Involvement of the Chemokine Receptor CXCR4 and Its Ligand Stromal Cell-Derived Factor 1α in Breast Cancer Cell Migration Through Human Brain Microvascular Endothelial Cells

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Abstract

In this study, we have characterized the signaling pathways mediated by CXCR4 in breast cancer cells and its role in breast cancer cell invasion and migration. Stromal cell-derived factor 1α (SDF-1α; CXCL12) stimulation of breast cancer cells resulted in phosphoinositide 3-kinase (PI-3K) activation, AKT phosphorylation, and activation of the FKHR1 transcription factor. In addition, SDF-1α induced activation of the focal adhesion kinase (FAK) as well as the migration of breast cancer cells. Expression of SDF-1α, the ligand of CXCR4, was about 2-fold higher in microdissected human breast epithelial cancer cells as compared with normal epithelial cells. Immunohistochemical analysis indicated that SDF-1α expression is consistently higher in primary breast tumor cells than in normal breast epithelial cells. Furthermore, SDF-1α induced blood vessel instability, through increased vascular permeability, resulting in the penetration of breast tumor cells through the human brain microvascular endothelial cells (HBMEC). Notably, the migration of breast cancer cells was inhibited by the PI-3K inhibitor, Wortmannin, and the Ca2+ inhibitor BAPTA/AM, indicating that transendothelial breast cancer cell migration induced by SDF-1α is mediated by activation of the PI-3K/AKT pathway and Ca2+-mediated signaling. Blockade of the CXCR4/SDF1 signaling pathway with anti-CXCR4 antibody also decreased transendothelial breast cancer cell migration as well as vascular permeability. This study focuses on novel interactions between highly relevant signaling pathways in breast cancer cells and brain microvascular endothelial cells and may provide insights into the molecular mechanisms of CXCR4/SDF-1α-mediated breast cancer metastasis to the brain.

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Introduction

Chemokines, which are a group of low molecular weight proteins, mediate several cellular functions. They play an important role in the regulation of hematopoiesis, leukocyte maturation, angiogenesis, trafficking, and in the homing of T and B lymphocytes as well as the development of lymphoid tissue (1-6).

On the basis of the arrangement of their first two of four conserved cysteine residues, the chemokine superfamily has been divided into four subfamilies: α (C-X-C), β (C-C), γ (C), and δ (C-X-X-C; refs. 1, 2, 6). Stromal cell-derived factor 1α (SDF-1α) is a member of the CXC or α-chemokine subfamily and is the only known ligand for the chemokine receptor CXCR4 (7-9). SDF1 is a highly conserved gene localized to chromosome 10q11.1 (10), and its mRNA is predominant in all tissues (10, 11). SDF1 was originally described as pre-B-cell growth-stimulation factor and is implicated in lymphocyte maturation (12). Human SDF1 is chemotactic for T lymphocytes, monocytes, and neutrophils (5); however, its expression is not restricted to the leukocyte lineage. High levels of SDF-1α expression were also found in the human pancreas, spleen, ovary, and small intestine, and lower expression levels were associated with the brain, colon, and placenta (10-13). Mice lacking the SDF1 gene die perinatally. These mice have severely impaired lymphopoiesis and abnormally low numbers of B-cell and myeloid bone-marrow precursors as well as a defective ventricular septum of the heart and defects in the central nervous system (14). These abnormalities suggest that SDF1 may play a role in diverse cellular aspects during morphogenesis and development.

SDF-1α interacts specifically with the seven-transmembrane G protein-coupled receptor, CXCR4 (15, 16). Expression of CXCR4 was observed in T lymphocytes, monocytes, and neutrophils (11), which mediates the chemotactic response to SDF1 by these cells. Expression of CXCR4 was also found in human neurons, cultured rodent neurons, glial cells (17, 18), microglial cells (19), and endothelial cells (20, 21). CXCR4 expression was observed to be up-regulated in glioblastomas (22, 23). In addition, CXCR4 plays a role in AIDS and is the cofactor necessary for CD4-mediated infection of T cells by HIV (8, 24-27). CXCR4/SDF1 interactions have been implicated in modulation of the immune response by inducing the
macrophage-mediated apoptosis of CD8 T cells (26). Interestingly, as observed in SDF1-deficient mice, mice lacking CXCR4 also exhibited hematopoietic, cardiac, and cerebellar defects (14, 28).

The expression of chemokine receptors, such as CXCR4 and CCR7, is tightly correlated with the metastatic properties of breast cancer cells (29). This study by Müller et al. showed that the level of CXCR4 is higher in malignant breast tumors than in their normal healthy counterparts, suggesting that its expression level correlates with increased metastasis-associated mortality. In vivo, neutralizing the interaction of CXCR4/SDF1 significantly impairs the metastasis of breast cancer cells and cell migration (29). Furthermore, Kato et al. (30) have shown that the expression of CXCR4 in surgically resected invasive ductal carcinomas (n = 79) is significantly correlated with the degree of lymph node metastasis. Another study has also described that breast cancer cells metastasized to the lungs express very high levels of CXCR4 as compared with the parental cells, suggesting the pivotal role of CXCR4 in breast cancer cell metastasis (31). These results are further substantiated by the fact that CXCR4 is one of the few genes that is up-regulated in bone-metastasized breast cancer cells (32). Consistent with these studies, knockdown of endogenous CXCR4 gene expression in breast cancer cells resulted in significant inhibition of breast cancer cell migration in vitro (33). In spite of its implication in cancer cell invasion and metastasis, little is known about CXCR4/SDF1 signaling events in breast cancer cells. Here, we demonstrate that the interaction of CXCR4 with its ligand, SDF-1, induced activation of the phosphoinositide 3-kinase (PI-3K)/AKT signaling pathway and the phosphorylations of focal adhesion kinase (FAK) and FKHR1 in breast cancer cells. Our results indicate that activation of the PI-3K/AKT signaling pathway by CXCR4/SDF1 is implicated in breast cancer cell migration through the human brain microvascular endothelial cells (HBMEC). In addition, we provide evidence for the differential expression of SDF-1 in normal and malignant breast tumor tissues. We propose here a molecular link between the CXCR4-mediated signaling pathway and breast cancer cell metastasis.

