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Chemotactic Effect of Leukotactin-1/CCL15 on Human Neutrophils

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Leukotactin-1 (Lkn-1)/CCL15 has been known as a potent chemoattractant of leukocytes. However, the precise function of Lkn-1 in human neutrophils has not been explained well. In the present study, we investigated the contribution of Lkn-1 in chemotactic activity of human neutrophils. Both CCR1 and CCR3 mRNA expressions are strongly expressed in human neutrophils but CCR2 protein expression was uniquely detected on the cell surface. Lkn-1 binding to CCR1 and CCR3 induced chemotactic activity of neutrophils. Chemotactic index of Lkn-1 was comparable to that of IL-8. MIP-1α/CCL3 binding to CCR1 and CCR5 has no effect on neutrophil migration. Cell migration, in response to Lkn-1, was blocked by pertussis toxin (Ptx), a G_o/G_i protein inhibitor, and U73122, a phospholipase C (PLC) inhibitor but not by protein kinase C inhibitor such as rottlerin, and Ro-31-8425. Taken together, our results demonstrate that Lkn-1 transduces the chemotaxis signal through G_o/G_i protein and PLC. This finding provides the molecular mechanism by which Lkn-1 may contribute to neutrophil movement into the site of inflammation.

Key Words: Leukotactin-1, Neutrophils, Chemotaxis

INTRODUCTION

Leukotactin-1 (Lkn-1)/CCL15, a member of the human CC chemokine family, has been known to bind CCR1 and CCR3, and chemoattracts leukocytes, including neutrophils, monocytes and lymphocytes (Youn et al., 1997). Lkn-1 is believed to play an important role in the development of inflammation and atherosclerosis (Youn et al., 1997; Yu et al., 2004). We previously demonstrated signal transduction induced by Lkn-1 after its binding to CCR1, seven transmembrane G protein-coupled receptor. However, the function of Lkn-1 and its associated signaling in human neutrophils remain to be characterized.

Neutrophils are short-lived and polymorphonuclear granulocytes. During early immune response against infection, neutrophils migrate from the blood into inflamed tissues, remove extracellular pathogens by phagocytosis and release of toxic mediators, and undergo apoptosis in the absence of survival stimuli. Regulation of neutrophil apoptosis plays an important role in the balance between immune defense and safe resolution of the inflammation (Webb et al., 2000; Maianski et al., 2004).

Chemokine and chemokine receptor regulate a variety of pathophysiological responses, including leukocyte recruitment in inflammatory response and cell differentiation in hematological malignancy (Murdoch and Finn, 2000; Laurence, 2006). Chemokine receptors are classified into two main groups. CC chemokine receptor (CCR) binding to CC chemokine includes CCR1 through CCR11 while CXC chemokine receptor (CXCR) interacting to CXC chemokine does CXCR1 through CXCR5 (Laurence, 2006). The role of CC chmokine in eosinophils and monocytes has been known well, but that of CC chemokine in neutrophils is still controversial (Lampinen et al., 2004; Baggiolini et al., 1997).

In the present study, we investigated the expression of CCRs and the chemotactic ability of Lkn-1 in human neutrophils.

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MATERIALS AND METHODS

1. Materials

RPMI 1640, fetal bovine serum (FBS), and Trizol were purchased from Life Technologies, Inc. (Gaithersburg, MD). Recombinant human Lkn-1/CCL15, MIP-1α/CCL3, IL-8/CXCL8, and anti-CCR1, anti-CCR2, anti-CCR3, anti-CCR4 and anti-CCR5 antibodies were obtained from R&D Systems (Minneapolis, MN). FITC-conjugated goat anti-mouse IgG and anti-rat IgG were obtained from Molecular Probes (Eugene, OR). Pertussis toxin (Ptx), U73122, rottlerin, and Ro-31-8425 were obtained from Calbiochem (San Diego, CA).

