



Apigenin protects ovalbumin-induced asthma through the regulation of GATA-3 gene

Jun-Rim Choi^{a,b,c,1}, Chang-Min Lee^{a,b,c,1}, In Duk Jung^{a,b,c}, Jun Sik Lee^{a,b,c}, Young-Il Jeong^d,
Jeong Hyun Chang^e, Hee-ju Park^f, Il-Whan Choi^g, Jong-Suk Kim^h, Yong Kyoo Shinⁱ,
Sung Nam Park^{a,b,c}, Yeong-Min Park^{a,b,c,*}

^a Department of Microbiology and Immunology, Pusan National University School of Medicine, Busan 602-739, South Korea

^b National Research Laboratory of Dendritic Cell Differentiation & Regulation, Pusan National University School of Medicine, Busan 602-739, South Korea

^c Medical Research Institute, Pusan National University School of Medicine, Busan 602-739, South Korea

^d Department of Microbiology, Pusan National University College of Nature Science, Busan 609-735, South Korea

^e Department of clinical laboratory Science, College of Health & Therapy, Daegu Haany University, Gyeong San 712-715, South Korea

^f Department of Pediatrics, Pusan National University School of Medicine, Busan 602-739, South Korea

^g Department of Microbiology, Inje University College of Medicine, Busan 614-735, South Korea

^h Department of Biochemistry, Chonbuk National University Medical School, Jeonju 561-180, South Korea

ⁱ Department of Pharmacology, Chungang University College of Medicine, Seoul 156-756, South Korea

ARTICLE INFO

Article history:

Received 16 February 2009

Received in revised form 23 March 2009

Accepted 26 March 2009

Keywords:

Apigenin

Asthma

GATA-3

MMP-9

AHR

ABSTRACT

Apigenin, a dietary plant-flavonoid has shown anti-inflammatory and anticancer properties, however the molecular basis of this effect remains to be elucidated. Thus we elucidated to anti-allergic effect of apigenin in ovalbumin (OVA)-induced asthma model mice. The OVA-induced mice showed allergic airway reactions. It included an increase in the number of eosinophils in bronchoalveolar lavage (BAL) fluid, an increase in inflammatory cell infiltration into the lung around blood vessels and airways, airway luminal narrowing, and the development of airway hyper-responsiveness (AHR). The administration of apigenin before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions. Accordingly, this study may provide evidence that apigenin plays a critical role in the amelioration of the pathogenetic process of asthma in mice. These findings provide new insight into the immunopharmacological role of apigenin in terms of its effects in a murine model of asthma.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Asthma is an inflammatory disease characterized by bronchial hyper-responsiveness that can proceed to life-threatening airway obstruction. The T helper 2 (Th2)-type cytokines interleukins-4 (IL-4), IL-5, and IL-13, produced by activated CD4⁺ T cells play a central role in the pathogenesis of asthma by controlling the key process of immunoglobulin E (IgE) production, growth of mast cells and the differentiation and activation of mast cells and eosinophils [1–5]. In contrast, Th1 cytokines such as interferon- γ (IFN- γ) and IL-12, which down-regulate Th2 responses, inhibit the development of allergic lung inflammation [6,7]. Thus, interventions that inhibit Th2 cytokines by enhancing Th1 cytokine production, may be useful in the treatment of allergic asthma [8]. OVA-induced asthma is characterized by AHR and

inflammation of the airways [9]. This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues [10,11]. These cellular infiltrates release various chemical mediators that can cause AHR [12,13]. Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and prolongation of airway inflammation. Inflammatory cells have to cross the basement membrane and move through connective tissue until they finally reach inflammatory sites, and require the involvement of adhesion molecules, cytokines, chemokine and enzymes including matrix metalloproteinases (MMPs) in this journey. MMPs are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix [14]. MMPs are produced by not only structural cells [15,16], but also inflammatory cells [17,18]. They are secreted as latent forms followed by proteolytic processing to active forms [19]. Of the MMP family, MMP-2 (gelatinase A, 72-kDa gelatinase) and MMP-9 (gelatinase B, 92-kDa gelatinase) are MMPs that share similar domain structures and in vitro matrix substrate specificities [20], and appear to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair [21,22]. Recruitment of

* Corresponding author. Department of Microbiology and Immunology & National Research Laboratory of Dendritic Cell Differentiation & Regulation, and Medical Research Institute, Pusan National University College of Medicine, Busan 602-739, South Korea. Tel.: +82 51 240 7557; fax: +82 51 243 2259.
E-mail address: immunpym@pusan.ac.kr (Y.-M. Park).

¹ These authors equally contributed.

