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S100A8 and S100A9 Secreted by Allergens in Monocytes Inhibit Spontaneous Apoptosis of Normal and Asthmatic Neutrophils via the Lyn/Akt/ERK Pathway

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단구에서 분비되는 S100A8과 S100A9의 Lyn/Akt/ERK 경로를 통한 정상인과 천식질환 호중구의 세포고사 억제 효과

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Der p 1 and Der p 2 are essential allergens of house dust mite associated with the development of asthma. In the present study, we examined whether Der p 1 and Der p 2 induce a release of S100A8 and S100A9 in monocytes, which are involved in the regulatory mechanism of neutrophil apoptosis. We found that Der p 1 and Der p 2 significantly increased the secretion of S100A8 and S100A9 in normal monocytes. Moreover, S100A8 and S100A9 strongly suppressed the spontaneous apoptosis of normal and asthmatic neutrophils. The inhibitory effect of S100A9 was stronger than that of S100A8, and asthmatic neutrophils showed a higher inhibitory effect than normal neutrophils. S100A8 and S100A9 induced activation of Lyn, Akt, and ERK in a time-dependent manner. These findings elucidate the roles of Der p 1 and Der p 2 in the interaction between monocytes and neutrophils, as well as contributing to our knowledge of the pathogenesis of allergic diseases.

Key words: Allergen, S100 protein, Monocyte, Neutrophil apoptosis

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INTRODUCTION

Allergic diseases including asthma, allergic rhinitis, and allergic conjuctivitis are caused by environmental, genetic, and immunological factors [1,2]. Asthma is an allergic disease characterized by airway obstruction, bronchial inflammation, and allergen-specific IgE. House dust mites were composed of *Dermatophagoides pteronissinus* and *Dermatophagoides farinae*, are closely related to asthma pathogenesis [3,4]. Der p 1 and Der p 2 are major allergens of *Dermatophagoides pteronissinus*. Der p 1 induces cleavage of protease-activated receptor (PAR), which results in allergic inflammation. Der p 1 also facilitates allergen invasion and increases IgE production [5,6]. Der p

2, a group II allergen from of *Dermatophagoides pteronissinus*, elicits an inflammatory process including secretion of cytokine such as IL-4, IL-6, and IL-8, and most asthmatic subjects were sensitized to Der p 2 [7,8].

S100A8 and S100A9 belong to the S100 family proteins and are constitutively expressed in monocytes and neutrophils [9-11]. They play as damage-associated molecular pattern (DAMP) via Toll-like receptor 4 (TLR4) and receptor for advanced glycation endproducts (RAGE), and triggers the pathogenesis of asthma, chronic obstructive pulmonary disease, colitis, rheumatoid arthritis, Alzheimer's disease, and tumor [12,13]. Our reports recently demonstrated that S100A8 and S100A9 induce cytokine secretion, which is involved in regulation of neutrophil apoptosis [14,15].

In this work, we studied the roles of Der p 1 and Der p 2 in production of S100A8 and S100A9 of normal monocytes, as well as constitutive neutrophil apoptosis of normal and allergic subjects due to S100A8 and S100A9 released by Der p 1 and Der p 2.

MATERIALS AND METHODS

1. Reagents

RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Gaithersburg, MD). Der p 1 and Der p 2 were obtained from INDOOR biotechnologies (Charlottesville, VA, USA). Antibodies against phospho-Lyn and phospho-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospho-Akt and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-S100A8 and S100A9 antibodies were obtained from Abnova (Taipei, Taiwan).

2. Normal and allergic subjects

Allergic asthma subjects were recruited from Konyang University Hospital. Asthmatic patients had mild to severe symptoms of the disease. The normal subjects had normal lung function, no history of asthma, and did not require medication. This study was approved by the Institutional Review Board of Eulji University for normal volunteers and by the Institutional Review Board of Konyang University for asthma patients. All participants in this study gave their written informed consent.

3. Isolation of neutrophils and monocytes, and and cell culture

Human monocytes and neutrophils were isolated from the peripheral blood of normal and asthmatic subjects using Ficoll-Hypaque (Amersham Phamacia Biotech, Buckinghamshire, UK) gradient centrifugation. CD16 microbeads magnetic cell sorting kit and a monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for neutrophil and monocyte isolation, respectively. The cells were washed after hypotonic lysis to remove erythrocytes. Neutrophils and monocytes were resuspended at 3×10^6/mL and 2×10^6/mL in RPMI 1,640 medium with 1% penicillin-streptomycin and 10% FBS (Life Technologies, Gaithersburg, MD, USA). This method routinely yielded greater than 97% neutrophil purity and more than 90% monocyte purity.

