

Different anti-apoptotic effects of normal and asthmatic serum on normal eosinophil apoptosis depending on house dust mite-specific IgE

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Abstract We investigated the effect of asthmatic serum on constitutive eosinophil apoptosis in normal subjects. Eosinophil apoptosis in normal subjects was inhibited by asthmatic serum but not normal serum. In a detailed analysis based on the presence of house dust mite (HDM) IgE, HDM IgE-positive (+) asthmatic serum was more effective for eosinophil apoptosis than that of HDM IgE-negative (−) asthmatic serum. HDM IgE+ asthmatic serum inhibited both HDM IgE− and HDM IgE+ normal eosinophil apoptosis, and HDM IgE− asthmatic serum suppressed eosinophil apoptosis of HDM IgE+ normal. HDM IgE− normal serum did not inhibit either HDM IgE− or HDM IgE+ normal eosinophil apoptosis, and HDM IgE+ normal serum inhibited HDM IgE+ normal eosinophil apoptosis. The kind of HDM IgE (*Dermatophagoides pteronissinus*-specific IgE and *Dermatophagoides farinae*-specific IgE) was not related to the effect of asthmatic serum on eosinophil apoptosis. Extracts of DP and DF, Der p1, and Der p2, were not effective for eosinophil apoptosis. HDM IgE+ asthmatic serum inhibited cleavage of procaspase 9 and procaspase 3. Asthmatic serum induced Akt and ERK phosphorylation, and ERK activation was suppressed by

AKTi. Taken together, asthmatic serum inhibited normal eosinophil apoptosis via PI3K/Akt/ERK cascade. The novel approach taken in this study provided better insight into HDM-associated anti-apoptotic mechanism of eosinophils in patients with asthma.

Keywords House dust mite-specific IgE · Eosinophils · Asthma · Constitutive apoptosis

Introduction

Asthma is a respiratory disease with an annual increase in prevalence. It is characterized by allergen-specific IgE, reversible airway obstruction, bronchial inflammation, and mucus hypersecretion. The subtype of asthma is composed of eosinophilic and neutrophilic asthma [1, 2]. The development of asthma is related to various factors such as environmental allergens and genetic modifications [3]. Exposure to house dust mite (HDM), particularly *Dermatophagoides pteronissinus* (DP) and *Dermatophagoides farinae* (DF), is one of the most fundamental causes in asthma pathogenesis [4]. HDM triggers an allergic response through a direct interaction with cell surface receptors and through HDM-specific IgE (HDM IgE) produced by B cells after exposure of the HDM [5, 6]. Patients with atopic asthma show an increased level of HDM IgE and total IgE in serum, in contrast to non-atopic asthmatics [7]. Atopic asthmatics have elevated eosinophils and mast cells [8]. Although the pathogenic mechanism of HDM IgE has been unveiled, the relationship between HDM IgE and eosinophil survival remains unknown.

Human eosinophils play an essential role in the pathogenesis of asthma. Peripheral blood eosinophils migrate to the lung and release mediators to induce lung inflammation

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and affect broncho-constriction and mucus secretion [3]. In addition, apoptosis to remove eosinophils is delayed by a variety of exterior ligands such as interleukin (IL)-5, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF) and retinoic acid [9–11]. Delayed eosinophil apoptosis may induce continuous tissue inflammation. The anti-apoptotic mechanism of eosinophils is involved in the PI3K/Akt pathway, the JAK/STAT pathway, and the caspase cascade [9, 12].

Based on the importance of HDM IgE, we investigated the effect of asthmatic serum on constitutive apoptosis in normal eosinophils and examined anti-apoptotic signaling due to asthmatic serum in normal eosinophils.

Materials and methods

Normal subjects and patients with asthma

Twenty-five patients with asthma were randomly recruited from Konyang University Hospital and showed peripheral eosinophilia. Twenty normal subjects were recruited as controls. The normal subjects had normal lung function (FEV1/FVC, 91.6 ± 4.9 %) compared to the lung function of the asthmatics (FEV1/FVC, 68.1 ± 11.0 %), had no history of asthma, and did not require medication. This study was approved by the Institutional Review Board of Eulji University for the normal volunteers and by the Institutional Review Board of Konyang University for the patients with asthma. All participants gave their written informed consent.

