Inhibition Effect of *Eucommia ulmoides* Leaf Extract on Interleukin 8 Production by A549 Cells

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Asthma is characterized by chronic inflammation of the airway mucosa. As *Eucommia ulmoides* Oliv. leaf extract (ELE) has been known to have anti-inflammatory properties, herein, we investigated the effect of ELE on interleukin (IL-) 8 production in A549 cells, a human airway epithelial cell line. The addition of ELE 1 h before tumor necrosis factor-alpha (TNFα) stimulation inhibited IL-8 production by A549 cells in a concentration-dependent manner. The addition of geniposidic acid, the main component of ELE, also inhibited IL-8 production. To further investigate the mechanism by which ELE inhibits IL-8 production, the effect of ELE or geniposidic acid on TNFα-stimulated p38 phosphorylation was examined by Western blotting. After 30 min of TNFα stimulation, p38 phosphorylation was inhibited by the addition of ELE or geniposidic acid, suggesting that ELE inhibited IL-8 production in TNFα-stimulated A549 cells by suppressing one of the signal transducers of p38 phosphorylation. These results indicate that ELE can be used as an effective measure against asthma, particularly neutrophilic asthma.

**Key words** *Eucommia ulmoides* leaf extract; asthma; interleukin 8; p38 phosphorylation; A549 cell

INTRODUCTION

Asthma is caused by chronic inflammation of the airway mucosa, with symptoms such as convulsive coughing, spitting, and stifling by slight irritation. The causes of inflammation involve allergens such as ticks, house dust, pet dandruff, and mold. In addition to allergens, exhaust gases in the atmosphere, overwork, and stress cause inflammation. Asthma can be classified as eosinophilic asthma and non-eosinophilic asthma (mainly neutrophilic asthma). 1, 2 In eosinophilic asthma, eosinophils, mast cells, and B cells activated by interleukin (IL-) 4, 5, 9, or 13 produced by T helper type 2 (Th2) cells play an important role. In addition, innate lymphoid cells (ILC2) produce IL-5 by releasing thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 secreted from the airway epithelium. Thereafter, more eosinophils are recruited and activated, and the allergic inflammation is exacerbated. 3, 4 In neutrophilic asthma, neutrophil production is increased by the infiltration of IL-8 secreted from the airway epithelium, causing severe airway inflammation. IL-17 produced by Th17 cells and ILC3 is also involved in the infiltration of neutrophils. 3, 4

IL-8 is a CXC chemokine known as a neutrophil chemotactic factor. In neutrophilic asthma, sputum IL-8, neutrophil esterase concentration, and mRNA levels of IL-8 and IL-1β are elevated compared to that in non-neutrophilic asthma. 5 Furthermore, in non-eosinophilic asthma, high levels of IL-8 are observed from blood neutrophils. 6 Stimulants such as viruses, tobacco, and pollutants induce chemotactic factors including IL-8 and accumulate neutrophils in the respiratory tract. In the asthma model, IL-17A and IL-17F produced from activated Th17 cells induce the production of neutrophil chemotactic factors such as CXCL1 and IL-8 from the airway epithelium. 7 Significant increases in Th17-related cytokines and chemokines such as CXCL1 and IL-8 are observed in the sputum of severe asthma patients with neutrophilia compared to that in other patients with severe inflammatory asthma without neutrophilia. 8

Previous reports have shown that *Eucommia ulmoides* Oliv. has various antioxidant, antihypertensive, and antihyperglycemic activities. 9–11 The hot water extract of *E. ulmoides* Oliv. leaves (ELE) contains various substances such as iridoids, phenol compounds, flavonoids, and terpenoids. 12 Geniposidic acid present in ELE has been known to relieve hypertension and hyperlipidemia 13 and suppress the production of inflammatory cytokines and infiltration of neutrophils in the colitis model. 14 Furthermore, administration of ELE decreased tumor necrosis factor-alpha (TNFα) levels in the plasma of rats on a high-fat diet. 15 Thus, it is conceivable that ELE has anti-inflammatory activity. Therefore, this study aimed to investigate the effect of ELE on asthma, especially neutrophilic asthma, by monitoring IL-8 production from cells of the human airway epithelial cell line, A549.

