Leukotactin-1-Induced Signal Transduction through CCR1 in Human Osteogenic Sarcoma Cells

In Sik Kim

The Graduate School

Yonsei University

Department of Biomedical Laboratory Science

Leukotactin-1-Induced Signal Transduction through CCR1 in Human Osteogenic Sarcoma Cells

A Dissertation

Submitted to the Department of Biomedical Laboratory Science and the Graduate School of Yonsei University

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

In Sik Kim

December 2002

This certifies that the dissertation of In Sik Kim is approved.

Thesis Supervisor: Yong Suk Ryang

Jong Bae Kim: Thesis Committee Member

Hyeyoung Lee: Thesis Committee Member

Doe Sun Na: Thesis Committee Member

Jesang Ko: Thesis Committee Member

The Graduate School

Yonsei University

December 2002

This dissertation is affectionately dedicated to my parents, my brothers, my wife and my daughters, who have encouraged me.

CONTENTS

LIST OF FIGURES

ABBREVIATION

BSA	: bovine serum albumin
C.I.	: chemotactic index
DMEM	: Dulbecco's modified Eagle's medium
EDTA	: ethylenediaminetetraacetic acid
EGTA	: ethyleneglycolbistetraacetic acid
EMSA	: electrophoretic mobility shift assay
ERK	: extracellular signal-regulated kinase
FACS	: fluorescence-activated cell sorter
FBS	: fetal bovine serum
GPCR	: G protein-coupled receptor
HOS	: human osteogenic sarcoma
Lkn-1	: leukotactin-1
MAP	: mitogen activated protein
MEK	: MAP kinase kinase
NF-κB	: nuclear factor-κappa B
PBS	: phosphate-buffered saline
PI-3 kinase	: phosphoinositide-3 kinase
PKC	: protein kinase C
PLA_2	: phospholipase A_2

PLC : phospholipase C

PLD : phospholipase D

PMSF : phenylmethylsulfonylfluoride

PTX : pertussis toxin

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ABSTRACT

Leukotactin-1-Induced Signal Transduction through CCR1 in HOS Cells

Leukotactin-1 (Lkn-1) is a recently cloned CC-chemokine that binds to the CCR1 and CCR3. Although Lkn-1 has been known to function as a chemoattractant for neutrophil, monocyte and lymphocyte, its cellular mechanism remains unclear. To understand the mechanism of Lkn-1-induced chemotaxis signaling, the chemotactic activities were examined in human osteogenic sarcoma (HOS) cells expressing CCR1. Cell migration experiment was performed to examine the molecules involved in Lkn-1-induced chemotaxis. Pertussis toxin, an inhibitor of G_i/G_o protein, and inhibitors of phospholipase C (PLC), and protein kinase C (PKC) blocked Lkn-1-induced chemotaxis. Especially, PKCδ specific inhibitor rottlerin inhibited the chemotactic activity of Lkn-1 indicating that Lkn-1-induced chemotaxis signal is tranduced through PKCδ. The activities of PLC and PKCδ were also enhanced by Lkn-1 stimulation. However, MEK inhibitor PD98059 did not inhibit the chemotaxis induced by Lkn-1 indicating that extracellular signal-related kinase (ERK) pathway is not involved in cell migration. Chemotactic

activity of Lkn-1 was inhibited by the treatment of cycloheximide and actinomycin D suggesting that newly synthesized proteins are needed for chemotaxis. The inhibitor of NF-κB inhibited Lkn-1-induced chemotaxis. Results from gel shift assay showed that the DNA binding activity of NF-κB was enhanced by Lkn-1 stimulation, and these results suggest that Lkn-1 transduces the signal through G_i/G_o protein, PLC, PKCδ, NF-κB and newly synthesized proteins for chemotaxis.

To understand the intracellular events following Lkn-1 binding to CCR1, the activities of signaling molecules in response to Lkn-1 were investigated in HOS cells expressing CCR1. Lkn-1-stimulated cells showed elevated phosphorylation of ERK1/2 with a distinct time course. ERK activation was peaked in 30 min and 12 h and pertussis toxin blocked Lkn-1-induced activation of ERK showing biphasic activation of ERK. PLC inhibitor and PKCδ specific inhibitor inhibited ERK activation in Lkn-1-stimulated cells. Dominant negative Ras inhibited activation of ERK. Immediate early response genes such as *c-fos* and *c-myc* were induced by Lkn-1 stimulation. Lkn-1 affected the cell cycle progression by cyclin D_3 induction. These results suggest that Lkn-1 activates the ERK pathway by transducing the signal through G_i/G_o protein, PLC, PKCδ and Ras, and it may play a role for cell proliferation, differentiation and regulation of gene expression for other cellular processes.

Keywords : Lkn-1, CCR1, PKC, PLC, NF-κB, ERK, chemokine, signal transduction, chemotaxis

CHAPTER I

Leukotactin-1-Induced Chemotaxis Signaling Through CCR1 in HOS Cells

I. INTRODUCTION

Chemokines are a large family of structurally homologous small proteins containing four to six conserved cysteines linked by disulfide bonds. Chemokines are often upregulated in inflammation and act mainly on leukocytes inducing migration and release responses. There are approximately 40 chemokines identified to date. Most chemokines have four characteristic cysteines, and they have been classified into CXC (α), CC (β), C (γ), and CX3C (δ) chemokines depending on the motif displayed by the first two cysteines. In recent years, chemokines have been studied actively, and it has been known that they play important roles in the biological functions of primary immune response (Banchereau *et al*., 1998), angiogenesis/angiostasis (Arenberg *et al*., 1997; Coughlin *et al*., 1998), lymphocyte and lymphoid organ development (Kim *et al*., 1998; Vicari *et al*., 1997), and antitumor activity/tumor progression (Hedrick *et al*., 1997; Luan *et al*., 1997).

Chemokine receptors belong to seven-transmembrane G-protein coupled receptors (GPCRs). Based on the chemokine class they bind, the receptors have been named as CXCR1, 2, 3, 4, and 5 which bind CXC chemokines, CCR1 through CCR9 which bind CC chemokines, XCR1 which binds the C chemokine, and CX3CR1 which binds the CX3C chemokine. GPCRs transduce signals to the inside of the cell through heterotrimeric G proteins

which are grouped into two families depending on the sensitivity to pertussis toxin (PTX). G_i/G_o family G proteins are sensitive to PTX whereas G_q family G proteins are known to be insensitive to this toxin (Venkatakrishnan *et al*., 2000). Activation of the GPCR-mediated signaling pathway involves the activation of several different effector molecules such as adenylate cyclase, phospholipase C (PLC), phospholipase D (PLD), phospholipase A_2 (PLA_2), phosphoinositide-3 (PI-3) kinase, protein kinase C (PKC) and Ca^{2+} mobilization which initiate a variety of cellular responses (Murphy, 1996; Wang *et al*., 1993). The epidermal growth factor (EGF) receptor is one of the protein tyrosine kinases that has been implicated in transducing signals initiated by GPCR-specific agonists (Daub *et al*., 1996). Recent studies indicate that non-receptor tyrosine kinases of the c-Src family are involved in GPCR-mediated Ras/mitogen-activated protein (MAP) kinase activation (Luttrell *et al*., 1997). Although chemokine receptors lack tyrosine kinase activity, they can stimulate the phosphorylation of cytoskeletal proteins, p130 Cas and paxillin (Dutt *et al*., 1998), and induce the activation of the related adhesion focal tyrosine kinase (Ganju *et al*., 1998), MAP kinases (ERK1/2, p38, and c-Jun kinase), and Janus kinase 2 (Mellado *et al*., 1998). Receptor activation is followed by receptor phosphorylation on multiple serine or threonine residue and subsequent desensitization of the receptor to further stimulation. Desensitized receptors are internalized into the early endosome

and undergo either endocytosis recycling or lysosomal degradation (Koenig *et al*., 1997; Zimmermann *et al*., 1999).

Recently, leukotactin-1 (Lkn-1), a member of the CC chemokine family, has been cloned and partially characterized (Youn *et al*., 1997). Lkn-1 which binds to CCR1 and CCR3 has chemoattraction for peripheral blood leukocytes including monocyte, lymphocyte, and neutrophil. Especially, chemotactic activity of Lkn-1 in neutrophil is unique among CC chemokines which generally do not attract neutrophil (Zhang *et al*., 1999). It may be associated that Lkn-1 is classified as C6 β-chemokine because it has two extra cysteines. Although Lkn-1 is believed to play an important role in the development of inflammation and allergic inflammatory diseases, its biological function and the mechanism of cell migration events remain to be characterized. CCR1 is a GPCR that is expressed by a variety of cells, including lymphocytes, monocytes, basophils, eosinophils, and neutrophils (Gao *et al*., 1997). CCR1-mediated signal transduction pathways have been proposed. For example, it has been reported that myeloid progenitor inhibitor factor 1 (MPIF-1) signal transduction includes binding to CCR1, transduction by G protein, effector function by PLC, PKC, calcium flux and PLA_2, and cytoskeletal remodeling (Bernardetta *et al*., 1999). Although CCR1 and Lkn-1 may play important roles in physiologic processes, little information is available regarding the Lkn-1-induced signal transduction pathway via CCR1

and the relationship of signaling events to the functional response of the cell.

The purpose of the study was to establish the mechanism of Lkn-1-induced chemotaxis signaling via CCR1 using human osteogenic sarcoma cells expressing CCR1 as a model system.

II. MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal bovine serum were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). PTX, PD98059, U73122, Ly294002, genistein, rottlerin, Ro-31-8425, SB202190, SN50, cycloheximide (CHX), and actinomycin D (ActD) were obtained from Calbiochem (San Diego, CA, USA). Quinacrine and rat tail collagen type I were products of Sigma (St. Louis, MO, USA). rLkn-1 was the kind gift of Greencross Life Science Corp. (Yongin, Korea). hMIP-1α, hMIP-1β, anti-CCR1 antibody and anti-CCR5 antibody were obtained from R&D Systems (Minneapolis, MN, USA). Antibody against PKCδ was purchased from Transduction Laboratories (Lexington, KY, USA). Antibodies against control mouse IgG and FITC-conjuated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [γ-^{32}P]ATP, myo-[2-^{3}H]inositol and ECL detecting reagent were purchased from Amersham Pharmacia Biotech. (Piscataway, NJ, USA). Stable HOS cells expressing CCR1 or CCR5 were the kind gift of Dr. O. M. Howard (National Cancer Institute, MD, USA) and were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and puromycin (0.5 μg /ml).

FACS analysis

HOS cells seeded into 100 mm dishes at 5×10^5 cells/dish were cultured in DMEM. Cells were harvested and washed with PBS buffer. After saturation of nonspcific binding sites with total rabbit IgG, cells were incubated at 4°C for 30 min with anti-CCR1 or anti-CCR5 antibody. Baseline staining was obtained by incubating mouse IgG instead of anti-CCR1 or anti-CCR5 antibody. Following incubation and washings, cells were incubated at 4°C for 30 min with FITC-conjugated goat anti-mouse IgG. Finally cells were washed and analyzed on a FACSort cytofluorimeter (Becton Dickinson, San Jose, CA, USA).

Chemotaxis assay

Migration of cells was monitored using a 48-well microchamber (Neuroprobe, Cabin John, MD). The lower wells were filled with 28 μl buffer alone or with buffer containing rLkn-1, hMIP-1α or hMIP-1β and the upper wells were filled with 50 μl of HOS cells at 5×10^5 cells/ml in RPMI 1640 containing 1% BSA and 30 mM HEPES. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuroprobe) with 10 μm pores that was precoated with RPMI 1640 containing rat tail collagen type I at 4°C overnight. After incubation for 3 h at 37°C, the filters were removed from the chamber, washed, fixed, and stained with Diff-Quick (Baxter, Deerfield,

IL). The cells of two randomly selected oil-immersed fields were counted using Axiovert 25 (Carl Zeiss, Jena, Germany) and Visus Image Analysis System (Foresthill Products, Foresthill, CA). The chemotactic index (CI) was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as CI > 2.

Measurement of PLC activity

HOS cells seeded into 35 mm dishes at 2×10^5 cells/dish were cultured in DMEM. The cells were labeled with myo-[2-^{3}H]inositol (2 mCi/ml) in inositol-free DMEM for 24 h. Subsequently, the labeled cells were washed and pre-treated with 20 mM LiCl for 15 min in DMEM containing 20 mM HEPES (pH 7.2) and 1 mg/ml BSA. Stimulation was initiated by the addition of Lkn-1 for different time, and terminated by the addition of ice-cold 5% $HClO_4$. After 30 min in an ice bath, extracts were centrifuged and diluted with distilled water, and applied to Dowex AG 1-X8 anion exchange column (Bio-Rad, Hercules, CA, USA). The column was then washed with 10 ml of distilled water followed by 10 ml of 0.06 M ammonium formate containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid.

PKC translocation assay

HOS cells seeded into 100 mm dishes at 5×10^5 cells/dish were cultured in DMEM. The cells were starved for 24 h in 0.5% FBS. After treatment with Lkn-1, the cells were harvested and washed three times with ice-cold phosphate-buffered saline (without Ca^{2+}). The cells then were resuspended in 200 µl of homogenization buffer A (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and protease inhibitors) and homogenized at 4°C by ten passages through a 21G needle fitted on a 1 ml plastic syringe. The homogenate was centrifuged at 1,000 × g for 5 min at 4°C. The supernatant was collected and centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in 100 µl of homogenization buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM EGTA, 1 mM PMSF, and protease inhibitors) and sonicated for 10 s. The suspension was centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was collected as a membrane fraction. Protein concentration of each sample was determined.

Western blot analysis

Cells were lysed in 10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and

protease inhibitors. Protein samples (15 μg of each) were separated by SDS-PAGE (10%) and transferred to nitrocellulose filters. The blots were incubated with antibodies and developed with ECL detecting reagent.