Measurement of serum HDM IgE

Serum was loaded into a Pharmacia Unicap 100 system (Pharmacia Unicap, Uppsala, Sweden). The DP or DF allergen covalently coupled to the cellulose solid-phase ImmunoCap and reacted with the specific IgE in the patient's serum specimen. After washing, enzyme-labeled antibodies against IgE were added to form a complex. The IgE concentration was measured by fluorescence. HDM (DP or/and Df) IgE+ is defined by >0.35 KU/L.

Eosinophil isolation and cell culture

Human granulocytes were isolated from heparinized peripheral blood of healthy subjects using Ficoll–Hypaque gradient centrifugation and the cells were washed after hypotonic lysis buffer to remove erythrocytes. Eosinophils were negatively separated from neutrophils using a CD16 microbead magnetic cell sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils were resuspended at 3×10^6 /ml in an RPMI 1640 medium (Life

Technologies, Inc., Gaithersburg, MD, USA) with 1 % penicillin–streptomycin and 10 % fetal bovine serum (Life Technologies). This method routinely yielded >97 % eosinophil purity as assessed by Cytospin counting of the cells.

Detection of constitutive apoptosis

An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was used to detect eosinophil apoptosis. After treatment with the DP and DF extracts (Korea National Arthropods of Medical Importance Resource Bank, Seoul, Korea), Der p1, or Der p2 (Indoor Biotechnologies, Charlottesville, VA, USA), isolated eosinophils were incubated with FITC-labeled Annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic eosinophils were analyzed using a FACSCalibur with CellQuest software (BD Biosciences) and were determined as the percentage of cells showing Annexin V+/PI– and Annexin V+/PI+.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-4, IL-5, and GM-CSF in normal and asthmatic sera were measured with a sandwich ELISA using the OptEIA™ Set human IL-4, IL-5, and GM-CSF (BD Biosciences).

Western blotting

After treatment with the asthmatic serum, the cells were harvested and lysed in extraction buffer (Intron Biotech., Seongnam, Korea). The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C. The supernatant was collected and the protein samples (50 µg/lane) were separated by SDS–polyacrylamide gel electrophoresis. The transferred membranes were incubated with anti-procaspase 3, anti-procaspase 9, anti-phospho-Akt, anti-Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-phospho ERK antibodies (Cell Signaling Technology, Beverly, MA, USA) and were developed with the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The same blot was stripped and reprobed with anti-ERK2 antibody (Santa Cruz Biotechnology) as an internal control.

Statistical analysis

All data are expressed as mean $\pm$ SE. Data were analyzed by one-way analysis of variance using the SPSS statistical software package, version 10.0 (SPSS Inc., Chicago, IL, USA). A *p* value < 0.05 was considered significant.

