FEBS Letters 580 (2006) 1883–1890

Epigallocatechin-3-gallate protects toluene diisocyanate-induced airway inflammation in a murine model of asthma

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Received 9 January 2006; revised 14 February 2006; accepted 16 February 2006

Available online 28 February 2006

Edited by Beat Imhof

Abstract **Epigallocatechin-3-gallate (EGCG), a major form of tea catechin, has anti-allergic properties. To elucidate the anti-allergic mechanisms of EGCG, we investigated its regulation of matrix metalloproteinase (MMP-9) expression in toluene diisocyanate (TDI)-inhalation lung tissues as well as TNF-α and Th2 cytokine (IL-5) production in BAL fluid. Compared with untreated asthmatic mice those administrated with EGCG had significantly reduced asthmatic reaction. Also, increased reactive oxygen species (ROS) generation by TDI inhalation was diminished by administration of EGCG in BAL fluid. These results suggest that EGCG regulates inflammatory cell migration possibly by suppressing MMP-9 production and ROS generation, and indicate that EGCG may be useful as an adjuvant therapy for bronchial asthma.**

Keywords: Epigallocatechin-3-gallate; Asthma; Toluene diisocyanate; Cytokines; Airway hyper-responsiveness; Matrix metalloproteinase

1. 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). Much evidence has suggested that ROS plays an essential role in the pathogenesis of airway inflammation [2–4]. The inflammatory cells recruited to the asthmatic airways are able to produce ROS. Evidence of an increase in 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.

Toluene diisocyanate (TDI)-induced asthma is characterized by AHR and inflammation of the airway [6]. This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues [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 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-dependent and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix [11]. MMPs are produced not only by structural cells [12,13], but also by 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 [19,20].

Tea (*Camellia sinensis* L.) is one of the most widely consumed beverages in the world, and 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 (EGCG″3Me) have been shown to inhibit type I allergy [25]. In addition, many inflammatory proteins expressed in asthmatic airways are regulated by NF-κB, including the TNF-α [26,27], all of which are closely involved in the pathogenesis of asthma. Recently, we have reported that EGCG

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Abbreviations: EGCG, epigallocatechin-3-gallate; TDI, toluene diisocyanate; ROS, reactive oxygen species; MMP-9, matrix metalloproteinase; AHR, airway hyper-responsiveness

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.052

suppresses the LPS-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and NF-κB [28]. It has also been reported that EGCG has an inhibitory effect on the gelatinolytic activity of MMP-2 [29]. In the present study, we investigated whether EGCG has an inhibitory effect against TDI-induced asthma in mice.

2. Materials and methods

2.1. Animals and experimental protocols

Female BALB/c mice, 6–8 weeks of age and free of murine specific pathogens, were obtained from the Orient (Seoul, Korea). All of the experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University College of Medicine. Mice were sensitized twice by 2 courses of intranasal administration of 3% TDI dissolved in ethyl acetate/olive oil (1:4) once per day for 5 consecutive days with a 3-week interval, as previously described [24,25]. Seven days later, the mice were challenged through the airways with 1% TDI dissolved in ethyl acetate/olive oil (1:4) by ultrasonic nebulization (NE-U12, Omron, Tokyo, Japan) (Fig. 1). To establish a control, mice were sensitized and challenged with the use of the same protocol but only with the solvent, ethyl acetate/olive oil (1:4). Bronchoalveolar lavage (BAL) was performed at 2 days after the TDI-challenge. After euthanasia with sodium pentobarbital (100 mg/kg body weight), pre-warmed 0.9% NaCl solution (500 μl) was slowly infused into the lungs and withdrawn. The total cell numbers were counted with a hemacytometer. 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.

2.2. Administration of EGCG

Mice were fed ad libitum 0.3% EGCG (TEAVIGO™, Roche Vitamins Ltd., Basel) in their drinking water with a light-protected bottle from last sensitization to 2 days after first challenge.

2.3. Measurement of intracellular ROS

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 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR), to label intracellular ROS. The cells were then immediately observed fluorescence-activated cell sorting (FACS) analysis (Beckton Dickinson, San Jose, CA).