Electrophoretic mobility shift assay (EMSA)

For the NF-κB gel shift assay, nuclear extracts were prepared from HOS/CCR1 cells. A double stranded oligonucleotide containing a consensus binding site for NF-κB (underlined), 5'-AGTTGAGGGGACTTTCCCAGGC-3', was obtained from Promega (Madison, WI). The oligonucleotide was 5'-end-labeled with [α-^{32}P]ATP using T4 polynucleotide kinase (Promega). Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad) as described by manufacturer's instruction. Nuclear extracts (15 μg of total protein) were incubated with radiolabeled probe for 20 min at room temperature, and protein-DNA complexes were separated from free probe by electrophoresis on a 4% native polyacrylamide gel in 0.5 × Tris-borate EDTA (TBE). DNA binding buffer contained 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 μg/ml of poly(dI-dC)•poly(dI-dC). Gels were pre-electrophoresed in 0.5 × TBE for 30 min prior to loading, and electrophoresis continued for approximately 3 h until the bromophenol blue dye approached the bottom of the gel. Dried gels were visualized by autoradiography. In competition

experiments, binding reactions were incubated with 10-fold molar excess of unlabeled NF-κB binding oligonucleotide or NFAT binding oligonucleotide for 20 min before addition of the radiolabeled oligonucleotide.

III. RESULTS

Expression of CCR1 and CCR5 in HOS cells

A stable HOS cell line expressing CCR1 (HOS/CCR1) has been established to investigate Lkn-1-induced chemotaxis signaling through CCR1 (Youn *et al*., 1997). A stable HOS cell line expressing CCR5 (HOS/CCR5) has been also established and used as a negative control for Lkn-1 stimulation (Youn *et al*., 1997). To examine the surface expression of CCR1 and CCR5 on HOS cells, FACS analysis was conducted. Figure I-1A and Figure I-1B show that CCR1 and CCR5 are expressed on the surface of HOS cells.

Lkn-1 induces cell migration in HOS cells expressing CCR1

To examine the chemotactic activity of Lkn-1 in HOS/CCR1 cells, cell migration assay was performed in a 48 well microchamber. The chemoattractant effect of Lkn-1 was comparable to that of MIP-1α in HOS/CCR1 cells, but MIP-1β did not affect migration of HOS/CCR1 cells (Figure I-2A). Lkn-1 showed the typical bell-shape curve in HOS/CCR1cell chemoattraction with the peak of the curve at 100 ng/ml and MIP-1α showed the maximum activity at 1 ng/ml. Lkn-1 did not show any chemotactic activity in HOS/CCR5 whereas MIP-1α and MIP-1β had chemoattractant effects in HOS/CCR5 (Figure I-2B). These data indicate that Lkn-1 shares

receptor CCR1 with MIP-1α but not with MIP-1β.

Lkn-1-induced chemotaxis is mediated via G_i/G_o protein

To evaluate the coupling mechanism of the Lkn-1 receptor and the nature of possible G proteins involved in the signaling events activated by Lkn-1, HOS/CCR1 cells were treated with PTX before the stimulation of Lkn-1. Figure I-3 shows that PTX had inhibitory effect on the Lkn-1 and MIP-1α-induced chemotaxis. In the presence of PTX, the number of the cells affected by Lkn-1 decreased to only 10 - 20% of the number that migrated in the absence of PTX. These data indicate that Lkn-1 exerts its effect through a receptor linked to PTX-sensitive G_i/G_o family G proteins.

Lkn-1-induced chemotaxis is mediated via PLC

To further characterize the chemotaxis signaling pathway implicated in response to Lkn-1, the possible involvement of PLC was investigated. Figure I-4 shows that Lkn-1and MIP-1α-induced cell migration was inhibited by the treatment of U73122. This result indicates that PLC is involved in Lkn-1-induced chemotaxis.

Lkn-1 increases PLC activity

Since PLC is involved in Lkn-1-induced chemotaxis, it was further tested

whether Lkn-1 enhances PLC activity. Exposure of HOS/CCR1 cells to Lkn-1 for different periods of time resulted in increased activity of PLC as assessed by measuring the [^{3}H]IP that was produced by PLC activity (Figure I-5A). Increased activation was observed within 5 min after addition of Lkn-1 to HOS/CCR1 cells, and continued to increase up to 1 hr, but no PLC activation was detected in HOS/CCR5. Furthermore, dose dependence of PLC activation by Lkn-1 was tested. PLC activity reached to a plateau at 200 ng/ml of Lkn-1 concentration. (Figure I-5B).

PKCδ is involved in Lkn-1-induced chemotaxis signaling

Since G_i/G_o protein and PLC participated in Lkn-1-induced chemotaxis, it was examined whether possible down-stream regulator of PKC is involved in the chemotaxis pathway of Lkn-1. As shown in Figure I-6, chemotactic activity of Lkn-1 and MIP-1α was inhibited in the presence of rottlerin but not in the presence of Ro-31-8425. Since rottlerin is a PKCδ specific inhibitor, this result indicates that PKCδ is involved in Lkn-1-induced chemotaxis among various PKC isoforms.

PKCδ is activated by Lkn-1

Translocation of PKC to a particulate fraction is the key step for the activation of this enzyme (Newton, 1995). Determination of PKC content in

membranes can reflect PKC activity. To investigate whether PKCδ is activated by Lkn-1 stimulation, the membrane/cytosol distribution of PKCδ was analyzed. PKCδ activity began to increase at 10 min after addition of Lkn-1 and the maximum activity of PKCδ was detected at 60 min by the Western blot analysis (Figure I-7). These results indicate that Lkn-1 transduces the signal to PKCδ for chemotaxis.

ERK is not involved in Lkn-1-induced signaling

To determine other signaling molecules contributing Lkn-1-induced chemotaxis, the respective effects of inhibitors of MEK (PD98059), p38 (SB202190), tyrosine kinase (genistein), PI-3 kinase (Ly294002), PLA_2 (quinacrine), and PLD (1-butanol) were tested on chemotactic activity of Lkn-1. Lkn-1-induced cell migration was not inhibited by the addition of these inhibitors suggesting that these molecules are not involved in Lkn-1-induced chemotaxis (Figure I-8).

Actinomycin D and cycloheximide inhibit the Lkn-1-induced chemotaxis

Since many cellular processes need newly synthesized proteins to carry out distinct functions, the effects of ActD and CHX were tested in Lkn-1-induced chemotaxis. As shown in Figure I-9, both inhibitors reduced the

chemotactic activity of Lkn-1 and MIP-1α to 20 - 30% of the control group indicating that chemotaxis induced by Lkn-1 needs newly synthesized proteins.

Actinomycin D and cycloheximide do not affect the expression of CCR1

Since ActD and CHX inhibit protein synthesis, it was examined whether ActD and CHX affect on CCR1 expression by FACS analysis. As shown in Figure I-10, both inhibitors did not inhibit the expression of CCR1 at the concentration of 10 μg /ml for 2 h treatment (Figure I-10). These data suggest that inhibition of Lkn-1-induced chemotaxis by both inhibitors is not associated with the reduction of CCR1 expression.

Lkn-1 enhances NF-κB activation

Inhibition of Lkn-1-induced chemotaxis by transcription/translation inhibitors prompted us to test the activation of transcription factors. Since NF-κB is activated by several chemokines (Ganju *et al*., 1998; Wang *et al*., 2001), it was investigated whether Lkn-1 induces the activation of NF-κB in HOS/CCR1 cells. EMSA was performed to evaluate the DNA binding activity of NF-κB in HOS/CCR1 cells treated with Lkn-1 for the indicated times by using a ^{32}P-labeled consensus NF-κB DNA binding sequence as a probe.

Figure I-11 shows that there are two shifted NF-κB nuclear complexes bound to the probe, and Lkn-1 induces an increase in the formation of theses complexes over time (0.5 - 4 h). To determine the specificity of NF-κB binding activity, nuclear extracts from 2 h time point were incubated with the labeled NF-κB binding probes in the absence or presence of a 10-fold molar excess of unlabeled NF-κB or NFAT binding competitor (Figure I-11). The competition experiments showed that NF-κB binding complex was competed with unlabeled NF-κB binding probes, but not with NFAT binding probes indicating that NF-κB binding activity is specific.

Lkn-1-induced NF-κB activation is mediated via G_i/G_o protein/PLC/PKCδ cascades

To investigate up-stream regulators of NF-κB, DNA binding ability of NF-κB was tested in the presence of inhibitors of possible signaling molecules. Inhibitors of G_i/G_o protein, PLC and PKCδ decreased NF-κB activation, demonstrating that these molecules are the up-stream regulators of NF-κB (Figure I-12). Especially, a PKCδ specific inhibitor rottlerin completely blocked the DNA binding of NF-κB at the concentration of 5 μM. However, Ro-31-8425 which does not inhibit PKCδ and PD98059 had no inhibitory effect on NF-κB activation.

Lkn-1-induced chemotaxis is mediated via NF-κB

Since Lkn-1 activates NF-κB, it was examined whether NF-κB is involved in Lkn-1-induced chemotaxis. Figure I-13 demonstrates that NF-κB inhibitor SN50 decreased the migrated cell number to 50 % of the control group at 36 μM. These data suggest that Lkn-1 enhances NF-κB activity, which is involved in the expression of many regulator proteins, and NF-κB is involved in Lkn-1-induced chemotaxis.

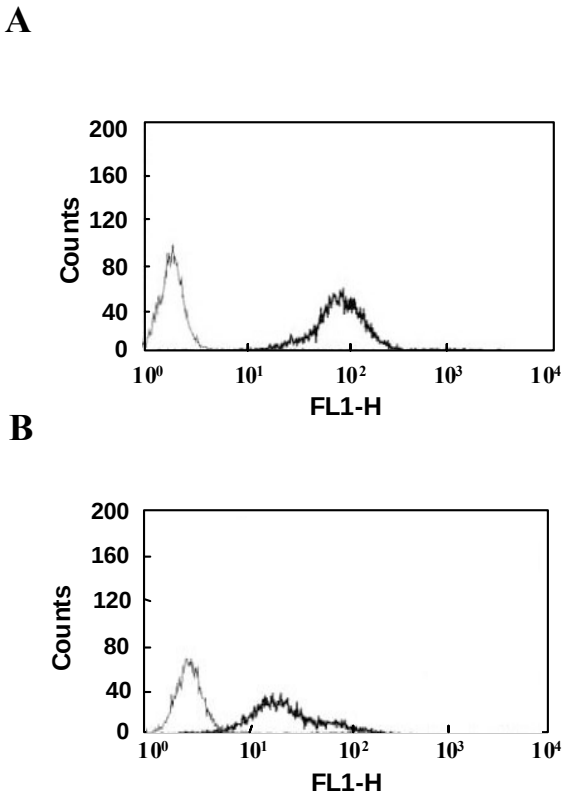


Figure I-1. Expression of CCR1 and CCR5 in HOS cells. HOS/CCR1 cells (A) or HOS/CCR5 cells (B) were harvested and analyzed by fluorescence-activated cell sorter using monoclonal anti-CCR1, anti-CCR5 antibodies (thick line) or control mouse IgG (thin line).

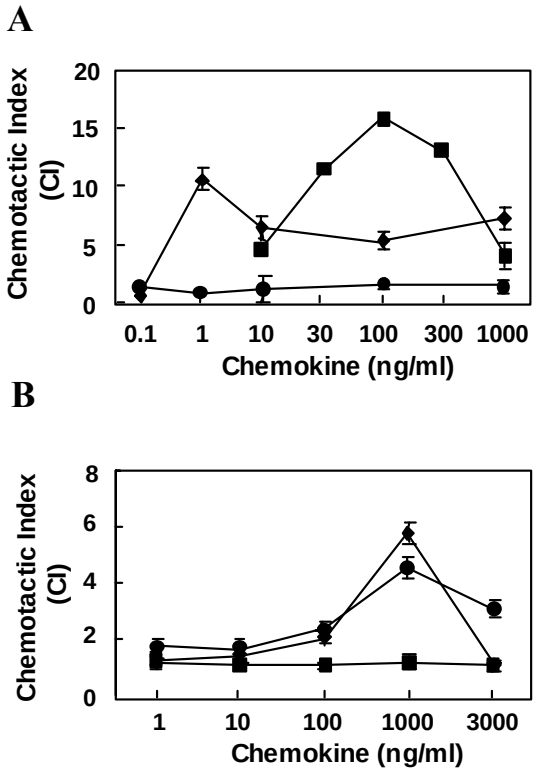


Figure I-2. Lkn-1 induces cell migration in HOS cells expressing CCR1. HOS/CCR1 cells (A) or HOS/CCR5 cells (B) were applied to the indicated concentrations of Lkn-1 (■), MIP1-α (♦) or MIP1-β (●) in microchamber and were allowed to migrate for 3 h. The number of cells that migrated was counted microscopically 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. Results are expressed as mean CI ± SEM of six replicate measurements from a single experiment and it is representative of three separate experiments.

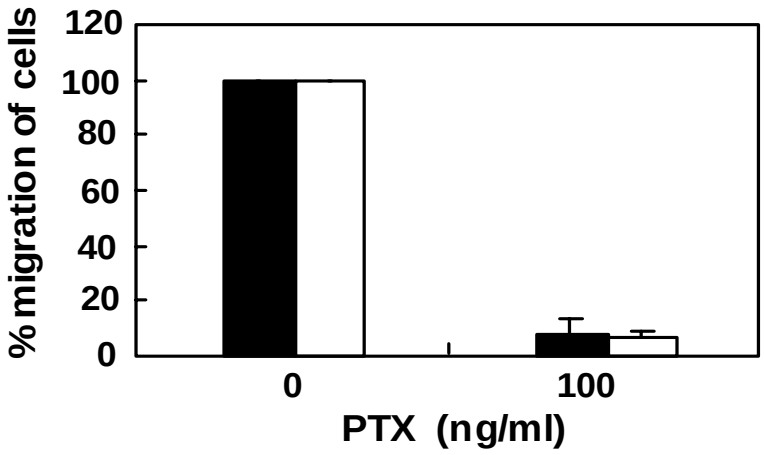


Figure I-3. Lkn-1-induced chemotaxis is mediated via G_i/G_o protein. HOS/CCR1 cells were pre-incubated in the absence (control) or presence of 100 ng/ml PTX for 16 h. Cell migration in response to Lkn-1 (100 ng/ml, black bar) or MIP-1α (1 ng/ml, white bar) was measured as described in Methods.

