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Role of thrombin in interleukin-5 expression from basophils

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Abstract

Interleukin-5 (IL-5) plays a key role in the pathogenesis of bronchial asthma. Thrombin is a procoagulant factor that has been also reported to participate in the inflammatory response by stimulating the secretion of cytokines. Interaction of inflammatory cells with airway epithelial cells may also promote the secretion of cytokines. However, the role of thrombin and cell-to-cell interaction in pathogenesis of allergic inflammation is unclear. In this study, we evaluated the role of thrombin and cell-to-cell interaction in the secretion of IL-5 from basophils. The human basophil cell line KU-812 was used in the assays. Thrombin and co-culture with alveolar epithelial cells significantly stimulated the secretion of IL-5 from KU-812 cells as compared to controls. Secretion of IL-5 was synergistically stimulated when KU-812 cells were incubated in the presence of both thrombin and alveolar epithelial cells. Co-culture of KU-812 cells with epithelial cells significantly increased the expression of tissue factor, an activator of coagulation activation, in a cell dose-dependent manner. Secretion of IL-5 from KU-812 basophils co-cultured with epithelial cells was significantly inhibited by LY294002, an inhibitor of phosphatidylinositol 3-kinase. These results suggest that thrombin and cell interaction with lung epithelial cells may augment the inflammatory response in allergic diseases by stimulating the secretion of IL-5 from basophils.

Keywords: Coagulation; Bronchial asthma; Allergy; Thrombin; Interleukin-5

Allergic diseases including bronchial asthma affect approximately 20% of the population and are, therefore, among the most frequent disorders treated by family practitioners, pediatricians, and general internists [1]. A great body of evidence suggests that inflammatory cytokines including interleukin (IL)-3, IL-4, IL-5, IL-6, IL-10, and IL-13 play a critical role in the pathogenesis of allergic inflammation [2,3]. IL-5 is produced by CD4+ T cells, basophils, mast cells, and airway epithelial cells [4]. IL-5 promotes allergic inflammation by promoting the production, activation, degranulation, and recruitment of eosinophils. IL-5 may also accelerate allergic responses by enhancing IL-4-induced production of IgE by normal B cells, by stimulating the secretion of inflammatory cytokines and the expression of MHC class II and adhesion molecules [5]. Another action of IL-5 that is also important in the allergic response is its effect on basophils; IL-5 may prime basophils for increased production of histamine and for leukotriene generation [5].

Thrombin is the effector enzyme of the coagulation system [6]. Thrombin results from the stepwise activation of coagulation factors via the extrinsic or intrinsic pathways. In addition to its function in thrombosis and hemostasis, thrombin is also able to affect the function of several cells [6]. Thrombin is an strong activator of platelets it may exert direct effects upon monocytes, endothelial cells,

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smooth muscle cells, lymphocytes, epithelial cells, macrophages, neural cells via its protease-activated receptor-(PAR)s [6]. Thrombin is able to promote inflammation by stimulating chemotaxis of monocytes and lymphocytes, the motility and proliferation of fibroblasts and smooth muscle cells, and the secretion of growth factors and proteases from the vascular endothelium, macrophages and epithelial cells [7]. Previous studies suggested that thrombin also play a role in allergic diseases, particularly in bronchial asthma [8,9]. IL-5 secretion is a crucial step for the induction of inflammation during the allergic response [5]. Whether thrombin affects the secretion of IL-5 is unknown. In the present study, to further expand the role of thrombin in the allergic reaction, the effect of thrombin on IL-5 secretion was evaluated using the human KU-812 basophil cell line.

Materials and methods

Reagents. RPM1 medium was purchased from Sigma (St. Louis, MO), fetal bovine serum (FBS) from Bio Whittaker (Walkersville, MD) and penicillin–streptomycin from Invitrogen (Grand Island, NY). Thrombin was purchased from WAKO (Osaka, Japan). All other chemicals and reagents used were of the best quality commercially available.

Cell culture. The human KU-812 basophil cells were purchased from Riken Bank (Tsukuba, Japan). KU-812 cells were cultured in RPM1 supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The A549 human alveolar epithelial cell lines were purchased from the American Type Culture Collection (Rockville, MD). A549 cells were cultured in DMEM containing 10% heat-inactivated FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 2% vitamin solution, 110 μg/ml sodium pyruvate, and 0.1 mM non-essential amino acids. All cells were cultured 37 °C in 75-cm flasks in an atmosphere composed of 5% CO2 and 95% air. Confluent cells were passaged after 5–7 days.

