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The Whole Extract of *Enterococcus faecalis* Has Suppressive Effect on the Allergic Responses in Asthmatic Mouse Model

Jeong Hyun Chang[1], EunJu Yang[1], Sun Nyoung Yu[2] and Soon-Cheol Ahn[2*]

[1]*Department of Clinical Laboratory Science, Daegu Haany University, Gyeongsan 38610, Korea*
[2]*Department of Microbiology and Immunology, Pusan National University School of Medicine, Yangsan 50612, Korea*

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Probiotics are usually defined as intestinal bacteria that provide healthy benefit to the host and may offer new therapeutic materials for the treatment of inflammatory diseases. *Lactobacillus, Bifidobacterium* and *Enterococcus* are known as typical probiotics. But, these bacteria have mostly a weak viability and thus decreased probiotics-mediated effects in the intestinal tract. Asthma is an inflammatory airway disease, which is characterized by the releases of inflammatory mediators including cytokine and IgE. They are mainly associated with the recruitment, activation and disregulation of specific inflammatory cells, especially mast cells, monocytes, T cells, eosinophils and neutrophils in asthma. We performed these studies as *in vitro* and *in vivo* test the human inflammatory cell lines and ovalbumin (OVA)-induced asthma mouse model. And then the inhibitory effects of *Enterococcus faecalis* whole extract on inflammatory responses were examined. For our examinations, the *E. faecalis* whole extract (Ef extract) was acquired from whole bacteria of *E. faecalis* using freeze/thawing after ultrasonication method. As results, OVA-mediated THP-1 cell viability was decreased by the treatment of Ef extract. In the asthmatic mouse model, Ef extract inhibited the infiltration of inflammatory cells into the inflammatory sites and blood. This whole extract may have anti-asthmatic effects associated with the regulation of IL-5 and IgE expression. It may also be a promising candidate in anti-allergic medicine for the treatment of asthma.

Key words : Asthma, *Enterococcus faecalis*, IgE, IL-5, inflammatory cell

Introduction

Asthma is an inflammatory disease of the lungs characterized by increased infiltration of leukocytes, especially eosinophils, into the airways and reduced respiratory function. The inflammation leads to bronchoconstriction, increased airway hyperresponsiveness, and mucus production [5]. In asthma, mast cells migrate into the inflammatory sites and are then activated by IgE, which is produced by activated plasma cells [18]. The activated mast cells secrete various pro-inflammatory mediators such as histamine, cytokines and prostaglandin, which induce eosinophilia and mucus production in lung tissue [1]. Eosinophils are infiltrated into the airways and act as primary effector cells by the release of specific granule proteins and reactive oxygen species [6]. T helper (Th) 2 cytokines induce elevation of IgE in serum and BAL fluid [7]. In Th2 cytokines, IL-4 plays important roles in immunoglobulin E (IgE) switching in B cells, mucus hypersecretion, and eosinophil infiltration into the lung tissues [10]. IL-5 promotes the proliferation and activation of eosinophils and increases eosinophil infiltration into the airways [16]. In addition to IL-5, the modulations of other cytokines are closely linked with the development of asthma. The prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries [2]. However, because the absence of proper treatment, we have been needed the development of therapeutic agents without adverse effect.

Normal flora is a microorganism that resides on a number of tissues and organs. In various normal flora, probiotics are usually defined as intestinal bacteria that provide healthy benefit to the host and may offer new therapeutic materials for the treatment of inflammatory diseases. *Lactobacillus*, known as common probiotics, reduced 50% of prevalence in early atopic dermatitis in children [8], and suppressed proliferation and inflammatory response of CD4+ T cells

***Corresponding author**
Tel : +82-51-510-8092, Fax : +82-55-382-8090
E-mail : ahnsc@pusan.ac.kr
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[22]. But, because these bacteria have almost a weak viability in the intestinal tract, *Lactobacillus*-mediated probiotic effects lead to be decreased. Other probiotics, *Enterococcus* may also be founded in vaginal tract and can contribute to the acidic environment. However, this bacteria can easily acquire an antibiotic resistance ability and then transmit resistance factor to other harmful bacteria [19]. For these reasons, WHO has avoided that *Enterococcus* is being used as a probiotics. Nevertheless, in other study, it has been reported that *Enterococcus* has suppressive effect on superantigen toxic syndrome toxin-1 induced IL-8 from vaginal inflammation [3].

