Original Article

Role of chemokine receptor CXCR2 expression in mammary tumor growth, angiogenesis and metastasis

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Abstract

Background: Chemokines and their receptors have long been known to regulate metastasis in various cancers. Previous studies have shown that CXCR2 expression is upregulated in malignant breast cancer tissues but not in benign ductal epithelial samples. The functional role of CXCR2 in the metastatic phenotype of breast cancer still remains unclear. We hypothesize that the chemokine receptor, CXCR2, mediates tumor cell invasion and migration and promotes metastasis in breast cancer. The objective of this study is to investigate the potential role of CXCR2 in the metastatic phenotype of mouse mammary tumor cells. Materials and Methods: We evaluated the functional role of CXCR2 in breast cancer by stably downregulating the expression of CXCR2 in metastatic mammary tumor cell lines Cl66 and 4T1, using short hairpin RNA (shRNA). The effects of CXCR2 downregulation on tumor growth, invasion and metastatic potential were analyzed in vitro and in vivo. Results: We demonstrated knock down of CXCR2 in Cl66 and 4T1 cells (Cl66-shCXCR2 and 4T1-shCXCR2) cells by reverse transcriptase polymerase chain reaction (RT-PCR) at the transcriptional level and by immunohistochemistry at the protein level. We did not observe a significant difference in in vitro cell proliferation between vector control and CXCR2 knock-down Cl66 or 4T1 cells. Next, we examined the invasive potential of Cl66-shCXCR2 cells by in vitro Matrigel invasion assay. We observed a significantly lower number (52 ± 5) of Cl66-shCXCR2 cells invading through Matrigel compared to control cells (Cl66-control) (182 ± 3) (P < 0.05). We analyzed the in vivo metastatic potential of Cl66-shCXCR2 using a spontaneous metastasis model by orthotopically implanting cells into the mammary fat pad of female BALB/c mice. Animals were sacrificed 12 weeks post tumor implantation and tissue samples were analyzed for metastatic nodules. CXCR2 downregulation significantly inhibited tumor cell metastasis. All the mice (n = 10) implanted with control Cl66 cells spontaneously developed lung metastasis, whereas a significantly lower number of mice (40%) implanted with Cl66-shCXCR2 cells exhibited lung metastases. Conclusions: Together, these results suggest that CXCR2 may play a critical role in breast cancer invasion and metastasis.

Keywords: CXC chemokines, CXCR2, metastasis, tumor growth

INTRODUCTION

Chemokines and chemokine receptors have been described as essential and selective mediators of leukocyte trafficking to inflammatory sites and to secondary lymphoid organs. Apart from their role in immune cell chemotaxis, chemokine signaling through their receptors plays a critical role in cancer.
CXCR2 signaling modulates angiogenesis and metastasis in various cancers; however, its function in breast cancer has not been elucidated to date. Understanding the role of CXCR2 in breast cancer metastasis will aid in the development of adjuvant therapies by targeting CXCR2.

CXCR2 is a G-protein coupled receptor[1] whose activity is controlled by the ligand it interacts with.[2,3] CXCR2 shares a significant homology with the chemokine receptor, CXCR1, but they differ considerably in their ligand specificity. CXCR2 interacts with a wide range of chemokines including CXCL8, CXCL1 (Groα), CXCL2 (Groβ), CXCL3 (Groγ) CXCL5 and CXCL6 (GCP-2), but the binding specificity for CXCR1 is limited to CXCL8 and CXCL6.[4] The mouse homologue mCXCR2 binds to the CXC ligands mCXCL1 (KC) and mCXCL2 (MIP2).[5,6] mCXCR2 can also be activated by human GRO chemokines CXCL1–CXCL3 and CXCL8.[7] Expression of CXCR1 and CXCR2 has been observed in several normal cells such as neutrophils and endothelial cells, and also in various tumor cells.[8–11] Both CXCR1 and CXCR2 were shown to bind CXCL8 with high affinity, and activation of CXCR1 and CXCR2 with CXCL8 can lead to cellular chemotaxis, cell growth and viability, angiogenesis, as well as transformation of cells. CXCR2 is expressed on a variety of tumor cells, including breast,[12] melanoma,[14] pancreatic,[15] and ovarian,[16] all cancers with a high metastatic index. In addition, it has been shown that activation of the CXCR2 receptor is a critical step in CXCL8-mediated angiogenesis.[17,18] Our laboratory has shown that CXCL8 is constitutively expressed in malignant melanoma and functions as an autocrine/paracrine growth, invasive and angiogenic factor.[19,20] Overexpression of CXCR2 and CXCR1 in otherwise nonmetastatic SBC-2 and A375P melanoma cells promotes tumor cell invasion and metastasis.[21,22]