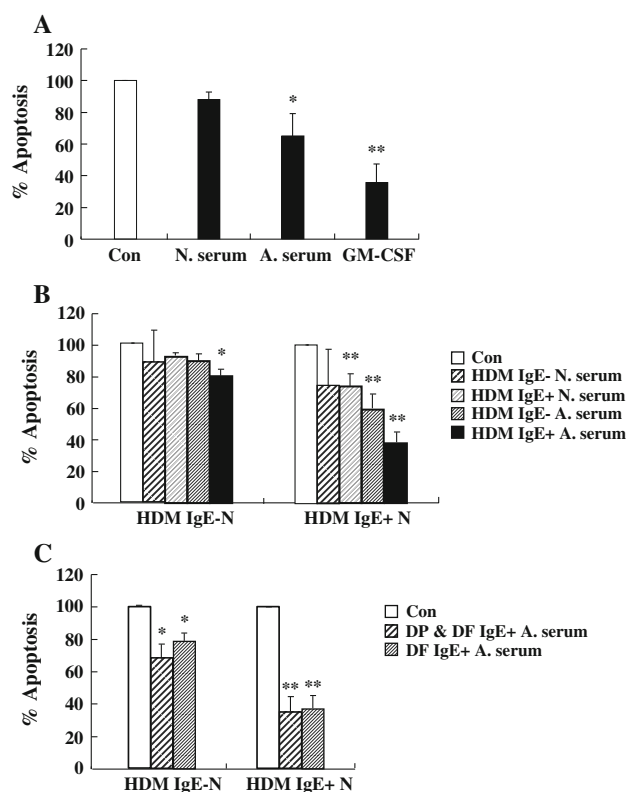


Fig. 1 HDM IgE is an essential factor in the regulation of eosinophil apoptosis by asthmatic serum. Eosinophils were isolated from peripheral blood of normal subjects ($n = 20$) and the cells were incubated for 48 h in the absence (Con) or presence of normal ($n = 20$) and asthmatic sera ($n = 25$) (serum:medium = 1:1), and GM-CSF (100 ng/ml). Apoptosis was analyzed by measuring the binding of Annexin V-FITC and PI. Data are expressed as mean $\pm$ SE and (upper panel) and are represented as dot plot data (lower panel). **b** Data are presented by classifying the results of (a), depending on the absence and presence of house dust mite (HDM) IgE in serum of normal and asthmatic subjects. **c** Data are presented by classifying HDM IgE+ serum-associated results of (b), depending on kind of HDM IgE, such as *D. pteronissinus* (DP) IgE and *Dermatophagoides farinae* (DF) IgE. Data are expressed as mean $\pm$ SE and are presented in relation to the control group, which was set to 100 %. * $p < 0.05$ and ** $p < 0.01$ indicate a significant difference between the control group and the serum-treated group or GM-CSF-treated group

Results

HDM IgE is an essential factor in the regulation of eosinophil apoptosis by asthmatic serum

Because patients with asthma show alterations in several proteins, we hypothesized that asthma is aggravated by inhibiting eosinophil apoptosis due to these alterations. We evaluated whether asthmatic serum, which can broadly indicate the protein alterations, would affect constitutive apoptosis of eosinophils from normal subjects. Asthmatic serum significantly suppressed normal eosinophil

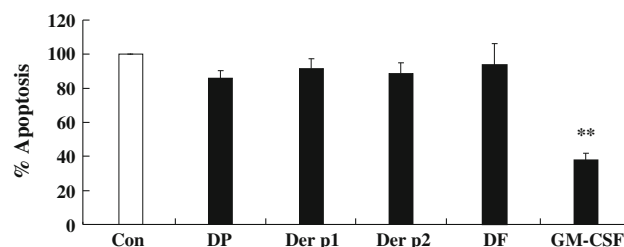


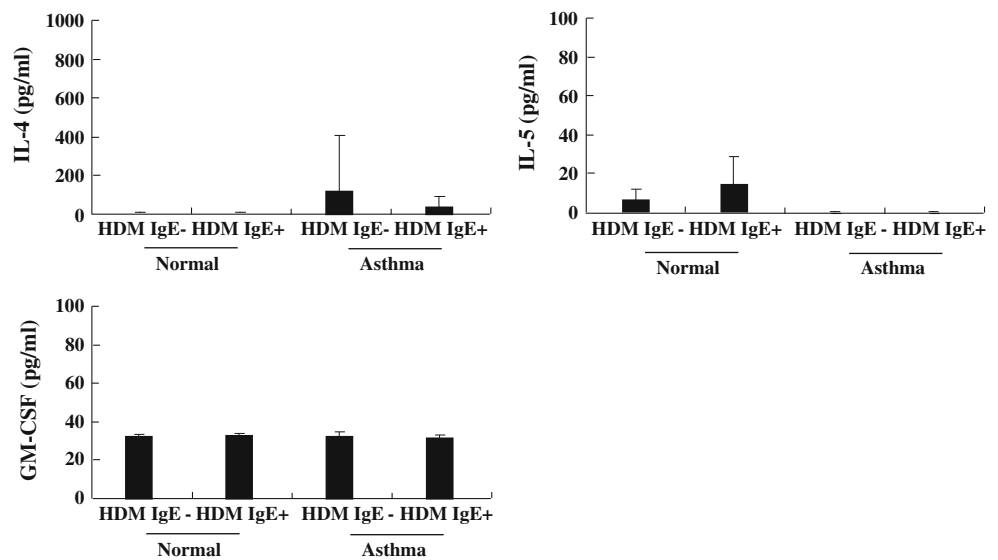
Fig. 2 HDM extracts have no effect on constitutive apoptosis in normal eosinophils. Eosinophils were isolated from peripheral blood of normal subjects ($n = 16$) and the cells were incubated for 48 h in the absence (Con) or presence of *D. pteronissinus* (DP) extract (10 μ g/ml), Der p1 (10 μ g/ml), Der p2 (10 μ g/ml), *Dermatophagoides farinae* (DF) extract (10 μ g/ml), and GM-CSF (100 ng/ml). Apoptosis was analyzed by measuring the binding of Annexin V-FITC and PI. Data are expressed as mean $\pm$ SE and are presented in relation to the control group, which was set to 100 %. ** $p < 0.01$ indicates a significant difference between the control group and the stimulator-treated group

