Epigallocatechin gallate improves airway inflammation through TGF-β1 signaling pathway in asthmatic mice

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Abstract. The present study aimed to investigate the effect of epigallocatechin gallate (EGCG) on airway inflammation in mice with bronchial asthma, and the regulatory mechanism of transforming growth factor (TGF)-β1 signaling pathway, so as to provide theoretical basis for research and development of a novel drug for clinical treatment. Mouse models of bronchial asthma were established and injected with dexamethasone and EGCG via the caudal vein. 7 days later, bronchoalveolar tissue was collected for hematoxylin and eosin staining. Determination of airway resistance (AWR) and lung function in mice was detected. Serum was separated for flow cytometric bead array to detect the changes in inflammatory factors. Bronchoalveolar lavage fluid was collected for eosinophil and neutrophil counts. Fresh blood was obtained for flow cytometry to determine the percentages of Th17 and Treg cells. Bronchovesicular tissue was utilized for western blot assay and reverse transcription-quantitative polymerase chain reaction to determine the proteins and transcription factors in the TGF-β1 pathway. EGCG 20 mg/kg significantly reduced asthmatic symptoms, lung inflammatory cell infiltration, and the inflammatory factor levels of interleukin (IL)-2, IL-6 and tumor necrosis factor (TNF)-α. In addition, it increased the percentage of Treg cells, and decreased the expression of TGF-β1 and phosphorylated (p)-Smad2/3 expression. Following the inhibition of the TGF-β1 receptor, the anti-inflammatory effect of EGCG disappeared, and the expression of TGF-β1 and p-Smad2/3 increased. EGCG attenuated airway inflammation in asthmatic mice, decreased the percentage of Th17 cells and increased the percentage of Treg cells. The anti-inflammatory effect of EGCG is achieved via the TGF-β1 signaling pathway.

Key words: asthma, EGCG, Th17 cells, Treg cells, TGF-β1 pathway

Introduction

Bronchial asthma is a chronic inflammatory airway disease involving eosinophils, mast cells, neutrophils, and airway epithelial cells (1-4), and is a common chronic respiratory disease, which seriously endangers human health. The pathogenesis of bronchial asthma is very complicated. Allergic airway inflammation is the key pathogenesis of bronchial asthma (5), is the basis of airway hyperresponsiveness and airway remodeling; the main cause of recurrent bronchial asthma and decreased lung function index is the persistence of airway inflammation (6). Inflammatory mediators and cytokines construct complex network structures (7), induce airway hyperresponsiveness in patients with bronchial asthma through multiple signaling pathways in the body, and lead to long-term chronic inflammation, thereby resulting in remodeling of airways and small airways (8).

Transforming growth factor (TGF)-β has a wide range of biological activities, participates in early embryonic development (9), cartilage and bone formation, inflammation, interstitial fibrosis, and regulation of immune and endocrine functions (10,11), and tumor formation and development (12,13). Eosinophils and bronchial epithelial cells are the major sources of TGF-β production in the bronchial asthma airway. Local TGF-β mainly acts as a proinflammatory cytokine to recruit eosinophils, lymphocytes, neutrophils and mast cells to the airway, enhances the inflammatory cell viability, and promotes their proliferation, differentiation and degranulation, so as to play an important role in chronic airway inflammation in bronchial asthma (14). Smads are an important intracellular TGF-β signal transduction and regulatory molecule, and can transfer TGF-β signals directly from the cell membrane into the nucleus. Smads protein as a substrate, which is phosphorylated (p) by intracellular kinase of the TGF-β receptor, can further traverse the cell membrane; Smads protein acts as an intracellular signaling molecule and a transcripton in genetics (15,16).

