Up-regulation of CXCR4 Expression in PC-3 Cells by Stromal-Derived Factor-1α (CXCL12) Increases Endothelial Adhesion and Transendothelial Migration: Role of MEK/ERK Signaling Pathway–Dependent NF-κB Activation

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Abstract

The chemokine stromal-derived factor-1α (SDF-1α/CXCL12) and its receptor, CXCR4, play a crucial role in adhesion and transendothelium migration (TEM) of prostate cancer cells. We tested the hypothesis that enhanced expression of CXCR4 in prostate cancer cells is dependent upon SDF-1α-mediated activation of nuclear factor-κB (NF-κB). SDF-1α increased the CXCR4 mRNA and protein expression in PC-3 cells but not in LNCaP cells. Similarly, SDF-1α enhanced the NF-κB-dependent transcriptional activity in PC-3 cells but not in LNCaP cells. SDF-1α increased PC-3 cell adhesion to the human umbilical vein endothelial cell monolayer and enhanced TEM, which was abrogated with anti-CXCR4 monoclonal antibody (mAb). Suppression of NF-κB activity in PC-3 cells by a mutant IκBα super-repressor adenoviral vector decreased the CXCR4 mRNA expression and inhibited adhesion and TEM. Transient overexpression of p65 subunit of NF-κB in PC-3 cells up-regulated CXCR4 receptor expression and increased the adhesion and TEM of these cells in response to SDF-1α gradient. Treatment of PC-3 cells with SDF-1α leads to nuclear translocation of NF-κB protein within 15 to 30 minutes, which correlated with IκBα phosphorylation. A p42/44 mitogen-activated protein kinase [MAPK, extracellular signal regulated kinase-1/2 (ERK-1/2)] biphasic activation pattern was observed in these cells at 15 minutes and 3 hours after SDF-1α treatment. Phosphorylation of IκB kinase α was observed within 30 minutes, which was blocked by PD98059 [MAPK kinase (MEK) inhibitor]. PD98059 cotreatment significantly inhibited SDF-1α-induced NF-κB reporter activity and CXCR4 receptor expression as shown by flow cytometry. These data suggest that SDF-1α-induced expression of CXCR4 in PC-3 cells is dependent on MEK/ERK signaling cascade and NF-κB activation. (Cancer Res 2005; 65(21): 9891-8)

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in American males and the incidence of prostate cancer is projected to increase significantly. In the United States, it is estimated that >232,090 men will be diagnosed with prostate cancer in 2005, and approximately >30,350 afflicted men will die of this disease (1). The symptomatic phase of prostate cancer is largely due to occurrence of metastasis to the bone, which is a complex and multistaged process. For disseminated prostate cancer cells to grow into overt metastasis in the bone, they must survive and proliferate in the vasculature or in the surrounding tissue after extravasation. To exit the vasculature, the prostate cancer cells must first adhere to the endothelium and subsequently move through the endothelial monolayer and underlying connective tissues.

It has been shown that SDF-1α (CXCL12) produced by bone marrow stromal cells and microvessel endothelial cells can facilitate the migration of hematopoietic progenitor cells (HPC) which express the chemokine receptor CXCR4 (2). CXCR4 is also found to be expressed on the surface of prostate cancer cells and may be involved in the metastasis process as well (3). In vitro adhesion assays have shown that pretreatment of prostate cancer cells with SDF-1α significantly increases their adhesion to osteosarcoma and bone marrow–derived endothelial cells (4). In this context, SDF-1α and CXCR4 seem critical molecular determinants for these events (5, 6). CXCR4 expression varies in different prostate cancer cell lines, and high levels of CXCR4 are linked to aggressive phenotypes in prostate cancer cells (7). Because SDF-1α is constitutively produced by stromal compartments in different tissues, CXCR4 expression on neoplastic cells may be important in tumor metastasis to different organs, similar to the migration potential of HPCs in different tissue compartments (8, 9). Signaling molecule associated with SDF-1α response is linked to the G-protein-coupled CXCR4 receptor, which activates the mitogen-activated protein kinases (MAPK) extracellular signal regulated kinase-1/2 (ERK-1/2), as well as protein kinase B, phosphatidylinositol 3-kinase (PI3K; refs. 10–13).

