Downregulation of CXCR4 by SDF-KDEL in SBC-5 cells inhibits their migration in vitro and organ metastasis in vivo

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Abstract. Metastasis is the principal cause of morbidity and mortality in cancer patients. The master genes that govern organ-selective metastasis remain elusive. We compared the expression levels of C-X-C chemokine receptor type 4 (CXCR4) in the human small cell lung cancer (SCLC) cells, SBC-5 and SBC-3, by flow cytometric analysis and found that CXCR4 was expressed at markedly higher levels in the SBC-5 cells which can produce multiple organ metastasis, particularly bone metastasis compared to the SBC-3 cells. Stromal-derived-factor-1 (SDF-1)-CXCR4 has been shown to regulate cell migration and metastasis in a variety of types of cancer; however, the roles of SDF-1-CXCR4 in the organ-selective metastasis of SCLC in vivo remain to be elucidated. Thus, in this study, we constructed a phenotype of SBC-5 cells in which CXCR4 was knocked out using the intrakine strategy and found that the downregulation of CXCR4 inhibited cell migration and invasion, but did not affect cell proliferation or apoptosis in vitro. In vivo experiments, the knockout of CXCR4 suppressed the development of metastatic lesions in the lungs, liver and bone, but did not decrease metastasis to the kidneys. Our data demonstrate that CXCR4 is a candidate gene involved in the development of metastatic lesions in specific organs, such as the lungs, bone and liver, which can secrete high concentrations of SDF-1, the sole ligand of CXCR4. Thus, CXCR4 may prove to be a promising target for the prevention and effective treatment of metastastic lesions due to SCLC.

Key words: SDF-1-CXCR4, intrakine, metastasis, small-cell lung cancer

Introduction

Metastasis is the principal cause of morbidity and mortality in cancer patients. It occurs during the late stages of tumor progression and is an exceedingly complex event (1). Of note, certain types of cancer show a predilection to metastasis to specific organs (2). In 1889, Stephen Paget set forth the ‘seed and soil’ hypothesis of metastasis, to explain the non-random pattern of metastasis (3). The theory states that metastasis is dependent on the cross-talk between selected cancer cells (the ‘seeds’) and specific organ microenvironments (the ‘soil’), and this theory still holds forth today. However, the precise molecular mechanisms, regulatory circuits and master metastasis-associated genes that govern these fatal changes remain elusive (4). Small cell lung cancer (SCLC) accounts for 15-20% of lung cancer cases and presents an aggressive clinical behavior characterized by rapid growth and spread to distant organs (5). Previously, Miki et al (6) examined the ability of various lung cancer cell lines to generate multiple organ metastasis, particularly bone metastasis by intravenously injecting the cancer cells into natural killer (NK) cell-depleted SCID mice; they found that the human the SCLC cell line, SBC-5 was the only one to generate bone metastasis; bone metastasis was not generated by the SBC-3 cells (the SBC-3 cell line was originally established from the bone marrow aspirate of a 24-year-old male patient with SCLC). Although the SBC-5 and SBC-3 cells have a similar genetic background, they differ in their potential to generate bone metastasis. In this study, we compared the surface expression of C-X-C chemokine receptor type 4 (CXCR4) proteins in SBC-5 and SBC-3 cells by flow cytometric analysis, and the results demonstrated that CXCR4 protein expression was markedly higher in the SBC-5 cells compared with the SBC-3 cells. It has previously been demonstrated that CXCR4 and stromal-derived-factor-1 (SDF-1) regulate migration and metastasis in certain types of cancer (7); however, the roles of SDF-1-CXCR4 in the organ-selective metastasis of SCLC remain to be elucidated.

The term intrakine (intracellular chemokine) refers to the strategy used to genetically silence a certain (cytokine) receptor. This is achieved by the fusion of the chemokine gene with an endoplasmic reticulum (ER) retention signal (KDEL), termed ‘intrakine’, which can bind to the newly synthesized protein and lead to its degradation.
chemokine receptor molecules within the cell and block their surface expression. This method was originally proposed as gene therapy for AIDS (8-10). Thereafter, it has been used as a tool for the functional analysis of chemokines and their receptors in vitro and in vivo (11).

