



In vitro anti-allergic activities of a newly concocted traditional Chinese medicine—The wheeze-relief formula

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Abstract

Asthma is one of the most common chronic diseases worldwide. Western medications such as glucocorticoids are effective therapeutic agents but may be associated with side effects. Traditional Chinese medicine (TCM) has been used for treating allergic diseases with observable clinical benefits. The present study investigated whether a novel TCM concoction, the wheeze-relief formula (WRF), possesses in vitro anti-allergic activities. We measured the effects of WRF on the release of eosinophil cationic protein (ECP) by human eosinophils using fluorescence enzyme immunoassay, expression of chemokine receptor CCR3 and adhesion molecule CD49d on eosinophils using immunophenotyping, cytokine induction from peripheral blood mononuclear cells (PBMC) using cytometric bead array (CBA), and the gene expression of cytokines and cytokine receptors using cDNA expression array. Results demonstrated that WRF dose-dependently and significantly: (1) suppressed ECP release from eosinophils activated with granulocyte macrophage-colony stimulating factor (GM-CSF) and platelet activating factor (PAF); (2) inhibited the expression of CCR3 and CD49d on PAF-activated eosinophils; and (3) attenuated the production of tumor necrosis factor α and gene expression of IL-2 receptor chain α (CD25) on house dust mite (Der p 1) activated PBMC. The above results suggest a possible anti-allergic role of WRF and provide a biochemical basis for further clinical trial on human subjects.

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1. Introduction

Asthma is a chronic inflammatory disease presented as recurrent and reversible airway obstruction, affecting millions of people worldwide. The pathogenesis of this disease is believed to be atopic in more than 50% adults and at least 80% of affected children (WHO, 2002). Bronchodilators and steroid inhalers are often effective in controlling mild to moderate asthma with minimal adverse reactions. However, the use of systemic steroid in severe and persistent cases shows only fair responses and is normally associated with serious side effects. Development of a

better and safer regime for the management of asthma is therefore urgently needed, at least to target at the development of steroid sparing medication.

On the basis that many useful drugs, including digoxin and aspirin, are originated from plants, herbal medicine should be a promising direction for the current drug search. In traditional Chinese medicine (TCM), herbs are widely used for treating diseases. In vivo studies have shown that TCM is able to reduce allergen-induced bronchial inflammation in mite-sensitized mice (Kao et al., 2000). Clinical trials have also demonstrated that TCM shows immunomodulatory activities to patients with allergic diseases such as atopic eczema (Sheehan and Atherton, 1992; Sheehan et al., 1992; Hon et al., 2004). The potential immunomodulatory efficacy of TCM in allergic asthma is therefore highly suggested.

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From the principle of TCM, the pathogenesis of asthma is due to the inability of ‘kidneys’ to nourish the lungs. Herbs that reverse such debilitating status are curative of asthma (Hsieh, 1996; Shen et al., 2003). The wheeze-relief formula (WRF) is a novel herbal concoction developed under this TCM principle with the ultimate goal to relieve wheezing and reduce frequency of attacks in asthmatic patients. The WRF consists of five herbs, namely, *Bulbus fritillariae cirrhosae* (Chuan Bei), *Cordyceps sinensis* Sacc (Dong Cong Xiao Cao), *Astragalus mongholicus* Bunge (Huangqi), *Radix stemonae* (Bai bu) and *Radix scutellariae* (Huang Qin). These herbs have been used in China for 2000 years and are known to be safe. *Bulbus fritillariae cirrhosae* is a bronchodilator with mucolytic effect (Huang, 1999). Being an immunomodulator, *Cordyceps sinensis* Sacc relaxes smooth muscle to achieve its anti-asthmatic effect (Kuo et al., 1996; Huang, 1999). *Astragalus mongholicus* Bunge exerts immunomodulatory, antioxidant and corticosteroid like effects (Huang, 1999; Lin et al., 2000). *Radix stemonae* and its alkaloids can suppress excitation of the respiratory center and inhibit the coughing reflex (Huang, 1999). *Radix scutellariae* exhibits antimicrobial, anti-inflammatory and sedative functions and is often used in bronchitis and chronic obstructive airway disease (Kong et al., 1993; Huang, 1999).

