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OVA로 유도된 생쥐 모델에서 EGCG의 천식 예방 효과

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Epigallocatechin-3-gallate, constituent of green tea, suppresses ovalbumin-induced inflammation in a murine model of asthma

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Abstract

Epigallocatechin-3-gallate (EGCG), a major form of tea epicatechin, has a variety of biological activities. It has recently been studied intensively as an antiallergic and anti-inflammatory agent. Thus we elucidated the anti-allergic effect of EGCG in the ovalbumin (OVA)-induced asthma model mice by examining the expression of matrix metalloproteinase (MMP)-9. Also airway reactivity to methacholine challenge and reactive oxygen species (ROS) generation in BAL fluid. mesured by the expression of iNOS mRNA in lung tissue and tumor necrosis factor (TNF) in BAL fluids. The OVA challenged mice showed allergic airway reactions, such as inflammatory chemokine (CCL5,CXCL10) in the airway and lung tissues. However mice administrated with EGCG had significantly reduced CCL5 and MMP-9 expression in lung tissues as well as TNF-α and IL-5 production in BAL fluid. these results suggest that EGCG regulates OVA-induced asthma model by reducing inflammatory chemokine expression and possibly by suppressing MMP-9.

Key words : ovalbumin, EGCG, MMP-9, CCL5, asthma

INTRODUCTION

Asthma is a chronic inflammatory lung disease characterized by airway hyper- responsiveness (AHR) to allergens, airway edema, and increased mucus secretion. A propensity to allergic responses, atopy, are associated with the development of asthma.[1] Oxidative stress is caused by a large variety of free radicals known as reactive oxygen species (ROS), [2-4] and many evidences have suggested that ROS play an essential role in the pathogenesis of airway inflammation. The inflammatory cells recruited to the asthmatic airway are able to produce ROS. Evidence of increased oxidative stress in asthma is also provided by the finding of defective endogenous antioxidant capacity in asthmatic patients. [5] For these reasons, antioxidant treatment of asthma has long been a promising subject of therapeutic strategy.

Ovalbumin (OVA)-induced asthma is characterized by AHR and airway inflammation .[6] This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues.

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[6-7] These cellular infiltrates release various chemical mediators which can cause AHR. [8-10] Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as an critical event in the development and prolongation of airway inflammation. Inflammatory cells have to cross the basement membranes and move through connective tissues 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.[11] MMPs are produced by not only structural cells,[12-13] but also inflammatory cells.[14-17] They are secreted as latent forms followed by proteolytic processing to active forms.[11] 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, [18] and appear to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair. [19-20] 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 important roles in the induction of airway inflammation. RANTES is a CCL5 chemokine that can induce chemotaxis and activation of eosinophils. Higher RANTES levels have been demonstrated in bronchoalveolar lavage fluid of patients with active asthma.

Tea (Camellia sinensis L.) is one of the most widely consumed beverages in the world, and it is known to contain various beneficial constituents such as epigallocatechin-3-gallate (EGCG). It has been demonstrated that EGCG exhibits various biological and pharmacological properties that have been reported to act in several antioxidative [21-22] and anticarcinogenic ways. [23-24] EGCG, which is the major catechin in tea leaves, apparently has the most essential role in these actions. Interestingly, EGCG and the O-methylated derivative of EGCG (EGCG3Me) have been shown to inhibit type I allergy. [25] In addition, many inflammatory proteins expressed in asthmatic airways are regulated by NF-B, including the TNF-α and adhesion molecules such as ICAM-1 and VCAM-1, [26-27] all of which are closely involved in the pathogenesis of asthma. Recently it has been reported that EGCG suppresses the LPS-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and NF-B. [28] It has also been reported that EGCG has an inhibitory activity on the gelatinolytic activity of MMP-2. [29] In this report, the subject of whether EGCG has an inhibitory effect against OVA-induced asthma in mice is investigated.