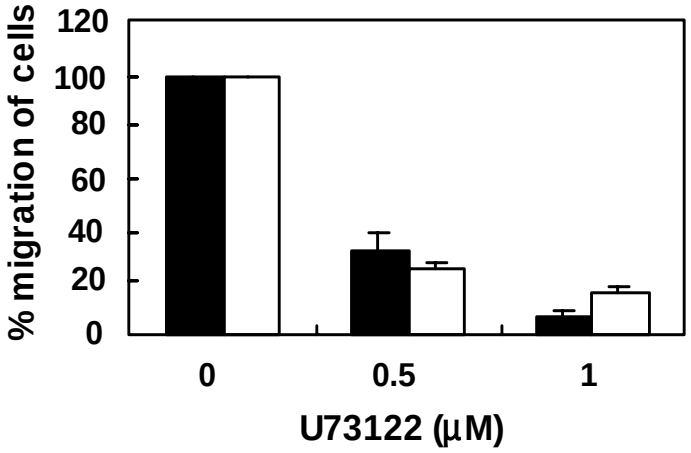
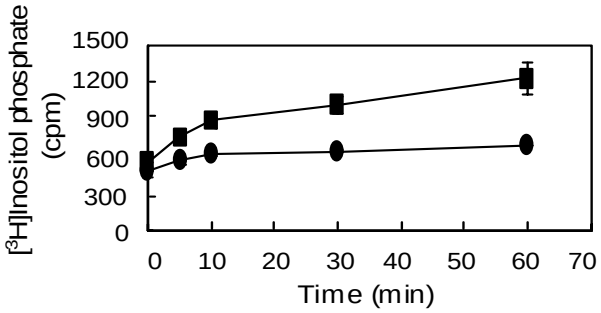


Figure I-4. Lkn-1-induced chemotaxis is mediated via PLC. HOS/CCR1 cells were pre-incubated in the absence or presence of U73122 for 30 min at 0.5 and 1 μM. Cell migration in response to Lkn-1 (100 ng/ml, black bar) or MIP-1α (1 ng/ml, white bar) was measured as described in Methods.

A



B

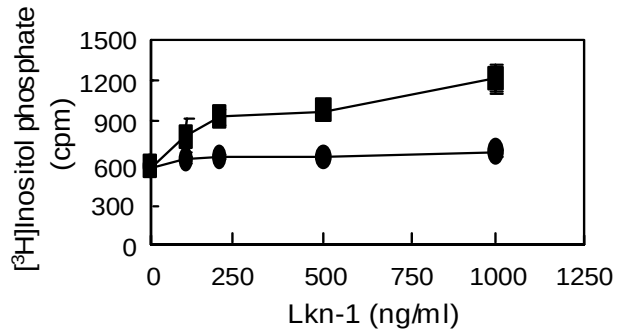


Figure I-5. Lkn-1 increases phospholipase C activity. HOS/CCR1 (■) or HOS/CCR5 (♦) cells were radiolabeled with myo-[2-^{3}H]inositol (2 μCi/ml) in inositol-free DMEM for 24 h. Stimulation was initiated by the addition of Lkn-1 at the indicated time (***A***) and at various concentrations (***B***). Results are expressed as the radioactivity in the total inositol phosphates. Each point represents the average value of three different experiments ± SEM.

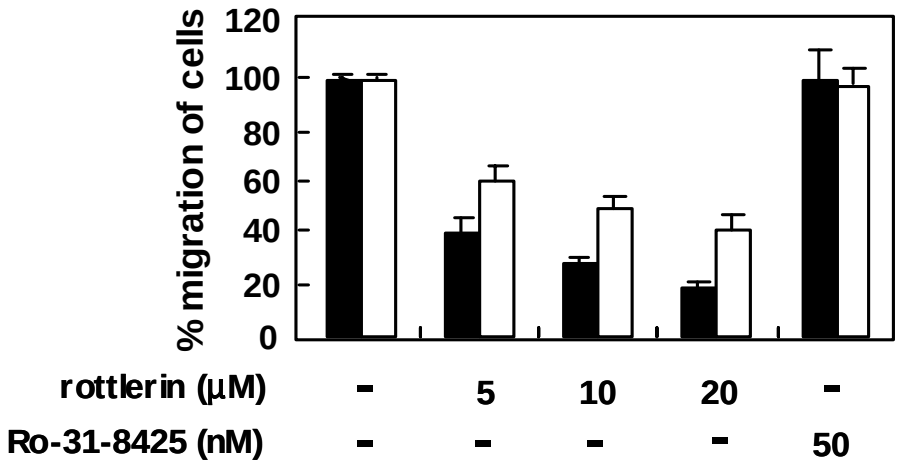


Figure I-6. PKCδ is involved in Lkn-1-induced chemotaxis signaling. HOS/CCR1 cells were pre-incubated in the absence or presence of rottlerin (5, 10 and 20 μM) and Ro-31-8425 (50 nM) for 30 min. Cell migration in response to Lkn-1 (100 ng/ml, black bar) or MIP-1α (1 ng/ml, white bar) was measured as described in Methods.

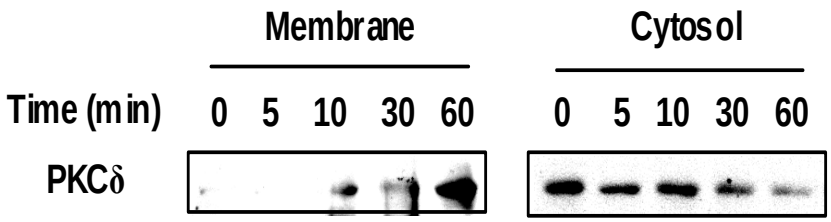


Figure I-7. PKCδ is activated by Lkn-1. Serum starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated time. Harvested cells were fractionated as described in Methods, then analyzed by 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. PKCδ translocation was detected by Western blotting with anti-PKCδ antibody.

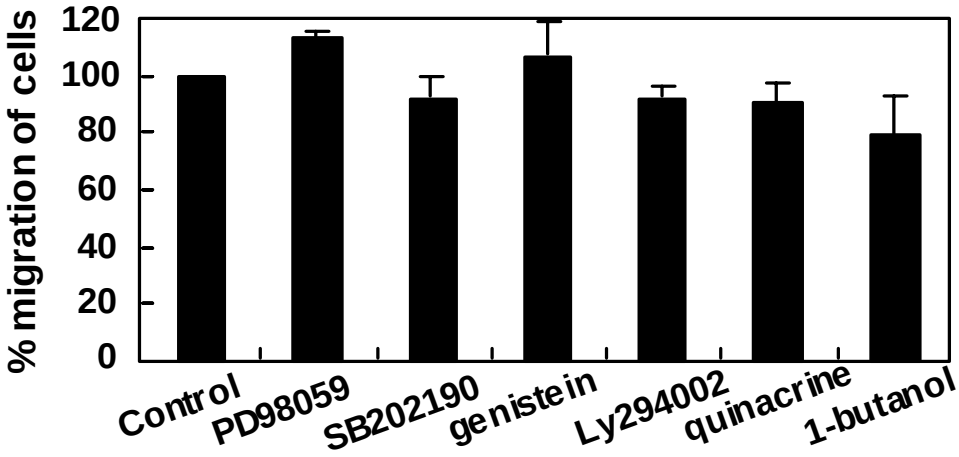


Figure I-8. ERK is not involved in Lkn-1-induced chemotaxis signaling. HOS/CCR1 cells were pre-incubated in the absence (control) or presence of PD98059 (50 μM), SB202190 (20 μM), genistein (10 μM), Ly294002 (10 μM), quinacrine (10 μM), 1-butanol (0.3%) for 30 min. Cell migration in response to Lkn-1 (100 ng/ml) was measured as described in Methods.

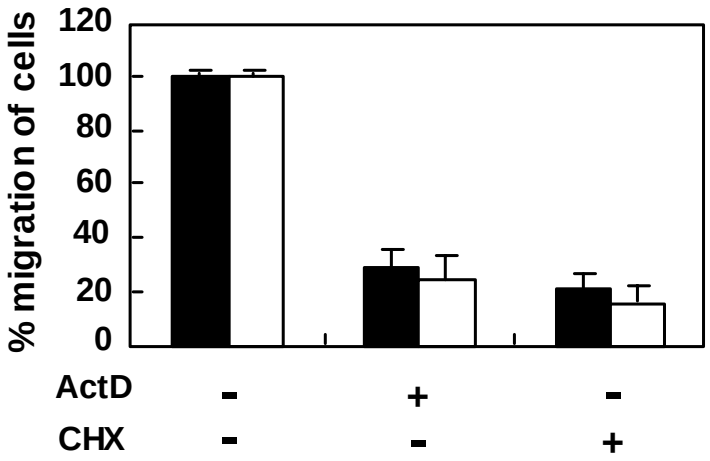


Figure I-9. Actinomycin D and cycloheximide inhibit Lkn-1-induced chemotaxis. HOS/CCR1 cells were pre-incubated in the absence or presence of 10 μg/ml ActD or 10 μg /ml CHX for 2 h. After addition of Lkn-1 (100 ng/ml, black bar) or MIP-1α (1 ng/ml, white bar), migration assay was performed as described in Methods.

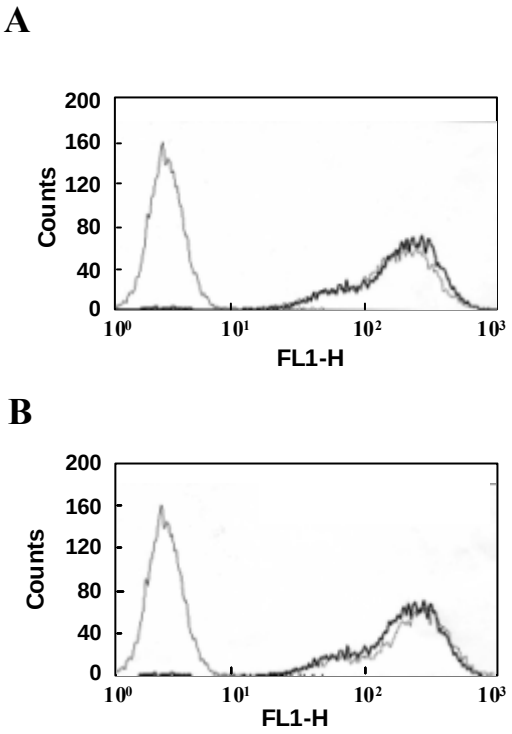


Figure I-10. Actinomycin D and cycloheximide do not affect the expression of CCR1. HOS/CCR1 cells were pre-incubated in the absence (A and B, thick line and thin line) or presence of 10 μg /ml ActD (A, dotted line) or 10 μg /ml CHX (B, dotted line) for 2 h and analyzed by fluorescence-activated cell sorter using monoclonal anti-CCR1 antibody (thick line and dotted line) or control mouse IgG (thin line) was performed as described in Methods.

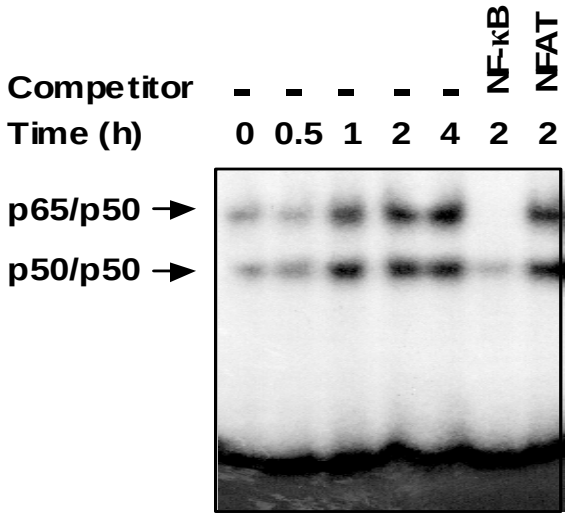


Figure I-11. Lkn-1 enhances NF-κB activity. Serum starved HOS/CCR1 cells were treated with 100 ng/ml Lkn-1 for the indicated time. Nuclear extracts (15 μg/lane) were prepared and subjected to EMSA for DNA binding activity of NF-κB with ^{32}P-end-labeled oligonucleotides in the absence or presence of 10-fold molar excess of unlabeled competitors. *NF-κB*, unlabeled NF-κB binding oligonucleotides; *NFAT*, unlabeled NFAT binding oligonucleotides; Arrows indicate the shifts corresponding to the position of NF-κB protein-DNA complexes.

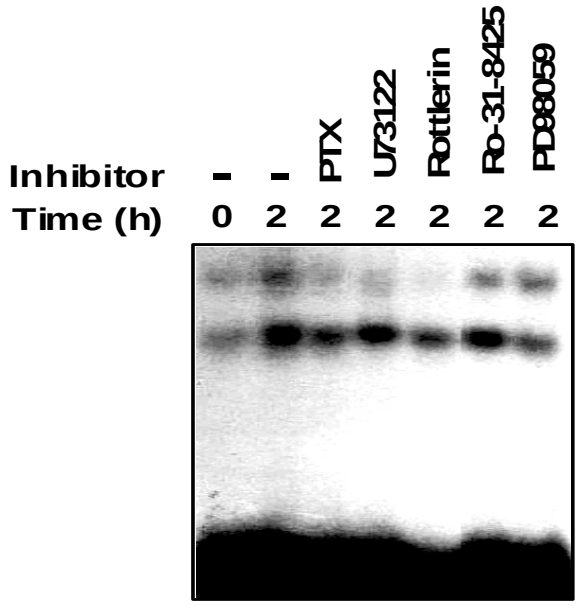


Figure I-12. Lkn-1-induced NF-κB activation is mediated via G_i/G_o protein/PLC/PKCδ cascades. Serum starved HOS/CCR1 cells were pre-incubated in the absence or presence of PTX (100 ng/ml), U73122 (10 μM), rottlerin (5 μM) and Ro-31-8425 (50 nM) for 30 min (16 h for PTX) and were treated with 100 ng/ml Lkn-1 for 2 h. Nuclear extracts (15 μg /lane) were prepared and subjected to EMSA.

