Biomedical Science Letters 2014, 20(4): 244~249
http://dx.doi.org/10.15616/BSL.2014.20.4.244
eISSN : 2288-7415

Original Article

Suppressive Effect of Arazyme on Neutrophil Apoptosis in Normal and Allergic Subjects

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Arazyme is a metalloprotease secreted by *Aranicola proteolyticus* that was previously shown to suppress cytokine expression of keratinocytes and endothelial cells and inhibit histopathological features in an atopic dermatitis-like animal model. However, the regulatory effects of arazyme in other allergic diseases have yet to be elucidated. In this study, we investigated whether arazyme is effective against neutrophil apoptosis in allergic diseases such as allergic rhinitis and asthma. Arazyme inhibited neutrophil apoptosis of normal subjects in a dose-dependent manner. However, the anti-apoptotic effect of arazyme was reversed by LY294002, an inhibitor of PI3K, AKTi, an inhibitor of Akt, PD98059, an inhibitor of MEK, and BAY-11-7085, an inhibitor of NF-κB. Arazyme induced activation of NF-κB via PI3K/Akt/ERK pathway. The anti-apoptotic effect of arazyme is associated with inhibition of cleavage of caspase 3 and caspase 9. Arazyme inhibited constitutive apoptosis of neutrophil in a dose-dependent manner in allergic subjects, and its mechanism was shown to be associated with PI3K/Akt/ERK/NF-κB. The results presented here improve our understanding of neutrophil apoptosis regulation and will facilitate development of drugs for treatment of allergic diseases.

Key Words: Arazyme, Allergic disease, Neutrophil apoptosis, Signal transduction

INTRODUCTION

Arazyme is a metalloprotease secreted by *Aranicola proteolyticus* (also known as *Serratia proteamaculans*), which is an aerobic Gram negative symbiotic bacterium that was isolated from the intestine of the spider *Nephila clavata* (Bersanetti et al., 2005; Kwak et al., 2007). Arazyme ameliorates acute liver injury by increasing expression of SMP30 and anti-oxidant proteins (Park et al., 2008). We recently revealed an anti-inflammatory effect of arazyme in both cells and animal models of atopic dermatitis (Kim et al., 2013; Kim et al., 2014a; Kim et al., 2014b). Arazyme suppresses the release of inflammatory cytokines and enhances the expression of skin barrier proteins, specifically filaggrin. In addition, arazyme decreases cytokine production and clinical features in the AD-like animal models, BALB/c and Nc/Nga mice.

Allergic diseases include atopic dermatitis, asthma and allergic rhinitis, which are caused by environmental problems, genetic factors and a deviation of immune response (Cookson WO and Moffatt, 2002; Bieber, 2008; Holgate and Polosa, 2008; Kang et al., 2014). Neutrophils and eosinophils are essential immune cells involved in the pathogenesis of allergic diseases (Lim et al., 1995; Holgate and Polosa, 2008). Persistent neutrophil survival is caused by

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altering constitutive apoptosis of neutrophils and results in aggravation of allergic diseases such as asthma (Scheel-Toellner et al., 2004; Monteseirin, 2009).

Based on the above reports, we examined the effects of arazyme on alteration of spontaneous apoptosis of neutrophils in normal and allergic subjects and its effective mechanism.

MATERIALS AND METHODS

Reagents

RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Gaithersburg, MD). Extract of house dust mite was obtained from Cosmo Bio (Tokyo, Japan). PI3K inhibitor (Ly294002), Akt inhibitor (AKTi), MEK inhibitor (PD98059), and NF-κB inhibitor (BAY-11-7085) were acquired from Calbiochem (San Diego, CA, USA). Antibodies against procaspase 3, procaspase 9, and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Enzyme purification