2. Preparation and culture of human neutrophils

Human neutrophils were isolated from heparinized peripheral blood of healthy volunteers by Ficoll-Hypaque solution (Amersham Pharmacia Biotechnology, Buckinghamshire, U.K.). After removal of erythrocytes using RBC lysis solution (Sigma, St. Louis, Mo), the neutrophils were washed, resuspended and cultured at a concentration of 3×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell viability was determined by the trypan blue exclusion test (> 95%). Neutrophil purity was above 97% as assessed by Wright staining of cytocentrifuged samples.

3. RT-PCR

RT-PCR was performed to evaluate relative quantities of mRNAs for CCR1, CCR2, CCR3, CCR4, and CCR5 in human neutrophils. Total RNA was extracted from the cells using Trizol reagent according to the manufacturer's instructions. For cDNA preparation, total RNA (2 μg) was incubated at 37℃ for 90 min using first-strand cDNA synthesis kit (Promega, Madison, WI). The cDNAs were denatured at 94℃ for 5 min and then amplified by 30~35 cycles of denaturation at 94℃C for 1 min, annealing at 58℃ for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 10 min. Primer sequences were done as described previously (Sato et al., 2003; Oba et al., 2005) (Table 1). GAPDH was used as an internal control for each PCR reaction. Final PCR products were separated on 1% agarose gel, and then visualized by ethidium bromide staining.

Table 1. Primers used for PCR

Target mRNA	Forward primer (5' → 3') Reverse primer (5' → 3')
CCR1	CTC TTC CTG TTC ACG CTT CC CCA AAT GTC TGC TCT GCT CA'
CCR2	AAC TCC TGC CTC CGC TCT AC TCA CTG CCC TAT GCC TCT TC
CCR3	TCT TCC TCG TCA CCC TTC CA GCT TCG TCC GCT CAC AGT CA
CCR4	CTT CCT GCC CCC ACT GTA TT TCT TCA CCG CCT TGT TCT TC
CCR5	TCC TGC CTC CGC TCT ACT GAA CTT CTC CCC GAC AAA
GAPDH	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA

4. Flow cytometry

Freshly isolated neutrophils were washed with PBS buffer containing 0.5% BSA. For blocking non-specific antibody binding, the cells were incubated with normal rabbit IgG. The cells were separated into new tubes and each tube was added with PBS buffer containing anti-CCR1, anti-CCR2, anti-CCR3, anti-CCR4 or anti-CCR5 antibodies. Baseline fluorescence was obtained by incubation at room temperature for 30 min with normal mouse IgG, instead of anti-CC chemokine receptor antibodies. After washing three times, the cells were incubated at 4℃ for 30 min with FITC-conjugated goat anti-mouse IgG or anti-rat IgG. Finally, the cells were washed and analyzed on a FACSort cytofluorimeter (Becton-Dickinson, Mountain View, CA). Ten thousand events were collected for each experiment. The surface expression of CCR1, CCR2, CCR3, CCR4 or CCR5 was evaluated as the relative mean intensity when the mean intensity of baseline fluorescence was set as 100%.

5. Chemotaxis assay

Cell migration was performed using a 48 well microchamber (Neuroprobe, Cabin John, MD). The lower wells were filled with 28 μl buffer alone or with buffer containing chemokine and a polyvinylpyrrolidone-free filter (Neuroprobe) with 5 μm pores was placed over them. The upper wells were filled with 50 μl of neutrophils at 2×10^6 cells/ml in RPMI 1640 containing 1% BSA and 30 mM HEPES. After incubation for 90 min at 37℃, the filters were removed from the chamber, washed, fixed, and stained with

Diff-Quick (Baxter, Deerfield, IL). The cells of two randomly selected fields per well were counted using Axiovert 25 (Carl Zeiss, Jena, Germany). The chemotactic index (CI) was calculated from the number of cells that migrated to the control. A significant chemotaxis was defined as CI > 2.