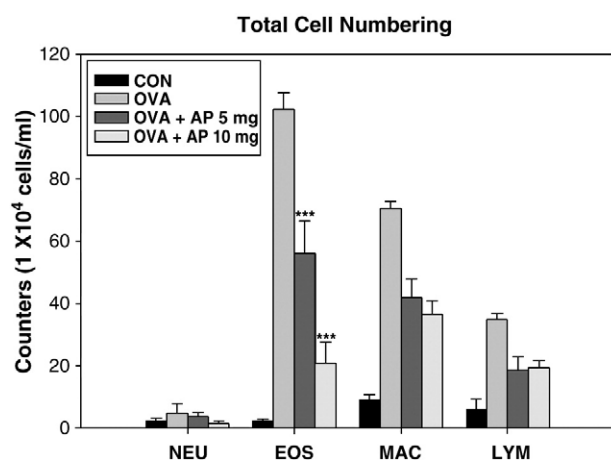


Fig. 1. Effect of apigenin on total and differential cellular components of BAL fluids of OVA-sensitized and OVA-challenged mice. Mice were treated with the PBS (CON), OVA plus apigenin 5 mg/kg/day, 10 mg/kg (OVA + AP 5 mg, OVA + AP 10 mg) and OVA (OVA), respectively, as described in Materials and methods. The BAL cells were collected 1 day after the OVA challenge. The different cell types were enumerated. The results were from one representative experiment out of 5 performed. This experiment used 5 mice ($n = 5$). *** $P < 0.001$ vs. OVA. NEU, neutrophil; EOS, eosinophil; LYM, lymphocyte; MAC, macrophages; TOT, total cell.

leukocytes from the circulating blood into tissues requires a series of cell adhesion molecules, such as ICAM-1 and VCAM-1, which are shown to play key roles in the induction of airway inflammation.

We reported in a previous work that a variety of phytochemicals exhibit profound immunoregulatory activities both *in vitro* and *in vivo*, particularly in DCs [23,24].

The flavonoids comprise a family of common phenolic plant pigments that have been identified as dietary anticarcinogens and antioxidants [25]. We reported in a previous study that a variety of phytochemicals exhibit profound immunoregulatory activity, particularly in the DC. Apigenin, one of the most common flavonoids, is found in a variety of fruits and vegetables, including onions, parsley, and oranges as well as chamomile tea, wheat sprouts, and certain seasonings [26]. Apigenin has demonstrated anti-inflammatory, anticarcinogenic, and free radical-scavenging activities in a variety of *in vitro* systems [27]. In a recent study, investigators identified apigenin as a potent inhibitor of the nuclear transcription factor nuclear factor- κ B (NF- κ B), which may perform a pivotal function in the regulation of cell growth, apoptosis, and the regulation of the cell cycle [28]. Studies using human leukemia cells as well as carcinoma cells in the breast, colon, and elsewhere have revealed that apigenin inhibits cell growth via the induction of cell cycle arrest and apoptosis [29]. It also attenuates proinflammatory cytokine production in LPS-stimulated peripheral blood mononuclear cells via the selective elimination of monocytes and macrophages, inhibits TNF-induced intercellular adhesion molecule-1 upregulation *in vivo*, and inhibits IL-1-induced prostaglandin synthesis and TNF-induced IL-6 and IL-8 production [30]. Moreover, it actively inhibits I κ B kinase activity, I κ B degradation, NF- κ B DNA protein-binding activity, NF- κ B luciferase activity, and mitogen-activated protein kinase (MAPK) activity.

It has been shown using animal models that allergic airway inflammation is increased by Th2 cytokine production and decreased Th1 cytokine production. In a recent study, it was suggested that T-bet might protect against asthma through increased expression of GATA-3 mRNA in asthmatic airways [31–35].

In this study, we have attempted to characterize the effects of a nontoxic concentration of apigenin in a murine model of asthma. Our findings demonstrated, for the first time, that apigenin treatment inhibited asthmatic syndrome, and suppressed the OVA-induced gelatinolytic activity of MMP-9, and the translocation of GATA-3 in the cytosol.

2. Materials and methods

2.1. Animals and experimental protocol

Female BALB/c mice, 6–8 weeks of age and free of murine-specific pathogens, were obtained from the Charles River Laboratories (Yokohama, Japan). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University Medical School. Mice were immunized intraperitoneally (i.p.) with 20 μ g of OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) on days 1 and 15. Mice were challenged for 30 min via the airway with OVA (5% OVA) each day from days 21–23 on consecutive days. BAL fluid was obtained at 24 h after the last challenge. At the time of lavage, the mice (6 mice in each group) were killed with an overdose of ether. The chest cavity was exposed to allow for expansion, after which the trachea was carefully incubated and the catheter secured with ligatures. Prewarmed saline solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4 °C. A part of each pool was then centrifuged, and the supernatants were kept at –70 °C until use.

2.2. Administration of apigenin

Mice were injected i.p. with 5 or 10 mg/kg/day in 200 μ l of apigenin (Sigma, St. Louis, Mo) each day from days 18 to 20 on consecutive days.