Although the convergence of signal transduction pathways following CXCR4 activation and stimulation of transcription factors in prostate cancer cells have not been delineated, the possible role of downstream activation of nuclear factor-κB (NF-κB) has been implicated from previous studies. NF-κB is constitutively active and expressed at high levels in metastatic prostate cancer cells. NF-κB is involved in the regulation of genes associated with pathologic processes like inflammation and cancer (14). Specific kinases, IκB kinase (IKK), phosphorylate the inhibitory α subunit, IκBα at serine residues 32 and 36, which is then ubiquitinated and targeted for proteolysis (15). This allows the NF-κB subunits to dimerize and translocate to the nucleus where it can regulate the prometastatic genes. Indeed, NF-κB has been shown to increase the expression of CXCR4 in MCF-7 and MDA-MB-231 breast cancer cells (16). It is also known that during fetal life and during marrow transplantation, HPCs "home" to the bone (17, 18) and SDF-1α expression in the
osteoblasts and marrow endothelial cells function as chemoattractant (19–21). It is therefore likely that metastatic prostate cancer may also use a similar pathway to localize to the bone marrow. In this study, we have shown that NF-κB activation is directly linked to up-regulation of the expression of CXCR4 in PC-3 cells. This report also delineates the signal transduction mechanism involved in SDF-1α-mediated activation of NF-κB and implicates a role of differential signaling in two different prostate cancer cell lines, the bone metastatic PC-3 and lymph node metastatic LNCaP cells, which may help elucidate the metastatic potential of certain prostate cancer cell clones.

Materials and Methods

Reagents. Recombinant human SDF-1α was obtained from Research Diagnostics, Inc. (Flanders, NJ). PD98059 was purchased from Calbiochem (La Jolla, CA) and LY-294002 was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Cycloheximide was obtained from Sigma (St. Louis, MO). Antibody to human CXCR4 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p65NF-κB, anti-IκBα, anti-IκK, anti-p-p21/44 MAPK, anti-phospho-IκK, and anti-phospho-IκBα were purchased from Cell Signaling Technology. Calcein AM dye was obtained from Molecular Probes (Eugene, OR). The reverse transcription-PCR (RT-PCR) primers for MAPK, anti-phospho-IκK, and anti-phospho-IκBα were purchased from Clonetics (BioWhittaker, Inc., Walkersville, MD) and were obtained from Clontech (Palo Alto, CA) and Cell Signaling Technology (La Jolla, CA) and LY-294002 was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Cycloheximide was obtained from Sigma (St. Louis, MO).

Cell culture. PC-3 and LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells were grown in Ham's F-12 and LNCaP cells were maintained in RPMI 1640. Media were washed in PBS and resuspended in 500 μL/mL for 30 minutes at 37°C. Cells were washed with RPMI and resuspended (250 μL) was determined using a Fx-800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT), with wavelengths set at absorbance maximum of 494 nm and emission maximum of 517 nm. The fluorescent-labeled prostate cancer cells were used in adhesion and TEM experiments with the HUVECs.

Adhesion assay. The adhesion of prostate cancer cells to HUVECs was directly linked to up-regulation of the expression of CXCR4 in PC-3 cells. The report also delineates the signal transduction mechanism involved in SDF-1α-mediated activation of NF-κB and implicates a role of differential signaling in two different prostate cancer cell lines, the bone metastatic PC-3 and lymph node metastatic LNCaP cells, which may help elucidate the metastatic potential of certain prostate cancer cell clones.

Fluorescent labeling of prostate cancer cells. The transient fluorescent labeling of prostate cancer cells with calcein AM was done by using the Vybrant cell adhesion assay protocol (Molecular Probes). Briefly, prostate cancer cells were washed with PBS and resuspended in RPMI without serum at 5 × 10⁵ cells/mL. Cells were incubated with the calcein AM solution (5 μM/mL) for 30 minutes at 37°C. Cells were washed with RPMI and fluorescence (250 μL) was determined using a Fx-800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT), with wavelengths set at absorbance maximum of 494 nm and emission maximum of 517 nm. The fluorescent-labeled prostate cancer cells were used in adhesion and TEM experiments with the HUVECs.

Transfections. Cells were transiently transfected with pCMV, p50, or pCMV-CXCR4 using electroporation technique (Gene Pulser, Bio-Rad). At 48 hours after transfection, RNA was isolated for RT-PCR and flow cytometric was carried for cell surface CXCR4 expression. NF-κB activity was assessed by cytokine-activated EPα-Luc and pCMV β-galactosidase. The luciferase activity was normalized to the β-galactosidase activity. Adenoviral transduction, cells were incubated for 24 hours with either an adenovirus expression construct or an adenovirus negative control and monitored in two-cell coculture model in Petri plates. The HUVECs were treated with adenovirus at a multiplicity of infection of 200 and the plates containing adhered prostate cancer cells were quantified by DQ-Quik staining. Prostate cancer cells were added to the upper chamber and transmigration towards an SDF-1α gradient in the lower chamber was monitored using calcein-labeled prostate cancer cells. In some experiments, prostate cancer cells were pretreated with SDF-1α (100 ng/mL) for 6 hours or transfected with NF-κB expression plasmids.