2.4. Cytokine measurements

Levels of TNF-α and IL-5 (Endogen Inc., Woburn, MA) in BAL fluids were measured with a specific mouse (m) TNF-α and IL-5 ELISA kit. The detection limit of the TNF-α and IL-5 assay were 5 pg/ml, and the assay is said by the manufacturer to be specific for TNF-α and IL-5.

2.5. Zymography

The gelatinolytic activity of MMP-9 was measured by gelatin zymography, as described previously [33,34]. Briefly, a total of 10 μl of BAL fluid was subjected to electrophoresis in 10% polyacrylamide gel containing gelatin (1 mg/ml). The gel was washed in 2.5% Triton X-100 to permit renaturation of gelatinases, and stained with Coomassie blue after overnight incubation. Destaining visualized clear zones of lysis against a blue background, indicating gelatinase activity [35].

2.6. Western blot analysis

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. A Western blot analysis was performed as described previously [18]. The samples were loaded to 10% SDS–PAGE gels and were separated at 120 V for 90 min. The blots were incubated with an anti-MMP-9 antibody diluted at a ratio of 1:800 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.

2.7. 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 [16]. The total RNA was reverse-transcribed to cDNA in a buffer. The single-strand cDNAs were used for the PCR amplification of MMP-9, ICAM-1, VCAM-1, or GADPH. The PCR amplification was performed by mixing 3 μl of RT reaction with 47 μl of buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, WI) and 30 pmol/L of specific primer pairs for mouse cDNAs of MMP-9, ICAM-1, VCAM-1, or GAPDH, according to published mouse gene sequences. All of the signals were visualized and analyzed by densitometric scanning (LAS-5000; Fuji Film, Tokyo, Japan).

2.8. Immunohistochemistry

At 48 h after the last challenge, lungs were removed from the mice after they were killed. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (4% formalin) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) neutral buffered formalin. 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 stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). The inflammation score was graded by three independent investigators who were not associated with this study. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere. A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by a thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

2.9. Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 3 days after the last challenge in an unrestrained conscious state, as described previously [2–4]. Mice were placed in a barometric plethysmographic chamber

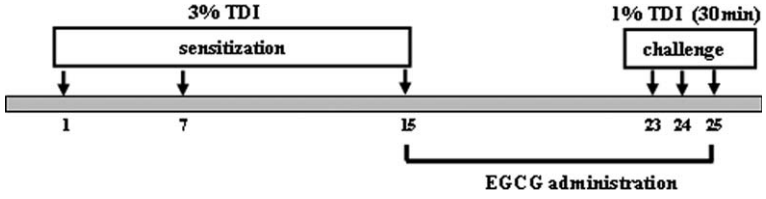

Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized three times by 3% TDI dissolved in ethyl acetate/olive oil (1:4) at a 1-week interval. Seven days later, the mice were challenged through the airways with 1% TDI dissolved in ethyl acetate/olive oil (1:4) by nebulization. To establish a control, mice were sensitized and challenged with the use of the same protocol but only with the solvent, ethyl acetate/olive oil (1:4). Mice were fed ad libitum 0.3% EGCG in their drinking water with a light-protected bottle from last sensitization to 2 days after first challenge.

(All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5–50 mg/ml, Sigma) 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 doses of a concentration of methacholine 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.

2.10. Statistics

Data are expressed as means ± S.D. 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 $P < 0.05$.

3. Results

3.1. EGCG decreases the increased numbers of inflammatory cells in BAL fluids of TDI-sensitized and TDI-challenged mice

The total cell numbers in BAL fluids were significantly increased, about 3-fold, compared to those in the control group 2 days after the last TDI challenge. The number of neutrophils, eosinophils, lymphocytes, and Macrophages in BAL fluids was increased 2.5-, 13.2-, 2-, and about 2-fold, respectively, compared to those in the control group at 2 days after the TDI challenge. Interestingly, the number of neutrophils, eosinophils, lymphocytes, and monocytes observed in BAL fluids in the EGCG-feeding group of mice decreased to the level of 1.6-, 1.9-, 2-, 1-fold of TDI-challenge group. The administration of the EGCG significantly reduced the increase in neutrophils, eosinophils, lymphocytes, macrophages and total cells elicited in the airway lumen 2 days after TDI inhalation (Fig. 2).

