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Identification of Novel Eosinophil Chemotactic Peptides Derived from *Dirofilaria immitis*

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Abstract

We previously cloned a cDNA encoding a neutrophil chemotactic factor of *Dirofilaria immitis* (DiNCF), and showed that Met-Phe-Lys could act as a functional epitope for neutrophil chemotactic activity. In the present study, we showed that recombinant DiNCF possessed chemotactic activities for both eosinophils and neutrophils. To identify a novel eosinophil chemotactic oligopeptide derived from DiNCF sequence, we synthesized a formylated tripeptide (fMet-Phe-Lys-OH) and its C-terminus derivatives (fMet-Phe-Lys-OMe, fMet-Phe-Lys-NH_2), and their chemotactic activities were examined. fMet-Phe-Lys-OH showed intense chemotactic activity for neutrophils but not for eosinophils. In contrast, fMet-Phe-Lys-OMe or fMet-Phe-Lys-NH_2 showed significant eosinophil chemotactic activity at 10^{-4} to 10^{-7} M. The neutrophil chemotactic activity of fMet-Phe-Lys-OMe or fMet-Phe-Lys-NH_2 was, however, less than 1/100 of fMet-Phe-Lys-OH in molar basis. These results suggest that Met-Phe-Lys potentially acts as a chemotactic factor for eosinophils as well as for neutrophils. C-terminal negative charge of the peptide likely repels the receptor molecule on eosinophils though it fits the receptor molecule on neutrophils.

Key words: *Dirofilaria immitis*; eosinophil; neutrophil; chemotaxis; peptide.

Introduction

Eosinophil chemotactic factor of anaphylaxis (ECF-A) is a well-characterized eosinophil chemotactic tetrapeptide derived from mast cells (Goetzl and Austen, 1975). Though various parasites also possess ECFs (Horii *et al.*, 1988), the molecular structure of those parasite-derived ECFs still remains unknown. As to the neutrophil chemotactic factors (NCFs) derived from parasites, we successfully purified a neutrophil chemotactic component from *D. immitis* adult worm with an apparent molecular weight of 14,000 (Horii *et al.*, 1986). Recently, we isolated a cDNA clone that encodes *D. immitis*-derived NCF (Owhashi *et al.*, 1993), and proposed Met-Phe-Lys as a candidate of the functional epitope for neutrophil chemotactic activity. Apart from the parasite-derived NCF, formylated Met-Leu-Phe is a most well-characterized neutrophil chemotactic peptide (Showell *et al.*, 1976). Similar to Met-Leu-Phe, N-terminus formylation of Met-Phe-Lys augmented the number of migrated neutrophils (Owhashi *et al.*, 1993) probably due to the favorable affinity for the ligand-receptor binding. However the role of carboxyl group of the peptide on leukocyte chemotaxis is unclear.

In this study, we examined the chemotactic activity of recombinant DiNCF for eosinophils. We also studied the effect of modulation of C-terminal carboxyl group of the formyl Met-Phe-Lys, and identified a novel eosinophil chemotactic peptide. The results suggested that C-terminal negative charge of fMet-Phe-Lys inhibits the eosinophil chemotactic activity, indicating the presence of distinctive receptor molecules on eosinophils from those on neutrophils.

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Materials and Methods

Mice

Female ddY mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and used at 8–10 weeks of age. Animals were housed in 23°C, 12 h light and dark conditions.

Eosinophils and neutrophils

For collection of eosinophil-rich peritoneal exudate cells, mice were infected intraperitoneally with 100 larvae of *Mesocestoides corti*. Three weeks later, peritoneal lavage was harvested. The cells were washed twice with PBS, and suspended in RPMI1640 medium (Gibco BRL, Gland Island, NY, USA) containing 2% fetal bovine serum (Gibco BRL). To remove adherent cells, the cell suspension was incubated in tissue culture flask (Nunc) at 37°C for 30 min. The non-adherent cells were used for eosinophil chemotaxis experiments. By this method, more than 1×10^7 eosinophils were collected from one mouse and the purity of eosinophils was 50–70%. Major contaminants were macrophages and neutrophils. Neutrophil-rich peritoneal exudate cells were obtained from normal ddY mice which received i.p. injection of 2 ml of 0.1% oyster glycogen (Nacalai Tesque, Kyoto, Japan) 2h before use. Neutrophil-rich cell suspensions (>90% neutrophils) from three animals were pooled, and used for the neutrophil chemotaxis experiments.