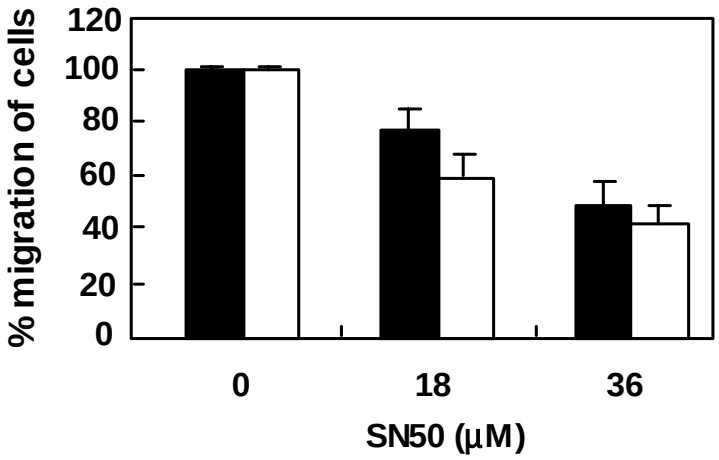


Figure I-13. Lkn-1-induced chemotaxis is mediated via NF-κB. HOS/CCR1 cells were pre-incubated with SN50 (18 and 36 μM) for 30 min and migrated toward 100 ng/ml Lkn-1 (black bar) or 1 ng/ml of MIP-1α (white bar). Cell migration in response to Lkn-1 or MIP-1α was measured as described in Methods.

IV. DISCUSSION

.

Despite substantial recent advances in the understanding of chemotaxis, the precise mechanism through which cells respond to a chemotactic gradient remains unclear. This study was performed to understand the mechanism of Lkn-1-induced chemotaxis signaling through CCR1 in HOS cells, and it has demonstrated that (1) Lkn-1 transduces the signal via PTX-sensitive G_i/G_o family G proteins. (2) Lkn-1 activates PLC and PKCδ in a time dependent manner. (3) Lkn-1 enhances the DNA binding activity of NF-κB. Cell migration assay showed that (4) G_i/G_o protein, PLC, PKCδ, NF-κB and newly synthesized proteins are involved in Lkn-1-induced chemotaxis, but ERK, p38, tyrosine kinases, PI-3 kinase, PLA_2, and PLD are not involved in the chemotaxis pathway of Lkn-1.

Since expression levels of CCR1 in resting neutrophils, lymphocytes, and monocytes were relatively low, CCR1 overexpression HOS cell line was used to examine the Lkn-1-stimulated signal transduction. The sensitivity to chemokines and cell migration activity of HOS/CCR1 cells were comparable to that of leukocytes from human PBMC (Youn *et al*., 1997 and Figure I –2).

Accumulating data have implicated that multiple signaling mechanisms exist to regulate cell migration. MAP kinase (Yebra *et al*., 1995; Klemke *et al*., 1997), PI-3 kinase (Ganju *et al*., 1998; Tapia *et al*., 1999; Turner *et al*., 1995)

and PKC (Pukac *et al*., 1998) signaling pathways have been shown to regulate the cell migration induced by chemokines or cytokines. Most of these signaling pathways are initiated with GPCR. Classical model for signal transduction by chemokines involves GPCR whose affinity state is increased by conformational changes induced by association with the GDP-bound state of a PTX-sensitive heterotrimeric G protein. Upon ligand binding, the activated receptor catalyzes exchange of GDP for GTP by the G protein α subunit, resulting in dissociation of α from $\beta\gamma$ subunits. In turn, $\beta\gamma$ activates a phosphoinositide-specific PLC leading to the accumulation of IP_3 and DAG in the cytoplasm. These products induce mobilization of calcium and activation of PKC (Murphy, 1994). Besides the classical model, other pathways for GPCR that have been studied include JAK/STAT, as well as both tyrosine and Ser/Thr kinases (Mellado *et al*., 2001). The data from cell migration assay showed that Lkn-1-induced chemotaxis was sensitive to PTX, indicating that G_i/G_o protein is involved in cell migration (Figure I-3). In addition, PLC and PKC participated in Lkn-1 signaling indicating that Lkn-1 transduces the signal through the classical chemokine signaling pathway (Figure I-4, 5 and 6). Among the various PKC isoforms, PKCδ was activated by Lkn-1 stimulation, and the PKCδ specific inhibitor rottlerin (Pongracz *et al*., 1999) blocked the chemotactic activity of Lkn-1 (Figure I-6). However, Ro-31-8425, a PKC inhibitor that does not inhibit PKCδ, showed no inhibitory activity of

Lkn-1-induced chemotaxis. As any other novel PKC, PKCδ is activated in a Ca^{2+}-independent manner by DAG, which is produced by activated PLC. This study is the first report that PKCδ is involved in chemokine signaling.

The transcription factor NF-κB is critical for the expression of multiple genes involved in inflammatory response and apoptosis (Barnes *et al*., 1997). Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) rapidly activate NF-κB in most cell types through the NIK/MEKK-IKK-IκB signal pathway (Barnes *et al*., 1997; Siebenlist *et al*., 1994). These cytokines induce NF-κB activation by modulating IκB phosphorylation, ubiquitination, and proteolytic degradation and by releasing functional NF-κB dimers to translocate to the nucleus (Israel, 2000). Chemotactic factors, such as the lipid mediator platelet-activating factor (Ye *et al*., 1996) and SDF-1 (Ganju *et al*., 1998), are also reported to activate NF-κB in leukocytes or murine pre-B cells, respectively. However, the mechanism of chemokine receptor activation of NF-κB is not clear. This study suggests that Lkn-1 can cause the activation of NF-κB. Lkn-1-induced cell migration was reduced, but not completely inhibited by the NF-κB inhibitor SN50, reflecting the involvement of NF-κB in Lkn-1-induced chemotaxis (Figure I-13). Through Lkn-1-induced activation of the NF-κB signaling pathway and possibly in conjunction with other signaling pathways, Lkn-1 probably promotes changes in cellular morphology, collectively known as

polarization, required for chemotactic responses.

It has been reported that NF-κB activation in TNF-α-stimulated neutrophils is mediated by PKCδ (Vancurova *et al*., 2001). At present, the downstream events regulated by PKCδ and leading to NF-κB activation and chemotaxis in Lkn-1-stimulated HOS/CCR1 cells are not known. One of the critical regulatory steps dictating IκB degradation and NF-κB activation is IκB kinase (IKK) and NF-κB inducing kinase (NIK) (Karin, 1999). Recent studies demonstrated that IKKβ is involved in NF-κB activation through PKCθ in T lymphocytes (Coudronniere *et al*., 2000; Lin *et al*., 2000; Sun *et al*., 2000). Therefore, the possible roles of IKK isoforms or other cellular molecules in Lkn-1-stimulated NF-κB activation are under investigation.

In conclusion, the present study provides the characterization of the chemotaxis signaling pathways activated by Lkn-1 in a model system of HOS/CCR1 cells, and has demonstrated that the binding of Lkn-1 to its G_i/G_o protein coupled receptor CCR1 leads to the specific activation of PLC, PKCδ, and NF-κB, and that the PLC/PKCδ/NF-κB pathway may mediate Lkn-1-induced chemotactic activity. While further studies are required to delineate the signaling pathways leading to chemotaxis through CCR1 stimulation by Lkn-1, this is the first report characterizing the chemotaxis signaling events in response to Lkn-1. Although Lkn-1-induced chemotaxis signaling was investigated in HOS/CCR1 cells as a model system, the preliminary data

using neutrophils showed the same results. From this information, we can begin to understand the molecular mechanism of cell migration and other cellular processes induced by Lkn-1 which are important events in both physiological and pathological processes.

CHAPTER II

Leukotactin-1-induced ERK activation is mediated via G_i/G_o protein/PLC/PKCδ/Ras cascades in HOS Cells

I. INTRODUCTION

Chemokines are a family of low-molecular weight proteins involved in leukocyte trafficking and many other immune responses (Rollins, 1997; Luster, 1998; Mellado *et al*., 2001). Significant advances have been made in recent years in understanding the role of chemokines in inflammatory diseases (Murdoch *et al*., 2000), hematopoiesis, angiogenesis, metastasis, tumor rejection (Wang *et al*., 1998), Th1/Th2 responses (Bonecchi *et al*., 1998), and HIV-1 infection (Cairns *et al*., 1998; Mellado *et al*., 1999). Chemokines are classified into four major groups depending on the positions of the first two cysteines. CXC (α) chemokines, with the first two cysteines separated by a nonconserved amino acid, function as a chemoattractant for neutrophils and T lymphocytes. CC (β) chemokines, in which the first two cysteines are adjacent, are chemotactic for monocytes, basophils, eosinophils, T lymphocytes, NK cells, and dendritic cells but usually do not affect neutrophils. The only known C (γ) chemokine, lymphotactin, is active on T lymphocytes and NK cells. The CX3C (δ) chemokine, fractalkine, stimulates chemotaxis of T lymphocytes and monocytes. Chemokine receptors have been known as a 7-transmembrane G protein-coupled receptor (GPCR) that transduces signals to the inside of the cell through heterotrimeric G proteins; mainly PTX-sensitive G_i/G_o protein family. Generally, activated G proteins

exert PLC activation leading to formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). In conjuction with intracellular Ca^{2+} elevated by IP_3, DAG activates various protein kinase C (PKC) isoforms. Chemokines also activate phospholipase A_2 (PLA_2) which is involved in chemotactic response (Dennis, 1994), and phosphoinositide-3 (PI-3) kinase activated by chemokines is implicated in integrin adhesiveness, cell migration and polarization (Dekker *et al*., 2000). Phospholipase D (PLD) exerts receptor internalization by chemokines through ARF and RhoA (Bacon *et al*., 1998), and G protein-coupled receptor kinase (GRK) mainly is associated with receptor desensitization after chemokine stimulation (Lefkowitz, 1993). Although chemokine receptors lack of tyrosine kinase activity, recent studies reported activation of tyrosine kinase pathway through them. For example, Janus kinase (JAK), which is included in non-receptor tyrosine kinase family, , zeta-associated protein (ZAP)-70 and the focal adhesion kinase (FAK) $pp125^{FAK}$ is activated by chemokine stimulation (Mark *et al*., 1998; Bacon *et al*., 1996).,

ERK is a group of widely distributed serine-threonine kinases which mediate mainly proliferative and mitogenic responses by activating transcription factors such as c-Fos and Elk-1 (Shyamala *et al*., 1998; Majka *et al*., 2000). ERK activation generally includes a cascade of events involving Ras, Raf, and ERK kinase. Several laboratories have established that

chemokines mediate ERK activation functioning cell adhesion by phosphorylating paxillin, and cell proliferation and differentiation through PI3-K and PKC (Brill *et al*., 2001; Minamiguchi *et al*., 2001)

Leukotactin-1 (Lkn-1) that belongs to the CC chemokine family has been known to bind CCR1 and CCR3, and to function on neutrophils, monocytes and lymphocytes (Youn *et al*., 1997). Since Lkn-1 has two extra cysteines, it is included in the C6 β-chemokine family. It is noteworthy that Lkn-1 shows chemotactic activity in neutrophils unlike other CC chemokines (Youn *et al*., 1997). Recent study indicates that Lkn-1 could be a novel mediator of atherosclerosis by inducing chemotaxis and the expression of pro-atherogenic cytokine and TF (Lee *et al*., 2002). Although Lkn-1 is believed to play an important role in leukocyte trafficking and the development of inflammation, the intracellular signaling mechanisms and functions of Lkn-1 are poorly defined.

The aim of the study was to investigate the intracellular events to induce biological actions following Lkn-1 binding to CCR1 using human osteogenic sarcoma cells expressing CCR1 (HOS/CCR1) as a model system.

II. MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal bovine serum were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Pertussis toxin (PTX), PD98059, U73122, rottlerin, and Ro-31-8425 were obtained from Calbiochem (San Diego, CA, USA). rLkn-1 was the kind gift of Greencross Life Science Corp. (Yongin, Korea). Antibodies against phospho-ERK1/2 was purchased from New England BioLabs (Beverly, MA, USA). Antibodies against ERK2, PKCα, c-Fos, c-Jun, c-Myc, Egr-1, cyclin D_3 and PCNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against PKCδ was purchased from Transduction Laboratories (Lexington, KY, USA). Myo-[2-^{3}H]inositol were purchased from Amersham Pharmacia Biotech. (Piscataway, NJ, USA).

Cell culture

Stable HOS cells expressing CCR1 or CCR5 were the kind gift of Dr. O. M. Howard (National Cancer Institute, MD, USA) and were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and puromycin (0.5 μg /ml).

Western blot analysis

Cells were harvested and washed three times with ice-cold phosphate-buffered saline. The cells then were resuspended in 100 μl of lysis buffer A (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, and protease inhibitors). The lysate was centrifuged at 8000g for 1 min at 4°C. The supernatant was collected as a cytosolic extract. The pellet was resuspended in 50 μl of lysis buffer B (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, .1 mM DTT, 25% glycerol and protease inhibitors). The suspension was centrifuged at 13000g for 15 min at 4°C. The supernatant was collected as a nuclear extract. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose filters. The blots were incubated with primary antibody and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same blot was stripped and reprobed with anti-ERK2 or β-actin antibody for use as an internal control.

PKC translocation assay

HOS cells seeded into 100 mm dishes at 5×10^5 cells/dish were cultured in DMEM. The cells were starved for 24 h in 0.5% FBS. After treatment with Lkn-1, the cells were harvested and washed three times with ice-cold

phosphate-buffered saline (without Ca^{2+}). The cells then were resuspended in 200 μl of homogenization buffer A (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and protease inhibitors) and homogenized at 4°C by ten passages through a 21G needle fitted on a 1 ml plastic syringe. The homogenate was centrifuged at 1,000 × g for 5 min at 4°C. The supernatant was collected and centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in 100 μl of homogenization buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM EGTA, 1 mM PMSF, and protease inhibitors) and sonicated for 10 s. The suspension was centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was collected as a membrane fraction. Protein concentration of each sample was determined.