2.3. Total cell counting

The total cell numbers were counted with a hemocytometer. Smears of BAL cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics of P.R. Inc, Aguada, PR) for differential cell counting. Two independent, blinded investigators counted the cells, using a microscope. Approximately 200 cells were counted in each of four different random locations.

2.4. Measurement of eosinophil peroxidase

The suspension of BAL cells and the pulmonary homogenates were frozen/thawed three times using liquid nitrogen and a water bath at 37 °C to obtain the EPO. The BAL fluid was centrifuged to 4 °C for 10 min and serially diluted in a 96-well plate (75 μ l/well) followed by the addition of 150 μ l of substrate (1.5 mM o-phenylenediamine and

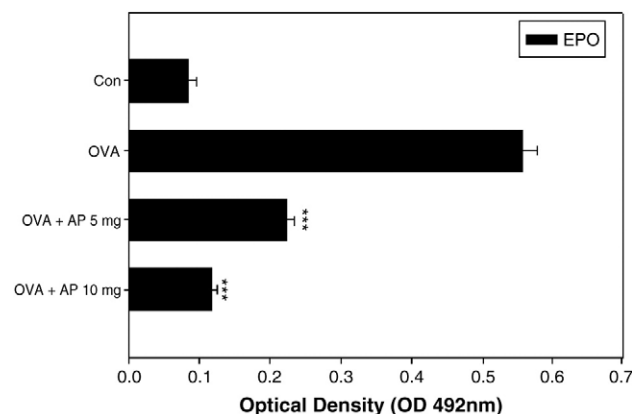


Fig. 2. Eosinophil peroxidase (EPO) activity in BAL fluids of OVA-sensitized and -challenged mice. EPO is an indicator of the numbers of eosinophil levels. The increased levels of EPO were significantly reduced about 60% (OVA + AP 5 mg/kg/day), 78% (OVA + AP 10 mg/kg/day) compared with OVA-treated mice. The results were from one representative experiment out of 5 performed. This experiment used 5 mice ($n = 5$). *** $P < 0.001$ vs. OVA.

6.6 mM H_2O_2 in 0.05M Tris–HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped by the addition of 75 μl of 30% H_2SO_4 , and the absorbance of the samples was determined at 492 nm on an ELISA reader.

2.5. Histopathology

At 48 h after the last challenge, lungs were removed from the mice after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass

slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI).

2.6. RNA preparation and quantitative RT-PCR

The total RNA from lung tissues was isolated with the use of a rapid extraction method (TRI-Reagent) (Invitrogen Life Technologies, CA, U.S.A), as previously described [14]. Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were GATA-3 sense 5'-GAG GTG GAC GTA CTT TTT AAC ATC G-3', GATA-3 antisense 5'-GGC ATA CCT GGC TCC CGT-3'. Cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis used the sequence detection software supplied with the instrument. The relative quantitation value is expressed as $2 \pm \text{DcT}$,