Results

Expression of CXCR4 in Breast Cancer Cells

To investigate the signaling pathways mediated by the CXCR4/SDF1 receptor ligand in breast cancer, we examined two breast cancer cell lines, MDA-MB-231 and DU4475, which are well characterized in terms of their metastatic potential and properties. MDA-MB-231 cells are invasive, and metastasize to lung from the primary mammary fat pad tumors of nude mice (34). The DU4475 cells were derived from a 70-year-old female patient with advanced breast cancer and are tumorigenic (35). Because the ability of these breast cancer cells to migrate in response to SDF1 suggested the functionality of the CXCR4 cell surface receptors, we, therefore, measured the cell-surface expression of CXCR4 on the MDA-MB-231 and DU4475 cells. DU4475 cells expressed significantly higher levels of CXCR4 (65%), as compared with the MDA-MB-231 cells (15%) (Fig. 1A). To determine whether CXCR4 was functional, we did a chemotactic assay. As shown in Fig. 1B, both the MDA-MB-231 and DU4475 cells migrated across the transwell membrane coated with fibronectin.

Activation of the PI-3K/AKT Pathway by SDF-1α

Previous studies showed that SDF-1α induced the activation of PI-3K in lymphocytes (36). Thus, we chose to analyze whether SDF-1α stimulates PI-3K in breast cancer cells. PI-3K activity was increased by SDF-1α treatment both in DU4475 and MDA-MB-231 cells (Fig. 2A). In the DU4475 cells, maximum activity was reached around 10 minutes of stimulation, and decreased by 30 minutes. Activation of PI-3K in the MDA-MB-231 cells was delayed as compared with the DU4475 cells. The maximum PI-3K activity in the MDA-MB-231 cells was observed 15 minutes after SDF-1α treatment, and declined by 30 minutes. AKT is a downstream target of PI-3K and is implicated in a variety of cellular functions, such as survival, transcription, and translation. Recent studies show evidence that AKT is also associated with extracellular matrix invasion by regulating matrix metalloproteinase 9 (MMP9; refs. 37, 38). Therefore, we examined whether SDF-1α activates AKT in breast cancer cells. The phosphorylation of AKT was monitored by immunoblotting with anti-phospho-AKT antibody. MDA-MB-231 cells, the phosphorylation of AKT was detected within 5 minutes of stimulation with SDF-1α, and decreased progressively by 30 minutes (Fig. 2B, left panel). In contrast, AKT was constitutively phosphorylated in DU4475 cells, although 5 minutes incubation with SDF-1α showed a modest increase in phospho-AKT activity (Fig. 2B, right panel). The level of PTEN is correlated with the constitutive activation of AKT (39). Therefore, we measured the level of PTEN by Western blot analysis in MDA-MB-231 and DU4475 cells, before and after SDF-1α stimulation. The level of PTEN was not significantly affected by the SDF-1α treatment (data not shown), suggesting that the activation of AKT in the MDA-MB-231 and DU4475 cells does not correlate with the level of PTEN.

Activation of FKHR1 by SDF-1α

FKHR1, which is regulated by the PI-3K pathway, is a direct target of AKT phosphorylation and is involved in the regulation of cell cycle progression and cell death (40). In MDA-MB-231 cells, the phosphorylation of FKHR1 was increased after treatment with SDF-1α. Its phosphorylation reached a maximum level by 10 minutes (Fig. 3A), and then gradually declined to the basal level by 30 minutes. In contrast, DU4475 cells showed a constitutive phosphorylation of FKHR1, which was unchanged in the presence of SDF-1α. One of the major target genes of FKHR1 is the Fas ligand, which is a mediator of cell apoptosis. On growth factor withdrawal, FKHR1 is unphosphorylated and translocates to the nucleus. Within the nucleus, FKHR1 activates its target genes, such as FasL. To examine whether SDF-1α stimulation regulates FasL expression in breast cancer cells, we transfected into MDA-MB-231 cells the FHRE-LUC reporter gene in which three canonical FHREs were inserted into the 5’ of a base promoter controlling the expression of the luciferase gene (40). Although we found that SDF-1α suppressed FasL promoter...
activity at the 4 and 48 hour time points (black bars) as compared with the untreated cells (white bars), its effect was minimal (Fig. 3B, top panel). We next compared apoptotic cell death between untreated and SDF-1α-treated MDA-MB-231 cells. Although the level of apoptosis was reduced at higher concentrations of SDF-1α (200 ng/mL), which is far above physiologic concentrations, its effect was still minimal (Fig. 3B, middle panel). An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was also used to measure changes in cell proliferation on SDF-1α stimulation. SDF-1α did not show any significant effects on cell proliferation (Fig. 3B, bottom panel). To detect apoptosis at the single cell level, we stained MDA-MB-231 cells using Annexin V in the presence or absence of SDF-1α. Consistent with the results in Fig. 3B, we could not detect any significant differences on the percentages of necrotic and apoptotic cells between the SDF-1α treated (10 and 100 ng/mL) and non-treated cells (data not shown). Although CXCR4/SDF-1α signaling has been described to have effects on cell growth and survival in other cell types, we were unable to detect effects on the in vitro growth and survival of the breast cancer cells used in our studies.

