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Novel co-operation between eotaxin and substance-P in inducing eosinophil-derived neurotoxin release

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Eosinophils, chemokines, and neuropeptides are thought to play effector roles in the pathogenesis of allergic diseases such as rhinitis. Eotaxin is a novel C–C chemokine with a potent and relatively specific eosinophil chemoattractant activity that binds selectively to CCR3 receptor, however, its activity in inducing eosinophil granules proteins release is poorly characterized. This study was performed to determine whether eotaxin primes eosinophil exocytosis and whether this co-operates with the sensory neuroimmune-axis. In the present communication, we show that 10 ng/ml eotaxin primed normal human eosinophil for exaggerated eosonophil-derived neurotoxin (EDN) release stimulated by 10^{-8} M Substance-P (SP). This novel priming was blocked by; 7B11 and Herbimycin A (HA), the CCR3 antagonist and the tyrosine kinase inhibitor, respectively. SDS-Page studies showed significant tyrosine phosphorylation of several protein residues induced by 10^{-8} M SP only after priming with 10 ng/ml eotaxin. These results demonstrate a novel co-operation between eotaxin and SP in inducing eosinophil cytotoxicity, which at least in part involves tyrosine kinases pathway(s).

Key words: Allergic inflammation, Eosinophils, Eotaxin, SP, EDN, Tyrosine phosphorylation

Introduction

Allergic rhinitis is a chronic inflammatory disease which is characterized by tissue eosinophilia. A number of allergic mediators act as chemoattractant for eosinophil, however, eotaxin the C–C chemokine which selectively binds to CCR3 receptor is a potent and relatively specific eosinophil chemoattractant and its role in rhinitis is well reported.[1]

After tissue infiltration with eosinophils, their cytotoxic function is activated by the proper stimuli to secrete a number of cationic proteins including the major basic protein, the eosinophil peroxidase, the eosinophil cationic protein and the eosinophil-derived neurotoxin. The release of these proteins contribute to the hypersensitivity reaction and to tissue damage of the airway.[2]

Among the proper stimuli of EDN release is the neuropeptide SP, which is released from the nasal sensory neurones.[3]

The current study was designed to investigate whether eotaxin possesses a priming activity for eosinophil exocytosis and whether this works in synergy with SP.

Materials and methods

Eosinophil purification

Eosinophils from healthy volunteers were purified from venous blood anticoagulated with heparin by Percoll discontinuous density gradients centrifugation. In short, heparanised blood was sedimented with 6% dextran for 30 min at room temperature. The leukocyte-rich plasma was collected and washed twice with Hank's balanced salt solution (HBSS) containing 2% fetal bovine serum (FBS). Eosinophils were then separated by Percoll discontinuous gradients centrifugation. Gradients consisted of a mixture of Percoll and HBSS adjusted to 2.5ml of 78%, 71% and 56% Percoll, respectively in 15 ml polystrene tubes, and recovered cells suspended in HBSS containing 2% FBS were overlaid. The tubes were then centrifuged at 400×*g* for 30 min. The pellet and the lowest band granulocytes were collected, and sedeminted red blood cells were removed by hypodense lysis. These lymphocyte- and monocyte-free granulocytes were incubated with CD16 immunomagnetic beads for 30 min to eliminate unwanted neutrophils. High purity >98% eosinophilic granulocytes were

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obtained by negative selection using magnetic cell sorting. Cell viabilities were >95% as judged by trypan blue exclusion.

Eosinophil degranulation and assay of EDN concentration

Freshly purified eosinophils were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and adjusted to 1×10^6 cells/ml. Eosinophil degranulation was induced by dilutions of either 10 ng/ml eotaxin (Funakoshi Co., Tokyo, Japan), or 10^{-8} M SP (Sigma, St Lous, MO) using 96 flat bottom plates (Nunc, Denmark). Briefly, 100 µl of each stimulus suspended in the same culture medium as eosinophils (RPMI) were added to 100 µl of the purified eosinophil, after 1 h incubation, supernatants were carefully collected. EDN concentrations in the supernatants were analysed by radioimmunoassay (RIA) using EDN RIA kits (Pharmacia, Uppsala, Sweden). The total content of EDN was measured by lysing eosinophils with 1% Nonidet P-40 (NP-40). As for the priming experiments, cells were incubated for 15 min with 10 ng/ml eotaxin, washed twice, resuspended in the culture medium and their EDN release induced by 10^{-8} M SP was tested in the same way as described above.

Western blotting

Cells were washed with Hepes-buffered saline containing 1 mM Na_3VO_4 (pH 7.4) at 4°C, and then lysed in triton X-100 lysis buffer (50 mM Hepes, pH 7.4, 1% triton X-100, 4 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 50 µg/ml aprotinin, 200 µM leupeptin, 50 µM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Lysates were subjected to SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes for western blotting.

Statistical analysis

Results are expressed as mean ±SEM and were analysed by paired *t*-test using statview software on an apple Macintosh computer. A value of $p<0.05$ was considered significant.