In the present study, to investigate the inhibitory effects of *Enterococcus faecalis* whole extract on asthmatic responses, its cytotoxic effects on the asthma-related cell was determined *in vitro*. The effects of *E. faecalis* whole extract on leukocyte infiltration, IgE level and IL-5 level were determined using *in vivo* Ovalbumin-specific asthma mouse model.

Materials and Methods

Preparation of *Enterococcus faecalis* whole extract

Enterococcus faecalis (ATCC® 19433TM) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). *E. faecalis* was grown overnight in brain-heart infusion broth (BD, Franklin lakes, NJ, USA) at 37℃. After 24 hr, cultured bacteria were concentrated by centrifugation at 20,000× g for 10 min. Cell pellets were washed three times with PBS and suspended in 1 ml of PBS. To obtain bacteria whole extract, *E. faecalis* suspension was disrupted ultrasonically at 20 kHz for 10 cycles of 30 sec (at 4°C). And then bacterial debris was heated at 75℃ for 30 sec, and lyophilized. The *E. faecalis* (Ef) whole extract was resuspended in PBS at a concentration of 10 mg/ml.

Cell viability

Cell viability of THP-1 cells was determined using an MTT assay kit (Roche, Penzberg, Germany). In brief, 100 µl of these cells (1×10^6 cells/ml) were seeded in 96-well plate and incubated for 24 hr after treatment with various concentrations (0, 30 and 60 µg/ml) of Ef extract and 3 µg/ml of dexamethasone (Dex), respectively. 10 µl of MTT solution (0.5 mg/ ml) was added, and the cells were incubated at 37℃ for 4 hr. 100 µl of solubilization solution was then added to each well. After 24 hr incubation, the optical densities of 96-well culture plate were measured using a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) at 540 nm. The optical density of untreated control cells was taken as 100% viability.

Induction of asthma in BALB/c mice

Six-week-old female BALB/c mice were obtained from DaehanBiolink Co. LTD (Seoul, Korea) and kept in an air-conditioned room at 22±1℃ and a humidity of 55±10%. The mice were divided into five groups (n=4) and the allergic lung inflammation of the mice in four groups was induced by ovalbumin (OVA) (Grade III) (Sigma-Aldrich, MO, USA) using the established protocol in a previous study [23]. Asthmatic mice groups were induced four times OVA challenges with PBS or Ef extract. Briefly, mice were sensitized via intra-peritoneal injection with 75 µg of OVA adsorbed to 2 mg of aluminum hydroxide (Alum; Sigma-Aldrich) in 200 µl of 0.9% sterile saline on day 1, 2, 8 and 9. For challenge, 50 µg of OVA was intra-nasally administered to the mice on day 15, 16, 22.

The asthma-induced mice were treated 10 times with oral injection of 30 mg/kg and 60 mg/kg of Ef extract and 3 mg/kg of Dex during 20 days, respectively. The normal group was sensitized and challenged with PBS without OVA and Ef extract treatment.

Measurment of airway hyperresponsiveness

And day 23 and airway hyperresponsiveness (AHR) was measured on day 24 and the mice were sacrificed on day 25. After last OVA challenges, airway responsiveness was monitored a change using whole-body plethysmography for animals (Allmedicus, Anyang, Korea) in response to aerosolized methacholine (Sigma-Aldrich). The enhanced pause (PenH) was evaluated at baseline (PBS) and after treatment with increasing doses of aerosolized methacholine (0-50 mg/ml) for measuring bronchoconstriction. The mice were permitted to acclimate for 3 min, exposed to nebulized PBS for 10 min, and then subsequently treated in methacholine using an ultrasonic nebulizer (Omron, Koyto, Japan). After each nebulization, the average of PenH values were measured during each 150 sec periods. All procedure for the handling and care of animals were approved by animal ethics committee of Pusan national university (ED-PNU2016-1056).

