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Short Communication

Mechanisms involved in human eosinophil chemotaxis induced by the newly cloned C–C chemokine eotaxin

Amr El-Shazly, Keisuke Masuyama, Masao Eura and Takeru Ishikawa

Department of Otorhinolaryngology, School of Medicine, Kumamoto University, Kumamoto, Japan

Abstract

The present study was performed in order to investigate the mechanism(s) involved in eotaxin-induced normal human eosinophil chemotaxis using a 48-well microchemotaxis chamber assay. Eotaxin, at a wide range of doses, induced eosinophil chemotaxis with optimal activity at 100 ng/mL. To elucidate the role of Ca^{2+} as a second messenger, eosinophils were depleted of intracellular Ca^{2+} which, *per se*, did not modify eosinophil chemotaxis. To gain insight of the possible intracellular signal transduction, we blocked pertussis toxin (PTX)-sensitive G_i proteins as well as several protein kinases. It was found that the inhibition of tyrosine kinase with herbimycin A and the inhibition of mitogen-activated protein kinase (MAPK) with MEK-1 inhibitor (PD98059) significantly blocked chemotaxis; however, inhibition of protein kinase C with staurosporine, protein kinase A with H-89 and G_i proteins with PTX did not affect chemotaxis. These results suggest a signal transduction pathway(s) involving Ca^{2+}-independent tyrosine kinase and MAPK activities.

Key words: calcium, chemotaxis, eosinophil, eotaxin, signaling.

Introduction

Eosinophils are recruited in large numbers and are thought to play a detrimental role in allergic disease.[1–3] Several eosinophil chemotaxins have been described; however, eotaxin the newly cloned chemoattractant cytokine that belongs to the β chemokine family, has gained a lot of attention due to its unique property of selectivity in attracting eosinophils, probably due to the restricted expression of the CCR3 on the cell type.[4] This novel 74 amino acid C–C chemokine has been identified in guinea pig and mouse, as well as in humans.[5–7] *In vivo*, eotaxin has been reported to cause the accumulation of eosinophils in the dermis of the guinea pig; in addition, increased mRNA expression in the lungs of postallergen-challenged sensitized guinea pigs has been described.[5,8] *In vitro*, activation of human eosinophils by eotaxin involves chemotaxis, actin polymerization, Ca^{2+} influx and the production of reactive oxygen species and it has been suggested that pertussis toxin (PTX)-sensitive G_i proteins, protein kinase C (PKC), tyrosine kinase and phosphatidyl-inositol-3-kinase were all involved in the signal transduction of human eosinophils following stimulation with eotaxin.[9] In the present *in vitro* study we investigated the intracellular signals involved in eotaxin-induced human eosinophil chemotaxis.

Methods

Reagents

Percoll solution was purchased from Pharmacia (Uppsala, Sweden). Herbimycin A, staurosporine, fura 2-AM and BAPTA-AM were obtained from Wako Pure Chemical Industries (Osaka, Japan). Pertussis toxin was purchased from Calbiochem–Novabiochem Corporation (La Jolla, CA, USA), MEK-1 inhibitor was obtained from Biolabs Inc. (New England, USA) and human eotaxin and H-89 were purchased from Funakoshi Co. (Tokyo, Japan).

Correspondence: Dr K Masuyama, Department of Otorhinolaryngology, School of Medicine, Kumamoto University, 1-1-1 Honjo, Kumamoto 860, Japan.
Email: <masuyama@gpo.kumamoto-u.ac.jp>

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Eosinophil purification

Eosinophils were separated from the heparin-anti-coagulated venous blood of healthy donors using Percoll discontinuous density gradient centrifugation. A high purity of > 98% eosinophilic granulocytes, as judged by Hinkelmann staining, was obtained by negative selection using CD-16 immunomagnetic beads and a magnetic cell sorting separator (Miltenyi Biotec, Bergisch Gladbach, Germany).[10] Eosinophil viability was evaluated by Trypan blue dye exclusion and was always > 95%.

