House Dust Mite Allergen Inhibits Constitutive Neutrophil Apoptosis by Cytokine Secretion via PAR2/PKC δ /p38 MAPK Pathway in Allergic Lymphocytes

Na Rae Lee¹, Ji-Sook Lee², In Sik Kim^{1,3}

¹Department of Biomedical Laboratory Science, School of Medicine, Eulji University, Daejeon 34824, Korea

²Department of Clinical Laboratory Science, Wonkwang Health Science University, Iksan 54538, Korea

³Department of Senior Healthcare, BK21 Plus Program, Graduate School, Eulji University, Daejeon 34824, Korea

알레르기 림프구에서 집먼지진드기 알러젠의 PAR2/PKC δ /p38 MAPK 경로를 통한 사이토카인 증가는 호중구의 세포고사를 억제시킨다

이나래¹, 이지숙², 김인식^{1,3}

¹을지대학교 대전캠퍼스 임상병리학과, ²원광보건대학교 임상병리과, ³을지대학교 대전캠퍼스 BK21 플러스 프로그램 시니어 헬스케어학과

Neutrophils and lymphocytes are essential inflammatory cells in the pathogenesis of allergy. In this study, we evaluated the role of house dust mite (HDM) in the interaction between allergic lymphocytes and neutrophils. The extract of *Dermatophagoides pteronissinus* (DP) showed a stronger anti-apoptotic impact on neutrophil apoptosis in the coculture of allergic neutrophils with allergic lymphocytes when compared with that in allergic neutrophils alone. DP increased IL-6, IL-8, MCP-1, and GM-CSF in allergic lymphocytes, and the increased cytokines were inhibited by rottlerin—an inhibitor of the protein kinase C (PKC) δ , as well as by SB202190—a p38 MAPK inhibitor. DP activated p38 MAPK in a time-dependent manner. The activation of p38 MAPK was suppressed by PAR2i, which is a protease-activated receptor (PAR) 2 inhibitor, and rottlerin. Both aprotinin—a serine protease inhibitor—and E64—a cysteine protease inhibitor—were not effective on cytokine secretion of lymphocytes. These results, despite increased cytokines in allergic lymphocytes via DP, did not show any differences between asthma and allergic rhinitis. Molecules, including cytokines, released by DP in lymphocytes inhibited the migration of neutrophils. This finding may further elucidate the pathogenic mechanism of allergic diseases due to HDM.

Key words: House dust mite, Cytokine, Neutrophils apoptosis, Allergy

Corresponding author: In Sik Kim
Department of Biomedical Laboratory Science
School of Medicine, Eulji University, 77,
Gyeryoung-ro 771 beon-gil, Jung-gu, Daejeon
34824, Korea
Tel: 82-42-259-1753
Fax: 82-42-259-1759
E-mail: orientree@eulji.ac.kr

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Introduction

Allergic diseases are characterized by elevated IgE in response to house dust mite (HDM) and inflammatory

responses, depending on survival and migration of leucocytes [1-3]. HDMs are mainly composed of *Dermatophagoides pteronissinus* (DP) and *Dermatophagoides farinae*. HDM exposure initiates allergic diseases, such as asthma, allergic

rhinitis, and atopic dermatitis, as well as aggravating symptoms of the diseases by increased inflammation [4,5]. The pathogenic mechanism is caused by allergen proteins contained in HDM, such as serine and cysteine proteases, lipid-binding protein, and chitinase [6,7]. It has been recently reported that regulation of neutrophil apoptosis due to HDM is important in the pathogenesis of asthma and allergic rhinitis [8-10].

Regulation of cytokine secretion in lymphocytes is an essential process of the pathogenesis of allergic diseases. Regarding allergic inflammation, IL-6 induces the transition from acute to chronic stage [11]. MCP-1/CCL2 and IL-8/CXCL8 play roles as chemoattractants, inducing monocytes and neutrophils, respectively [12,13]. Moreover, GM-CSF induces the differentiation of granulocyte precursors [9]. These cytokines are involved in the proliferation and anti-apoptotic action of neutrophils [9,10].