Stimulation of cells with thrombin and its receptor. KU-812 cells were serum-starved overnight and then stimulated with varying concentrations of thrombin or PAR-1 agonist and conditioned media were sampled after 24 of stimulation. The samples were centrifuged at 10,000 rpm for 5 min and stored at −80 °C until analysis.

Effect of co-culture of KU-812 and A549 cells on IL-5 secretion. A549 and KU-812 cells were cultured up to confluency and then serum-starved overnight. KU-812 cells were then cultured in the presence of A549 cells for 24 h and cell supernatants were separated by centrifugation at 2000 rpm for 10 min. Supernatants were then stored at −80 °C until use.

Effect of co-culture of KU-812 and A549 cells on tissue factor activity. A549 cells were cultured in 48-well microplates in the same conditions as described above. Tissue factor activity was determined as factor X activation by factor VIIa/tissue factor complex. After overnight serum-starvation, the cells were co-cultured overnight with varying number of KU-812 cells in DMEM without fetal bovine serum. KU-812 cells were then discarded and washed with Hepes-buffered saline. A549 cells were then incubated in the presence of a mixture of 50 μL of 4 nM factor VIIa, 50 μL of 1 μM factor X, and 150 μL of Hepes-buffered saline containing 5 mM $CaCl_2$ for 60 min at room temperature. The reaction was stopped by adding 10 μL of 100 mM EDTA and generated factor Xa was determined using 100 μM S-2222. Color development was determined by measuring the absorbance of 405 nm with EAR 340 microplate reader (SLT-Lab, Salzburg, Austria).

Biochemical analysis. The concentration of IL-5 in cell supernatant was measured using an enzyme immunoassay kit specific for human IL-5 purchased from BD Biosciences Pharmigen (San Diego, CA) following the manufacturer's instructions.

Statistical analysis. Data are expressed as means ± the standard error (SE) unless otherwise specified. The statistical difference between variables was calculated by analysis of variance with *post hoc* analysis using Fisher's predicted least significant difference test. Statistical analyses were carried out using the StatView 4.1 package software (ABACUS CONCEPTS, Berkeley, CA, USA) for the Macintosh. A $p < 0.05$ was considered as statistically significant.

Results

Co-culture of KU-812 and A549 cells stimulates tissue factor activity

A549 cells were cultured in the presence of varying number of KU-812 cells for 24 h and then tissue factor activity was measured. The activity of tissue factor was significantly increased on the surface of A549 cells after co-culture with KU-812 cells in cell number-dependent manner (Fig. 1A). This result suggests that interaction of alveolar epithelial cells and basophils induces activation of the coagulation cascade.

Effect of thrombin on IL-5 secretion

The cells were stimulated with several concentrations of thrombin for 24 h and cell supernatants were sampled at

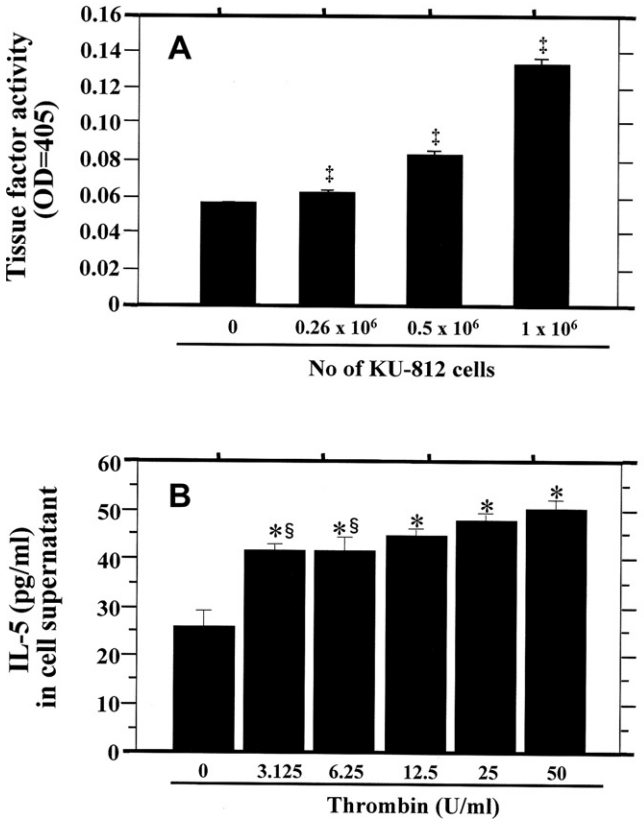

Fig. 1. Expression of tissue factor after co-culture of KU-812 cells with A549 cells and stimulation of IL-5 secretion by thrombin. Co-culture with KU-812 cells significantly promotes the expression of tissue factor from A549 cells in a cell number-dependent manner. Thrombin stimulates the secretion of IL-5 from KU-812 cells in a dose-dependent manner. $^{*}p < 0.0001$ versus control; $^{\S}p < 0.05$ versus 25 and 50 U/ml; $^{\ddagger}p < 0.001$ versus control.