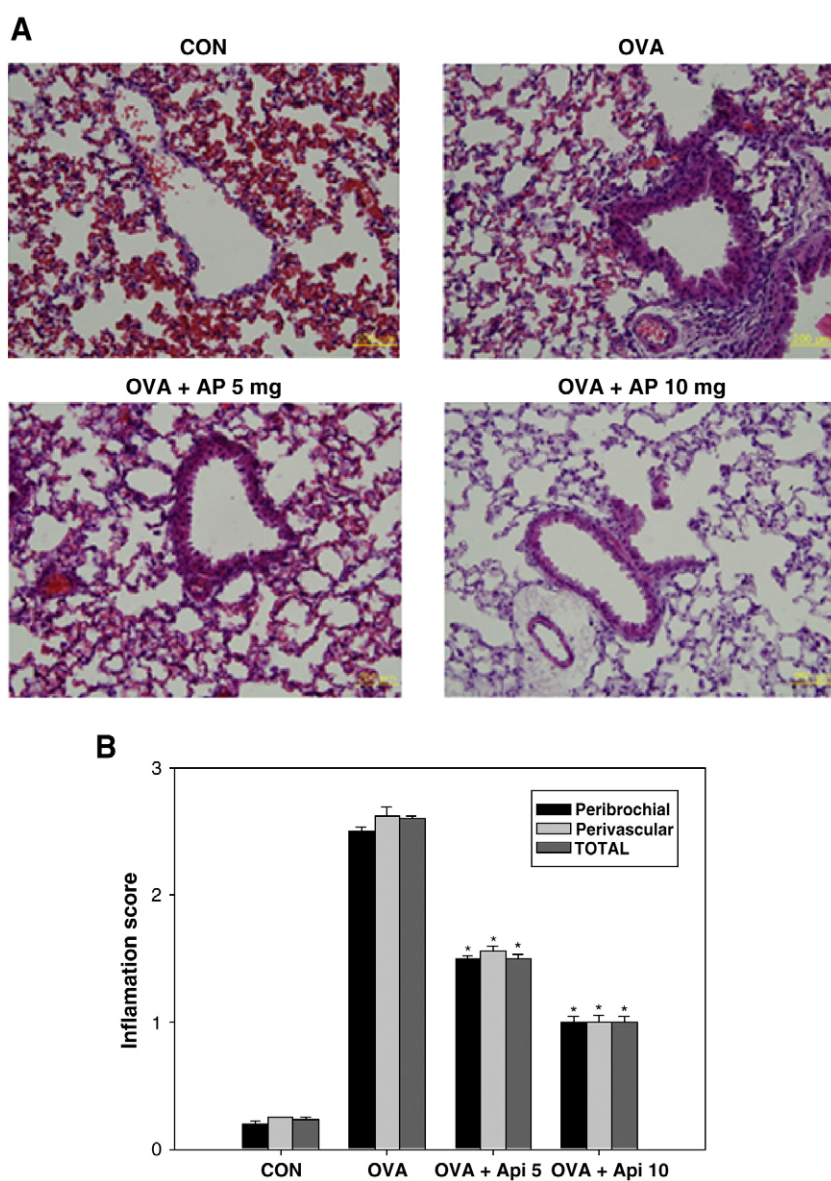


Fig. 3. Apigenin inhibits lung inflammation and inflammatory cells infiltration. Mice were sensitized and challenged as described in Materials and methods. Sections were obtained from the lungs of mice receiving the control (CON), OVA plus apigenin 5 mg/kg/day (OVA + AP 5 mg), OVA plus apigenin 10 mg/kg/day (OVA + AP 10 mg) and OVA (OVA). Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining ($\times 200$). (B) Bars represent mean $\pm$ SEM from 6 independent experiments. * $P < 0.05$ vs OVA.

where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

2.7. Measurement of Th1/Th2 cytokines and IgE levels

Levels of IL-4 and IL-5 were quantified in the supernatants of BAL fluids by enzyme immunoassays performed according to the manufacturer's protocol of the manufacturer (IL-4, IL-5; R&D Systems, Inc., Minneapolis, MN). IgE in sera by enzyme immunoassays according to the manufacturer's protocol (R&D Systems; Minneapolis, MN).

2.8. Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 24 h after the last challenge in an unrestrained conscious state, as described previously [32]. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5 to 50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Enhanced pause (Penh), calculated as $(\text{expiratory time/relaxation time} - 1) \times (\text{peak expiratory flow/peak inspiratory flow})$, according to protocol of the manufacturers, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Results were expressed as the percent increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values were averaged for 3 min after each nebulization and evaluated.

2.9. Zymography

Gelatin zymography was used to assess the activity of MMP-9. The supernatant was diluted three times with nonreducing loading buffer (400 mmol/L Tris-HCl, 5% sodium dodecyl sulfate, 20% glycerol, 0.006% bromophenol blue). 15 μ L of equal amounts of the sample were mixed with the loading buffer, and proteins were separated by polyacrylamide gel electrophoresis (0.75 mm; constant current 20 mA) consisting of an 8% sodium dodecyl sulfate solution with 1% gelatin (Bio-Rad). The gels were incubated in a renaturing buffer (2.5% Triton X-100 buffer) for 30 min to remove the sodium dodecyl sulfate. After rinsing, the gels were incubated (37 °C for 20 h) in an enzyme activation buffer (50 mmol/L Tris-HCl [pH 7.3], 200 mmol/L NaCl, and 0.02% Tween 20). The gels were then stained with Coomassie brilliant blue R250 stain and destained (5% methanol, 7% acetic acid in PBS solution), and the gelatinolytic activity was detected as clear bands. The molecular weight of the gelatinolytic bands was estimated relative to the prestained molecular-weight markers (see BluePlus2 Prestained Standard; Invitrogen, Carlsbad, CA).

2.10. Densitometric analysis and statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean $\pm$ S.E. M. ANOVA was used to compare experimental groups to control values while comparisons between multiple groups were performed using Tukey's Multiple Comparison test. Statistical significance was indicated by a *P* value less than 0.05. $P^{***} < 0.001$, $P^{**} < 0.05$, $P^* < 0.01$.

3. Results

3.1. Apigenin reduces inflammatory cells in BAL fluids

Numbers of total cells, eosinophils, lymphocytes, and macrophages in BAL fluids were increased significantly at 24 h after OVA inhalation

