Regulation of Cellular Proliferation, Cytoskeletal Function, and Signal Transduction through CXCR4 and c-Kit in Small Cell Lung Cancer Cells

Takashi Kijima, Gautam Maulik, Patrick C. Ma, Elena V. Tibaldi, Ross E. Turner, Barrett Rollins, Martin Sattler, Bruce E. Johnson, and Ravi Salgia

Department of Adult Oncology [T. K., G. M., P. C. M., R. E. T., B. R., M. S., B. E. J., R. S.], and Department of Immunobiology [E. V. T.], Dana-Farber Cancer Institute, and Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02115, and Division of Hematology and Oncology, Tufts-New England Medical Center, Boston, Massachusetts 02111 [P. C. M.]

ABSTRACT

The regulation of biological functions including cell growth, viability, migration, and adhesion of small cell lung cancer (SCLC) cells depends largely on the autocrine or paracrine stimulation of growth factor receptors and chemokine receptors. Stem cell factor (SCF) and its receptor c-Kit have been identified as important regulators of SCLC viability and are coexpressed in approximately 40–70% of SCLC specimens. In vitro, the inhibition of c-Kit tyrosine kinase activity by the small molecule tyrosine kinase inhibitor STI571 (Gleevec) abrogates cell growth. We have investigated the role of c-Kit and chemokine receptors in the regulation of cell migration and adhesion of SCLC cells. CXCR4, the chemokine receptor for stromal cell-derived factor-1α (SDF-1α), was found to be the major chemokine receptor commonly expressed in all of the 10 SCLC cell lines tested. SCF and SDF-1α increased cellular proliferation over a course of 72 h in both the c-Kit- and the CXCR4-positive NCI-H69 SCLC cell line. Recently, SDF-1α and CXCR4 have been shown to be important regulators of migration and metastasis in breast and ovarian cancer. We found that SDF-1α dramatically increased cell motility and adhesion in CXCR4-expressing NCI-H446 SCLC cells. In addition, SDF-1α altered cell morphology with increased formation of filopodia and neurite-like projections. In NCI-H69 SCLC cells, SCF and SDF-1α cooperatively induced morphological changes and activated downstream signaling pathways. Treatment of NCI-H69 cells with STI571 specifically inhibited the c-Kit signaling events of Akt and p70 S6 kinase, whereas SDF-1α signaling events of Akt and p70 S6 kinase was normal. In contrast, the phosphatidylinositol 3-kinase inhibitor, LY294002, prevented these cells from adhering and completely blocked SCF- and/or SDF-1α-induced Akt or p70 S6 kinase phosphorylation. These results demonstrate that the CXCR4 receptor is functionally expressed in SCLC cells and may, therefore, be involved in the pathogenesis of SCLC in vivo. Inhibition of both the CXCR4 and the c-Kit downstream events could be a promising therapeutic approach in SCLC.

INTRODUCTION

SCLC is characterized by overexpression of several RTKs. Some of these RTKs are proto-oncogenes and key regulators for cell growth, differentiation, survival, and motility. Developing novel therapeutic agents targeting these RTKs looks attractive, and this has just begun to be identified in SCLC (1, 2). We have recently identified c-Met and c-Kit RTKs to be important in SCLC. The c-Kit receptor, a proto-oncogene product with a M₆ of 145,000, is a class III RTK similar to c-Fms, Flt3, and platelet-derived growth factor receptor. c-Kit protein contains five immunoglobulin-like domains in the extracellular region, a transmembrane domain, and a cytoplasmic domain with two kinase domains separated by a kinase insert (3–5). Approximately 40–70% of SCLC tumor specimens and cell lines coexpress c-Kit and its natural ligand, SCF; and the SCF/c-Kit pathway is functional in an autocrine or a paracrine fashion in SCLC (6–12). c-Kit receptor can be inhibited by a variety of inhibitors, including the novel tyrosine kinase inhibitor STI571 (13–17). In hematopoietic cells, c-Kit receptor has been shown to interact with a variety of molecules, including chemokine receptors (18–21).

