO. To determine the effect of EOCO on the production of Th chemical mediators, PBMCs were cultured in NECM for 72 h. The levels of IL-5, IFN-γ, and TNF-α were found to be significantly increased in the NECM treated with DP (IL-5: 12.5 ± 6.2 pg/mL; IFN-γ: 28.4 ± 13.6 pg/mL; and TNF-α: 186.1 ± 46.7 pg/mL) and DF (IL-5: 14.0 ± 4.6 pg/mL; IFN-γ: 24.9 ± 12.7 ; and TNF-α, 317.7 ± 96.7 pg/mL) compared with nontreated NECM (IL-5: 7.4 ± 2.7 pg/mL; IFN-γ: 16.5 ± 7.2 pg/mL; and TNF-α: 35.2 ± 16.5 pg/mL). When the nasal epithelial cells were pretreated with EOCO, only TNF-α production from PBMCs was found to be significantly inhibited (Fig. 4).

Fig. 2 Influence of essential oil extracted from *Chamaecyparis obtusa* (EOCO) on the epithelial cell derived cytokine production induced by *Dermatophagoides pteronyssinus* (DP) and *Dermatophagoides farina* (DF) from nasal epithelial cells. DP and DF induced IL-25, IL-33, and TSLP production was significantly suppressed by EOCO treatment in a dose-dependent manner. NC; negative control, NT; nontreated, *, $p < 0.05$ compared with NC, †; $p < 0.05$ compared with NT, $n = 7$.

4 Discussion

Nasal epithelial cells comprise the first mucosal defense organ against external stimuli by innate and adaptive immune responses. They play an active role in inflammation through the production of various chemical mediators. The DP and DF are the major source of inhaled allergens

Fig. 3 Effects of essential oil extracted from *Chamaecyparis obtusa* (EOCO) on the expression of transcription factors by treatment with 100 $\mu\text{g/mL}$ of *Dermatophagoides pteronyssinus* (DP) and *Dermatophagoides farinae* (DF). Treatment with 0.1% EOCO significantly suppressed the expression of DP and DF induced NF- κ B, phosphorylated NF- κ B, and phosphorylated C-Jun from nasal epithelial cells. NC; negative control, *, $p < 0.05$ compared with NC, † ; $p < 0.05$ compared with DP or DF, $n = 5$.

Fig. 4 Effects of essential oil extracted from *Chamaecyparis obtusa* (EOCO) on the production of chemical mediators in peripheral blood mononuclear cells (PBMcs) by nasal epithelial cell conditioned media (NECM) treated with *Dermatophagoides pteronyssinus* (DP) and *Dermatophagoides farina* (DF). Levels of IL-5, TNF- α and IFN- γ were significantly increased by NECM and EOCO only significantly inhibited the production TNF- α from PBMcs. NC; negative control, NT; nontreated, *; $p < 0.05$ compared with NC, †; $p < 0.05$ compared with NT, $n = 5$.

that can develop allergic airway inflammation. They contain various proteins, enzymes, and other macromolecules. The proteases of HDM disrupt the physical barrier of airway epithelial cells and induce the production of chemical mediators through interaction with PAR-2, TLR-4, or other receptors^{10, 15}. In the present study, we observed that both DP and DF induced the production of the Th2 immune-modulating epithelial cell derived cytokines IL-25, IL-33, and TSLP from nasal epithelial cells. This production of chemical mediators was associated with the NF- κ B, AP-1, and p38 pathway. Pretreatment of nasal epithelial cells with EOCO effectively suppressed HDM-induced epithelial cell derived cytokine production and NF- κ B and AP-1 expression.

Phytotherapy represents an alternative practice of using plant or animal extracts as health promoting agents with varying benefits and lack of side effects¹⁶. Phytoncides, or essential oils, contain volatile compounds emitted by plants to protect from the harmful external environment. Studies have reported that essential oils contribute to the improvement of various disorders by exerting antibacterial, antifungal, anti-oxidative, and anti-inflammatory activities^{17, 18}. EOCO inhibited lipopolysaccharide-induced inflammation through the control of prostaglandin and TNF- α gene expression¹⁹. EOCO suppressed the production of IL-6 and IL-8 from bronchial epithelial cells through the inhibition of the expression and activity of NF- κ B and AP-1. In the present study, HDM-induced epithelial cell derived cytokine production was significantly suppressed by treatment with microencapsulated EOCO in a dose-dependent manner. Plain essential oil is volatile and unstable, whereas the microencapsulation of essential oil can protect the active compounds from environmental factors, inhibit the degradation or evaporation, and increase the solubility in water²⁰. We observed that a concentration of EOCO less than 0.1% had no influence on the survival of nasal epithelial cells treated with HDM. *C. obtusa* plain oil was approximately 10 times toxic to nasal epithelial cells than microencapsulated EOCO (data not shown).