We postulated that the WRF could exert anti-allergic activities on patients with atopic asthma. In the present study, we conducted cellular experiments on eosinophils and lymphocytes in preparation for clinical trials.

2. Methodology

2.1. Wheeze-relief formula

The WRF was formulated under the supervision of the Clinical Research Center on Chinese Medicine, the Chinese University of Hong Kong, Hong Kong. The formula was composed of equal amounts in dry weight of *Bulbus fritillariae cirrhosae* (Chuan Bei), *Cordyceps sinensis* Sacc (Dong Cong Xiao Cao), *Astragalus mongholicus* Bunge (Huangqi), *Radix stemonae* (Bai bu) and *Radix scutellariae* (Huang Qin). Dried powder of the WRF was manufactured and supplied by the Hong Kong Institute of Biotechnology based on Good Manufacturing Practice (GMP). In accordance with the traditional protocol of TCM preparation, the water-soluble active ingredients of the WRF were isolated by heating at 80 °C for 4 h in autoclaved deionized water (volume ratio 1:30). Supernatant of the WRF was obtained by centrifugation (5000 × *g*, 10 min). The extracted content was subsequently lyophilized, re-constituted in phosphate-buffered saline (PBS, Gibco™ Invitrogen Corp., CA, USA) and finally stored at –20 °C. The extraction efficiency was calculated by the formula [(Weight of final hot water extracts/Weight of starting raw TCM) × 100%]. The preparation was filtered through 0.2 µm polyethersulfone filter before use.

2.2. Endotoxin level

The level of Gram negative bacterial endotoxin contaminated in WRF was measured by colorimetric method using

QCL-1000® Chromogenic *Limulus* Amebocyte Lysate kit (Bio-Whittaker Inc., Walkersville, MD, USA), which detects as low as 0.1 Endotoxin Unit (EU)/mL in a given sample by comparing to the endotoxin standard curve (Wong et al., 2004).

2.3. Cell isolation

Eosinophils and peripheral blood mononuclear cells (PBMC) were isolated from human buffy coat (Blood Transfusion Center, Hong Kong Red Cross, Hong Kong) by density gradient separation using Percoll™ solution and Ficoll-Paque™ Plus solution (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden), respectively. Purification of eosinophils was further enhanced by CD16 magnetic beads using magnetic cell sorting system. Both eosinophils and PBMC were resuspended at 1×10^6 cells/mL in RPMI 1640 medium (Gibco™ Invitrogen Corp., CA, USA) supplemented with 10% fetal bovine serum (Hyclone Co., UT, USA) for further assays.

2.4. In vitro cytotoxicity of WRF

Eosinophils or PBMC (1×10^6 cells/mL) were treated with various concentrations of WRF for 18 h. The in vitro cytotoxic effect of WRF on the cells was assessed by trypan blue exclusion method and was calculated by comparing the number of viable cells to the total number of cells.

2.5. Release of eosinophilic cationic protein (ECP)

Eosinophils (1×10^6 cells/mL) were primed with recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) (50 ng/mL) (Pepro Tech EC Ltd., London, UK) for 30 min and then by platelet activating factor (PAF) (10 µM) (Calbiochem Corp., CA, USA) for 1 h. The activated eosinophils were then cultured with various concentrations of WRF for another 18 h. Supernatant of the eosinophil culture was collected by centrifugation and was stored in tubes that contains 0.3% *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB) to neutralize cationic charges of the eosinophilic cationic protein (ECP). The amount of ECP synthesized was then measured by fluorescence enzyme immunoassay (FEIA; CAP analyzer, Pharmacia Diagnostics AB, Uppsala, Sweden).

