Journal of Life Science **2009** Vol. 19. No. 6. 735~743 ©JLS / ISSN 1225-9918

Naringin Protects Ovalbumin-induced Asthma through the Down-regulation of MMP-9 Activity and GATA-3 Gene

Chang-Min Lee, Jeong Hyun Chang[1], In Duk Jung, Young-Il Jeong, Noh Kyung Tae, Hee-ju Park[2], Jong-Suk Kim[3], Yong Kyoo Shin[4], Sung Nam Park and Yeong-Min Park*

Department of Microbiology and Immunology & National Research Laboratory of Dendritic Cell Differentiation & Regulation, and Medical Research Institute, Pusan National University School of Medicine, Busan 602-739, South Korea
[1]*Department of clinical laboratory Science, College of Health & Therapy, Daegu Haany University, Gyeong San 712-715, South Korea*
[2]*Department of Pediatrics, Pusan National University School of Medicine, Busan 602-739, South Korea*
[3]*Department of Biochemistry, Chonbuk National University Medical School, Jeonju 561-180, South Korea*
[4]*Department of Pharmacology, Chungang University College of Medicine, Seoul 156-756, South Korea.*

Received April 2, 2009 / Accepted June 22, 2009

The common word flavonoids is often used to classify a family of natural compounds, highly abundant in all higher plants, that have received significant therapeutic interest in recent years. Naringin is associated with a reduced risk of heart disease, neurodegenerative disease, cancer and other chronic diseases; however the molecular basis of this effect remains to be elucidated. Thus we attempted to elucidate the anti-allergic effect of Naringin in ovalbumin (OVA)-induced asthma model mice. The OVA-induced mice showed allergic reactions in the airways. These 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 Naringin before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions. Accordingly, this study may provide evidence that Naringin 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 Naringin in terms of its effects on asthma in mice.

Key words : Naringin, asthma, GATA-3, AHR, IgE

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 [7,15,32-34]. 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 [13,39]. Thus, interventions that inhibit Th2 cytokines by enhancing Th1 cytokine production, may be useful in the treatment of allergic asthma [36]. OVA-induced asthma is characterized by AHR and inflammation of the airways [20]. This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues [4,21]. These cellular infiltrates release various chemical mediators that can cause AHR [5,16]. 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 [27]. MMPs are produced by not only structural cells, [26,40] but also inflammatory cells [18,23]. They are secreted as latent forms followed by proteolytic processing to active forms [8]. 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* ma-

***Corresponding author**
Tel : +82-51-240-7557, Fax : +82-51-243-2259
E-mail : immunpym@pusan.ac.kr

trix substrate specificities [35], and appear to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair [22,29]. Recruitment of 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 [17,41].

The flavonoids comprise a family of common phenolic plant pigments that have been identified as dietary anticarcinogens and antioxidants [6]. We reported in a previous study that a variety of phytochemicals exhibit pro-found immunoregulatory activity, particularly in the DC. Naringin, 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 [9]. Naringin has demonstrated anti-inflammatory, anticarcinogenic, and free radical-scavenging activities in a variety of in vitro systems [2]. In a recent study, investigators identified Naringin as a potent inhibitor of the nuclear transcription factor nuclear factor-κB (NF-κB), which may perform a pivotal function in the regulation of cell growth, apoptosis, and the regulation of the cell cycle [12]. Studies using human leukemia cells as well as carcinoma cells in the breast, colon, and elsewhere have revealed that Naringin inhibits cell growth via the induction of cell cycle arrest and apoptosis [14]. 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 up-regulation in vivo, and inhibits IL-1-induced prostaglandin synthesis and TNF--induced IL-6 and IL-8 production [11]. Moreover, it actively inhibits IkB kinase activity, IkB degradation, NF-kB DNA protein-binding activity, NF-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 [3,24,28,31,37].

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

Materials and Methods

Animals and experimental protocol

Female BALB/c mice, 6-8weeks 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 μg of OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) on day 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 hr 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.

Administration of Naringin

Mice were injected i.p with 3 or 6 mg/kg/day in 200 μl of Naringin (Sigma, St Louis, Mo) each day from days 18-20 on consecutive days.