Arazyme was purified as previously described (Kwak et al., 2007). Briefly, extracellular fractions were collected by centrifugation of the culture medium or by filtration using a 0.2 μL membrane filter (Pall Life Sciences, Port Washington, NY). Chromatography was performed on a DEAE-cellulose column equilibrated with 50 mM potassium phosphate buffer (pH 7.6). Bound proteins were eluted with a concentration gradient of sodium chloride ranging from 0.1 to 0.5 M at a flow rate of 400 mL/h, and each fraction was concentrated with a 10 kD cassette membrane (Pall Life Sciences). Thereafter, the protein solution was loaded at a flow rate of 20 mL/h onto a Sephadex G-75 column previously equilibrated with 50 mM potassium phosphate buffer (pH 7.8). Fractions including proteolytic activity were concentrated with the 10 kD cassette membrane and stored at -20℃.

Normal subjects and asthmatic patients

Patients with allergic disease such as allergic rhinitis or/and asthma were recruited from the Department of Pediatrics at Eulji University Hospital. Patients with allergic rhinitis patients had general nasal symptoms for more than 4 days a week during more than 4 consecutive weeks. Allergic status was based on the presence of positive results of a skin prick test or multiple allergen simultaneous test (MAST) to common allergens. Pathients suffering from asthma had mild to severe symptoms of the disease. The normal subjects had no history of allergic diseases such as allergic rhinitis, asthma, and atopic dermatitis 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 Eulji University Hospital for allergic patients. Written informed consent was obtained from study participants and from parents of children in this study. All participants in this study gave their written informed consent.

Neutrophil isolation and cell culture

Human neutrophils were isolated from the heparinized peripheral blood of healthy persons and asthmatics using Ficoll-Hypaque gradient centrifugation and a CD16 microbeads magnetic cell sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were washed after hypotonic lysis to remove erythrocytes and then resuspended at 3×10^6/ml in RPMI 1640 medium with 1% penicillin-streptomycin and 10% FBS. Counting the cells on cytospin revealed that this method routinely yielded greater than 97% neutrophil purity.

Detection of apoptosis

An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was used to detect neutrophil apoptosis. Isolated neutrophils were incubated with an FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic neutrophils were analyzed using a FACSCalibur with CellQuest software (BD bioscience) and were determined as the percentage of cells showing annexin V+/PI- and annexin V+/PI+. For the morphological estimation of neutrophil apoptosis, neutrophils were cytocentrifuged and stained with Wright staining solution.

Western blotting

After being treated with arazyme, neutrophils were harvested and then lysed in 50 μL lysis buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, and protease inhibitors). They were centrifuged at 12,000 g for 15 min at 4℃. The protein samples (50 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the proteins were transferred to nitrocellulose filters. The blots were incubated with anti-caspase 3 or anti-caspase 9 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.

NF-κB p65 transcription factor assay

The DNA-binding activity of NF-κB was evaluated using EZ-Detect™ transcription factor kits for NF-κB p65 (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. DNA-binding specificity was assessed using wild type or mutant NF-κB oligonucleotides. Chemiluminescent detection was performed using a luminometer.

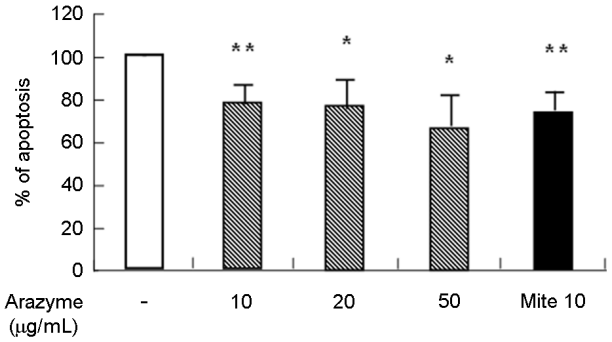

Fig. 1. Arazyme delays constitutive neutrophil apoptosis in normal subjects. Neutrophils were isolated from the peripheral blood of normal subjects (n=4) and then incubated for 24 h in the absence (Con) and presence of house dust mite (Mite) (10 μg/mL) or arazyme in the indicated concentration Apoptosis 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.

