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Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells

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Small-cell lung cancer (SCLC) is an aggressive, rapidly metastasizing neoplasm. The chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) is constitutively secreted by marrow stromal cells and plays a key role for homing of hematopoietic cells to the marrow. Here, we report that tumor cells from patients with SCLC express high levels of functional CXCR4 receptors for the chemokine CXCL12. Reverse transcriptase–polymerase chain reaction and flow cytometry demonstrated CXCR4 mRNA and CXCR4 surface expression in SCLC cell lines. Immunohistochemistry of primary tumor samples from SCLC patients revealed high expression of CXCR4. CXCL12 elicited CXCR4 receptor endocytosis, actin polymerization, and a robust activation of phospho-p44/42 mitogen-activated protein kinase in SCLC cells. Furthermore, CXCL12 induced SCLC cell invasion into extracellular matrix and firm adhesion to marrow stromal cells. Stromal cell adhesion of SCLC cells was significantly inhibited by the specific CXCR4 antagonist T140, pertussis toxin, antivascular cell adhesion molecule-1(VCAM-1) antibodies, and CS-1 peptide, demonstrating the importance of CXCR4 chemokine receptor activation and $\alpha 4\beta 1$ integrin binding, respectively. In addition, CXCL12 enhanced the adhesion of SCLC cells to immobilized VCAM-1, demonstrating that CXCR4 chemokine receptors can induce integrin activation on SCLC cells. As SCLC has a high propensity for bone marrow involvement, our findings suggest that CXCR4 chemokine receptors and $\alpha 4\beta 1$ integrins play a critical role in the interaction of SCLC cells with stromal cells in the tumor microenvironment.
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Introduction

Small-cell lung carcinoma (SCLC) constitutes 20–25% of cases of lung cancer, currently the leading cause of death from malignant diseases in Western societies. SCLC is a highly fatal cancer due to early and widespread metastasis and development of resistance to chemotherapy (Ihde *et al*., 1993; Hoffman *et al*., 2000). Despite initial sensitivity to chemotherapy, SCLC almost invariably relapses, and the patients 2-year survival rate remains less than 5% (Ihde, 1992). New therapeutic strategies that target SCLC dissemination and resistance mechanism therefore are urgently needed. Recent studies indicate that tumor metastasis is not a random process, but shares many similarities with leukocyte trafficking (Muller *et al*., 2001). Constitutively secreted chemokines play a key role in trafficking and homing of hematopoietic cells. The chemokine stromal-cell-derived factor-1 (SDF-1 or CXCL12 according to a new chemokine classification system (Zlotnik and Yoshie, 2000) is a CXC chemokine constitutively expressed by marrow stromal cells and other stromal cells of mesenchymal origin. CXCL12 binds to the G-protein-coupled receptor termed CXCR4 that was designated CD184 during the most recent Human Leucocyte Differentiation Antigen Workshop (Mason *et al*., 2001). Hematopoietic stem cells (HSCs) are uniquely selective in their migratory response to CXCL12 (Wright *et al*., 2002), which regulates the specific homing of HSC in marrow microenvironment. Involvement of the CXCL12/CXCR4 axis in the dissemination and marrow homing of malignant hematopoietic and nonhematopoietic cells has been demonstrated recently by our group and other investigators (Burger *et al*., 1999; Geminder *et al*., 2001; Muller *et al*., 2001; Robledo *et al*., 2001; Taichman *et al*., 2002).

Metastases to the skeletal system are common in lung cancer, and particularly in SCLC. However, in terms of seeding metastases, two distinct compartments have to be differentiated, namely the bone marrow and the osseous tissue. Clinically, metastasis to these different sites can be distinguished by hematopoietic abnormalities or bone pain, respectively. SCLC has a striking tendency to metastasize to the bone marrow, whereas

other lung cancer types preferentially metastasize to the osseous tissue (Kristensen *et al*., 1996). Moreover, bone marrow metastasis is an important negative prognostic factor in SCLC (Lassen *et al*., 1995). Owing to the importance of the CXCR4/CXCL12 axis in bone marrow homing and the high propensity of SCLC cells for marrow infiltration, we hypothesized that SCLC cells express CXCR4 chemokine receptors that could mediate the homing of SCLC cells to the marrow.

According to the multistep paradigm of leukocyte transendothelial migration, a sequential engagement of integrins and subsequent activation via chemokine receptors is required for emigration and tissue homing (Springer, 1994). SCLC cells express various α and β integrin chains that allow for interactions with the tumor microenvironment (Sethi *et al*., 1999). Integrin-mediated interaction of SCLC cells with accessory cells or extracellular matrix (ECM) enhances tumorigenicity and confers resistance to chemotherapeutic agents as a result of β1 integrin-stimulated tyrosine kinase activation, as demonstrated by Sethi *et al*. (1999). We therefore also examined whether CXCR4 chemokine receptor activation cooperates with integrin binding during adhesion to stroma or immobilized vascular cell adhesion molecule-1 (VCAM-1) molecules. Our results demonstrate that SCLC cells express functional CXCR4 chemokine receptors that play a role in adhesive interactions with stromal cells.

Results

SCLC cell lines expression of CXCR4 mRNA and surface protein

CXCL12 is a powerful chemoattractant cytokine (chemokine) that stimulates directional migration of hematopoietic and nonhematopoietic cells. We examined SCLC cell lines for expression of the CXCL12 receptor (CXCR4) by reverse transcriptase–polymerase chain reaction (RT—PCR) and flow cytometry, respectively. CXCR4 mRNA was detected in all SCLC cell lines tested (Figure 1). Flow cytometry revealed high-level expression of CXCR4 on NCI-H69, NCI-H146, NCI-N592, NCI-H446, and NCI-H345, and relatively lower levels of CXCR4 on NCI-H82 and NCI-H510A SCLC cells (Figure 2).

