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Effect of Korean folk medicine 'SecSec' on inflammatory cytokine secretion in HMC-1 cells

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SUMMARY

"SecSec" has been used for the purpose of prevention and treatment of throat diseases such as sore throat, cough, bronchial asthma and allergic asthma in Korea. However, its effect in experimental models remains unknown. To investigate the biological effect of SecSec, we examined cytotoxicity and secretion of inflammatory cytokines on human leukemic mast cell line, HMC-1, stimulated with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187. SecSec by itself had no cytotoxicity on HMC-1. When SecSec (1 mg/ml) was added, the secretion of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and granulocyte macrophage-colony stimulating factor (GM-CSF) was significantly inhibited about 47.20%, 25.55%, and 46.43%, respectively on PMA plus A23187-stimulated HMC-1 cells. But SecSec did not inhibit IL-8 secretion. These findings may help understanding the mechanism of action of this medicine leading to control activated mast cells on allergic inflammatory condition like asthma.

Key words: Asthma; Human mast cell line HMC-1; TNF-α; GM-CSF; IL-6; IL-8

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways in which many cells and elements play a role. The chronic inflammation is associated with increased airway hyperresponsiveness leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning (Barnes, 2000). The chronic airway inflammation of asthma is unique in that the airway wall is infiltrated by lymphocytes of the T-helper (T_h) type 2 phenotype, eosinophils, macrophages/monocytes, and mast cells (Barnes, 1995). Many of the effector cells, including mast cells in asthma, produce a variety of cytokines (Hamid *et al*., 2003). Cytokines are small secretory proteins produced by a wide variety of stimulated cells. They are extremely potent, act via specific receptors and function to amplify and regulate local immune responses. Much of what is known about the importance of cytokines in allergic inflammation comes from research on the pathogenesis of allergic asthma (Bittleman and Casale, 1994). The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, and IL-8 have been reported to be significantly high in bronchial asthma patients (Stankiewicz *et al*., 2002). Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics (Broide *et al*., 1992; Oh *et al*., 1999).

SecSec is a korean onomatopoeic word to

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express wheeze in asthma. SecSec was constituted five herbs. Its materials have been used for the purpose of prevention and treatment of throat diseases such as sore throat, cough, bronchial asthma, and allergic asthma in Korea. This study deals with an anti-inflammatory activity of SecSec on HMC-1 cells. The inhibitory effect of SecSec on the secretion of inflammatory cytokine, TNF-α, IL-6, IL-8, and GM-CSF was investigated in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-stimulated HMC-1 cells.

MATERIALS AND METHODS

Preparation of SecSec

SecSec was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h was filtered, lyophilized, and kept at 4°C. The SecSec water extract powder was dissolved in PBS and filtered with 0.2 μm syringe filter. SecSec include 10 g, 4 g, 3 g, 2 g and 1 g of *Cordyceps sinensis*, *Schisandra chinensis*, *Liriope platyphylla*, *Platycodon grandiflorum* and *Glycyrrhiza uralensis*, respectively.

Reagents

Cell culture medium, Iscove's Modified Dulbecco's Media (IMDM) was purchased from Gibco BRL (Grand Island, NY, USA). PMA, A23187, avidin-peroxidase, 2,2'-azio-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) and other reagents were obtained from sigma (St. Louis, MO, USA). Anti-human TNF-α/GM-CSF antibody (Ab), biotinylated anti-human TNF-α/GM-CSF Ab, and recombinant human TNF-α/GM-CSF were purchased from R&D Systems. Anti-human IL-6/IL-8 Ab, biotinylated anti-human IL-6/IL-8 Ab, and recombinant human IL-6/IL-8 were purchased from Pharmingen (Minneapolis, MN, USA).

Culture of HMC-1 cells

Human leukemic mast cell line (HMC-1) were grown in IMDM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-5} M mercaptoethanol and 10% heat-inactivated FBS at 37°C in 5% CO_2 and 95% humidity. Cells were treated SecSec for 30 min prior to stimulation with 50 nM PMA plus 1 μM A23187 and incubated at 37°C for 8 h - 24 h.

MTT assay

Cell viability was determined by the MTT assay. Briefly, 500 μl of HMC-1 cells suspension (5×10^5 cells) was cultured in 4-well plates for 8 h after treatment by 1 mg/ml concentration of SecSec. 50 μl of MTT solution (5 mg/ml) was added and the cells were incubated at 37°C for an additional overnight. After washing the supernatant out, the insoluble formazan product was dissolved in dimethysulfoxide. Then, optical density of 96-well culture plates was measured using a microplate reader at 540 nm.