3.2. EGCG inhibits ROS generation in BAL fluids

Oxidative stress occurs in many allergic and immunologic disorders. Number of studies have shown an increased production of ROS in asthma, AR and AD, which contribute, in part, to tissue injury at sites of inflammation [36]. Respiratory burst of eosinophils activated by eotaxin has been demonstrated, leading in ROS production [37,38]. 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 TDI inhalation compared with the levels after saline inhalation (Fig. 3). To investigate whether ROS generation on EGCG affects, we detected 3.3 μM 2′,7′-dichlorofluorescein diacetate (DCFDA). When treated with EGCG, ROS levels was lower (18.69 ± 0.7%) than for TDI-induced levels of ROS (39.28 ± 0.8%), suggesting that the administration of EGCG had a reducing effect on ROS generation.

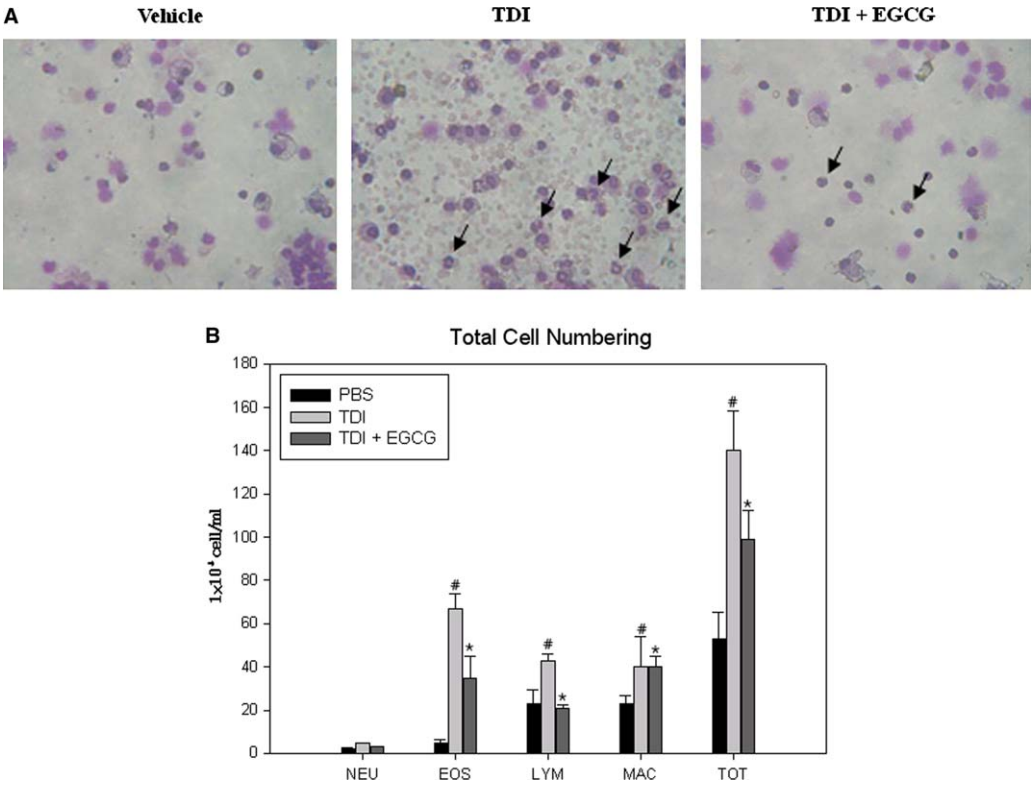

Fig. 2. Effect of EGCG on total and differential cellular components of BAL fluids of TDI-sensitized and TDI-challenged mice. Mice were treated with the vehicle (PBS), TDI plus EGCG (TDI + EGCG), and TDI (TDI), respectively, as described in Section 2. The BAL cells were collected 2 days after the TDI challenge. The different cell types were enumerated (A) and the numbers of each cellular component were counted (B). The results were from one representative experiment out of 5 performed. This experiment used 5mice ($n = 5$). $^{\#}P < 0.05$ vs. SAL + SAL, $^{*}P < 0.05$ vs. OVA + SAL. NEU, neutrophil; EOS, eosinophil; LYM, lymphocyte; MAC, macrophages; TOT, total cell.