Chemokines are small cytokine-like peptides that direct various subsets of hematopoietic cells to home-specific anatomical sites through interaction with their G protein-coupled receptors (22, 23). CXCR4 is a seven-transmembrane G protein-coupled receptor and is also known as a coreceptor for HIV (24–26). SDF-1α, the natural ligand for CXCR4, is a member of the CXC chemokine family that has chemotactic activity for hematopoietic progenitor cells (24, 27–29). In hematopoietic cells, it has been shown that c-Kit and CXCR4 interact to provide homing to the bone marrow. SCF and SDF-1α can cooperatively enhance migration and proliferation potency in hematopoietic CD34⁺ progenitor cells (18–21). Recently, CXCR4 has been shown to play an important role in migration and metastasis of solid tumors such as breast, ovarian cancers (30, 31). Thus far, the role of interaction between chemokine receptors and cytokine receptors has not been defined for solid tumors such as SCLC.

In this report, we show that CXCR4 is the major chemokine receptor in SCLC and that SDF-1α can increase proliferation, cell adhesion, motility and change morphology and also cooperate with SCF to induce the downstream signaling targets Akt and p70 S6 kinase. By using the novel tyrosine kinase inhibitor STI571, c-Kit signaling events were inhibited, whereas SDF-1α-mediated activation of Akt or p70 S6 kinase was normal in the treated cells. In contrast, the PI3-K inhibitor, LY294002, prevented these cells from adhering and completely blocked SCF- and/or SDF-1α-induced Akt or p70 S6 kinase phosphorylation. It is known that stromal cells in the bone marrow and the lymph node produce considerable amounts of SCF and SDF-1α, and, with the expression of c-Kit and CXCR4 in SCLC, this could be a potential mechanism of metastasis.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Ten SCLC cell lines (NCI-H69, NCI-H82, NCI-H128, NCI-H146, NCI-H209, NCI-H249, NCI-H345, NCI-H446, NCI-H510, and NCI-H526) were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Cellgro) supplemented with 10% (v/v) FCS. MO7e cells were maintained as described before (32). Cells were deprived of growth factors by incubation in RPMI 1640 containing 0.5% (w/v) BSA (Sigma, St. Louis, MO) for 18 h. In some experiments, cells were treated with 5 μM STI571 (Gleevec; kindly provided by Novartis Pharmaceuticals, Basel, Switzerland) or 25 μM LY294002 (Sigma). Recombinant human SDF-1α and SCF (BioSource International, Inc., Camarillo, CA) were used in the condition as indicated below.

Cell Viability Assay. NCI-H69 cells (1 × 10⁵/ml) were cultured in serum free (0.5% BSA) or serum containing (0.5, 1, 5, and 10% FCS) media with or without 100 ng/ml SCF and/or SDF-1α. Viable cells were counted by trypan...
blue dye exclusion. Student’s t test was used for the statistical analysis and differences were considered statistically significant at P < 0.05.

RPA. The specific mRNA for chemokine receptors was detected using the hCR-6 multiprobe template set (RiboQuant; PharMingen, San Diego, CA) according to the manufacturer’s protocol. This set contains DNA templates for CXCR-1, -2, -3, and -4; BLR-1/CXCR5; BLR-2/CCR7; and V28/CX3CR1; as well as ribosomal protein L32 and GAPDH as controls. RNase-protected probes were resolved on a denaturing 5% acrylamide-urea sequencing gel and identified by autoradiography.

Fluorescence-activated Cell Sorting Analysis. Cells (1 × 10^5) were washed three times in PBS containing 0.5% BSA (PBS buffer), then incubated for 30 min at 4°C with 10 μg/ml phycoerythrin-conjugated mouse-antihuman CXCR4 monoclonal antibody or phycoerythrin-labeled mouse control IgG2b (R&D Systems Inc., Minneapolis, MN). After washing the cells twice with PBS buffer to remove unbound antibodies, the stained cells were resuspended in 300 μl of PBS and analyzed by FACScan using Cell Quest software (Becton Dickinson Labware, Franklin Lakes, NJ).