Materials and Methods

1. Animals and experimental protocol

Female BALB/c mice, 6-8 weeks of age and free of murine-specific pathogens, were obtained from the Orient (Seoul, Korea), All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized on days 1, 8 and 15 by intraperitoneal injection of 20 g ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 l, as described previously.(Fig. 1). The mice were challenged for 30 min with an aerosol of 5% (w/v) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed 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 intubated 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 4C. A part of each pool was then centrifuged, and the supernatants were kept at -70C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin. The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc. Aguada, PR) in order to examine the cell differentials. Two independent investigators, not associated with this study, counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Interinvestigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

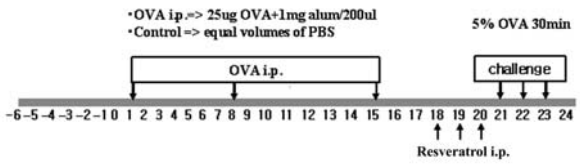

Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized on days 1, 8 and 15 by intraperitoneal injection of OVA emulsified in 1mg of aluminum hydroxide. Seven days later, the mice were challenged for 30 min with an aerosol of 5(w/v)% OVA in saline (or with saline as a control) using an ultrasonic nebulizer. Mice were fed ad libitum 0.1%, 0.2% EGCG in their drinking water with a light-protected bottle from 7 days before sensitization to 2 days after challenge.

2. Administration of EGCG

Mice were fed ad libitum 0.1%, 0.2% EGCG (TEAVIGOTM, Roche Vitamins Ltd., Basel) in their drinking water with a light-protected bottle from 7 days before sensitization to 3 days after challenge.

3. Measurement of intracellular reactive oxygen species

ROS were measured by a method described previously with modification[30-32]. BAL fluids were washed with phosphate-buffered saline (PBS). To measure intracellular ROS, cells were incubated for 10 min at room temperature with PBS containing 3.3 M 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR), to label intracellular ROS. The cells were then immediately observed under fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

4. Immunhistochemistry

At 48 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, 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 m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). Inflammation score was graded by three independent investigators who were not associated with this study.

5. RNA isolation and RT-PCR

The total RNA from lung tissues was isolated with the use of a rapid extraction method (TRI-Reagent), as previously described. [14] The total RNA was reverse-transcribed to cDNA in a buffer. The single-strand cDNAs were used for the PCR amplification of MMP-9, RANTES, CXCL10, iNOS or GADPH. The PCR amplification was performed by mixing 3 l of RT reaction with 47 l of buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, Wis) and 30 pmol/L of specific primer pairs for mouse cDNAs of MMP-9, RANTES, CXCL10, iNOS or GAPDH, according to published mouse gene sequences. All of the signals were visualized and analyzed by densitometric scanning (Imager-1000, NTSC, America).

6. 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. [16] The samples were loaded to 10% SDS-PAGE gels and were separated at 120 V for 90 minutes. The blots were incubated with an antiMMP-9 antibody diluted at a ratio of 1:800, (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4C. The membranes were stripped and reblotted with anti-actin antibody (Sigma) to verify the equal loading of protein in each lane.

7. Measurement of cytokines

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

8. Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 48hrs after the last challenge in an unrestrained conscious state, as described previously[15, 20, 21]. 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) were 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 the manufacturers' protocol, 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 percentage increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values averaged for 3 min after each nebulization were evaluated.

9. Densitometric analysis and statistics

Data are expressed as mean SD. Statistical comparisons were performed by a 1-way analysis of variance, followed by the Fisher test. Significant differences between groups were determined by the unpaired Student t test. Correlations were calculated by means of the Spearman rank test. The statistical significance was set at $P < 0.05$.

Results

1. Effect of EGCG on cellular changes in BAL fluids

Numbers of total cells, eosinophils, lymphocytes, and macrophages in BAL fluids were increased significantly at 24 h after OVA inhalation compared with the numbers after saline inhalation (Fig. 2). The increased numbers of these cells were significantly reduced by the administration of EGCG.