Reverse transcription-PCR. Total RNAs were isolated using the Trizol method (Life Technologies, Carlsbad, CA). PCR for CXCR4 was done using an assay kit from Promega (Madison, WI), and a thermal cyclor from Perkin Elmer (Shelton, CT; model 9600). Total RNAs (~2 μg) were used in each reverse transcription reaction mixture (50 μL), containing MMLV-RT (0.5 unit), RNase inhibitor (0.1 unit), oligo-dT (1 μg), and deoxyribonucleotides (deoxynucleotide triphosphates, 1 mmol/L each), and incubated for 30 to 60 minutes at 42°C. The PCR amplifications were carried out at the conditions specified for each primer pair, by using Taq DNA polymerase (0.5 unit/100 μL). The cycling conditions of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 30 cycles followed by a 10-minute extension at 72°C were used for CXCR4. The PCR products were viewed by electrophoresis on a 2.0% agarose gel containing ethidium bromide, along with a DNA molecular weight marker (Boehringer Mannheim, Mannheim, Germany). Band intensities were monitored by a Bio-Rad (Hercules, CA) UV Gel Documentation system, and a semiquantitative analysis was done by comparing relative band intensities of GAPDH.

Western immunoblot. For Western blot analysis, cells were starved in serum-free Ham's F-12 for 2 hours and stimulated with 100 ng/mL SDF-1α for the indicated period of time at 37°C. The treated cells were lysed in 200 μL of lysis buffer and fractionated by a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane by electrophoresis for 1 hour. After soaking in blocking buffer (1× TBS, 0.1% Tween 20, and 5% casein), membranes were incubated with primary antibody overnight at 4°C. Blots were developed using horseradish peroxidase–linked secondary antibody and a chemiluminescent detection system (LumiGLO, KPL, Gaithersburg, MD).

Flow cytometric analysis. To monitor the CXCR4 protein levels, PC-3 and LNCaP cells were fixed in 4% formaldehyde diluted in PBS and permeabilized using 0.1% Triton X-100 in PBS. FITC-conjugated anti-CXCR4 antibody (1:400) was added and incubated at 4°C for 1 hour. Cells were washed in PBS and resuspended in 500 μL PBS before analysis using a Beckman Coulter FACS-Calibur (Miami, FL). A total of 10,000 events were analyzed.

Transient transfection and adenoviral transduction. Cells were transfected with pCMV, p50, or pCMV-CXCR4 using electroporation technique (Gene Pulser, Bio-Rad). At 48 hours after transfection, RNA was isolated for RT-PCR and flow cytometric was carried for cell surface CXCR4 expression. NF-κB activity was assessed by cotransfecting pNFκB-Luc and pCMV β-galactosidase. The luciferase activity was normalized to the β-galactosidase activity. Adenoviral transduction, cells were incubated for 24 hours with either an adenovirus expression vector for the dominant-negative IκB super-repressor (pAxCAmIκB-M) or the wild-type (pAxCAmIκBα) construct (multiplicity of infection = 100). Transduction efficiency was monitored by an Adv-β-galactosidase reporter construct followed by in situ staining of the percentage of blue cells.

Statistical analysis. All experiments were done in duplicate or triplicate cultures. The results obtained from at least three independent experiments are expressed as ±SE. Statistical analysis was done using the Statview software. The two-tailed Student's t test was used to determine whether the samples are representative of a known experimental condition, and one-way ANOVA was used to compare among three or more groups. P < 0.05 was considered significant.