CXCR4 chemokine receptors are expressed in primary tumors from patients with SCLC

CXCR4 expression in primary tumors from SCLC patients was demonstrated by immunohistochemistry. In all samples from patients with SCLC ($n=10$) immunoreactivity with anti-CXCR4 monoclonal antibodies (mAbs) was detected (Figure 3a and b). This staining was specific, as demonstrated by staining SCLC tumor samples with isotype control antibodies (Figure 3c). In contrast to SCLC samples, tissue samples from patients with NSCLC ($n=10$) did not display or very low CXCR4 surface expression by means of immunohistochemistry in the 10 primary tumor samples tested (Figure 3d).

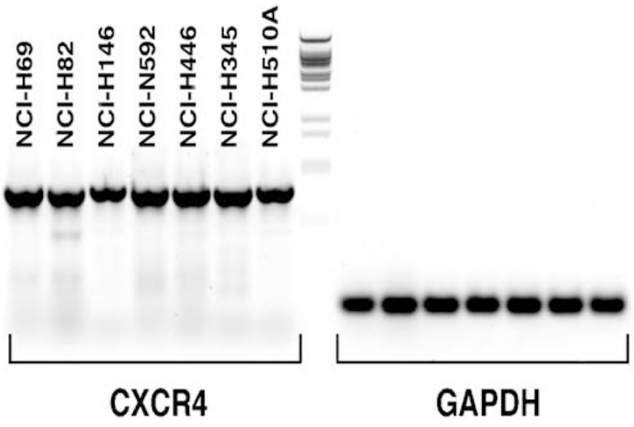

Figure 1 RT–PCR analysis for CXCR4 expressed by different SCLC cell lines. A CXCR4 PCR product of the expected size (1.058 bp) was generated using cDNA generated from NCI-H69, NCI-H82, NCI-H146, NCI-N592, NCI-H446, NCI-H345, and NCI-H510A in lanes 1–7, respectively. Similarly, a GAPDH PCR product was generated using cDNA from NCI-H69, NCI-H82, NCI-H146, NCI-N592, NCI-H446, NCI-H345, and NCI-H510A in lanes 9–15, respectively. Lane 8 contains DNA fragments of known size, allowing for the calibration of the migration distances

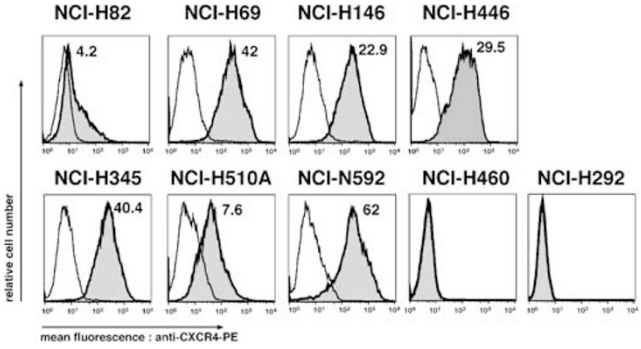

Figure 2 Expression of CXCR4 by different SCLC and NSCLC cell lines. Displayed are fluorescence histograms depicting the relative red fluorescence intensity of SCLC cells (seven different cell lines) and NSCLC (NCI-H460 and NCI-H262) stained with anti-CXCR4 mAbs (shaded histograms) compared with that of the same cells stained with a PE-conjugated isotype control mAb of irrelevant specificity (open histograms). The mean fluorescence intensity ratio of each specifically stained cell population is displayed above the histograms

CXCL12 induces dose-dependent CXCR4 receptor endocytosis

Receptor endocytosis is a characteristic capability of chemokine receptors that allows for continuous sampling of chemoattractants, permitting the cells to follow a chemotactic gradient. Figure 4 depicts the mean fluorescence of the SCLC cell lines NCI-H345 and NCI-H69 stained with anti-CXCR4 mAbs after incubation with increasing concentrations of SDF-1α or medium alone. We found that CXCR4 receptor endocytosis was dose dependent with highest levels of endocytosis at the highest SDF-1 concentration of 1000 ng/ml.