Adhesion Assay. The wells of a 96-well tissue culture plate (Corning-Costar, Cambridge, MA), precoated with 10 μg/ml human plasma FN or humancoll. IV (Life Technologies, Inc., Rockville, MD) overnight at 4°C, were washed with PBS twice and blocked for 1 h at 37°C with RPMI 1640 containing 0.2% BSA (adhesion media) before plating cells. NCI-H446 cells (3 × 10^5) were washed twice, resuspended in the adhesion media with or without SDF-1α (100 ng/ml), and plated onto uncoated, FN-coated, or col. IV-coated wells. Unattached cells were removed after incubation for 2 h at 37°C by gentle washing with adhesion media. The relative number of attached viable cells was determined by the MTT colorimetric assay (Sigma) following the instruction manual. Student’s t test was used for the statistical analysis of the attached cell number, and differences were considered significant at P < 0.05.

Analysis of Cell Motility by TLVM. NCI-H446 cells were plated on cell culture dishes and placed into a temperature-controlled chamber at 37°C in an atmosphere of 5% CO₂. The cells were examined by TLVM using an Olympus IX70 inverted microscope. Omega temperature controlled device, DVC1310 digital video camera, and QED Camera with Standalone 145 software. SDF-1α (100 ng/ml) was added into the culture after 6 h and images were recorded for another 10 h. Digital video images were saved every 90 s, and cell movement or morphological changes were analyzed with the NIH Image Analysis pro-

Fig. 1. CXCR4 and c-Kit are expressed in SCLC cell lines. In A, the expression of chemokine receptor mRNAs in 10 SCLC cell lines was determined by RPA. All of the SCLC cell lines expressed CXCR4 mRNA at various levels with no detectable mRNA for other chemokine receptors. The human megakaryoblastic cell line MO7e, as a control, expressed CXCR3 as well as CXCR4 mRNA. Yeast tRNA (lane 2) and human control RNA-2 (lane 3) were used as the negative and positive control for CXCR4, respectively. In B, expression of CXCR4 protein was confirmed by flow cytometric analysis in these cell lines. CXCR4 protein was highly expressed in NCI-H209 and NCI-H446 cells. In C, the expression of c-Kit was evaluated by immunoblotting. Six of 10 SCLC cell lines expressed various levels of c-Kit with high expression in the MO7e control cells (upper panel). The blots were stripped and reprobed with antibody against β-actin as internal control (lower panel). kDa, M, in thousands.
Fig. 2. SCF and SDF-1α induce proliferation of NCI-H69 cells. NCI-H69 cells (1 × 10^4/ml) were cultured in serum-free (0.5% BSA) or serum-containing (10% FCS) media with or without 100 ng/ml SCF and/or SDF-1α. Viable cells were counted by trypan blue dye exclusion test every 24 h and shown as the mean (data points) ± SD (bars) from three independent experiments. Without serum, neither SCF nor SDF-1α showed any effect on cell survival. On the other hand, in media containing 10% FCS, cell proliferation was significantly induced by SCF (∼21.5%, \( P = 0.0373 \)) and SDF-1α (∼26.6%, \( P = 0.0133 \)) separately or in combination (∼26.6%, \( P = 0.0133 \)) at 48 h compared with untreated control. SCF and SDF-1α conferred an increase in viable cell number (16.5%, \( P = 0.0164 \), and 15.5%, \( P = 0.0184 \), respectively, and 20.0%, \( P = 0.0322 \), combined) at 72 h. There was no additive or synergistic effect seen with both the cytokine and chemokine combined. *, statistically significant (∼21.5% or ∼26.6% versus ∼0%, \( P < 0.05 \)).

**RESULTS**

**CXCR4 Is Ubiquitously Expressed and c-Kit Is Variably Expressed in SCLC Cell Lines.** The expression of chemokine receptor mRNAs in SCLC cells was determined by RPA. All of the 10 SCLC cell lines tested expressed CXCR4 mRNA at various levels with no detectable mRNA for other chemokine receptors. The human megakaryoblastic cell line M07e, as a control, expressed CXCR3 as well as CXCR4 (Fig. 1A). Expression of CXCR4 protein was confirmed by flow cytomteric analysis in these SCLC cell lines and M07e. The expression of CXCR4 was highest in NCI-H209 and NCI-H446 cells (Fig. 1B).

The expression of c-Kit in these cell lines was evaluated by immunoblotting, and 6 of 10 SCLC cell lines tested expressed variable levels of c-Kit with high expression in the M07e control cells (Fig. 1C).