**Role of CXCR4/SDF-1α in Breast Cancer Metastasis**

Cell adhesion is a critical step in the migration of tumor cells, and is regulated by a cytoskeletal complex of focal adhesion components. Thus, we examined whether focal adhesion proteins, such as FAK and related focal adhesion kinase (RAFTK), are stimulated by SDF-1α. FAK phosphorylation was measured by immunoprecipitation with anti-FAK antibody and by Western blot analysis with anti-phosphotyrosine (4G10) antibody. We observed a rapid phosphorylation of endogenous FAK in the MDA-MB-231 cells on SDF-1α stimulation (Fig. 4A, left panel). In contrast, DU4475 cells showed a constitutive phosphorylation of FAK, even without SDF-1α stimulation (Fig. 4A, right panel). Next, to examine the role of FAK phosphorylation in breast cancer cell migration, we introduced FAK COOH-terminal domain (FRNK) into MDA-MB-231 cells. FRNK is a dominant-negative inhibitor, which promotes FAK dephosphorylation and inhibits cell motility (41). FRNK is expressed endogenously under the control of an alternative intronic promoter (41). The expression of FRNK was confirmed by GFP expression (Fig. 4B) as well.

**FIGURE 1.** Expression of CXCR4 in MDA-MB-231 and DU4475 cell lines. **A**, CXCR4 expression at the surface of breast cancer cells. Cells were incubated with anti-CXCR4 antibody (12G5; gray line) or isotype control (black line) and stained with FITC-conjugated anti-mouse IgG. Similar results were obtained in two additional experiments. **B**, Transmigration of MDA-MB-231 and DU4475 cells on SDF-1α treatment. The transmigration of cells on SDF-1α treatment was measured in a transwell assay as described in Materials and Methods. For MDA-MB-231 cells, the filters were coated with fibronectin (10 μg/mL). To enumerate the migrated cells, cells were stained using a Hema3 kit. Columns, mean of four separate experiments; bars, SD.
as by Western blot analysis (Fig. 4C). The anti-FAK antibody cross-reacted with endogenous FAK and virus-expressing FRNK (Fig. 4C). As seen in Fig. 4D, overexpression of FRNK reduced the migration of MDA-MB-231 cells on SDF-1α stimulation, demonstrating that FAK phosphorylation is implicated in SDF-1α/CXCR4-mediated breast cancer cell migration. Interestingly, we did not notice any significant phosphorylation of another focal adhesion component, RAFTK, in either the MDA-MB-231 or DU4475 cells (data not shown).

Analysis of SDF-1α Secretion

Constitutive activation of the FAK, AKT, and FKHRL1 signaling pathways in DU4475 cells prompted us to test whether the DU4475 cells secrete SDF-1α, and the possibility that autocrine effects might mediate CXCR4/SDF-1α signaling in these cells. Thus, we measured secreted SDF-1α in the conditioned medium collected from cultured DU4475 cells. We found that the concentration of SDF-1α was below physiologically effective levels (of >18 pg/mL) in the cells (data not shown; based on three independent experiments), indicating that the constitutive activation of the CXCR4/SDF-1α signaling pathways in DU4475 cells is not due to autocrine effects mediated by CXCR4/SDF-1α.

Expression of SDF-1α in Microdissected Human Normal Breast Epithelial Cells and Breast Tumor Specimens

To examine whether primary breast tumor cells express higher levels of SDF1, perhaps creating an autocrine loop that enhances tumor growth and invasive behavior, we analyzed by reverse transcription (RT)-PCR the expression of SDF-1α in microdissected breast cancer cells. SDF1 and CXCR4 autocrine loops have been described in glioblastomas and neuroblastomas and were found to be correlated with the malignant progression of tumors (19, 22, 42, 43). In contrast, SDF-1α is not an autocrine but rather a paracrine factor in normal cells and pancreatic tumors (44). Because breast tumors are histologically and biochemically heterogeneous and might also contain normal breast epithelial cells, we used microdissection techniques to isolate mRNA from breast tumor epithelial cells. SDF-1α expression was analyzed in invasive breast carcinoma cells or adjacent normal breast tissue, followed by semi-quantitative RT-PCR. SDF1 mRNA was clearly detected in four normal breast tissues and three malignant breast tissues (Fig. 5A, top panel). However, SDF1-Actin ratios were much higher in malignant breast epithelial cells as compared with normal breast cells (4.9058 versus 2.4925, P < 0.01; Fig. 5A, bottom panel). In the microdissected tissues, tumor cells showed higher expression of SDF-1α as compared with the normal adjacent cells [2.2-fold in patient 1 (T1/N1); 1.5-fold in patient 2 (T2/N2); Fig. 5A, bottom panel].