2.6. Expression of chemokine receptor and adhesion molecule

Eosinophils (1×10^6 cells/mL) were primed with PAF (10 µM) for 30 min and were incubated with various concentrations of WRF for another 18 h. The treated cells (5×10^5 cells) were then harvested and washed with fluorescence-activated cell sorting (FACS) medium by centrifugation at $300 \times g$ for 5 min at 4 °C. The cell pellet was re-suspended in 100 µL FACS medium. Human pooled serum (3%) was incubated with the cells for 20 min at 4 °C to block the non-specific binding sites. To stain for the chemokine receptors on eosinophils, the cells were incubated

with fluorescein isothiocyanate (FITC)-conjugated cysteine-cysteine chemokine receptor (CCR) 3-specific mouse anti-human monoclonal antibody or the corresponding fluorescein-conjugated mouse IgG isotype for 30 min at 4 °C in dark. To stain for the adhesion molecule, the cells were first incubated with purified CD49d-specific mouse anti-human monoclonal antibody or the corresponding mouse IgG isotype for 30 min at 4 °C, followed by the addition of a FITC-conjugated anti-mouse secondary antibody for another 30 min in dark at 4 °C. The cells were then washed by centrifugation and were finally resuspended in 400 μ L paraformaldehyde (1%). The cell surface antigens were analyzed by flow cytometry (FASCalibur, Becton Dickinson Corp., CA, USA) using the CellQuest™ programme (BD Pharmingen Corp., CA, USA). A total of 10,000 events (gated to exclude non-viable cells) were collected in the log mode and were expressed as histograms of relative fluorescence intensity.

2.7. Cytokine assays using cytometric bead array (CBA)

Human PBMC (1×10^6 cells/mL) were pre-activated with the allergen recombinant house dust mite *Dermatophagoides pteronyssinus* protein (Der p 1, 10 μ g/mL) for 1 h before treating with various concentration of WRF for 18 h. T helper (Th) cells

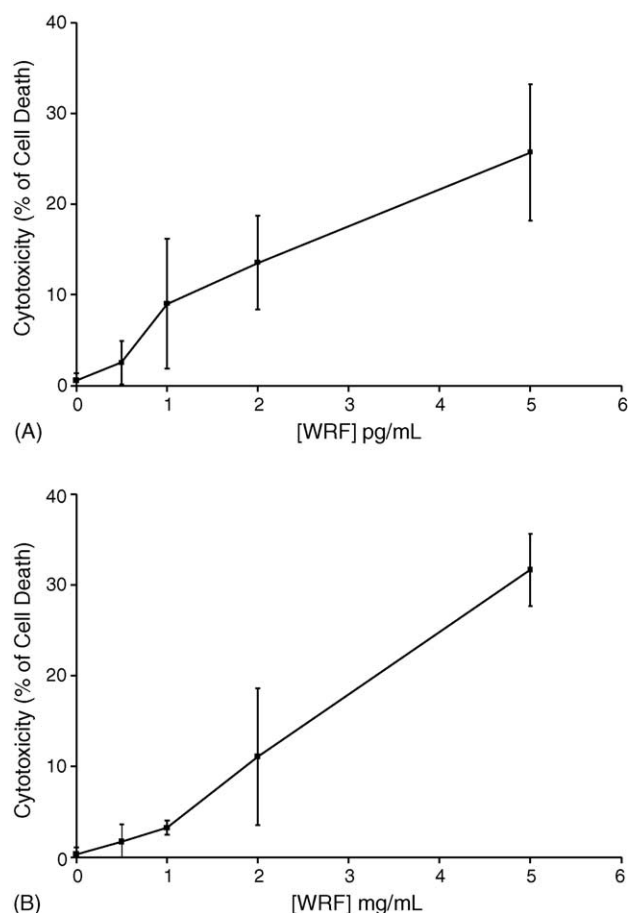


Fig. 1. In vitro cytotoxic effect of WRF on: (A) eosinophils and (B) PBMC. Results are presented by mean $\pm$ S.D. of triplicate experiments.

type 1 and 2 cytokines interleukin (IL)-2, IL-4, IL-5, IL-10, interferon (IFN) γ and tumor necrosis factor (TNF) α in the culture supernatant were measured by human Th1/Th2 cytokine cytometric bead array (CBA; BD Pharmingen) using flow cytometer (FACSCalibur, Becton Dickinson).