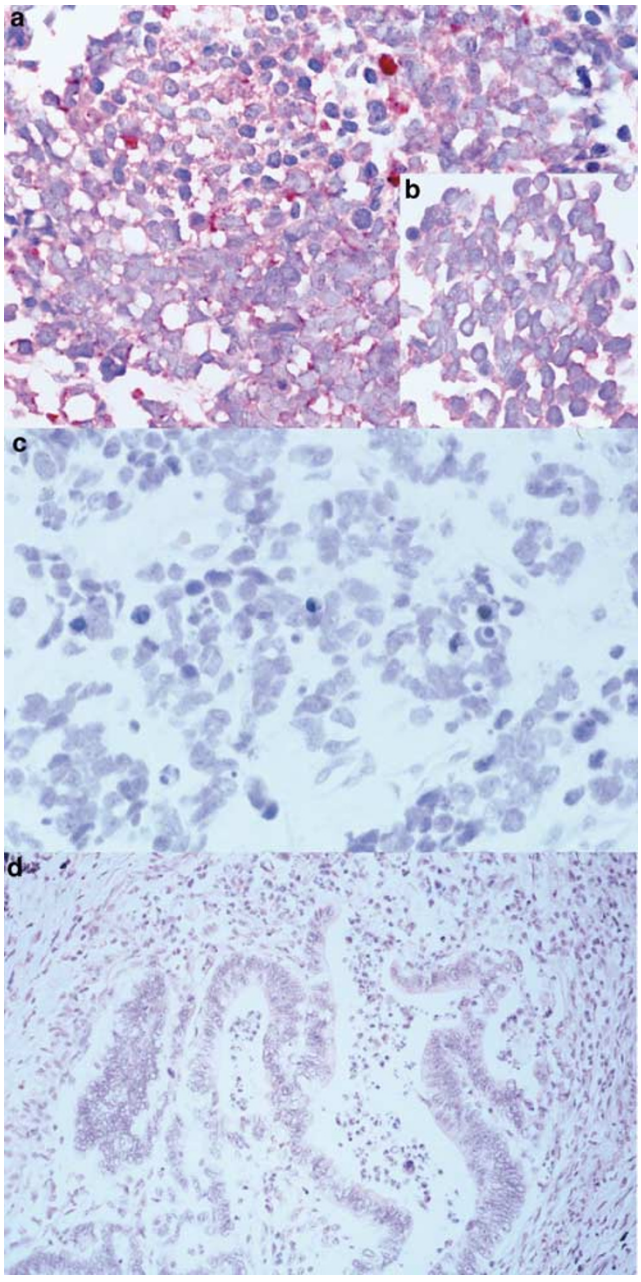

Figure 3 CXCR4 immunohistochemistry of SCLC and NSCLC. Tumor tissue samples from patients diagnosed with SCLC or NSCLC were subject to immunohistochemical examination for CXCR4 expression. After incubation with anti-CXCR4 mAbs (12G5) or mouse monoclonal isotype control MOPC-21, tissues were stained using the APAAP method. Specimens were lightly counterstained with hematoxylin and were photographed at $\times 400$ or $\times 600$ magnification. Displayed are sections from a representative patient with SCLC that display immunoreactivity with anti-CXCR4 mAbs ((**a**) $\times 400$ magnification, (**b**) $\times 600$, (**c**) isotype control, $\times 400$)), and a representative patient with NSCLC that is CXCR4 negative (**d**) $\times 400$)

CXCL12 induces actin polymerization in SCLC

Cytoskeletal reorganization is a prerequisite for motility and migration (Moser *et al*., 1998). To evaluate the ability of SDF-1 to induce changes in the actin cytoskeleton of SCLC, we examined for changes in the

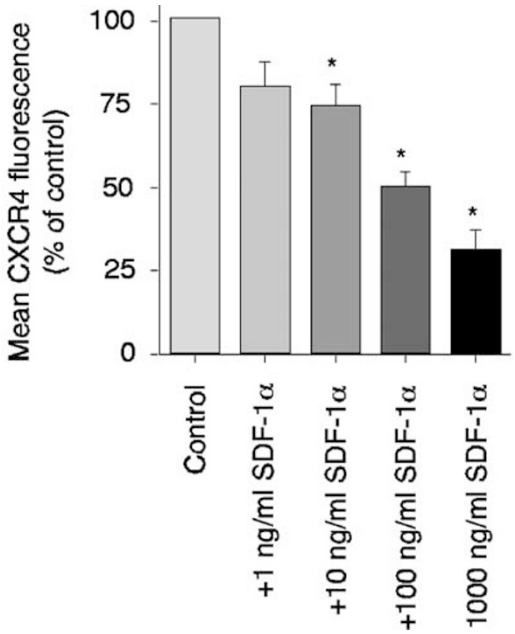

Figure 4 SDF-1/CXCL12 induces CXCR4 receptor endocytosis in SCLC cells. SCLC cells were incubated with synthetic CXCL12 for 1 h at 37°C at the concentrations indicated. Mean relative CXCR4 surface expression of the seven different SCLC cell lines after incubation with 0, 1, 10, 100, and 1000 ng/ml CXCL12 are shown. Significant receptor endocytosis was induced by 10 ng/ml and higher concentrations

reorganization of filamentous actin (F-actin) in response to 200 ng/ml SDF-1α. Cells were serum starved overnight and displayed a disorganized cytoskeleton. As shown in Figure 5, SDF-1α caused a cytoskeletal rearrangement with stress fiber formation due to CXCR4 activation, which starts 1–2 min after stimulation and persists for about 30 min.

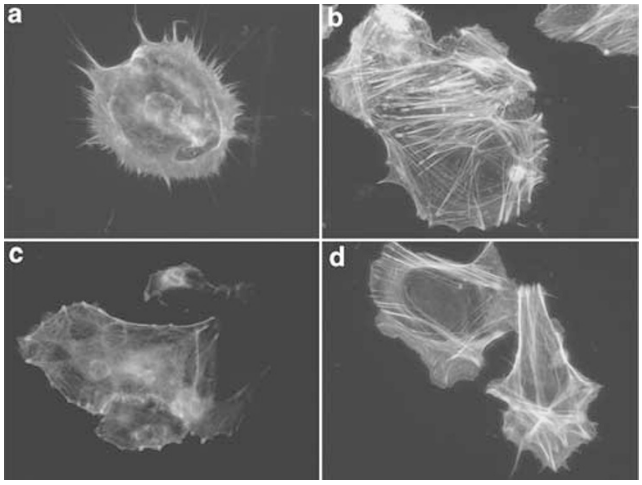

Figure 5 SDF-1/CXCL12 induces actin polymerization in SCLC cells. NCI-H82 SCLC cells were seeded on poly-L-lysine-coated chamber slides and stained with FITC-conjugated phalloidin to visualize the actin cytoskeleton. Figure 5(**a**) and (**c**) depict the actin staining of resting NCI-H82 cells with relatively few cytoplasmatic actin fibers. In contrast, stimulation with CXCL12 for 10 min induces stress fiber formation in NCI-H82 SCLC cells, as displayed in Figure 5(**b**) and (**d**)

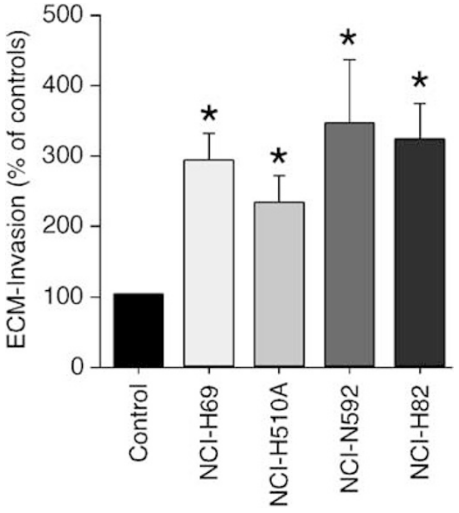

Figure 6 SDF-1/CXCL12 induces ECM invasion by SCLC cells. SCLC cells were incubated for 48 h with 200 ng/ml CXCL12 separated by a micropore filter coated with ECM proteins. The coated filters then were removed, stained, and SCLC cell invasion was quantified microscopically. The control represents random invasion of the individual SCLC cell lines into ECM without CXCL12 stimulation. The bars represent the mean (±s.e.m.) relative invasion of the four SCLC cell lines displayed on the *x*-axis into ECM in response to CXCL12. The ECM invasion is displayed as a relative value, as indicated on the *y*-axis, to account for differences in random migration of the individual cell lines. The asterisks above the error bars indicate significant differences in comparison to the controls with *P*-values that are <0.05