several time intervals for measurement of IL-5. The results showed that thrombin significantly stimulates the protein expression of IL-5 from KU-812 cells in a dose-dependent manner (Fig. 1B).

Co-culture of KU-812 and A549 cells stimulates IL-5 secretion

KU-812 cells were cultured in the presence of A549 cells for 24 h and then IL-5 was measured in cell supernatant. IL-5 was significantly increased when basophils were co-cultured with A549 cells as compared to A549 or KU-812 cell alone (Fig. 2A). This result suggests that interaction of alveolar epithelial cells and basophils induces the secretion of IL-5.

IL-5 secretion from KU-812 cells co-cultured with alveolar epithelial cells in the presence of thrombin

KU-812 cells were co-culture in the presence of A549 cells and several concentrations of thrombin for 24 h. Cell supernatants were separated and IL-5 was measured. The results showed a synergistic stimulation of IL-5 expression from basophils culture in the presence of A549 cells and thrombin. Thrombin stimulated the secretion of IL-5 in a dose-dependent manner (Fig. 2B).

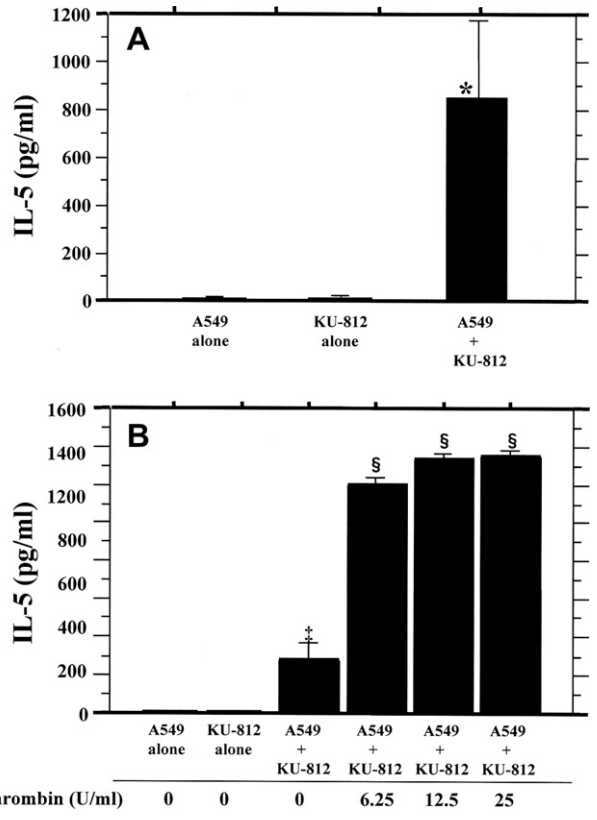

Fig. 2. Effect of co-culture of KU-812 and A549 cells in the presence of absence of thrombin on IL-5 secretion. Significant increase in the secretion of IL-5 was observed when both KU-812 and A549 cells were co-culture. This effect synergistically increases when both cells were co-culture in the presence of several concentrations of thrombin. $^{*}p < 0.01$ versus A549 and KU812 cells alone; $^{\ddagger}p < 0.0005$ versus A549 cells alone and KU812 cells alone without thrombin; $^{\S}p < 0.0001$ versus A549 co-cultured with KU812 in the absence of thrombin.

Effect of PI3 kinase inhibition on IL-5 secretion from KU-812 cells co-culture with A549 cells

KU-812 cells were cultured in the presence of A549 cells and LY294002, an inhibitor of PI-3-kinase. LY294002 significantly suppressed the secretion of IL-5 from KU-812 co-cultured with A549 cells (Fig. 3). This result suggests that the PI3 kinase is involved in the mechanism of IL-5 secretion from basophils during their interaction with alveolar epithelial cells.