Chemotaxis assay

Chemotaxis experiments were performed using a 48-well microchemotaxis chamber and a 5 μm pore polycarbonate membrane filter, as described previously.[11,12] Briefly, 29 μL of the chemotactic agent was placed in the lower compartment of the chamber, while 50 μL purified eosinophils, suspended in RPMI 1640 containing 2% fetal calf serum (FCS) and adjusted to 10^6 cells/mL, was placed in the upper compartment. After incubation for 90 min at 37°C, 5% CO_2, the filter was removed, fixed and stained with Diff-Quick set (International Reagents Corp., Kobe, Japan). As for the checkerboard assay, both the upper and lower compartments contained different dilutions of eotaxin. Results of the chemotaxis experiments are expressed as eosinophils/5 selected high power fields (5 h.p.f.).

Ca^{2+}-depleted eosinophils

Ca^{2+}-depleted eosinophils were obtained by incubating 10^7 cells/mL with 30 μmol/L of the calcium chelating agent BAPTA-AM in test medium (composition (in mmol/L): NaCl 130; $NaHCO_3$ 5; KCl 4.6; glucose 5; EGTA 2; HEPES 20) for 30 min at 37°C in the dark. After washing the cells, they were suspended in the Ca^{2+}-free buffer and their chemotactic response against eotaxin was then tested.

Statistical analysis

Values are expressed as the mean ± SEM. Statistical analysis was performed by analysis of variance (ANOVA) except for Fig. 3, which was analyzed by paired Student's *t*-test. Statistical significance was indicated by $P < 0.05$.

RESULTS

A wide range of doses of eotaxin attracted normal eosinophils in modified Boyden chambers, with optimal activity at 100 ng/mL (Fig. 1). A checkerboard assay was done to clarify whether eotaxin also exhibited chemokinetic activity. Whenever cells migrate in response to concentration gradients of chemotactic molecules, it is termed chemotaxis or directed cell migration, whereas chemokinesis represents random migration without the requirement of concentration gradient. As can be seen in Fig. 2, eotaxin not only showed chemotactic activity, but also exhibited chemokinetic activity. Nonetheless, the chemotactic activity was more significant than the chemokinetic activity.

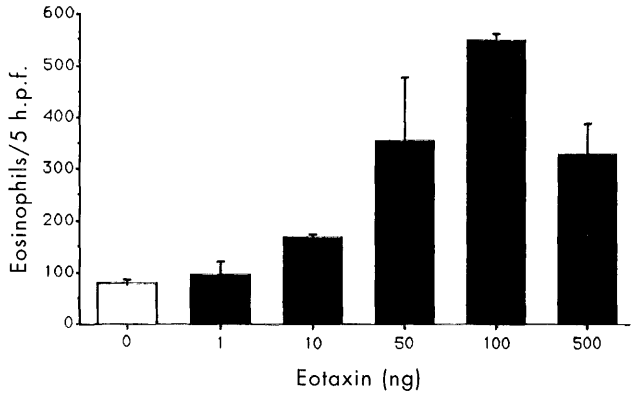

Fig. 1 Induction of normal human eosinophil chemotaxis by eotaxin. Each bar represents the mean ± SEM of six independent experiments, each performed in triplicate ($P < 0.05$, 10–500 ng eotaxin).

[Eotaxin] above filter

[Eotaxin] below filter

ng/mL	10	50	100
10	**167.3**	**257.3**	**316**
50		**233.3**	
100			**240.3**

Fig. 2 The checkerboard analysis of eotaxin on normal human eosinophils. Values represent the mean of migrated cells in triplicate filters.

The optimal dose of eotaxin induced chemotaxis of Ca^{2+}-depleted eosinophils equally potently as in non-depleted cells (Fig. 3). In depleted cells, preliminary studies showed an initial $[Ca^{2+}]_i$ of 20 nmol/L, as judged by fura 2-AM assay using the equation established by Poenie *et al.*,[13] which renders the depletion assay technically reliable (data not shown).

Eosinophil treatment with PTX, staurosporine or H-89 did not affect eosinophil chemotaxis against eotaxin; however, treatment of cells with herbimycin A and MEK-1 inhibitor resulted in a significant block of eotaxin-induced eosinophil chemotaxis, as can be seen in Table 1. Cell viability was checked after each treatment to rule out cytotoxic effects and was always > 95% as judged by Trypan blue exclusion.