apoptosis, whereas normal serum had no effect (Fig. 1a). We also classified normal subjects by the absence and presence of serum HDM IgE, which includes DP-specific IgE (DP IgE) and DF-specific IgE (DF IgE). Surprisingly, approximately 45 % of the normal subjects were HDM IgE+, whereas 72 % of asthmatic subjects were HDM IgE+. HDM IgE+ asthmatic serum inhibited apoptosis of HDM IgE+ and HDM IgE- normal eosinophils. HDM IgE- asthmatic serum inhibited apoptosis in HDM IgE+ normal eosinophils but not apoptosis in HDM IgE- normal eosinophils. The anti-apoptotic effect of HDM IgE+ asthmatic serum was stronger than that of HDM IgE- asthmatic serum. HDM IgE+ normal serum inhibited apoptosis only in HDM+ normal eosinophils. The anti-apoptotic effect of asthmatic serum on HDM IgE+ normal eosinophils was stronger than that on HDM IgE- normal eosinophils (Fig. 1b). HDM IgE+ subjects showed DP IgE+ DF IgE+, or DF IgE+. No difference was observed between the inhibitory effect of DP IgE+ DF IgE+ serum and DF IgE+ serum (Fig. 1c). GM-CSF was used as the positive control in this experiment. These results indicate that asthmatic serum inhibited eosinophils and that the inhibitory effect was affected by the presence of HDM IgE.

The HDM extract has no effect on constitutive apoptosis in normal eosinophils

The importance of HDM IgE led us to examine whether the HDM extract and HDM-associated with protein could directly affect apoptosis in normal eosinophils. Extracts of DP and DF were not effective for eosinophil apoptosis (Fig. 2). DP-specific proteins such as Der p1 and Der p2 also had no effect on eosinophil apoptosis. Thus, we investigated the presence of eosinophil anti-apoptotic

Fig. 3 Levels of IL-4, IL-5, and GM-CSF are not different between normal and asthmatic serum. Serum was collected from normal ($n = 20$) and asthmatic subjects ($n = 25$). Eosinophils were isolated from peripheral blood of normal subjects, and the cells were incubated for 48 h in the absence (Con) or presence of normal and asthmatic sera. Serum was measured by ELISA. Data are mean $\pm$ SE



factors such as IL-4, IL-5, and GM-CSF in serum. Although the IL-4 concentration was higher in asthmatic serum than that in normal serum, the IL-4 level was low (Fig. 3). Additionally, IL-5 and GM-CSF levels were very low in all experimental groups. We also examined whether stimulation by HDM IgE+ serum would induce IL-4, IL-5, or GM-CSF, but these cytokines were not detected in the supernatant following treatment with normal or asthmatic serum. Our results indicate that IL-4, IL-5, and GM-CSF are not involved in the inhibitory effect of asthmatic serum on eosinophil apoptosis.

HDM IgE+ asthmatic serum delays the decrease in procaspase 3 and procaspase 9 during eosinophil apoptosis

Both caspase 3 and caspase 9 are essential pro-apoptotic proteins, and these proteins are activated after cleavage by a constitutive apoptosis signal. As shown in Fig. 4, procaspase 3 and procaspase 9 expression decreased 24 and 48 h after constitutive apoptosis began. These results indicate that HDM IgE+ serum inhibited cleavage or activation of caspase 3 and caspase 9.