Statistical analysis

Data are expressed as the mean ± SD. Statistical differences were analyzed using a paired *t*-test for two-group comparisons. SPSS statistical software, version 10.0 (SPSS, Chicago, IL) was used for statistical analysis. A significant value was considered as $P < 0.05$.

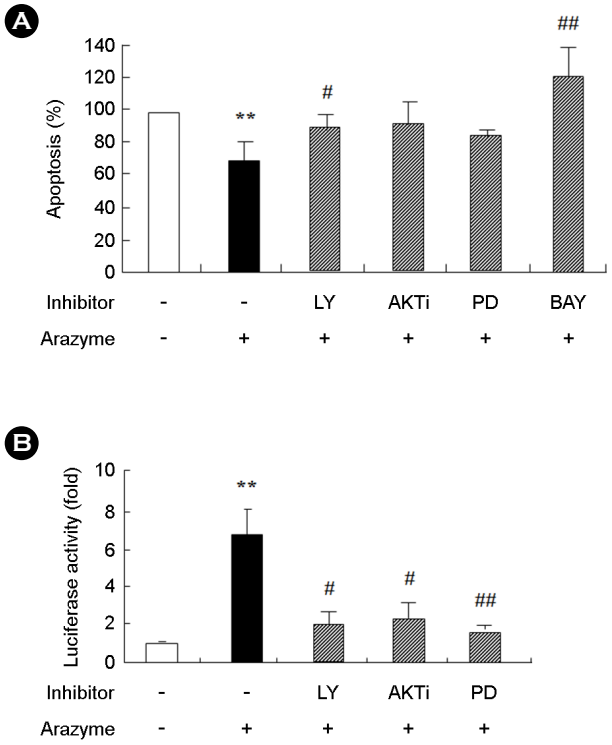

Fig. 2. Arazyme induces suppression of neutrophil apoptosis via activation of the PI3K/Akt/ERK/NF-κB pathway. (A) Normal neutrophils (n=4) were pre-treated for 1 h with and without 10 μM Ly294002 (LY), 10 μM AKTi, 10 μM PD98059 (PD) and 10 μM BAY-11-7085 (BAY), after which the cells were incubated for 24 h in the absence and presence of arazyme (10 μg/mL). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented relative to the control, which was set at 100% of the means ± SD. **$P < 0.01$ indicates a significant difference between the control and arazyme-treated groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ represent a significant difference between the arazyme-treated group and the inhibitor-treated group. (B) Normal neutrophils were pre-treated for 1 h with and 10 μM Ly294002 (LY), 10 μM AKTi, and 10 μM PD98059 (PD) and then incubated with arazyme (10 μg/mL) for 8 h. The nuclear fraction was extracted, and the NF-κB DNA binding activity was evaluated using an EZ-Detect™ transcription factor kit. **$P < 0.01$ indicates a significant difference between the control and arazyme-treated groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ represent a significant difference between the arazyme-treated group and the inhibitor-treated group.