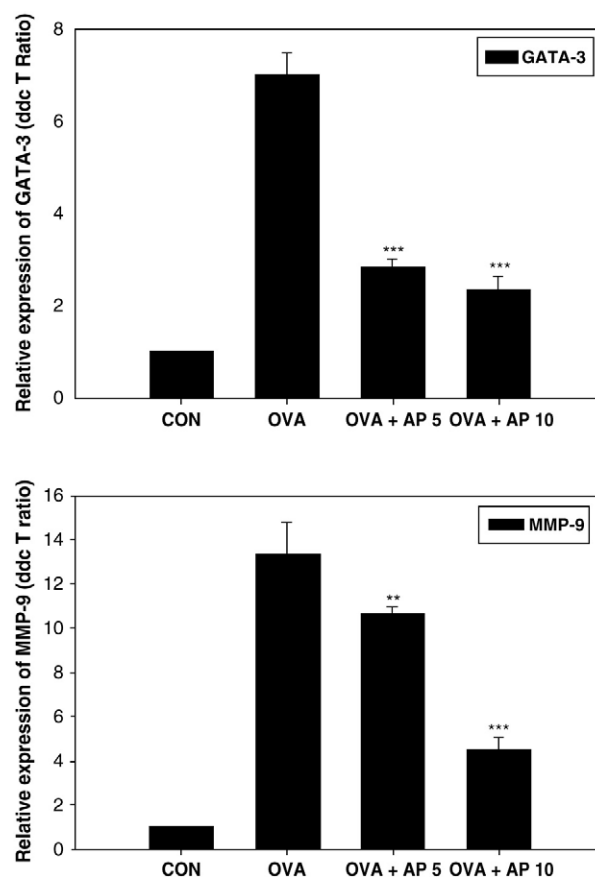


Fig. 4. Effect of apigenin on MMP-9, GATA3 mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 24 h after the last challenge in saline-inhaled mice administered saline (CON), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered apigenin 5 mg/kg/day (OVA + AP 5) and OVA-inhaled mice administered apigenin 10 mg/kg/day (OVA + AP 10). Data represent means $\pm$ S.E.M. from 5 independent experiments. $***P < 0.001$, $**P < 0.01$.

compared with the numbers after saline inhalation (Fig. 1). The increased numbers of eosinophils were significantly reduced by the administration of apigenin.

3.2. Apigenin decreases the level of eosinophils in BAL fluids

The levels of eosinophils in BAL fluids were significantly increased at 24 h after OVA inhalation compared with the levels after saline inhalation (Fig. 2). The increased levels of these cells were significantly reduced by about 59.76% (AP 5 mg) and 78.77% (AP 10 mg) upon administration of apigenin.

3.3. Apigenin ameliorates pathological changes of OVA-induced asthma

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells, including eosinophils infiltrated around the bronchioles as compared with the control (Fig. 3). Mice treated with apigenin showed marked reductions in the infiltration of inflammatory cells in the lung tissues. Total lung inflammations were increased significantly at 24 h after OVA inhalation compared with the scores after saline inhalation. The total lung inflammations were significantly reduced by the administration of apigenin. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after OVA inhalation compared with scores after saline inhalation (Fig. 3B). The increased peribronchial, perivascular, and total lung inflammation after OVA inhalation were significantly decreased by the administration of apigenin.

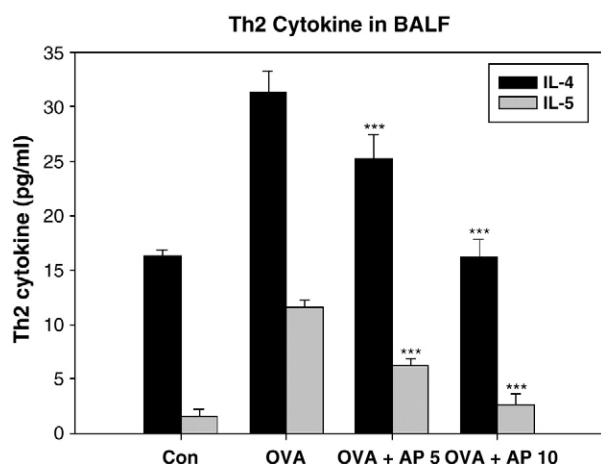


Fig. 5. The effect of apigenin treatment on Th2 cytokine. OVA-sensitized mice were treated as described in Materials and methods. BAL fluid was performed 4 h after the last airway challenge as described by the manufacturer. IL-4, IL-5 cytokine levels in the BAL fluids were measured by ELISA Kit. Data represent mean $\pm$ SEM from 6 independent experiments *** P <0.001 vs. OVA, ** P <0.01 vs. OVA

These results suggest that apigenin inhibits OVA-induced inflammation in the lungs, including the influx of eosinophils.

3.4. Apigenin decreases MMP-9 and GATA-3 mRNA levels in lung tissues of OVA-sensitized and -challenged mice

RT-PCR analysis revealed that expression of MMP-9, T-bet, and GATA3 mRNA in lung tissues was significantly increased at 24 h after OVA inhalation compared with the levels after saline inhalation (Fig. 4). The increased mRNA expression of MMP-9, GATA-3 was decreased by the administration of apigenin. Conversely, apigenin treatment resulted in a slightly increase in Th1 transcription factor T-bet.