In the present study, we knocked down CXCR4 expression in SBC-5 cells using the intrakine strategy and evaluated the biological behavior of these cells, including proliferation, apoptosis, cell cycle progression, invasion and migration in vitro. In addition, we used the multiple organ metastasis model of human SCLC cells to study organ metastasis in vivo. Our results revealed that CXCR4 was a candidate gene involved in metastasis to specific organs, such as the lungs, liver and bone. However, the silencing of CXCR4 did not affect the proliferation and apoptosis of the SBC-5 cells in vitro. CXCR4 may prove to be a promising target for the prevention and effective treatment of metastatic lesions due to SCLC.

Materials and methods

Cell culture. The human SCLC cell lines, SBC-5 and SBC-3, were gifts from Professor Saburo Sone and Professor Seiji Yano (Tokushima University, Tokushima, Japan). They were maintained at 37°C with 5% CO₂ in RPMI-1640 supplemented with 10% (v/v) heated-inactivated fetal bovine serum (FBS; both from Gibco, Gaithersburg, MD, USA), 100 U/ml streptomycin and 100 U/ml penicillin.

Flow cytometric analysis of CXCR4 expression in SBC-5 and SBC-3 cells. SBC-5 and SBC-3 cells (or stably transfected SBC-5/S-K and SBC-5/neo cells) were collected and washed with phosphate-buffered saline (PBS) supplemented with 0.5% BSA. The cells were then resuspended to a final concentration of 4x10⁵ cells/ml, 25 µl of which were extracted for staining. In brief, this was followed by the addition of 10 µl carboxyfluorescein-conjugated mouse anti-human CXCR4 (clone 12G5) monoclonal antibodies (FAB170F; R&D Systems, Minneapolis, MN, USA) and incubation for 30 min at 4°C. Subsequently, the cells were washed with PBS to remove the unreacted antibodies and were then resuspended in 200-400 µl of PBS. The cell surface expression of CXCR4 was measured using a flow cytometer (BD Biosciences, San Jose, CA, USA) using a 488 nm wavelength laser excitation. The cells expressing CXCR4 were fluorescently stained, with the intensity of staining determined by flow cytometry using the Annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA, USA). Absorbance was detected at reference wavelengths of 490 and 630 nm using an ELISA plate reader (Multiskan MK3; Thermo Fisher Scientific, Inc., Waltham, MA, USA). This assay was performed 3 times.

In vitro cell proliferation assay. To measure the cell proliferation of the SBC-5/S-K and SBC-5/neo cells, cells at 80% confluence were harvested and placed into 96-well plates (1,000 cells/well). Each day 1 plate was used for MTT assay. A total of 20 µl MTT solution (5 mg/ml) was added to each well followed by incubation at 37°C for 4 h. Subsequently, the MTT solution was removed and 150 µl of dimethyl sulfoxide (DMSO) were added to dissolve the formazan crystals. Absorbance was detected at reference wavelengths of 490 and 630 nm using an ELISA plate reader (Multiskan MK3; Thermo Fisher Scientific, Inc., Waltham, MA, USA). This assay was performed 3 times.

Soft agar colony formation assay. Twenty-four-well plates were covered with 0.6% agar in RPMI-1640 medium containing 10% FBS to prevent the attachment of the cells to the plastic substratum. Cell suspensions (1x10³ cells/well) of the SBC-5/S-K and SBC-5/neo cells with 0.3% agar were prepared and seeded on the foundation agar. After 2 weeks of incubation at 37°C, the colonies containing at least 50 cells were counted under an inverted microscope (IX53; Olympus). All assays were performed 3 times.

Cell apoptosis and cell cycle analysis. Cell apoptosis was determined by flow cytometry using the Annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA, USA). Cells (1x10⁵) were collected and washed twice with cool PBS. The cells...
were then resuspended in 1X binding buffer. Annexin V-FITC and PI were then added and the cells were incubated at room temperature for 15 min in the dark. The cells were again washed with cool PBS twice. Finally, 500 µl PBS were added to the mixture which was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell cycle distribution was determined by the following steps: the cells were resuspended at 1x10^6 cells/ml, fixed with 75% ethanol at 4°C overnight, washed and resuspended with cool PBS. Subsequently, 5 µl RNase (10 mg/ml) were added, and the cells were fixed for 1 h at 37°C; 100 µg/ml propidium iodide in a 0.1% sodium citrate/0.1% Triton X-100 solution was then added, followed by incubation for 30 min at room temperature in the dark. After the cells were washed, the analysis of cellular DNA content was carried out using a flow cytometer (BD Biosciences, San Jose, CA, USA) at an excitation wavelength of 488 nm. The distribution of these cells in 3 major phases of the cell cycle (G1, S and G2 phase) was analyzed using CellQuest and ModFit software (BD Biosciences, San Jose, CA, USA).