Results

Stromal-derived factor-1α stimulation induced CXCR4 expression in PC-3 cells. We monitored the levels of CXCR4 transcripts in PC-3 and LNCaP cells with or without SDF-1α stimulation by semiquantitative RT-PCR assays. The data showed...
that PC-3 cells constitutively expressed 2- to 3-fold higher levels of CXCR4 mRNA compared with LNCaP cells (Fig. 1A). Within 6 hours following SDF-1α treatment, CXCR4 transcript levels increased by 4- to 6-fold in PC-3 cells but not in LNCaP cells. The enhanced CXCR4 gene expression returned to basal level within 24 hours after stimulation. However, even at this time point, a higher level of CXCR4 expression was observed in PC-3 cells compared with LNCaP cells. These findings suggested that increased CXCR4 expression in PC-3 cells, both constitutive and inducible, may play a direct role in the enhanced levels of CXCR4 expression observed in PC-3 cells. The protein expression of CXCR4 receptor was monitored using flow cytometry (Fig. 1B). Similar to the RT-PCR data, PC-3 cells expressed a higher (6- to 8-fold) constitutive level of CXCR4 protein compared with LNCaP cells. Exposure to SDF-1α for 12 hours showed a 4- to 6-fold increase in CXCR4 protein levels in PC-3 cells compared with untreated cells, whereas in LNCaP cells, there was no significant change in CXCR4 expression.

**Stromal-derived factor-1α–induced CXCR4 expression increases human umbilical vein endothelial cells adhesion and transendothelial migration of PC-3 cells.** For the adhesion studies, PC-3 and LNCaP cells were treated with increasing concentrations of SDF-1α for 6 hours and cocultured with HUVECs grown in 24-well culture plates for 30 minutes. Non-adherent prostate cancer cells were removed and percent change in prostate cancer cell adhesion was monitored by fluorescent dye analysis to determine the effects of SDF-1α on adhesion of prostate cancer cells to HUVECs. We observed a concentration-dependent inductive effect of SDF-1α (0-200 ng/mL) upon exposure of PC-3 cells but not of LNCaP cells (Fig. 1C). Even in the absence of stimulation, PC-3 cells were more adherent to HUVECs compared with LNCaP cells. Exposure to SDF-1α showed a significant (P < 0.05) increase (175-200%) in adhesion of PC-3 cells; however, adhesion of LNCaP cells was not significantly changed following their SDF-1α stimulation. The SDF-1α-induced (100 ng/mL) adhesion could be significantly blocked by coincubation of PC-3 cells with mAb to CXCR4. A slight decrease in basal level of PC-3 adhesion was also observed in the presence of antibody to CXCR4.

We monitored the effects of SDF-1α-induced CXCR4 expression in PC-3 cell TEM via a confluent HUVEC monolayer (Fig. 1D). The unstimulated or SDF-1α prestimulated (6 hours) PC-3 cells were fluorescent labeled and cultured with HUVEC in transwell culture plates, and percent change in TEM were monitored after 6 hours of coincubation. To observe the transmigratory potential of a chemokine gradient, SDF-1α was added to the lower chamber. The SDF-1α-stimulated cells showed a slight increase in TEM. Increase in the percentage of unstimulated PC-3 cells transmigrating towards an SDF-1α concentration gradient was also observed. However, the combined exposure of PC-3 cells to SDF-1α and presence of the chemokine in the lower chamber significantly increased TEM. Interestingly, a proportional increase in transmigration was not observed when SDF-1α was present in the upper chamber of the transwell. Furthermore, the increase in TEM was suppressed upon incubation of the coculture with CXCR4 antibodies added to the upper chamber.

**Suppression of nuclear factor-κB activity decreased both CXCR4 expression and endothelial adhesion of PC-3 cells.** NF-κB activity is closely related to regulation of critical genes involved in cancer metastases (22). Overexpression of the transdominant-negative mutant subunit of the inhibitory component of NF-κB (IκBα) suppresses nuclear translocation of the DNA-binding subunits of NF-κB and abrogates NF-κB-mediated signaling in different cell types (23). To monitor the role of NF-κB in CXCR4 expression in prostate cancer cells, we introduced adenoviral vectors coding for either the wild type
or the trans-dominant-negative mL-Bo into prostate cancer cells. We then monitored CXCR4 gene expression by RT-PCR (Fig. 2A) and change in endothelial adhesion of the prostate cancer cells (Fig. 2B). The introduction of trans-dominant-negative mutant of l-Bo suppressed (2- to 3-fold) CXCR4 gene expression in PC-3 cells but not in LNCaP cells (Fig. 2A). Furthermore, a significant decrease (30-40%) in endothelial adhesion of PC-3 cells expressing the trans-dominant-negative mutant was also observed (Fig. 2B), whereas mL-Bo did not significantly affect endothelial adhesion of LNCaP cells. These findings suggested a direct role of NF-κB signaling pathway in regulating CXCR4 expression specifically in the bone-metastasized PC-3 cells but not in the lymph node-metastasized LNCaP cells.