**SCF and SDF-1α Induce Proliferation of NCI-H69 Cells.** The effect of SCF and SDF-1α on viability in NCI-H69 cells was analyzed. As shown in Fig. 1, NCI-H69 cells express both c-Kit and CXCR4, and, thus, these cells were used for many of the experiments. Without serum, neither SCF nor SDF-1α showed any effect on cell survival. On the other hand, in media containing 10% FCS, cell proliferation was significantly induced by SCF (21.5%; \( P = 0.0373 \)) and SDF-1α (26.6%; \( P = 0.0133 \)) separately or in combination (26.6%; \( P = 0.0133 \)), at 48 h compared with untreated control. SCF and SDF-1α conferred an increase in viable cell number at 72 h also (16.5%; \( P = 0.0164 \) and 15.5%; \( P = 0.0184 \), respectively, and 20.0%, \( P = 0.0322 \), combined). Even though SCF and SDF-1α individually induced proliferation of NCI-H69 cells, there was no additive or synergistic effect seen with both the cytokine and the chemokine combined (Fig. 2). Similar results were observed for different concentrations of FCS tested (0.5, 1, and 5% FCS; data not shown), implicating the importance of SCF and SDF-1α in the proliferation of NCI-H69 SCLC cells.

**SDF-1α Regulates Adhesion, Motility, and Cell Shape in NCI-H446 SCLC Cells.** CXCR4 has been shown to be important in the homing of hematopoietic cells to the bone marrow, the spleen, and the lymph nodes (28, 29, 33). Neutralization of CXCR4 has been reported to decrease the frequency of lymph node metastases of breast cancer cells in vivo (30). Cytoskeletal functions such as increased cell motility, adhesion to extra cellular matrix proteins, morphological change, and movement, are crucial for cancer cells to metastasize. To determine the effect of SDF-1α on cell motility and adhesion, NCI-H446 cells were used that express high amounts of CXCR4 and grow in an anchorage-dependent fashion. In an adhesion assay, FN (3.84-fold; \( P = 0.0002 \)) and col. IV (2.98-fold; \( P = 0.0124 \)) were found to increase the adhesion of NCI-H446 cells compared with the uncoated surface (Fig. 3). In conjunction with this, SDF-1α stimulation further increased the attachment 3.14-fold on the uncoated surface (\( P < 0.0001 \)) but did not significantly enhance FN- and col. IV-mediated adhesion.
In addition to cell adhesion, SDF-1α also markedly increased the motility of NCI-H446 cells (Fig. 4, A and B) with higher speed (Fig. 4C). Morphological changes from round to polygonal shape, including the formation of neurite-like projections, increased membrane ruffling, and more frequent filopodia and uropods formations, were observed in response to SDF-1α (Fig. 4). Filopodia formation in the presence of SDF-1α occurred much more frequently (13.14 versus 2.86 times/h/cell) with a longer existing period (6.09 versus 3.75 min/filopodium). Uropods formation was observed in four of seven (57.1%) of the SDF-1α treated cells; however, only one cell of seven (14.3%) untreated cells showed uropods. The lasting period per uropod also became much longer by SDF-1α stimulation (12.2 versus 5.0 min/uropod).

PI3-K Regulates SDF-1α-induced Cell Motility of NCI-H446 SCLC Cells. We tested whether the PI3-K inhibitor LY294002 can inhibit the SDF-1α-induced cell motility in NCI-H446 SCLC cells. NCI-H446 cells either were left untreated or were treated with SDF-1α (100 ng/ml) in the absence or presence of LY294002 (25 μM). Phase-contrast pictures were taken at the 24-h time point. Most of the untreated cells kept their rounded shape and formed clusters, and nearly one-half of them attached weakly to the bottom of the dish (Fig. 5a). With SDF-1α, almost all of the cells tightly adhered to the bottom of the dish, and neurite-like projections were induced in many cells (Fig. 5b). In the presence of LY294002, more than 90% of the cells were floating with rounded shape despite SDF-1α treatment, although they could form clumps (Fig. 5, c and d).

