Inhibitory effects of Schizandrae Fructus on eotaxin secretion in A549 human epithelial cells and eosinophil migration

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Abstract

Eosinophilia have been implicated in a broad range of diseases, most notably allergic conditions (e.g. asthma, rhinitis and atopic dermatitis) and inflammatory diseases. These diseases are characterized by an accumulation of eosinophils in the affected tissue. Defining the mechanisms that control the recruitment of eosinophil is fundamental to understanding how these diseases progress and identifying a novel target for drug therapy. Accordingly, this study was conducted to evaluate the regulatory effect of Schizandrae Fructus (SF) on the expression of eotaxin, an eosinophil-specific chemokine released in respiratory epithelium following allergic stimulation, as well as its effects on eosinophil migration.

To accomplish this, human epithelial lung cells (A549 cell) were stimulated with a combination of TNF-\(\alpha\) (100 ng/ml) and IL-4 (100 ng/ml) for 24 h. The cells were then restimulated with TNF-\(\alpha\) (100 ng/ml) and IL-1\(\beta\) (10 ng/ml) to induce the expression of chemokines and adhesion molecules involved in eosinophil chemotaxis for another 24 h. Next, the samples were treated with various concentrations of Schizandrae Fructus (SF) (1, 10, 100, 1000 \(\mu\)g/ml) or one of the major constituents of SF, schizandrin (0.1, 1, 10, 100 \(\mu\)g/ml), after which following inhibition effect assay was performed triplicates in three independence.

The levels of eotaxin in secreted proteins were suppressed significantly by SF (100 and 1000 \(\mu\)g/ml, \(p<0.01\)) and schizandrin (10 and 100 \(\mu\)g/ml, \(p<0.01\)). In addition, SF (1, 10, 100 and 1000 \(\mu\)g/ml) decreased mRNA expression levels in A549 cells significantly (\(p<0.01\)). Eosinophil recruitment to lung epithelial cells was also reduced by SF, which indicates that eotaxin plays a role in eosinophil recruitment. Furthermore, treatment with SF suppressed the expression of another chemokine, IL-8 (0.1 and 1 \(\mu\)g/ml SF, \(p<0.01\), as well as intercellular adhesion molecule-1 (10 and 100 \(\mu\)g/ml SF, \(p<0.01\)) and vascular cell adhesion molecule-1 (0.1 and 1 \(\mu\)g/ml SF, \(p<0.05\)), which are all related

**Abbreviations:** ERK, extracellular signal-regulated kinases p42/p44; HOG, p38/MAPK; HPLC, high-performance liquid chromatography; ICAM-1, intercellular adhesion molecule-1; IL-4, interleukin-4; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MAPK, mitogen-activated protein kinases; MTS, [3-(4; 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; SF, Schizandrae Fructus; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); VCAM-1, vascular cell adhesion molecule-1.

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to eosinophil migration. Taken together, these findings indicate that SF may be a desirable medicinal plant for the treatment of allergic diseases.

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**Keywords:** Schizandrae Fructus; Eosinophil migration; Eotaxin; Allergic diseases

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**Introduction**

Because exposure to environmental hazards is inevitable, outbreaks of allergic diseases such as allergic asthma, atopic dermatitis, and allergic rhinitis have increased. Indeed, in the United States more than 50 million people suffer from allergic diseases each year, costing the US health care system approximately $18 billion annually (Elsner et al. 2004). As a result, many academic and industrial studies have been conducted to define disease mechanisms and develop therapies to treat or prevent the symptoms of allergies.

The initial stage of asthmatic symptoms is airway inflammation, in which eosinophils play a crucial role (Kay 1991). Eosinophils are present in excess in the airways of asthma patients; however, their accumulation decreases with subsidence of the symptoms of asthma. During an asthma attack, eosinophils selectively migrate and adhere to vascular endothelial cells, after which they migrate into the airways in response to chemokine recruitment. Once in the airway, they infiltrate and cause inflammation (Djukanovic et al. 1992).