Analysis of malignant and benign breast cancer specimens has shown that tumor cells express both CXCR1 and CXCR2 in all malignant samples compared to only 50% of benign ductal epithelial cells expressing these receptors.[12] Differential expression of CXCR2 was also observed in human breast cancer cell lines. Malignant breast cancer cell lines, such as MDA MB-231, MDA MB-436 and SKBR3, express high levels of CXCR2, whereas MCF-7 and T47-D cell lines, which are non-malignant, have lower CXCR2 expression.[23] Estrogen receptor (ER) negative breast tumors displayed higher CXCR2 1208C/T polymorphisms in cancer patients, and may affect disease progression.[24] These data suggest that CXCR2 expression in breast cancer tissue might be associated with progression and development of metastasis. To date, the role of CXCR2 in breast cancer metastasis is not clear. In this report, our data provide evidence for the role of CXCR2 expression in mammary tumor progression. Silencing CXCR2 expression in mammary tumor cells impairs their ability to metastasize to lung and also inhibits tumor angiogenesis and invasiveness.

MATERIALS AND METHODS

Cell culture
Murine mammary adenocarcinoma cell lines differing in their metastatic potential, 4T1 (highly metastatic), Cl66 (moderately metastatic), Cl66M2 (poorly metastatic), and BMI164 (a bone metastatic variant of Cl66), were used in this study.[25,26] Cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Mediatech, Hendon, VA, USA) with 5% serum (Biowhitaker, Walkersville, MD, USA) or 5% fetal bovine serum (FBS), 1% vitamins, 1% l-glutamine and 0.08% gentamycin (Invitrogen, Carlsbad, CA, USA).

Generation of shRNA-expression plasmids and CXCR2 knock-down cells
Silencing of CXCR2 gene expression was achieved using short hairpin RNA (shRNA) technology. shRNAs targeting CXCR2:(2sh-CCC CAA TAC AGC AAA CTG GCG GAT) and control shRNA targeting a sequence unrelated to known mouse genome sequences were used. 4T1 and Cl66 cells were stably transfected with control and CXCR2 shRNA plasmid vectors using lipofectamine reagent (Invitrogen) following the manufacturer’s protocol. Stably transfected clones of 4T1 (4T1-control, and 4T1-shCXCR2) and Cl66 (Cl66-control and Cl66-shCXCR2) cells expressing either shCXCR2 or vector control were isolated and maintained in medium supplemented with 1000 µg/ml of G418-sulfate (Invitrogen). To avoid clone-specific effects, pooled cultures were used for all the experiments.

mRNA expression analysis
Analysis of gene expression was performed using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as described.[27] Briefly, cDNA was synthesized from 5 µg total RNA using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT) primer. Two microliters of first strand cDNA (1:10 dilution) was amplified. The following primer sequences were used: CXCR2, 5′-ACT TTT CCG AAG GAC CGT CT-3′ (forward) and 5′-GTA ACA GCA TCC GCC AGT TT-3′ (reverse); CXCL-1, 5′-TGG CTT CTC TGT GCA GGC CT-3′ (forward) and 5′-GTC GTG TTG GAC ACT TAG TGG TCT C-3′ (reverse). For internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CGC ATT TGG TCG TAT TGG G-3′ (forward) and 5′-TGG TTT TGG AGG AGT CTC GC-3′ (reverse) was used. Amplified products were resolved...
through a 1% agarose gel containing ethidium bromide and analyzed using an Alpha Imager gel documentation system (AlphaInnotech, San Leandro, CA, USA).

**CXCR2 protein expression by immunofluorescence**

Cells were plated in 8-well chamber slides. After 24 h of incubation at 37°C, cells were fixed in cold acetone and methanol followed by incubation at −20°C for 10 min. After washing twice with phosphate buffer saline (PBS), the cells were blocked with antibody diluent (BD Biosciences, San Jose, CA, USA) for 30 min at room temperature (RT); anti-CXCR2 antibody (SC-683, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:100 dilution and incubated for 2 h at RT. Cy3 conjugated anti-rabbit antibody was used at 1:200 dilution and incubated for 30 min at RT. Cells were further washed with PBS and mounted with vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA). The stained cells were analyzed for CXCR2 surface expression using confocal microscopy (UNMC core facility).

**Cell proliferation assay**

Cells were seeded in 96-well plates at low density (1000 cells/well). Following overnight adherence, cells were incubated with media alone or medium containing different serum concentrations for 72 h. Cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay as described earlier.[28,29] The differences in absorbance were compared in vector control transfected cells and CXCR2 knock-down cells.