In vitro migration and invasion assay. To detect cell migration in vitro, 24-well Transwell plates containing filters of 8.0 µm pore size (Costar, Cambridge, MA, USA; Matrigel uncoated) were used. The SBC-5/S-K and SBC-5/neo cells (5x10^4) in RPMI-1640 medium containing 0.1% BSA (Sigma-Aldrich Co., St. Louis, MO, USA) were placed into the upper chamber of the wells and RPMI-1640 containing 10% FBS was added to the lower chamber. The Transwell plates were incubated for 36 h. The filters were fixed with 10% formalin, and stained with crystal violet. The cells on the upper surface of the filters were removed by swabbing with a cotton swab and the cells migrated to the lower surface were counted under a microscope (IX53; Olympus) at x200 magnification. Ten fields for each sample were counted and analyzed. All assays were performed 3 times.

Cell invasion assays were performed using Matrigel-coated (BD Biosciences, Le Pont-de-Claix, France) 24-well Transwell plates. The other steps were the same as those used for the migration assay.

Determination of metastasis in vivo. All animal experiments were performed according to the Guidelines on Animal Experimentation formulated by the Forth Military Medical University, Xi'an, China. The SBC-5/S-K and SBC-5/neo cells were harvested and only a single cell suspension with ≥90% cell viability were used. NOD-SCID mice at 3-4 weeks of age (from the Institute of Biochemistry and Cell Biology of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, China) were divided into 2 groups and each group consisted of 2 male and 3 female mice. The SBC-5/S-K and SBC-5/neo cells (1x10^6/300 µl) were injected into the tail vein of the mice, which were maintained under specific pathogen-free conditions. After 5 weeks, the mice were anesthetized and bone metastases were visualized by X-ray images. The number of osteolytic bone metastases on the X-ray images was evaluated independently by 2 investigators (Dr Haichuan Su and Professor Minzhang Tang, Department of Oncology, Tangdu Hospital, The Fourth Military Medical University, Xi'an, China).

Subsequently, the mice were sacrificed by anesthesia and all the major organs were removed. The number of metastatic lesions >0.5 mm in diameter on the surface of the major organs was counted macroscopically. The lungs were fixed in Bouin's solution for 24 h. The major organs with metastatic lesions were fixed in 10% formalin. The bone specimens were decalcified in 10% EDTA solution for 1 week and then embedded in paraffin.

Statistical analysis. The Wilcoxon rank sum test was used to determine the significance of the differences in the number and incidence of metastatic lesions in multiple organs/tissues (bone, lungs, liver and kidneys) between 2 groups, and the other data were analyzed by variance analysis or the t-test. Statistical tests were performed using SPSS software version 13.0.0 (SPSS Inc., Chicago, IL, USA). A value of P<0.05 was considered to indicate a statistically significant difference and all statistical tests performed were two-sided.

Results

Expression levels of CXCR4 in SBC-5 cells are higher than those in SBC-3 cells. We determined the expression levels of CXCR4 in the SBC-5 and SBC-3 cells by flow cytometric analysis and found that the CXCR4 expression rate was 53.04±1.35% in the SBC-5 cells and 25.91±0.78% in the SBC-3 cells (P=0.000002) (Fig. 1). This assay indicated that the surface expression of CXCR4 in the SBC-5 cells was markedly higher than that in the SBC-3 cells.

Expression of the recombinant fusion protein SDF-KDEL in SBC-5/S-K cells. After we confirmed the recombinant plasmid PCMV-S-K by colony PCR and DNA sequencing (Fig. 2), the PCMV-S-K vector and the PCMV mock vector were transfected into the SBC-5 cells and the stably transfected cells were designated as SBC-5/S-K and SBC-5/neo cells, respectively. Of these cells, 80% were GFP-positive within 24 h after transfection (Fig. 3A). We examined the location of the recombinant fusion protein, SDF-KDEL, in the SBC-5/S-K and SBC-5/neo cells by immunofluorescence staining. SDF-1 was mainly expressed in the cytoplasm of the SBC-5/S-K cells, particularly in the perinuclear region (Fig. 3C). However, the expression of SDF-1 was not detected in the SBC-5/neo cells (Fig. 3B).

