Targeting CXC motif chemokine receptor 4 inhibits the proliferation, migration and angiogenesis of lung cancer cells

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Abstract. An increasing volume of data indicates that disrupting the interaction between CXC motif chemokine receptor 4 (CXCR4) and its specific ligand, CXC motif chemokine 12 (CXCL12), may reduce tumor growth and metastasis. However, the translation from bench to bedside must be performed with extreme caution, as the CXCR4/CXCL12 axis is crucial for the normal development and maintenance of tissues and organs. In the present study, Cell Counting Kit-8 and Transwell migration assays were used to detect in vitro proliferation and chemotaxis of CXCR4-expressing A549 cells, a cell strain originating from human non-small-cell lung cancer (NSCLC), with or without the presence of AMD3100, a small-molecule inhibitor specific to CXCR4 signaling. In a xenograft model established by injecting nude mice with A549 cells, tumor growth, CXCR4 expression and microvessel density (MVD) in the tumor mass were determined through tumor size measurements and immunohistochemical staining following intraperitoneal administration of AMD3100 or vehicle. The results demonstrated that CXCR4 blockade inhibited the proliferation of A549 cells and their migration towards CXCL12 in vitro. Tumor growth, CXCR4 expression and MVD were markedly reduced in nude mice treated with AMD3100 compared with mice treated with the vehicle. In conclusion, the present data demonstrated that CXCR4 targeting impaired NSCLC cell growth, angiogenesis and metastatic spread, indicating that it may represent a novel treatment strategy for NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide in males and females. Non-small-cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases (1,2). Despite continuous endeavors to develop novel treatment strategies, the majority of patients with lung cancer exhibit a poor prognosis (1,2). Basic biological behaviors of cancer cells, including migration, angiogenesis and cross-talk between cancer cells and their microenvironment, serve critical roles in tumor growth and metastasis; chemokines are considered to be specific major modulators in these biological processes (3).

Chemokines, also referred to as chemotactic cytokines, are proteins secreted into the blood circulation that mediate numerous physiological and pathological processes primarily associated with cell homing and migration through interactions with the chemokine receptors present on target cells (4). Accumulating data have indicated that tumor growth, invasion and metastasis may be facilitated by the overexpression of chemokine receptors on cancer cells (3), among which CXCR4 is the most extensively investigated. CXCR4, a type of 7-transmembrane G protein-coupled receptor, is the cognate receptor for stromal cell-derived factor-1 (CXCL12) and is expressed on naive T cells, natural killer cells, dendritic cells and monocytes (5). Recently, it was also identified to be overexpressed in several different types of human cancer, such as breast and pancreatic cancer (6). Meta-analyses (7,8) and clinical (9,10) have demonstrated that CXCR4 expression is associated with organ-specific metastasis and poor survival in patients with NSCLC. An increasing number of studies have elucidated the mechanisms by which chemokine receptors are involved in cancer progression, resulting in the design of corresponding antagonists (11), several of which have already been identified to be effective in certain tumor types according to preliminary (12,13). However, the data are conflicting and the knowledge is incomplete regarding the complexity and diversity of the biological effects induced by CXCR4-CXCL12 (3).

In the present study, A549, a cell strain originating from human NSCLC, was used as an in vitro model. The interaction of CXCR4 with CXCL12 was blocked by a CXCR4 antagonist, AMD3100, followed by evaluation of A549 cell proliferation and migration towards CXCL12 with Cell Counting Kit-8.
(CCK-8) and Transwell migration assays. In a preclinical model developed by inoculating nude mice with A549 cells, tumor size and microvessel density (MVD) were compared between experimental and control mice. In addition, the effect of AMD3100 administration on CXC4R expression was also determined by joint evaluation of the extent and intensity of immunohistochemical (IHC) staining. The results of the present study demonstrated targeting CXC4R decreased the proliferation, migration and angiogenesis of lung cancer cells.