4. Flow cytometry

After treatment with Der p 1 or Der p 2, human monocytes were harvested and washed twice with PBS. The cells were then fixed with 100 μL of 0.37% paraformaldehyde solution for 15 min at room temperature. Following removal of the fixing solution, the cells were added to 100 μL of 0.2% Triton X-100 in PBS and incubated for 3 min. Next, the cells were washed twice with PBS buffer containing 0.5% BSA, after which non-specific antibody binding was reduced by incubating the cells with normal rabbit IgG. The cells were subsequently separated into new tubes, to which PBS buffer containing anti-S100A8 and anti-S100A9 antibodies was added. Baseline fluorescence was obtained by incubation with normal mouse IgG instead of anti-S100 protein antibodies. After washing three times, the cells were incubated at 4°C for 30 min with FITC-conjugated goat anti-mouse IgG (Molecular Probes; Eugene, OR, USA). Finally, the cells were washed and analyzed on a FACSort cytofluorimeter

(Becton Dickinson). The mean intensity of untreated cells was considered 100%. Alteration of intracellular S100A8 and S100A9 expression after Der p 1 or Der p 2 stimulation was evaluated as the mean intensity of Der p 1 or Der p 2-treated cells/the mean intensity of untreated cells ×100.

5. Production of recombinant S100A8 and S100A9 proteins

In our previous report, the cDNA of human S100A8 and S100A9 was cloned into pET28 expression vector (Merck Millipore, Darmstadt, Germany) [16]. Recombinant S100A8 and Millipore.

S100A9 expression was then induced with 1 mM isopropyl β-D-thiogalactoside in *E. coli* BL21 (DE3, Merck Millipore) for 4 h and 16 h at 37°C, respectively. Thereafter, the bacteria were centrifuged at 5,000 g for 10 min and the pellet was lysed in BugBuster Protein Extraction reagent (Merck Millipore).

6. 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 incubated with FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic neutrophils were analyzed using a FACSCalibur flow cytometer with the CellQuest software (BD Bioscience) and reported as the percentage of cells showing annexin V+/PI- and annexin V+/PI+.

7. Western blotting

After being stimulated with S100A8 or S100A9, neutrophils were harvested and lysed in a lysis buffer. The homogenate was then centrifuged at 10,000 g for 1 min at 4°C, and the supernatant was collected. The protein samples (50 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis. The transferred membranes were incubated with anti-phospho-Lyn, anti-phospho-Akt, and anti-phospho ERK1/2 antibodies and then developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The same blot was stripped and reprobed with anti-ERK2 antibodies for use as an internal control.

8. Statistical analysis

Data were expressed as the means±SD 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 version 10.0 (SPSS, Chicago, IL, USA), and a p-value <0.05 was considered to indicate statistical significance.

RESULTS

1. Der p 1 and Der p 2 increase the secretion of S100A8 and S100A9 in normal monocytes

We examined, for the first time, the production of S100A8 and S100A9 due to Der p 1 and Der p 2 in normal monocytes. Treatment with Der p 1 and Der p 2 at the concentrations of 10 μg/mL for 24 h increased the release of S100A8 and S100A9 of normal monocytes (Figure 1). These results indicate that the effects of house dust mite on cytokine release is associated with Der p 1 and Der p 2.

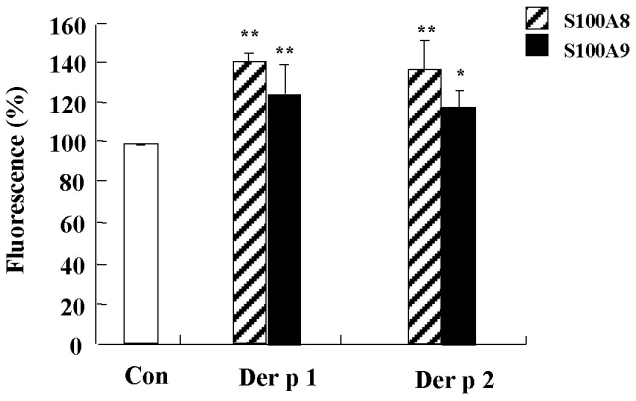

Figure 1. Der p 1 and Der p 2 increase the secretion of S100A8 and S100A9 in normal monocytes. Monocytes isolated from normal were incubated in the absence or presence of Der p 1 or Der p 2 at the concentrations of 10 μg/mL for 24 h, after which the expression of S100A8 and S100A9 was measured by flow cytometry as described in the materials and methods section.