6. Statistical analysis

Data are expressed as means ± SD. Statistical differences were analyzed using the paired t-test for two-group comparisons. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for statistical analysis. A significant value was defined as $P<0.01$.

RESULTS

1. mRNA and protein expression of CC chemokine receptors in human neutrophils

We first examined mRNA and surface protein expression of CCRs, CCR1 through CCR5, in neutrophils by performing RT-PCR and flow cytometry. As shown in Fig. 1 and Fig. 2, both CCR1 and CCR3 mRNAs were strongly expressed, but protein expression of the receptors was not detected on the cell surface. Protein expression of CCR2 was weakly detected. Surface protein expression of other chemokine receptors, CCR4, and CCR5 was not detected. These results indicate that the difference between CCR mRNA and protein expression may be caused by different translation process of individual CCR mRNA.

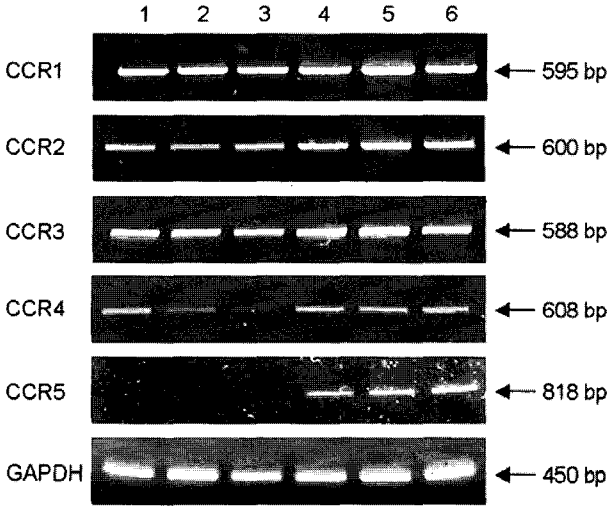

Fig. 1. mRNA expression of CC chemokine receptors in human neutrophils. Total RNA was extracted from isolated neutrophils. mRNA expression of CC chemokine receptors was analyzed by RT-PCR using CCR1, CCR2, CCR3, CCR4 or CCR5 primer as described in the materials and methods section. GAPDH was used as an internal control. Data are expressed as representative of six individual experiments (1-6).