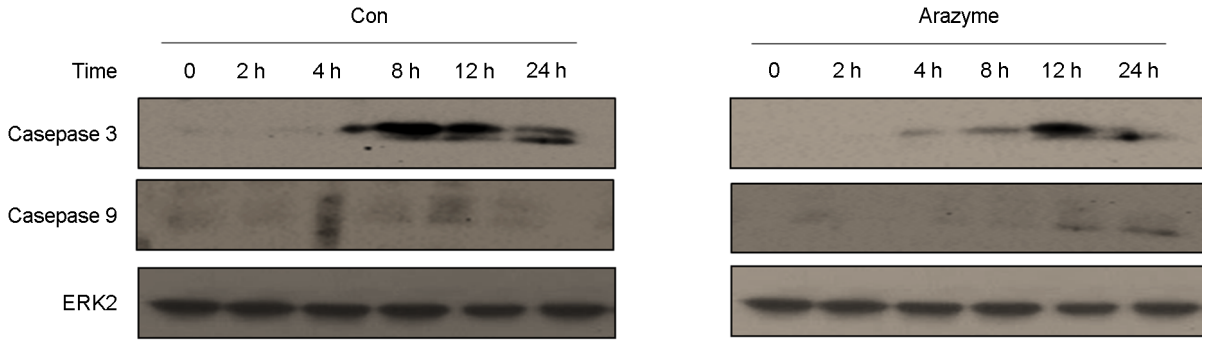

Fig. 3. Arazyme induces inhibition of the caspase9/3 pathway. Normal blood neutrophils were incubated with arazyme (10 μg/mL) for the indicated time. Caspase 9 and caspase 3 were detected by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control.

RESULTS

Arazyme delays constitutive neutrophil apoptosis in normal subjects

To investigate the effects of arazyme on neutrophil apoptosis, we first evaluated whether it alters the regulation of neutrophil apoptosis. Arazyme suppressed neutrophil apoptosis in a dose-dependent manner (Fig. 1). The anti-apoptotic effect is comparable to the effect of hose dust mite as a positive control (Kim et al., 2014c). These results indicate that arazyme has an anti-apoptotic effect on neutrophil apoptosis.

DP induces suppression of neutrophil apoptosis via activation of the PI3K/Akt/ERK/NF-κB pathway and inhibition of the caspase9/3 pathway

Since arazyme inhibits neutrophil apoptosis, we next examined how arazyme tranduces the anti-apoptotic signal. Ly294002, AKTi, PD98059, and BAY 11-7085 blocked the inhibitory effects on neutrophil apoptosis due to arazyme (Fig. 2A). NF-κB is activated at 8 h in neutrophils in contrast to other cells (Yang et al., 2012; Ledoux and Perkins, 2014). Arazyme induced NF-κB activation at 8 h and this activation was inhibited by Ly294002, AKTi, and PD98059 (Fig. 2B). As shown in Fig. 3, The cleavage of procaspase 9 and procaspase 3 increased after constitutive apoptosis began, Arazyme inhibited the cleavage of procaspase 9 and procaspase 3 (Fig. 3).

Arazyme have anti-apoptotic effects in allergic neutrophils

Because arazyme delays neutrophil apoptosis of normal subjects, we examined whether arazyme alters the constitutive apoptosis of allergic neutrophils comparable to normal neutrophil apoptosis. Arazyme inhibited neutrophil apoptosis and the anti-apoptotic effect was suppressed by Ly294002, AKTi, PD98059, and BAY 11-7085. These results indicate that arazyme prolongs neutrophil survival of allergic subjects as comparable to normal subjects and the anti-apoptotic effect is involved in PI3K/Akt/ERK/NF-κB pathway.

DISCUSSION

Arazyme is a metalloprotease that induces anti-inflammatory responses such as downregulation of cytokine and reactive oxygen species (ROS) production (Kim et al., 2013; Kim et al., 2014b). We recently found arazyme to be a useful drug candidate for treatment of atopic dermatitis (Kim et al., 2014a). In the present study, we examined the effects of arazyme in neutrophil apoptosis to evaluate its role as a protease in neutrophil apoptosis and determine if it had the potential for use in treatment of other allergic diseases such as asthma or AR. Metalloprotease binds to protease activated receptors (PARs), which include a distinctive four-member family of seven transmembrane G protein-coupled receptors (Zhang et al., 2014). A variety of proteases are associated with the pathological processes of