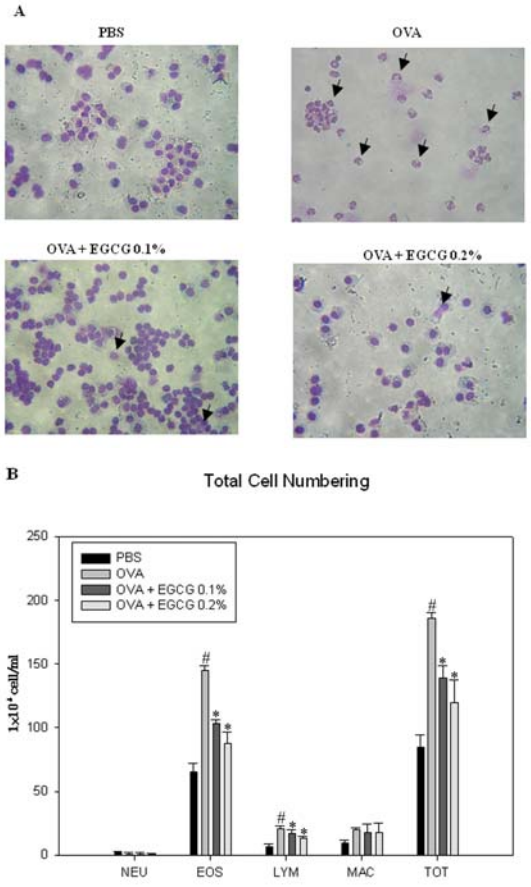

Fig. 2. Effect of EGCG on total and differential cellular

components of BAL fluids of OVA-sensitized and OVA-challenged mice. (A) Mice were treated a with the PBS (PBS), OVA plus EGCG (OVA + EGCG), and OVA (OVA), respectively, as described in Materials and Methods. The BAL cells were collected 2 days after the OVA challenge. The different cell types were enumerated. (B) The arrow is pointed at Eosinophils. The results were from one representative experiment out of 5 performed. This experiment used 5 mice. #P < 0.05 vs. SAL, *P < 0.05 vs. OVA. NEU, neutrophil; EOS, eosinophil; LYM, lymphocyte; MAC, macrophages; TOT, total cell.

2. Effect of EGCG on ROS generation in BAL fluids

We examined the effect of EGCG on ROS generation, as described in Fig. 3. For this experiment, sampling was performed at 72 h after the last challenge. ROS generation in BAL fluids was increased significantly at 72 h after ova inhalation compared with the levels after saline inhalation (Fig. 3). To investigate whether ROS generation on EGCG affects, we detected 3.3 M 2',7'-dichlorofluorescein diacetate (DCFDA). When treated with EGCG, ROS levels was lower than for ova-induced levels of ROS, suggesting that the administration of EGCG had a reducing effect on ROS generation.

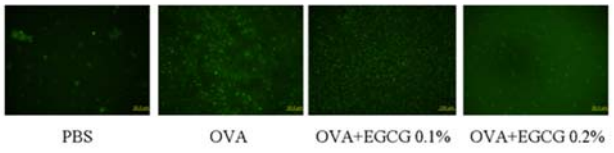

Fig. 3. Effect of EGCG on ROS levels in BAL fluids of OVA-sensitized and OVA-challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (PBS), OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered EGCG (OVA + EGCG). The BAL fluids were stained with PBS containing 3.3 M DCFDA, and analyzed using fluorescence microscopy. ROS levels was lower than for OVA-induced levels of ROS, suggesting that the administration of EGCG had a reducing effect on ROS generation.

3. Effect of EGCG on 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. 4). Mice treated with EGCG showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 24 h after OVA inhalation compared with the scores after saline inhalation (Fig. 4). The increased peribronchial, perivascular, and total lung inflammation were significantly reduced by the administration of EGCG. These results suggest that EGCG inhibits antigen-induced inflammation in the lungs, including the influx of eosinophils.