2.8. cDNA expression array of inflammatory cytokines and cytokine receptors

Total RNA was extracted from the untreated or WRF treated PBMC using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Non-radioactive GEArray™ Q-series kits (Superarray Bioscience Corp., Bethesda, MD, USA) were used in conjunction with the GEArray™ probe synthesis (AmpoLabeling-LPR) kit (Superarray Bioscience Corp.) to quantify the gene expression profiles of the treated cells. An aliquot of total RNA (3 μ g) was used as the template to synthesize biotinylated cDNA probes. The GEArray™ Q-series membranes dotted with tetra-spot cDNA fragment of 96 human inflammatory cytokine and receptor genes were hybridized for 18 h with the cDNA probes. The labeled biotin on the membrane was detected by chemiluminescent method and the luminescence intensities of hybridized cDNA probes were analyzed using BioRad Quantity One™ software (Bio-Rad Laboratories, CA, USA). The relative amount of a given gene transcript was estimated by comparing its signal intensity with that derived from glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Wong et al., 2004).

2.9. Statistical analysis

Student's *t*-test was used to compare the effectiveness of WRF in the present study. All statistical analyses were performed using Microsoft® Excel 2002 (Microsoft Corporation,

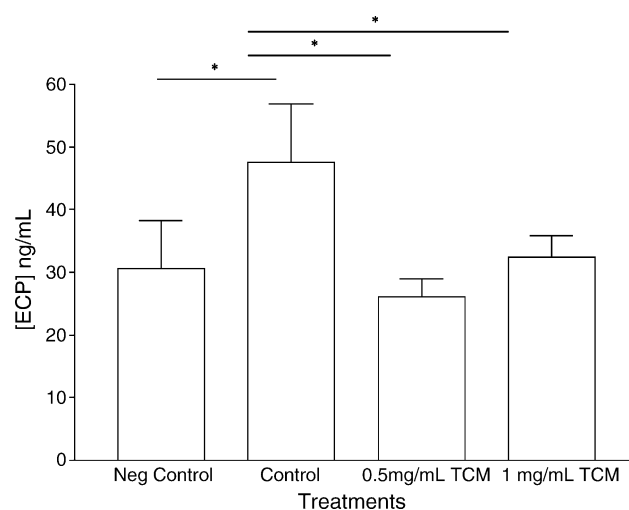


Fig. 2. In vitro effects of WRF on the degranulation of activated eosinophils. Values shown in the box-plots are from five replicate experiments. Statistical analysis was performed using Student's *t*-test. **P* < 0.05, significantly different from unstimulated cells. Negative control, eosinophils only; Control, eosinophils activated with PAF and GM-CSF; WRF, PAF and GM-CSF activated eosinophils treated in the respective concentration of WRF.

WA, USA). A probability $P < 0.05$ was considered as significantly different.

3. Results

3.1. Extraction efficiency and endotoxin level

The extraction efficiency of the water-soluble active ingredients in WRF was found to be $48.7 \pm 3.6\%$. Undetectable endotoxin level was found for WRF at concentrations < 5 mg/mL. Since the lowest detection limit of the assay kit was 0.1 EU/mg, the amount of endotoxin present in the experimenting drug was considered negligible when used at < 5 mg/mL.

3.2. Cytotoxicity of WRF on eosinophils and PBMC

In vitro cytotoxicity of WRF was determined to establish a suitable test-dose range in the present study. Fig. 1 demonstrates that WRF < 2 mg/mL retained viability of eosinophils and PBMC $> 80\%$. A test-dose range of WRF < 2 mg/mL was therefore selected for the functional assays of pharmacological parameters.

3.3. In vitro effects of WRF on the release of ECP from eosinophils

As shown in Fig. 2, WRF suppressed the release of ECP from GM-CSF- and PAF-activated eosinophils. Results demonstrated that WRF at 500 μ g/mL and 1 mg/mL could significantly

inhibit the stimulated eosinophils from degranulation (both $P < 0.05$).

3.4. In vitro effects of WRF on the expression of CCR3 and CD49d on eosinophils

Fig. 3 shows that WRF could dose-dependently down-regulate the expression of chemokine receptor CCR3 and adhesion molecule CD49d on PAF-activated eosinophils. There were statistical differences when comparing the expression of CCR3 and CD49d of WRF-treated cells to their respective control in triplicate experiments ($P < 0.05$).

3.5. In vitro effects of WRF on the synthesis of IL-5, IL-10 and TNF α of PBMC

The inducing effects of WRF on the synthesis of cytokines were studied. Results showed that WRF could significantly reduce the liberation of TNF α from Der p 1-activated PBMC ($P < 0.05$) (Fig. 4). Production of the cytokines IL-5 and IL-10 from the stimulated PBMC were also suppressed by WRF, although the difference could not reach statistical significance (Fig. 4).