Chemotaxis assay

Chemotactic activity for eosinophils or neutrophils was measured according to the method described previously (Owhashi and Ishii, 1982) with slight modifications in these peptides. In brief, multi-well micro-chemotaxis chambers (Neuro Probe, Bethesda, MD, USA) were equipped with Millipore membrane filters (Millipore Co., Bedford, MA, USA) with pore size of 3 μm. The concentration of indicator cells was adjusted to 1×10^6 with RPMI 1640 medium containing 2% fetal bovine serum. Incubation was performed at 37°C for 90 min. The membranes were stained according to Litt's procedure. The numbers of migrated neutrophils were counted in randomly selected 10 high-power fields (hpf) at 400 magnification as described previously (Owhashi and Ishii, 1982). The chemotactic activity was expressed as the mean ± SEM of 4 experiments.

Preparation of recombinant DiNCF

Expression vector pGEM-EX, which allows in-frame ligation of DNA to yield gene 10 fusion proteins, was purchased from Promega (Madison, WI, USA). A cDNA clone that encodes DiNCF (pD4) (Owhashi *et al.*, 1993) was subcloned at the Eco RI site of pGEM-EX. The recombinant plasmid was introduced into *Escherichia coli* JM109 (DE3) (Promega), and expression was induced by isopropyl-β-D-thiogalactopyranoside (Takara Shuzo, Kyoto, Japan). Insoluble fraction of bacterial proteins was prepared (Kleid *et al.*, 1981).

Peptide synthesis

Formyl Met-Phe-Lys-OH or fMet-Phe-Lys-NH_2 was prepared by Fmoc-based solid phase peptide synthesis (Atherton and Sheppard, 1989). fMet-Phe-Lys-OMe was prepared by a mixed anhydride method in solution (Day and Freer, 1979). Purification of the peptides after deprotection was achieved by reverse phase high-performance liquid chromatography.

Results

Recombinant DiNCF was examined for the chemotactic activity for eosinophils or neutrophils. As shown in Fig. 1, a fusion protein of DiNCF and gene 10 showed a significant chemotactic activity for eosinophils as well as for neutrophils in a dose-dependent fashion. In contrast, control gene 10 protein showed marginal chemotactic activities for both eosinophils and neutrophils.

In the previous study, we showed that Met-Phe-Lys (residue 99–101) is a functional epitope for neutrophil chemotactic activity of DiNCF (Owhashi *et al.*, 1993). To examine whether Met-Phe-Lys could also exhibit a chemotactic activity for eosinophils, fMet-Phe-Lys-OH, fMet-Phe-Lys-OMe and fMet-Phe-Lys-NH_2 were synthesize and examined for chemotactic activities for eosinophils and neutrophils. As shown in Fig. 2, the eosinophil chemotactic activity of fMet-Phe-Lys-OH was hardly detectable at any concentrations of the peptide so far as we examined. In contrast, both fMet-Phe-Lys-