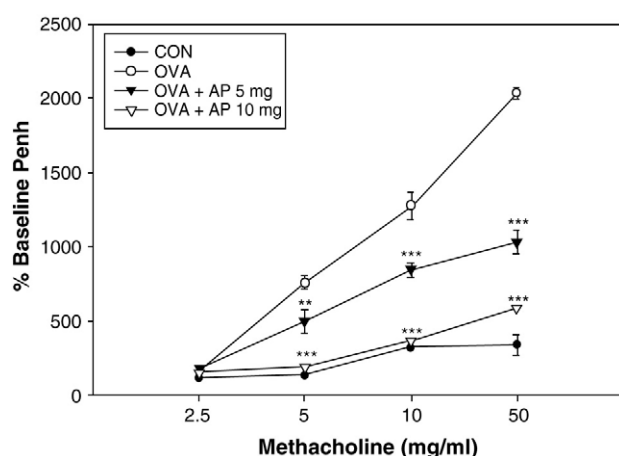


Fig. 6. The effect of apigenin on airway responsiveness in OVA-sensitized and OVA-challenged mice. Airway responsiveness was measured at 24 h after the last challenge in saline-inhaled mice administered PBS (PBS), OVA-sensitized mice administered saline (OVA) and OVA-sensitized mice administered apigenin (OVA + AP 5 mg/kg/day, 10 mg/kg/day). Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with PBS, then with increasing doses (2.5 to 50 mg/ml) of methacholine for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization during which Penh values were determined. Data represent means $\pm$ S.E.M. from 5 independent experiments. *** P <0.001, ** P <0.01.

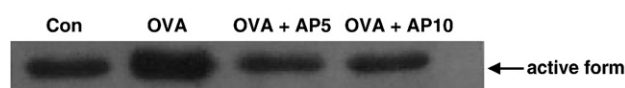


Fig. 7. The effect of apigenin on MMP-9 production in lung tissues of OVA-sensitized and OVA-challenged. Sampling was performed at 48 h after challenge with the PBS (Con), OVA (OVA), and OVA plus apigenin 5 or 10 mg/kg/day (OVA + AP 5 or 10 mg) and analyzed by zymography. All of the groups of the experiment showed active form of MMP-9.

3.5. Apigenin reduces levels of Th2 cytokine (IL-4, IL-5) in lung tissues of OVA-sensitized and -challenged mice

BAL fluids were obtained 4 h after the last airway challenge. The levels of IL-4 and IL-5 in the BAL fluids were significantly increased by airway challenge with OVA when compared with that with that of the control. The administration of apigenin reduced the concentration of IL-4, IL-5 secretion (Fig. 5). The levels of Th2 cytokines, IL-4 and IL-5, were found to be increased in OVA-sensitized and -challenged mice, but that of the Th1 cytokine, IFN- γ (Data not shown) was not changed as compared to saline-sensitized and -challenged mice. These results indicate that apigenin treatment inhibits Th2 cytokine levels in the BAL fluids.

3.6. Apigenin decreases airway hyper-responsiveness

Airway responsiveness was assessed as the percent increase of Penh in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Penh was shifted to the left compared with that of control mice (Fig. 6). In addition, the percent Penh produced by methacholine administration (at doses from 2.5 mg/ml to 50 mg/ml) increased significantly in the OVA-sensitized and -challenged mice compared with the controls. OVA-sensitized and -challenged mice treated with apigenin showed a dose-response curve of percent Penh that shifted to the right compared with that of untreated mice. The shift was dose-dependent. These results indicate that apigenin treatment reduces OVA-induced airway hyper-responsiveness.

3.7. Apigenin inhibits MMP-9 gelatinolytic activity

OVA-challenge induced a marked induction of matrix metalloproteinase-9 activity in BAL fluids in comparison to control mice (Fig. 8). When the administration apigenin 5 mg/kg/day, 10 mg/kg/day this increased MMP-9 gelatinolytic activity was significantly inhibited (Fig. 7). These results indicate that apigenin regulates MMP-9 gelatinolytic activity in lung tissues.

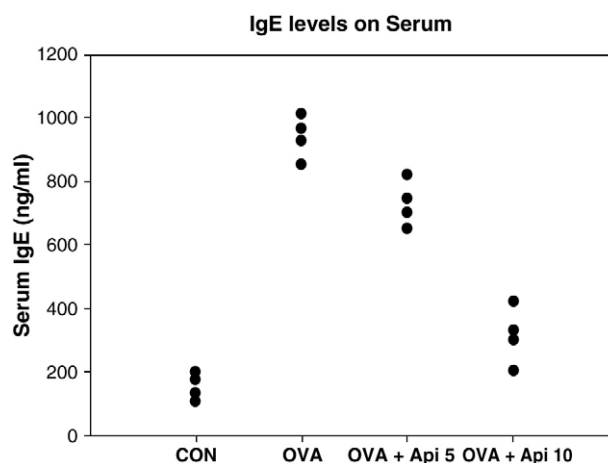


Fig. 8. The effect of apigenin on IgE levels in serum of OVA-sensitized and OVA-challenged. Blood was collected by cardiac puncture to measure serum IgE. All experiments were analyzed using ELISA (n =4). Values shown are means $\pm$ SEM * P <0.05.