Many natural products used in traditional oriental medicine are reportedly good agents for the treatment of asthma (Lima-Landman et al. 2007). For example, it has been suggested that Moutan Cortex Radicis reduced eotaxin secretion (Kim et al. 2007). However, despite their remarkable ability to treat asthma, most natural products have not been widely used in western societies, because little is known about the modes of action at the molecular level. One such product, Schizandrae Fructus (SF) is the fruit of *Schizandra chinensis* Baill. SF, which is an oriental herb that contains schizandrin as one of its major constituents, is used by traditional oriental clinicians to treat several diseases including hepatitis (Liu 1989; Liu and Lesca 1982), and cancer (Li 1991). Accordingly, there have been several studies conducted to evaluate the molecular mechanisms responsible for the anti-tumor effects (Huang et al. 2004), effects on cycloheximide-induced amnesia (Hsieh et al. 1999), and inhibitory effects on human articular cartilage and chondrocytes (Choi et al. 2006a) that are exerted by SF. However, few studies have been conducted to evaluate the effects of SF on airway-related diseases. Therefore, we evaluated the effects of SF on asthma while focusing on its ability to recruit eosinophils.

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**Cell culture**

A549 cells, human type II-like epithelial lung cells, were obtained from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). These cells were cultured in 100 mm tissue culture plates (Corning, Corning, NY, USA) in RPMI medium (Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 100 U/ml penicillin–streptomycin (Invitrogen, Rockville, MD, USA) at a density of 1 × 10⁶ cells/ml. The plates were incubated at 37°C under 100% humidity and 5% CO₂. The cells were sub-cultured every 3-4 days to maintain a density of 1 × 10⁶ cells/ml.

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**Preparation of SF and schizandrin**

SF powder granules were purchased from Sun Ten Pharmaceutical (Taipei, Taiwan) and schizandrin was purchased from Wako Pure Chemical Industries, Ltd., Japan. One hundred mg of SF powder was added to 10 ml of DW, while 1 mg of schizandrin was added to 1 ml of DW. The mixtures were then stirred overnight at room temperature. Each sample was then centrifuged for 10 min at 3000 rpm (Eppendorf, Hamburg, Germany), after which the supernatant was removed and sterilized by passing it through a 0.22 μm syringe filter. The stocks of SF or schizandrin were then diluted to various concentrations and used in the subsequent experiments.

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**Endotoxin test (LAL test)**

Endotoxin toxicity was determined using a quantitative, Limulus Amebocyte Lysate (LAL) QCL-1000 test (CAMBREX, Harriman, NY, USA) using the microplate method described by the manufacturer. The kit included vials of standard *E. coli* O111:B4 containing a defined number of endotoxin units (EU). A standard curve ranging from 0 to 1.0 EU/ml was constructed by plotting the OD405 nm versus EU per absorbance unit. SF was also assayed in duplicate and the concentration was plotted against OD405 nm. The absorbance of p-nitroaniline released from the substrate was measured.
using the Microplate Reader (GENios, TECAN, USA). All equipments used were pyrogen-free and all experiments were conducted in triplicate.

Quantitative chromatographic analysis

HPLC analysis was conducted according to the slightly modified methods of Wagner et al. (1996). HPLC analysis was conducted using the Waters system (Waters Co., Milford, MA, USA) with a 717+ autosampler, 2996 photodiode array detector (PDA) 2487 dual λ absorbance detector, and 1525 binary HPLC pump, and Waters Millennium³² System (Waters Co., Milford, MA, USA) were used for data acquisition and integration. HPLC grade and other reagents (J.T. Baker Co., Ltd., Phillipsburg, NJ, USA) were used for HPLC analysis. All solvents were filtered and degassed before use.