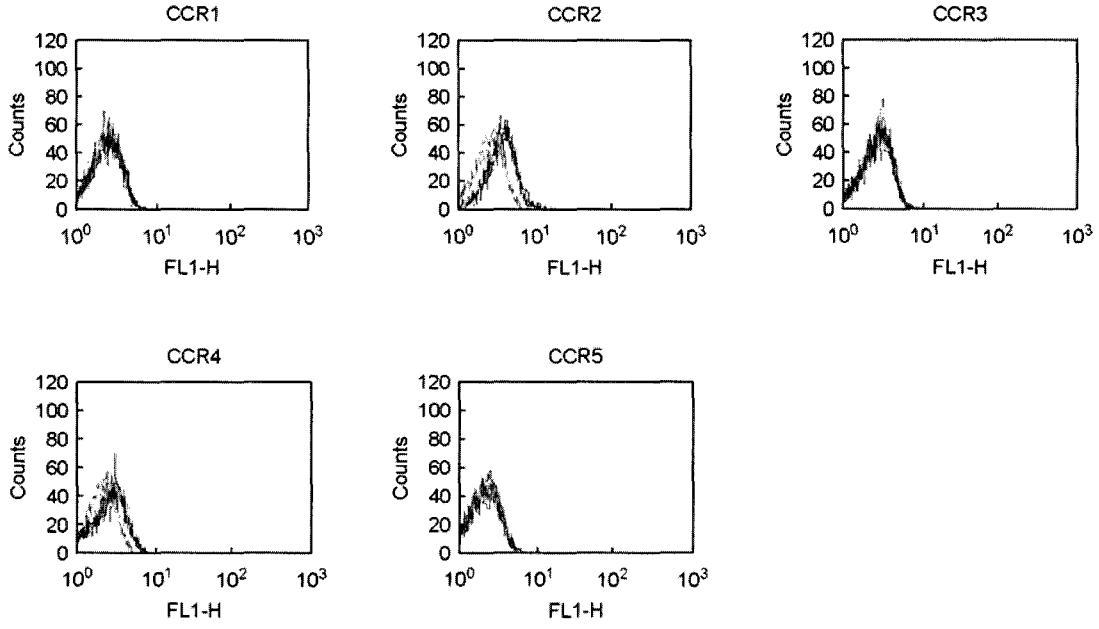

Fig. 2. Surface protein expression of CC chemokine receptors in human neutrophils. Neutrophils were isolated and analyzed by fluorescence-activated cells sorter using anti-CCR1, anti-CCR2, anti-CCR3, anti-CCR4 or anti-CCR5 antibodies (thick line). Baseline fluorescence was obtained by incubating normal mouse IgG instead of anti-CCR antibodies (thin line). Data are expressed as representative of three individual experiments.