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.

Histopathology

At 48h after the last challenge, lungs were removed from

the mice after they had been sacrified. 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).

RNA preparation and real-time 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 15s 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±DcT, where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

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). Levels of IgE were quantified in the supernatants of whole blood by enzyme immunoassays according to the manufacturer's protocol (R&D Systems; Minneapolis, MN).

Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 24 hr after the last challenge in an unrestrained conscious state, as described previously. [24] 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 (expiratory time/relaxation time-1) × (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.

Western blot analysis

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer plus a protease inhibitor cocktail (Sigma, St Louis, Mo) to obtain extracts of lung proteins. A western blot analysis was performed as described previously [40]. The samples were loaded to 10% SDS-PAGE gels and were separated at 120 V for 90 minutes. The blots were incubated with an anti MMP-9 antibody, anti GATA-3 diluted at a ratio of 1:800, (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were stripped and reblotted with anti-actin antibody (Sigma) to verify the equal loading of protein in each lane.

Densitometric analysis and statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean±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.

Results

Naringin reduces inflammatory cells in BAL fluids

Numbers of total cells, eosinophils, lymphocytes, and macrophages in BAL fluids were increased significantly at 24 hr after OVA inhalation compared with the numbers after saline inhalation (Fig. 1). The increased numbers of eosinophils were significantly reduced by the administration of Naringin.

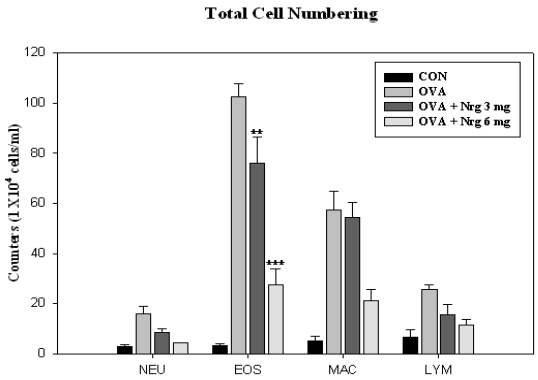

Fig. 1. Effect of Naringin on total and differential cellular components of BAL fluids of OVA-sensitized and OVA-challenged mice. Mice were treated with the PBS (CON), OVA plus Naringin 3 mg/kg/day, 6 mg/kg (OVA + Nrg 3 mg, OVA + Nrg 6 mg) and OVA (OVA), respectively, as described in Materials and Methods. 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.

Naringin 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. 2). Mice treated with Naringin showed marked reductions in the infiltration of inflammatory cells in the lung tissues. Total lung inflammations were increased significantly at 24 hr after OVA inhalation compared with the scores after saline inhalation. The total lung inflammations were significantly reduced by the administration of Naringin. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 hr after OVA inhalation compared with scores after saline inhalation (Fig. 2). The increased peribronchial, perivascular, and total lung inflammation after OVA inhalation were significantly decreased by the administration of Naringin. These results suggest that Naringin inhibits OVA-induced inflammation in the lungs, including the influx of eosinophils.

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

Real-time PCR analysis revealed that expression of

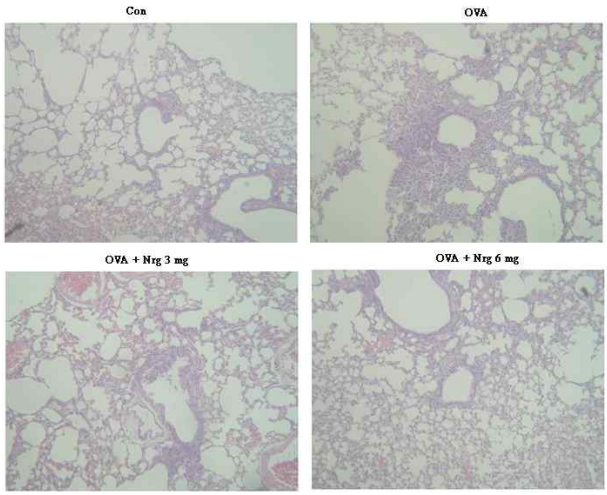

Fig. 2. Naringin inhibits lung inflammation and inflammatory cells infiltration. Mice were sensitized and challenged as described in Materials and Methods. Sections were obtained from the lungs of mice receiving the control (CON), OVA plus Naringin 3 mg/kg/day (OVA + Nrg 3 mg), OVA plus Naringin 6 mg/kg/day (OVA + Nrg 6 mg) and OVA (OVA). Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining (x200).