At present, the effective drugs for bronchial asthma are glucocorticoids, but the hormone drugs cause many side effects, so it is still urgent to find effective therapeutic agents. Epigallocatechin gallate (EGCG) is a water-soluble component extracted from green tea polyphenols, has high antioxidant activity and can protect the cells and DNA from damage (17,18).
Previous studies have confirmed that EGCG has a wide range of pharmacological effects, and has obvious preventive and therapeutic effects on anti-inflammation (19), antivirus (20), anticancer (21), ulcerative colitis (22), and autoimmune diseases (23). In this study, mouse models of bronchial asthma were established by OVA combined with aluminum hydroxide gel. This study aimed to observe the effect of EGCG on bronchial asthma and to investigate the regulatory mechanism of TGF-β1 signaling pathway so as to provide theoretical basis for clinical prevention and treatment of bronchial asthma and the research and development of new drug.

**Materials and methods**

**Animals and groups.** Forty Balb/c mice, 6 weeks old, male, weighing 20-23 g, were obtained from Experimental Animal Center of China Medical University in China (production license no. SCXK (Liao)-2013-0001, application license no. SYXK (Liao)-2013-0007). This study was approved by the Animal Welfare and Ethics Committee of China Medical University Laboratory (no. 2016099).

The mice were randomly assigned to control group (n=8), asthma model group (AS group; n=8), dexamethasone group (DX group; asthma + 0.5 mg/kg DX; n=8), high-dose EGCG group [HEGCC group; asthma + 20 mg/kg/day; this dose selected came from reference (24) EGCC; n=8], low-dose EGCG group (LEGCG group; asthma + 10 mg/kg/day EGCG; n=8), LY 364947 (ab141890; Abcam, Cambridge, UK) inhibitor group (LYEGCG group; asthma + 40 µg/kg/day EGCG + 20 mg/kg/day LY 364947; n=8). Mice were given a normal diet and water, and the following housing conditions: Ambient temperature of 20-26˚C, 40-70% relative humidity, 12-h light/dark cycle. The mice in the control group were daily treated with 5% OVA saline inhalation for 20 min, for 7 days. Except the normal control group, mice in other groups were intraperitoneally injected with an equal volume of physiological saline via the caudal vein. All experiment repeated three times.

**Establishment of mouse models of asthma.** Except the normal control group, mice in other groups were intraperitoneally injected with 0.1 ml antigen solution [0.1 g/ml OVA +1 g/ml Al(OH)3]. The mice were immunized again on day 8 of the experiment. On day 15, the mice were placed in closed container, treated with 5% OVA saline inhalation for 20 min, for 7 days. The provocative status was observed. The mice in the control group were given an equal volume of physiological saline via the caudal vein. All experiment repeated three times.

**Sample collection.** On day 23 after model establishment, mice in each group were injected with dexamethasone and EGCG via the caudal vein. 7 days later, venous blood was collected from mice and serum was separated for cytokometric bead array. A T-shaped incision was made on the site of trachea, and a 22G indwelling needle was inserted for tracheal intubation. Physiological saline 0.4 ml was injected, repeatedly three times. Bronchoalveolar lavage fluid was collected for eosinophil count. Mouse bronchoalveolar tissue was dissociated. Some were fixed in 10% formalin, and the other was used for western blot assay and qRT-PCR.

**Determination of airway resistance (AWR) and lung function in mice with asthma.** At 24 h of final atomization, AWR was detected using mouse lung function instrument. The mice were anesthetized with sodium pentobarbital (50 mg/kg). The 22G indwelling needle was inserted for tracheal intubation. After connecting to the mouse lung function instrument, the mice were placed on the heating plate of a sealed incubator. A respirator was used for assisted respiration. 12.5, 25 and 50 mg/ml of methacholine were infused intravenously. The changes in AWR (cm H2O s/l) and lung function index [peak expiratory flow (PEF) and the ratio of forced expiratory volume in 0.4 sec to forced vitia capacity (FEV0.4/FVC)] were measured. The AWR was R (baseline) when inhaled PBS. The AWR of inhaling methacholine at different concentrations was R (response). The maximum of the total AWR at each concentration was taken and converted to the fold increase during PBS provocation as the index to assess AWR according to the following formula: Fold increase of R = [R (response) - R (baseline)]/R (baseline).