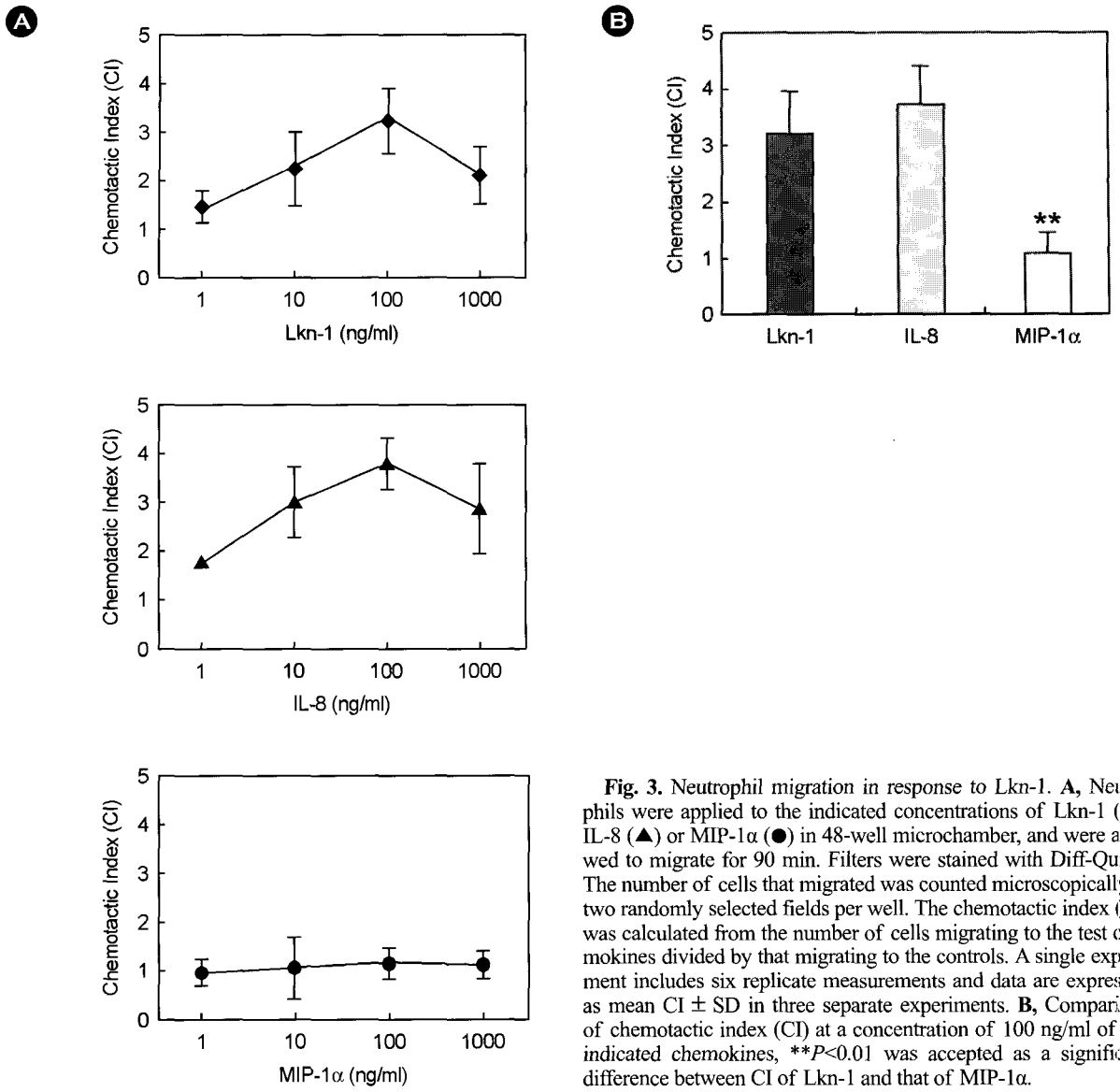

Fig. 3. Neutrophil migration in response to Lkn-1. **A,** Neurophils were applied to the indicated concentrations of Lkn-1 (◆), IL-8 (▲) or MIP-1α (●) in 48-well microchamber, and were allowed to migrate for 90 min. Filters were stained with Diff-Quick. The number of cells that migrated was counted microscopically in two randomly selected fields per well. The chemotactic index (CI) was calculated from the number of cells migrating to the test chemokines divided by that migrating to the controls. A single experiment includes six replicate measurements and data are expressed as mean CI ± SD in three separate experiments. **B,** Comparison of chemotactic index (CI) at a concentration of 100 ng/ml of the indicated chemokines, $^{**}P<0.01$ was accepted as a significant difference between CI of Lkn-1 and that of MIP-1α.

2. Neutrophil migration in response to Lkn-1

Although CCR1 and CCR3 expressions were not detected in neutrophils, we examined whether Lkn-1 induces neutrophil migration by performing a chemotaxis assay. Lkn-1 showed the typical bell-shape curve in neutrophil chemoattraction with the peak of the curve at a concentration of 100 ng/ml and IL-8 also showed the maximum activity at a concentration of 100 ng/ml (Fig. 3A). However, MIP-1α binding to CCR1 and CCR5 did not show significant chemotactic activity in neutrophils. Chemotactic index of Lkn-1 was comparable to that of IL-8 at the peak concentration (Fig. 3B). These data indicate that Lkn-1 differentially triggers neutrophil chemotaxis unlike MIP-1α.

3. Involvement of G_o/G_i protein and phospholipase C (PLC) in Lkn-1-induced chemotaxis

To better understand how Lkn-1 induces neutrophil migration, we investigated signaling proteins associated with this mechanism using specific signal inhibitors. Since CCR is a seven G protein-coupled receptor, neutrophils were pre-treated with Ptx, a G_o/G_i protein inhibitor, before the stimulation of Lkn-1. In the presence of Ptx, the number of the cells migrated by Lkn-1 significantly decreased to 54% of the number that chemoattracted in the absence of Ptx (Fig. 4) ($P<0.01$). To further characterize the chemotaxis signaling in response to Lkn-1, we investigated the possible implication of PLC and PKC. Fig. 4 shows that Lkn-1-