Materials and methods

Reagents. A CCK-8 kit was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA). The Transwell chamber was purchased from Corning Life Sciences (Corning, NY, USA). AMD3100 was supplied by MedChem Express (cat. no. HY-10046; Monmouth Junction, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO) for the cell assay and in sterile PBS for animal administration. Recombinant human CXC12 protein was purchased from R&D Systems, Inc. (cat. no. 350-NS; Minneapolis, MN, USA). Monoclonal rabbit anti-human CXC4R antibody was provided by Abcam (cat. no. ab181020; Cambridge, UK). Mouse anti-human CD34 monoclonal antibody (cat. no. GM716502), horseradish peroxidase (HRP)-labeled anti-rabbit/mouse secondary antibody (cat. no. GP016129) and DAB coloring agent (cat. no. GK500510A) were all purchased from Gene Tech Biotechnology Co. Ltd. (Shanghai, China).

Animals and cell lines. The A549 human alveolar adenocarcinoma cell line was a gift from Professor Liu Ming (Guangzhou Institute of Respiratory Diseases, Guangzhou, China) and maintained in RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA, cat. no. 10099-141), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO2 incubator. Female BALB/c nude mice (n=10, 4-6 weeks old, 15-18 g) were provided by Beijing Vital River Laboratory Animal Technology Company (Beijing, China) and housed under specific pathogen-free conditions at 25±2°C in a 12 h night/dark cycle. All animals were fed sterilized rodent food, and has unrestricted access to food and water. They were cared for in accordance with the Animal Welfare Act guidelines under an animal protocol approved by Guangzhou Medical University Animal Care and Use Committee. For experimental end-points, tumor-bearing mice were euthanized prior to the subcutaneous tumor reaching 20 mm in any direction, but all animals exhibiting signs including restriction of mobility, the inability to feed, pressure on internal organs or sensitive regions of the body or a body condition score (14) of <2 were euthanized, even if the maximum tumor size had not been reached.

Cell proliferation assay. The cell proliferation assay was performed using a CCK-8 kit, according to the manufacturer's protocol. Briefly, A549 cells grown to 80% confluence were rinsed with 0.25% Trypsin/EDTA solution (Thermo Fisher Scientific Inc.) and 2 ml of trypsin solution was added to detach the cells. The detachment was monitored under an inverted microscope for 2-5 min at a magnification of x100. A total of 8 ml of RPMI 1640 complete medium were added to cease cell detachment and cells were washed twice with phosphate-buffered saline (PBS) followed by centrifugation at 4°C, 100 x g for 5 min. The cells were suspended in complete medium and plated in 96-well plates at a density of 10,000 cells/well and cultured for 12 h in RPMI-1640 growth medium containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO2 incubator. The culture supernatant was removed and growth medium with AMD3100 was added to a final concentration of 2 µmol/l for an additional 0, 24, 48, 72 or 96 h culture. An equal volume of growth medium with vehicle alone was added to the control wells. Each medium was added to triplicate wells. Prior to the end of the culture (4 h), AMD3100-containing medium was replaced with 110 µl growth medium containing 10 µl CCK-8 solution. Following a 4 h incubation, the optical density (OD) of each well was determined at 450 nm with an enzyme-linked immunometric meter (BIOBASE-EL10A, BioBase, Jinan, China), and the average OD values were calculated from triplicate wells. The experiment was repeated 3 times independently.

Chemotaxis assay. A549 cells grown to 80% confluence were harvested as described previously and resuspended in growth medium composed of 2 µmol/l AMD3100 under the aforementioned culture conditions. The cells treated with vehicle alone (DMSO) were used as the control. After 48 h, all cells were serum-starved and cultured for 12 h, followed by assessing chemotaxis to CXCL12. A chemotaxis assay was performed in triplicate in 24-well Transwell chambers with polycarbonate membranes of 8-µm pore size. Briefly, 2x104 A549 cells in 100 µl serum-reduced RPMI-1640 medium containing 1% bovine serum albumin (MedChemExpress) were added to the upper chamber of each well, and 700 µl RPMI-1640 medium containing 10% FBS and 10 ng/ml soluble CXCL12 was added to the lower chamber. Chemotaxis was allowed at 37°C in a 5% CO2 incubator for 12 h. Cells adhering to the upper surface of the membrane were removed with a cotton swab. Cells that had migrated through the filter and adhered to the lower surface of the membrane were fixed by 4% paraformaldehyde at room temperature (RT) for 30 min and stained with 0.1% crystal violet solution at RT for min. Following continuous washing 3 times with running water for 5 min each, the membranes were removed from the Transwell inserts and Image-Pro Plus software (Ver. 6.0, Media Cybernetics, Inc., Rockville, MD, USA) was used to count the cells under the light microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan) in 6 randomly selected fields at a magnification of x400.