**Hematoxylin and eosin (H&E) staining.** Bronchovesicular tissue of mice was dehydrated, permeabilized, embedded in wax, and sliced into sections. The sections were stained with hematoxylin for 5 min, washed with PBS, differentiated with ethanol hydrochloride for 3 sec, stained with eosin for 2 min, and mounted with neutral resin. The changes in bronchovesicular tissue were observed under a light microscope.

**Cytometric bead array.** After fully mixed Th1, Th2, Th17 and Treg cells-related cytokines interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17a, tumor necrosis factor (TNF)-α, and IFN-γ protein microspheres, the remaining procedures were carried out in accordance with the kit instruction (560485, BD Biosciences, Franklin Lakes, NJ, USA). After establishing standard curves, 50 µl serum was added in the detected tube and microsphere assay reagent was added at room temperature in the dark for 2 h. The samples were centrifuged and the supernatant was discarded. The microspheres were resuspended, and determined using flow cytometry. Results were analyzed with FCAP Array V3 software.

**Eosinophil and neutrophil count using bronchoalveolar lavage fluid.** Totally 10 µl lavage fluid was placed in cell count plate to quantify the number of cells. After centrifugation, cell sediment was resuspended with moderate amount of PBS. Cells suspension (100 µl) was loaded. After the residual liquid was got rid of, cells were dried in the open air, fixed in alcohol for 1 min, and received Diff-Quik staining. 400 larupcutes were counted under a microscope. The percentages of neutrophils and eosinophils were calculated.

**Flow cytometry.** Peripheral blood mononuclear cells in mice were isolated and adjusted to a concentration of 1x10⁶, and coated in a 6-well plate. PMA (20 ng/ml), ionomycin (1 µg/ml) and monensin (2 nmol/ml) were added at 37˚C in 5% CO2 incubator for 5 h. Afterwards, cells were incubated with FITC-anti-CD4⁺ (554843; BD Biosciences) and PE-cy7-anti-CD25⁺ (552880; BD Biosciences) antibody at room temperature in the dark for 30 min, washed with PBS, fully mixed with 500 µl fixation/permeabilization solution at 4˚C in the dark for 45 min. After washing with 1xBD perm/wash buffer, cells were incubated with APC-anti-IL-17a (17-7177-81; eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and PE-anti-Foxp3⁺
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(A18690; eBioscience; Thermo Fisher Scientific, Inc.) at 4°C in the dark for 50 min, washed with PBS, and resuspended with 400 µl PBS. The samples were determined using flow cytometry and analyzed with Flow Jo V10 software.

**RT-qPCR.** The lung of mice was completely triturated and treated with 1 ml TRIzol reagent (15596018; Invitrogen; Thermo Fisher Scientific, Inc.). RNA was extracted in accordance with the reagent instruction, reverse transcribed into first-strand DNA (4387406; Invitrogen; Thermo Fisher Scientific, Inc.). Real-time quantitative fluorescence PCR was performed in accordance with the instruction of RT-PCR kit (204057; Qiagen GmbH, Hilden, Germany). The relative gene expression data were analyzed with the 2^ΔΔCq method (25). The primers used for real-time PCR are listed in Table I.

**Western blot assay.** Mouse bronchial lung tissue was placed in RIPA lysate containing proteinase inhibitor on the ice for 30 min, and centrifuged. The supernatant was collected. Bicinchoninic acid assay was applied to quantify proteins. These proteins were subjected to electrophoresis and transferred onto the PVDF membrane. The membrane was blocked and incubated with TGF-β1 (ab92486; Abcam), Smad2/3 (ab202445; Abcam), and p-Smad2/3 (ab63399; Abcam) antibody at 4°C overnight, and washed with TBST. The PVDF membrane was incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 2 h. The proteins were visualized using enhanced chemiluminescence kit and gel imaging system. Absorbance values were analyzed using Image Tools.