HDM IgE+ asthmatic serum requires the activation of PI3K, Akt, and ERK to suppress eosinophil apoptosis

To identify intracellular signaling molecules associated with the delay in apoptosis induced by HDM IgE+ serum, we examined the alterations in eosinophil apoptosis using specific inhibitors of PI3K, Akt, and ERK. Ly294002, a PI3K inhibitor, AKTi, an Akt inhibitor, and PD98059, a MEK inhibitor, blocked the suppressive effect on eosinophil apoptosis induced by

HDM IgE+ serum (Fig. 5a). To confirm the activation of the associated signal proteins after treatment with HDM IgE+ serum, we investigated the alteration in Akt and ERK phosphorylation. HDM IgE+ serum induced Akt and ERK phosphorylation in a time-dependent manner (Fig. 5b, c). ERK activation by HDM IgE+ serum was inhibited by AKTi (Fig. 5d). These data indicate that HDM IgE+ serum transduces the delay in eosinophil apoptosis through the PI3K/Akt/ERK pathway.

Discussion

Asthma is an inflammatory disease characterized by an increase of eosinophils in the respiratory organs [13]. Eosinophils are key cells in asthma pathogenesis, and they have been identified as an essential target for asthma treatment [14–16]. The increased number of eosinophils in the lungs of asthmatic subjects is associated with the regulation of eosinophil recruitment, chemotaxis, activation, survival, and apoptosis. In particular, prolonged eosinophil survival is involved in the dysregulation of eosinophil apoptosis [17]. We hypothesized that unknown or complex factors in asthmatic serum can inhibit eosinophil apoptosis as compared to those in normal serum. IL-4, IL-5, and GM-CSF are key molecules that regulate eosinophil apoptosis and differentiation [12]. We examined the association between the anti-apoptotic effect of asthmatic serum and IL-4, IL-5, and GM-CSF, but no difference in the cytokines was detected between asthmatic and normal serum (Fig. 3). The cytokines were not detected in the supernatant after treatment with asthmatic serum. These results indicate that asthmatic serum suppresses normal eosinophil apoptosis by

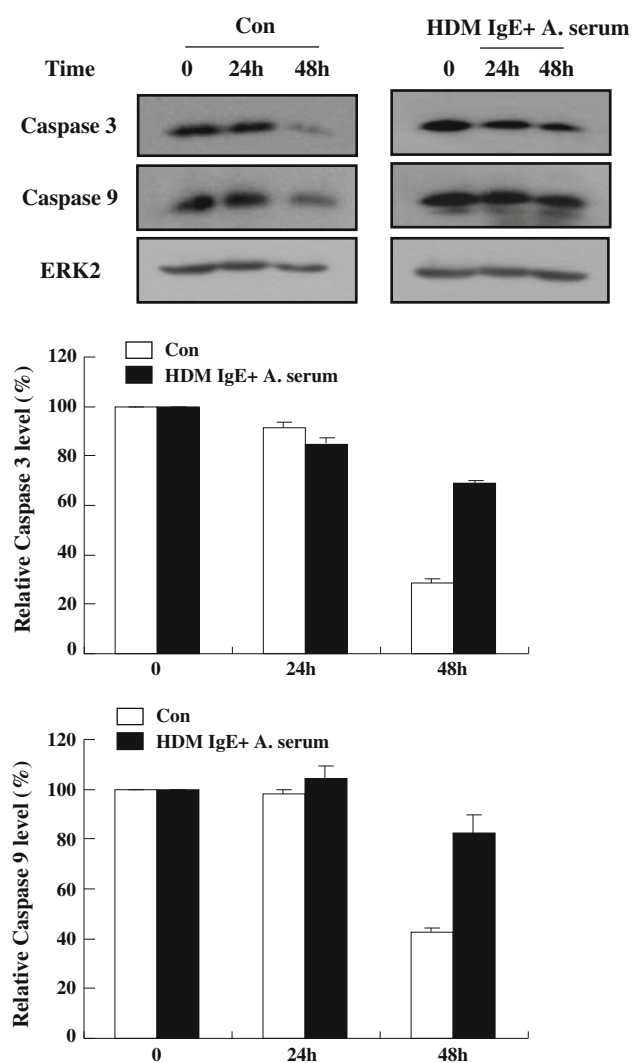


Fig. 4 HDM IgE+ asthmatic serum delays the decrease in procaspase 3 and procaspase 9 during eosinophil apoptosis. Normal (HDM IgE+) eosinophils were incubated with HDM IgE+ serum (serum:medium = 1:1) for the indicated time. Procaspase 3 and procaspase 9 were detected by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibody as an internal control. Densitometric data are expressed in mean $\pm$ SE of three independent experiments and are presented relative to the negative control, which was set at 100 % (lower panel)