CXCR4 and c-Kit Cooperatively Induce Morphological Changes in NCI-H69 SCLC Cells. The role of interaction between CXCR4 and c-Kit in SCLC has not been shown previously. Both CXCR4- and c-Kit-positive NCI-H69 cells were either untreated or treated with SDF-1α (100 ng/ml) and/or SCF (100 ng/ml), in the absence or presence of STI571 (5 μM). Then, phase-contrast pictures were taken after 8 h (Fig. 6). Morphological changes began to be apparent from 4 h, and the changes plateaued after 8 h (data not shown). Neurite-like actin formations were observed in response to SCF (Fig. 6c), and this morphological change was more apparent as projections when treated with SDF-1α (Fig. 6, e and g). Neurite-like projections, induced by SDF-1α alone, still formed even in the presence of STI571 (Fig. 6f). Interestingly, STI571-treated NCI-H69 cells that were stimulated with both SDF-1α and SCF could not form any neurite-like structure (Fig. 6h). STI571 inhibited the morphological changes only in the presence of SCF (Fig. 6, b, d, and h). These results suggest that there are important interactions between CXCR4 and c-Kit in SCLC in terms of cell motility (34). The inhibition of the active c-Kit receptor by STI571 may lead to the inhibition of other active receptors such as CXCR4.


**CXCR4 AND c-Kit IN SCLC CELLS**

**DISCUSSION**

In this report, we have investigated the expression of CXCR4 and c-Kit in SCLC cell lines and determined the biological and biochemical effects of stimulating the two pathways with their ligands. We showed that in the 10 SCLC cell lines tested, all of the cell lines express the CXCR4 receptor and 6 of 10 cell lines express the c-Kit receptor. Initially, we have shown that there was a proliferative advantage with SCF and SDF-1α stimulation in NCI-H69 cells with FCS in the media; whereas, when the survival of NCI-H69 cells was determined with SCF and SDF-1α without FCS, there was no significant antiapoptotic advantage observed. This would imply that both SCF and SDF-1α in these cells are important for cell growth. Interestingly, it has been shown that in the murine myeloid interleukin 3-dependent hematopoietic 32D cell line, SDF-1α has an inhibitory role in growth (41).

Chemoactive receptors and cytokine receptors are crucial in the homing mechanisms of hematopoietic cells and the metastasis of solid tumors such as breast cancer and ovarian cancer. The initial step toward metastasis is increased cell motility and migration of cancer cells. Our results show that the SDF-1α stimulation of NCI-H446 cells leads to increased adhesion to uncoated surface and increased cell motility. At this time, we do not know the relevance of this in vivo. The enhancement of cell motility by SDF-1α is characterized by the increased formation of filopodia, uropods, and neurite-like projections with at least 25 ng/ml SDF-1α and 10 ng/ml SCF (data not shown). Phosphorylation of Akt (Ser 473) and p70 S6 kinase (Thr 389) occurred in response to SDF-1α within 5 min and in response to SCF within 2.5 min.

**STI571 and LY294002 Inhibit Signal Transduction of CXCR4 and c-Kit Pathways.** Small molecular inhibitors, STI571 (targeting c-Kit) and LY294002 (targeting PI3-K), were used to determine the effects on downstream signaling by SDF-1α and SCF in NCI-H69 cells. Cells were left untreated or were pretreated with STI571 (5 μM) or LY294002 (25 μM) overnight in serum-starved media and subsequently stimulated with 50 ng/ml SCF and/or SDF-1α for 15 min. Cooperative phosphorylation of Akt at Ser 473/Thr 389 and p70 S6 kinase at Thr 389 was induced by SCF and SDF-1α (Fig. 8). STI571 pretreatment inhibited SCF- but not SDF-1α-induced phosphorylation. Expressions of Akt, p70 S6 kinase, and c-Kit were not affected by any of these treatments (Fig. 8A). In contrast, LY294002 pretreatment blocked SDF-1α- as well as SCF-induced phosphorylation of Akt and p70 S6 kinase (Fig. 8B).

**SDF-1α and SCF Independently Regulate Phosphorylation of Akt and p70 S6 Kinase.** PI3-K is important in the regulation of cellular proteins and Ser/Thr phosphorylation and may also contribute to PI3-K is important in the regulation of cellular proteins and Ser/Thr phosphorylation and may also contribute to Akt and p70 S6 kinase. Several tyrosine-phosphorylated bands between Mr 60,000 and Mr 120,000 were identified within 15 min of SDF-1α (50 ng/ml) stimulation. On the other hand, maximal tyrosine phosphorylation of proteins at Mr 60,000–90,000 and Mr 110,000–145,000 occurred within 2.5–7.5 min in response to SCF (50 ng/ml). We also performed dose-response studies and found that optimal phosphorylation of cellular proteins was obtained.