Collection of blood and serum

At day 25 after the first ovalbumin (OVA) sensitization, the mice were sacrificed. And the blood was collected by

heart puncture. After blood sampling, the blood smear films were prepared for leukocyte differential counting and stained with Wright staining solution. The cells in blood smear film were divided into neutrophils, eosinophils, basophils, monocyte and lymphocyte according to general leukocyte morphology and staining features. The percentage of each cell was determined by counting 300 cells per slide. Also, blood were concentrated by centrifugation at 2,000× g for 10 min and the supernatant (serum) was stored at -70℃ until analysis of IL-5 and immunoglobulin E (IgE).

Collection of bronchoalveolar lavage (BAL) fluid

After the mice were sacrificed, BAL fluid was collected by lavage of the lung via the trachea with 1 ml of PBS. After three lavages, the total cells were separated from the supernatant of BAL fluid. The BAL fluid were used for the determination of total cells and differential counting. Total cell number was counted using a Neubauer hemocytometer. And the cells were cytospined and stained with Wright stain solution for differential counting. The cells in BAL fluid were divided into neutrophils, eosinophils, lymphocyte, and alveolar macrophage according to general leukocyte morphology and staining features. The percentage of each cell was determined by counting 300 cells per cytospined slide.

Determination of cytokine concentrations

In the serum, the concentrations of IL-5 and the total IgE were measured using OptEIA Set mouse IL-5 and IgE (BD, Franklin lakes, NJ, USA) according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve of indicated protein.

Statistical analysis

All data were expressed as the means ± S.E.M. Data were analyzed by one-way ANOVA or Student's *t* test using the SPSS statistical software package, Version 10.0 (SPSS Inc., Chicago, IL, USA). A *p* value less than 0.05 was considered significant.

Results

Ef extract has the inhibitory effect on cellular inflammation induced by OVA

Prior to examining the effect of Ef extract on asthma mouse model, it was investigated whether Ef extract has a cytotoxic effect. For this examination, we used the THP-1 cell, the human monocytic cell line. Monocytes act on the pathogenesis of asthma. The survival rate of THP-1 cell was not changed after treatment with Ef extract for 24 hr in a dose-dependent manner (Fig. 1A). These results indicate that Ef extract used in this study have no cytotoxic effect on the cells. Also Ef extract considerably decreases the elevated rate of survival by OVA in THP-1 cells (Fig. 1B). Dex was used