unknown factors or an interactive reaction with known or unknown factors. A variety of survival factors transduce the anti-apoptotic signaling pathways including the PI3K, MAPK, PKC and caspase-mediated pathways [9, 12, 18]. A recent paper suggested the importance of caspase recruitment domain family, member 11 (CARD11), which plays an essential role in the differentiation of both neuronal and immunologic tissues [19]. We investigated alterations in caspase and survival signal proteins due to asthmatic serum to evaluate anti-apoptotic mechanism of

asthmatic serum. Asthmatic serum delayed cleavage of procaspase 3 and procaspase 9, and induced ERK activation via the PI3K/Akt pathway (Fig. 4). Although our results revealed the detailed mechanism of the anti-apoptotic effect due to asthmatic serum, the exact molecule that functions as an anti-apoptotic factor in asthmatic serum remains to be elucidated. Additionally, the anti-apoptotic effect of asthmatic serum has not been studied in asthmatic eosinophils. Further investigations are required, and our laboratory is currently investigating the different molecules between normal and asthmatic serum.

Atopy is one of the strongest risk factors for allergic diseases such as asthma. Allergen-specific IgE, specifically HDM IgE, is consistently involved in the development of asthma and its exacerbation and, thus, is used in the diagnosis, treatment, and management of asthma [20, 21]. Because DP IgE and DF IgE are known as HDM IgE, which is closely associated with asthma pathogenesis, we measured DP IgE and DF IgE in serum of normal and asthmatic subjects [4, 22]. HDM IgE+ (DP IgE+ DF IgE+ or DF IgE) asthmatics included 72 % of asthmatic subjects, and this value was higher than that in a recent study on Asian asthmatics [23]. DP IgE+ serum was not detected in asthmatic subjects who participated in this study. HDM IgE+ normal subjects, who do not have asthmatic symptoms, were 45 % of the normal subjects. These results indicate that the presence of HDM IgE is important in normal subjects as well as in asthmatic subjects. As shown in Fig. 1, HDM IgE+ asthmatic serum was more effective for inhibiting eosinophil apoptosis compared to that of HDM IgE– asthmatic serum. Additionally, apoptosis of eosinophils in HDM IgE+ normal subjects was more strongly suppressed by asthmatic serum than that from HDM IgE– normal subjects. However, the DP and DF extracts had no effect on eosinophil apoptosis (Fig. 2). The production of HDM IgE by exposure to HDM may be closely involved in the delay in eosinophil apoptosis. Although IgE inhibits neutrophil apoptosis, the direct action of IgE on eosinophil apoptosis has not been reported [24]. We will study whether HDM IgE functions as a direct blocker of eosinophil apoptosis or whether another factor produced by HDM IgE acts as an effective molecule for apoptosis.

In summary, asthmatic serum delayed constitutive apoptosis in normal eosinophils compared to that of normal serum. This delay in apoptosis was strongly associated with the presence of HDM IgE in asthmatic serum. The anti-apoptotic signal induced by HDM IgE+ serum was involved in the PI3K/Akt/ERK cascade in normal eosinophils. These results may contribute to a better understanding of the differential regulation of constitutive eosinophil apoptosis between normal subjects and patients with asthma.