Transient transfection

HOS cells were grown in DMEM with 10% heat-inactivated fetal bovine serum. One day after seeding cells into 35 mm dishes (2×10^5 cells), cells were transfected with 0.5 μg of mammalian expression vectors of dominant-negative RasN17, pSV-SPORT-H/RasN17 and dominant-negative Raf, pcDNA3.0/CAAX Raf-1 using LipofectAMINE 2000 reagents (Life Technologies) according to the manufacturer's instructions. Plasmid

pCMV/β-gal was included to monitor the transfection efficiency. The total amount of DNA was maintained at 1 μg with an empty vector pcDNA3.0. Twelve hours post transfection, cells were serum starved for 24 h and treated with Lkn-1. Cells were harvested after Lkn-1 treatment and protein extracts were prepared by reporter lysis buffer (Promega). One to five μg of protein was assayed for β-galactosidase activities.

Cell cycle analysis

HOS cells seeded into 60 mm dishes at 2×10^5 cells/dish were cultured in DMEM. After 24 h starvation, cells were treated with Lkn-1 (100 ng/ml). Harvested cells were fixed with 70 % ethanol and incubated for 1 h at 4°C. The fixed cells were stained with a solution containing RNase and propidium iodide at 50 μg/ml. After incubation for at least 30 min at 37°C, cells were analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA). The cell cycle distribution was evaluated on DNA plots by ModFit LT software (Verity Software House Inc., Topsham, ME, USA).

III. RESULTS

Lkn-1 stimulates ERK in HOS/CCR1 cells with a distinct time course

Since several chemokines have been shown to transduce the signal to MAP kinase for chemotactic activity and other cellular processes, this study first focused on the MAP kinase pathway, which is activated in response to many soluble factors including chemokines and regulates different cellular functions (Venkatakrishnan *et al*., 2000). To examine the effects of Lkn-1 on ERK activation, HOS/CCR1 cells were treated with Lkn-1 (100 ng/ml) for various periods of time before being lysed and subsequently analyzed for the activation status of ERK1 and ERK2 by immunodetection with a specific anti-phospho p42/p44 ERKs antibody. As shown in Figure II-1A, Lkn-1 stimulated both ERK1 and ERK2 activities with a peak at 30 min and a basal level recovered within 1 h. At later time points, a second peak of ERK phosphorylation was also observed around 12 h following the beginning of stimulation. Thus, ERK activation in response to Lkn-1 showed biphasic differential activation kinetics. Dose dependence of ERK activation by Lkn-1 was examined and maximum ERK activation was detected at a Lkn-1 concentration of 800 ng/ml (Figure II-1B).

PTX inhibits Lkn-1-induced ERK activation

To evaluate the coupling mechanism of the Lkn-1 receptor and the nature of possible G proteins involved in the signaling events activated by Lkn-1, HOS/CCR1 cells were treated with PTX before the stimulation of Lkn-1. As assessed by Western blotting with antibody against dual-phosphorylated ERK1/2, PTX inhibited the early and the delayed peaks of ERK1/2 activation induced by Lkn-1 confirming that Lkn-1 exerts its effect through a receptor linked to a PTX-sensitive G_i/G_o family G proteins (Figure II-2).

U73122 inhibits Lkn-1-induced ERK activation

Classical model for signal transduction by chemokines involves the activation of a phosphoinositide-specific PLC leading to the accumulation of inositol-1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) in the cytoplasm. To investigate whether PLC is the up-stream signal molecule of the ERK pathway activated by Lkn-1, Western blot analysis was performed in the absence or presence of PLC inhibitor. Figure II-3 shows that Lkn-1-induced ERK1/2 phosphorylation was inhibited by the pretreatment of U73122 and PD98059.

PKCδ is involved in the Lkn-1-induced ERK pathway

Activation of PLC by mitogenic signals is known to hydrolyze

phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce two second messengers, DAG and IP_3. DAG activates PKC, whereas IP_3 stimulates the release of Ca^{2+} from internal stores (Nishizuka, 1984). Since G_i/G_o protein and PLC participated in ERK activation by Lkn-1 stmulation (Figure II-4 and Figure II-5), it was examined whether possible down-stream regulator PKC is involved in the ERK pathway of Lkn-1. As shown in Figure II-4A, Lkn-1-induced ERK activation was inhibited in the presence of rottlerin but not in the presence of Ro-31-8425. Since rottlerin is a PKCδ specific inhibitor, this result indicates that PKCδ is involved in the ERK pathway of Lkn-1. The membrane/cytosol distribution of PKCδ was analyzed to investigate whether PKCδ is activated by Lkn-1 stimulation. PKCδ activity began to increase at 10 min after addition of Lkn-1, and the maximum activity of PKCδ was detected at 60 min by the Western blot analysis (Figure II-4B). However, PKCα activity was not increased by the treatment with 100 ng/ml of Lkn-1. These results indicate that PKCδ is involved in the Lkn-1-induced ERK pathway.

Lkn-1-dependent ERK activation is inhibited by dominant negative mutant of Ras

To examine whether the Ras/MEK pathway or the Raf/MEK pathway responds to Lkn-1-stimulation, HOS/CCR1 cells were transiently transfected

with either a dominant-negative Ras mutant plasmid or a dominant-negative Raf mutant plasmid. As shown in Figure II-5, Lkn-1-stimulated ERK activation was efficiently inhibited by dominant-negative RasN17. However, transfection with dominant-negative Raf did not affect Lkn-1-induced ERK activation. These data indicate that the Ras/MEK pathway is activated by Lkn-1 stimulation.

Lkn-1 induces the expression of immediate early response genes

Since Lkn-1 activates the ERK pathway, it may participate in the regulation of cellular processes such as cell proliferation, differentiation, and cytokine production. To understand whether the genes involved in these cellular processes are induced by Lkn-1 stimulation, the expression levels of several immediate early response genes such as *c-fos, c-jun, c-myc,* and *egr-1* were determined in Lkn-1-stimulated HOS/CCR1 cells. As shown in Figure II-6, Lkn-1 enhanced protein expression levels of c-Fos, c-Myc and Egr-1 with a distinct time course. However, protein expression of *c-jun* was not increased by Lkn-1 treatment. These results indicate that *c-fos, c-myc* and *egr-1* but not *c-jun* are involved in Lkn-1-induced cellular events.

ERK is involved in the expression of immediate early response genes by Lkn-1.

To examine whether ERK activation is associated with the elevated expression of the immediate early response genes. HOS/CCR1 cells were treated with PD98059 before the stimulation of Lkn-1. Protein expression levels of c-Fos and c-Myc were not affected by Lkn-1 treatment (Figure II-7). These results indicate that Lkn-1-dependent ERK activation induces expression of c-Fos and c-Myc, and that *c-fos* and *c-myc* but not *c-jun and egr-1* are involved in Lkn-1-induced cellular events.

Effect of Lkn-1 on cell cycle progression

To examine the effect of Lkn-1 on cell cycle progression, FACS analysis was performed. It was found that Lkn-1 increased both S and M phase populations and decreased G1 phase population by 48 h after treatment (Figure II-8).

Lkn-1 enhances expression level of cyclin D_3

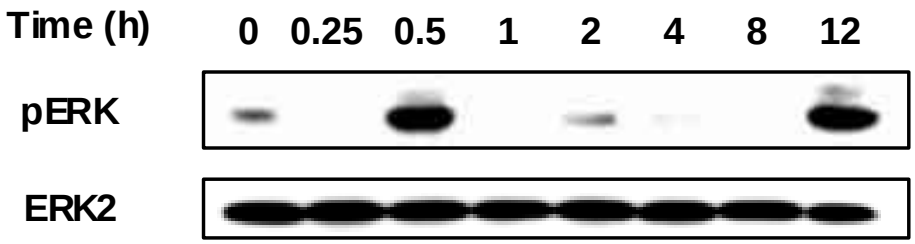
Since Lkn-1 affected the cell cycle progression at S and M phases, the levels of cell cycle regulators was examined. It was found that Lkn-1 increased the amount of cyclin D_3 but did not affect on the amount of PCNA (Figure II-9). These data indicate that Lkn-1 probably regulates the cell cycle

progression by induction of cell cycle-related molecules.

Schematic diagram of possible Lkn-1-induced signaling pathway

To examine Lkn-1-mediated signaling through CCR1, stable HOS/CCR1 cells were used. Lkn-1 binds to CCR1, which mediates signaling through activation of G_i/G_o protein, resulting in phospholipase C activation with subsequent inositol 1,4,5-trisphosphate and diacylglycerol generation, and Ca^{2+}-independent PKCδ activation. This mechanism may be common upstream pathway in subsequent ERK and NF-κB activation. The ERK activation through Ras may regulate the cell proliferation, differentiation, and various chemokine production, while the NF-κB activation is responsible for Lkn-1-induced chemotaxis and expression of genes related to cell movement.

A



B

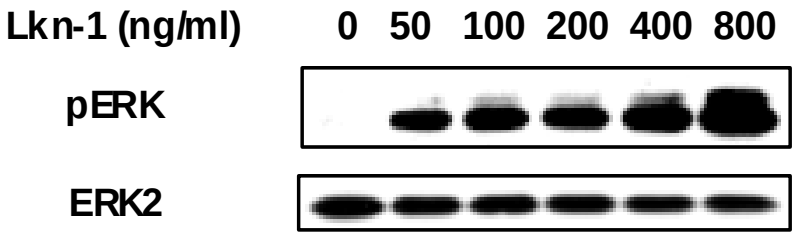


Figure II-1. Lkn-1 stimulates ERK in HOS/CCR1 cells with a distinct time course. HOS/CCR1 cells were serum starved with 0.5% serum for 24 h and were stimulated with 100 ng/ml Lkn-1 for the indicated time up to 12 h (A). For dose dependence experiments, serum starved HOS/CCR1 cells were stimulated with indicated concentrations of Lkn-1 for 30 min (B). Proteins were extracted, separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. ERK1/2 phosphorylation level was detected by Western blotting with anti-pERK1/2 antibody. The membrane was stripped and reprobed with ERK2 antibody as an internal control.

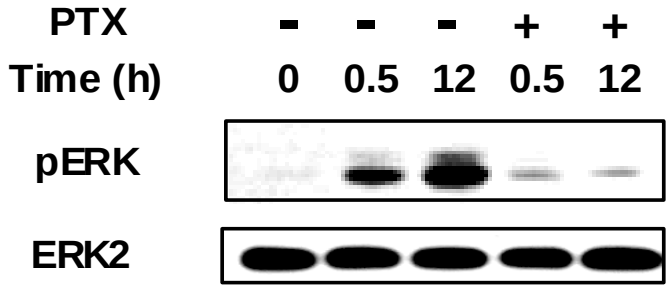


Figure II-2. PTX inhibits Lkn-1-induced ERK activation.

Serum starved HOS/CCR1 cells were pre-incubated in the absence (control) or presence of 100 ng/ml PTX for 16 h. ERK activation in response to Lkn-1 (100 ng/ml) was determined as described in Fig. II-1. Proteins were separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. ERK1/2 phosphorylation level was detected by Western blotting with anti-pERK1/2 antibody. The membrane was stripped and reprobed with ERK2 antibody as an internal control.

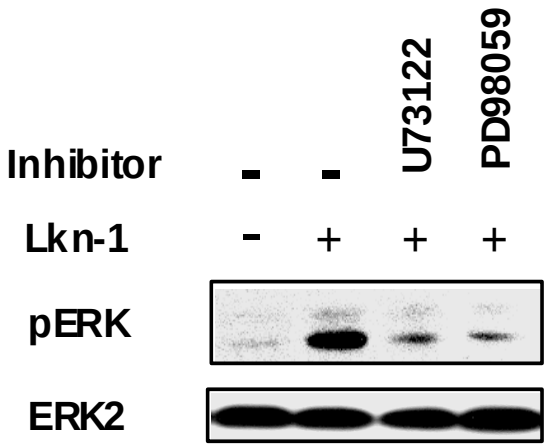


Figure II-3. U73122 inhibits Lkn-1-induced ERK activation. Serum starved HOS/CCR1 cells were pre-incubated with U73122 (10 μM) and PD98059 (50 μM) for 30 min followed by the stimulation with 100 ng/ml Lkn-1 for 30 min. Proteins were separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. ERK1/2 phosphorylation level was detected by Western blotting with anti-pERK1/2 antibody. The membrane was stripped and reprobed with ERK2 antibody as an internal control.

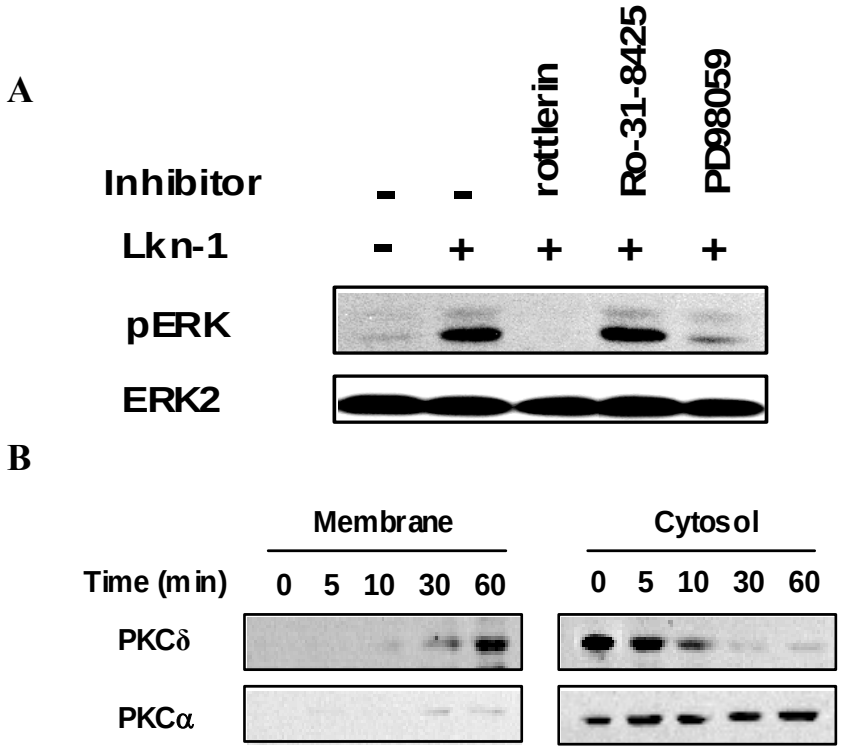


Figure II-4. PKCδ is involved in Lkn-1-induced signaling. (A) Serum starved HOS/CCR1 cells were pre-incubated with rottlerin (5 μM), Ro-31-8425 (50 nM), and PD98059 (50 μM) for 30 min followed by the stimulation with 100 ng/ml Lkn-1 for 30 min. Proteins were separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. ERK1/2 phosphorylation level was detected by Western blotting with anti-pERK1/2 antibody. The membrane was stripped and reprobed with ERK2 antibody as an internal control. (B) Serum starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated time. Harvested cells were fractionated as described in Methods, then analyzed by Western blotting with anti-PKCδ and anti-PKCα antibodies.