To analyze the SF, it was accurately weighed to 0.9621 g and dissolved in 10 ml of methanol. The sample was ultrasonic for 60 min and centrifuged at 15,000 rpm for 10 min. The supernatant of the sample was filtered through a 0.45 μm syringe filter (PVDF, GELMAN, USA).

For the quantitative analysis of S. chinensis, schisandrol A, schisandrol B and schisandrin B were purchased from Chroma-Dex, diluted to 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/ml and then used as standards. The separation was conducted using a reverse phase system (XTerra™ C₁₈, 4.6 × 150 mm i.d., 5 μm, Waters Co., Milford, MA, USA) with a gradient mobile phase consisting of A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water). The elution program was set as follows: from 40% A/60% B to 80% A in 30 min at a flow rate of 1.0 ml/min with an injection volume of 25 μl and an UV detection wavelength of 250 nm.

Cytotoxicity assays

Changes in the cell viability in response to SF treatment was measured to determine if SF was toxic to A549 cells. To accomplish this, cells were plated in a 96-well flat-bottomed well plate at a density of 2 × 10⁵ cells/well. The well plate was then incubated for 24 h at 37 °C under 100% humidity and 5% CO₂. Next, SF stock was diluted in RPMI media (Invitrogen, Rockville, MD, USA) to concentrations of 0.1, 1, 10, 100 and 1000 μg/ml, and then used as a standard. The separation was conducted using a reverse phase system (XTerra™ C₁₈, 4.6 × 150 mm i.d., 5 μm, Waters Co., Milford, MA, USA) with a gradient mobile phase consisting of A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water). The elution program was set as follows: from 40% A/60% B to 80% A in 30 min at a flow rate of 1.0 ml/min with an injection volume of 25 μl and an UV detection wavelength of 250 nm.

Real-time RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the user manual. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis (data not shown), and the concentration of total RNA was quantified by determination of optical density at 260 nm (OD260), using spectrophotometer (DU 500, Beckman Instruments, Inc., Fullerton, CA, USA). Subsequently, the total RNA was reverse transcribed for cDNA synthesis, using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Applied science, Indianapolis, USA) as guided in manual. Real-time PCR was performed on a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR Green I as the dsDNA-specific binding dye and continuous fluorescence.
monitoring. Amplification was carried out in a reaction mixture with a total volume of 25 µl that contained 2 × PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 µl of 1:4 diluted cDNA and 5 µM of one of the following primer sets: GAPDH: 5'-TTC ACC ATG GAG AAG GC-3'/5'-GGC ATG GAC TGT GGT CAT GA-3'; Eotaxin: 5'-CCT CCA ACA TGA AGG TCT CC-3'/5'-GTT AAA GCA GCA GTG GGT TG-3'; ICAM-1: 5'-TGA GGA AGC ATC TCC AAG CTG GCC GTG-3'; VCAM: 5'-TTC ACC AGG CCA CCA CTC ATC-3'/5'-ATG ACT TCC AAG CTG GCC GTG-3'; Eosinophil migration assay

EoL-1 cells, a human eosinophilic cell line, were obtained from the RIKEN cell bank (Wako Pure Chemical Industries, Tokyo, Japan) and cultured under the same conditions as A549 cells until they were confluent. The cell-free culture supernatants were then collected and subjected to an eosinophil migration assay using a QCM™ Chemotaxis 96-well (5 µM) Cell Migration Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. These assays are ideal for assessing the effects of pharmacological compounds on tumor cells, and for analyzing the migratory capacity of multiple cell lines in parallel. QCM chemotaxis cell migration assays are built on the platform of a multiwell plate that includes a 24- or 96-well migration chamber and a feeder tray. Each chamber contains a microporous polycarbonate membrane of a specified pore size. Both sides of the membrane are uncoated. Cells of interest are pipetted onto the top of the insert, after which migratory cells move through the pores of the membrane and cling to the bottom of the polycarbonate membrane in response to a chemoattractant loaded into the bottom feeder tray. The migrated cells are detached, lysed, and labeled with a fluorescent dye that exhibits strong fluorescence when bound to cellular nucleic acids. Finally, the sample fluorescence is measured using a fluorescence microplate reader with an excitation of 480 nm and an emission of 520 nm.