**Overexpression of nuclear factor-κB (p65 subunit) increases both CXCR4 expression and adhesion of PC-3 cells.**

The transcriptional activation of NF-κB occurs via heterodimerization of its subunits, p50 and p65 (24). Because our study suggested a crucial role of the NF-κB pathway in CXCR4 expression in PC-3 cells, we further investigated whether one or both of the NF-κB subunits are important in this process. PC-3 cells were transfected with either control vector or the p65 or p50 expression vectors. As positive controls, parallel groups were also transfected with the CXCR4 expression vector. CXCR4 mRNA expression was monitored in the transfected cells by RT-PCR (Fig. 2C). The RT-PCR data showed that overexpression of the p65 (Rel-A) subunit of NF-κB significantly up-regulated (2- to 3-fold) CXCR4 gene expression in PC-3 cells. Flow cytometric analysis of cell surface CXCR4 expression in PC-3 cells also showed increased levels of CXCR4 protein in p65-transfected cells (data not shown). Thus, p65 overexpression enhanced CXCR4 expression in PC-3 cells. However, introduction of the p50 subunit of NF-κB did not significantly affect CXCR4 expression. The transfected PC-3 cells were also used for adhesion assays (Fig. 2D). The PC-3 cells transfected with either the CXCR4 or the NF-κB p65 expression plasmids showed a significant increase (160-175%) in adhesion to HUVECs. In contrast, no detectable percent change in adhesion was observed following introduction of the p50 subunit. These findings suggested that increase in NF-κB p65-mediated signaling in PC-3 cells enhanced CXCR4 levels, which may be directly linked to their increased adhesive properties towards HUVECs.

**Stromal-derived factor-1α stimulation regulates temporal activation of nuclear factor-κB in PC-3 cells.** We monitored the SDF-1α-induced NF-κB activation in PC-3 cells by Western immunodetection of p65 and I-Bo and by transient transfection assays using an NF-κB reporter plasmid (Fig. 3). SDF-1α stimulation increased nuclear levels of NF-κB p65 in a biphasic manner (Fig. 3A). The p65 subunit increased within 15 to 30 minutes following SDF-1α stimulation, which was followed by a decrease to basal levels within 2 hours and a second phase of up-regulation at 3 to 4 hours following stimulation (Fig. 3A).

To determine SDF-1α-induced activation of NF-κB, kinetic analysis of total I-Bo and phospho-I-Bo was conducted by Western blot analysis (Fig. 3B). Activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of I-Bo. Phospho-I-Bo levels increased by 4-fold at 30 minutes after SDF-1α stimulation, with maximal increase observed between 2 and 3 hours, suggesting that the activation occurred within minutes following addition of the ligand to the culture. A simultaneous decrease in total I-Bo levels was also observed within 2 hours. SDF-1α-induced I-Bo activation correlated with activation of NF-κB as early as 15 minutes (Fig. 3B).

We also examined the effect of SDF-1α (100 ng/mL) treatment of PC-3 cells at different time intervals on NF-κB-dependent transcriptional activity (Fig. 3C). PC-3 cells were transfected with NF-κB luciferase reporter construct and treated with SDF-1α for 1, 4, 6, and 18 hours before harvesting. The normalized relative luciferase activity values showed that SDF-1α treatment significantly increased (8- to 10-fold) NF-κB activity within 4 hours (Fig. 3C). The relative fold induction in NF-κB activity showed a
activity was normalized to treated with SDF-1.

Columns, set at 1.0, and are an average of duplicate experiments of the same clone. Temporal effect of SDF-1.

**Suppression of mitogen-activated protein kinase/extracellular signal-regulated kinase signaling decreased stromal-derived factor-1α–induced CXCR4 expression and transendothelial migration of PC-3 cells.** Flow cytometric analysis showed that SDF-1α-induced CXCR4 protein expression was significantly inhibited by cotreatment of PC3 cells with PD98059 but not LY-294002 (Fig. 5A). SDF-1α-induced CXCR4 protein expression (3.32 ± 2.8) was significantly enhanced compared with untreated group (8.2 ± 3.6). PD98059 cotreatment suppressed the CXCR4 expression (18.6 ± 2.2) in PC-3 cells; in contrast, LY-294002 (PI3K inhibitor) cotreatment did not change the CXCR4 expression (Fig. 5A). We also monitored TEM of SDF-1α-treated PC-3 cells in the presence and absence of PD98059 in response to SDF-1α (10 ng/mL). SDF-1α pretreatment for 6 hours

peak level at 3 to 4 hours which returned to basal levels within 18 hours following SDF-1α stimulation.