**Cell motility and invasion assay**

To investigate the effect of silencing CXCR2 expression on cell migration, cells (1 × 10⁴ cells/well) in serum free media were plated in the top chamber of noncoated polyethylene terephthalate membranes (6-well insert; 8 µm pore size; Becton Dickinson, Franklin Lakes, NJ, USA). For invasion, cells (1 × 10⁴ cells/well) were plated onto Matrigel-coated transwell chambers (24-well insert; 8 µm pore size; Corning Costar Corp., Cambridge, MA, USA) in serum free media. The bottom chamber contained 1.0 ml serum containing media. The cells were incubated for 24 h at 37°C and cells that did not pass through the membrane pores were removed. Migrated cells were stained using Hema 3 kit (Fisher Scientific Company L.L.C., Kalamaoo, MI, USA) per the manufacturer’s instructions. Cells were counted in 10 random fields (200X) and expressed as the average number of cells per field of view. The data were represented as the average of three independent experiments.

**Tumor growth and spontaneous metastasis analysis**

Female BALB/c mice (6–8 weeks old) were purchased from the National Cancer Institute and maintained under specific pathogen-free conditions. All procedures performed were in accordance with institutional guidelines and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Cl66-control or Cl66-shCXCR2 cells (1 × 10⁶ in 0.1 ml of Hank’s balanced salt solution) were injected orthotopically in the mammary fat pad (MFP) to study tumor growth and spontaneous metastasis. Tumor growth was measured twice weekly until the mice were sacrificed.

In the spontaneous mouse metastasis model, the primary tumor was surgically removed when the tumors reached 0.4 mm³ in size. Subsequently, MFP implanted tumor group was sacrificed at the end of 45 days after implantation. Tumor volume was calculated using the formula π/6 × (smaller diameter)² × (larger diameter). Tumors recovered from mice were fixed in 4% paraformaldehyde, embedded in paraffin and processed for histopathological evaluation and immunohistochemistry.

**Tumor microvessel density, cell proliferation and apoptosis analysis**

Immunohistochemical analysis was performed to determine microvessel density as previously described.[30] In brief, 6-µm-thick tumor sections were deparaffinized by xylene and ethanol (Biogenex, San Roman, CA, USA) and blocked for 30 min. Tumor sections were incubated overnight in a humid chamber with the following primary antibodies: antibody to proliferating cell nuclear antigen (anti-PCNA; Santa Cruz Biotechnology), biotinylated anti-GS-IB4 isolecint from *Griffonia simplicifolia* (Vector Laboratories) or anti-cleaved caspase-3 (Cell Signaling Technologies, Danvers, MA, USA). Corresponding biotinylated secondary antibody was used (except for GS-IB4) at RT. Immunoreactivity was detected using the ABC Elite kit and DAB substrate (Vector Laboratories) as per the manufacturer’s instructions. A reddish brown precipitate in the cytoplasm indicated a positive reaction. Negative controls had all the reagents except the primary antibody. The number of microvessels was quantitated microscopically with a 5 × 5 reticle grid (Klarmann Rulings, Litchfield, NH, USA) using a 400× objective (250 µm total area).

**Statistical analysis**

Differences between the groups were compared applying the unpaired two-tailed *t*-test using SPSS software (SPSS Inc., Chicago, IL, USA). *In vivo* analysis was done using the Mann–Whitney *U*-test. All the values are expressed as mean ± SEM. A *P* value of equal to or less than 0.05 was considered statistically significant.
RESULTS

Silencing of CXCR2 expression in metastatic mammary carcinoma cell lines

To evaluate the role of CXCR2 signaling in breast cancer progression and metastasis, we examined CXCR2 and CXCL1 expression in four mammary carcinoma cell lines with different metastatic potential. We observed constitutive expression of CXCL1 in all cell lines examined [Figure 1a]. CXCR2 expression was observed in Cl66, 4T1, and Cl66M2 cells [Figure 1a].

To investigate the functional significance of CXCR2 expression in mammary tumor growth and metastasis, we knocked down its expression using a pSuper.neo vector expressing shRNA targeting CXCR2. Quantification of CXCR2 mRNA expression by RT-PCR in control and shCXCR2 transfected Cl66 cells showed more than 50% reduction in the mRNA and protein (immunofluorescence) levels in Cl66 cells transfected with shCXCR2 vector (Cl66-shCXCR2) as compared to vector control transfected cells [Figure 1b, c]. Similarly, we observed significant inhibition of CXCR2 mRNA and protein expression (immunofluorescence) in shCXCR2 transfected 4T1 cells (4T1-shCXCR2) cells [Figure 1d].