**Statistical analysis.** All data were analyzed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA), and expressed as the mean ± SD. Paired comparison was conducted using Student’s t-test. Multiple comparisons were used one-way analysis of variance with Student-Newman-Keuls test. A value of Ρ<0.05 was considered statistically significant.

**Results**

**EGCG improves provocative symptoms in asthmatic mice.** After asthma was provoked by 5% OVA atomization, the mice presented shortness of breath, restlessness, back upright, repeated scratching head, horripilation and slow action. The symptoms of mice from the DX group were improved, but their activities did not obviously increase. Their actions were still sluggish. In the HEGCG and LEGCG groups, the respiration in mice was smooth; sluggish action improved; the degree of pruritus and cyanosis were relieved. H&E staining (Fig. 1) demonstrated that in the asthma group, bronchial and alveolar tissues were disorganized and some structures disappeared; a large number of inflammatory cells infiltrated in the bronchial mucosa; and the arrangement of the airway epithelium was irregular. After treatment with EGCG, the infiltration of inflammatory cells was obviously alleviated, and

| Primer (5′ → 3′) | Primer (5′ → 3′) |
|------------------|------------------|
| RORγT Forward    | GCACCCGCTGAGAGGGCT |
| Reverse          | CGAACCAGCCGCAACCGA |
| Foxp3 Forward    | TACACTTATGGGCTCTCA |
| Reverse          | CCTCCTGAGAGCCTCAGG |
| GAPDH Forward    | AGGGTCCAGACAGCCACT |
| Reverse          | TGAGATGGCCCTTACAC |

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RORγT, RAR-related orphan receptor T; Foxp3, Forkhead box protein P3.

Figure 1. Established asthmatic mice model and treated with dexamethasone (0.5 mg/kg), high-dose EGCG (20 mg/kg) and low-dose EGCG (10 mg/kg). On day 30 after model establishment and treatment, mice lung tissue was collected. Lung tissue was fixed, dehydrated, paraffin embedded, sliced. H&E staining observed the pathology alteration (magnification, x40). EGCG, epigallocatechin gallate; AS, asthmatic; LEGCG, low-dose EGCG; HEGCG, high-dose EGCG; DX, dexamethasone group; H&E, hematoxylin and eosin.
the therapeutic efficacy of high dose was more obvious than that of low dose.

**EGCG lessens AWR in asthmatic mice.** Lung function tests in mice showed that pulmonary function parameters PEF, FEV₀.₄/FVC in asthma group were decreased than those in control group (P<0.05 vs. control), respiratory rate was increased (P<0.05 vs. control); LEGCG group, HEGCG group and DX group pulmonary function parameters PEF, FEV₀.₄/FVC were increased and respiratory rate was decreased (P<0.05 vs. asthma group; Fig. 2A). After provocation with different concentrations of methacholine, AWR was significantly increased in the asthma group, but significantly decreased in the DX group (P<0.05 vs. asthma group; Fig. 2B). AWR was significantly reduced in the HEGCG group (P<0.05 vs. asthma group; P>0.05 vs. DX group). These data indicated that EGCG 20 mg/kg via caudal vein injection could remarkably mitigate AWR and improve lung function in asthmatic mice.

**EGCG improves the number of eosinophils and neutrophils in bronchoalveolar lavage fluid of mice.** The number of eosinophils and neutrophils was significantly increased in the asthma group (P<0.05 vs. control) (Fig. 3). The number of eosinophils and neutrophils was significantly decreased in the DX group (P<0.05 vs. asthma group). The number of eosinophils and neutrophils was significantly diminished in the HEGCG group (P<0.05 vs. asthma group). Although LEGCG group also can significantly improve the number of neutrophils and eosinophils, the effect of LEGCG group was no significant effect of HEGCG group. These findings further suggested that EGCG 20 mg/kg could noticeably improve the number of inflammatory cells in bronchoalveolar lavage fluid of asthmatic mice.