2. S100A8 and S100A9 induced by Der p 1 and Der p 2 suppress spontaneous apoptosis of normal and allergic neutrophils

Because Der p 1 and Der p 2 increase the expression of S100A8 and S100A9 associated with neutrophil survival, we investigated whether S100A8 and S100A9 inhibit constitutive neutrophil apoptosis or not. As shown in Figure 2 and 3, S100A8 and S100A9 were significantly effective on inhibition of normal and allergic neutrophil apoptosis, despite the different degree of inhibition ($p<0.05$). These results indicate that the effect of house dust mite on cytokine secretion of monocytes is involved in inhibition of neutrophil apoptosis.

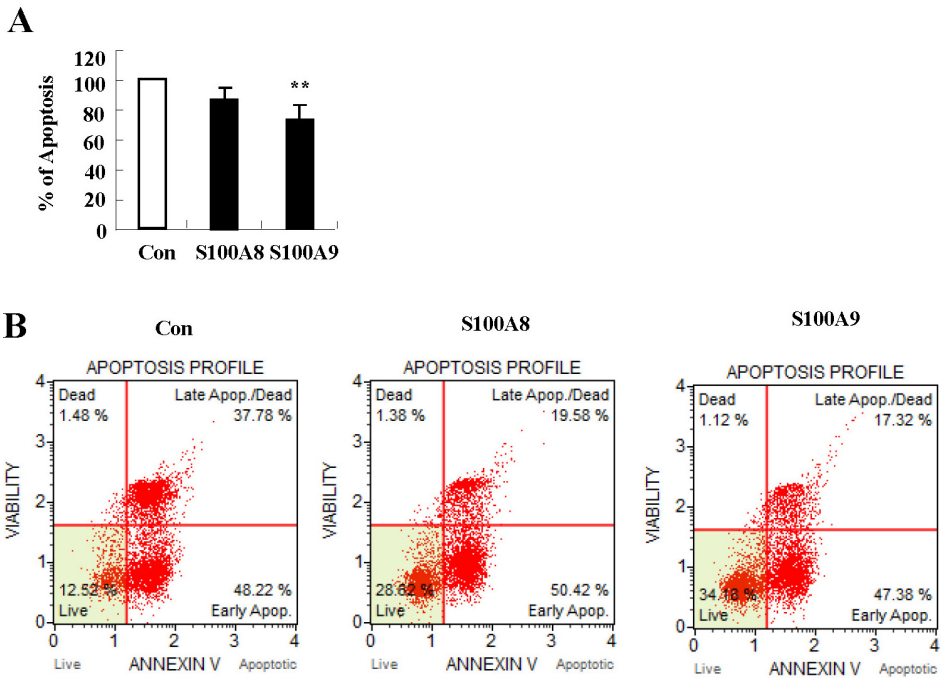

Figure 2. S100A8 and S100A9 induced by Der p 1 and Der p 2 suppress spontaneous apoptosis of normal neutrophils. (A) Normal neutrophils were incubated with 10 μg/mL S100A8 or 10 μg/mL S100A9 for 24 h. Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means±SD and are presented relative to the control, which was set at 100%. *$p<0.05$ and **$p<0.01$ indicate a significant difference between the control and stimulator-treated groups. (B) Flow cytometry dot plots are presented .