Previous studies showed that mesenchymal cells (stroma) adjacent to tumors are activated and produce several cytokines. These cytokines include SDF-1α, which acts on epithelial cells that express CXCR4, thereby regulating cell migration and metastasis. We carried out immunohistochemistry to examine SDF-1α localization. We could not differentiate tumor tissue from normal tissue based on SDF-1α expression in the stroma cells (data not shown). However, five of six breast tumor epithelial cells investigated in this study were positive for SDF-1α expression (staining average 1.5 to 2; Fig. 5B). In contrast, SDF-1α was expressed at lower levels in three of five normal breast epithelial cells (staining average 1), or were negative for SDF-1α expression (2 of 5). These results are in agreement with the RT-PCR analysis.
(Fig. 5A), which showed that tumor epithelial cells consistently express higher levels of SDF-1α relative to the levels in normal mammary epithelial cells. The increased expression of SDF-1α may favor ligand-dependent activation, thus, creating an autocrine loop in the CXCR4-positive tumor cells in primary tissues.

SDF-1α Increases the Migration of MDA-MB-231 Cells Through PI-3K Signaling

SDF-1α, after binding to CXCR4, activates multiple signaling molecules, including PI-3K, mitogen-activated protein kinase (MAPK), AKT kinase, NF-κB as well as Crk (36, 45, 46). SDF-1α also activates the Gi or Gq signal

FIGURE 3. Phosphorylation of FKHRL1 by SDF-1α and its effects on cell survival. A. MDA-MB-231 and DU4475 cells were serum starved and stimulated with SDF-1α as described in Fig. 2. At the indicated times, cells were harvested and the cell extracts prepared. Whole cell extracts were resolved by SDS-PAGE and immunoblotted with antibody directed against phospho-FKHRL1 (P-FKHRL1; top panel). The total level of protein was assayed by immunoblotting using anti-FKHRL1 antibody (bottom panel). To ensure that the marked band was indeed P-FKHRL1 antibody (bottom panel). To ensure that the marked band was indeed P-FKHRL1, 20% serum-treated (+) or untreated (-) NIH/3T3 cell lysates were run on the same gel. B. Top panel, MDA-MB-231 cells were co-transfected with the FasL reporter plasmid and pcDNA3-lacZ as an internal control. Cells were serum starved for 48 hours, then incubated without (white bars) or with (black bars) 100 ng/mL SDF-1α. Cells were harvested at the indicated times, and luciferase activity was measured. The FasL luciferase activity of the non-treated (white bar) groups at each time point was set as an index (=1), respectively. Columns, mean of two independent experiments done in triplicate; bars, SD. Middle panel, MDA-MB-231 cells were serum starved for 48 hours, then stimulated with various concentrations of SDF-1α as indicated. Cell death was measured using an ELISA Cell Death Detection kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer’s instructions. Columns, mean of three separate experiments; bars, SD. Bottom panel, MDA-MB-231 cells were serum starved for 48 hours, then stimulated for 24 hours with various concentrations of CXCL12/SDF-1α as indicated. Cell proliferation was measured using an MTT assay. MTT conversion was spectrophotometrically quantified at 550 nm. Columns, mean of three experiments; bars, SD.
transduction cascade, which leads to a transient increase in intracellular calcium (47). The activation of these signaling molecules is implicated in cell motility and chemotactic responsiveness. Therefore, we examined the role of these signaling molecules in the migration of breast cancer cells using a series of inhibitors. MDA-MB-231 cells were treated with LY294002 (LY), PD98059 (PD), chelerythrine chloride (CH), or tetramethylbenzidine (TMB) for 30 minutes before the SDF-1α stimulation. None of the inhibitors affected cell viability under these conditions (data not shown). As shown in Fig. 6A, the suppression of PI-3K activation by the specific PI-3K inhibitor, LY294002, and the blockage of the release of intracellular Ca2+ by TMB, correlated with a reduction in SDF-1α-induced cell migration. None of the inhibitors affected cell viability under these conditions (data not shown). As shown in Fig. 6A, the suppression of PI-3K activation by the specific PI-3K inhibitor, LY294002, and the blockage of the release of intracellular Ca2+ by TMB, correlated with a reduction in SDF-1α-induced cell migration. These results strongly suggest that the migration of breast cancer cells induced by SDF-1α is mainly associated with PI-3K, and Ca2+ mobilization, but not with mitogen-activated protein kinase or PKC.

SDF-1α Increases the Penetration of MDA-MB-231 Cells Across an HBMEC Monolayer

Next, we examined whether SDF-1α is implicated in breast cancer cell penetration through HBMEC. Disruption of the blood-brain barrier is a hallmark of many central nervous system pathologies. HBMEC constitute the major component of the blood-brain barrier and are, therefore, critical in maintaining its structural and functional integrity. We used HBMEC because of their direct application as a model system for the in vivo human blood-brain barrier (48, 49). The HBMEC used in this study formed tubular-like networks
inhibited the transendothelial migration of the MDA-MB-231 cells at 50 ng/mL, suggesting that the engagement of SDF-1α to CXCR4 is necessary for breast cancer cells to penetrate the HBMEC monolayer.