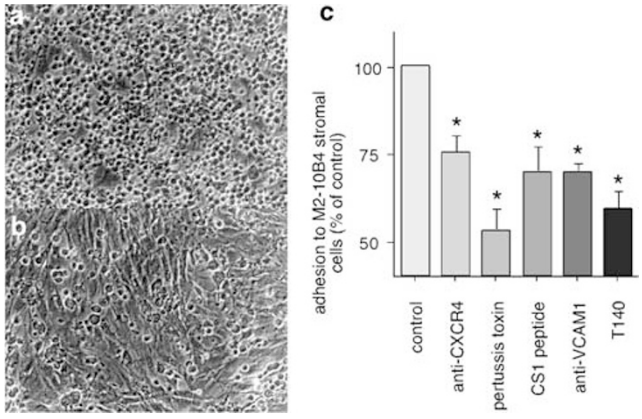

Figure 7 SCLC cell adhesion to M2-10B4 marrow stromal cells involves CXCR4 chemokine receptor activation. (**a**) NCI-H82 SCLC cells adhere to stromal cells after incubation on confluent M2-10B4 cells. Nonadherent SCLC cells were removed by vigorously washing the plate prior to taking phase contrast micrographs at ×200 magnification. (**b**) Pertussis toxin pretreatment significantly reduced the adhesion of SCLC cells to stromal cells. (**c**) Inhibition of SCLC cell to marrow stromal cells. Prior to the addition of a representative SCLC cell line (NCI-N592) to the stromal cell layer, SCLC cells were pretreated with different inhibitors that are displayed on the *x*-axis. The bars represent the mean (+s.d., $n=4$) relative adhesion of the SCLC cells to stromal cells after pretreatment with the inhibitors in comparison to untreated controls (100%)

CXCL12 enhances migration into an ECM layer

A prerequisite for a CXCR4/CXCL12-mediated homing of SCLC cells to the bone marrow is the migration and ability to invade ECM in response to SDF-1. To test the biological relevance of CXCR4 expression on SCLC cells, cell invasion was studied using a reconstituted ECM on filters of a Boyden chamber system. Invading cells migrate through and attach to the bottom of the membrane, whereas noninvading cells remain in the upper chamber and are removed prior to staining of the membranes for cell counting. SDF-1 activation resulted in an increased ECM invasion of SCLC cell lines that was 2.9±0.4-fold (NCI-H69; $n=12$), 2.3±0.4-fold (NCI-H 510A; $n=4$), 3.4±0.9-fold (NCI-N592; $n=4$), or 3.2±0.5-fold (NCI-H82; $n=4$) compared to unstimulated controls.

CXCL12 induces adhesion to stromal cells

M2-10B marrow stromal cells secretes high levels of bioactive CXCL12 (Burger *et al*., 1999). To further investigate the role of CXCR4/CXCL12 interaction for bone marrow metastasis of SCLC, we investigated whether SCLC cells can interact with marrow stromal cells that are considered the predominant source of CXCL12 *in vivo*. Coculture of SCLC cells with marrow stromal cells induced firm adhesion of the SCLC cells to the stromal cell layer. Figure 7a depicts phase-contrast micrographs of untreated (a) or pertussis toxin pretreated (b) NCI-H82 cells cultured on M2-10B4 stromal cells after vigorous washes to remove nonadherent cells in order to illustrate the effects of inhibited SCLC cell adhesion to stromal cells. As demonstrated by the inhibition studies displayed in Figure 7c, this adhesion is dependent on CXCR4/CXCL12 interaction, because it can significantly be inhibited by a specific CXCR4 inhibitor (T140), by mAbs to CXCR4, and by pertussis toxin that inhibits $G_i\alpha$ signalling of G-protein-coupled receptors, such as CXCR4. SCLC cell adhesion was also significantly inhibited by synthetic CS-1 peptide that blocks $\alpha4\beta1$ integrin (VLA4, CD 49d) binding or by anti-VCAM-1 mAbs. The data summarized in Figure 7c represent the mean (±s.e.m.) relative adhesion of NCI-N592 SCLC cells to marrow stromal cells in comparison to untreated NCI-N592 controls from four independent experiments.

CXCL12 activates α4β1 integrins on SCLC cells and enhances adhesion to immobilized VCAM-1

In order to extravasate to a target tissue, blood-borne cells must firmly arrest on vascular endothelium and then transmigrate. Surface-bound SDF-1 and vascular cellular adhesion molecule-1 (VCAM-1/CD106) expressed on bone marrow endothelium are critical molecules for hematopoietic progenitor cell homing to the marrow (Imai *et al*., 1999; Peled *et al*., 1999, 2000). We find that SCLC cells can adhere to immobilized VCAM-1 (CD106). This adherence was significantly enhanced by coimmobilized SDF-1/CXCL12 in NCI-N592, NCI-H345, and NCI-H82 (Figure 8a). Control experiments, in which adhesion of SCLC cells in the presence of immobilized SDF-1 was analysed on