Role of thrombin receptor on IL-5 secretion from KU-812 cells

The cellular effects of thrombin are mediated by its receptors PAR-1, PAR-3, and PAR-4. We first investigated by RT-PCR whether thrombin receptors are present in KU-812 cells. KU-812 cells expressed all three thrombin receptors PAR-1, PAR-3, and PAR-4 (Fig. 4A). To investigate whether thrombin-mediated stimulation of IL-5 expression is mediated by PAR-1, the KU-812 cells were stimulated with PAR-1 agonist. The results showed that PAR-1 agonist stimulates the secretion of IL-5 from KU-812 cells (Fig. 4B). These findings suggest that thrombin promotes IL-5 secretion from KU-812 cells via its PAR-1 receptor.

Discussion

Allergic reaction such as bronchial asthma is characterized by the infiltration of inflammatory cells including mast

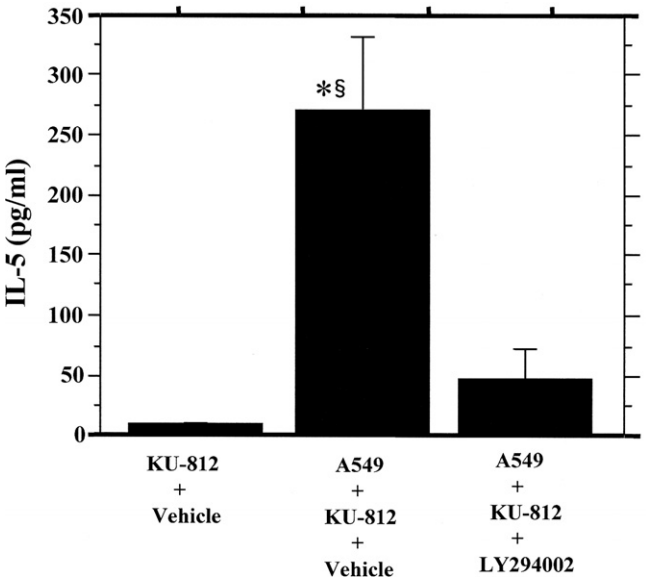

Fig. 3. Effect of PI3 kinase inhibitor on IL-5 secretion. IL-5 secretion during co-culture of A549 and KU-812 cells was significantly inhibited by the PI3 kinase inhibitor LY294002. $^{*}p = 0.002$ versus KU812 cells + vehicle; $^{\S}p = 0.006$ versus A549 + KU-812 + LY294002.