CXCR2 knock down did not modulate cell proliferation in vitro

We examined whether knock down of CXCR2 in mammary tumor cells modulated cell proliferation. Vector control and shCXCR2 transfected Cl66 and 4T1 cells were incubated in medium with or without serum. We did not observe any significant difference in in vitro cell proliferation in Cl66-control and Cl66-shCXCR2 cells [Figure 2a]. Similarly, we observed no difference in 4T1-control and 4T1-shCXCR2 cell proliferation (data not shown).

CXCR2 knock down inhibits mammary tumor cell invasiveness

Next, we tested the hypothesis that CXCR2 signaling in breast cancer cells promotes tumor cell invasion. To test the role of CXCR2 signaling in tumor cell invasion, we performed an in vitro Matrigel invasion assay using Cl66-shCXCR2 and Cl66-control cells. Knocking down CXCR2 expression in...
Cl66 tumor cells significantly inhibited the ability of these cells to invade through Matrigel coated basement membrane [Figure 2b]. We quantified the invasiveness of the tumor cells by enumerating the number of cells that invaded Matrigel and migrated to the other side of the membrane. We observed a 72% reduction in Matrigel invasion in CXCR2 knock-down Cl66 cells compared to Cl66-control cells [Figure 2b].

**Silencing of CXCR2 expression inhibits spontaneous lung metastasis**

To test whether the *in vitro* results can also be observed during *in vivo* invasion of tumor cells, we implanted both Cl66-shCXCR2 and Cl66-control tumor cells in the MFP of female BALB/c mice. Tumor growth was monitored twice weekly and we did not find any significant difference in the tumor volume between the groups [Figure 3].

Primary tumors in the MFP were surgically removed at 21 days post implantation. This model allows us to evaluate the role of CXCR2 signaling in spontaneous metastasis of breast cancer cells. At the time of necropsy, we found a significant difference in the development of gross lung metastatic nodules between the groups [Figure 4a]. All the mice in the control group developed lung metastases, whereas only 40% of the mice in the Cl66-shCXCR2 group had gross lung metastases [Figure 4b]. We examined the lung sections for micrometastatic lesions, and we observed numerous metastatic nodules in lungs from mice with control tumors, but the CXCR2 knock-down group had significantly fewer lung micrometastases [Figure 4c]. Together, these data demonstrate a significant role for CXCR2 signaling in the modulation of the metastatic phenotype in mammary tumor cells.

**Silencing of CXCR2 increases apoptosis but does not alter in vivo proliferation**

Primary tumors from Cl66-control and Cl66-shCXCR2 injected mice were analyzed for *in situ* cell proliferation and apoptosis using immunohistochemistry. We did not observe a significant difference in PCNA-positive proliferating tumor cells in Cl66-control and Cl66-shCXCR2 tumors [Figure 5a]. Interestingly, the frequency of apoptotic caspase-3 positive cells was significantly higher in Cl66-shCXCR2 tumors as compared to Cl66-control tumors [Figure 5b]. These data, in combination with our *in vitro* data, suggest that knock down of CXCR2 has little or no effect on tumor cell proliferation, but rather modulates survival of mammary tumor cells.

**Silencing of CXCR2 expression inhibits angiogenesis**

Tumor-induced angiogenesis is important for tumor
growth and progression. The effect of CXCR2 silencing on tumor-induced angiogenesis was analyzed by isolectin B4 immunostaining on Cl66-shCXCR2 and Cl66-control tumors. Microscopic analysis of tumor tissue immunostained for blood vessels showed a significantly lower number of microvessels in Cl66-shCXCR2 tumors compared to Cl66-control tumors [Figure 5c].
DISCUSSION

Numerous studies have demonstrated that chemokines and chemokine receptors regulate a variety of biological functions in several tumors, including tumor cell proliferation, angiogenesis and metastasis. Expression of certain chemokine receptors and chemokines confers not only a proliferative advantage to tumor cells but also the ability to form metastases. CXCR2, a G-protein coupled receptor (GPCR), binds to ELR+ chemokines and plays an important role in the chemotaxis of immune cells and tumor-induced angiogenesis. CXCR2 binds to its ligand CXCL8 with high affinity. Expression of CXCR2 has been shown in several tumor types. The role of CXCR2 in breast cancer progression and metastasis remains unclear. In this report, we have shown that CXCR2 signaling is important in tumor cell invasion, angiogenesis and spontaneous lung metastasis formation.