**SDF-1α Increases the Permeability of the HBMEC Monolayer Through Calcium and PI-3K Signaling**

Endothelial cell retraction induces the breakdown of intercellular junctions and leads to an increase in vascular permeability. As shown in Fig. 6C, SDF-1α increased the permeability of the HBMEC monolayer as compared with the untreated control. This effect was blocked by the CXCR4 antibody (Fig. 6C). Furthermore, the increase in vascular permeability on SDF-1α treatment was abolished by the Ca²⁺ antagonist, BAPTA/AM, and the PI-3K inhibitor, Wortmannin, but not by the MEK inhibitor, PD98059. These results indicate that calcium and PI-3K mediate the increase in vascular permeability induced by CXCR4/SDF-1α.

**Discussion**

CXCR4 expression has been implicated in breast tumor cell metastasis, yet the actual mechanism of this effect is poorly understood. While it is clear that CXCR4 expression in breast tumor epithelial cells cannot account for all of their highly metastatic characteristics, higher CXCR4 expression in breast cancer cell lines and primary breast tumor epithelial cells is correlated with higher cell motility and metastatic potentiality (29). In this study, we have investigated the CXCR4/SDF1 signaling pathway in the breast cancer cell lines, DU4475 and MDA-MB-231. Both of these cell lines are CXCR4 positive and highly metastatic. Our results showed that SDF-1α activates the PI-3K/AKT signaling pathway in MDA-MB-231 breast cancer cells. While DU4475 breast cancer cells are more prominent in terms of CXCR4 expression, they were excluded from our further studies because high basal activity of AKT was found in these cells even in the absence of SDF-1α. The PI-3K signaling pathway is overactivated in a wide range of tumor types (50). A recent study reported that the PI-3K/AKT pathway is constitutively active in MDA-MB-231 cells even without any stimulation (51). We also observed constitutive activation of the PI-3K/AKT signaling pathway in MDA-MB-231 cells under culture conditions with serum. However, the present study was carried out under serum-starved conditions to avoid activation of signaling pathways by serum components. In addition, we observed the differential tyrosine phosphorylation of FAK in MDA-MB-231 and DU4775 cells. Some investigators have reported that fibroblasts derived from FAK-deficient mice exhibit reduced cell motility, and that reexpression of FAK restores its motility, thereby suggesting its pivotal role in cell migration (52). Others have shown that phosphorylation of FAK at Y397 results in activation and recruitment of Src family kinases and other signaling molecules, such as Cas, Grb2, and PI-3K (53). Physical and functional associations of FAK and PI-3K have also been shown in several types of cell

**FIGURE 5.** SDF-1α expression in normal and tumor breast tissues. A. Top panel: RT-PCR analysis of SDF-1α (top lanes) and Actin (bottom lanes) expression in various microdissected tumor (T) and normal (N) tissues. Note that the matching numbers (i.e., N1 versus T1) denote normal and tumor tissues from the same patient. (The tissue samples without matching numbers are from different patients.) Semi-quantitative RT-PCR was performed using primers specific for SDF-1α. DNA products were electrophoresed on 2% agarose gels. Total RNAs (2 µg) were subjected to reverse transcription and PCR amplification. DNA products were electrophoresed on 2% agarose gels. Actin expression was shown as the control. The figure is representative of two additional RT-PCR experiments on the same set of tissue samples. Actin, human Actin. Bottom panel, the fold changes of SDF-1α in the tissues shown above were calculated by densitometry using actin expression as the normalized control. B. Immunohistochemical staining of SDF-1α in paraffin-embedded human breast tissues. Tissue sections were stained with SDF-1α. The dark gray staining in the tumor tissue indicates SDF-1α protein expression (staining average of 2). All sections were counterstained with H&E. The figure is representative of six tumor and five normal tissue specimens.

lines and primary cells. Whether the differential phosphorylation of FAK by SDF-1α in the two cell lines that we studied contributes to the differential adhesive characteristics of these cell lines, awaits further investigation. The discrepancy that was found in RAFTK activation, as compared with a previously published study, could be the result of differences in the chosen starvation and culture conditions (54).

PI-3K activation is closely correlated with cell motility and migration. Our chemotactic assay using a variety of inhibitors showed that SDF-1α mediates breast cancer cell migration through the activation of PI-3K as well as through the release of Ca²⁺. While our study showed that the p85/p110 class of PI-3K is implicated in CXCR4-mediated breast cancer cell migration, the involvement of p110γ awaits further studies (55). In addition, we observed that AKT, a downstream target of PI-3K, is phosphorylated on SDF-1α stimulation. AKT plays a critical role in promoting cell survival by phosphorylating and inactivating components of the apoptotic machinery, such as BAD, caspase-9, and FKHR L1 (40). Phosphorylated FKHR L1 is retained in the cytoplasm, and is thereby sequestered from its target gene, Fasl. Although our study showed that SDF-1α phosphorylates FKHR L1 in breast cancer cells, its effects on the Fasl ligand promoter were minimal. In fact, SDF1 has shown various effects depending on its target cells. While SDF-1α induces T-cell apoptosis via up-regulation of the Fas/Fasl ligand pathway (56), it has been shown to promote the survival of CD34 hematopoietic progenitor cells (57). In lympho-hematopoietic cells, SDF-1 activates the mitogen-activated protein kinase p42/44 pathway and the PI-3K/AKT axis, but does not directly effect their proliferation or survival (58). Our results showed that SDF-1 is not a major regulator of survival or apoptosis, at least in the breast cancer cell lines that we tested.