Stromal-derived factor-1α–induced nuclear factor-κB activation is dependent on IkB kinase phosphorylation via mitogen-activated protein kinase/extracellular signal-regulated kinase signaling cascade. NF-κB activation requires activation of IKK via its phosphorylation (P-IKK). SDF-1α-induced P-IKK-α levels within 10 to 15 minutes (data not shown) and reached peak at 30 minutes following treatment of PC-3 cells with SDF-1α (Fig. 4A). Preincubation of PC-3 cells with a specific inhibitor of MAPK kinase (MEK), PD-98059 (50 μmol/L), abrogated SDF-1α-induced IKK-α phosphorylation (Fig. 4A). The IKK-α phosphorylation and NF-κB activation may also occur via the MAPK pathway. A similar but more rapid biphasic effect of SDF-1α on MAPK-1/2 (ERK-1/2) levels was indeed observed (Fig. 4B). Western analysis of the MAPK p42 and p44 levels in SDF-1α-treated PC-3 cells showed an increase within 15 minutes, which waned to basal levels within 60 minutes and were up-regulated again 2 to 3 hours following stimulation with SDF-1α. In PC-3 cells transfected with NF-κB-luc, SDF-1α-induced reporter activation was also blocked by PD98059 (50 μmol/L), which suggested that early activation of NF-κB by SDF-1α is dependent on the MEK/ERK pathway (Fig. 4C).

Relative fold change was based on densitometric analysis of bands, with vehicle-treated control samples being set at 1.0, and are an average of duplicate experiments of the same clone. Temporal effect of SDF-1α

**Figure 3.** A. SDF-1α-induced NF-κB nuclear translocation in PC-3 cells. PC-3 cells were serum starved for 12 hours and incubated with or without SDF-1α for the indicated periods of time. Whole cell and nuclear extracts were then prepared and subjected to Western blot analysis using p65NF-κB antibodies. B, additional aliquots of the same whole cell extracts were subjected to anti-IkBα Western blot analysis. Numbers represent fold change based on densitometric analysis of bands, with vehicle-treated control samples being set at 1.0, and are an average of duplicate experiments of the same clone. C, temporal effect of SDF-1α on NF-κB transcriptional activity in PC-3 cells. PC-3 cells were cotransfected with NF-κB-luc and pFR-luc constructs and treated with SDF-1α (100 ng/mL) for 1, 4, 6, and 18 hours. Relative luciferase activity was normalized to β-galactosidase activity to correct for variability in transfection efficiency. The results were analysed as relative fold induction. Columns, mean of triplicate experiments; bars, ± SE. **, P < 0.01 [SDF-1α (4 hours) compared with untreated group].

**Figure 4.** A. Effect of SDF-1α on IKKα protein levels and its phosphorylation in PC-3 cells. Whole cell extracts were prepared from SDF-1α-treated PC-3 cells for the indicated period of time. IKKα phosphorylation was then analysed by anti-phospho-IKKα (pIKKα) Western blot. The effect of PD98059 on SDF-1α-induced IKKα phosphorylation was also monitored. B, PC-3 cells were preincubated with PD98059 (50 μmol/L) for 1 hour and treated with SDF-1α for 30 minutes. Kinetic analysis of SDF-1α-induced ERK-1/2 (p42/p44 MAPK) expression. Aliquots from same whole cell extracts were prepared and subjected to anti-p42/p44 MAPK Western blotting. Numbers represent fold change based on densitometric analysis of bands, with vehicle-treated control samples being set at 1.0, and are an average of duplicate experiments of the same clone. C, PD98059 cotreatment suppressed SDF-1α–induced CXCR4 expression and transendothelial migration in PC-3 cells. Whole cell extracts were prepared from SDF-1α–stimulated PC-3 cells expressing CXCR4 and treated with PD98059 (50 μmol/L) for 4 hours in the presence or absence of PD98059 (50 μmol/L). Relative luciferase activity was normalized to β-galactosidase activity to correct for variability in transfection efficiency. Columns, mean of three independent experiments; bars, ± SE. **, P < 0.01 [SDF-1α compared with SDF-1α + PD98059].
The semiquantitative RT-PCR assay and treatment of PC-3 cells but not of LNCaP cells in increasing their via activation of NF-κB.