**Fig. 5.** PI3-K regulates SDF-1α-induced cell motility in NCI-H446 SCLC cells. NCI-H446 cells were either left untreated (a and c) or treated (b and d) with SDF-1α (100 ng/ml) in the absence (a and b) or presence (c and d) of LY294002 (25 μM) for 24 h. Then phase-contrast pictures were taken and shown. Most of the untreated cells kept a rounded shape and formed clusters, and nearly one-half of them attached weakly to the bottom of the dish (a). With SDF-1α, almost all of the cells tightly adhered to the bottom of the dish and neurite-like projections were induced in many cells (b). In the presence of LY294002, more than 90% cells were floating with rounded shape despite SDF-1α treatment and even though they could form clumps (c and d).

**Fig. 6.** CXCR4 and c-Kit interact to induce morphological changes in NCI-H69 SCLC cells. NCI-H69 cells were cultured in serum-starved medium with (b, d, f, and h) or without (a, c, e, g, and STI571 (5 μM), with SDF-1α (100 ng/ml; c, f, g, and h), and/or SCF (100 ng/ml; c, d, g, and h) for 8 h. Then phase-contrast pictures were taken and shown. SCF induced the neurite-like actin formation (c, arrows) and they were abrogated by STI571 treatment (d). SDF-1α induced the longer neurite-like projections (c, arrows) and they were abrogated by STI571 treatment (f). Interestingly, NCI-H69 cells stimulated with both SDF-1α and SCF could not form any morphological changes in the presence of STI571 (h).

with at least 25 ng/ml SDF-1α and 10 ng/ml SCF (data not shown). Phosphorylation of Akt (Ser 473) and p70 S6 kinase (Thr 389) occurred in response to SDF-1α within 5 min and in response to SCF within 2.5 min.
CXCR4 by a neutralizing antibody impaired the experimental metastasis of breast cancer cells to regional lymph nodes and to the lung in vivo (30). Robledo et al. (42) have most recently shown that CXCR3 and CXCR4 are expressed in melanoma cells. The chemokine Mig, a ligand for CXCR3, activated the small GTPases RhoA and Rac1 and induced a reorganization of the actin cytoskeleton and cell migration. Both Mig and SDF-1α triggered modulation of integrin VLA-4 (α7β3)- and VLA-5 (α6β1)-dependent cell adhesion to FN. Scotton et al. (31) have also reported that CXCR4 was expressed in 4 of 6 ovarian cancer cell lines and 8 of 10 primary ovarian tumors. AMD3100, a CXCR4 antagonist that is currently under clinical trial for HIV/AIDS, would be a potential therapeutic molecule to use in SCLC (43).

c-Kit is expressed in ~40–70% of SCLCs and is functionally stimulated by its ligand SCF (6–11). Using transwell migration assays, SCF has been shown to act as a chemotactic signal (9). Through in vitro modeling, it has been hypothesized that SCF, in synergy with SDF-1α, can mobilize hematopoietic CD34+ stem cells from the bone marrow to peripheral organs such as the spleen (18–20). We show that, in using NCI-H69 SCLC cells, SCF and SDF-1α can act cooperatively to enhance not only cell motility but also signal transduction as well as increased migratory movements. This change in morphology and motility in NCI-H446 cells is in contrast to Ba/F3 cells (interleukin-3-dependent pre-B cells expressing CXCR4) stimulated with SDF-1α. The Ba/F3 cells do have increased cellular migration and increased membrane ruffling but have less dramatic change in morphology in response to SDF-1α (33). This may reflect the probability that hematopoietic cells have a different response to chemokines as compared with their response to solid tumors such as SCLC.