**EGCG lessens inflammatory reaction in asthmatic mice.** Cytometric bead array results demonstrated that serum IL-2 and IFN-γ levels were significantly decreased in the asthma group (P<0.05 vs. control) (Fig. 4). IL-4, IL-6 and TNF-α levels were significantly increased (P<0.05 vs. control). These results verified that the imbalance of Th1/Th2 cells was found in asthmatic mice. After treatment with EGCG, IL-2 and IFN-γ levels were significantly increased (P<0.05 vs. asthma group); IL-4, IL-6 and TNF-α levels were significantly diminished (P<0.05 vs. asthma group). EGCG could effectively improve the imbalance of Th1/Th2 cells in asthmatic mice. We further analyzed IL-17a and IL-10 levels. Results found that IL-17a levels were significantly increased in the asthma group (P<0.05 vs. control). After treatment with EGCG, IL-17a levels were
significantly reduced; IL-10 levels were significantly diminished in the asthma group (P<0.05 vs. control). After treatment with EGCG, IL-10 levels were significantly increased (P<0.05 vs. asthma group). These results suggest that EGCG may improve Th17/Treg balance. These data further confirmed that EGCG effectively improved the inflammatory reaction in asthmatic mice.

**EGCG improves the proportion of Th17/Treg cells in asthmatic mice.** As we all know, Th17 cells secrete IL-17a, Treg cells secrete IL-10, so the proportion of Th17 cells and Treg cells in mice was determined by flow cytometry. The percentage of Th17 cells was significantly increased in the asthma group (P<0.05 vs. control; Fig. 5A), but the percentage of Treg cells was significantly reduced (P<0.05 vs. control) (Fig. 5B). After treatment with EGCG, the percentage of Th17 cells was significantly decreased (P<0.05 vs. asthma group); the percentage of Treg cells was significantly increased (P<0.05 vs. asthma group). After treatment with EGCG, Th17/Treg ratio was significantly decreased (P<0.05 vs. asthma group).
EGCG improves inflammatory reaction in asthmatic mice through TGF-β1 signaling pathway.

To confirm the action mechanism of EGCG, we used TGF-β1 receptor inhibitor LY 364947. Cytometric bead array results showed that after inhibition of TGF-β1 receptor, the protective effect of EGCG disappeared (Fig. 6A). The expression of proinflammatory cytokines such as IL-6 was significantly increased after inhibition of TGF-β1 receptor (Fig. 6A). The expression of anti-inflammatory factors such as IL-10 was significantly increased after inhibition of TGF-β1 receptor (Fig. 6A). Western blot assay results demonstrated that TGF-β1 and P-smad2/3 expression was significantly increased in the lung of mice in the asthma group (P<0.05 vs. control; Fig. 6B). After treatment with EGCG, TGF-β1 and P-smad2/3 expression was significantly reduced (P<0.05 vs. asthma group). After inhibition of TGF-β1 receptor, TGF-β1 and p-smad2/3 expression was significantly increased (P<0.05 vs. EGCG group). These data suggested that EGCG improved the airway inflammation in asthmatic mice through TGF-β1 signaling pathway.

Discussion

This study established mouse models of asthma, and found that EGCG remarkably mitigated the airway inflammation in asthmatic mice, reduced the number of eosinophils and neutrophils in bronchoalveolar lavage fluid, diminished the percentage of Th17 cells, and increased the number of Treg cells, thereby exerting anti-inflammatory effect through TGF-β1 signaling pathway.