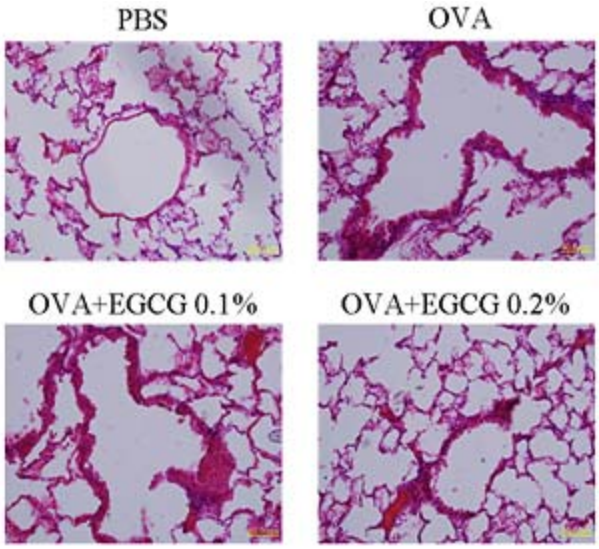

Fig. 4. EGCG inhibits lung inflammation. Mice were sensitized and challenged as described in Materials and Methods. Sections were obtained from the lungs of mice receiving the vehicle (PBS), OVA plus EGCG (OVA + EGCG), and OVA (OVA), respectively. Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining (x 200).

4. Effect of EGCG on expression of RANTES and MMP-9 mRNA and protein levels in lung tissues and BAL fluids of OVA-sensitized and -challenged mice

RT- PCR analysis revealed that expression of RANTES, CXCL10, iNOS and MMP-9 mRNA in lung tissues was increased significantly at 24 h after OVA inhalation compared with the levels after saline inhalation (Fig. 5).

The increased mRNA expression of these adhesion molecules and chemokines was decreased by the administration of EGCG. Western blot analysis revealed that levels of MMP-9 in the lung tissues were increased significantly at 24 h after OVA inhalation compared with the levels after saline inhalation. The increased levels of MMP-9 protein were significantly reduced by the administration of EGCG. (Fig. 6)

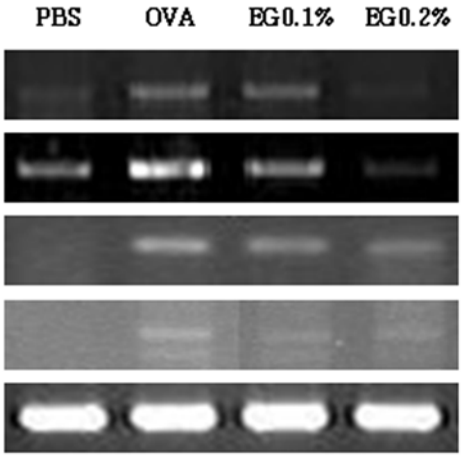

Fig. 5. Effect of EGCG on CCL5, CXCL10, iNOS and MMP-9 mRNA expression in lung tissues of OVA-sensitized and challenged mice. Sampling was performed at 24 h after the last challenge in saline-inhaled mice administered saline (PBS), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered EGCG 0.1% (EG0.1%) and OVA inhaled mice administered EGCG 0.2% (EG0.2%).

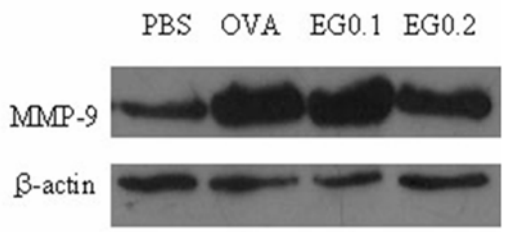

Fig. 6. Effects of EGCG administration on the expression MMP-9 protein levels in the lungs of OVA-challenged mice. OVA-sensitized mice were pretreated (or not) with EGCG before the OVA challenge. The cell extracts were prepared from the lungs of the experimental mice 24h after challenge as well as from the lung tissue of a naive control mouse. The extracts were subjected to immunoblot analysis with antibodies to MMP-9; the blots were reprobed with antibodies to -actin to confirm the consistent application of samples densitometric analyses were also performed. The effect of EGCG on MMP production in lung tissues of OVA-sensitized and OVA-challenged mice was investigated.

5. Effect of EGCG on levels of IL-5 and TNF- α in lung tissues of OVA-sensitized and -challenged mice

The regulation of cytokine production is largely mediated by NF-B activation, and the inhibition of this expression and activation could lead to a reduction in the inflammatory cytokine production. To determine cytokine level in the BAL fluid, IL-5 and TNF- α were determined by ELISA. BAL fluids were obtained 4 h after the last airway challenge. The levels of IL-5 and TNF- α in the BAL fluids were significantly increased by airway challenge with OVA when compared with that with saline as control. The administration of EGCG reduced the concentration of IL-5 and TNF- α secretion by 41% and 56%.(Fig. 7).