Downregulation of CXCR4 in SBC-5/S-K cells compared with SBC-5/neo cells. To examine the biological activity of the produced SDF-KDEL fusion protein, the expression of CXCR4 in the SBC-5/S-K cells was monitored by flow cytometry. The CXCR4 expression rates in the SBC-5/S-K and SBC-5/neo cells were 10.08±1.49 and 50.50±2.31%, respectively (P=0.000006) (Fig. 4). These results indicated that the cells transfected with the recombinant plasmid, pCMV-S-K, presented with a significantly reduced expression of CXCR4 on the cell surface.

Downregulation of CXCR4 has no effect on cell proliferation. Subsequently, the effects on cell proliferation of the downregulation of CXCR4 in the SBC-5 cells were analyzed by MTT assay and soft agar colony formation assay. From the cell proliferation curve, we concluded that the proliferation of the SBC-5/S-K cells did not differ significantly from that of the SBC-5/neo cells (Fig. 5A). Furthermore, the colonies formed by these 2 types of cells also showed no differences (210.75±10.89 and 226.25±13.31, P=0.4; Fig. 5B). These results
suggested that the downregulation of CXCR4 had no effect on the proliferation of the SBC-5 cells. 

*Downregulation of CXCR4 does not affect the apoptotic rate of SBC-5 cells.* The analysis of cell apoptosis revealed that the
apoptotic rates of the SBC-5/S-K and SBC-5/neo cells were 0.19±0.0967 and 0.2275±0.15%, respectively (P=0.84; Fig. 6). This assay indicated that the downregulation of CXCR4 did not affect the apoptotic potential of the SBC-5 cells.
Downregulation of CXCR4 does not affect the cell cycle distribution of SBC-5 cells. The percentage of SBC-5/S-K and SBC-5/neo cells in the G1 phase of the cell cycle was 49.64±5.56 for SBC-5/S-K and 44.78±1.75 for SBC-5/neo, respectively. These results indicated that the downregulation of CXCR4 did not affect the cell cycle distribution of SBC-5 cells.

Downregulation of CXCR4 inhibits the migration and invasion of SBC-5 cells. Through preliminary experiments, we found that there was a marked difference in the number of SBC-5/S-K and SBC-5/neo cells that had migrated through the insert (33±1.73 for SBC-5/S-K and 80.2±4.2 for SBC-5/neo, P=0.000006; Fig. 8A). The number of invaded SBC-5/S-K and SBC-5/neo cells was 19.2±0.86 and 39.6±1.96, respectively (P=0.00001; Fig. 8B). These results indicated that the downregulation of CXCR4 significantly decreased the invasion and migration capability of the SBC-5 cells.

Downregulation of CXCR4 inhibits metastasis in vivo. Finally, we examined the role played by CXCR4 in vivo. At the 5th week after inoculation, paralysis (possibly caused by spinal cord compression and bone metastases in the hind limbs) occurred in 1/5 mice in the SBC-5/S-K group and in 5/5 mice in the SBC-5/neo group. In addition, bone metastasis occurred in 3/5 mice in the SBC-5/S-K group, but in 100% of the mice in the SBC-5/neo group (Table II). The number of metastatic lesions observed in the bone, liver and lungs in the SBC-5/S-K group was significantly decreased compared with that in the SBC-5/neo group (P=0.01; Table II); however, the number of metastatic lesions observed in the kidneys did not differ significantly the 2 groups (P>0.05). These results revealed that the downregulation of CXCR4 significantly inhibited specific metastasis to the bone, liver and bone. The images of bone metastatic lesions are shown in Fig. 9.

Discussion

Chemokines are a large family of small, structurally related heparin-binding proteins classified as XCL, CXCL, CCL and CX3CL1 subfamilies depending on the number and spacing of conserved cysteine residues near the N-terminus. Chemokines interact with seven-transmembrane G protein-coupled chemokine receptors. More than 40 chemokines and 18 chemokine receptors have been discovered, and some chemokines bind to
multiple chemokine receptors or vice versa (12). Chemokines were noted initially for their ability to stimulate the directional migration of nearly all classes of leukocytes (13). Recent evidence indicates that members of the chemokines and their receptors may play critical roles in tumorigenesis and/or metastasis (14).