SDF1 and CXCR4 expressions are correlated with tumor progression and metastasis. Both SDF1 and CXCR4 show higher expression levels with increasing tumor grades in glioblastomas, suggesting that the CXCR4/SDF1 signaling axis may contribute to angiogenesis and the modulation of immune response (18, 43). In our study, the quantitation of SDF-1α expression in primary breast tissues using RT-PCR showed that breast tumor epithelial cells exhibit higher SDF-1α expression than normal breast epithelial cells. The differential expression of SDF-1α between tumor and normal cells was further substantiated by RT-PCR, using cDNA from laser micro-dissected breast tissue samples. In agreement with the RT-PCR results, the expression of SDF-1α protein was low in normal

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** A. Effects of various inhibitors on SDF-1α-induced chemotaxis in MDA-MB-231 cells. MDA-MB-231 cells were pretreated with various inhibitors: 10 μmol/L PD98059 (PD), 20 μmol/L LY294002 (LY), 20 μmol/L tetramethylbenzidine (TMB), and 10 μmol/L chelerythrine chloride (CHL). Cell migration was then assayed on SDF-1α (30 ng/mL) transwells. Cells on the lower surface of the membrane were stained and counted as described in Materials and Methods. Cell death was minimal under these conditions and did not contribute to the inhibition of cell migration. Columns, mean of two experiments; bars, SD. UN (unstimulated), cells without SDF-1α treatment. B. Transendothelial migration of MDA-MB-231 cells across an HBMEC monolayer. HBMEC were added to fibronectin-coated 24-well Transculture inserts. The monolayers were pretreated for 30 minutes with 10 μg/mL of CXCR4 antibodies (CXCR4 Ab) or with the same amount of control antibodies (Control Ab). Dil-labeled MDA-MB-231 cells were added to the apical chamber, and then SDF-1α was added evenly to the apical and basolateral chambers. After incubation for 6 hours, the migrating cells were counted under a fluorescent microscope. Normal mouse IgG was used as a control antibody. A representative experiment out of two experiments is shown. Columns, mean; bars, SD. UN (unstimulated), cells without SDF-1α treatment. C. Permeability change in the HBMEC monolayer on SDF-1α treatment. HBMEC were added to fibronectin-coated 24-well Transculture inserts. After the removal of culture medium, 0.4 mL of the fresh culture medium containing [³H]inulin (1 Ci) was added to the apical chamber. The basolateral chamber was filled with 0.6 mL of the same medium without [³H]inulin and then SDF-1α (50 ng/mL) was added to the apical and basolateral chambers. The monolayers were treated with antibodies as described above. For the inhibitor studies, the monolayers were pretreated for 30 minutes with the indicated inhibitors before the addition of SDF-1α, 1 μmol/L PD98059 (PD), 1 μmol/L Wortmannin (WMN), and 10 μmol/L BAPTA. Medium from the basolateral chamber was collected and the amount of [³H]inulin passing across the monolayers was determined by scintillation counting. Columns, mean values of total counts per minute from three separate experiments; bars, SD. UN (unstimulated), cells without SDF-1α treatment.
breast epithelial cells and elevated in breast tumor epithelial cells, as determined by immunohistochemical analysis. Although the difference in SDF-1α expression between primary tumor and normal tissues was not striking, both the RT-PCR and immunohistochemical analysis showed a consistently higher level of SDF-1α expression in the tumor tissues. It is intriguing to investigate whether the actual differences in SDF-1α levels, which we found between primary tumor and normal tissues, could contribute to the differential metastasis and cell motility of the breast epithelial cells in vivo. Breast cancer cells preferentially metastasize to the lymph node, bone marrow, lung and liver, all of which contain higher levels of SDF-1α as compared with other organs (29). It is also well known that breast cancer cells often infiltrate the blood-brain barrier and cause brain cancer. Our studies showed that SDF-1α promotes the transmigration of breast cancer cells through brain endothelial cells. Notably, these migrations were affected by the PI-3K inhibitor, Wortmannin, but not by the mitogen-activated protein kinase inhibitor, PD98059. This indicated that the transendothelial cell migration was mediated by the activation of PI-3K/AKT, which was triggered by the CXCR4/SDF1 interaction. In addition, SDF-1α treatment modulated the permeability of the HBMEC, which may affect the integrity of the blood-brain barrier. Taken together, it is conceivable that the CXCR4/SDF-1α signaling axis, via PI-3K and AKT, may contribute to the invasive and metastatic behavior of breast cancer cells. Although SDF1 is not abundantly present in adult brain, it is selectively expressed in the developing and mature central nervous system. SDF1 protein was identified by immunoblotting and/or immunocytochemistry in most brain regions where these transcripts were detected (59). Functional CXCR4 and SDF1 are expressed in glial and neuronal cells (17-19, 22), and increase the intracellular calcium level in cultured astrocytes and cortical neurons on receptor/ligand stimulation, or as stated otherwise. Furthermore, the SDF1 mRNA signal increases during the formation of the hippocampal dentate gyrus and stays high in this region throughout life (59). This selective presence of SDF1 in the central nervous system could be correlated with the potential of breast cancer cells to metastasize to the brain. It would be of interest to assess whether breast cancer cells preferentially metastasize to the specific areas of the brain that express relatively high levels of SDF-1α, as compared with other brain areas. While infiltration of breast cancer cells into the brain is a more complicated process than can be explained by the data presented here, our findings suggest that the activation of PI-3K/AKT by the CXCR4/SDF1 axis may contribute to this brain infiltration as well as to changes in vascular permeability, which lead to breast epithelial cell metastasis through the brain microvascular endothelial cells.