Bronchial asthma is a common and frequent chronic inflammatory reaction, frequently occurs in autumn and has the characteristics of recurrent and repeated attacks. Glucocorticoids are still the best drug for the treatment of
Figure 6. In order to further verify the mechanism of EGCG, TGF-β1 receptor inhibitor which is LY 364947 (40 µg/kg/day) was used. The inflammatory factors and TGF-β1 pathway-related protein were detected. Data collected from control group, AS group, HEGCG group and LYEGCG group. (A) The levels of inflammatory factor by CBA. (B) TGF-β1 pathway-related protein expression by western blot analysis. *P<0.05, compared with control group. #P<0.05, compared with AS group. $P<0.05, compared with HEGCG group. EGCG, epigallocatechin gallate; AS, asthmatic; LEGCG, low-dose EGCG; HEGCG, high-dose EGCG; DX, dexamethasone group.
Bronchial asthma; its effect is only to relieve the symptoms associated with asthma, and hormonal treatment is associated with many side effects (25). Therefore, finding effective treatment remains imminent. EGCG is a water-soluble component extracted from green tea polyphenols, and has high antioxidant activity (26). Previous studies demonstrated that antioxidant activity of EGCG was 30 times that of vitamin C and vitamin E (27). It has been found that EGCG can affect apoptosis, autophagy or (and) block the flow of calcium ions in cells, which achieve anti-inflammatory and anti-apoptotic effects (28-30). The present study showed that EGCG could mitigate OVA-mediated airway inflammation in mouse models of asthma, which was consistent with the results of the study by Yu et al (31).

Bronchial asthma is a chronic airway inflammation that is involved in a variety of inflammatory cells. Bronchial asthma was considered to be a disease associated with abnormal differentiation of CD4+ T lymphocytes (32). CD4+ T cells, including Th1, Th2, Th17, and Treg subsets, are regulators of inflammatory reaction of bronchial asthma. The function of these lymphocytes is mainly achieved by secreting cytokines (33). Th17 cells are one of the effectors of host defense, are named for their production of cytokine IL-17a, which has specific effects. Th17 can secrete cytokines such as IL-22, IL-17f and CCL20 at the same time, activate immune cells and non immune cells, and participate in the invasion of extracellular bacteria (34). Treg is a new type of CD4+ T lymphocytes, can effectively control or inhibit the function of related immune cells, regulate immune response, and thus playing an important regulatory role in the immune system (35). Results from this study verified that EGCG could reduce the percentage of Th17 cells, increase the percentage of Treg cells, and then suppress inflammatory reaction.

The association between TGF-β and airway remodeling in bronchial asthma has been studied extensively, considering that TGF-β is an important inflammatory factor for airway remodeling (36,37). TGF-β combines with type II receptor to form a dimer. After conformation changes, it is recognized by the type I receptor and combined to form trimer. Simultaneously, type I receptor is activated by phosphorylation and activates its substrate, amplifying the signal and sending it downstream (38). Smads are an important intracellular TGF-β signal transduction and regulatory molecule, can transfer TGF-β signals directly from the cell membrane to the nucleus. Many studies have suggested that Smad2, Smad3 and Smad7 in Smad protein family are involved in TGF-β signal transduction and play an important role in the formation of airway remodeling in bronchial asthma (39). TGF-β antibody antagonist could adjust the expression of TGF-β signaling pathway by Smad2/3 expression, and then regulate airway remodeling (40). In the present study, We established the model of bronchial asthma and found that EGCG has the effect of reducing airway asthama. However, the mechanism of EGCG is unclear. We suspect that may be related to the TGF-beta signaling pathway, after the inhibition of TGF-β1 receptor, the protective effect of EGCG disappeared; IL-6, TNF-α and IL-17a levels increased; but IL-10 levels significantly decreased. These findings further confirmed that EGCG acts through TGF-β1 signaling pathway.

In conclusion, EGCG mitigated AWR, lessened airway inflammation, reduced the number of eosinophils and neutrophils in bronchoalveolar lavage fluid, suppressed the percentage of Th17 cells, and increased the percentage of Treg cells. This protective effect is achieved via the TGF-β1 signaling pathway. This will provide a theoretical basis for the clinical treatment of asthma and the research and development of new drugs. This study has many deficiencies, for example, whether EGCG plays a role through binding to TGF-β1 receptor or by which specific mechanism EGCG regulates TGF-β signal pathway needs further study.

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Competing interests
The authors declare that they have no competing interests.

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