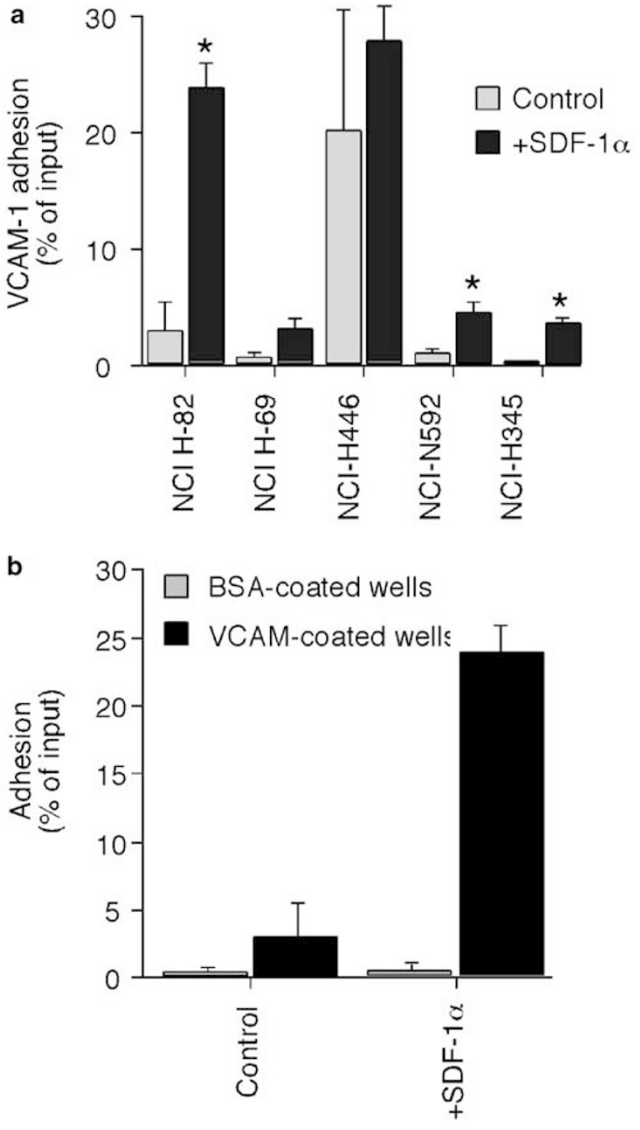

Figure 8 (**a**) SCLC cell adhesion to immobilized VCAM-1 is enhanced by CXCL12. Coimmobilized CXCL12 induced a significant increase in SCLC cell adhesion to VCAM-1 in three out of five SCLC cell lines, as indicated by the asterisks depicted above the bars with *P*-values <0.05. The black bars represent the relative proportion of adhering SCLC cells in comparison to SCLC cell adhesion to VCAM-1 without CXCL12 (gray bars). (**b**) Adhesion of SCLC cells (NCI-H82) is dependent on immobilized VCAM-1. Wells were precoated with either BSA (open bars) or VCAM-1 (solid bars) and CXCL12 was coimmobilized (right-hand bars). The bars represent the mean relative proportion of adherent SCLC cells

VCAM-1- or BSA-coated plates demonstrate that this adhesion was dependent on VCAM-1 (Figure 8b).

CXCL12 induces p42/44 mitogen-activated protein kinase (MAPK) activation in SCLC cells

Engagement of CXCR4 by SDF-1/CXCL12 induced a robust activation of p42/44 MAPK. Figure 9a displays a representative time course of p44/42 MAPK activation (1, 5, 15, and 30 min) after stimulation of NCI-H146

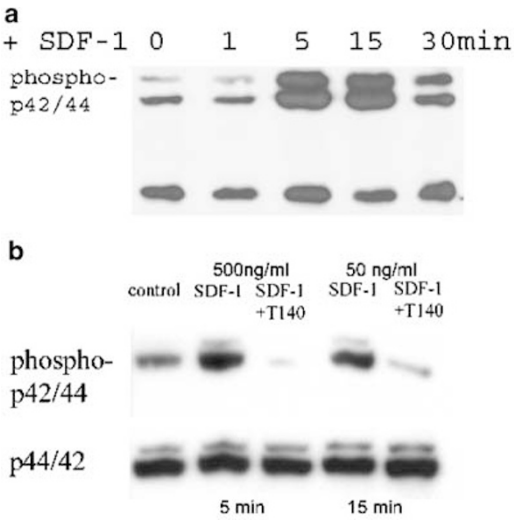

Figure 9 CXCL12 induces p42/44 MAPK activation in SCLC cells. (**a**) SCLC cells (NCI-H146) were incubated with 200 ng/ml SDF-1α and cell lysates of equal amounts of cells were prepared at the indicated time points. (**b**) SCLC cells (NCI-H146) were preincubated with 100 μg/ml T140 for 30 min before stimulation with SDF-1α (500 or 50 ng/ml) for 5 and 15 min, respectively. Western blot analysis was performed using phospho-specific p42/44 MAPK antibodies

SCLC cells with 200 ng/ml SDF-1. These data are representative for MAPK activation experiments performed with four different SCLC cell lines (NCI-H510A, NCI-N592, NCI-H69, and NCI-H146). Cell lysates were blotted with a phospho-specific p42/44 antibody, which show a strong increase in p42/44 activation upon stimulation with 200 ng/ml SDF-1/CXCL12 with a maximum 5–15 min after stimulation. As shown in Figure 9b, the phosphorylation of p42/44 can be inhibited by using the specific CXCR4 inhibitor T140. In the experiment displayed, two different concentrations of SDF-1 were used for stimulation, as described in the figure legend.

Discussion

A major function of chemokines is the recruitment of leukocytes to sites of infection and inflammation. Moreover, constitutively secreted chemokines regulate the trafficking and homing of leukocytes (Butcher and Picker, 1996; Baggiolini, 1998; Zlotnik and Yoshie, 2000). Recently, it has been proposed that chemokine receptors also play a critical role in determining the metastatic destination of tumor cells (Geminder *et al*., 2001; Moore, 2001; Muller *et al*., 2001; Murphy, 2001; Robledo *et al*., 2001; Taichman *et al*., 2002). SCLC is characterized by an early and widespread metastasis. Residual tumor cells resistant to the initial chemotherapy are responsible for relapses, treatment failure, and death of SCLC patients. Interactions with accessory cells within the tumor microenvironment can regulate, for example via integrin activation, the resistance of SCLC cells to cytotoxic drugs (Sethi *et al*., 1999). In this