3.8. Apigenin decreases IgE levels in serum

Because Th2 cytokines promote airway inflammation in asthma through increased IgE levels, we investigated the expression of IgE in serum. IgE favors to Th2 in airway inflammation, we measured how far apigenin modulated the levels of serum IgE in OVA mice. As shown in Fig. 8, the levels of serum IgE were found to be significantly increased in OVA mice compared with those of PBS mice (CON). However, administration of apigenin significantly decreased the levels of serum IgE. These data indicate that apigenin modulates Th1/Th2 levels in an OVA-induced asthma model (Fig. 8).

4. Discussion

This study is the first to provide experimental evidence demonstrating that apigenin inhibits OVA-induced airway inflammation in a murine model of asthma. Apigenin profoundly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation. We also demonstrated that apigenin regulates the Th1/Th2 balance, which can be mediated by the level of GATA3 levels.

Based on animal studies, the immunological processes involved in airway inflammation of asthma are characterized by the proliferation and activation of T cells of the subtype Th2 CD4⁺. Ultimately, mediators lead to degranulation of effector/proinflammatory cells with the release of mediators and oxidants, which lead to the injury and inflammation noted in asthma. OVA-induced asthma has been recognized as a disease that results from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen [1,3]. In our experiment, we demonstrate that OVA-induced asthma increased levels of eosinophil infiltration, eosinophils peroxidase activity, eotaxin, and the thicknesses of bronchial wall and area of smooth muscle. But they were significantly decreased by administration of apigenin.

It was recently demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes [21]. Additionally, an MMP inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma [22]. In our murine model of asthma, intraperitoneally injected mice with 5 mg/kg/day, 10 mg/kg/day apigenin to evaluate the effect of apigenin on the expression of MMP-9, T-bet and GATA3 mRNA. In this study, apigenin reduced levels of MMP-9, GATA3 in lung tissues of apigenin treated mice.

T-bet, a member of the T-box family of transcription factors, is a master determinant of Th1 lineage [36,37]. Indeed, T-bet deficient mice exhibit a profound lack of Th1 immune responses [38] and ectopic expression of T-bet in murine Th2 cells directs activation of IFN- γ , as well as the upregulation of IL-12R β [38,39]. Th1 cytokines are known to inhibit allergic responses [39]. Six members (GATA-1 to GATA-6) of this family have been identified in avians, with homologues in mammals and avians. Based on their expression profile and structure, the GATA proteins may be classified as haematopoietic (GATA-1 to GATA-3) [40,41] or nonhaematopoietic (GATA-4 to GATA-6). Naive CD4⁺ T cells express low levels of GATA-3 mRNA. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is downregulated in cells differentiating along the Th1 pathway [35].

Our data demonstrate that apigenin reduces the increased levels of GATA3 mRNA in OVA-sensitized and -challenged mice (Fig. 5). Also, it suggest that apigenin treatment is a novel, selective way to simultaneously suppress GATA-3 in asthmatic reactions in vivo. Also, we examined Th1/Th2 cytokine production in BAL fluid cells. Apigenin reduces the increased levels of IL-4, Th2 cytokine production in OVA-sensitized and -challenged mice. Taken together these suggest that GATA-3 might be a candidate gene for asthma, and a regulator of Th1/Th2 balance.

In conclusion, our results strongly indicate that apigenin reduces allergic airway inflammation due to the alteration of Th1/Th2 polarization via the suppression of GATA-3. Therefore our data suggests that apigenin might offer a new therapeutic approach to allergic airway diseases.

Acknowledgements

This work partly was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-511-E00006) and the Korea Science and Engineering Foundation through National Research Laboratory Program Grant (ROA-2005-000-10008-0)