Nude mice lung cancer model. For the xenograft model, 1x106 A549 cells were inoculated subcutaneously in the right flank of each BALB/c nude mouse. The length and width of the subcutaneous tumor were measured regularly and the tumor volumes were calculated using the formula: Volume=(length x width^2)/2. Tumors were not permitted to exceed 2 cm in diameter; all mice only developed one subcutaneous tumor. Once the tumor width had reached 0.5 cm, the mice were randomly divided into two groups (n=5/group). The mice in the two groups received intraperitoneal administration of either AMD3100 (1.25 mg/kg body weight; treatment group) or vehicle (100 µl sterile PBS; control group) twice daily.
IHC staining for CXCR4 and CD34 expression. Staining procedures were performed according to the antibody manufacturer's protocol. Briefly, tissues embedded in paraffin were cut into 4-μm sections, deparaffinized and rehydrated in a graded alcohol series (100, 95, 80 and 70%, for 1 min each at RT), prepared from absolute alcohol (cat. no. 64-17-5; Shanghai Macklin Biochemical Co., Ltd., Shanghai, China). Following antigen retrieval in sodium citrate buffer (10 mM, pH 6.0; Haoran Bio-Pharma Co., Ltd., Shanghai, China), endogenous peroxidase was blocked by 3% H₂O₂ in methanol for 10 min at RT. Tissue sections were then blocked with normal goat serum (cat. no. ab7481; Abcam) for 30 min at 37°C and then incubated with rabbit anti-human CXCR4 (1:400 dilution) or mouse anti-human CD34 (1:200 dilution) at 4°C overnight. Subsequent to washing (1X PBS, three times, 5 min each), the tissue sections were incubated with HRP-labeled goat anti-rabbit/mouse antibody (1:100 dilution) at 37°C for 15 min. The tissue sections were incubated with the freshly prepared DAB coloring agent (50-100 μl) at room temperature for 3-10 min, examined under a microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan) at a magnification of x200 and counterstained with 0.2% hematoxylin for 2 min at RT.

Scoring of CXCR4 expression. CXCR4 expression was quantified as previously described (16,17), with modifications. In brief, two scoring systems were used to quantify CXCR4 staining. The percentage of positive tumor cells was referred to as staining extent (E), which was graded on a scale of 0-3 as follows: 0, ≤5%; 1, 6-20%; 2, 21-50%; and 3, >50%. Staining intensity (I) was also graded on a scale of 0-3 as follows: 0, none; 1, weak staining (+); 2, moderate staining (++); and 3, strong staining (+++). The product of E x I, referred to as the EI score, ranged from 0 to 9, and was used to quantify the CXCR4 expression of each section. In view of the heterogeneous expression of CXCR4 in tumor tissues, the total EI score for any section was calculated as a sum of EI scores for 2-3 different fields of view.

Microvessel counting. Microvessel counting was performed as previously described (18) under a microscope (at x40 and x100 magnification, DM2500, Leica Microsystems Inc.,Wetzlar, Germany) and pictures were taken by a color camera (TK-C9501EC, Victor Company of Japan, Limited). In brief, the slides were examined at low magnification (x40 and x100) to identify the highest vascular density areas (hot spots) within the tumor, and then 3-4 areas of highest neovascularization were selected for counting at a magnification of x200 (0.739 mm²/field). The mean number of microvessels of these areas was recorded as the MVD level. Any brown-stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells and other connective tissue elements, was considered as a single countable microvessel. Vessel lumina and red blood cells were not necessary for defining a microvessel. Macrovessels, characterized by thick muscular walls or with lumina >8 red blood cells in diameter (~50 μm), were excluded from the count.