3.6. In vitro effects of WRF on the gene expression of PBMC

As shown in Fig. 5, the gene expression of IL-2 receptor IL-2R α was markedly heightened in the Der p 1-activated PBMC. The addition of WRF could significantly reduce the

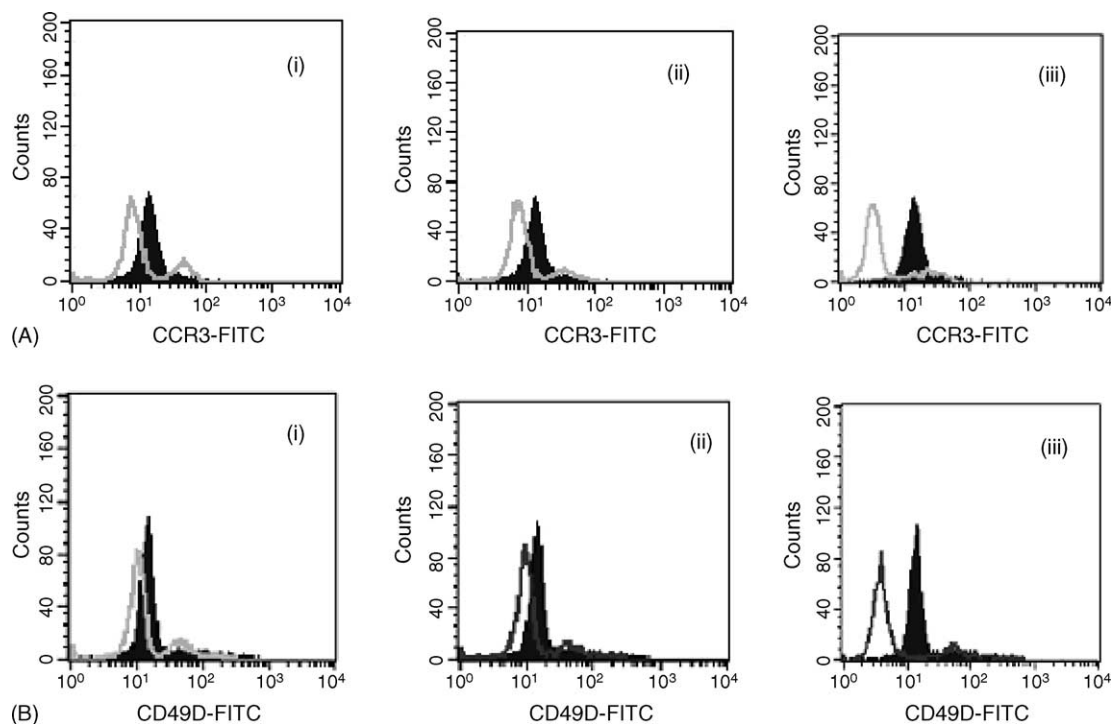


Fig. 3. Representative results showing the in vitro effects of WRF on the cell surface expression of: (A) chemokine receptor CCR3 and (B) adhesion molecule CD49d on eosinophils. Solid peak = PAF-stimulated eosinophils; line peak = WRF at: (i) 400 μ g/mL; (ii) 800 μ g/mL; (iii) 1 mg/mL. The experiments were performed in triplicate and similar results were obtained.