Recent studies indicate that CXCR4 is particularly important in breast cancer invasion and metastasis. Down-regulation of CXCR4 by inducible small interfering RNA was found to inhibit breast cancer cell invasion in vitro (33). In addition, vascular endothelial growth factor (VEGF) was shown to promote breast carcinoma invasion in an autocrine manner by regulating CXCR4 expression (63). NF-κB also promoted breast cancer cell migration and metastasis by inducing the expression of CXCR4 (31), and the CXCR4 antagonists (T140 analogues) resulted in the inhibition of breast cancer metastasis (64). Lastly, expression of CXCR4 was correlated with lymph node metastasis in human invasive ductal carcinomas (30). Thus, understanding CXCR4/SDF1 signaling in breast cancer cells will lead to greater insights into the molecular mechanisms of breast cancer metastasis and to the design of therapies based on the blocking of the CXCR4/SDF1-mediated signaling pathway in breast cancer.

Materials and Methods

Materials and Antibodies

Media, antibiotics, and glutamic acids were from Mediatech (Herndon, VA). Recombinant SDF-1α was purchased from Peprotech (Rocky Hill, NJ) and used at 100 ng/mL. The following antibodies were obtained as indicated: anti–phospho-AKT (ser473), anti-AKT, anti–phospho-FKHR (ser256), and anti-FKHR were purchased from Cell Signaling (Beverly, MA). Anti-FAK and anti-RAFTK were generated in our lab as described (65). Anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). Anti–SDF-1α (clone 79014.111) and anti-CXCR4 monoclonal (clone 12G5) antibody were from R&D Systems (Minneapolis, MN). Anti-PTEN polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), PY20 was obtained from Transduction Laboratories, Inc. (Lexington, KY). Normal and malignant breast tissue sections were from the Cooperative Human Tissue Network (Philadelphia, PA). The human Fas ligand promoter (FHRE-luciferase reporter) was kindly provided by Dr. Greenberg (Children’s Hospital, Boston, MA) and the microdissected breast tissues were provided by Dr. Sgroi (Massachusetts General Hospital, Boston, MA).

Tissue Culture

MDA-MB-231 and DU4475 cells were purchased from the American Type Culture Collection. The MDA-MB-231 cell line was cultured in DMEM containing 10% fetal calf serum. The DU4475 cell line was maintained in RPMI containing 20% fetal calf serum. Cells were serum-starved for 24 to 48 hours before SDF-1α stimulation, or as stated otherwise.

Immunohistochemical Analysis

Breast tissues were fixed in 10% formalin and embedded in paraffin, after which sections were cut and placed on glass slides. For the immunohistochemical studies, tissue sections were deparaffinized and rehydrated through an alcohol series. For the SDF1 staining, a Histostain-SP kit (Zymed, South San Francisco, CA) was used as per the manufacturer’s instructions. Tissue sections were incubated with SDF-1α monoclonal antibody (clone 79014.111, R&D Systems) at 25 μg/mL for 30 minutes. Slides were washed in PBS and incubated with a biotinylated secondary antibody, then incubated for 20 minutes with streptavidin-peroxidase conjugate. The colorimetric detection was done using 3,3′-diaminobenzidine (DAB) substrates. All steps were carried out at room temperature in a humidified chamber. All of the stainings were reviewed by two independent investigators.
Chemotactic Assays

Cells were added to fibronectin (50 μg/mL)-coated transwell inserts (Costar Corp., Cambridge, MA). The MDA-MB-231 cells were starved overnight in serum-free media before their application to 8-μm-pore-size transwell inserts. The DU4475 cells were passed twice through a 30-gauge needle to make a single cell suspension, and then added to 12 μm transwell inserts (Costar). Cells were suspended into the upper chamber at a final concentration of 7 × 10^5/mL in 500 μL of RPMI 1640. Serially diluted recombinant SDF-1α was added to the lower chamber. After 3 to 6 hours of incubation, the cells on the upper surface of the filters were removed by wiping with cotton swabs, and the migrated cells on the lower chamber were fixed and stained using a Hema3 kit (Biochemical Sciences Inc., Swedesboro, NJ), according to the manufacturer’s instructions. Cellular transmigration was enumerated in four separate microscopic fields per field.

Fluorescence-Activated Cell Sorting Staining Analysis

Cells were washed, resuspended in ice-cold PBS containing 0.1% bovine serum albumin, and then incubated for 30 minutes with mouse monoclonal CXCR4 antibody (Clone; 12G5) and 30 minutes with anti-mouse secondary antibody conjugated to FITC or TRITC (Jackson ImmunoResearch, West Grove, PA). Analysis was done in a FACScan (Becton Dickinson, San Jose, CA).