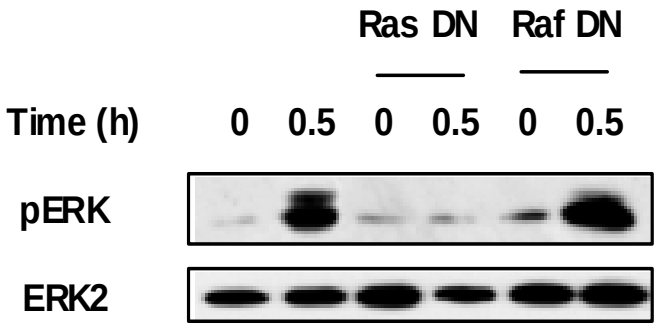


Figure II-5. Ras is involved in Lkn-1-induced signaling. HOS/CCR1 cells were transfected with dominant-negative Ras or Raf plasmid (0.5 μg). The pCMV/β-gal plasmid (0.2 μg) was included as an internal control for normalization of transfection efficiency. The total amount of DNA was maintained at 1 μg with the pcDNA3.0 plasmid. After transfection, cells were serum-starved and treated with Lkn-1 (100 ng/ml) for 30 min. Proteins were separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. ERK1/2 phosphorylation level was detected by Western blotting with anti-pERK1/2 antibody. The membrane was stripped and reprobed with ERK2 antibody as an internal control.

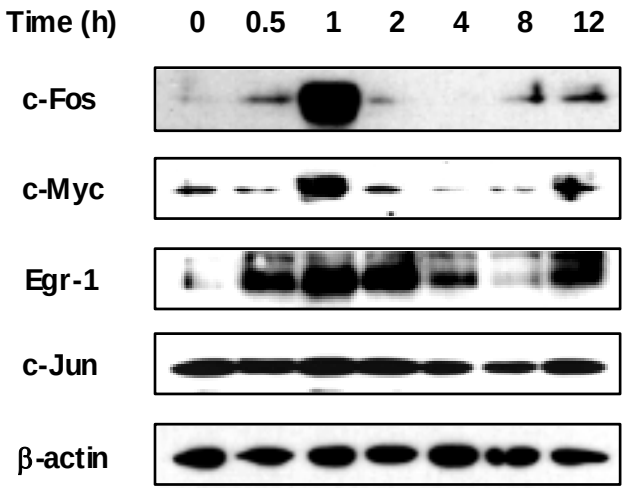


Figure II-6. Lkn-1 induces the expression of immediate early response genes. Serum starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated time. Nuclear extracts were prepared as described in Methods, then separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. Protein expression levels of c-Fos, c-Jun, c-Myc, and Egr-1 were detected by Western blot analysis. β-actin was used as an internal control.

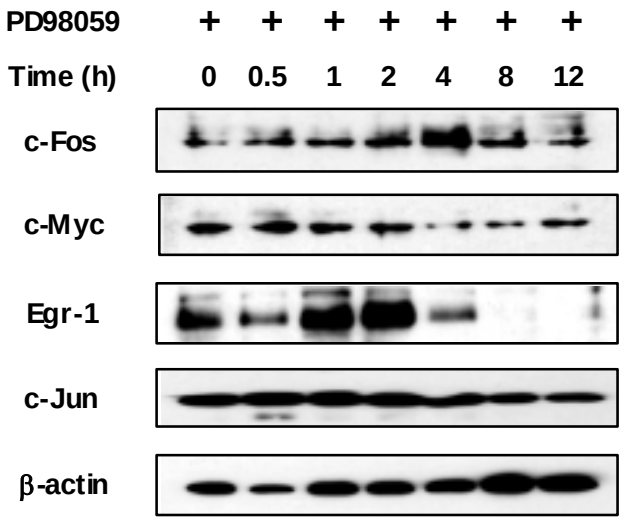


Figure II-7. ERK is involved in the expression of immediate early response genes by Lkn-1. Serum starved HOS/CCR1 cells were pre-incubated with PD98059 (20 μM) for 30 min followed by the stimulation with 100 ng/ml Lkn-1 for the indicated time. Nuclear extracts were prepared as described in Methods, then separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. Protein expression levels of c-Fos, c-Jun, c-Myc, and Egr-1 were detected by Western blot analysis. β-actin was used as an internal control.

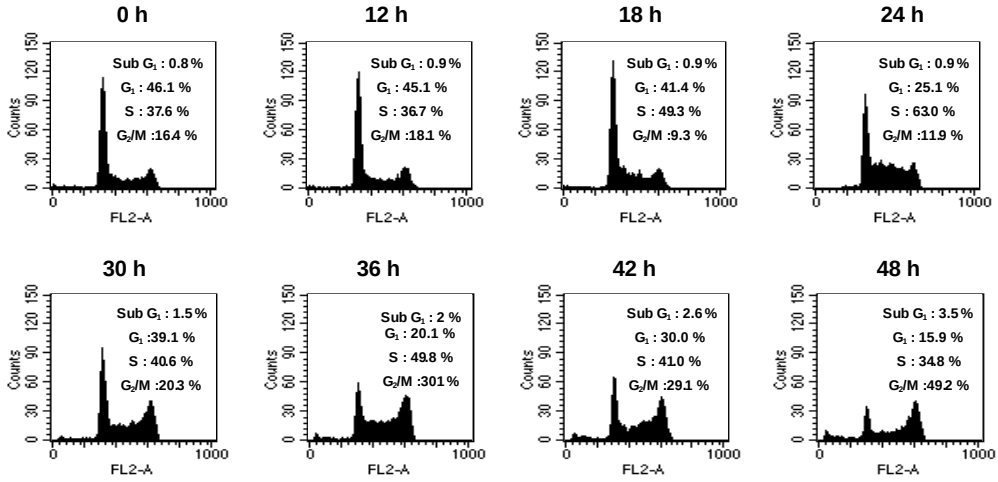


Figure II-8. Effect of Lkn-1 on cell cycle progression. Serum starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated time. The cell cycle distribution in G_1, S or G_2/M phase was determined by FACS analysis. The x-axis shows DNA content; and y-axis shows the number of cells

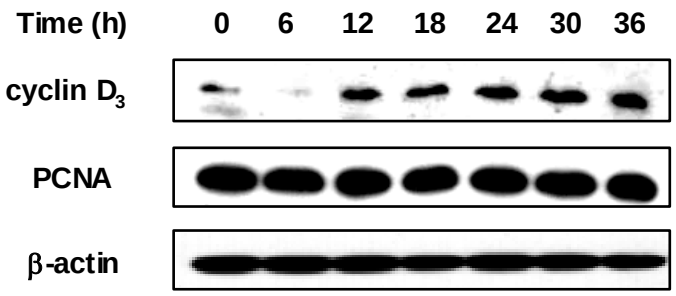


Figure II-9. Lkn-1 enhances expression level of cyclin D_3. Serum starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated time. Nuclear extracts were prepared as described in Methods, then separated on 10% SDS-polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. Protein expression levels of cyclin D_3 and PCNA were detected by Western blot analysis. β-actin was used as an internal control.

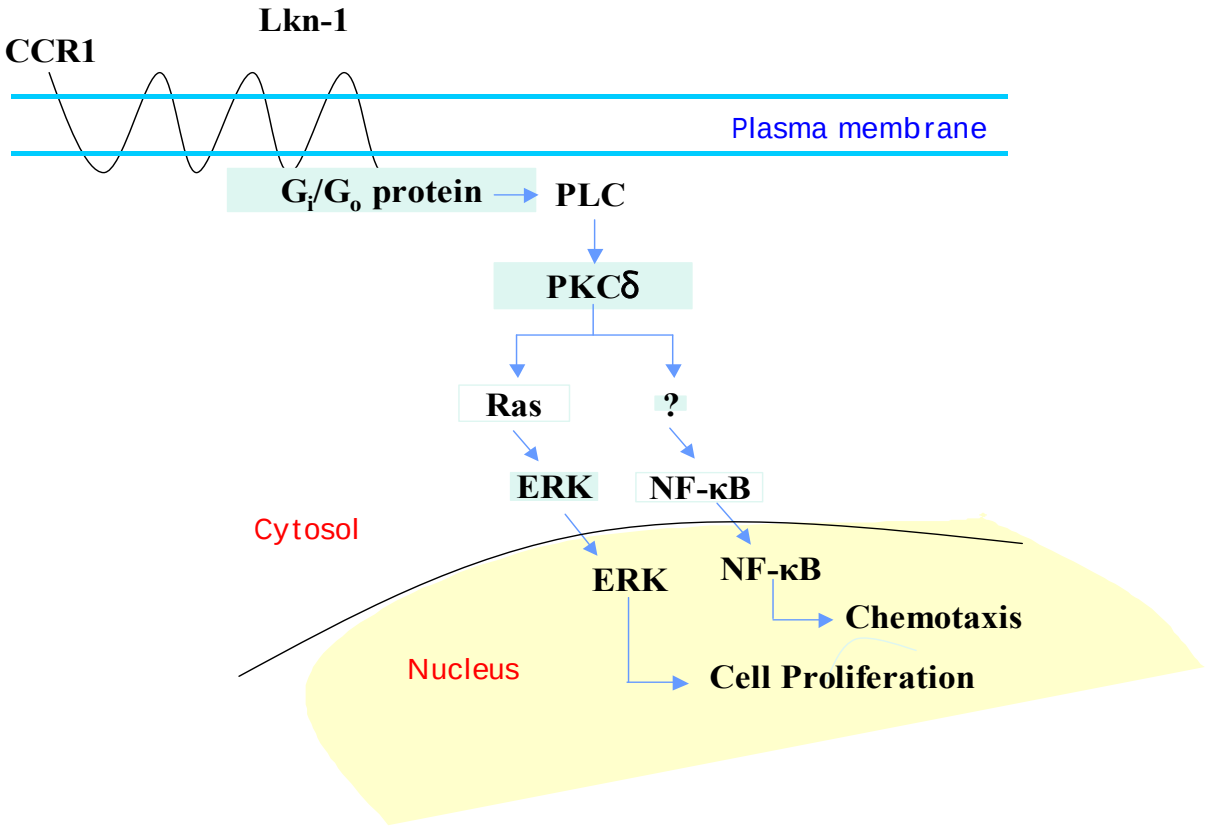


Figure II-10. Schematic diagram of possible Lkn-1-induced signaling pathway. Lkn-1 activates ERK via Gi/Go protein, PLC, and PKCδ. ERK pathway may regulate the cell proliferation, differentiation, and induction of chemokine gene expression. Lkn-1-induced NF-κB activation shares the same pathway with ERK that includes Gi/Go protein, PLC, and PKCδ. NF-κB pathway is responsible for Lkn-1-induced chemotaxis and expression of genes related to cellular events.

IV. DISCUSSION

In this contribution, the mechanism of Lkn-1-induced intracellular signal transduction through CCR1 in HOS cells was characterized. It has been demonstrated that (1) Lkn-1 activates ERK with a distinct time course and the activation is biphasic. (2) Lkn-1 transduces the signal via PTX-sensitive G_i/G_o family G proteins. (3) Lkn-1 activates PLC in a time dependent manner. (4) The δ subtype of PKC is involved in the Lkn-1-induced ERK pathway. (5) The Ras/MEK pathway is activated by Lkn-1 stimulation. (6) Immediate early response genes such as *c-fos* and *c-myc* are involved in Lkn-1-induced cellular events. (7) Lkn-1 affects the cell cycle progression by cyclin D_3 induction.

The MAP kinase cascade is one of the most frequently studied signal tranduction systems and is known to participate in multiple cellular functions, such as proliferation, differentiation, survival, and locomotion (Lewis *et al*., 1998). Since many chemokines are dependent on the MAP kinase pathway to biologically function, this study first focused on MAP kinase activation in response to Lkn-1 to understand intracellular signaling pathway induced by Lkn-1. Figure II-1 showed that Lkn-1 enhanced the phosphorylation of ERK1/2 indicating that the ERK pathway is involved in Lkn-1-induced signaling. Different time kinetics of ERK activation was observed in HOS/CCR1 cells in response to Lkn-1 with the presence of a late peak

appearing 12 h following stimulation. A biphasic activation of ERK has been previously reported in cells synchronized in the M phase and subsequently released, compatible with the presence of peaks of ERK activation in the G_1 and G_2/M phases, respectively (Tamemoto *et al*., 1992). Along these lines, interfering with ERK activation has recently been shown to delay the progression of cycling cells through G_2 (Wright *et al*., 1999). These data indicate that, upon stimulation with agonists, cells that progress through the cell cycle show a second peak of ERK activation, whereas the response of those that do not proliferate is characterized by a early single peak (Bonacchi *et al*., 2001). Activation of ERK has been observed in response to a wide number of agonists in different cell types, and chemokine receptors other than CCR1 have recently been associated with activation of this pathway (Venkatakrishnan *et al*., 2000; Tilton *et al*., 2000). Although activation of the ERK signaling pathway has been shown to promote cell motility by regulating gene expression (Yebra *et al*., 1995), this is not the case in Lkn-1-induced cell migration in HOS cells. Chapter I in this study showed that inhibition of ERK did not inhibit Lkn-1-induced migration in HOS cells. These results imply that the ERK signaling pathway is not involved in cell migration induced by Lkn-1 in HOS cells. The ERK pathway is probably involved in cell proliferation and differentiation by regulating the cell cycle regulators, and in regulation of gene expression for other cellular processes. The involvement of the MAP

kinase pathway in cell proliferation, differentiation, and inflammatory cytokine production has been shown in hematopoietic cells (Adachi *et al*., 2000). In addition, there is a report that IL-8 activates ERK although the regulation of cell migration by IL-8 is independent of ERK activation (Knall *et al*., 1997).