Assay of cytokine secretion

Cytokine, TNF-α, IL-6, IL-8, and GM-CSF, secretion was measured by modification of an enzyme-linked immunosorbant assay (ELISA) as described previously (Kim *et al*., 2003). HMC-1 cells were cultured with IMDM plus 10% FBS. The cells were sensitized with PMA (50 nM) plus A23187 (1 μM) for 8 - 24 h in the absence or presence of SecSec. The ELISA was performed by coating 96-well plates (Nunc, Denmark) with 1 μg/well of monoclonal antibody with specificity for TNF-α, IL-6, IL-8, and GM-CSF respectively. Before use and between subsequent steps in the assay, the coated plates were washed three times with PBS containing 0.05% Tween-20. All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, recombinant cytokines were added to serum previously determined to be negative for endogenous cytokines. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human TNF-α, IL-6, IL-8, and GM-CSF. After 1 hour, the

assay plates were exposed to enzyme, avidin-peroxidase for 30 min. And then substrate was added to the wells. Optical density readings were made within 10 min of the addition of the substrate on a Versamax (Molecular Devices, USA) with a 405 nm filter.

Statistical analysis

Each datum represents the mean ± SEM of the different experiments under the same conditions. The Student's *t*-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of SecSec on the cell viability in HMC-1 cells

To test the cytotoxicity of SecSec, we performed MTT assay in HMC-1 cells. Figure 1 shows the cell viability at 8 h incubation after treatment with SecSec (1 mg/ml). SecSec by itself had no cytotoxicity on HMC-1 cells.

Effect of SecSec on inflammatory cytokine secretion in PMA plus A23187-stimulated HMC-1 cells

To determine whether SecSec can modulate PMA plus A23187-induced TNF-α, IL-6, and IL-8 secretion, the cells were pretreated with various concentrations of SecSec for 30 min prior to PMA plus A23187 stimulation. Culture supernatants were assayed for TNF-α, IL-6, and IL-8 protein levels by using ELISA method. The SecSec did not affect TNF-α,

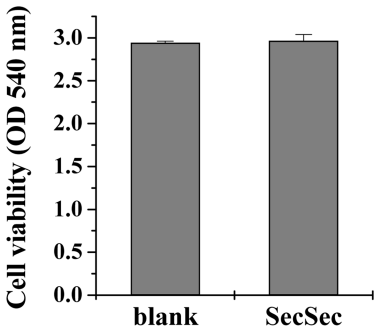

Fig. 1. Effect of SecSec on the cell viability in HMC-1 cells. Cell viability was evaluated by MTT assay 8 h after SecSec treatment (1 mg/ml) in HMC-1 cells. Data represent the mean ± SEM of three independent experiments.

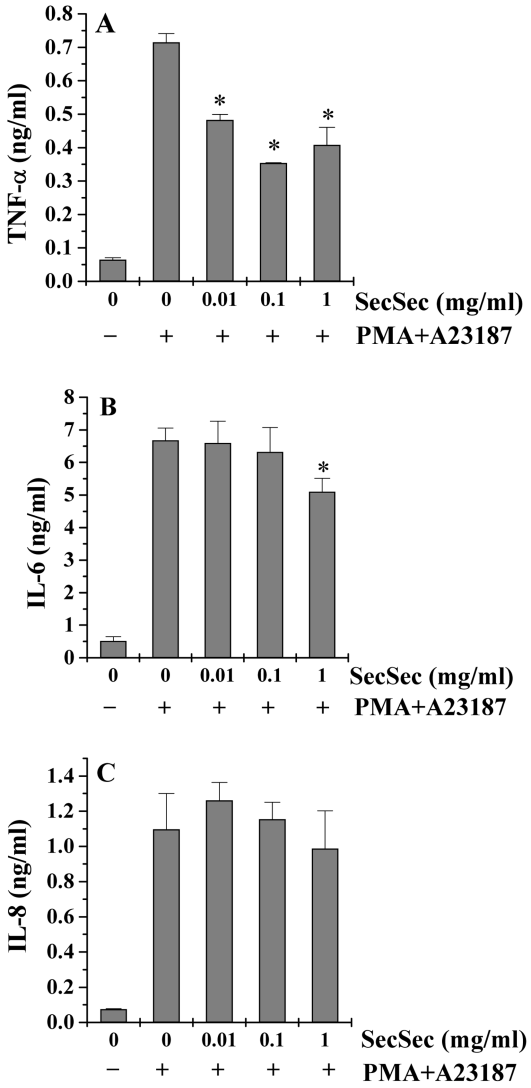

Fig. 2. Effect of TNF-α (A), IL-6 (B), and IL-8 (C) secretion by SecSec in PMA plus A23187-stimulated HMC-1 cells. Cells were pretreated with SecSec for 30 min and then challenged with PMA plus A23187 for 8 h. TNF-α, IL-6, and IL-8 concentrations were measured from cell supernatants using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments. *P<0.05; significantly different from the PMA plus A23187 treatment group.