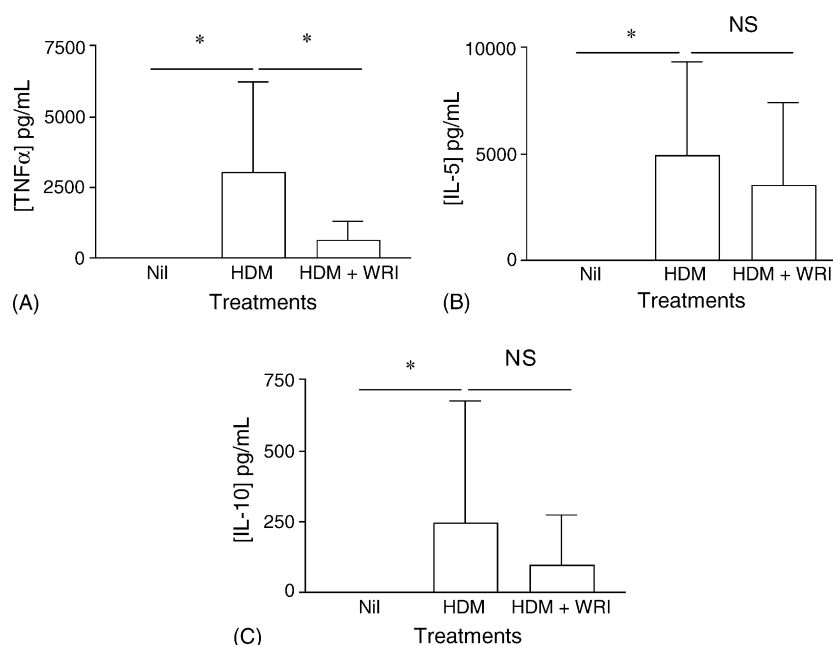


Fig. 4. In vitro effects of WRF on the induction of: (A) TNF α ; (B) IL-5; and (C) IL-10 from PBMC. PBMC (1×10^6 cells/mL) were left untreated, stimulated with house dust mite (HMD) allergen Der p 1 (10 μ g/mL) or treated with HMD Der p 1 (10 μ g/mL) and WRF (1 mg/mL) together for 18 h. Culture supernatant was collected for cytokine assay using CBA. Values shown in the box-plots are from five separate experiments. Statistical analysis was performed using Student's *t*-test. * $P < 0.05$, significantly different; NS, no significant difference was observed.

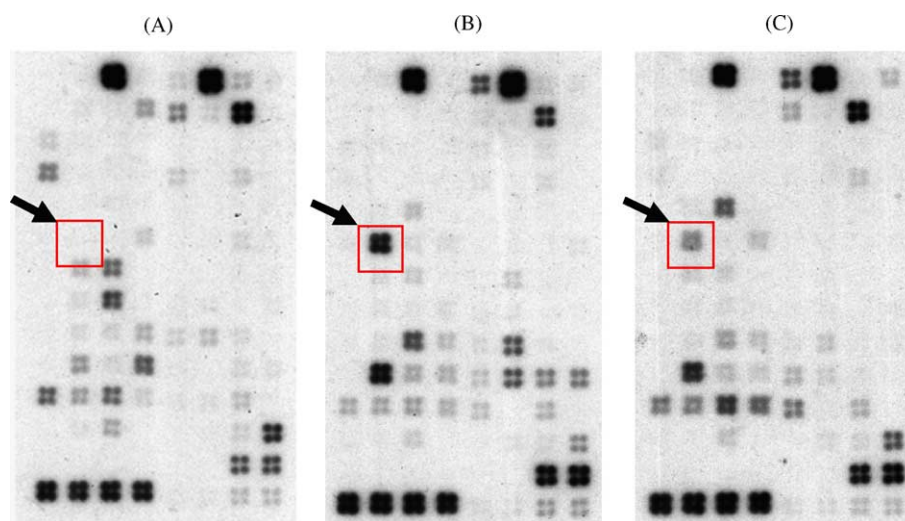


Fig. 5. Gene expression of PBMC after stimulation with WRF. Cells (1×10^6 cells/mL) were: (A) left untreated; (B) stimulated with Der p 1 (10 μ g/mL); or (C) treated with Der p 1 (10 μ g/mL) and WRF (1mg/mL) together for 24 h. Data shown in this image are from the human inflammatory cytokines and receptors GEArray and are representatives from three separate experiments. Gene pointed with arrow, IL-2R α .

expression of IL-2 α by $72.4 \pm 0.6\%$ in triplicate experiments ($P < 0.05$).

4. Discussion and conclusion

This is the first report investigating the in vitro immunomodulatory activities of WRF. Here we described eosinophil and PBMC inhibition as the two mechanisms contributing to the anti-allergic activity of WRF.