Statistical analysis. All data were presented as mean ± standard error of the mean. GraphPad Prism v. 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for graph plotting and statistical analysis. Relative cell viability was compared using a two-way analysis of variance, followed by Bonferroni’s multiple comparison test. The Mann-Whitney U test was used for comparison between two means. P<0.05 was considered to indicate a statistically significant difference.

Results

CXCR4 blockade effectively inhibits in vitro proliferation of A549 cells and their migration towards CXCL12. AMD3100, a selective small-molecule inhibitor of CXCR4 signaling, was added into the culture medium of A549 cells, and cell viability and migration were observed. As shown in Fig. 1A, cell viability in the control group increased rapidly with increases in culture time and until the endpoint of incubation (>6-fold enhancement in relative cell viability). Regarding the AMD3100-treated group, relative cell viability exhibited a markedly slower increase, followed by a decline 96 h later (P<0.05). At the end of the incubation, the cell viability was increased by a little more than 1-fold. CXCR4-expressing A549 cells migrate towards their specific ligand, CXCL12 (6); this phenomenon is referred to as chemotaxis. However, when blocked by AMD3100, the number of A549 cells that migrated through the chamber membrane was markedly reduced compared with vehicle control (P=0.001; Fig. 1B). The crystal violet staining demonstrated that cells that had migrated through the filter and adhered to the lower surface of the membrane were increased in the vehicle group (Fig. 1C) compared with the AMD3100 group (Fig. 1D).

CXCR4 blockade decelerates tumor growth in nude mice with NSCLC. To determine whether AMD3100 was able to decelerate tumor growth in vivo, a NSCLC xenograft model was established by subcutaneously injecting nude mice with human A549 cells. Mice bearing xenograft tumors were treated with AMD3100 or vehicle, and the tumor volumes were measured. At the endpoint of the study (Day 19), all mice were sacrificed and larger tumor masses were macroscopically observed in vehicle-treated mice than AMD3100-treated mice (Fig. 2A). In addition, tumor growth curve showed that tumor growth was significantly slower in the AMD3100-treated mice compared with the vehicle group (P=0.0247; Fig. 2B).

CXCR4 expression and vascularization are inhibited by an antagonist. Tumor xenografts were removed from euthanized mice and CXCR4 expression in tumor cells and MVD in the tumor mass were additionally analyzed by IHC staining. It was demonstrated that CXCR4 expression was localized to the cell membranes (Fig. 3A and B). Tumor sections from AMD3100-treated mice exhibited significantly decreased total EI scores of CXCR4 expression compared with those from...
control mice (P=0.0179; Fig. 3C), the EI score is considered to be positively associated with antigen expression level (16). Subsequently, the microvessels in the tumor mass were visualized by CD34 antigen staining (Fig. 3D and E). By calculating microvessel numbers per mm² slice, a decreased MVD was observed in AMD3100-treated mice compared with that in control mice (P=0.0159, Fig. 3F). In summary, these data indicate that CXCR4 targeting is likely to downregulate tumor expression of CXCR4 and impair tumor vascularization.