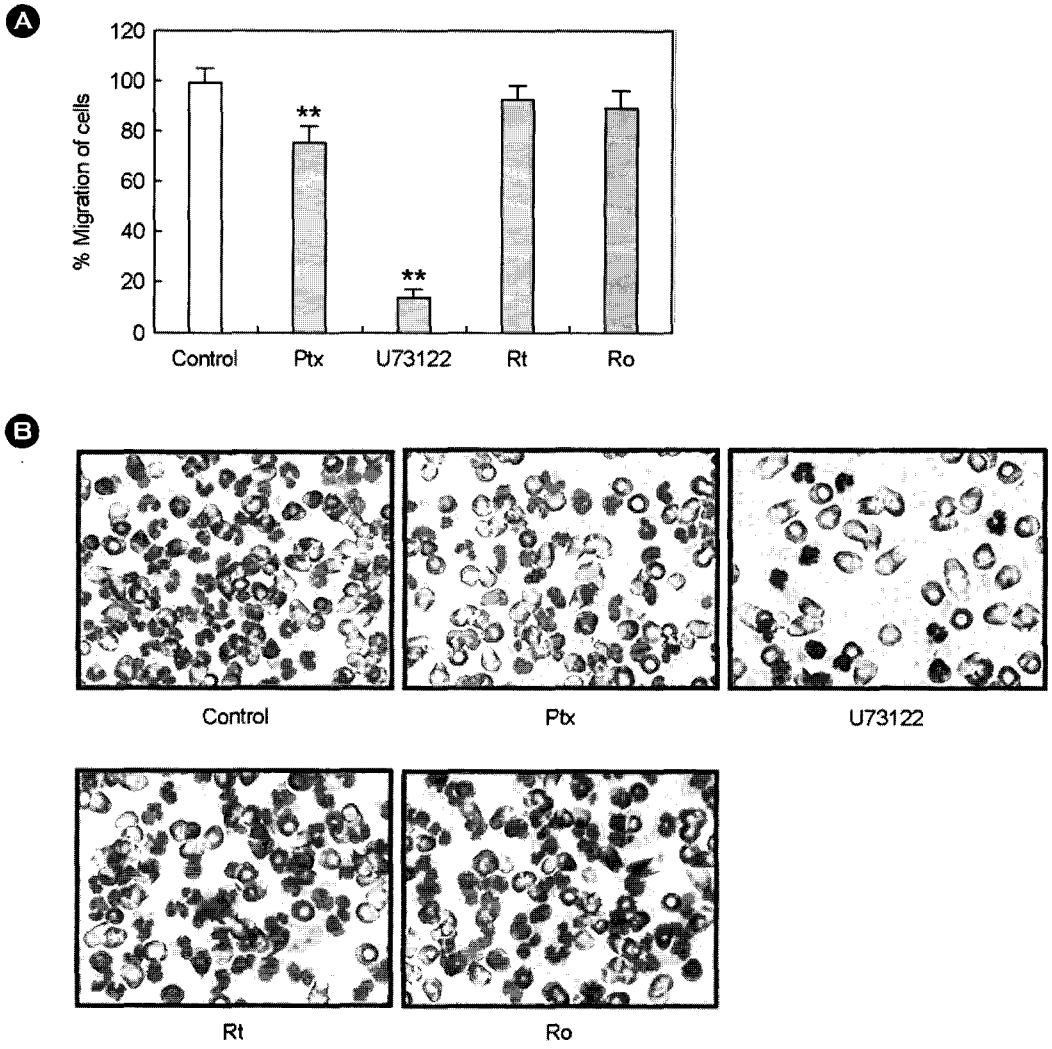

Fig. 4. Effect of pertussis toxin, G_o/G_i protein inhibitor and U73122, PLC inhibitor on neutrophil chemotaxis in response to Lkn-1. **A,** Neutrophils were pre-treated in the absence (Control; open bar) or presence (closed bar) of 100 ng/ml pertussis toxin (Ptx), 10 μM U73122 (U73122), 5 μM rottlerin (Rt), or 200 nM R0-31-8425 (Ro). The cells were applied to 100 ng/ml Lkn-1 in a 48-well microchamber and were allowed to migrate for 90 min. Filters were stained with Diff-Quick. The number of cells that migrated was counted microscopically in two randomly selected fields per well. Data are expressed as means ± SD in two individual experiments, and presented in relation to the negative control, which was set at 100%. **The significant difference was $P<0.01$. **B,** Photograph of the migrated cells after pre-treatment in the absence (Control) or presence of 100 ng/ml pertussis toxin (Ptx), 10 μM U73122 (U73122), 5 μM rottlerin (Rt) or 200 nM Ro-31-8425 (Ro).