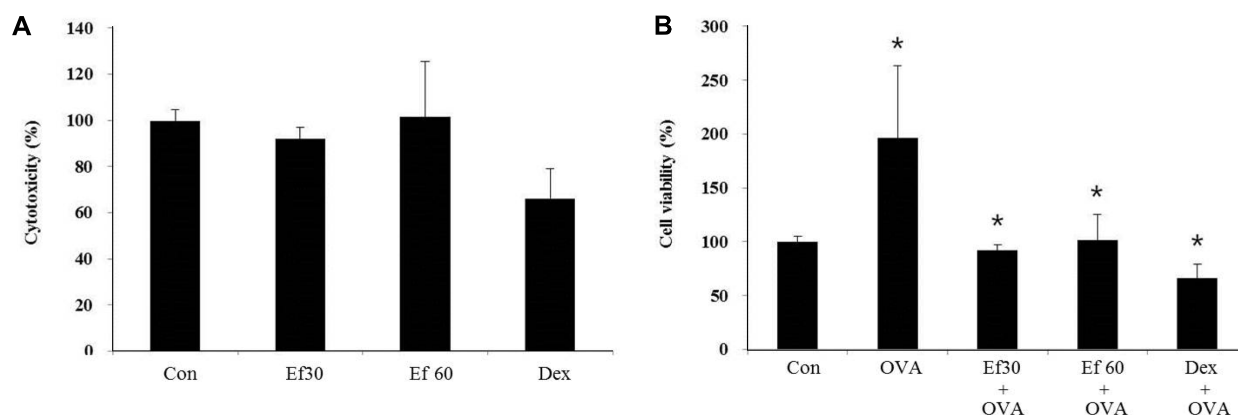

Fig. 1. The effect of Ef extract on the viability of THP-1 cells. THP-1 cells were seeded into 96-well plate at 5×10^4 cells/100 µl per well. (A) These cells were treated with Ef extract at the concentration of 30 µg/ml (Ef 30), 60 µg/ml (Ef 60), or with 1 µg/ml of dexamethasone (Dex) for 24 hr. (B) THP-1 cells were pre-treated with the Ef extract at the concentration of 30 µg/ml (Ef-30), 60 µg/ml (Ef 60), or with 1 µg/ml of dexamethasone (Dex) for 1 hr. And then the cells were treated with 100 µg/ml of OVA for 24 hr. The cell viability was measured by MTT assay as described in Materials and Methods section. The data were expressed as the relative ratio to the absorbance of the untreated cells (Con), which was set at 100%. All data were expressed as the means ± S.D. of three independent experiments. *p<0.05 indicates statistically significant differences between the control group .

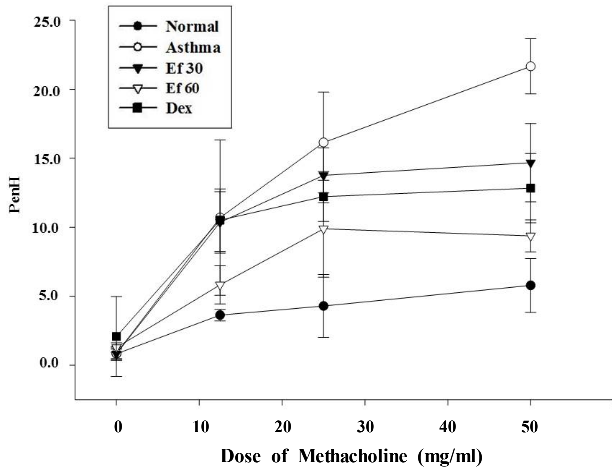

Fig. 2. The effect of Ef extract on airway hyperresponsiveness in OVA-induced asthma mouse model. The OVA-induced asthma model mice were divided into 5 groups with 30 mg/kg (Ef 30), 60 mg/kg (Ef 60), or 3 mg/kg of dexamethasone (Dex) after the sensitization and the negative control group (Normal) without drug administration. The PenH was evaluated at baseline and after aerosolized administration with increasing doses of methacholine (0, 12.5, 25, 50 mg/ml). All data were expressed as the means ± S.D. of three independent experiments.

as a positive control for its inhibitory effects.

Ef extract inhibits the infiltration of eosinophils and neutrophils in a mouse model of asthma

To examine the pathogenesis in asthma, we induced asthma in BALB/c mice using OVA and evaluated the number of eosinophils in the BAL fluid. In a mouse model of allergic asthma, we compared the effects of Ef extract, delivered by means of nebulization only or oral administration. Both Ef extract treatments were equally efficient at reduction AHR to methacholine, as measured on the basis of whole-body plethysmography. In OVA-sensitized and -challenged mice, the dose-response curve of Penh value was shifted to the upper of the image compared with that of normal mice (Fig. 2). As shown in Fig. 2, related to asthma group, AHR to methacholine was reduced in Ef extract treated mice.

In addition, the alteration of cellular components, especially eosinophils, in the airway is an important step in the pathogenesis of asthma. In BAL fluid of normal subject, the macrophage is the most abundant cell and there are very little other cells, including neutrophil and eosinophils. However, differential leukocyte counting showed that neutrophil and eosinophils were increased in asthma-induced mouse group (Fig. 3). In the blood of asthmatic mouse, inflammatory cells, such as eosinophils, neutrophils and basophils, were also increased (Fig. 4). Infiltration of these inflammatory cells into BAL fluid and blood were significantly inhibited by Ef extract (Fig. 3 and Fig. 4). Dex was used as a positive control for its inhibitory effects on asthmatic response. Dex is one of the anti-inflammatory drugs used in the treatment of asthma and its action is associated with inhibition of pro-inflammatory transcription factors.