CXCR4 is by far the most common chemokine receptor that has been demonstrated to be overexpressed in a broad array of human cancer tissues, but its expression is low or absent in many normal tissues (15). Its sole ligand, CXCL12, was found mainly in 2 isoforms $\alpha$ and $\beta$, and isoform $\alpha$ is constitutively produced in multiple tissues, including lung, liver and bone tissue (16). There is growing evidence that CXCR4 and SDF-1 regulate migration and metastasis in various types of cancer (17). It has been reported that high levels of CXCR4 expression positively correlate with bone metastasis in breast cancer patients (18). GST-NT21MP, an antagonist of CXCR4 was identified to inhibit the progression of breast cancer (19). Therefore, targeting CXCR4 may be a promising strategy for the treatment of human cancer. The overexpression of CXCR4 in pancreatic cancer, melanoma and neuroblastoma plays an important role in the progression and organ-specific metastasis (20-23). Thus, CXCR4 is considered one of several genes which contribute to bone metastasis in cancer (24). Endothelial cell-derived CXCL12 may trigger the integrin activation to promote the adhesion of cancer cells to the extracellular matrix or accessory cells within the tumor microenvironment. Of note, cancer cells can be retained in the metastatic microenvironment which may confer protection against chemotherapy, and which may be responsible for residual disease and relapses (25,26).

In this study, the expression of CXCR4 was knocked down using the intrakine strategy with the recombinant plasmid, PCMV-S-K. It was confirmed that the downregulation of CXCR4 significantly inhibited invasion and migration in vitro and metastasis in vivo. Although the number of metastatic lesions observed in the lungs, bone and liver was decreased in the mice injected with PCMV-S-K cells, metastasis to the kidneys was not suppressed. These results indicated that SDF-1-CXCR4 mediated organ-specific metastasis in SCLC. The mechanisms of action of the SDF-1-CXCR4 axis remain unclear, and thus future studies are required on this issue.

Whether CXCR4 is involved in the survival and proliferation of tumor cells remains controversial, perhaps as this is tumor dependent. The activation of extracellular signal-regulated kinase and Akt can both potentially contribute to the survival and growth of tumor cells (27,28). SDF-1-CXCR4 has been implicated in the organ-specific metastases of many types of cancer, including bone-specific metastasis. The bone marrow is a hypoxic microenvironment and is rich in hypoxia-inducible factor-1 (HIF-1). HIF-1$\alpha$ and CXCR4 co-operate to regulate the adaptation and survival of cancer- and metastasis-initiating cells (29). In addition, CXCR4 expression can be upregulated by the hypoxia response element, HIF-1$\alpha$ (30,31). In a relay multistep navigation process, the hypoxia-HIF-1$\alpha$-CXCR4 pathway may regulate trafficking in and out of hypoxic tissue microenvironments, which is in favor of cell proliferation, migration, invasion and the formation of tumor/metastases.

Metastases arise following the spread and subsequent growth of cancer cells from a primary site to distant tissues. There are likely to be some special requirements for the formation of metastasis, as evidenced by the existence of a class of genes termed ‘metastasis-related genes’, which regulate the development of metastatic tumors, but have relatively little effect on the growth of primary lesions. In view of these

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**Table II. Metastatic lesions formed in NOD-SCID mice following inoculation with SBC-5/S-K and SBC-5/neo cells for 5 weeks.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Bone Incidence N</th>
<th>Lung Incidence N</th>
<th>Liver Incidence N</th>
<th>Kidney Incidence N</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBC-5/S-K</td>
<td>3/5 (0-3)$^a$</td>
<td>5/5 (2-5)$^a$</td>
<td>5/5 (2-4)$^a$</td>
<td>5/5 (4-6)</td>
</tr>
<tr>
<td>SBC-5/neo</td>
<td>5/5 (4-6)</td>
<td>5/5 (7-9)</td>
<td>5/5 (7-11)</td>
<td>5/5 (3-7)</td>
</tr>
</tbody>
</table>

The incidence of metastases in mice and the number of metastatic lesions in bone, lungs, liver and kidneys at the 5th week after inoculation of the 2 groups of cells. Values are shown as the means ± SD. $^a$P=0.01 compared with the control group (Wilcoxon rank sum test).

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![X-ray images of osteolytic bone metastases](image_url)

**Figure 9. X-ray images of osteolytic bone metastases formed following inoculation of the cancer cells for 5 weeks.** (A and B) Bone metastatic lesions in the SBC-5/neo group; (C and D) bone metastatic lesions in SBC-5/S-K group.
biological functions mediated by CXCR4, it can be concluded that CXCR4 is a gene involved in metastasis in SCLC, and the blockade of the interaction between SDF-1 and CXCR4 may lead to the development of a novel therapeutic strategy for the prevention and treatment of organ-specific metastases in SCLC.

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References