RT-PCR Analysis

Two micrograms of RNA from each source was reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase and by using oligo(dT) primers. PCR amplification was done using AdvanTaq PCR kits (Clontech, Palo Alto, CA). The amplification protocol for SDF-1α was 1 × 1 minute at 95°C; 25 × (95°C, 30 seconds, 68°C, 1 minute); 1 × 68°C, 3 minutes using the following primers: sense: 5'-CGCGGCTTCGTTCCTCAAGCAGGGAAAG-3' and antisense: 5'-CTTGTATATAAAAGCTTTCTCCAGGTACT-3'. PCR products were electrophoresed on 2% agarose gel. The quantity of cDNAs of each tissue was controlled by PCR with human Actin primer. Levels of expression were quantitated using an automated digitizing system (UN-SCAN-IT, Silk Scientific Co., Orem, UT).

ELISAs

SDF-1α concentration was measured using a commercially available ELISA (R&D Systems). MDA-MB-231 and DU4475 cells were grown in DMEM containing 0.1% fetal bovine serum. The cells were grown to confluence and after 3 days, aliquots of the culture supernatants were assayed for the expression level of SDF-1α. The mean minimum detectable dose is 18 pg/mL. To measure apoptotic cell death, a Cell Death Detection ELISA kit (Roche) was used in accordance with the manufacturer’s instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. Briefly, MDA-MB-231 cells were serum-starved for 48 to 72 hours, then stimulated for 24 hours with various concentrations of SDF-1α. Cell lysates were placed into streptavidin-coated microplates, and a mixture of anti–histone-biotin and peroxidase-conjugated anti-DNA was added, followed by incubation. After washing, the bound nucleosomes were detected by anti-DNA peroxidase monoclonal antibody for 90 minutes at room temperature. After the addition of substrate, the absorbance was read with an ELISA reader at 405 nm.

PI-3K Assay

Cell lines were serum-starved for 24 to 48 hours, then stimulated with 100 ng/mL SDF-1α. At the indicated times, cells were harvested and the cell extracts prepared. PI-3K activity was measured as described previously (66).

Western Blot Analysis and Immunoprecipitation

Cells were lysed on ice for 30 minutes in immunoprecipitation buffer containing protease inhibitor cocktail tablets (Complete, Mini, Roche; 50 mmol/L Tris (pH 7.6), 1 mmol/L EDTA, 1% NP40, 150 mmol/L NaCl, 0.25% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 0.8 μg/mL Aprotinin, Na3VO4, 1 mmol/L NaF). Cell lysates were clarified by centrifugation and then incubated with protein G-Sepharose and primary antibodies at 4°C overnight. The beads were washed three times with SNNTE buffer [5% sucrose, 1% NP40, 50 mmol/L Tris (pH 7.4), 500 mol/L NaCl, 5 mmol/L EDTA], and then boiled in the presence of SDS sample buffer. Protein extracts were separated on SDS-PAGE gel, transferred to polyvinylidene difluoride membrane, blocked with 3% bovine serum albumin, and reacted with primary antibody overnight at 4°C. Phosphorylations of FAK, RAFTK, AKT, and FKHRL1 were detected using commercial mouse phospho-specific monoclonal antibodies or rabbit phospho-specific polyclonal antibodies, followed by horseradish peroxidase–conjugated goat anti-mouse IgG or donkey anti-rabbit IgG as a secondary antibody (Pharmacia Biotech, Piscataway, NJ).

Transendothelial Migration Assay

The transendothelial migration assay was done as described previously (48). Briefly, HBMEC were obtained from Cell Systems, Inc. (Kirkland, WA) and were grown confluent onto gelatin-coated 100-mm dishes. HBMEC were cultivated in CS-3.47 growth medium (Cell Systems, Inc.). HBMEC were added to fibronectin-coated 24-well Transculture inserts with pore sizes of 8 μm (Costar) and grown for 5 days in 5% CO2 at 37°C. The monolayers were pretreated for 30 minutes with CXCR4 antibodies or with control antibodies. Forty thousand DiI-labeled MDA-MB-231 cells were added to the apical chamber, and then SDF-1α was added evenly to the apical and basolateral chambers. After incubation for 6 hours, the apical chamber was fixed by 3.7% formaldehyde and washed extensively with PBS. The apical side of the apical chamber was scraped gently with cotton wool. Only the migrating tumor cells were observed by fluorescent microscopy and counted from 10 random fields of 200× magnification.

Permeability Assay

Approximately 100,000 HBMEC were added to fibronectin-coated 24-well Transculture inserts and grown for 5 days in 5% CO2 at 37°C. After the removal of culture medium, 0.4 mL of the fresh culture medium containing [3H]inulin (1 μCi) was
added to the apical chamber. The basolateral chamber was filled with 0.6 mL of the same medium without [3H]inulin and then SDF-1α (50 ng/mL) was added to the apical and basolateral chambers. For the inhibitor studies, the monolayers were pretreated for 30 minutes with various inhibitors before the addition of SDF-1α. After incubation, 30 μL of medium from the basolateral chamber were collected and the amount of [3H]inulin passing across the monolayers was determined by scintillation counting.

**Data Analysis and Statistics**

Results are shown as the mean ± SD of at least two experiments each. A value of P < 0.05 was considered significant.

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**References**

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