Ef extract decreases the release of total IL-5 and IgE in serum in a mouse model of asthma

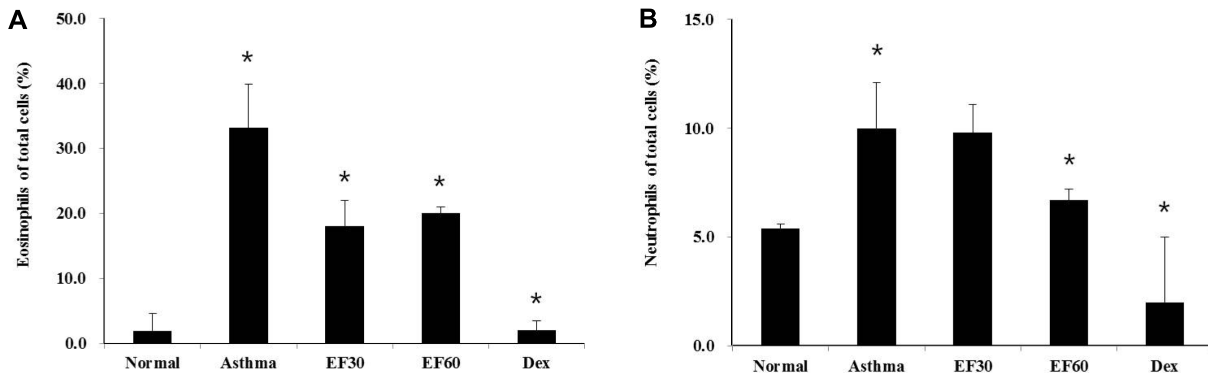

Fig. 3. The effect of Ef extract on the recruitment of inflammatory cells into BAL fluid in OVA-induced asthma mouse model. (A) The rate of eosinophils in total cells of BAL fluid. (B) The rate of neutrophils in total cells of BAL fluid. The OVA-induced asthma model mice were divided into 5 groups with 30 mg/kg (Ef 30), 60 mg/kg (Ef 60), or 3 mg/kg of dexamethasone (Dex) after the sensitization and the negative control group (Normal) without drug administration. The recovered cells in BAL fluid were used for differential counting after Wright's staining. The results were the means ± S.D. of 4 independent experiments. *$p<0.05$ indicates statistically significant differences between the normal group and asthma group, or between the asthma group and Ef extract-treated group. (*$p<0.05$)