study, we demonstrate that SCLC cells express functional CXCR4 chemokine receptors for the chemokine SDF-1 (CXCL12). SCLC cell lines or primary SCLC tumor cells express CXCR4 mRNA and surface protein, respectively. Stimulation of SCLC cells by the CXCR4 ligand CXCL12 triggers responses that are similar to leukocyte responses to chemoattractants, such as CXCR4 receptor endocytosis, actin polymerization, and migration into ECM. As such, SCLC cells display a response pattern to CXCL12 similar to leukocyte activation by chemoattractants. *In vivo*, CXCL12 is constitutively secreted by stromal cells within the marrow microenvironment and other tissues that represent destinations of SCLC cell metastasis such as liver, lung, and lymph nodes (Muller *et al*., 2001). As such, stromal cell-derived CXCL12 can activate CXCR4 chemokine receptors on SCLC cells. Several recent studies indicate that the CXCR4/CXCL12 axis is the principal mechanism for marrow homing of normal or malignant hematopoietic cells. HSCs are uniquely selective in their migratory response to CXCL12, necessary for the specific homing of circulating HSC, as well as for the retention of HSC in marrow microenvironment (Wright *et al*., 2002). CXCR4- or CXCL12-deficient mice display severe defects in hematopoiesis due to premature release of immature hematopoietic cells into the circulation (Nagasawa *et al*., 1996, 1998; Ma *et al*., 1999). We and others recently characterized the importance of CXCR4 receptors for marrow homing in hematologic malignancies, such as B-cell chronic lymphocytic leukemia (Burger *et al*., 1999), multiple myeloma (Sanz-Rodriguez *et al*., 2001), or acute myelogenous leukemia (Mohle *et al*., 1998). Involvement of the CXCR4 chemokine receptor in bone marrow metastasis of nonhematopoietic malignancies has been suggested by recent studies that demonstrated functional CXCR4 receptors on breast cancer (Muller *et al*., 2001), prostate cancer (Taichman *et al*., 2002), melanoma (Robledo *et al*., 2001), and neuroblastoma cells (Geminder *et al*., 2001). In accordance with these reports on tumors that frequently show bone marrow involvement, our observation that SCLC cell lines or primary tumor samples express CXCR4 receptors provides new mechanism to explain the distinct metastatic pattern in lung cancer. SCLC preferentially metastasizes to the bone marrow, whereas bone metastases in NSCLC generally involve the osseous tissue. However, in contrast to our observation that primary NSCLC cells display no or very low detectable CXCR4 surface receptors by means of immunohistochemistry (Figure 3d), Phillips *et al*. (2003) found functional expression of CXCR4 on NSCLC cell lines and primary tumors and performed studies that suggest a role of the SDF-1/CXCR4 axis in NSCLC metastasis. Compared to the CXCR4 expression levels in SCLC cells, as determined by flow cytometry (Figure 2), NSCLC cells displayed relatively low surface expression in Phillips' study. Moreover, the MAPK activation seen in NSCLC by Phillips *et al*. (2003) was rather weak when compared to the robust activation seen in SCLC (Figure 9a). As such, our lack of CXCR4 detection in NSCLC cells by immunohistochemistry may be due to a relatively lower CXCR4 expression in NSCLC cells along with a lower sensitivity of our immunohistochemistry method.

Marrow stromal cells are a dominant source of CXCL12 *in vivo* and retain their capacity to secrete high amounts of this chemokine *in vitro* (Burger *et al*., 1999). Coculture of SCLC cells with marrow stromal cells induces adhesion of SCLC that could be inhibited by anti-CXCR4 mAbs, the specific CXCR4 inhibtior T140, pertussis toxin, anti-VCAM-1 antibodies, or CS1 peptide that blocks $\alpha4\beta1$ integrin binding. Furthermore, CXCL12 enhances the adhesion of SCLC cells to immobilized VCAM-1, demonstrating that CXCL12 can trigger integrin binding. Interactions with a specialized tumor microenvironment, involving adhesive interactions with accessory cells or ECM and resulting in integrin activation favor the survival of SCLC cells and could explain the partial responses and local recurrence of SCLC often seen clinically after chemotherapy. Integrins are a family of transmembrane adhesion receptors composed of noncovalently linked α and β subunits. Integrins exist in distinct activation states, which exhibit different affinities for ligand. The $\alpha4\beta1$ and $\alpha5\beta1$ integrin adhesion molecules (VLA-4 and VLA-5) and their respective ligands on bone marrow endothelium and stromal cells (VCAM-1 and fibronectin) play a key role for the homing of hematopoietic cells to the marrow (Simmons *et al*., 1992; Teixido *et al*., 1992; Papayannopoulou *et al*., 1995; Verfaillie, 1998). We found that $\alpha4$ and $\beta1$ integrin subunit expression is high in SCLC cells, but $\beta7$ is absent that makes $\alpha4\beta1$ integrins (VLA-4) the exclusive ligand for VCAM-1. In addition, we demonstrated that CXCL12 induces increased adhesion of SCLC cells to immobilized VCAM-1, which indicates a cooperation between chemokine receptors and integrins in modulating SCLC cell adhesion. An increased $\alpha4\beta1$ (VLA4) integrin-mediated adhesion to VCAM-1 or fibronectin induced by CXCL12 was demonstrated recently for hematopoietic progenitors (Peled *et al*., 2000; Hidalgo *et al*., 2001) and has been implicated in modulation of stem cell trafficking and homing within the marrow microenvironment, but has not yet been shown in nonhematopoietic cells such as SCLC. These data indicate that CXCR4 receptors and $\alpha4\beta1$ integrins play a major role in trafficking and heterotypic adhesion of SCLC cells within the tumor microenvironment.