NF- κ B and AP-1 are key transcription factors associated with the induction and regulation of chemical mediators involved in inflammatory immune responses. These transcription factors are regulated by different mechanisms, and there is an overlap of the signaling pathway leading to the activation of NF- κ B and AP-1, which implies that they can influence each other and function cooperatively²¹. HDM contain several proteases, such as cysteine, serine, or aspartic proteases, which can interact with pattern-recognition receptors of the cell membrane. Studies have shown that HDM induce the production of inflammatory cytokines from respiratory epithelial cells through PAR-1, PAR-2, TLR-2, and TLR-4^{15, 22}. Interaction of allergens with PAR or TLR was found to induce the expression of NF- κ B, AP-1, and MAPK in various immune cells^{22, 23}. HDM can interact with and induce the production of chemical mediators from nasal epithelial cells through the NF- κ B, AP-1, and MAPK pathway. In the present study, we observed that EOCO significantly inhibited the HDM-induced epithelial cell derived cytokine production through the inhibition of the phosphorylated NF- κ B and AP-1 activity from the nasal epithelial cells.

Type-2-cell mediated immune stimuli induce the production of IL-25, IL-33, and TSLP that activate dendritic cells to promote the Th2 immune response through the activation of type 2 innate lymphoid cells (ILC2s), basophils, eosinophils, and mast cells^{2, 24}. IL-25, IL-33, and TSLP also promote the differentiation of ILC2s. Because, HDM induced the production of IL-25, IL-33, and TSLP from nasal epithelial cells, which could induce the Th2 immune response, we cultured PBMcs with NECM. The NECM produced by HDM treatment induced the production of IL-5, IFN- γ , and TNF- α from PBMcs, which implied that NECM induced not only Th2 inflammation but also Th1 inflammation in the nasal mucosa. We also measured the level of IL-25, IL-33, and TSLP in NECM. HDM could induce the production of several other chemical mediators such as granulocyte macrophage colony stimulating factor (GM-CSF), IL-1 β , IL-6, and IL-8¹⁵. These chemical mediators

may influence both Th1 and Th2 immune responses. NECM, which was pretreated with EOCO, suppressed only TNF- α production from PBMCs, which implies that EOCO may have a strong anti-inflammatory effect. The NECM produced by nasal epithelial cells, which was pretreated with EOCO, did not suppress the production of IL-5 and IFN- γ from PBMCs. Although EOCO treated with NECM did not affect the production of IL-5 and IFN- γ from PBMCs, we could not conclude that EOCO could not affect Th1 or Th2 immune responses. This is because we did not investigate the direct effect of EOCO on the production of chemical mediators from PBMCs.

The results of our study demonstrate that DP and DF induced the production of IL-25, IL-33, and TSLP from nasal epithelial cells through the NF- κ B and AP-1 pathway. The production of HDM-induced nasal epithelial cell derived cytokines was significantly suppressed by EOCO in a dose-dependent manner. The NECM produced by HDM-induced nasal epithelial cells enhanced Th1 and Th2 responses and the proinflammatory cytokine production from PBMCs. However, pretreatment of nasal epithelial cells with EOCO significantly suppressed only TNF- α production from PBMCs. These findings suggest that EOCO inhibits the production of HDM-induced chemical mediators by suppressing the phosphorylation of NF- κ B and AP-1. Although we cannot conclude that EOCO has anti-allergic effects, EOCO possesses strong anti-inflammatory properties. Further studies are required to determine in more detail the anti-inflammatory mechanism of EOCO, the immunomodulatory properties, and the optimal effective concentration for the development of a novel therapeutic strategy against airway inflammatory diseases.

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Conflict of Interest

All authors have no conflicts of interest to disclose.

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