Eosinophils mediate allergic diseases such as asthma by their infiltration into the affected tissues (Wardlaw et al., 1995). The

recruitment of eosinophils is regulated by chemokines and an increased expression of adhesion molecules on the eosinophil surface (Henricks et al., 1997). Activated eosinophils degranulate in the asthmatic lungs to release granular toxic proteins including eosinophil cationic protein (Thomas and Warner, 1996). ECP causes surfactant dysfunction which subsequently results in airway obstruction (Hohlfeld et al., 2004). The present study therefore measured the effects of WRF on the eosinophil expression of chemokine receptor CCR3 and adhesion molecule CD49d and the synthesis of ECP to investigate our hypothesis that WRF has potential anti-allergic effect at least in vitro.

The chemokine receptor CCR3 is expressed in high number on eosinophils and is the exclusive binding site for the eosinophil chemoattractant CC chemokine eotaxin. The CCR3-eotaxin interaction mediates eosinophil migration into the airway (MacKay, 2000). On the other hand, the adhesion molecule CD49d activate eosinophil adherence onto the lung endothelium, whereas eosinophil survival is enhanced and superoxide synthesis is up-regulated (Nagata et al., 1995a,b; Thomas and Warner, 1996). The present study has demonstrated that WRF reduced the expression of CCR3 and CD49d on PAF-activated eosinophils. Eosinophil migration and the subsequent adherence, enhanced survival and release of cytotoxic superoxide in the airway were therefore suppressed. Previous reports have suggested that CCR3 and CD49d antagonists are likely to be safe and effective therapeutic agents for allergic diseases and asthma (Heath et al., 1997; Singh et al., 2004). Being a CCR3 and CD49d antagonist, WRF may be beneficial to the asthmatic patients.

Numerous reports have suggested that ECP released from the activated eosinophils is a marker for asthma and its plasma concentration correlates closely with the disease severity (Kandil et al., 2003; Joseph-Bowen et al., 2004). Furthermore, airway obstruction in asthma may be contributed by the surfactant dysfunction caused by ECP (Hohlfeld et al., 2004). The present study has demonstrated that ECP synthesis was attenuated in WRF-treated eosinophils, suggesting WRF may potentially reduce the severity of attacks in asthma patients.

On the other hand, T helper type 2 (Th2) lymphocytes orchestrate the allergic inflammation by producing a profile of cytokines. We therefore included cytokines and cytokine receptors as the immune parameters in the present in vitro study. It has been shown that the inflammatory cytokines TNF α (Finotto et al., 1994; Mueller et al., 1996; Yoshida et al., 1996), Th2 cytokines IL-10 (Wong et al., 2001) and IL-5 (Mueller et al., 1996) are generally elevated in the asthmatics. By adding the house dust mite allergen Der p 1, our in vitro system has successfully mimicked the asthma condition in which the liberation of these Th2 cytokines is augmented. Introduction of WRF into the in vitro system down-regulated the synthesis of TNF α ($P < 0.05$), IL-5 and IL-10 ($P > 0.05$). Specifically, TNF α is an important chemoattractant for the recruitment of eosinophils into the lungs (Sabatini et al., 2002; Lampinen et al., 2004). It is also a potent modulator of immune and inflammatory response. IL-5 uniquely and specifically participates in the control of eosinophil production and differentiation (Lampinen et al., 2004). Attenuated synthesis of TNF α and IL-5 could therefore relieve allergic responses caused by eosinophils. Although the down-regulation of IL-5 by WRF did not reach statistical significance, there was obvious observable change when compared the WRF treated cells to the control. Furthermore, the modulation of IL-10 by WRF did not reach statistical significance, implying that this anti-inflammatory cytokine has not been strikingly down-regulated to lift inflammatory responses.

Th2 cells play a critical role in the pathogenesis of allergy and asthma. CD25 or IL-2 receptor chain α is the marker for Th cell proliferation and differentiation. In the present study, we demonstrated that WRF could significantly down-regulated the gene expression of CD25 in PBMC, implicating that WRF may

exert its anti-allergic activities by inhibiting the differentiation and maturation of Th2 cells.

The above in vitro results suggested that the novel WRF may employ potential anti-allergic activities, possibly by inhibiting the inflammatory mechanisms triggered by allergen-activated eosinophils and Th2 cells. The pending clinical trial on allergic asthma patients is therefore safe and feasible to proceed.

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