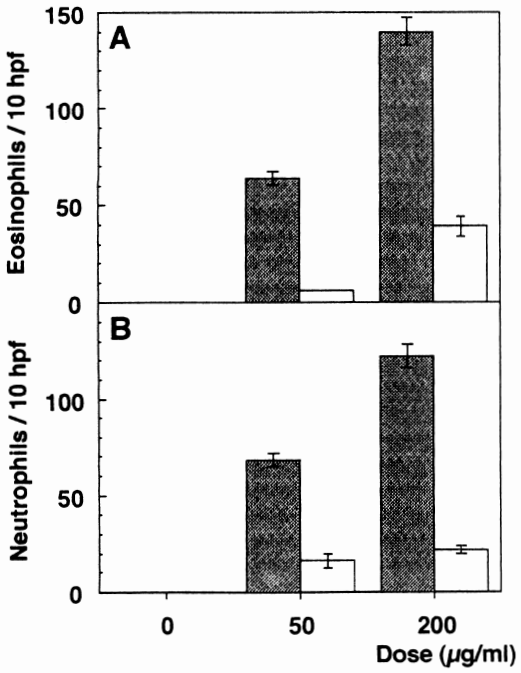

Fig. 1 Chemotactic activity of recombinant NCF derived from *D. immitis*. Recombinant fusion protein of DiNCF and gene 10 (shaded column), and gene 10 only (open column) was examined for chemotactic activity for eosinophils (panel A) or neutrophils (panel B) at 200 μg/ml (4×10^{-6} M fusion protein) or 50 μg/ml (1×10^{-6} fusion protein).

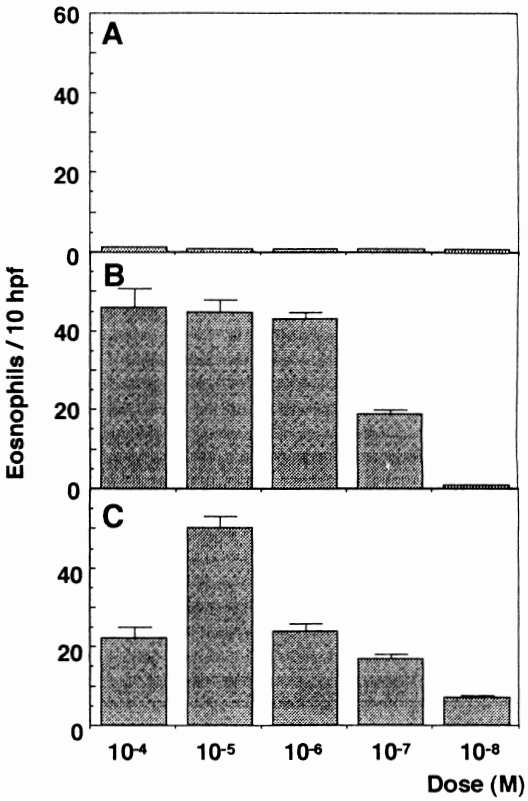

Fig. 2 Eosinophil chemotactic activity of fMet-Phe-Lys-OH and its derivatives. (A) fMet-Phe-Lys-OH, (B) fMet-Phe-Lys-OMe or (C) fMet-Phe-Lys-NH_2 were examined for neutrophil chemotactic activity. Spontaneous migration yielded at average of 2±1 of eosinophils per 10 hpf.

OMe and fMet-Phe-Lys-NH_2 showed a significant eosinophil chemotactic activity peaked around 10^{-5} M (Fig. 2). In addition, they exhibited a chemotactic activity also for monocytes, whereas fMet-Phe-Lys-OH showed a marginal chemotactic activity (data not shown). As shown in Fig. 3, the peak of neutrophil chemotactic activity of fMet-Phe-Lys-OH was identified at 10^{-7} to 10^{-8} M. On the other hand, fMet-Phe-Lys-OMe or fMet-Phe-Lys-NH_2 showed at least 100-fold reduction of the neutrophil chemotactic activity in molar basis when compared with fMet-Phe-Lys-OH.

Discussion

We have shown that various parasites themselves possess ECFs and NCFs (Horii *et al.*, 1986; 1988; Owhashi and Ishii, 1982). Recently, we successfully cloned a cDNA encoding *D. immitis*-derived NCF (Owhashi *et al.*, 1993). However, the molecular structure of the parasite-derived ECFs is still unknown.