Discussion

CXCR4 belongs to the superfamily of G protein-coupled receptors (GPCRs) that possess seven transmembrane domains (5). CXCR4 is constitutively identified and widely expressed by numerous cell types, including the majority of hematopoietic cell types in the blood and bone marrow, vascular endothelial cells, Langerhans cells, neurons and neuronal stem cells (4). Through binding to CXCL12, which is widely expressed in multiple organs, including the colon, liver, brain, lungs, heart, kidney and spleen, CXCR4 is extensively involved in various physiological functions, including embryonic hematopoiesis, organogenesis, vascularization and immune surveillance, and pathological processes, including inflammation, tumorigenesis and wound healing (4). The roles of chemokine/chemokine receptor interactions in cancer biology have been attracting an increasing amount of attention. As it is overexpressed in >23 different types of human cancer (4), CXCR4 is considered
to contribute to tumor growth, angiogenesis, metastasis and resistance to treatment (6,12). With regards to lung cancer, clinical data indicated that CXCR4 overexpression was significantly associated with poorer progression-free survival time and overall survival rate (OS) (8,9,19). A number of experimental studies demonstrated that in vitro or in vivo inhibition of CXCR4/CXCL12 interaction via CXCR4 blockade (13,15,20,21) attenuated NSCLC cell growth, migration, angiogenesis and metastasis and increased the sensitivity of cancer cells to chemotherapy (22). It appears that this axis may represent a promising target for the development of novel anticancer chemokine-based therapeutics. In fact, multiple agents targeting CXCR4/CXCL12 signaling in cancer are currently being developed (4), among which the CXCR4 antagonist AMD3100 is the most extensively investigated (12). AMD3100, also referred to as plerixafor, was initially studied as an anti-HIV agent, and it was then identified to increase the white blood cell count in the blood and mobilize stem cells from the bone marrow (12). In 2008, AMD3100 gained Food and Drug Administration approval for mobilization of hematopoietic stem cells for bone marrow transplantation, rather than for cancer treatment (23).

Despite the collective aforementioned evidence mentioned supporting the potential efficacy of CXCR4 inhibition for the treatment of cancer, there remains controversy and uncertainty regarding the role of the CXCR4/CXCL12 axis in cancer growth and metastasis. For example, regarding the prognostic value of CXCR4 overexpression, a number of studies concluded that CXCR4 expression was associated with unfavorable prognosis (8-10,13). However, a number of studies also reported different, even opposite conclusions: CXCR4 was detected on the cell membrane, in the cytoplasm and in the nucleus of gastric cancer and NSCLC cells, and strong CXCR4-positive nuclear staining was associated with a significantly improved OS rate (24,25), although the underlying mechanisms remain unclear. In addition, there are data indicating that CXCR4 expression exerted no significant effect on patient survival and was not significantly associated with any other clinicopathological variables (26). Furthermore, increased levels of CXCR4 expression in the tumor cells in comparison with tumor cells with no or low levels of CXCR4 expression from the patients with lung adenocarcinoma, were even identified to be an independent predictor of an improved prognosis in patients with lung adenocarcinoma (27), whereas epigenetic silencing of CXCR4 expression facilitated metastasis and progression of cervical cancer by causing loss of cell-to-cell adhesions (28). Additionally, contradictory data have also been demonstrated regarding the association between CXCL12 expression and clinical outcome (26,29). Despite laboratory data supporting the inhibition of tumor growth and metastasis by CXCL12 expression (30,31), it is reasonable to hypothesize that the CXCR4/CXCL12 axis may exhibit an important antitumor role under certain conditions (32). Discrepancies in observations indicate that our knowledge of certain aspects of this chemokine axis remains incomplete, and additional investigations are required. The therapeutic strategies that block CXCR4 signaling must undergo continued evaluation prior to clinical application, due to the ubiquitous expression of CXCR4 in normal tissues and the functional importance of CXCR4-CXCL12 interaction.

In our previous study (33), 61 patients with NSCLC were analyzed with IHC staining for CXCR4 expression. An increased CXCR4 expression rate was observed in tumor tissues compared with normal lung tissue and a positive association of CXCR4 expression with tumor stage and lymph node metastasis was identified. Despite the lack of data on the association between CXCR4 expression and prognosis, it is well-established that a more advanced tumor stage is associated with poorer survival (9). In the present study, the data indicated that CXCR4 blockade markedly attenuated in vitro cell proliferation and migration towards CXCL12, which was consistent with the aforementioned observations described by a number of studies (4,11-13). In addition, in the present study a xenograft tumor model was established to mimic patients with NSCLC. Following continuous intraperitoneal administration of a CXCR4 antagonist, tumor growth in nude mice was demonstrated to be markedly decreased compared with control mice due to the pronounced inhibition of CXCR4 expression and neovascularization in the tumor mass.