For this study, EoL-1 cells were starved in serum-free media containing 0.2% bovine serum albumin 1 day prior to the assay. After at least 8 h of starvation, the cells were counted and then plated onto the upper well of the kit at a density of 1 × 10^6 cells/ml in 100 µl of media. In the lower well of the kit, 150 µl of the previously collected supernatants were added. The upper well was then set onto the lower well, after which the lid was closed and the wells were incubated for 4 h. Next, the media in the upper well was removed, a new lower well was filled with 100 µl of cell detachment buffer (Millipore, Billerica, MA, USA) and the upper well was applied to the new lower well. The wells were then incubated for an additional 30 min with gentle stirring. A new 96-well flat-bottomed well plate (Corning, Corning, NY, USA) that contained 75 µl of supernatant from the first lower well, 75 µl of cell detachment buffer (included in the kit; Millipore, Billerica, MA, USA) from second lower well, and 50 µl of dye solution (dye:lysis buffer = 1:75) was then prepared. Next, 150 µl was transferred onto the appropriate well plate, after which the absorbance at 484/527 nm was measured using a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland). All steps were performed in triplicate in three independent experiments.

Statistical analysis

Statistical analysis of the data was conducted using the Prism 3.02 software (GraphPad Software Inc., CA, USA). All data were presented as the means ± SEM, and one-way ANOVA was used for multiple comparisons. Results with a p < 0.05 were considered to be statistically significant.

Results and discussion

HPLC spectrum of SF

The relationship between the concentration and peak area was measured using the minimum square method (R^2 value). The standard calibration curves of schisandrol A, schisandrol B and schisandrin B were Y = 41214280X + 65684.1 (R^2 = 0.9983), Y = 13902960X + 12889.03 (R^2 = 0.9994) and Y = 2482371X + 21539.89 (R^2 = 0.9992), respectively. The average concentrations of schisandrol A, schisandrol B and schisandrin B in SF were determined to be 0.3072 ± 0.0054 mg/g (n = 3), 0.3282 ± 0.0028 mg/g (n = 3) and 0.1711 ± 0.0001 mg/g (n = 3), respectively, using the formulas listed above (Figs. 1A and B).

Cell toxicity of SF on A549 cells

To exclude the possibility that the reduction in the level of cotaxin and other chemokines in the A549 cells was due to the direct toxicity of SF against these cells,
we tested the toxicity of various concentrations of SF. MTS assays revealed that SF did not exert any cytotoxicity against A549 cells, regardless of the concentration used (Fig. 2).

**SF and schizandrin inhibit the secretion of eotaxin protein**

We evaluated the suppression of eotaxin secretion in response to treatment with various concentrations of SF and schizandrin (Figs. 3A and B). To accomplish this, A549 cells were pre-stimulated with TNF-α, IL-4, and IL-1β to induce the production of chemokines and adhesion molecules. Treatment with SF at concentrations of less than 10 μg/ml resulted in a reduction of eotaxin secretion of less than 50%. However, the decrease in eotaxin secretion reached 64% in response to treatment with higher concentrations of SF (100 and 1000 μg/ml), which was significantly lower than the secretion of eotaxin by the control cells (**p < 0.01).
Furthermore, eotaxin secretion was decreased by 40% (**p<0.01) in response to treatment with 10 μg/ml schizandrin, and by over 81% (**p<0.01) after treatment with 100 μg/ml schizandrin; however, schizandrin was less active than SF. The results of a cytotoxicity test and an LAL test revealed that SF had no effect on cell viability and was in an endotoxin-free state in the cultures (data not shown); therefore, the results presented above imply that SF and schizandrin suppress eotaxin secretion and that schizandrin is one of the primary constituents of SF responsible for the suppression of eotaxin secretion.