We have reported that HDM induces the cytokines associated with neutrophil survival in lymphocytes, which regulate neutrophil apoptosis via the PAR2/PI3K/Akt/ERK/NF- κ B pathway [14]. In this study, we examined another PAR2-mediated signal mechanism due to HDM in allergic lymphocytes. We also investigated the difference of cytokine secretion due to HDM between asthma and rhinitis, as well as the effects of secreted molecules induced by HDM on neutrophil chemotaxis.

Materials and Methods

1. Reagents

RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Gaithersburg, MD, USA). DP was obtained from Cosmo Bio (Tokyo, Japan). Rottlerin and SB202190 were purchased from Calbiochem (San Diego, CA, USA). ENMD-1068, a PAR2 antagonist (PAR2i), was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The protease inhibitors, aprotinin and E64 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-p38 MAPK and p38 MAPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Normal and allergic subjects

A total of 27 allergy patients were recruited from Eulji University Hospital. Allergic status was based on the presence of positive results of a skin prick test ($\geq 2+$), multiple allergen simultaneous test (MAST) ($\geq$ class 2), or evaluation of specific HDM IgE using the Pharmacia Unicap 100 system (Pharmacia, Uppsala, Sweden) for common allergens (≥ 100 IU/ml). The normal subjects ($n=3$) had no history of asthma or allergic rhinitis. This study was approved by the Institutional Review Board of Eulji University for normal volunteers and the Institutional Review Board of Eulji University Hospital for allergic patients [2015-07-022]. All participants in this study gave their written informed consent.

3. Isolation of lymphocytes and neutrophils and cell culture

Human lymphocytes and neutrophils were separated from the heparinized peripheral blood (10 mL) of healthy persons and allergic subjects using Ficoll-Hypaque gradient centrifugation. A CD16 microbeads magnetic cell sorting kit and a monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for neutrophil and lymphocyte separation, respectively. The cells were washed after hypotonic lysis to remove erythrocytes. Neutrophils and lymphocytes were resuspended at 3×10^6 /ml and 2×10^6 /ml in RPMI 1640 medium with 1% penicillin-streptomycin and 10% FBS.

4. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6, IL-8, GM-CSF, and MCP-1 in a cell supernatant were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) using OptEIA™ Set human IL-6, IL-8, GM-CSF, and MCP-1 (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

5. Detection of apoptosis

An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was used for detection of neutrophil apoptosis. Isolated neutrophils were treated with DP, then incubated with FITC-labeled annexin V and propidium iodide (PI) for 15 min at room

temperature. Apoptotic neutrophils showing annexin V+/PI- and annexin V+/PI+ were analyzed using a FACSCalibur flow cytometer with the CellQuest software (BD bioscience) and were reported as the percentage.

6. Western blotting

After being treated with DP, cells were harvested and lysed in Ceti lysis buffer (TransLab, Daejeon, Korea). The lysate was centrifuged at $12,000 \times g$ for 15 min at $4^{\circ}C$, after which the supernatant was collected. The protein samples (50 μg /lane) were separated by SDS-polyacrylamide gel electrophoresis, after which they were transferred to membranes and

incubated with anti-phospho p38 MAPK antibodies and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same blot was stripped and reprobed with anti-p38 MAPK antibodies for use as an internal control.

7. Chemotaxis assay

The activity of cell migration was estimated using a 48-well microchamber (Neuroprobe, Gaithersburg, MD, USA). The lower wells were filled with 28 μL buffer alone or with buffer containing IL-8. A polyvinylpyrrolidone (PVP)-free filter with 5 μm pore size was placed over each lower well. Cell

Fig. 1. The secretion of IL-6, IL-8, MCP-1, and GM-CSF is inhibited by rottlerin and SB202190 in allergic lymphocytes. (A) Allergic neutrophils or neutrophils and lymphocytes (1:1 ratio) ($n=4$) were incubated for 24 hr in the absence and presence of DP (10 $\mu g/mL$). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. (B) Allergic lymphocytes ($3 < n < 5$) were pre-treated for 1 hr with and without PAR2i, 5 μM rottlerin (Rt), and 20 μM SB202190, after which the cells were incubated for 24 hr in the absence and presence of DP (10 $\mu g/mL$). The supernatant was collected and analyzed by ELISA. Data are expressed as the means $\pm$ S.E.M. * $p < 0.05$ and ** $p < 0.01$ indicate a significant difference.

suspension was added with 50 μ L of neutrophils at 2×10^6 cells/mL to the upper wells. The assembled chamber was incubated for 90 min at 37°C in a humidified incubator. Non-migrated cells adhering to the upper surface of polycarbonate filter were removed with a filter wiper. The filter was dried, fixed, and stained with Diff-Quick (Baxter, McGaw Park, IL, USA). The cells from four randomly selected fields per well were counted, and a single experiment includes six replicate measurements.