The remodeling of membrane phospholipids by PLA_2, PLD and PLC is an essential step in the signal transduction pathways associated with the response of leukocytes to CC chemokines. It has been reported that CC chemokines activate PLA_2 and the release of arachidonic acid in human monocytes (Locati *et al*., 1994; Dennis, 1994). PLD activation by chemokines has been reported (Bacon *et al*., 1998), although its significance is speculative and details of its regulation in leukocytes remain to be elucidated. PLC activation was described in this study. G proteins are grouped into two families depending on the sensitivity to PTX. G_i/G_o family G proteins are sensitive to PTX whereas G_q family G proteins are insensitive to this toxin (Venkatakrishnan *et al*., 2000). Most of chemokines are known to be affected by the treatment of PTX. The phosphorylation of ERK in Lkn-1-stimulated HOS/CCR1 cells is inhibited by PTX. ERK activation was also inhibited in the presence of specific inhibitors of PLC and PKCδ demonstrating that Lkn-1 activates the ERK pathway through the conventional signaling pathway. PKC is a family of serine/threonine protein kinases consisting of 11 isoforms,

which are divided into three groups on the basis of their biochemical properties and sequence homologies (Mellor *et al*., 1998). The different PKC isotypes may have specific roles in signal transduction (Schonwasser *et al*., 1998). The δ subtype of PKC belongs to novel PKC group and is activated by DAG in a calcium-independent manner (Mischak *et al*., 1998; Soltoff *et al*., 1995). Although PKCδ is one of the major isoforms expressed in hemopoietic cells (Mischak *et al*., 1998), the involvement of PKCδ in chemokine-induced signaling pathway has not been reported in any chemokines. Figure II-4 showed that PKCδ was activated by Lkn-1 stimulation, but PKCα activity was not affected by Lkn-1 stimulation. PKCδ specific inhibitor rottlerin (Pongracz *et al*., 1999) blocked Lkn-1-induced ERK activation whereas Ro-31-8425, a PKC inhibitor that does not inhibit PKCδ, showed no inhibitory effect of ERK activation by Lkn-1. The involvement of PKCδ in Lkn-1-induced signaling pathway is distinct from other chemokine signal transduction reported so far. Although it has been reported that PLCβ2 and PLCβ3 are the PLC isoforms that are activated by chemoattractants in mouse neutrophils (Li *et al*., 2000), the specific PLC isoform activated by Lkn-1 stimulation in HOS/CCR1 cells has not been elucidated in this study. The detailed kinetics of PLC and PKC activation is under investigation using various PLC and PKC isoforms.

Lkn-1 induced NF-κB activation (Figure I-11) as well as the ERK

pathway (Figure II-1), and ERK is involved in activating NF-κB (Tuyt *et al*., 1999). However, Lkn-1-induced activation of NF-κB was independent of the ERK pathway because an inhibitor of MEK (PD98059) had no effect on Lkn-1-enhanced NF-κB activation (Figure I-12). This result is in agreement with a recent report showing that Raf-mediated NF-κB activation is independent of the MEK1/ERK cascade (Baumann *et al*., 2000). PKCδ activated by Lkn-1 may be an upstream molecule of both pathways, and activate different molecules which transduce the signal to either the ERK or NF-κB pathway (Figure II-10).

In conclusion, it has been demonstrated that CC chemokine Lkn-1 activates the ERK signaling pathway in a model system of HOS/CCR1 cells. Upon binding of Lkn-1 to its G_i/G_o protein coupled receptor CCR1, the ERK pathway is activated through the specific activation of PLC/PKCδ/Ras/ERK. The G_i/G_o protein/PLC/PKCδ/Ras/ERK pathway may mediate Lkn-1-induced cellular events including proliferation, differentiation and regulation of gene expression.

CONCLUSIONS

In this study, Lkn-1-induced chemotaxis signaling and intracelluar event including ERK through CCR1 in HOS cells have been investigated.

1. Lkn-1 tranduces the signal through PTX-sensitive G_i/G_o family G protein.

   In the presence of PTX, Lkn-1-induced chemotaxis was blocked. This result indicates that PTX-sensitive G_i/G_o family G protein is associated with Lkn-1-induced cell migaration.

2. Lkn-1 activates PLC and PKCδ in a time dependent manner.

   When HOS/CCR1 cells were treated with Lkn-1, PLC activity increased in both a time and dose dependent manner. PKCδ was translocated to membrane in response to Lkn-1 indicating that PKCδ is activated by Lkn-1.

3. Lkn-1 increases DNA binding activity of NF-κB.

   NF-κB movement to nucleus was enhanced by Lkn-1 treatment indicating that NF-κB is activated by Lkn-1. The specificity of NF-κB binding activity was confirmed by the competition experiment using unlabeled NF-κB binding sequence.

4. PLC, PKCδ, NF-κB and newly synthesized proteins are involved in Lkn-1-induced chemotaxis.

PLC inhibitor U73122 and PKCδ specific inhibitor rottlerin blocked Lkn-1-induced cell migration. In addition, NF-κB, transcription and translation inhibitors decreased cell movement in response to Lkn-1.

5. ERK, p38, tyrosine kinases, PI-3 kinase, PLA_2 and PLD are not associated with Lkn-1-induced chemotaxis events.

Inhibitors of ERK, p38, tyrosine kinases, PI-3 kinase, PLA_2 and PLD did not affect the chemotactic activity of Lkn-1 indicating that these molecules are not involved in Lkn-1-induced chemotaxis.

6. Lkn-1 activates ERK with a distinct time course and the activation is biphasic.

Lkn-1 activated both ERK1 and ERK2 with a peak at 30 min and a basal level recovered within 1 h. At later time points, a second peak of ERK phophorylation was also observed around 12 h.

7. PTX-sensitive G_i/G_o family G protein, PLC and PKCδ are involved in the Lkn-1-induced ERK pathway.

ERK phosphorylation decreased in the presence of PTX, U73122, and rottlerin. This result indicates that ERK activation is mediated via G_i/G_o family G protein, PLC and PKCδ.

8. Lkn-1 activates the Ras/MEK pathway.

ERK activation by Lkn-1 was inhibited by dominant negative mutant of Ras but not by dominant negative mutant of Raf. MEK inhibitor PD98059 also blocked ERK activation. Therefore, Ras and MEK are upstream signaling molecules of Lkn-1-induced ERK activation.

9. Immediate early response genes such as *c-fos* and *c-myc* are involved in Lkn-induced cellular events.

Lkn-1 enhanced protein expression levels of c-Fos, c-Myc and Egr-1 with a distinct time course. Especially, the induction of c-Fos and c-Myc is blocked by PD98059. This result indicates that c-Fos and c-Myc are involved in ERK activation by Lkn-1

10. Lkn-1 affects the cell cycle progression by cyclin D_3 induction.

Lkn-1 increased both S and M phase populations and decreased G1 phase population by 48 h after treatment. Lkn-1 increased the amount of cyclin D_3 but did not affect on the amount of PCNA. These results indicate

that Lkn-1 probably regulates the cell cycle progression by induction of cell cycle-related molecules.

In summary, Lkn-1-induced chemotaxis signaling and ERK activation are mediated via G_i/G_o protein/PLC/PKCδ cascades in HOS/CCR1 cells. Lkn-1 activates ERK through Ras, which may regulate the cell proliferation, differentiation, and various chemokine production, while Lkn-1 induced NF-κB activation is responsible for Lkn-1-induced chemotaxis and expression of genes related to cell movement.

REFERENCES

Adachi, T., Choudhury, B.K., Stafford, S., Sur, S., and Alam, R. (2000) The differential role of extracellular signal-regulated kinases and p38 mitogen-activated protein kinase in eosinophil functions. *J. Immunol*. **165,** 2198-2204.

Arenberg, D.A., Polverini, P. J., Kunkel, S.L., Shanafelt, A., Hesselgesser, J., Horuk, R., and Strieter, R.M. (1997) The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. *J. Leukocyte Biol.* **62**, 554-562.

Bacon, K.B., Szabo, M.C., Yssel, H., Bolen, J.B., and Schall, T.J. (1996) RANTES induces tyrosine kinase activity of stably complexed p125FAK and ZAP-70 in human T cells. *J. Exp. Med*. **184,** 873-882.

Bacon, K.B., Schall, T.J., and Dairaghi, D.J. (1998) RANTES activation of phospholipase D in Jurkat T cells: Requirement of GTP-binding proteins ARF and RhoA. *J. Immunol.* **160,** 1894-1900.

Banchereau, J., and Stinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245-252.

Baumann, B., Weber, C.K., Troppmair, J., Whiteside, S., Israel, A., Rapp, U. R., and Wirth, T. (2000) Raf induces NF-κB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. *Proc.*

Natl. Acad. Sci. U. S. A. **97,** 4615-4620.

Bernardetta, N., Tiffany, H.L., Bong, G.W., Yourey, P.A., Morahan, D.K., Li, Y., Murphy, P.M., and Alderson, R.F. (1999) Characterization of the signal transduction pathway activated in human monocytes and dendritic cells by MPIF-1, a specific ligand for CC chemokine receptor 1. *J. Immunol.* **162,** 435-444.

Bonecchi, R., Bianchi, G., Panina-Bordignon, P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A., and Sinigaglia, F. (1998) Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* **187,** 129-134.

Bonacchi, A., Romagnani, P., Romanelli, R.G., Efsen, E., Annunziato, F., Lasagni, L., Francalanci, M., Serio, M., Laffi, G., Pinzani, M., Gentilini, P., and Marra, F. (2001) Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J. Biol. Chem.* **276,** 9945-9954.

Brill, A., Hershkoviz, R., Vaday G.G., Chowers, Y.C., and Lider, O. (2001) Augmentation of RANTES-induced extracellular signal-regulated kinase mediated signaling and T cell adhesion by elastase-treated fibronectin. *J. Immunol*. **166,** 7121-7127.

Cairns, J.S., and D'Souza, M.P. (1998) Chemokines and HIV-1 second receptors: the therapeutic connection. *Nat. Med.* **4,** 563-568.

Coughlin, C.M., Salhany, K.E., Wysocka, M., Aruga, E., Kurzawa, H., Chang, A.E., Hunter, C.A., Fox, J.C., Trinchieri, G., and Lee, W.M.F. (1998) Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. *J. Clin. Invest.* **101,** 1441-1452.

Dekker, L.V., and Segal, A.W. (2000) Signals to move cells. *Science* **287,** 962-985.

Dennis, E.A. (1994) Diversity of group types, regulation, and function of phospholipase A2. *J. Biol. Chem.* **269,** 13057-13060.

Dutt, P., Wang, J.F., and Groopman, J.E. (1998) Stromal cell-derived factor-1α and stem cell factor/kit ligand share signaling pathways in hemopoietic progenitors: A potential mechanism for cooperative induction of chemotaxis. *J. Immunol.* **161,** 3652-3658.

Ganju, R.K., Dutt, P., Wu, L., Newman, W., Avraham, H., Avraham, S., and Groopman, J.E. (1998) β-chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood* **91,** 791-797.

Ganju, R.K., Brubaker, S.A., Meyer, J., Dutt, P., Yang, Y., Qin, S., Newman, W., and Groopman, J. E. (1998) The α-Chemokine, stromal cell-derived factor-1α, binds to the transmembrane G-protein-coupled

CXCR-4 receptor and activates multiple signal transduction pathway. *J. Biol. Chem.* **273,** 23169-23175.

Gao, J.L., Wynn, T.A., Chang, Y., Lee, E.J., Broxmeyer, H.E., Cooper, S., Tiffany, H.L., Westphal, H., Kwon-Chung, J., and Murphy, P.M. (1997) Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med.* **185,** 1959-1968.

Hedrick, J.A., Saylor, V., Figueroa, D., Mizoue, L., Xu, Y., Menon, S., Abrams, J., Handel, T., and Zlotnik, A. (1997) Lymphtactin is produced by NK cells and attracts both NK cells and T cells in vivo. *J. Immunol.* **158,** 1533-1540.

Kim, C.H., Pelus, L.M., White J.R., and Broxmeyer, H.E. (1998) Differential chemotactic behavior of developing T cells in response to thymic chemokines. *Blood* **91,** 4434-4443.

Knall, C., Worthen, G.S., and Johnson, G.L. (1997) Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases *Proc. Natl. Acad. Sci. U. S.A.* **94,** 3052-3057.

Koenig, J.A., and Edwardson, J.M. (1997) Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* **18,** 276-287.

Lee, W.H., Kim, S.H., Jeong, E.M., Choi, Y.H., Kim, D.I., Cho, Y.S., Kwon, B.S., and Park, J.E. (2002) A novel chemokine, Leukotactin-1 induces chemotaxis, pro-atherogenic cytokines, and tissue factor expression in atherosclerosis. *Atherosclerosis* **161,** 255-60.

Lefkowitz, R.J. (1993) G protein-coupled receptor kinases. *Cell* **74,** 409-12.

Lewis, T.S., Shapiro, P.S., and Ahn, N.G. (1998) Signal transduction through MAP kinase cascades. *Adv. Cancer. Res.* **74,** 49-139.

Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V., and Wu, D. (2000) Roles of PLC-β2 and -β3 and PI3Kγ in chemoattractant-mediated signal transduction. *Science* **287,** 1046-1049.

Locati, M., Zhou, D., Luini, W., Evangelista, V., Mantovani, A., and Sozzani, S. (1994) Rapid induction of arachidonic acid release by monocyte chemotactic protein-1 and related chemokines. Role of Ca2+ influx, synergism with platelet-activating factor and significance for chemotaxis. *J. Biol. Chem.* **269,** 4746-4753.

Luan, J., Shattuck-Brandt, R., Haghnegahdar, H., Owen, J.D., Strieter, R., Burdick, M., Nirodi, C., Beauchamp, D., Johnson, K.N., and Richmond, A. (1997) Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumor progression. *J. Leukocyte Biol.* **62,** 588-597.