References

- [1] Corrigan CJ, Kay AB. T cells and eosinophils in the pathogenesis of asthma. *Immunol Today* 1992;13:501–7.
- [2] Karp M, Oker-Blom C. A streptavidin–luciferase fusion protein: comparisons and applications. *Biomol Eng* 1999;16:101–4.
- [3] Punnonen J, Aversa G, Cocks BG, de Vries JE. Role of interleukin-4 and interleukin-13 in synthesis of IgE and expression of CD23 by human B cells. *Allergy* 1994;49:576–86.
- [4] Renz H, Bradley K, Saloga J, Loader J, Larsen GL, Gelfand EW. T cells expressing specific V beta elements regulate immunoglobulin E production and airways responsiveness in vivo. *J Exp Med* 1993;177:1175–80.
- [5] Saito H, Hatake K, Dvorak AM, Leiferman KM, Donnenberg AD, Arai N, et al. Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc Natl Acad Sci U S A* 1988;85:2288–92.
- [6] Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J Exp Med* 1993;177:573–6.
- [7] Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwaru D, Takatsu K, et al. Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice. *Am J Respir Cell Mol Biol* 2004;31:62–8.
- [8] Sur S, Lam J, Bouchard P, Sigounas A, Holbert D, Metzger WJ. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *J Immunol* 1996;157:4173–80.
- [9] Mapp CE, Boschetto P, Zocca E, Milani GF, Pivrotto F, Tegazzin V, et al. Pathogenesis of late asthmatic reactions induced by exposure to isocyanates. *Bull Eur Physiopathol Respir* 1987;23:583–6.
- [10] Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033–9.
- [11] Mapp CE, Boschetto P, Dal Vecchio L, Maestrelli P, Fabbri LM. Occupational asthma due to isocyanates. *Eur Respir J* 1988;1:273–9.
- [12] Busse WW, Calhoun WF, Sedgwick JD. Mechanism of airway inflammation in asthma. *Am Rev Respir Dis* 1993;147:S20–24.
- [13] Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991;87:893–910.
- [14] Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* 1997;378:151–60.
- [15] Murphy G, Docherty AJ. The matrix metalloproteinases and their inhibitors. *Am J Respir Cell Mol Biol* 1992;7:120–5.
- [16] Yao PM, Maitre B, Delacourt C, Buhler JM, Harf A, Lafuma C. Divergent regulation of 92-kDa gelatinase and TIMP-1 by HBECS in response to IL-1 β and TNF- α . *Am J Physiol* 1997;273:L866–874.
- [17] Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immunol* 1995;154:4379–89.
- [18] Mautino G, Oliver N, Chanez P, Bousquet J, Capony F. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 1997;17:583–91.
- [19] Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996;14:288–95.
- [20] Stahle-Backdahl M, Inoue M, Guidice GJ, Parks WC. 92-kD gelatinase is produced by eosinophils at the site of blister formation in bullous pemphigoid and cleaves the extracellular domain of recombinant 180-kD bullous pemphigoid autoantigen. *J Clin Invest* 1994;93:2022–30.
- [21] Matrisian LM. Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet* 1990;6:121–5.
- [22] Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997;17:519–28.
- [23] Yoon MS, Lee JS, Choi BM, Jeong YI, Lee CM, Park JH, et al. Apigenin inhibits immunostimulatory function of dendritic cells: Implication of immunotherapeutic adjuvant. *Mol Pharmacol* 2006;70:1033–44.
- [24] Kim GY, Cho H, Ahn SC, Oh YH, Lee CM, Park YM. Resveratrol inhibits phenotypic and functional maturation of murine bone marrow-derived dendritic cells. *Int Immunopharmacol* 2004;4:245–53.
- [25] Chen CC, Chow MP, Huang WC, Lin YC, Chang YJ. Flavonoids inhibit tumor necrosis factor- α -induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor- κ B: structure–activity relationships. *Mol Pharmacol* 2004;66:683–93.

- [26] Duthie G, Crozier A. Plant-derived phenolic antioxidants. *Curr Opin Clin Nutr Metab Care* 2000;3:447–51.
- [27] Kim HP, Mani I, Iversen L, Ziboh VA. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins Leukot Essent Fatty Acids* 1998;58:17–24.
- [28] Hastak K, Gupta S, Ahmad N, Agarwal MK, Agarwal ML, Mukhtar H. Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. *Oncogene* 2003;22:4851–9.
- [29] Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol Carcinog* 2000;28:102–10.
- [30] Hougee S, Sanders A, Faber J, Graus YM, van den Berg WB, Garssen J, et al. Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. *Biochem Pharmacol* 2005;69: 241–8.
- [31] Bian T, Yin KS, Jin SX, Zhang XL, Zhou JY, Ma XQ, et al. Treatment of allergic airway inflammation and hyperresponsiveness by imiquimod modulating transcription factors T-bet and GATA-3. *Chin Med J (Engl)* 2006;119:640–8.
- [32] Montefort S, Holgate ST. Adhesion molecules and their role in inflammation. *Respir Med* 1991;85:91–9.
- [33] Nakamura Y, Ghaffar O, Olivenstein R, Taha RA, Soussi-Gounni A, Zhang DH, et al. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *J Allergy Clin Immunol* 1999;103:215–22.
- [34] Parronchi P, De Carli M, Manetti R, Simonelli C, Sampognaro S, Piccinni MP, et al. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. *J Immunol* 1992;149:2977–83.
- [35] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655–69.
- [36] Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 2002;3: 549–57.
- [37] Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002;295:338–42.
- [38] Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001;292:1907–10.
- [39] Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995;182:1527–36.
- [40] Li XM, Chopra RK, Chou TY, Schofield BH, Wills-Karp M, Huang SK. Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J Immunol* 1996;157:3216–9.
- [41] Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 1998;9:745–55.