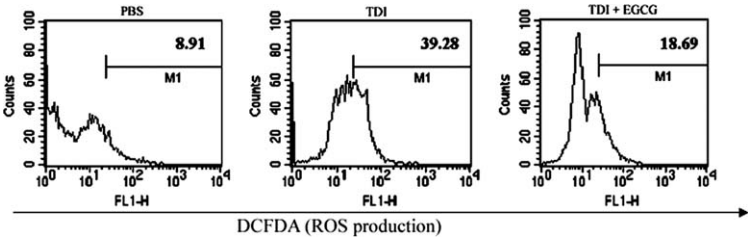

Fig. 3. Effect of EGCG on ROS levels in BAL fluids of TDI-sensitized and TDI-challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (PBS), TDI-inhaled mice administered saline (TDI), and TDI-inhaled mice administered EGCG (TDI + EGCG). The BAL fluids were stained with PBS containing 3.3 μM DCFDA, and analyzed using flow cytometry. ROS levels was lower (18.69 ± 0.7%) than for TDI-induced levels of ROS (39.28 ± 0.8%), suggesting that the administration of EGCG had a reducing effect on ROS generation. The histogram shown are gated on BAL cells. Data represent means ± S.E.M. from 6 independent experiments.

3.3. EGCG inhibits infiltration of inflammatory cells in airways

The results of histological examination of lung tissues paralleled the cell numbers in BAL fluid. A marked influx of inflammatory cells into the airway and around blood vessels, as well as airway luminal narrowing by secreted mucus, was observed in the TDI-sensitized group (Fig. 4). Administration of EGCG resulted in a marked inhibition of cellular infiltration into the airways and pulmonary blood vessels (Fig. 4).

3.4. EGCG reduces expression of MMP-9 mRNA and protein in lung tissues and decreases TNF-α and IL-5 production in the BAL fluids of TDI-sensitized and TDI-challenged mice

The administration of EGCG significantly reduced the increase in expression of MMP-9 mRNA in the lung tissues 48 h after TDI inhalation (Fig. 5A). The administration of EGCG also reduced the increase expression of MMP-9 protein in lung tissues (Fig. 5B). Also the administration of EGCG significantly reduced the increased expression of MMP-9 gelatinolytic activity, as well as the level of pro-MMP-9 in lung tissues 2 days after TDI inhalation (Fig. 5C). EGCG administration also dramatically reduced the increase in TNF-α and IL-5 production in BAL fluids 48 h after TDI inhalation (Fig. 6).

3.5. EGCG reduces AHR

Airway responsiveness was assessed as a percent increase of Penh in response to increasing doses of methacholine. In TDI-sensitized and TDI-challenged mice, the dose–response curve of percent Penh shifted to the left compared with that of control mice (Fig. 7). In addition, the percent Penh produced by methacholine administration (at doses from 2.5 to 50 mg/ml) increased significantly in the TDI-sensitized and TDI-challenged mice compared with the controls. TDI-sensitized and TDI-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 TDI-induced AHR.