MATERIALS AND METHODS

**Reagents** ELE (Lot No. 180724M; geniposidic acid content: 5.898%) was provided by Kobayashi Pharmaceutical Company (Osaka, Japan). Two tons of *Eucommia* leaves (Sichuan, China) were boiled in 10 t of water at 90°C for 1 h. The extract was filtered and concentrated and then powdered by vacuum-drying (yield: 18%). Geniposidic acid was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, U.S.A.). Asperuloside was purchased from ChemFaces (Wuhan, Hubei, China). Recombinant human TNF-α was purchased from Peprotech (Rocky Hill, NJ, U.S.A.). Anti-phospho-Syk, anti-Syk, anti-phospho-p38, anti-phospho-c-Jun, anti-phospho-JNK, horseradish peroxidase (HRP) conjugated anti-β-Actin, and HRP conjugated anti-rabbit im-
munoglobulin G (IgG) were purchased from Cell Signaling Technologies (Danvers, MA, U.S.A.).

Cells The human airway epithelial cell line A549 was purchased from the Japanese Collection of Research Resources Cell Bank (Ibaraki, Japan). A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient Mixture F-12 Ham medium supplemented with 10% fetal bovine serum, 20 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Induction of IL-8 Production A549 cells (1 × 10⁵ cells/mL) were dispensed into 96-well microplates at 200 µL/well and cultured overnight at 37°C. The next day, the supernatant from each well was discarded; the cells were washed and 100 µL/well of DMEM/Nutrient Mixture F-12 Ham medium supplemented with 0.1% bovine serum albumin (BSA) (0.1% BSA medium) was added to each well. Then, 50 µL/well of ELE, geniposidic acid, or asperuloside diluted to each concentration in 0.1% BSA medium was added to each well and incubated for 1 h at 37°C. After incubation, 50 µL/well of TNF-α (200 ng/mL) diluted in 0.1% BSA medium was added to each well to stimulate the cells. The culture supernatant on the third day of culture was collected and used for IL-8 measurement.

Enzyme-Linked Immunosorbent Assay (ELISA) IL-8 levels in cell culture supernatants were assayed using Human IL-8/CXCL8 DuoSet ELISA (R&D Systems, Minneapolis, MN, U.S.A.).

Western Blot Analysis Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing both protease and phosphatase inhibitors (Roche, Indianapolis, IN, U.S.A.). Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). Equal amounts of protein were loaded onto a 12% sodium dodecyl-sulfate polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked using 5% skim milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Then, the membranes were incubated with HRP conjugated secondary antibodies for 1 h at room temperature and detected by enhanced chemiluminescence reagents using Amersham Imager 680 blot and gel imager (GE Healthcare, Buckinghamshire, U.K.).

Statistical Analysis Results are expressed as mean ± standard deviation (S.D.). The statistical significance of the difference between groups was determined by one-way ANOVA followed by Tukey’s test. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

We examined the anti-inflammatory mechanisms of ELE using the human airway epithelial cell line, A549. IL-8 levels markedly increased in the supernatants of A549 cells on day 3 after TNF-α stimulation (Fig. 1). A549 cells were treated with ELE 1 h before TNF-α stimulation. ELE significantly inhibited IL-8 production from TNF-α-stimulated cells in a dose-dependent manner.

Treatment with 10 and 100 µM geniposidic acid significantly inhibited IL-8 production from TNF-α-stimulated cells (Fig. 2). Asperuloside is another major component of ELE. We also examined the effect of asperuloside on IL-8 production from TNF-α-stimulated cells. Asperuloside had no inhibitory effect.
In conclusion, the present results suggest that ELE can be effective against asthma, especially neutrophilic asthma.

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Conflict of Interest The authors declare no conflict of interest.

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