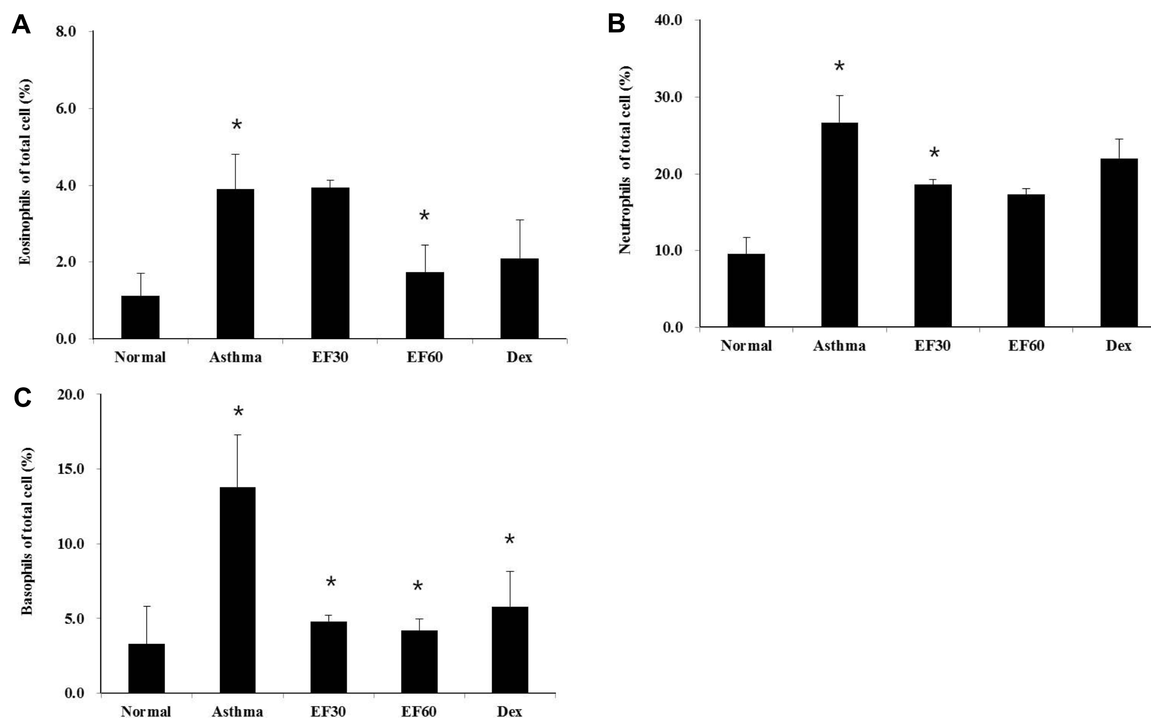

Fig. 4. The effect of Ef extract on the recruitment of inflammatory cells into blood in OVA-induced asthma mouse model. (A) The rate of eosinophils in the total blood cells. (B) The rate of neutrophils in the total blood cells. (C) The rate of basophils in the total blood cells. The OVA-induced asthma model mice were divided into 5 groups with 30 mg/kg (Ef 30), 60 mg/kg (Ef 60), or 3 mg/kg of dexamethasone (Dex) after the sensitization and the negative control group (Normal) without drug administration. The slides of blood smear were used for differential counting after Wright's staining. The results were the means ± S.D. of 4 independent experiments. *p<0.05 indicates statistically significant differences between the normal group and asthma group, or between the asthma group and Ef extract-treated group.

Since the activation and recruitment of inflammatory cells, especially eosinophils, are particularly modulated by IL-5 [6], the IL-5 expression in serum was evaluated. The IL-5 level increased after asthma induction and the increased expression was remarkably inhibited by the Ef extract (Fig. 5). These results indicated that the inhibitory effects of Ef extract on eosinophilia and the infiltration of inflammatory cells are associated with the modulation of IL-5 release.

It has been reported that IgE is associated with the pathogenesis of allergic asthma. To examine the effect of Ef extract on the level of total IgE, the total IgE in the serum were analyzed using ELISA. The total IgE in the serum were considerably increased in the asthmatic control as compared to the normal group (Fig. 6). As shown in Fig. 6, Ef extract suppressed the protein level of IgE in the serum.

Discussion

Asthma is an inflammatory disease of the airways characterized by infiltration of various inflammatory cells, reversible airway obstruction and bronchial hyperresponsiveness [4]. As the prevalence of asthma increases around the world, the development of an effective therapeutic drug for asthma was needed. Recent studies have reported the anti-allergic mechanism regulated by several herbs both *in vitro* and *in vivo* models [9, 13, 14, 20]. In various probiotics, the whole extract of bacteria has been widely used as a therapeutic agent in various inflammatory diseases. In this study, the used Ef extract suppressed the ovalbumin OVA-induced THP-1 cell viability without cytotoxicity (Fig. 1A and Fig. 1B). OVA is the protein purified in egg white and acts as a representative stimulator for inflammatory reaction and allergic reaction of airway hyper-responsiveness [11]. THP-1 is the human monocytic cell line and these monocytic cells, such as alveolar macrophages in the lung tissue, are activated by allergen and then produce inflammatory factors (chemokines, cytokines and toxic molecules). In these processes, alveolar macrophages contribute to the airway inflammation and remodeling responses during asthma [12].