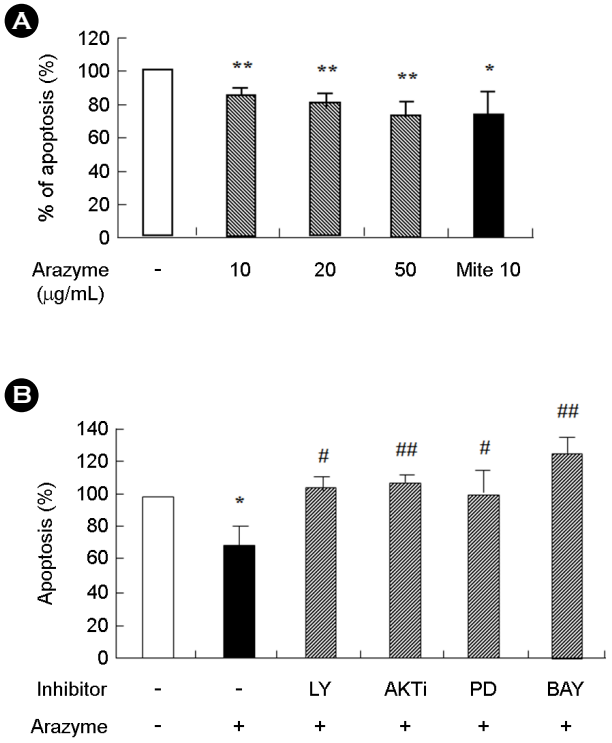

Fig. 4. Arazyme have anti-apoptotic effects in allergic neutrophils. (A) Neutrophils were isolated from the peripheral blood of normal subjects (n=3) and then incubated for 24 h in the absence (Con) and presence of house dust mite (Mite) or arazyme in the indicated concentration Apoptosis was analyzed 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) Normal neutrophils (n=4) were pre-treated for 1 h with and without 10 μM Ly294002 (LY), 10 μM AKTi, 10 μM PD98059 (PD) and 10 μM BAY-11-7085 (BAY), after which the cells were incubated for 24 h in the absence and presence of arazyme (10 μg/mL). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented relative to the control, which was set at 100% of the means ± SD. $*P < 0.05$ indicates a significant difference between the control and arazyme-treated groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ represent a significant difference between the arazyme-treated group and the inhibitor-treated group.

inflammation, including cytokine production, movement of immune cells, and tissue damage. As shown in Figs. 1 and 4, arazyme inhibited constitutive apoptosis of neutrophils in normal and allergic subjects. The anti-apoptotic effects of arazyme are involved in the PI3K/Akt/ERK/NF-κB pathway and the caspase 9/3 pathway (Fig. 2 and 3). The plasticity of the neutrophil life span occurs via blockage of apoptosis by extracellular factors including GM-CSF, tumor necrosis factor-α (TNF-α) and CCL2 released from various cells (Simon, 2003; Luo and Loison; 2008; Yang et al., 2012). Protease may be involved in survival of immune cells such as neutrophils.

In addition, arazyme may aggravate the pathogenesis of allergic diseases. However, we elucidated anti-inflammatory effect of arazyme in keratinocytes and endothelial cells (Kim et al., 2013; Kim et al., 2014b). Arazyme has also been shown to effectively alleviate clinical features of atopic dermatitis in an atopic dermatitis-like mouse model. The above results are contrary to the results of the present study. Allergic diseases are complex conditions caused by various and complicated mechanisms (Bieber, 2008; Holgate and Polosa, 2008). Although arazyme inhibited survival of neutrophils, we cannot determine whether it has harmful effects in allergic diseases. The number of patients with allergic diseases was limited in the present study, and atopic dermatitis, allergic rhinitis and asthma have common and different pathogenic mechanisms. The exact mechanism of arazyme in allergic diseases remains to be elucidated and is the subject of ongoing study.

In summary, arazyme inhibits neutrophil apoptosis via the PI3K/Akt/ERK/NF-κB pathway and the caspase 9/3 pathway. The results presented herein will facilitate development of arazyme as a therapeutic drug for the treatment of allergic diseases.

Acknowledgements

This paper was supported by Wonkwang Health Science University in 2014.

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