MMP-9, T-bet, and GATA3 mRNA in lung tissues was significantly increased at 24 hr after OVA inhalation compared with the levels after saline inhalation (Fig. 3). The increased mRNA expression of MMP-9, GATA-3 was decreased by the administration of Naringin.

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

BAL fluids were obtained 4 hr 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 Naringin reduced the concentration of IL-4, IL-5 secretion (Fig. 4). 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 Naringin treatment inhibits Th2 cytokine levels in the BAL fluids.

Naringin decreases airway hyper-responsiveness

Airway responsiveness was assessed as the percent increase of Penh in response to increasing doses of methacholine.

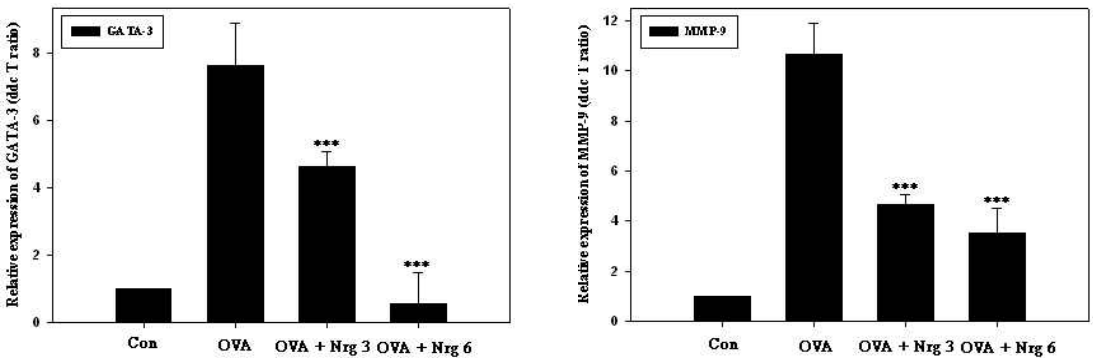

Fig. 3. Effect of Naringin on MMP-9, GATA3 mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 24 hr after the last challenge in saline-inhaled mice administered saline (CON), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered Naringin 3 mg/kg/day (OVA+Nrg 3) and OVA inhaled mice administered Naringin 6 mg/kg/day (OVA+Nrg 6). Data represent means±S.E.M. from 5 independent experiments. ***P<0.001.

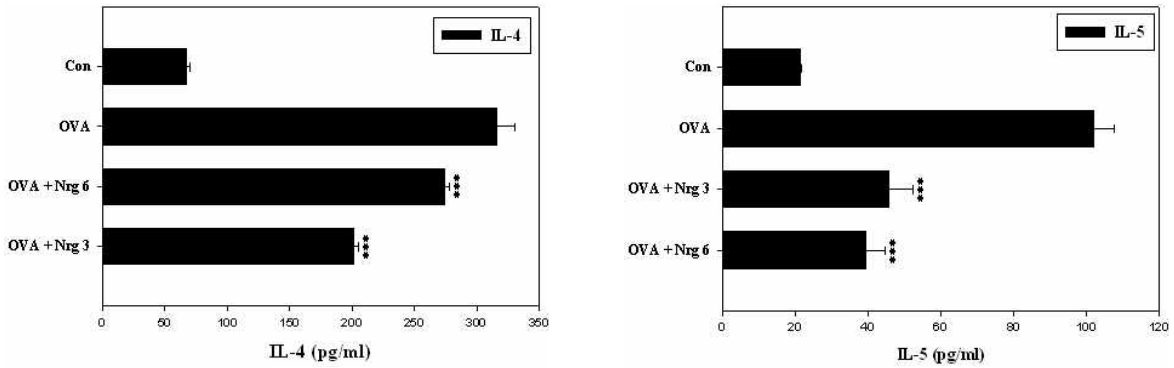

Fig. 4. The effect of Naringin treatment on Th2 cytokine. OVA- sensitized mice were treated as described Material and Method. BAL fluid was performed 4 hrs after the last airway challenge as described by the manufacturer. IL-4, IL-5 cytokine levels in the BAL fluids were measured by ELISA Kit. Data represent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

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. 5). 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 Naringin 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 Naringin treatment reduces OVA-induced airway hyperresponsiveness.