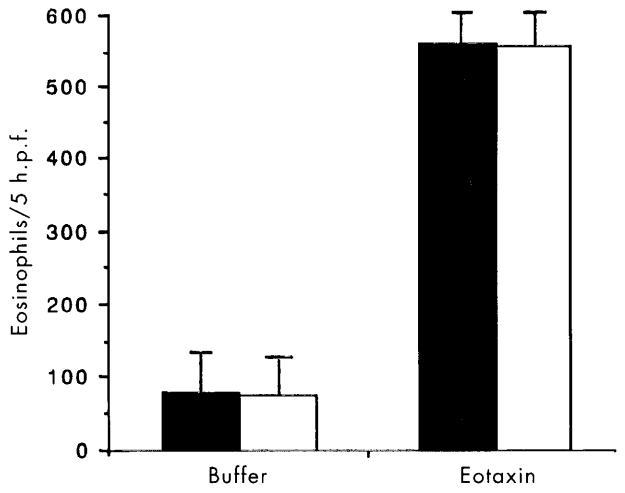

Fig. 3 Comparison of the eosinophilotactic activity of 100 ng/mL eotaxin on Ca^{2+}-depleted eosinophils (□) compared with non-depleted eosinophils (■). Results are the mean ± SEM of six different experiments performed in triplicate.

Table 1. Effect of pertussis toxin, staurosporine, H-89, MEK-1 inhibitor and herbimycin A on 100 ng/mL eotaxin-induced eosinophil chemotaxis

Pretreatment	Eosinophils/5 h.p.f.
Control (buffer)	563 ± 13
PTX (1000 ng/mL)	567 ± 11
Staurosporine (10^{-7} mol/L)	551 ± 45
H-89 (10^{-6} mol/L)	564 ± 47
MEK-1 inhibitor (10^{-7} mol/L)	386 ± 22*
Herbimycin A (10^{-6} mol/L)	280 ± 34†

Eosinophils were pretreated with buffer, pertussis toxin (PTX) or various kinase inhibitors for 1 h, followed by washing of the cells and resuspension in the culture medium. Eosinophil chemotaxis against 100 ng/mL eotaxin was then tested as described in Methods. Results represent the mean ± SEM of nine different experiments, each performed in triplicate. †$P < 0.01$, *$P < 0.05$ compared with control cells pretreated with buffer only.

Discussion

The newly cloned eotaxin has proven to be a very potent and selective activator of eosinophils. In the present study, we investigated the mechanism(s) involved in human eosinophil directed cell migration against this novel chemokine. In an earlier study, pretreatment of eosinophils with PTX blocked the respiratory burst activation of human eosinophils by eotaxin;[9] however, in the present study, PTX did not modify chemotaxis, indicating that other G protein(s) rather than the PTX-sensitive G_i protein may be involved in chemotaxis signaling.

Staurosporine, at a dose that inhibited the respiratory burst induced by eotaxin in human eosinophils,[9] and H-89, at a dose that ensured a potent inhibitory action against cAMP-dependent protein kinase,[14] did not modify eosinophil chemotaxis, which eliminates the possibility of involvement of PKC and protein kinase A pathways in eotaxin-induced eosinophil chemotaxis. Similarly, the observation that depletion of intracellular $[Ca^{2+}]_i$ did not affect chemotaxis renders the involvement of other Ca^{2+}-dependent pathways, such as phospholipase A_2 and phospholipase D, unlikely.[15,16] However, herbimycin A, the potent tyrosine kinase inhibitor,[17,18] and MEK-1 inhibitor[19–22] significantly blocked chemotaxis. Taken together, our results suggest the involvement of Ca^{2+}-independent tyrosine kinase and mitogen-activated protein kinase activation in eotaxin signaling for chemotaxis, which correlates well with the fact that phosphorylation of 102 and 122 kDa proteins on the tyrosine residues of human eosinophils induced by interleukin (IL)-5, granulocyte–macrophage colony stimulating factor and IL-3 were Ca^{2+} independent.[23] Nonetheless, further studies are needed to detect protein phosphorylation mediated by eotaxin in the presence and absence of $[Ca^{2+}]_i$. These studies are currently underway in our laboratory.

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