Increased TEM of the PC-3 cells (60-80%), which was completely blocked by PD98059 cotreatment (Fig. 5B). However, PD98059 treatment alone in the absence of SDF-1α did not affect TEM of PC-3 cells.

Discussion

Recent evidence suggests that SDF-1/CXCR4 axis plays a pivotal role in triggering bone metastasis. The chemotactic effect of SDF-1α is known to enhance the TEM of both tumor cells and hematopoietic cells (25). The local release of SDF-1α from bone marrow endothelial cells as well as a concentration gradient generated via their production by the marrow stromal cells may act in conjunction to enhance the TEM of prostate cancer cells. The chemokine, SDF-1α is constitutively produced by endothelial cells, fibroblasts, and osteoblasts in the bone marrow (26) and may play an important role in bone metastasis of prostate cancer cells. The current study shows that there is a significant increase in PC-3 cell adhesion to HUVECs following stimulation with SDF-1α. Two different phenotypes of prostate cancer cells, the bone metastasis--derived PC-3 cells and lymph node metastatic LNCaP cells were investigated to delineate the molecular mechanisms that may be involved in their ability to metastasize to the bone. The findings implicate a crucial role of the CXCR4/SDF-1α signaling in both recruitment and TEM of PC-3 cells towards the underlying marrow stroma and suggest a preferential adhesion of PC-3 cells to the endothelial cells and their enhanced transmigration in response to the chemokine gradient.

SDF-1α has been shown to induce NF-κB activation in primary astrocytes and Kaposi's sarcoma spindle cells (27). We hypothesized that SDF-1α-induced CXCR4 gene up-regulation is mediated via activation of NF-κB in bone metastatic PC-3 cells. We observed that there is a concentration-dependent effect of SDF-1α treatment of PC-3 cells but not of LNCaP cells in increasing their adhesion to HUVECs. The semiquantitative RT-PCR assay and flow cytometric data clearly showed that PC-3 cells constitutively express CXCR4. Our observation that within 6 hours following SDF-1α treatment there was a 4- to 6-fold increase in CXCR4 message in PC-3 cells but not in the LNCaP cells suggest that a specific signaling in bone metastatic prostate cancer cells may lead to their enhanced CXCR4 expression showing responsiveness to the chemokine.

SDF-1α and CXCR4 are overexpressed in several neoplasms that metastasize to the bone marrow, such as breast cancer, neuroblastoma, and several leukemias (28, 29). The transcription factors that regulate SDF-1α and CXCR4 expression in prostate cancer cells are yet to be confirmed. NF-κB has been shown to regulate several prometastatic and antimetastatic factors (30). In this study, we explored whether NF-κB has a direct role in regulation of CXCR4 expression in two different prostate cancer cell lines. The present data suggest that overexpression of super-repressor IκBα mutant suppressed CXCR4 gene expression in PC-3 cells but not in LNCaP cells. This was also supported by the findings that transient transfection of NF-κB expression plasmid (p65 subunit) up-regulated CXCR4 expression in PC-3 cells. Our data clearly show that SDF-1α enhances transcriptional activity of NF-κB in an IκBα-dependent manner, which in turn would up-regulate expression of CXCR4, an effect that was reversed by a dominant-negative mutant IκBα. These data clearly suggest that suppression of NF-κB results in inhibition of SDF-1α-stimulated expression of CXCR4.

The differential response to SDF-1α treatment may be attributed to constitutively active NF-κB in PC-3 cells. It is reported that constitutive activation of NF-κB is inversely related to the androgen receptor status of the cells (31). Prominent constitutive NF-κB activation was observed in prostate cancer cell lines lacking androgen receptor expression (PC-3), whereas only very low levels of NF-κB activity was seen in androgen responsive LNCaP cell line (32). The detailed mechanisms responsible for constitutive NF-κB activation in prostate cancer cells remain unclear, but some studies have implicated constitutive activation of IKK complex as a possible mechanism (33). Thus, the half-life of IκBα protein is greatly reduced in prostate cancer cells with constitutive NF-κB activity compared with the cells with basal activity. Thus, despite the expression of CXCR4 receptor in androgen responsive LNCaP cells, the resistance to SDF-1α treatment is most likely associated with suppression of NF-κB activity in this cell line.