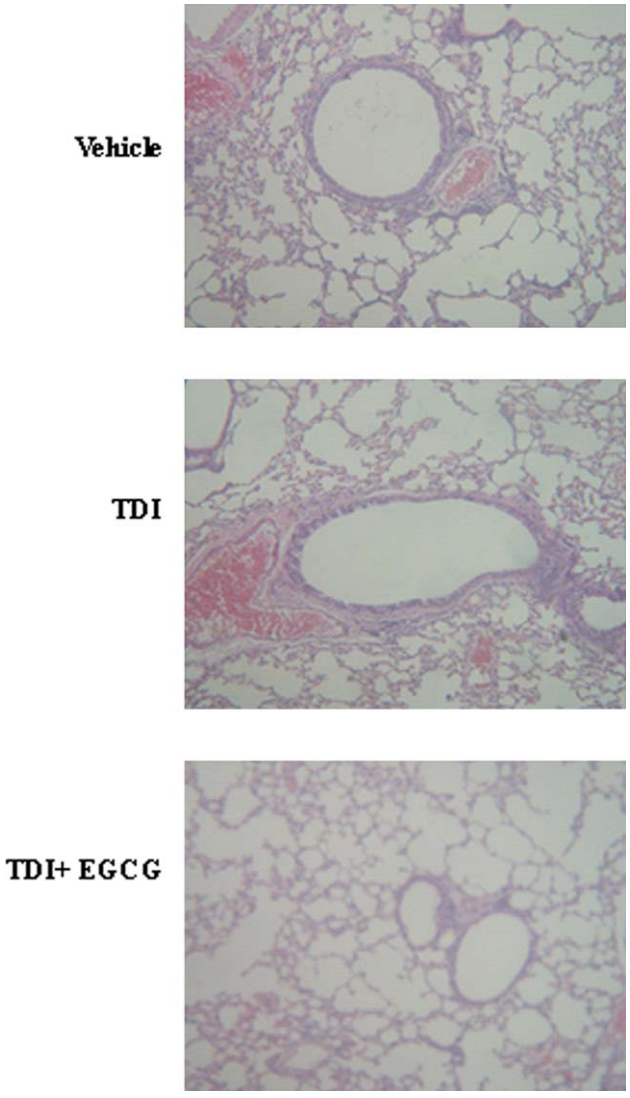

Fig. 4. EGCG inhibits lung inflammation. Mice were sensitized and challenged as described in Section 2. Sections were obtained from the lungs of mice receiving the vehicle (PBS), TDI (TDI), and TDI plus EGCG (TDI + EGCG), respectively. Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining (100×).

4. Discussion

This study is the first to provide experimental evidence demonstrating that EGCG inhibits TDI-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