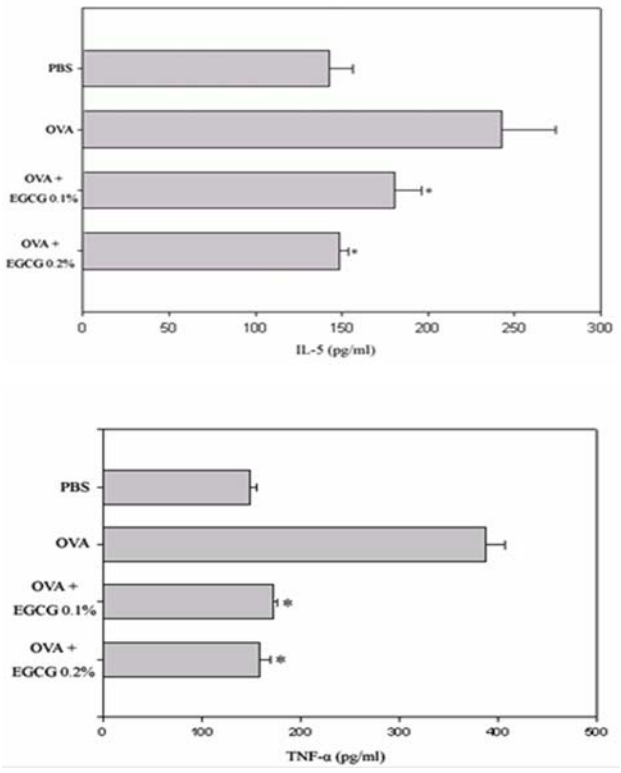

Fig. 7.The effect of TNF- and IL-5 on BAL fluid cytokines. OVA-sensitized mice were treated as described in Fig.1. (A) Bronchoalveolar lavage (BAL) was performed 4 h after the last airway challenge as described in the manufacturer. TNF- cytokine levels in the BAL fluids were measured by ELISA Kit. (B) The mice were bled 4 h after the last airway OVA challenge. Data represent mean SEM from 4 independent experiments. *P<0.05 vs. OVA

6. Effect of EGCG on airway hyper-responsiveness

Airway responsiveness was assessed as a percent increase of Penh in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Penh shifted to the left compared with that of control mice (Fig. 8). 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 EGCG 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 EGCG treatment reduces OVA-induced airway hyperresponsiveness.

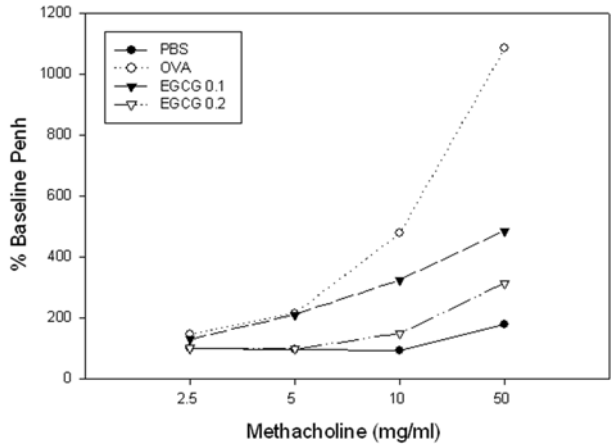

Fig. 8. Effect of EGCG on airway responsiveness in OVA-sensitized and OVA-challenged mice. Airway responsiveness was measured at 72 h after the last challenge in saline-inhaled mice administered PBS (PBS), OVA-sensitized mice administered saline (OVA) and OVA-sensitized mice administered EGCG (OVA + EGCG). 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 mean 6 independent experiments.

Discussion

This study is the first in providing experimental evidence demonstrating EGCG inhibition of OVA-induced airway inflammation in a murine model of asthma. EGCG profoundly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation.