We have explored the regulatory mechanism of NF-κB activation in response to SDF-1α in bone metastatic PC-3 cells. Our data have shown that expression of p65 NF-κB increased within 15 to 30 minutes following SDF-1α treatment. Interestingly, p65 expression showed a biphasic pattern, initial up-regulation decreased to basal level within 2 hours, and the second phase of up-regulation was observed at 3 to 4 hours. The activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of IκBα (34). Comparison of the levels of total and phosphorylated forms of IκBα in extracts from SDF-1α-treated PC-3 cells showed that total IκBα was reduced after 1 hour of SDF-1α stimulation with maximal decay between 2 and 3 hours. At the same time, the levels of phospho-IκBα were found to be maximal at 2 to 3 hours of SDF-1α stimulation. These results suggest that SDF-1α-induced NF-κB expression correlated with degradation of total IκBα and induction of phospho-IκBα. However, one of the key target genes regulated by NF-κB is its inhibitor IκBα. A feedback inhibition pathway for control of IκBα
gene transcription and down-regulation of transient activation of NF-κB activity has been described (35–37). Additionally, it has been shown that proinflammatory cytokines induce biphasic NF-κB activity, consisting of a transient phase mediated through IκBα followed by a persistent phase mediated through IκBβ (38). Hoffmann et al. have shown that IκBβ/NF-κB signaling has biphasic characteristics: IκBα mediates rapid NF-κB activation and strong negative feedback regulation, resulting in an oscillatory NF-κB activation profile, whereas IκBβ and IκBε respond more slowly to IKK activation and act to dampen the long-term oscillations of the NF-κB response (39). Based on these observations, it is possible that the bimodal induction of NF-κB activation by SDF-1α in PC-3 cells is related to transient inhibition caused by IκBα. Alternatively, it remains to be seen whether the second phase of NF-κB activation by NF-SDF-1α is associated with IκBβ and/or IκBε phosphorylation. Thus, the possibility of involvement of another component in SDF-1α or NF-κB signaling for CXCR4 activation in PC-3 cells certainly warrants further investigation.

ERK-1/2 MAPK mediates signals for many growth factors and G-protein-coupled receptors. Activation of ERK-1/2 MAPK by G-protein-coupled receptors occurs via Raf/MEK-1/2/ERK-1/2 cascade, which can be blocked by a specific chemical inhibitor, PD98059 (40, 41). Consistent with our results, SDF-1α activates ERK-1/2 MAPK in several cell types (42), including epitheloid (43) and ovarian (44) carcinoma cells. However, we observed a biphasic activation of MAPK levels in PC-3 cells; the p42/p44 MAPK levels showed an increase in expression within 15 minutes, which waned to basal levels within 60 minutes and were up-regulated again at 2 hours following SDF-1α stimulation. We have shown that SDF-1α stimulation of PC-3 cells induced IKKα activation by up-regulating the phosphorylated form of IKKα. However, unlike in hematopoietic progenitor cells (45), early SDF-1α activation of ERK-1/2 could be responsible for activation of NF-κB in PC-3 cells. Activation of IKKα was found to be sensitive to a pharmacologic inhibitor, PD98059, a specific inhibitor of MEK-1 and MEK-2 phosphorylation. Additionally, we also show that PD98059 effectively blocked SDF-1α-induced NF-κB activation by reporter assays. Accordingly, our data suggest that IKKβ/NF-κB activation is a downstream target to MEK-1/2 induced signaling by SDF-1α in PC-3 cells.

The role of SDF-1α-induced MEK/ERK signaling was investigated by monitoring CXCR4 expression. Our data showed that SDF-1α-induced CXCR4 protein expression was significantly suppressed by PD98059 and that NF-κB p65 subunit regulates CXCR4 gene expression in PC-3 cells. The functional role of CXCR4 receptor in adhesion to endothelial cells and TEM of PC-3 cells further attests to its vital role in bone metastasis. These data provide evidence of increased metastatic potential of osteotropic PC-3 cells by enhancing expression of CXCR4 upon exposure to SDF-1α in a MEK/ERK/NF-κB-dependent manner. In summary, we have shown that following SDF-1α treatment of PC-3 cells, MEK activation occurs within 15 minutes leading to IKK and IκBα phosphorylation. This leads to nuclear localization of NF-κB followed by transcription and expression of CXCR4. These findings suggest novel targets for therapeutic intervention to suppress bone metastasis of prostate cancer cells.

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Up-regulation of CXCR4 Expression in PC-3 Cells by Stromal-Derived Factor-1 α (CXCL12) Increases Endothelial Adhesion and Transendothelial Migration: Role of MEK/ERK Signaling Pathway –Dependent NF-κB Activation

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