In the present study, we showed that the recombinant DiNCF possesses chemotactic activities for both neutrophils and eosinophils (Fig. 1). Concerning this, Horii *et al.* (1988) showed that crude *D. immitis* extract exhibits an intense chemotactic activity for neutrophils whereas it shows a weak chemotactic activity for eosinophils. This discrepancy may be due to the difference of the source of indicator cells used in chemotaxis assays, or effect of the coexistence of other neutrophil chemotactic components in the crude *D. immitis* extract that would result in the relative weakness for eosi-

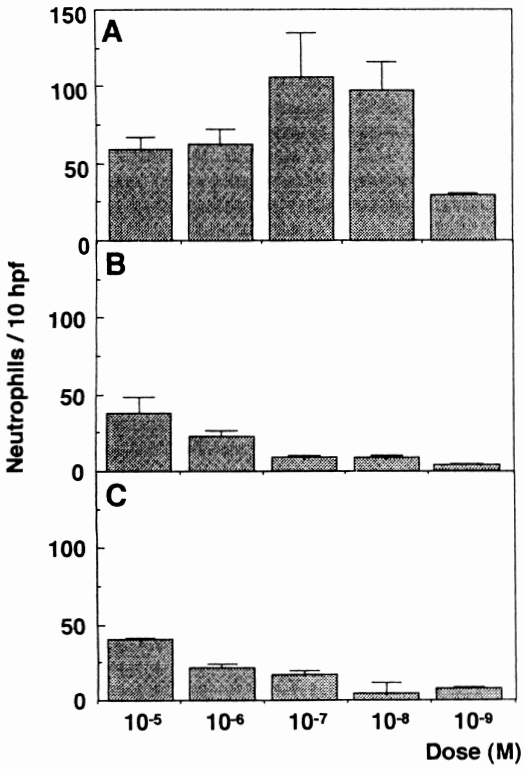

Fig. 3 Neutrophil chemotactic activity of fMet-Phe-Lys-OH and its derivatives. (A) fMet-Phe-Lys-OH, (B) fMet-Phe-Lys-OMe or (C) fMet-Phe-Lys-NH_2 was examined for neutrophil chemotactic activity. Spontaneous migration yielded at average of 4±2 of neutrophils per 10 hpf.

nophil chemotaxis in comparison with neutrophil chemotaxis.

We previously showed the NCF activity of fMet-Phe-Lys-OH, and proposed Met-Phe-Lys (residue 99–101) as the functional epitope of DiNCF for neutrophil chemotaxis (Owhashi *et al.*, 1993). In the present study, we examined the possibility that the same moiety of the molecule also possesses an important role for eosinophil chemotaxis. To confirm this, we synthesized fMet-Phe-Lys-OH, fMet-Phe-Lys-OMe and fMet-Phe-Lys-NH_2, and examined for chemotactic activities. Both fMet-Phe-Lys-OMe and fMet-Phe-Lys-NH_2 showed an intense chemotactic activity for eosinophils (Fig. 2) but they showed a weaker neutrophil chemotactic activity than fMet-Phe-Lys-OH did. This fact suggests that Met-Phe-Lys potentially acts as a functional epitope also for eosinophils. It seems likely that C-terminal negative charge of the peptide repels the receptor molecule on eosinophils even though it fits for the receptor molecule on neutrophils. Similar to our findings, Ho *et al.* (1978) showed that the C-terminal carboxyl modifications of formylated chemotactic peptides resulted in a remarkable reduction in the chemotactic activity for neutrophils, whereas a general increase of the chemotactic activity for monocytes. In contrast, Freer *et al.* (1982) showed higher neutrophil chemotactic activity of benzyl esters and benzylamide derivatives of tripeptides than their free acid counterparts.

It has been shown that the receptor molecules for fMet-Leu-Phe resolve into two isoforms (Boulay *et al.*, 1990), and the sequence comparisons revealed the features of a subfamily of G-protein coupled receptors (Boulay *et al.*, 1990; Thomas *et al.*, 1990, Ye *et al.*, 1993). In the present study, we showed that the blocking of a C-terminal negative charge of fMet-Phe-Lys resulted in the remarkable reduction of neutrophil chemotaxis and appearance of eosinophil chemotaxis. This fact indicates that eosinophils possess distinctive receptors for Met-Phe-Lys from those on neutrophils. It is possible that a positively charged group(s) of the receptor molecules where a negatively charged carboxyl group of the formyl peptides interacts on neutrophils may be replaced with a negatively charged group(s) on eosinophils.

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