Sethi *et al*. (1999) reported that adhesion of SCLC cells to ECM via $\beta1$ integrins activates SCLC cells tyrosine kinase and protects SCLC cells from chemotherapy-induced apoptosis. They found that adhesion to ECM or a 'feeder' layer of fibroblasts is essential for SCLC cell expansion and resistance to chemotherapy. Consistent with their concept that signals from the tumor microenvironment confer survival signals to SCLC cells, we found that stimulation of SCLC cells with synthetic CXCL12 induced a rapid, transient activation of p44/42 MAPK (ERK 1/2), a key signaling pathway for promoting cell survival through transcription-dependent and -independent mechanisms (Xia *et al*.,

1995; Nebreda and Gavin, 1999; Chang and Karin, 2001).

Most recently, Kijima *et al*. (2002), reported that SCLC cells express functional CXCR4 chemokine receptors, as demonstrated by RT–PCR, flow cytometry, cell motility and proliferation assays, and Akt and S6K phophorylation in response to synthetic CXCL12/SDF-1. Their studies have been performed using several SCLC cell lines, whereas this study includes CXCR4 immunohistochemistry on primary tumor samples from patients with SCLC or NSCLC (Figure 3), indicating that CXCR4 is expressed in SCLC *in vivo* and therefore might be of clinical relevance in this disease. In this study, we furthermore report that CXCL12 induces invasion of SCLC cells into ECM (Figure 6) and integrin activation on SCLC cells. CXCL12 induced invasion and cooperation between CXCR4 and integrin activation are findings that provide novel insights into the mechanism of SCLC cell motility. Stromal cells are a major source of CXCL12 *in vivo*. We demonstrate that CXCL12 constitutively secreted by stromal cells induces adhesion of SCLC cells to marrow stromal cells. Another distinct finding in our study is that CXCL12 induces a robust, transient activation of p44/42 MAPK that was almost completely blocked by CXCR4 antagonists (Figure 9). Collectively, these observations provide novel insights into the molecular mechanism of SCLC cell activation by accessory cells in the tumor microenvironment, and supports Sethi *et al*'s. (1999) concept that such interactions are involved in regulation of SCLC cell motility and survival (as outlined above).

Future studies will have to evaluate whether antagonists of CXCR4 activation on SCLC cells, such as T140 and its derivates, can affect SCLC cell survival and/or chemosensitivity. Additional studies will furthermore have to define whether signals from CXCR4 receptors synergize with those induced by integrin activation in regulating the organization of the cytoskeleton, transcriptional activation, cell proliferation, and drug resistance in SCLC. Such approaches may lead to new therapeutic avenues for patients with SCLC.

Materials and methods

Cell culture, chemokines, antibodies, and flow cytometry

The SCLC cell lines NCI-H82, NCI-H69, NCI-H146, NCI-H446, NCI-H345, NCI-H510A, NCI-H592, belonging to the classic subclass of SCLC, and the murine stromal cell line M2-10B4 were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. The cells were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS) and penicillin–streptomycin–glutamine (Gibco-BRL, Grand Island, NY, USA). Prior to functional assays, cells were transferred to RPMI-1640 medium supplemented with 5 μg/ml insulin, 10 μg/ml transferrin, 30 nM sodium selenite, and 0.25% bovine serum albumin (BSA), as described earlier (Sethi *et al*., 1999).

Synthetic human SDF-1α was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The following mAbs specific for human surface antigens were used: anti-CXCR4–phycoerythrin (PE), clone12G5, anti-CD49d, anti-VCAM-1 (CD106), and the appropriate isotype controls from PharMingen (San Diego, CA, USA). For flow cytometry, the cells were adjusted to a concentration of 5×10^6 cells/ml in RPMI-1640 with 0.5% BSA. A total of 5×10^5 cells/ml were stained with saturating antibody concentrations for 30 min at 4°C, washed three times, and then analysed on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA). Flow cytometry data were analysed using the FlowJo 3.3 software (Tree Star, Inc, San Carlos, CA, USA). The cyclic peptide inhibitor containing the minimal CS1-VLA-4 binding motif 'LDV' (H-CWLDVC-NH2) was kindly provided by Dr NJ Zvaifler (UCSD, La Jolla, CA, USA), and was used as described earlier (Boyle *et al*., 2000; Burger *et al*., 2001). The specific CXCR4 inhibitor T140, a 14-residue peptide, was used as described earlier (Tamamura *et al*., 1998). T140 possesses the highest inhibitory activity of all CXCR4 antagonists that have been reported to date.

CXCR4 RT–PCR

RNA was isolated from SCLC cell lines (1×10^7 cells each) using the Qiagen RNAeasy kit (Qiagen, Santa Clarita, CA, USA). RNA then was used for first-strand cDNA synthesis with the superscript reamplification system (Gibco-BRL, Rockville, MD, USA), according to the manufacturer's instructions. The following CXCR4-specific primers were used: 5′ primer, GGA GAA TTC TTA CCA TGG AGG GGA TCA; 3′ primer, GGA GAA TTC AGC TGG AGT GAA AAC TTG. The annealing temperature was 58°C and the reaction proceeded for 30 cycles. To control for the presence of intact RNA, RT–PCR for human GAPDH was performed, as described (Burger *et al*., 2001).

Immunohistochemistry

Tissue samples of primary tumors from patients suffering from SCLC ($n = 10$) and NSCLC ($n = 10$) were fixed in formalin and embedded in paraffin. Sections (3 μm) were dewaxed in xylene and rehydrated through graded alcohol to water. Antigen retrieval was carried out by boiling the samples for 3 min in EDTA buffer, followed by pretreatment with 0.05% Protein K (Sigma-Aldrich, Taufkirchen, Germany). Antigens were stained using the alkaline phosphatase–antialkaline phosphatase (APAAP) method (DAKO, Hamburg, Germany) as described by the manufacturer. Monoclonal anti-CXCR4 antibodies (12G5, Pharmingen, San Diego, CA, USA) were used at a concentration of 1 : 60. MOPC-21 mouse ma's (Sigma) were used as an isotype control.