It was demonstrated previously that CXCR4 engagement facilitates cancer cell proliferation, survival, angiogenesis and migration by stimulating multiple downstream signaling pathways, including the phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (12). AMD3100 prevented CXCR4 from binding to CXCL12 by occupying the ligand-binding pocket, thereby blocking the generation and downward transduction of activation signals (12). Cancer cell migration was previously identified as one of the key steps of distant cancer spread (34). CXCR4-expressing lung cancer cells migrated to tissues or organs with a high level of CXCL12, including lymph nodes, contralateral lung, liver and brain, finally forming metastatic loci (10,34). The CXCR4 antagonist was previously demonstrated to attenuate tumor metastasis by disrupting the interaction between CXCR4 and CXCL12 (15). Of note, the current immunostaining data demonstrated that CXCR4 blockade significantly downregulated CXCR4 expression by tumor tissues. Based on this result, it is reasonable to infer that decreased CXCR4 expression resulted in weakened CXCR4-CXCL12 interaction and resultant decreased metastasis. At present, little is known about how AMD3100 downregulates CXCR4 expression. Theoretically, CXCR4-associated downstream signaling pathways, including PI3K/Akt, MAPK and Janus kinase/Signal transducer and activator of transcription, all promote anti-apoptosis; therefore, apoptosis induction by signal inhibition is likely to be involved in this downregulation effect. In fact, previous data demonstrated that AMD3100 resulted in increased tumor apoptosis and necrosis (35), although knockdown of CXCR4 by small interfering RNA did not affect tumor cell apoptosis induced by chemotherapeutic agents (36). In the present study, large necrotic areas were observed in tumor tissue sections from mice treated with AMD3100 (data not shown), however the apoptosis data were not obtained and this result requires additional investigation. In addition, it must be mentioned that in the present study CXCR4 expression was only localized to cell membranes, which was not consistent with previous studies (24,25). In the experiment with human NSCLC tissues, positive CXCR4 staining was not observed in the nucleus, but in the cell membrane or the cytoplasm. This result may partly
explain our conclusion that high CXCR4 expression was associated with advanced tumor stage, in that patients with nuclear CXCR4 expression were identified to have an improved OS rate compared with those with membranous or cytoplasmic expression (24). Neovascularization is a key process in cancer survival and metastatic spread (15, 18). The CXCR4/CXCL12 axis was recognized as an important modulator of the angiogenesis/angiostasis balance (37). MVD, a measure of tumor angiogenesis, was reported by various studies (18, 38, 39) to be closely associated with the incidence of metastases and clinical outcome. By means of immunostaining against the CD34 antigen, a sensitive biomarker for blood vessels, the present study demonstrated a marked reduction of MVD in the tumor masses following treatment with the CXCR4 antagonist. Based on this result, a similar conclusion may be drawn that the CXCR4 blockade may decrease metastasis through inhibition of angiogenesis, in accordance with the findings of previous studies (11, 15).

In summary, the data of the present study lead to the conclusion that CXCR4 expression facilitates tumor growth and metastasis, and its targeting may induce antitumor effects to a certain extent by impairing tumor proliferation, migration and angiogenesis. However, despite the encouraging results of the present study and other preclinical data, it is too early to draw definitive conclusions on the role of the CXCR4/CXCL12 axis and its inhibition for cancer treatment. Increased efforts should be focused on elucidating conflicting aspects and improving the understanding of this axis, in order that targeted therapies may be safely applied in clinical settings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WH conceived the experimental design and wrote the manuscript, and WH, TY, XHG, RZQ, XDZ and WDL conducted the experiments. WH and TY analyzed the obtained data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were performed in accordance with the Animal Welfare Act guidelines under an animal protocol approved by Guangzhou Medical University Animal Care and Use Committee (Guangzhou, China).
Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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