8. Statistical analysis

Data were expressed as the means $\pm$ S.E.M. Statistical differences were analyzed using a paired t-test for two-group comparisons and one-way ANOVA for comparison of more than two groups. All analyses were conducted using the SPSS statistical software package (Version 10.0, Chicago, IL, USA), and a p value < 0.05 was considered to indicate statistical significance.

Results

1. The secretion of IL-6, IL-8, MCP-1, and GM-CSF induced by DP was involved in PKC δ and p38 MAPK in allergic lymphocytes

To the best of our knowledge, this study is the first to confirm that secretory cytokines of DP treated-lymphocytes are related to neutrophil survival before investigating a PAR2-mediated signal mechanism induced by DP on cytokine secretion of allergic lymphocytes. Allergic neutrophils were cocultured with allergic lymphocytes, and the cells were treated with DP. Spontaneous neutrophil apoptosis was strongly suppressed by the coculture of neutrophils with

lymphocytes after DP treatment, as compared with neutrophils alone (Fig. 1A). We then examined the alteration of cytokine after treatment with signal inhibitors. Both rottlerin and SB202190 significantly inhibited the production of IL-6, IL-8, MCP-1, and GM-CSF increased by DP ($p < 0.05$) (Fig. 1B). Given these results, we examined the association of PKC δ and p38 MAPK with the effect of DP. p38 MAPK was phosphorylated by DP in a time-dependent manner, and the activation was inhibited by PAR2i and rottlerin (Fig. 2). In addition, both aprotinin—a serine protease inhibitor—and E64—a cysteine protease inhibitor—had no effect on the alteration of cytokines induced by DP in normal and allergic lymphocytes; however, DP was closely associated with protease activity (Fig. 3).

2. The effect of DP on cytokine release in allergic lymphocytes was not different between asthma and allergic rhinitis

Since the pathogenic mechanisms of allergic diseases are different depending on the individual allergy, we examined the difference of increased cytokines in the lymphocytes of asthma and allergic rhinitis. As shown in Fig. 4, there was a lack of difference between asthma and allergic rhinitis in the cytokine production of lymphocytes by DP.

3. The secretory molecules produced by allergic lymphocytes inhibited the neutrophil chemotaxis in response to IL-8

Since the released cytokines due to DP alter neutrophil apoptosis and cell migration plays an important role in allergic inflammation, we examined whether the released molecules due to DP affect neutrophil migration. Supernatant

Fig. 2. DP activates p38 MAPK via PAR2 and PKC δ in allergic lymphocytes. (A) Normal lymphocytes were incubated with DP (10 μ g/mL) for the indicated time. (B) Normal lymphocytes were pre-treated for 1 hr with and without PAR2i (B) and 5 μ M rottlerin (Rt), after which harvested cells were lysed and phosphorylation of p38 MAPK in the lysates was detected by Western blotting.

Fig. 3. The secretion of IL-6, IL-8, MCP-1, and GM-CSF due to DP is not associated with protease activity. Allergic (A) and normal (B) lymphocytes (n=3) were pre-treated for 1 hr with and without 50 µg/mL E64 and aprotinin (AP), after which the cells were incubated for 24 hr in the absence and presence of DP (10 µg/mL). The supernatant was collected and analyzed by ELISA. Data are expressed as the means±S.E.M. * $p<0.05$ and ** $p<0.01$ indicate a significant difference.

from allergic lymphocytes inhibited the neutrophil migration and DP-treated supernatant inhibited the movement more strongly (Fig. 5).