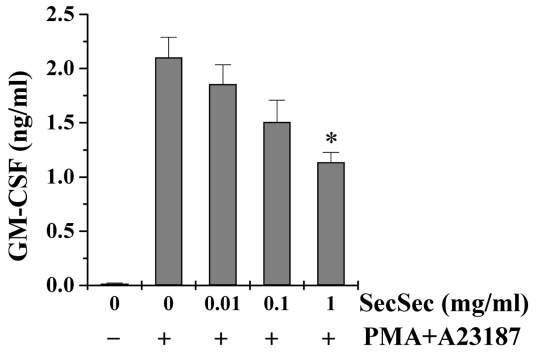

Fig. 3. Effect of GM-CSF secretion by SecSec in PMA plus A23187-stimulated HMC-1 cells. Cells were pretreated with SecSec for 30 min and then challenged with PMA plus A23187 for 24 h. GM-CSF concentrations were measured from cell supernatants using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments. **P*<0.05; significantly different from the PMA plus A23187 treatment group.

IL-6, and IL-8 secretion in the absence of PMA plus A23187 (data not shown). However, in PMA plus A23187-stimulated cells, TNF-α ($P < 0.05$) and IL-6 ($P < 0.05$) secretion was decreased by treatment of SecSec (Fig. 2A, B). But SecSec treatment did not show a significant difference in its IL-8 secretion (Fig. 2C).

Effect of SecSec on GM-CSF secretion in PMA plus A23187-stimulated HMC-1 cells

GM-CSF is a crucial cytokine for the maturation of various cell types and regulates allergic airway inflammation (Gajewska *et al*., 2003). To determine whether SecSec can modulate PMA plus A23187-induced GM-CSF secretion, the cells were pretreated with 0.01 - 1 mg/ml SecSec for 30 min prior to PMA plus A23187 stimulation. Culture supernatants were assayed for GM-CSF protein levels by using ELISA method. The SecSec did not affect GM-CSF secretion in the absence of PMA plus A23187 (data not shown). However, in PMA plus A23187-stimulated cells, GM-CSF secretion was significantly decreased by treatment of SecSec in a dose-dependent manner ($P < 0.05$). The observations are given in Fig. 3.

DISCUSSION

The present data demonstrated anti-inflammatory effect of SecSec in HMC-1 cells. This study was designed to examine the effects of TNF-α, IL-6, IL-8, and GM-CSF on HMC-1 cells because these cytokines have powerful inflammatory effects and are released by activated mast cells. In PMA plus A23187-stimulated HMC-1 cells, pretreatment of SecSec inhibited inflammatory cytokine such as TNF-α, IL-6, and GM-CSF on HMC-1 cells. In addition, SecSec did not directly affect HMC-1 cell viability. However, we did not observe an inhibitory effect of SecSec on IL-8 secretion.

The mast cell contains potent mediators, including histamine, heparin, proteinases, leukotrienes and multifunctional cytokines, these have potential contributions to the processes of inflammation and also play important role in asthma (Bradding *et al*., 1999). HMC-1 cells activated by PMA and A23187 are useful *in vitro* model system for studying of multifunctional effects of the immune and inflammatory reactions (Hosoda *et al*., 2002; Kim *et al*., 2003). We experimented on HMC-1 cells with SecSec and showed SecSec inhibited inflammatory cytokines such as TNF-α, IL-6, and GM-CSF from PMA plus A23187-stimulated HMC-1 cells.

TNF-α may have an important amplifying effect in asthmatic inflammation, and potently stimulates airway epithelial cells to produce cytokines (Cromwell *et al*., 1992; Kwon *et al*., 1994; Shah *et al*., 1995). Infusion of TNF-α causes increased airway responsiveness in Brown-Norway rats, and normal human subjects (Kips *et al*., 1992; Thomas *et al*., 1995). IL-6 is also released in asthma. There is evidence for increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge, and increased basal release, compared with non-asthmatic subjects (Gosset *et al*., 1991; Broide *et al*., 1992). GM-CSF plays a pivotal role in inflammatory and immunologic processes (Gajewska *et al*., 2003). Release of GM-CSF in the airway can mediate acute inflammatory responses as well as

initiate and perpetuate local immune responses. Elevated levels of GM-CSF, derived from epithelial cells, macrophages, and mast cells, have been demonstrated to increase eosinophil activation and survival in asthmatics (Masuda *et al*., 1992; Vignola *et al*., 1999). GM-CSF stimulates the recruitment and activation of eosinophils via β-integrin-mediated adhesion to epithelial and endothelial cells (Jagels *et al*., 1991) and prolongs eosinophil survival via inhibition of apoptosis (Gasson, 1991; Yousefi *et al*., 1997). Moreover, mouse models of asthma and diesel-induced hyperresponsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyper-responsiveness (Yamashita *et al*., 2002; Lei *et al*., 1998). Recently, the regulation of pro-inflammatory cytokines may be important possibility for treatment of asthma (Babu *et al*., 2004; Barnes, 2004).

This study investigated the effects of SecSec in an *in vitro* experimental model. Overall our findings suggest that SecSec has various regulatory effects, which might explain its beneficial effect in the treatment of inflammatory diseases like asthma.

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