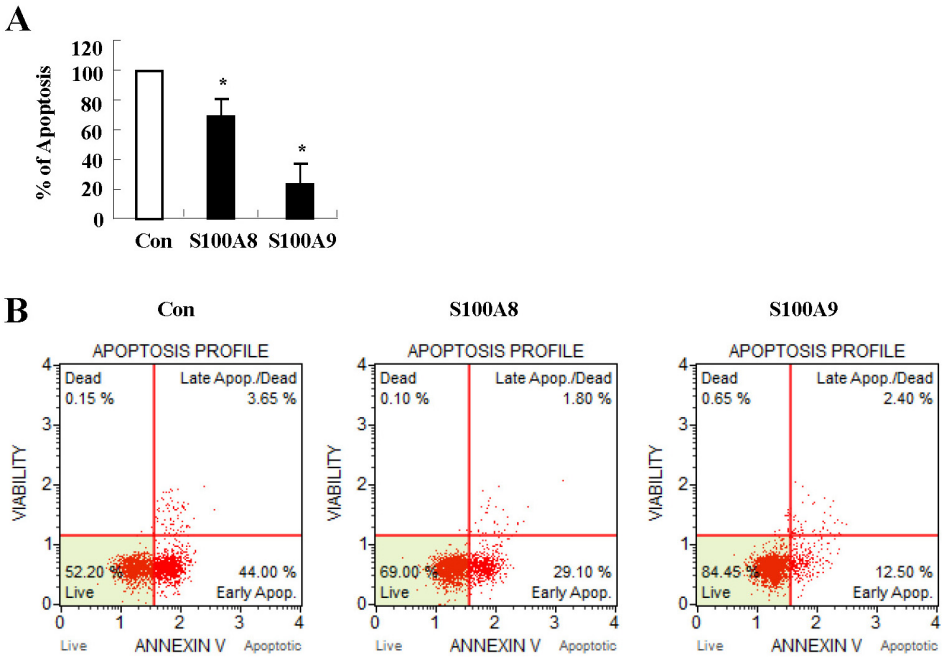

Figure 3. S100A8 and S100A9 induced by Der p 1 and Der p 2 suppress spontaneous apoptosis of asthmatic neutrophils. (A) Asthmatic neutrophils were incubated with 10 μg/mL S100A8 or 10 μg/mL S100A9 for 24 h. Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means±SD and are presented relative to the control, which was set at 100%. *$p<0.05$ indicates a significant difference between the control and stimulator-treated groups. (B) Flow cytometry dot plots are presented.

3. S100A8 and S100A9 have anti-apoptotic effects on neutrophils via Lyn, Akt, and ERK

Because secretory molecules of monocytes, S100A8 and S100A9, after Der p 2 treatment are involved in neutrophil apoptosis, we investigated the exact anti-apoptotic mechanism of S100A8 and S100A9 in neutrophils. As shown in Figure 4, S100A8 and S100A9 induced the phosphorylation of Lyn, Akt, and ERK in a time-dependent manner. These results indicate that Lyn, Akt, and ERK arre essential signal molecules in inhibitory effects of S100A8 and S100A9 on neutrophil apoptosis.

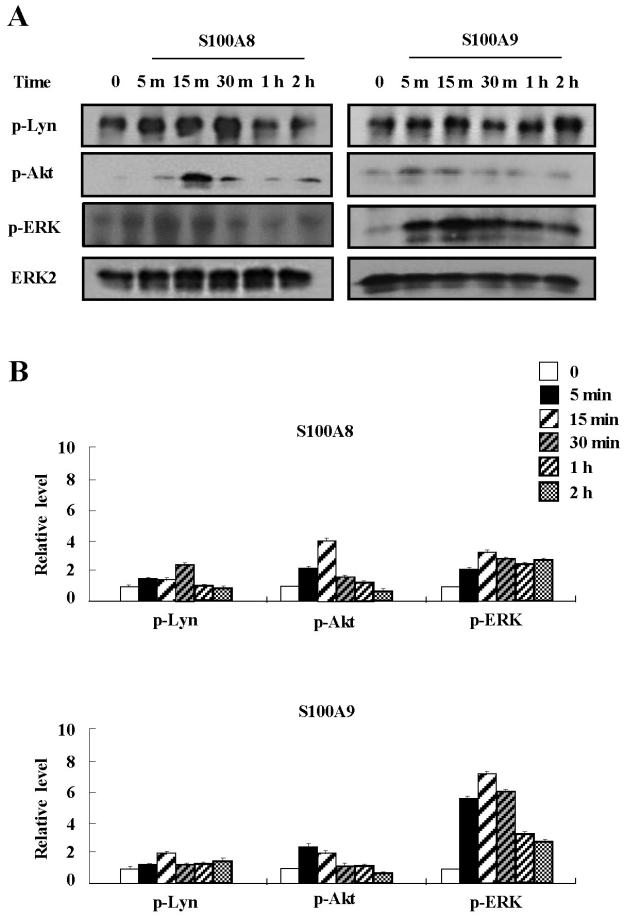

Figure 4. S100A8 and S100A9 have anti-apoptotic effects on neutrophils via Lyn, Akt, and ERK. (A) Normal blood neutrophils were incubated with S100A8 (10 μg/mL) or S100A9 (10 μg/mL) for the indicated time. Phosphorylation of Lyn, Akt and ERK in the lysates was detected by Western blotting. (B) Densitometric data are presented relative to the negative control (lower panel).