CXCR4 receptor endocytosis assay

Receptor downregulation was performed as described (Burger *et al*., 1999). Briefly, SCLC cells each were adjusted to a concentration of 5×10^6 cells/ml in RPMI-1640 with 0.5% BSA. The cells were incubated with SDF-1α at increasing concentrations for 1 h at 37°C in 5% CO_2. Cells were washed with a 20-fold volume of ice-cold buffer without FCS, stained with saturating antibody concentrations of PE-labeled anti-CXCR4 mAbs for 30 min on ice, washed two times with ice-cold buffer, and then were analysed by flow cytometry.

Actin-polymerization assay

Actin-polymerization assay was performed as described earlier (Barak *et al*., 1980; Schraufstatter *et al*., 2001). Briefly, NCI-H82 and NCI-H446 SCLC cells were seeded on Labtech-eight-well-chamber slides precoated with 0.01% poly-L-lysine Solution (Sigma, St Louis, MO, USA). NCI-H82 and NCI-H446 cells were serum starved over night and then were

stimulated with 200 ng/ml SDF-1α at different time points. To stop the reaction, cells were placed at 4°C and fixed for 20 min in 3% paraformaldehyde in PBS, permeabilized for 5 min with 0.2% Triton X-100, incubated with 5 mU/ml FITC–phalloidin (Molecular Probes) for 30 min, washed three times with PBS and mounted with Antifade (Molecular Probes). Fluorescence microscopy was performed on a Zeiss Axiovision 2.0 microscope with the axioplan 2.0 software to obtain digital images.

ECM invasion assay

Migration of SCLC cells into micropore filters coated with ECM proteins was performed according to the manufacturer's instructions (Cell invasion kit, Chemicon Int. Inc., Temecula, CA, USA). Briefly, SCLC cells at a concentration of 1×10^6 were placed in the top chamber of a two-chamber assay system and incubated for 48 h with or without 200 ng/ml SDF-1 placed in the lower chamber. The two chambers are separated by a micropore filter (8 μm pore size) coated with ECM proteins. The ECM layer occludes the membrane pores, blocking noninvasive cells from migrating through, but allowing the chemokine to penetrate through the filter to activate the cells. Invasive cells migrate into the coated filter. After the incubation period the coated filters were removed, stained, and SCLC cell invasion was quantified microscopically by counting the cells that had migrated into the filters. All filters were counted independently by two people.

Phospho-p44/42 MAPK assay

The p44/42 MAPK assays were performed as described earlier (Burger *et al*., 2000). Briefly, SCLC cells were serum starved for 4 h, and lysates from 1×10^6 cells per sample were prepared after stimulation with 200 ng/ml SDF-1α at the indicated time points. Equal amounts of protein were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto PDVF membranes. Western blot analysis was performed using p44/42 and phospho-p44/42 antibodies that specifically recognize the phosphorylated form of p44/42 MAPK protein (Cell Signalling Technology, Inc., Beverly, MA, USA). Immunoreactive bands were visualized using horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody and the enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany).

SCLC–stromal cell adhesion assay

In order to quantify the adhesion and to analyse the effects of inhibitors on SCLC cell adhesion to stromal cells, we modified our recently reported adhesion assay (Burger *et al*., 1999, 2001) as outlined below. The murine stromal cell line M2-10B4 that secretes high amounts of SDF-1 (Burger *et al*., 1999) was seeded the day before the assay onto collagen-coated 24-well plates at a concentration of 1.5×10^5 cells per well in RPMI-1640 supplemented with 10% FCS. In order to distinguish SCLC cells from stromal cells during the flow cytometry-based analysis of SCLC cell adhesion to stromal cells, SCLC cells were prelabeled with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions. For inhibition studies, SCLC cells were pretreated with 100 ng/ml pertussis toxin (Calbiochem, La Jolla, CA, USA), 10 μg/ml anti-CXCR4 antibodies (PharMingen), 10 μg/ml anti-VCAM antibodies (R&D Systems, Minneapolis, MN, USA), 100 μg/ml T140, or 50 μg/ml CS-1 peptide, for 30 min at 37°C, and seeded on the stromal cell layer at a concentration of 1×10^6 cells per well. The plates were incubated for 4 h at 37°C in 5% CO_2. After the incubation period, SCLC cells that had not formed firm adhesion to the stromal cells were removed by vigorously washing the wells with RPMI three times. The stromal cell layer with adhering SCLC cells was detached with trypsin/EDTA solution (Gibco/BRL, Grand Island, NY, USA). Cells were then resuspended in 0.5 ml medium for counting by flow cytometry. A gate was set using the PKH26 labeling of the SCLC to exclude stromal cells from the counts. Duplicate samples were counted at high flow for 20 s to determine the relative number of cells that adhered to the stromal cells. In addition, the adhesion of SCLC cells to stromal cells and the integrity of the stromal cell layer was assessed by phase-contrast microscopy and documented photographically at $\times 200$ magnification. Initial adhesion experiments were performed using the seven SCLC cell lines described above. For inhibition studies, NCI-N592 SCLC cells were used.

SCLC cell adhesion to immobilized VCAM-1

In all, 96-well plates (Nalge Nunc International, Rochester, NY, USA) were coated with recombinant human VCAM-1 (R&D Systems, Minneapolis, MN, USA) prepared at 1.0 μg/ml in bicarbonate buffer (pH 9.0) and incubated overnight at 4°C. Wells were then washed with bicarbonate buffer and coated with recombinant human SDF-1 (R&D Systems) prepared at 1.0 μg/ml in bicarbonate buffer for 30 min at room temperature or with the buffer alone. Wells were subsequently washed and blocked with 20% FBS in PBS for 1 h at 37°C, followed by washing with Hank's balanced salt solution (HBSS) supplemented with HEPES. Cells resuspended at 10^5 per 50 μl of adhesion medium (HBSS buffered with HEPES and supplemented with 0.5% BSA) were added in triplicate to the wells and allowed to settle for 30 min at 37°C, followed by four washes with adhesion medium to remove nonadhered cells. The number of adhered cells was determined using CyQUANT Cell Proliferation Kit (Molecular Probes, Eugene, OR, USA) and fluorescence of the samples was measured by a Microtiter Plate Fluorometer (DYNEX Technologies, Chantilly, VA, USA).

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