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+.[36] Ultimately, mediators lead to degranulation of effector/proinflammatory cells with the release of mediators and oxidants, which consequently lead to the injury and inflammation noted in asthma. ROS such as superoxide, hydrogen peroxide, and possibly hydroxyl radicals contribute to inflammatory changes in the asthmatic airway.[30-31] In support of this concept is the high levels of ROS and oxidatively modified proteins in airways of asthmatics [32-33]. High levels of ROS are produced in the lungs of asthmatic patients by activated inflammatory cells, i.e., eosinophils, alveolar macrophages, and neutrophils.

Recently, EGCG and O-methylated derivative of EGCG (EGCG3Me) have been shown to suppress FcRI, a high affinity IgE receptor that plays a key role in a series of acute and chronic allergic reactions such as atopic dermatitis, bronchial asthma, allergic rhinitis, and food allergy, expression in human basophilic KU812 cells. [25] It has also been reported that EGCG suppresses IgE-mediated histamine and leukotriene C4 release via blockade of reactive oxygen synthesis.[34] In addition, EGCG has an inhibitory effect on the gelatinolytic activity of MMP-2 through enhancement of MMP-2 binding to the tissue inhibitor of metalloproteinase-2 (TIMP-2).[29]

OVA-induced asthma has been recognized as a disease resulting from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen. [6-7] There is an increasing evidence that cytokine-inducible leukocyte-endothelial adhesion molecules

are important in the recruitment and migration of leukocytes to the sites of inflammation. [35-36] Cell adhesion molecules such as ICAM-1 and VCAM-1 are expressed on endothelial cells and are markedly upregulated on the bronchial vascular endothelium of subjects with asthma. [37-38] In this murine model of OVA-induced asthma, the expression of MMP-9 mRNA as well as the protein levels were increased in the lung tissues after the OVA challenge. These effects were the main causes of migration of the inflammatory cells, including eosinophils, to the asthmatic airways. Therefore, these data suggest that MMP-9 may play an important role in inducing and prolonging OVA-induced asthma. Previous studies have demonstrated that the production of matrix-degrading enzymes, such as MMP-9, is essential for extravasation of leukocytes and recruitment to the affected sites. [39-40] Recently, it was demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes. 28) In addition, an MMP inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. [41]
In this murine model of asthma, we fed 0.1%, 0.2% EGCG in the drinking water to evaluate the effect of EGCG on the expression of MMP-9, TNF-α, and IL-5. It has been reported that EGCG is better absorbed when administered through drinking fluid than intragastric (i.g) administration, that most of the ingested EGCG apparently does not get into the blood, and that the absorbed EGCG is preferentially excreted through the bile to the colon. [37] It was also reported that tmax and Cmax were 85.5 min and 19.8 ng/ml respectively when EGCG was given to rats at a dose of 75 mg/kg. Our current EGCG dose (169.2 mg/kg/day) was determined by extrapolating from previous reports. [42-43] Prediction of the dosage requirement for humans still requires accessible in vitro biological data and other absorption estimates obtained from in vitro animal models. Therefore, further studies exploring in vitro/in vivo correlation and inter-species scaling would provide a more accurate estimation of concentrations/dose of EGCG and its activity in humans. A better-designed clinical study is necessary to determine the safety and efficacy of EGCG in human asthmatic subjects.
In this study, EGCG reduced levels of inflammatory cell migration in lung tissues of OVA-sensitized and -challenged mice. These effects of EGCG may explain why the administration of EGCG significantly reduced the increase in neutrophils, eosinophils, lymphocytes, monocytes, and in total cells, as elicited in the airway lumen 1 days after OVA inhalation (Fig. 2). It has long been postulated that the expression of MMP-9 is regulated by cytokines, especially TNF-α. 44-45) Out data demonstrates that EGCG reduces the increased numbers of inflammatory cells -the source of TNF-α in the airways- then decreases the increased levels of TNF-α in BAL fluids of OVA-sensitized and OVA-challenged mice (Fig. 6).
In conclusion, these results strongly indicate that EGCG reduces the pathologic lung damage by the suppressing ROS generation through inhibition of inflammatory cells migration by reducing MMP-9 expression and also by suppressing TNF-α and IL-5 production. This study also supports the evidence that EGCG might offer a new therapeutic approach to allergic airway diseases.

Acknowledgement

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