DISCUSSION

House dust mites contain a variety of allergen proteins, which function as cysteine and serine proteases, MD-like molecule, α-amylase, and chitinase [17,18]. Der p 1 and Der p 2 are representative allergens and play as important roles in the pathogenesis of asthma [4,19]. We recently demonstrated that S100A8 and S100A9 act as anti-apoptotic factors in neutrophils [4]. In the present study, we investigated that Der p 1 and Der p 2 regulate neutrophil apoptosis by inducing the production of S100A8 and S100A9 in monocytes.

Asthma consists of neutrophilic and eosinophilic subtypes, depending on pathological features. Neutrophilic asthma is characterized by a persistence of airway neutrophilia. Dysregulation of neutrophil apoptosis is one of the most important causes in the pathogenesis of neutrophilic asthma [2,20]. As shown in Figures 1-3, Der p 1 and Der p 2 enhanced the secretion of S100A8 and S100A9 in monocytes comparable to the results of our previous report [20]. S100A8 and S100A9 are effective on suppression of neutrophil apoptosis (Figure 2, 3). The constitutive apoptosis of normal and allergic neutrophils is inhibited by lymphocyte activation due to Der p 1 and Der p 2 [16,19]. The activated lymphocytes secret the cytokines such as IL-6, IL-8, MCP-1, and GM-CSF, which were essential inhibitory molecules of neutrophil apoptosis. Taken together, alteration of neutrophil apoptosis may be affected by various cytokines such as IL-6, IL-8, S100A8 and S100A9 secreted by monocytes and lymphocytes. The exact mechanism due to Der p 1 and Der p 2 remains to be elucidated. Further study is needed to examine the Der p 1 and Der p 2-mediated signaling related to S100 protein expression and anti-apoptotic signaling.

Since S100A8 and S100A9 have inhibitory effects on neutrophil apoptosis, the fact leads us to examine the exact signal mechanism. As shown in Figure 4, S100A8 and S100A9 inhibit neutrophil apoptosis through Lyn, Akt, and ERK. House dust mite regulates neutrophil apoptosis via TLR4, Lyn, PI3K, Akt, ERK, and NF-κB [15,16]. Anti-apoptotic signaling mediated by MCP-1 is involved in the

PI3K/Akt/ERK/NF-κB cascade in normal neutrophils [21] Leptin delays neutrophil apoptosis via ERK/NF-κB pathway [22]. Based on above results, Lyn, Akt, and ERK are essential proteins in suppression of neutrophil apoptosis. Because Der p 1 and Der p 2 are not directly effective on neutrophil apoptosis in normal and asthmatic subjects [16], Der p 1 and Der p 2 indirectly regulate neutrophil apoptosis of normal and asthmatic subjects by increasing S100A8 and S100A9 in monocytes.

요 약

Der p 1과 Der p 2는 알레르기 질환과 관련된 집먼지 진드기의 핵심적인 알러젠이다. 본 연구에서는 Der p 1과 Der p 2가 단구에서 S100A8과 S10A9을 분비시키는지를 확인하였고, 분비된 S100A8과 S10A9이 호중구의 세포고사 조절기전에 작용하는지를 연구하였다. Der p 1과 Der p 2는 정상인의 단구에서 S100A8과 S10A9을 유의하게 증가시켰고, S100A8과 S10A9은 정상인과 알레르기 질환 호중구의 자발적 세포고사를 억제시켰다. 호중구의 Lyn, Akt, ERK는 S100A8과 S10A9을 시간별로 처리하였을 때 활성화하였다. 본 연구를 통하여 단구와 호중구에서 Der p 1과 Der p 2의 역할을 규명하였고, 나아가 관련된 알레르기 병인기전을 이해하는데 유용할 것이다.

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