Naringin inhibits MMP-9, GATA-3 production in the lung tissue

OVA-challenge induced a marked induction of matrix metalloproteinase-9, GATA-3 activity in BAL fluids in comparison to control mice (Fig. 6). When the administration Naringin 3 mg/kg/day, 6 mg/kg/day this increased MMP-9, GATA-3 production was significantly inhibited (Fig. 6).

Naringin 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 Naringin modulated the levels of serum IgE in OVA mice. As shown in Fig. 9, the levels of serum IgE were found to be significantly increased in OVA mice compared with those of PBS mice (CON). However, administration of Naringin significantly decreased the levels of serum IgE. These data indicate that Naringin modulates Th1/Th2 levels in an OVA-induced asthma model (Fig. 7).

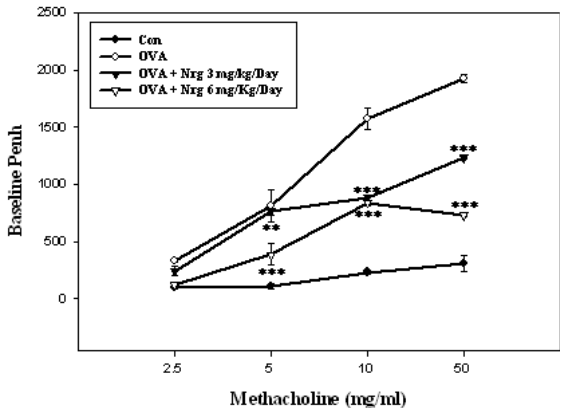

Fig. 5. The effect of Naringin on airway responsiveness in OVA-sensitized and OVA-challenged mice. Airway responsiveness was measured at 24 hr after the last challenge in saline-inhaled mice administered PBS (Con), OVA-sensitized mice administered saline (OVA) and OVA-sensitized mice administered Naringin (OVA+Nrg 3 mg/kg/day, 6 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±S.E.M. from 5 independent experiments. ***P<0.001, **P<0.01.

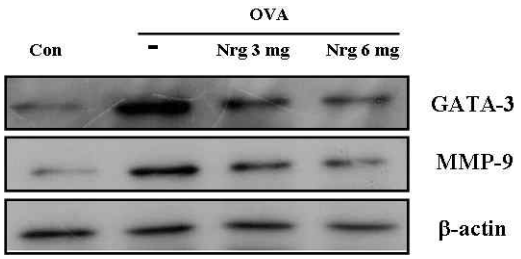

Fig. 6. The effect of Naringin on MMP-9 production in lung tissues of OVA-sensitized and OVA-challenged. Sampling was performed at 48 hrs after challenge with the PBS (Con), OVA (OVA), and OVA plus Naringin 3 or 6 mg/kg/day (OVA+Nrg 3 or 6) and analyzed by western blotting. All of the groups of the experiment showed MMP-9 production, but the OVA plus Naringin group only showed the active form of MMP-9.

Discussion

This study is the first to provide experimental evidence demonstrating that Naringin inhibits OVA-induced airway inflammation in a murine model of asthma. Naringin profoundly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation. We also

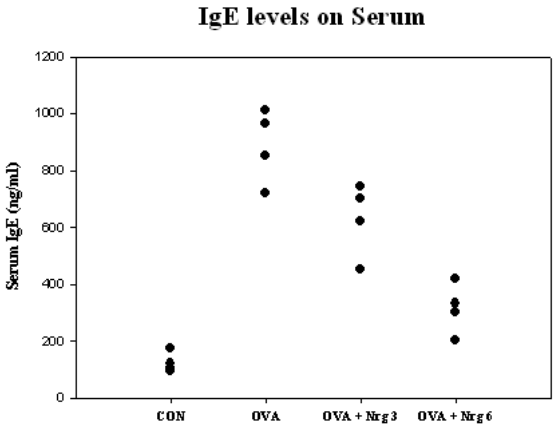

Fig. 7. The effect of Naringin on IgE levels in serum of OVA-sensitized and OVA-challenged. Blood was collected by cardiac puncture to measure serum IgE. All experiments were analyzed using ELISA (n=4).