We determined whether Ef extract has an inhibitory effect

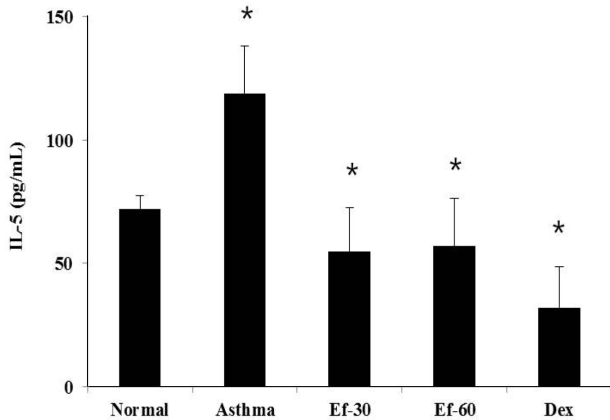

Fig. 5. The effect of Ef extract on the level of IL-5 in OVA-induced asthma mouse model. The OVA-induced asthma model mice were divided into 5 groups with 30 mg/kg (Ef 30), 60 mg/kg (Ef 60), or 3 mg/kg of dexamethasone (Dex) after the sensitization and the negative control group (Normal) without drug administration. The serum was collected, the cells in the blood were eliminated. The protein expression of IL-5 in serum was analyzed by ELISA. The results were expressed as the means ± S.D. of 4 independent experiments. *$p<0.05$ indicates statistically significant differences between the normal group and asthma group, or between the asthma group and Ef extract-treated group.

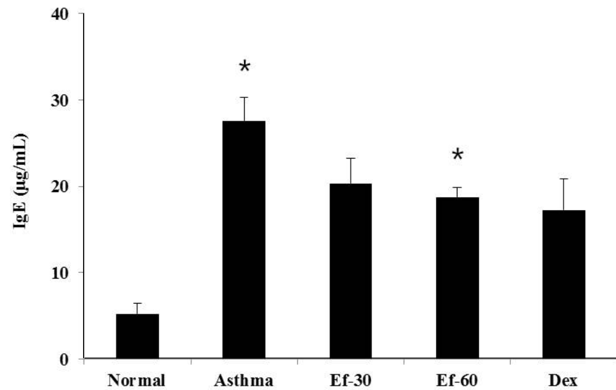

Fig. 6. The effect of Ef extract on the level of IgE in OVA-induced asthma mouse model. The OVA-induced asthma model mice were divided into 5 groups with 30 mg/kg (Ef 30), 60 mg/kg (Ef 60), or 3 mg/kg of dexamethasone (Dex) after the sensitization and the negative control group (Normal) without drug administration. The serum was collected, the cells in the blood were eliminated. The protein expression of IgE in serum was analyzed by ELISA. The results were expressed as the means ± S. D. of 4 independent experiments. *$p<0.05$ indicates statistically significant differences between the normal group and asthma group, or between the asthma group and Ef extract-treated group.

on the OVA-induced inflammatory reaction. To confirm of anti-inflammatory effect of Ef extract, we induced asthma in BALB/c mice using OVA and treated Ef extract by oral injection. As a result, the number of inflammatory cells, including eosinophils and neutrophils, in the lung tissues were elevated in asthmatic mice, where were recovered by treatment with the Ef extract (Fig. 3). Also, eosinophils, neutrophils and basophils were increased in the blood of asthmatic mice (Fig. 4) and its inflammatory cells were suppressed by treatment with the Ef extract (Fig. 4). In allergic diseases, an imbalance of Th1 and Th2 is important in the pathogenesis processes. Th2 cytokines produced by Th2 cells are typically increased in the serum and lung tissue of allergic disease and have important roles on infiltration of inflammatory cells, eosinophil activation, production of IgE and the release of allergic mediators [21]. Among these Th2 cytokines, IL-5 in the serum was strongly elevated in asthmatic mice but was diminished by the Ef extract (Fig. 4). IL-5 plays a pivotal role in the maturation and recruitment of eosinophils into the airways [17]. Eosinophils are known as effector cells in allergic disease by cytokine release, cytotoxic granule proteins and tissue-damaging superoxide [16].