The functional expression of chemokine receptors has recently been described in several other solid tumors. CXCR4 and CCR7 are highly expressed in breast cancer and melanoma cells. In breast cancer cells, their respective ligands, SDF-1α and CCL21, mediated actin polymerization and pseudopodia formation and, subsequently, induced chemotactic and invasive responses in vitro. The neutralization of

![Fig. 7. SDF-1α and SCF independently regulate phosphorylation of Akt and p70 S6 kinase. Starved NCI-H69 cells were stimulated with SDF-1α (30 ng/ml; A) or SCF (50 ng/ml; B) for 0–60 min before lysis. Cell lysates were applied to a 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes. The membrane was probed with the monoclonal antibodies against phosphorytrosine (4G10) and β-actin and the polyclonal antibodies against Akt (pSer 473) and p70 S6 kinase ([S6K] (pThr 389)]. SDF-1α and SCF induced time-dependent tyrosine phosphorylation of cellular proteins and Ser/Thr phosphorylation of Akt and S6K. Several tyrosine-phosphorylated bands (arrows) were identified between M, 40,000 and M, 120,000 within 15 min of SDF-1α stimulation (A). On the other hand, maximal tyrosine phosphorylation of proteins at M, 60,000–90,000 and M, 110,000–145,000 (arrows and brackets) occurred within 2.5–7.5 min in response to SCF (B). Phosphorylation of Akt (Ser 473) and S6K (Thr 389) occurred in response to SDF-1α within 5 min (A) and in response to SCF within 2.5 min (B). kDa, M, in thousands.

![Fig. 8. STI571 and LY294002 inhibit signal transduction of CXCR4 and c-Kit pathways. NCI-H69 cells were left untreated or were pretreated with STI571 (5 μM; A) or LY294002 (25 μM; B) overnight in serum-starved medium and, subsequently, were stimulated with SDF-1α (50 ng/ml) and/or SCF (50 ng/ml) for 15 min before lysis. Lysates were processed as in Fig. 7, and the membrane was probed with the phosphospecific or regular antibodies against Akt and p70 S6 kinase (S6K) and with the anti-c-Kit antibody. Cooperative phosphorylation of Akt at Ser 473/Thr 308 and S6K at Thr 389 was induced by SDF-1α and SCF (A and B). STI571 pretreatment inhibited SDF-1α-induced phosphorylation. Expressions of Akt, S6K, and c-Kit were not affected by any of these treatments (A). In contrast, LY294002 pretreatment blocked SDF-1α- as well as SCF-induced phosphorylation of Akt and S6K (B).]
by phosphorylating Akt and p70 S6 kinase. Both SCF and SDF-1α signal through PI3-K-dependent pathways that converge in the activation of the Ser/Thr kinases Akt and p70 S6 kinase. Phosphorylation of Akt on Thr 308 and Ser 473 or of p70 S6 kinase on Thr 229 correlates with their activation and occurs through direct phosphorylation by the phosphoinositide-dependent kinase-1 (PDK-1; Refs. 40 and 44). p70 S6 kinase regulates cell cycle progression through phosphorylation of the S6 protein of the 40S ribosomal subunit (40). Akt controls the activation of several downstream molecules that regulate cell survival and apoptosis, including Forkhead transcription factors, Caspase 9, or Bad (45–47). This suggests that, in addition to activated tyrosine kinase receptors, CXCR4 in SCLC cells may well play an important role in tumor progression.

We have used small-molecule inhibitors to dissect out the pathways related to the downstream signaling events of c-Kit and CXCR4. c-Kit activity is inhibited by STI571 in SCLC and in gastrointestinal stromal tumors (14–16, 48). Through using STI571, we have shown that the cell motility of NCI-H69 cells was abrogated in SCF/SDF-1α-treated cells. This would imply that c-Kit/CXCR4 pathways cooperate to induce cell motility; however, the signal transduction pathway in SCF/SDF-1α-treated cells was abolished only in the c-Kit pathway with STI571 treatment. Thus, the changes in morphology and motility in relation to biochemical changes in the phosphorylation of Akt and p70 S6 kinase are probably two separate events. Also from our data, when c-Kit pathway is inhibited by STI571, the CXCR4 signal transduction pathway is still functional. However, when the PI3-K pathway is inhibited with LY294002, the c-Kit and CXCR4 signal transduction pathways are both inhibited. This would implicate PI3-K as an important intermediate messenger molecule in the signal transduction of c-Kit and CXCR4. PI3-K is also important to inhibit small cell lung cancer growth by the quinoxaline tyrphostins. Cancer Res., 57: 370–376, 1997.


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