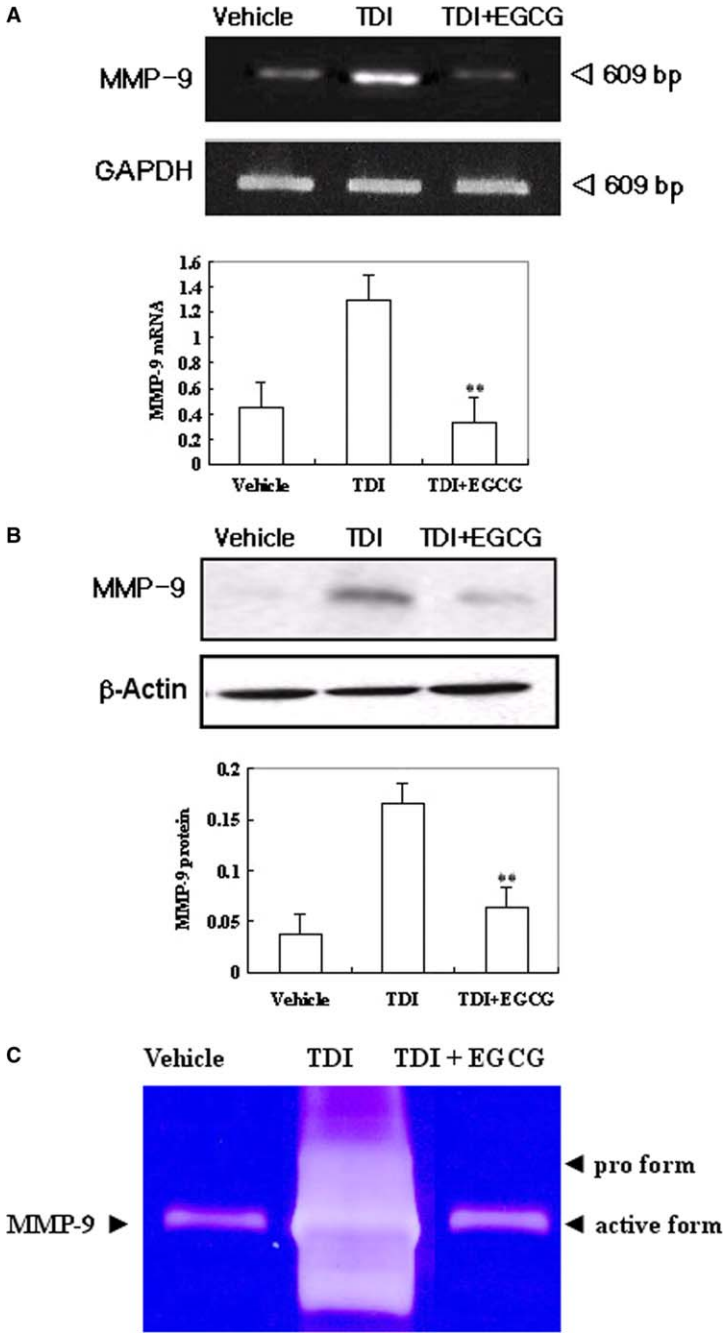

Fig. 5. Effects of EGCG administration on the expression of MMP-9 in the lungs of TDI-challenged mice. TDI-sensitized mice were pretreated (or not) with EGCG before the TDI challenge. The cell extracts were prepared from the lungs of the experimental mice 48 h after challenge as well as from the lung tissue of a naive control mouse. The extracts were subjected to RT-PCR for MMP-9 mRNA analysis (A) and densitometric analyses were performed. Also, the extracts were subjected to immunoblot analysis with antibodies to MMP-9; the blots were reprobed with antibodies to β-actin to confirm the consistent application of samples (B), densitometric analyses were also performed. The effect of EGCG on MMP production in lung tissues of TDI-sensitized and TDI-challenged. Sampling was performed at 48 h after challenge with the PBS (lane 1), TDI (lane 2), or TDI plus EGCG (lane 3) and analyzed by zymography. All of the groups of the experiment showed MMP-9 production, but the TDI plus EGCG group only showed the active form of MMP-9 (C). Data represent means ± S.E.M. from 6 independent experiments. $^{**}P < 0.05$ vs. OVA.

Th2 CD4+ [39]. 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. ROS such as superoxide, hydrogen peroxide, and possibly hydroxyl radicals contribute to inflammatory changes in the asthmatic airway [36–39]. In support of this concept are the high levels of ROS and oxidatively modified proteins in the airways of asthmatics [40,41,43,44]. 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 derivatives of EGCG (EGCG″3Me) have been shown to suppress FcεRI, a high-affinity IgE receptor that plays a key role in a series of acute

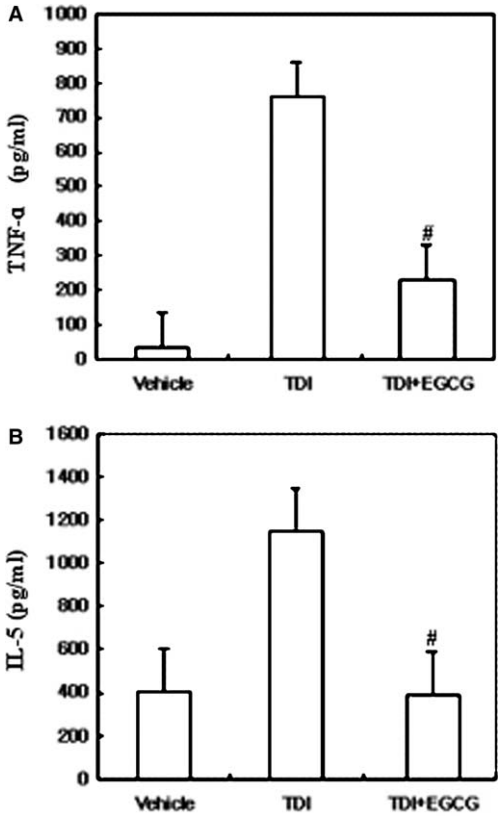

Fig. 6. The effect of TNF-α and IL-5 on BAL fluid cytokines. TDI-sensitized mice were treated as described in Fig. 1. (A) Bronchoalveolar lavage (BAL) was performed 4 h after the last airway challenge as described by the manufacturer. TNF-α cytokine levels in the BAL fluids were measured by ELISA Kit. (B) The mice were bled 12 h after the last airway TDI challenge. The detection limit of the IL-5 assay was 5 pg/ml, and the assay is assured by the manufacturer to be specific for the IL-5 ELISA kit. Data represent means ± S.E.M. from 5 independent experiments. $^{\#}P < 0.05$ vs. OVA.