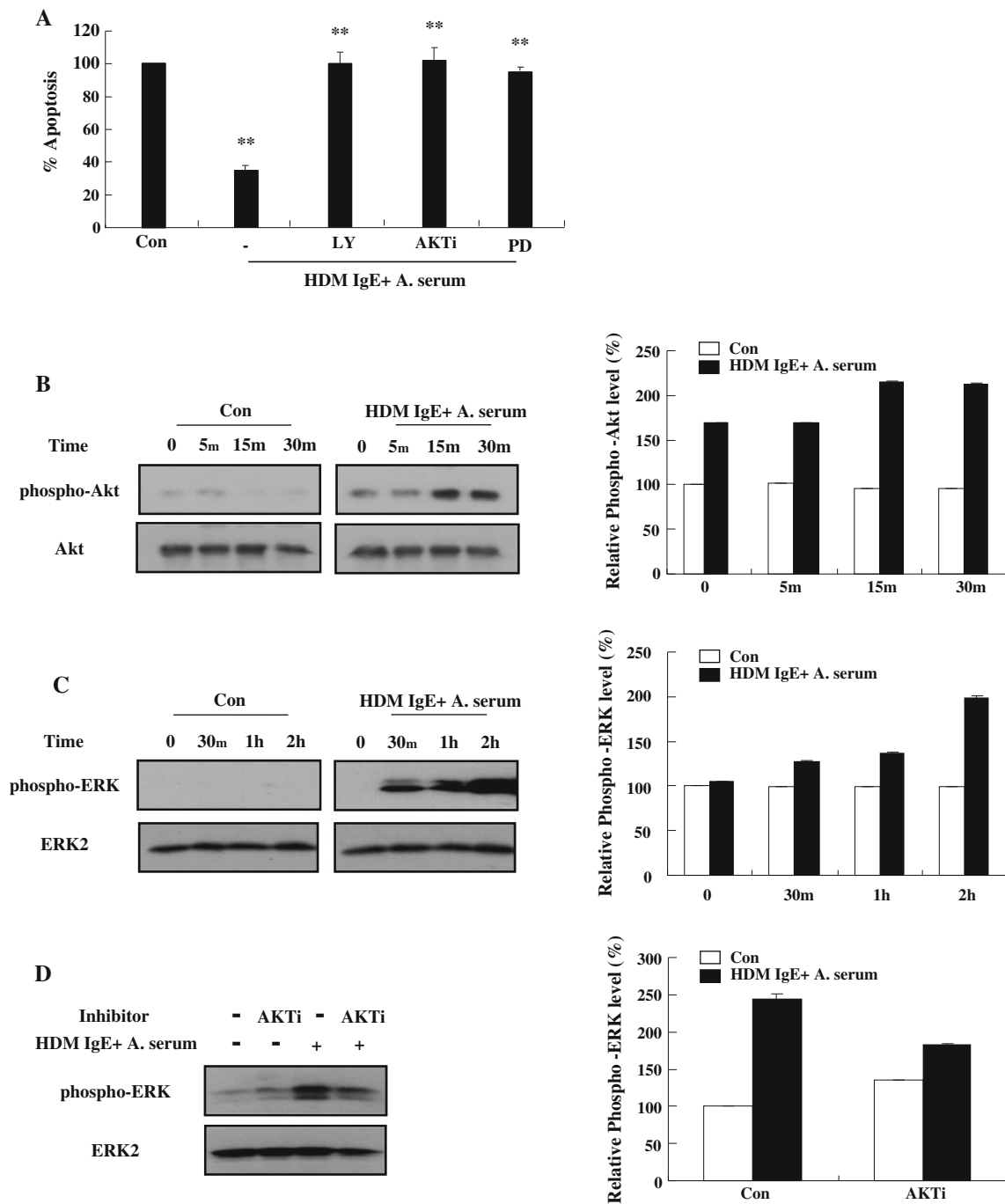


Fig. 5 HDM IgE+ asthmatic serum requires the activation of PI3K, Akt, and ERK for suppression of eosinophil apoptosis. **a** Normal (HDM IgE+) eosinophils were pre-treated for 1 h with or without 10 μ M LY294002 (LY), 10 μ M Akt inhibitor (AKTi), and 10 μ M PD98059 (PD) ($n = 3$). The cells were incubated for 24 h in the presence and absence of HDM IgE+ asthmatic serum (serum:medium = 1:1). Apoptosis was analyzed by measuring the binding of Annexin V-FITC and PI. **b, c** Normal (HDM IgE+) eosinophils were incubated with HDM IgE+ asthmatic serum for the indicated time. Akt phosphorylation (**b**) and phospho-ERK (p-ERK) (**c**) in the lysates

were detected by Western blotting. **d** Normal eosinophils were incubated with HDM IgE+ asthmatic serum for 1 h after a pre-treatment with 10 μ M of the Akt inhibitor AKTi. Harvested cells were lysed, and the p-ERK in the lysates was detected by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control. Densitometric data are expressed in mean $\pm$ SE of three independent experiments and are presented relative to the negative control, which was set at 100 % (right panel of **b–d**)

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