**SF inhibits the expression of eotaxin mRNA**

To determine if the reduction of eotaxin secretion occurred due to a reduction in gene expression level, we measured the expression of eotaxin mRNA in cells that were treated with varying concentrations of SF (Fig. 4). GAPDH mRNA expression was also quantified to normalize the expression of eotaxin. The mRNA levels decreased upon SF treatment at all tested concentrations (approximately 80–90%), which clearly demonstrates that SF inhibits the expression of eotaxin mRNA.

**SF inhibits the expression of IL-8, ICAM-1, and VCAM-1 mRNA**

We performed additional assays to determine if SF regulates the expression of mRNA encoding proteins other than eotaxin that are also involved in eosinophil migration. To accomplish this, we treated cells with two concentrations of SF extract (0.1 and 1 μg/ml). Although the patterns and the extent of the changes differed, all genes evaluated showed a decrease in mRNA expression. Specifically, IL-8 mRNA expression was clearly suppressed by both concentrations of SF, and this suppression occurred in a dose-dependent fashion (77% and 96%; Fig. 5A). ICAM-1 mRNA expression was also clearly inhibited by approximately 80–90% in response to both concentrations of SF (Fig. 4B). Finally, VCAM-1 was reduced in a dose-dependent fashion in response to treatment with SF (72% and 81%; Fig. 5C).

**SF treatments lead to the suppression of eosinophil migration**

Eosinophil migration was measured fluorescently based on the optical density (OD). The supernatant of pre-stimulated A549 cells that did not receive any SF treatment was used to normalize the OD value of other groups. The cell migration reduction rates were as follows: 23% with 0.1 μg/ml SF, 51% with 1 μg/ml SF,
and 58% with 10 μg/ml SF (Fig. 6). These results demonstrate that treatment of A549 cells with SF led to the suppression of eosinophil migration.

Eotaxin is a major eosinophil-specific chemokine that is released in the respiratory epithelium following allergic stimulation. Eotaxin not only causes the migration of eosinophils, but is also generated within eosinophils, which increases the level of migration (Jose et al. 1994). The expression of eotaxin is regulated by mitogen-activated protein (MAP) kinases (MAPK). In addition to eotaxin regulation, the MAPK cascade participates in airway remodeling by inducing many other chemokines. The MAPK super family has been divided into the following three groups: extracellular signal-regulated kinases p42/p44 (ERK), p38/MAPK (HOG), and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). Eotaxin is induced in response to cytokine stimulation via NF-κB and p38/MAPK (Wong et al. 2005), whereas ICAM-1 is induced via ERK and JNK (Krunkosky and Jarrett 2006), VCAM-1 is induced via NF-κB and p38/MAPK (Kim et al. 2006), and IL-8 is induced via NF-κB and p38/MAPK (Tsai et al. 2001).

SF is an important medicinal plant in Korea, and schizandrin is one of the major constituents of the fruit of SF that has been reported to have antioxidant activity (Choi et al. 2006b). In this study, we investigated the molecular mechanism of the effects of SF against asthma. Our results revealed that SF inhibits cytokine-induced eotaxin in human lung epithelial cells more effectively than schizandrin. These results indicated that schizandrin was one of the major eotaxin secretion suppressors of SF, but that another constituent of SF must also be a potent inhibitor of eotaxin. SF did not exert any cytotoxicity in its endotoxin-free state (data not shown), which indicates that it is a safe medicinal
Taken together, these findings indicate that SF regulates several factors involved in eosinophil chemotaxis.

It should also be noted that eotaxin, VCAM-1 and IL-8 are all regulated by the NF-κB and p38/MAPK signaling pathway. Therefore, further study is needed to determine if SF effects this signaling pathway and, if so, the mechanism by which SF generates this effect should be elucidated.

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