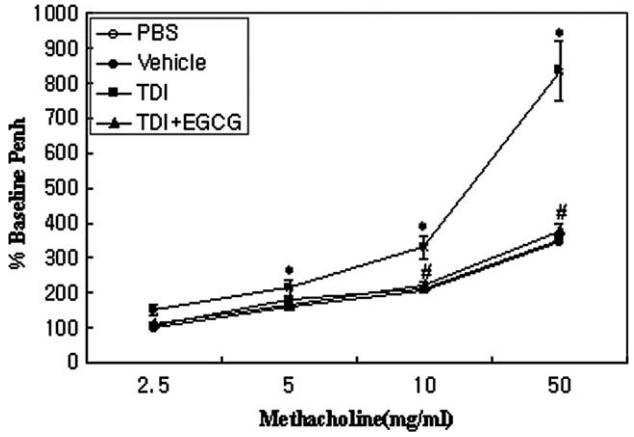

Fig. 7. Effect of EGCG on airway responsiveness in TDI-sensitized and TDI-challenged mice. Airway responsiveness was measured at 72 h after the last challenge in saline-inhaled mice administered PBS (PBS), ethyl acetate/olive oil-inhaled mice administered PBS (Vehicle), TDI-inhaled mice administered saline (TDI) and TDI-inhaled mice administered EGCG (TDI + 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 (2.5–50 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 6 independent experiments. $^{*}P < 0.05$ vs. PBS, $^{\#}P < 0.05$ vs. OVA.

and chronic allergic reactions such as atopic dermatitis, bronchial asthma, allergic rhinitis, and food allergies expressed in human basophilic KU812 cells [25]. It has also been reported that EGCG suppresses IgE-mediated histamine release and leukotriene C4 via the blockade of reactive oxygen synthesis [42]. 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].

TDI-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 increasing evidence that cytokine-inducible leukocyte-endothelial adhesion molecules are important in the recruitment and migration of leukocytes to the sites of inflammation [43,44]. 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 [45,46]. Previous studies have demonstrated that the production of matrix-degrading enzymes, such as MMP-9, is essential for leukocytes extravasation and recruitment to the affected sites [15,19]. 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 TDI-induced asthma [47].

In this murine model of asthma, we fed 0.3% EGCG into 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 (IG) administration, that most of the ingested EGCG apparently does not get into the blood, that and the absorbed EGCG is preferentially excreted through the bile to the colon [42]. It was also reported that t_{max} and C_{max} were 85.5 min and 19.8 ng/ml, respectively, when EGCG was given to rats intragastrically at a dose of 75 mg/kg. Our current EGCG dose (169.2 mg/kg/day) was determined by extrapolating from previous reports [48,49]. 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/doses of EGCG and its effect 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 the expression of MMP-9 in lung tissues of TDI-sensitized and TDI-challenged mice. These effects of EGCG may explain why the administration of EGCG significantly reduced the increase in neutrophils, eosinophils, lymphocytes, macrophages, and the total cells elicited in the airway lumen 2 days after TDI inhalation (Fig. 2). It has long been postulated that the expression of MMP-9 is regulated by cytokines, especially TNF [50,51]. Our data demonstrate that EGCG reduces the increased numbers of inflammatory cells (the source of TNF- α) in the airways and then decreases the increased levels of TNF-α in BAL fluids of TDI-sensitized and TDI-challenged mice (Fig. 6).

Taken together, our results strongly indicate that EGCG reduces the pathologic lung damage due to the suppression of ROS generation through the inhibition of inflammatory cells migration by reducing MMP-9 expression and also by suppressing TNF-α and IL-5 production. This study also supports evidence that EGCG might offer a new therapeutic approach to allergic airway diseases.

Acknowledgement: This work was partially supported by the Korea Science and Engineering Foundation through National Research Laboratory Program Grant M10500000008-05J000000810.

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