demonstrated that Naringin 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 [7,32]. In our experiment, we demonstrate that OVA-induced asthma increased levels of eosinophil infilteration, eosinophils peroxidase activity, eotaxin, and the thicknesses of bronchial wall and area of smooth muscle. But they were significantly decreased by administration of Naringin.

It was recently demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes [22]. 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 [29]. In our murine model of asthma, intraperitoneally injected mice with 3 mg/kg/day, 6 mg/kg/day Naringin to evaluate the effect of Naringin on the expression of MMP-9 and GATA3 mRNA. In this study, Naringin reduced levels of MMP-9, GATA3 in lung tissues of Naringin treated mice.

T-bet, a member of the T-box family of transcription fac-

tors, is a master determinant of Th1 lineage [1,38]. Indeed, T-bet deficient mice exhibit a profound lack of Th1 immune responses [25] and ectopic expression of T-bet in murine Th2 cells directs activation of IFN-γ, as well as the upregulation of IL-12Rβ [10,25]. Th1 cytokines are known to inhibit allergic responses [25]. 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) [19,30] 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 [37].

Our data demonstrate that Naringin reduces the increased levels of GATA3 mRNA in OVA-sensitized and -challenged mice (Fig. 5). Also, it suggests that Naringin 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. Naringin 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 Naringin reduces allergic airway inflammation due to the alteration of Th1/Th2 polarization via the suppression of GATA-3. Therefore our data suggests that Naringin might offer a new therapeutic approach to allergic airway diseases.

Acknowledgement

This work was supported for two years by Pusan National University Research Grant.

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초록 : Naringin에 의한 천식치료 효과연구

이창민 · 장정현[1] · 정인덕 · 정영일 · 노경태 · 박희주[2] · 김종석[3] · 신용규[4] · 박성남 · 박영민*

(부산대학교 의학전문대학원 미생물학 및 면역학교실, [1]대구한의대학교 임상병리학과, [2]부산대학교 소아과학교실, [3]전북대학교 생화학교실, [4]중앙대학교 해부학교실)

Naringin은 레몬, 오렌지에서 발견되는 flavonoid계열에 속하는 물질로 여러 식물과 과일에 다량 함유되어 있다. 항암, 항산화 작용을 하는 것으로 알려져 있는 Naringin을 ovalbumin (OVA)으로 유도한 천식(asthma) 생쥐모델을 이용하여 치료효과를 알아 보았다. 기관지 폐포 세척액을 회수하여 백혈구의 수적 변화, 제2형 협조T세포(Th2 cell)가 생산하는 IL-4, IL-5의 생산에 미치는 영향과 폐조직에서 matrix metalloproteinase (MMP)-9 활성을 측정하였다. 또한, 최근에 Th1/Th2 전사인자로서 GATA-3가 밝혀졌는데 이번 실험에서 Naringin이 ovalbumin (OVA)으로 유도한 천식(asthma) 생쥐모델에서 Th1, Th2 싸이토카인과 유전자 발현을 조절할 수 있는가에 대하여 알아보았다 그 결과 기관지 폐포 세척액에서 OVA로 감작하여 천식을 유도한 실험군에서는 호산구의 현저한 증가, Th2 형 싸이토카인(IL-4, IL-5)의 증가가 관찰되었다. 그러나 Naringin을 투여한 그룹에서는 OVA의 감작에 의하여 증가한 각종 염증성 지표들이 감소하거나 정상화 되었다. 또한 OVA에 의하여 증가된 기도저항성이 Naringin 투여에 의하여 감소하였으며 폐조직의 염증성 소견도 뚜렷하게 감소되었다. 이와 같은 연구 결과는 Naringin이 천식의 치료에 유용하게 쓰일 수 있음을 시사해준다.