Previous studies have shown that malignant breast cancer specimens express CXCR2 and its expression is higher in malignant breast cancer cells compared to non-malignant cells. We observed constitutive expression of CXCR2 and its ligand CXCL1 in all the mammary tumor cells analyzed. In our study, we aimed to address the functional significance of CXCR2 expression in metastatic mammary tumor cells. We used shRNA technology to target CXCR2 expression in Cl66 mammary tumor cells (Cl66-shCXCR2) and achieved significant reduction in the CXCR2 mRNA expression. Cl66-shCXCR2 cells were used to determine the effect of CXCR2 downregulation on tumor cell proliferation and invasion. We have observed that knock down of CXCR2 expression in tumor cells significantly reduced their ability to invade Matrigel-coated basement membrane in a Boyden chamber. Failure of the Cl66-shCXCR2 cells to invade Matrigel indicates that CXCR2 expression is important for the invasive capability of the tumor cells. This is in accordance with our previous observations in melanoma tumors. However, downregulation of CXCR2 did not affect the tumor cell proliferation.

Our observations in our in vitro system paralleled the in vivo orthotopic mouse model. Mice were orthotopically implanted with Cl66-shCXCR2 or Cl66-control into the MFP. We did not find a significant difference in tumor size between the groups, but the incidence of lung metastasis formation was significantly reduced in mice with Cl66-shCXCR2 tumors compared to mice with Cl66-control tumors. This is in contrast to our previous report demonstrating that knock down of either CXCR2 or CXCR1 inhibited human melanoma growth in vitro and in vivo. Reports from other laboratories have also demonstrated that blocking CXCR2 signaling using antibody neutralization or pharmacological antagonists inhibited tumor growth in vivo. All these reports have used human xenograft models. In this report, we have used a syngenic murine tumor model to study the functional significance of CXCR2 signaling. The difference in the observed results here could be due to the aggressive nature of the syngenic tumors used in this study.

More importantly, we have observed that the number of metastatic lung nodules formed by Cl66-shCXCR2 tumors was significantly lower and also smaller in size as compared to metastases in Cl66-control tumor bearing mice. Our in vitro studies suggest a difference in the invasive potential of CXCR2 knock-down cells, which could alter metastatic behavior. Earlier observations have also shown a significant role for CXCR2-dependent pathways in regulating the metastatic phenotype. It is interesting to note that in this mammary tumor cell model, knock down of CXCR2 had no effect on primary tumor growth and in situ cell proliferation but had an effect on cell survival (as observed by increased apoptosis) and invasiveness. Recently, we have observed that attenuation of CXCR2 signaling using small molecule antagonists to CXCR2 had no effect on human colon cancer primary tumor growth, but significantly inhibited liver metastases. Similarly, we observed enhanced apoptosis in tumors treated with CXCR2 antagonists as compared to control group. Our present report clearly indicates that CXCR2 expression in mammary tumor cells promotes invasion and metastasis.

Angiogenesis is one of the essential steps for tumor growth and metastasis in tumors that grow beyond a certain size. Our immunohistochemical analysis of microvessel density on primary tumor sections clearly indicates a significant reduction in tumor-induced angiogenesis in Cl66-shCXCR2 tumors compared to control tumors. Though we did not find a significant difference in tumor size between the groups, their ability to form lung metastases was significantly inhibited in Cl66-shCXCR2 tumors. These observations indicate that CXCR2 expression by mammary tumor cells promotes neovascularization and also lung metastasis. Similar observations have been made in earlier reports demonstrating inhibition of neovascularization following attenuation of CXCR2 signaling using genetic and pharmacological interventions. Blocking the function of CXCR2 by specific antibodies or inhibiting its downstream signaling using inhibitors of ERK and PI3K could be used to inhibit angiogenesis induced by ELR+ CXC chemokines and would be an interesting adjuvant in cancer therapy. It has been reported that blocking CXCR2 inhibited pancreatic cancer cell induced angiogenesis. Anti-CXCR2 antibodies significantly reduced tumor volume, tumor cell proliferation...
index and microvessel density in an orthotopic nude mouse pancreatic cancer model.\(^{45}\)

In conclusion, our data provide evidence for the role of CXCR2 expression in mammary tumor progression. Silencing CXCR2 expression in mammary tumor cells impairs their ability to metastasize to the lung and also inhibits tumor angiogenesis and invasiveness. CXCR2 blocking using small molecular antagonists or receptor antagonists could have therapeutic importance in reducing metastatic disease progression in breast cancer.

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