In addition, the elevated IgE in the early asthmatic response induces the degranulation of mast cells by cross-linkage of allergen-specific IgE, and this process is an important step in the development of asthmatic responses [15]. The total IgE in serum increased after the induction of asthma (Fig. 6). The total IgE was significantly suppressed by Ef extract in the serum (Fig. 6). Although the exact mechanism for this activity is unclear, these results suggest that Ef extract blocks specific IgE induction by certain allergens. Ef extract may provide a useful therapeutic approach for the treatment of allergic airway diseases.

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초록 : 천식 마우스 모델의 알러지 반응에서 *Enterococcus faecalis* 전체 추출물의 억제 효과

장정현[1], 양은주[1], 유선녕[2], 안순철[2]*
([1]대구한의대학교 임상병리학과, [2]부산대학교 의학전문대학원 미생물학 및 면역학 교실)

프로바이오틱스(probiotics)는 숙주에 이로운 작용을 하는 장내세균으로 주로 알려져 있으며 꾸준히 염증치료의 새로운 치료물질로 주목 받고 있다. 그러나 *Lactobacillus*나 *Bifidobacterium*과 같은 전형적인 프로바이오틱스는 장내에서 꾸준히 생존하기 어렵고 그로 인해 매개할 수 있는 다양한 효능이 줄어든다. 반면, *Enterococcus*는 다양한 물질을 분비하고 생물학적 활성을 지니고 있는 프로바이오틱스이긴 하나 동시에 기회감염균으로 항생제 내성능을 쉽게 획득할 수도 있어 사용을 자제할 것을 권고하고 있으며, Vancomycin 내성인자를 원래부터 가지고 있어서 이 인자의 발현과 함께 타 독성균주에 전달할 가능성이 있다. 그러나 본 연구에서는 이러한 *Enterococcus*의 추출물을 이용하여 항염증 물질을 확인해보고자 하였다. 특히, 호흡기계 질환인 천식의 치료효능을 보기 위해 human monocytic cell line인 THP-1 세포를 이용한 *in vitro* test와 천식 유발 BALB/c mice를 이용한 *in vivo* test를 시행하였다. 천식은 사이토카인과 IgE 등 세포 염증성 물질을 분비하고 호산구, 호중구, 호염기구, 비만세포, 단구, T 세포 등 다양한 염증 세포가 폐 부위로 침윤되는 복합적인 염증 질환으로 잘 알려져 있다. 본 연구에서는 우선 *Enterococcus faecalis* 에서 초음파 처리를 통해 균체성분을 분해한 다음 동결건조하여 추출물을 획득하였다. *E. faecalis*에서 추출한 추출물(Ef extract)을 PBS에 부유시킨 다음, 염증유발을 위해 ovalbumin (OVA)를 처리한 THP-1 세포에 처리한 결과, OVA에 의해 증가한 THP-1 세포의 생존율이 감소하였다. 또한 OVA에 의해 천식이 유발된 BALB/c mice에서 염증부위인 폐의 세척액을 채취하여 폐 조직 내 세포 변화를 관찰하였다. 그 결과, Ef extract에 의해 호산구와 호중구가 Ef extract 농도에 따라 점차 감소하였고 혈액 내에서도 Ef extract에 의해 호산구, 호중구, 호염기구가 감소하였다. 이는 Ef extract에 의해 염증세포로부터 IL-5의 분비를 억제하고 IgE의 양을 감소시켜 이와 관련된 염증세포의 침윤 및 증가를 억제한 것으로 사료된다. 이를 통해 Ef extract 가 천식 동물모델에서 항염증효과가 있음을 관찰하였지만 Ef extract의 구성 물질 분석 및 작용 메커니즘에 대한 추가연구를 통해 천식 치료물질로의 가능성을 규명할 것이다.