Results and Discussion

A primer is an agent at non functionally stimulatory dose(s), prepare the cell, making it ready to function to suboptimal dose from the proper stimuli. Accordingly, 10 ng/ml eotaxin was chosen for the following experiments since it is the dose which does not directly induce functional response i.e. EDN release[4]

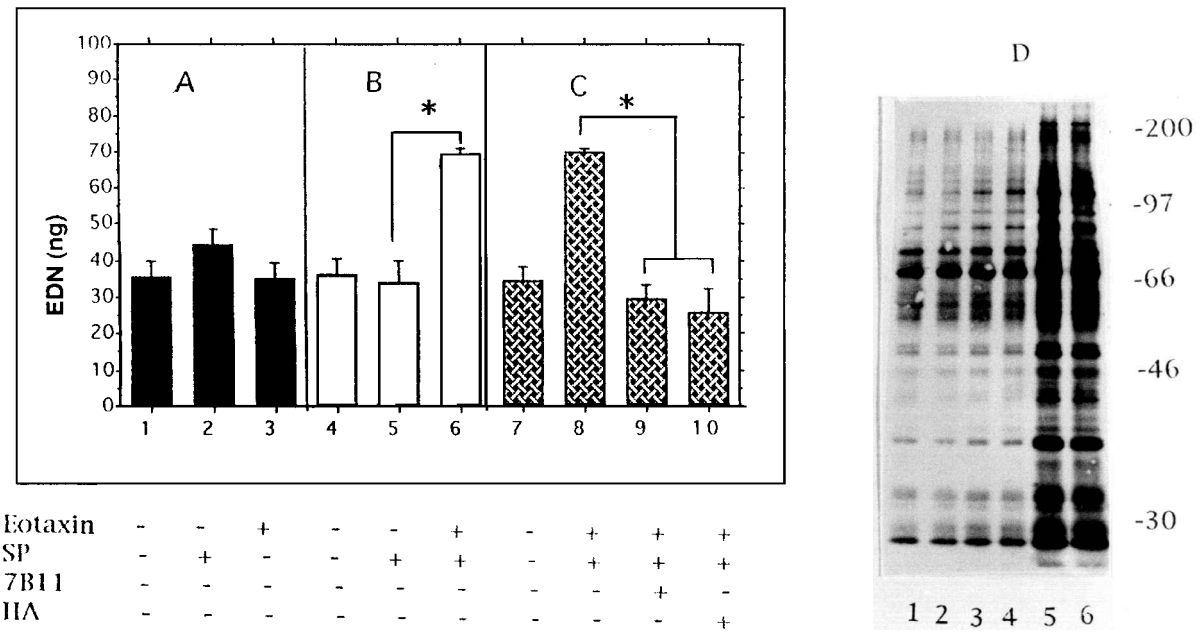

FIG. 1. Relationship between the priming activity of eotaxin in EDN release and tyrosine phosphorylation. (A) EDN secretagouge activity of 10 ng/ml eotaxin and 10^{-8} M SP. Results are of a 1 h degranulation assay and represent – SEM of 6 independent experiments performed in duplicate. (B) Priming activity of 10 ng/ml eotaxin in EDN release stimulated by 10^{-8} M SP. Asterisk indicates $p=0.0001$. Results are of 8 independent experiments performed in duplicate, – SEM. (C) Modulation of eotaxin priming activity in EDN release by 7B11 and herbimycin A. Asterisk indicates $p=0.0001$. Results are of 6–8 independent experiments performed in duplicate, – SEM. (D) Association of tyrosine phosphorylation with CCR3 stimulation. Normal human eosinophils were pre-incubated for 15 min at 37¡C with 10 ng/ml eotaxin (lanes 5 and 6) or for 15 min with buffer only (lanes 3 and 4), cells were washed three times followed by 10^{-8} M SP stimulation for a further 30 min at 37¡C. Lanes 1 and 2 represent control cells stimulated with buffer only. Equal amounts of Triton X-100 cell lysates were analysed by western blotting with 4G10 mAb for phosphorylation. Molecular size markers are indicated on the right (kDa). Experiment shown is one representative of three independent experiments performed in duplicate.

(Fig. 1A) and thus was ideal for the priming experiments, similarly 10^{-8} M SP which is suboptimal stimulatory dose[3] (Fig. 1A) was tested in the following experiments to demonstrate whether synergy exists between eotaxin and SP. As can be seen in Fig. 1B, 10^{-8} M SP caused a significant EDN release only after the cells were first primed with 10 ng/ml eotaxin (compare bar 5 with bar 6).

Recently there is increasing evidence that tyrosine kinases are involved in EDN release pathway(s).[5] Therefore we next blocked CCR3 with 7B11 and tyrosine kinases with herbimycin A, before priming eosinophil with eotaxin. It was shown that both agents significantly blocked the priming activity of eotaxin (Fig. 1C, compare bars 9 and 10 with bar 8). To further gain insight into the role of tyrosine phosphorylation in eotaxin priming activity, we studied the phosphorylation pattern in normal eosinophils by SP in the same dynamics as the pharmacological assay i.e. in the presence and absence of 10 ng/ml eotaxin. As can be seen in Fig. 1D, 10^{-8} M SP did not induce any tyrosine phosphorylation (Compare lanes 3 and 4 with lanes 1 and 2), however, the same dose caused a significant phosphorylation of several protein residues, only after priming the cells with 10 ng/ml eotaxin (compare lanes 5 and 6 with lanes 3 and 4). It is of note that 10 ng/ml eotaxin stimulation did not cause any tyrosine phosphorylation (data not shown).

These findings suggest that low dose eotaxin stimulation of CCR3 may prime human eosinophil cytotoxic functional response against SP through a change in the signal transduction which at least in part involves tyrosine kinases.

We propose that eotaxin at low non functional stimulatory dose primes eosinophil for exaggerated inflammatory response by SP. If so, our data demonstrate a new indirect property of eotaxin in activating eosinophil exocytosis and provide a novel model of synergism at the cellular and molecular levels in hypersensitivity disease, which could be a potential target for therapy.

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