Luster, A.D. (1998) Mechanisms of Disease: Chemokines-chemotactic

cytokines that mediate inflammation. *N. Engl. J. Med.* **338,** 436-445.

Majaka, M., Ratajczak, J., Kowalska, M.A., and Ragajczak, M.Z. (2000) Binding of stromal derived factor-1alpha (SDF-1alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur. J. Haematol.* **64,**164-72.

Mellado, M., Rodriguez-Frade, J.M., Aragay, A., del Real, G., Martin, A.M., Vila-Coro, A.J., Serrano, A., Mayor, F.J., and Martinez, A.C. (1998) The chemokine monocyte chemotactic protein 1 triggers Janus Kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J. Immunol.* **161,** 805-813.

Mellado, M., Rodriguez-Frade, J.M., Vila-Coro, A.J., Martin de Ana, A., and Martinez-A, C. (1999) Chemokine control of HIV-1 infection. *Nature* **400,** 723-724.

Mellado, M., Rodriguez-Frade, J.M., Manes, S., and Martinez-A, C. (2001) Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu. Rev. Immunol.* **19,** 397-421.

Mellor, H., and Parker, P.J. (1998) The extended protein kinase C superfamily *Biochem. J.* **332,** 281-292.

Minamiguchi, H., Kimura, T., Urata, Y. Miyazaki, H., Bamba, T., Abe, T., and Sonoda, Y. (2001) Simultaneous signaling through c-mpl, c-kit and CXCR4 enhances the proliferation and differentiation of human megakaryocyte progenitors: possible roles of the PI3-K, PKC, and MAPK pathways. *Br. J. Haematology* **115,** 175-185.

Mischak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F., and Mushinski, J.F. (1998) Mouse protein kinase C-.delta. the major isoform expressed in mouse hemopoietic cells: sequence of the cDNA, expression patterns, and characterization of the protein. *Biochemistry* **30,** 7925-7931.

Murdoch, C., and Finn, A. (2000) Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95,** 3032-3043.

Murphy, P.M. (1996) Chemokine receptors: structure, function and role in microbial pathogenesis. *Cytokine Growth Factors Rev.* **7,** 47- 64.

Newton, A.C. (1995) Protein kinase C. Seeing to domains. *Curr. Biol*. **5,** 973-976.

Nishizuka, Y. (1984) Studies and perspectives of protein kinase C. *Science* **233,** 305-312.

Pongracz, J., Webb, P., Wang, K., Deacon, E., Lunn, O.J., and Lord, J.M. (1999) Spontaneous neutrophil apoptosis involves Caspase 3-mediated activation of protein kinase C-δ. *J. Biol. Chem.* **274,** 37329-37334.

Schonwasser, D.C., Marais, R.M., Marshall, C.J., and Parker, P.J. (1998) Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.* **18,** 790-798.

Shyamala, V., and Khoja, H. (1998) Interleukin-8 receptors R1 and R2 activate mitogen-activated protein kinases and induce c-fos, independent of Ras and Raf-1 in Chinese hamster ovary cells. *Biochemistry* **37,** 15918-15924.

Soltoff, S.P., and Toker, A. (1995) Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase C in salivary gland epithelial cells. *J. Biol. Chem*. **270,** 13490-13495.

Tamemoto, H., Kadowaki, T., Tobe, K., Ueki, K., Izumi, T., Chatani, Y., Kohno, M., Kasuga, M., Yazaki, Y., and Akanuma, Y. (1992) Biphasic activation of two mitogen-activated protein kinases during the cell cycle in mammalian cells. *J. Biol. Chem.* **267,** 20293-20297.

Tilton, B., Ho, L., Oberlin, E., Loetscher, P., Baleux, F., Clark-Lewis, I., and Thelen, M. (2000) Signal transduction by CXC chemokine receptor 4: stromal cell–derived factor 1 stimulates prolonged protein kinase B and extracellular signal–regulated kinase 2 activation in T lymphocytes. *J. Exp. Med.* **192,** 313-324.

Tuyt, L.M., Dokter, W.H., Birkenkamp, K., Koopmans, S.B., Lummen, C.,

Kruijer, W., and Vellenga, E. (1999) Extracellular-regulated kinase 1/2, Jun N-terminal Kinase, and c-Jun are involved in NF-κB-dependent IL-6 expression in human monocytes. *J. Immunol.* **162,** 4893-4902.

Venkatakrishnan, G., Salgia, R., and Groopman, J. (2000**)** Chemokine Receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. *J. Biol. Chem.* **275,** 6868-6875.

Vicari, A.P., Figueroa, D.J., Hedrick, J.A., Foster, J.S., Singh, K.P., Menon, S., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Bacon, K.B., and Zlotnik, A. (1997) TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity* **7,** 291-301.

Wang, D., and Richmond, A. (2001) Nuclear Factor-κB activation by the CXC chemokine melanoma growth-stimulatory activity/growth-regulated protein involves the MEKK1/p38 mitogen-activated protein kinase pathway *J. Biol. Chem.* **276,** 3650-3659.

Wang, J.M., Hishinuma, A., Oppenheim, J.J., and Matsushima, K. (1993) Studies of binding and internalization of human recombinant monocyte chemotactic and activating factor (MCAF) by monocytic cells. *Cytokine* **5,** 264-275.

Wang, J.M., Deng, X., Gong, W., and Su, S. (1998) Chemokines and their role

in tumor growth and metastasis. *J. Immunol. Methods* **220,** 1-17.

Wong, M., and Fish, E.N. (1998) RANTES and MIP-1α activate Stats in T cells. *J. Biol. Chem.* **273,** 309-314.

Wright, J.H., Munar, E., Jameson, D.R., Andreassen, P.R., Margolis, R.L., Seger, R., and Krebs, E.G. (1999) Mitogen-activated protein kinase kinase activity is required for the G_2/M transition of the cell cycle in mammalian fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11335-11340.

Yebra, M., Filardo, E.J., Bayna, E.M., Kawahara, E., Becker, J.C., and Cheresh, D.A. (1995) Induction of carcinoma cell migration on vitronectin by NF-kappa B- dependent gene expression. *Mol. Biol. Cell* **6,** 841-850.

Youn, B.S., Zhang, S.M., Lee, E.K., Park, D.H., Broxmeyer, H.E., Murphy, P. M., Locati, M., Pease, J.E., Kim, K.K., Antol, K., and Kwon, B.S. (1997) Molecular cloning of leukotactin-1: a novel human beta-chemokine, a chemoattractant for neutrophils, monocytes, and lymphocytes, and a potent agonist at CC chemokine receptors 1 and 3. *J. Immunol.* **159,** 5201-5205.

Zhang, S.M., Youn, B.S., Gao, J.L., Murphy, P.M., and Kwon, B.S. (1999) differential effects of leukotactin-1 and macrophage inflammatory protein-1α on neutrophils mediated by CCR1. *J. Immunol.* **162,** 4938-4942.

Zimmermann, N., Conkright, J.J., and Rothenberg, M.E. (1999) CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J. Biol. Chem.* **274,** 12611-12618.

국문 요약

사람 골수종 세포에서 CCR1 을 통한 Leukotactin-1의 신호 전달 기전에 대한 연구

Leukotactin-1 (Lkn-1)은 최근에 클론된 CCR1과 CCR3에 결합하는 CC chemokine이다. Lkn-1이 중성구, 단핵구, 림프구의 화학주성제로서 역할을 하는 것으로 알려져 있으나, 세포내 기전은 명확하게 밝혀지지 않았다. 본 실험에서는 Lkn-1에 의해 유도되는 화학주성 신호전달과정을 이해하기 위해서 CCR1을 발현하는 사람 골수종세포에서 화학주성 활성도를 연구하였다. Lkn-1에 의해 유도되는 화학주성에 관련된 분자들을 조사하기 위해서 세포유주실험을 수행하였다. G_i/G_o 단백질의 억제제인 pertussis toxin과 인지질 가수분해효소 C와 단백질 인산화효소 C의 억제제는 Lkn-1에 의해 유도되는 화학주성을 감소시켰다. 특히 단백질 인산화 효소 Cδ의 특이적 억제제인 rottlerin은 Lkn-1의 화학주성 활성도를 억제시켰는데, 이것은 Lkn-1의 화학주성 신호전달과정에서 단백질 인산화효소 Cδ가 관여하는 것을 나타낸다. 인지질 가수분해효소 C와 단백질 인산화효소 C의 활성도는 Lkn-1의 자극에 의해서 증가하였다. 그러나, MEK 억제제인 PD98059는 화학주성 활성도를 억제시키지 않아서 ERK가 관여하지 않는다는 것을 보여주었다. Lkn-1에 의한 화학주성 활성도는 cyclonheximide와 actinomycin D에 의해서 억제되는데 이는 화학주성에 새로운 단백질의 합성이 필요하다는 것을 나타낸다. 새로운 단백질 합성을 위해

서는 전사인자의 활성화가 요구되는데, 이 중에서 가장 잘 알려져 있는 NF-κB가 관련하는지를 조사하였다. NF-κB의 억제제는 화학주성 활성도를 감소시켰고, gel shift assay 결과 Lkn-1의 자극에 의해서 NF-κB의 DNA 결합능력이 증가되었다. 이러한 결과는 Lkn-1에 의한 화학주성 신호전달이 G_i/G_o 단백질, 인지질 가수분해효소 C, 단백질 인산화효소 Cδ, NF-κB을 거쳐 새로운 단백질합성으로 이어지는 것을 나타낸다.

Lkn-1이 CCR1에 결합한 후에 일어나는 세포내 신호전달에 대한 연구를 위해서 CCR1을 발현하는 사람 골수종 세포주의 신호분자 활성도를 실험하였다. Lkn-1에 의해 자극받은 세포는 시간별로 인산화된 ERK1/2를 증가시켰다. ERK의 활성도는 30분과 12시간에 최고점에 이르렀고, pertussis toxin에 의해서 ERK의 활성도가 감소하였다. 인지질 가수분해효소 C와 단백질 인산화효소 Cδ의 억제제도 또한 ERK의 활성도를 감소시켰다. *c-fos*, *c-myc*, *egr-1* 같은 immediate early response genes은 Lkn-1에 의해 유도되어졌다. Lkn-1이 G_i/G_o 단백질, 인지질 가수분해효소, 단백질 인산화효소 Cδ를 통하여 ERK를 활성화시키는데 이러한 결과는 세포증식과 분화 그리고 유전자발현조절과 같은 다른 세포내 과정에 Lkn-1에 의해 유도되어진 ERK 활성화가 관여할 수 있다는 가능성을 제시한다.

핵심어: Lkn-1, CCR1, 인지질 가수분해효소 C, 단백질 인산화효소 C, NF-κB, ERK, 케모카인,신호전달, 화학주성

감사의 글

인생에 있어서 무엇을 해야 저에게 의미가 있을까라고 물음을 던진날이 엊그제 같은데 벌써 박사논문을 썼다는게 믿기지 않습니다. 그동안 열심히 하고자 노력했던 많은 날들이 머릿속에 떠오릅니다. 한편으로는 부족했던 나를 생각하며 그러나 한편으로는 열심히 하려고 하며 힘들어했던 기억들이 생각납니다. 이제 공부의 시작점에 도달했다고 생각합니다. 앞으로는 지금까지 배운 것을 활용하고 또한 이것을 밑거름으로 하여 과학에 이바지할 수 있는 사람이 되고 싶습니다. 지금의 제가 있기까지 도와주신 분들이 많이 있습니다.

학문적인 조언과 깨우침을 아끼지 않으시며 석사때부터 저를 지도해주신 양용석 교수님, 과학적인 논리와 연구에 대해서 부족한 저를 일깨워 주신 나도선 교수님, 부족한 저를 항상 자상하게 가르쳐주시며 연구자의 갈 길을 보여주신 고제상 교수님께 깊은 감사를 드리며, 바쁘신 와중에서도 심사를 하시며 아낌없는 관심을 가져주신 김종배, 이혜영 교수님께 다시 한번 감사드립니다. 학부때부터 박사과정까지 늘 가르침을 주신 오옥두, 김태우, 박용석 교수님께도 감사드립니다.

주위에서 도와주신 분들이 너무 많이 있습니다. 제 옆에서 깨우쳐주고 나아갈 길을 항상 얘기해주신 김윤석, 윤혜영 선배님께 감사드립니다. 석사때부터 저에게 조언을 아끼지 않으신 조윤경, 임지애, 장재호 선배님께도 감사드립니다. 실험심에서 많을 것을 도와준 호중이, 성욱씨, 혜진씨,

이용진 선생님에게도 고맙다는 말을 전하고 싶습니다. 저희과 박사과정에서 같이 공부하던 최현일, 김성수, 심문정, 최태현, 이태섭, 김홍성, 박상욱 선배님과 근식형, 현석이, 승주 후배에게도 감사드립니다. 석사과정에서 열심히 공부하고 있는 대종이, 정혁이, 종철이, 은숙이, 명민이, 규상이, 지영이,인수도 많은 도움이 되었습니다. 석사때 같이 공부했던 선후배님과 저희과를 위해 항상 노력하시는 임병혁선생님께도 이 글을 빌어 감사드립니다. 이번에 같이 졸업하게된 엄용빈 선배님께도 축하와 감사를 보내드립니다.

제가 이 자리에 있기까지 사랑으로 밀어주신 부모님, 항상 큰 힘이 되어준 형, 형수님, 누나, 매형, 건강하고 씩씩한 조카 재현이, 준현이, 병준이에게 고마움을 전하고 싶습니다. 장인어른의 빠른 쾌유를 바라며 저를 위해 항상 염려와 걱정해주신 장모님, 처형, 동서, 처남 경훈이, 기훈이에게도 감사드립니다.

공부하느라 얘기키우느라 고생하면서도 항상 제 옆에서 큰 힘이 되어준 나의 사랑하는 아내 지숙이, 맑은 웃음으로 나를 위로해주며 기쁘게 해준 딸 서정이, 유정이에게 다시한번 고마움과 사랑한다는 말을 전하고 싶습니다.