induced cell migration is blocked by the pre-treatment with U73122, a PLC inhibitor. PKC inhibitors, including rottlerin and Ro-31-8425 have no inhibitory effect on Lkn-1-induced chemotaxis. These data indicate that Lkn-1 triggers chemotactic effect via a PTX-sensitive G_o/G_i protein and PLC, following its binding to a receptor.

DISCUSSION

Although Lkn-1 plays an important role in immune responses, including leukocyte recruitment and antigen presentation, the function of Lkn-1 in human neutrophils has not been elucidated clearly (Youn et al., 1997; Lee et al., 2005). In this study, we examined the expression of CC chemokine receptors in neutrophils and cell migration in response to of Lkn-1. We demonstrated that (1) protein expression of CCR-2 can be weakly found in neutrophils, but those of other chemokine receptors, CCR1, CCR3, CCR4 and CCR5 cannot be detected. (2) Lkn-1 induces neutrophil chemoattraction, whereas MIP-1α does not. (3) Lkn-1 transduces the chemotaxis signal via Ptx-sensitive G_o/G_i protein and PLC.

CC chemokine has a chemotactic effect on monocytes,

eosinophils and T cells, but it does not generally act on neutrophils because CCR is not expressed in neutrophils (Miller MD and Krangel, 1992; Baggiolini et al., 1994; Ben-Baruch et al., 1995; Sozzani et al., 1995). However, CC chemokines, including myeloid progenitor inhibitory factor-1 (MPIF-1) and liver and activation-regulated chemokine (LARC) induce neutrophil chemotaxis (Patel et al., 1997; Hieshima et al., 1997). In this respect, there is a controversy in fact that CC chemokine functions in neutrophils. As such, we first examined the expression of CCRs in neutrophils. Surface protein expression of CCR1 and CCR3 binding to Lkn-1 was not found in neutrophils by performing flow cytometry (Fig. 1 and 2). These results are in good agreement with previously published data (Bonecchi et al., 1999). However, Lkn-1 induced chemotactic activity in neutrophils despite non-detection of CCR1 and CCR3 while another CC chemokine, MIP-1α did not (Fig. 3). The same has been recently reported in the differential regulation of chemokine (Kim et al., 2005). Lkn-1 differs from other CCR1-dependent chemokines because it includes six conserved cysteines (Youn et al., 1997). This structural difference may be one of the possible causes of differential neurophil migration in response to Lkn-1.

After binding to chemokine receptor, chemokine initiates the release of GDP bound to the α subunit of G-protein receptors, leading to the rapid binding of GTP and the subsequent dissociation of β/γ subunits. These β/γ subunits activate a variety of intracellular cascades, including Janus kinase (JAK)/STAT, PKC and MAPK (mitogen-activated protein kinase) signaling pathways (Mellado et al., 2001; Kim et al., 2003; Kim et al., 2005). To better explain Lkn-1-induced chemotaxis signaling, the involvement of G protein and PLC in neutrophil migration induced by Lkn-1 chemotaxis was examined using chemotaxis assay. As shown in Fig. 4, Lkn-1-induced chemoattraction is blocked by the presence of Ptx and U73122. These results indicate that Lkn-1 mediates the chemotaxis signal through G_o/G_i protein and PLC.

Moreover, we have characterized that Lkn-1-induced chemotaxis signaling. Lkn-1 activates neutrophil migration, whereas another CC chemokine, MIP-1α does not. Cell migration induced by Lkn-1 is mediated via G_o/G_i protein and PLC. This study may provide a clue to elucidate the molecular mechanism of neutrophil recruitment into inflamed tissues induced by Lkn-1.

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