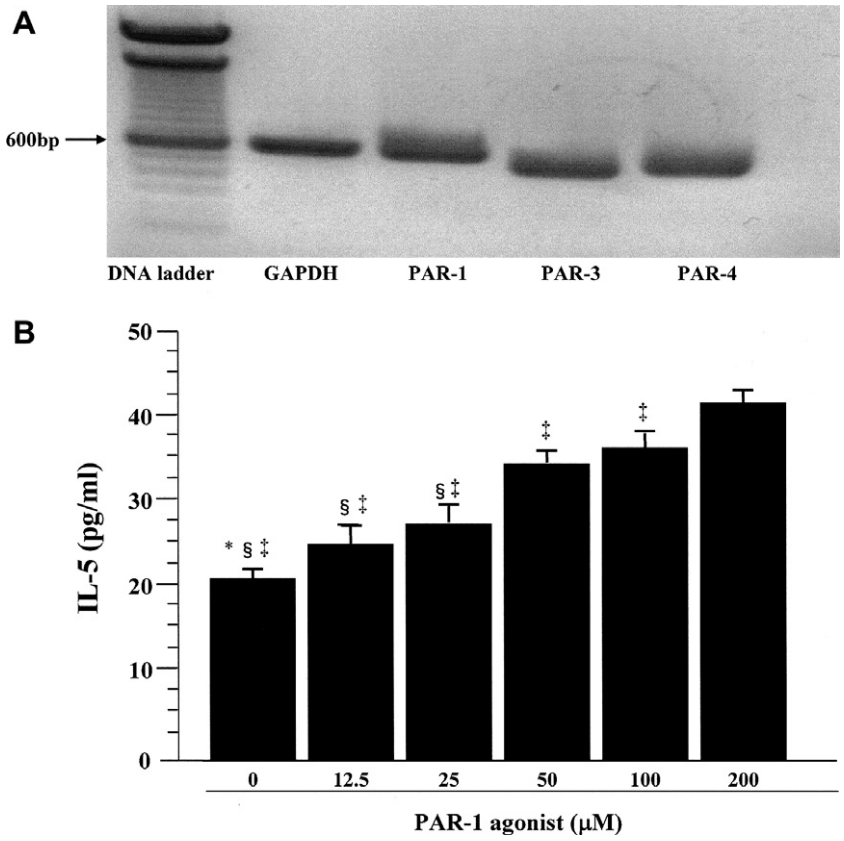

Fig. 4. Expression of protease-activated receptor (PAR)s and effect of PARs on the expression of IL-5 in basophils. Expressions of PAR-1, PAR-3, and PAR-4 were detected in basophils. PAR-s agonist induced significant expression of IL-5 from basophils. $^{*}p < 0.01$ versus 25, 50, 100, and 200 μM; $^{\S}p < 0.005$ versus 50, 100, and 200 μM; $^{\ddagger}p < 0.05$ versus 200 μM.

cells, lymphocytes, eosinophils, and basophils [1,2]. The early stage of the allergic response is characterized by the appearance and activation of mast cells followed by a chronic stage during which infiltration of lymphocytes, eosinophils, and basophils occur [10]. Of these, basophils can foster the inflammatory response during allergy because, like mast cells, they are endowed with high-affinity receptors for IgE antibody, which after cross-linking leads to the release of potent inflammatory mediators such as histamine and leukotriene (LT) C4 [10]. Basophils can also be activated by several cytokines and chemokines including IL-1β, IL-3, and IL-5, after binding to their receptors; these interleukins can potentiate the antigen- or anti-IgE-driven release of histamine and LTC4 from basophils [10]. In addition, it has been shown recently that basophils are also capable of secreting cytokines such as IL-4 and IL-5 [11]. IgE-mediated activation was reported to regulate the expression of these cytokines [10]. We recently demonstrated that the concentration of thrombin, the effector enzyme of the coagulation system, is abnormally increased in the lung of asthmatic patients and in animal models of bronchial asthma [8,9]. The role of thrombin in basophil-mediated allergic inflammation is unknown. In the present study, we showed for the first time that thrombin through its receptor is able to stimulate the secretion of IL-5 from basophils, suggesting that activation of the coagulation may also share a role in allergy-related inflammatory responses.

The mechanism of coagulation activation in the airways is not well understood. The main initiator of coagulation activation is tissue factor, which once expressed on the cell surface binds to activated factor VII and activates the extrinsic pathway of coagulation system [6]. A number of cells including monocytes/macrophages, endothelial, and epithelial cells can express tissue factors when they are stimulated with inflammatory cytokines such tumor necrosis factor-α, interleukin-1β, lipopolysaccharide, and growth factors [6]. During allergic inflammation such as that occurring during bronchial asthma increased concentration of inflammatory cytokines and growth factors can be detected in the lung [1,2]. Therefore, cytokine-mediated stimulation may mediate tissue factor expression and subsequent coagulation activation and increased thrombin generation in the lung during allergic responses. Increased cell-to-cell interaction between lung epithelial cells and inflammatory may also take place during lung inflammatory responses. In the present study, we found that interaction between basophils and alveolar epithelial cells enhances the expression of tissue factor, suggesting that this cell-to-cell interaction may also cause increased coagulation activation during allergic inflammation.

Another novel finding of our present study is that cell-to-cell interaction between basophils and alveolar epithelial cells increases the secretion of IL-5 from basophils. The exact mechanism of this finding was not clarified in this study but several factors including adhesion molecules, integrin receptors and the CD40 receptor and its ligand CD154 may be involved in the communication between cells to enhance cytokine expression [12]. Further, co-culture of basophils and epithelial cells in the present of the PI3 kinase inhibitor LY294002 significantly inhibited the secretion of IL-5 from basophils, suggesting that increased IL-5 secretion during cell-to-cell interaction is mediated by PI3 signaling. Interestingly, thrombin enhances several folds the effect of basophil and lung epithelial interaction on IL-5 secretion in a dose-dependent manner; this result suggests that cell-to-cell interaction in a microenvironment with increased coagulation activation may enhance IL-5-mediated inflammatory responses.

In brief, the results of this study showed for the first time that thrombin promotes allergic responses by stimulating the secretion of IL-5 from basophils alone and from basophils interacting with lung epithelial cells. This study also showed that cell-to-cell interaction stimulates tissue factor expression and that PI3 signaling is involved in the cell-to-cell-mediated secretion of IL-5.

Acknowledgments

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