Discussion

House dust mite (HDM) is an important allergen that evokes and aggravates allergic diseases, including asthma and allergic rhinitis. The pathogenic mechanisms of these

diseases are related to the dysregulation of various cytokines released from lymphocytes and survival of inflammatory leucocytes, specifically neutrophils [3-5]. As shown in Figs. 1 and 2, DP increased the cytokines released, which were involved in neutrophil survival. This mechanism was accomplished by the PAR2/PKCδ/p38 MAPK pathway. PAR2 is closely involved in allergic disease. Thymic Stromal Lymphopoietin (TSLP), increased by PAR2 activation, induces or aggravates atopic dermatitis [15]. Transient PAR2 lowers

Fig. 4. The secretion of IL-6, IL-8, MCP-1, and GM-CSF due to DP is not different between asthma and allergic rhinitis. Asthma and allergic rhinitis lymphocytes ($n=10$) were pre-treated for 1 hr with and without DP (10 µg/mL), after which the cells were incubated for 24 hr and 48 hr in the absence and presence of DP (10 µg/mL). The supernatant was collected and analyzed by ELISA. Data are expressed as the means $\pm$ S.E.M.

Fig. 5. The secretory molecules produced by allergic lymphocytes inhibit neutrophil chemotaxis in response to IL-8. Allergic lymphocytes ($4 < n < 6$) were incubated in the absence or presence of 10 µg/mL of DP for 24 hr and supernatant was collected. Neutrophils were treated with the supernatant, and a chemotaxis assay was performed. Data are presented relative to the control, which was set at 100% of the means $\pm$ S.E.M. ** $p < 0.01$ indicates a significant difference.

the bronchospasm by relaxing the airway smooth muscle. However, chronic PAR2 activation induces pro-inflammatory responses [16]. Studies on the association between PAR2 with PKC δ is lacking in the literature. PKC δ is involved in PAR2-mediated signal mechanism for the production of thromboxane in platelets [17]. The activation of PKC β and PKC δ triggers basolateral PAR2-induced chloride secretion [18]. Our results show that ERK1/2 is not a downstream molecule of PKC δ , but p38 MAPK, which is activated by DP in a time-dependent course (Fig. 2). These results indicate that the novel PAR2/PKC δ /p38 MAPK pathway is an important signal mechanism in allergic diseases.

Apoptosis and movement of leucocytes are essential steps in the pathogenic mechanism of allergic diseases. HDM induces allergic diseases and aggravates the clinical features

and symptoms of the diseases by altering the mechanisms of apoptosis and chemotaxis [19,20]. DP suppressed neutrophil apoptosis in the coculture of allergic neutrophils and lymphocytes. Moreover, it also inhibited the migration of neutrophils (Fig. 1A and Fig. 5). The molecules secreted by lymphocyte with no treatment also suppressed the chemotaxis of neutrophils, and the inhibitory effect was weaker than that of DP-treated supernatant. We believe that DP stops the movement of neutrophils and delays neutrophil apoptosis in inflammatory sites by activating lymphocytes. In summary, DP increases IL-6, IL-8, MCP-1, and GM-CSF via the PAR2/PKC δ /p38 MAPK pathway in allergic lymphocytes, and the secreted cytokines delay constitutive neutrophil apoptosis. These findings may shed light on the complex pathogenic mechanism of allergic diseases by understanding the interaction between lymphocytes and neutrophils.

요 약

본 연구에서는 집먼지 진드기 추출물은 호중구에 단독으로 작용하는 것보다, 림프구와 호중구의 공동배양에서 호중구의 세포고사를 더 억제시켰다. 집먼지 진드기는 알레르기 질환의 림프구에서 IL-6, IL-8, MCP-1, GM-CSF의 분비를 증가시켰다. 집먼지 진드기에 의해 증가된 사이토카인은 protein kinase C δ 의 억제제인 rottlerin과 p38 MAPK의 억제제인 SB202190에 의해서 감소하였다. 집먼지 진드기에 의해 활성화된 p38 MAPK은 protease-activated receptor (PAR2)의 억제제, rottlerin, SB202190에 의해서 억제되었다. Serine protease 억제제인 aprotinin과 cysteine protease 억제제인 E64은 림프구의 사이토카인의 증가와 관련이 없었다. 또한 집먼지 진드기에 의해 증가된 사이토카인의 변화는 천식과 알레르기 비염 환자에서 차이가 없었다. 림프구에서 집먼지 진드기에 의해서 분비되는 분자들은 호중구의 유주운동을 억제시켰다. 본 연구를 통하여 집먼지진드기에 의해 유발되는 알레르기 질환의 병인기전을 규명하는데 유용한 결과가 될 것이다.

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