CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model

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Abstract

Breast tumor cells express the chemokine receptor CXCR3, which binds the ligands CXCL9, CXCL10, and CXCL11. CXCR3 and other chemokine receptors may mediate tumor metastasis by supporting migration of tumor cells to sites of ligand expression including the lymph nodes, lungs, and bone marrow. We examined the relationship of CXCR3 expression to clinical outcome in 75 women diagnosed with early-stage breast cancer. We detected CXCR3 in malignant epithelium from all tumors. Twelve percent were weakly positive and 64% had moderate levels of CXCR3. Strong CXCR3-positive staining was observed in 24% of tumors. Kaplan-Meier survival curves showed that high CXCR3 expression was associated with poorer overall survival; the unadjusted hazard ratio was 1.56 and it was marginally significant ($P = 0.07$). When interactions between lymph node status and CXCR3 were considered, the adjusted hazard ratio for CXCR3 was 2.62 ($P = 0.02$) for women with node-negative disease at diagnosis, whereas the hazard ratio for CXCR3 was not significant for those with node-positive disease. CXCR3 gene silencing inhibited lung colonization and spontaneous lung metastasis from mammary gland–implanted tumors in a murine model. The size or growth rate of the locally growing tumors was not affected. The implanted tumors in a murine model. The size or growth rate of the locally growing tumors was not affected. The chemokine superfamily is composed of ~40 low molecular weight cytokines that bind a family of 18 to 22 G-protein–coupled receptors. Since the first description of chemokine receptors on malignant cells (1), an extensive literature has developed describing the expression and function of chemokine receptors in many malignancies. CXCR4 and CCR7 expression, in particular, is associated with more aggressive disease in many malignancies (1, 2). Considerably less is known about the role of the related chemokine receptor, CXCR3, which binds the ligands CXCL9, CXCL10, and CXCL11. We previously reported that CXCR3 is expressed on murine mammary tumor cells and that a small molecular weight pharmacologic antagonist of CXCR3 effectively limited breast tumor metastasis (3). Limited data are available about the expression of CXCR3 in primary breast cancers, and nothing is known about the relationship of CXCR3 to clinical characteristics. The current study examines the relationship of CXCR3 expression to clinical outcome in women diagnosed with early-stage breast cancer. Specific genetic targeting of CXCR3 on murine mammary tumor cells was also used to examine the role of the tumor cell chemokine receptor on metastatic potential. These studies further indicate that CXCR3 is a prognostic indicator in breast cancer and is a potential therapeutic target.

Materials and Methods

Cells

Line 66.1 and 410 tumor cells were derived from a spontaneously occurring mammary adenocarcinoma in a BALB/cfC3H mouse. Line 410.4 was derived from a pulmonary tumor of a mouse implanted with line 410 tumor cells. Both lines 66.1 and 410.4 are highly tumorigenic and metastatic following s.c. or i.v. injection into syngeneic BALB/c mice. Cells are grown in DMEM, supplemented with 10% fetal bovine serum (Gemini Bio-products), 1.5 mg/mL sodium bicarbonate, 2 mmol/L L-glutamine, 100 μmol/L nonessential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin in a 10% CO2 atmosphere.

Construction and Characterization of CXCR3 Gene Silenced Cell Lines

Two independent strategies were used to silence CXCR3 gene expression. Using Lipofectamine (Invitrogen), line 410.4 and 66.1 tumor cells were transfected with control plasmid (p5m2c, MSCV-retroviral vector expressing a...
nonsilencing shRNA) or two different plasmids expressing shRNAs directed to murine CXCR3; a second panel of clones was derived from cells transfected with either the TRC pLKO.1 lentiviral vector or TRC shRNA to CXCR3 (all from Open Biosystems, Inc.) and selected in puromycin (Sigma Chemical Co.). Multiple stable transfectants were analyzed for expression of CXCR3 mRNA by standard reverse transcription-PCR protocols using CXCR3 primers (forward, 5'-aaaaaagacacctctcctca-3'; reverse, 5'-tcgaacctcactcaca-3') or glyceraldehyde-3-phosphate dehydrogenase–specific primers. Cell lysates were prepared and immunoblotted with rabbit antibody to CXCR3 (Zymed Invitrogen).

Migration Assay

Tumor cells loaded with calcein AM (Molecular Probes) were placed in the upper chamber of collagen- and fibronectin-coated inserts (Millipore Corp.) and placed in 24-well plates containing base medium (negative control), 5% fetal bovine serum (positive control), or IP-10 (Pepro-Tech). After 24 h, the degree of migration was assessed by fluorescence reading at 485 nm.

In vivo Studies

Local tumor growth and spontaneous metastasis were evaluated by injecting $3 \times 10^5$ viable tumor cells s.c. proximal to the right abdominal mammary gland of syngeneic female mice. Tumor diameters were measured with a caliper twice weekly and mice were euthanized on an individual basis when the s.c. tumor measured 18 mm in diameter or earlier if the mouse was moribund. The lungs were weighed and surface tumor colonies were quantified in a blinded fashion under a dissecting microscope. Experimental metastasis was evaluated by injecting $1 \times 10^5$ viable tumor cells i.v. into the lateral tail vein of syngeneic female mice. All mice were euthanized on day 21 posttransplantation or earlier if the mice were moribund. Lungs were examined for tumor colonies as above.

Mice

BALB/cByJ and BALB/c-Ifngm1Ts (IFNγ−/−) female mice were purchased from The Jackson Laboratory. For studies requiring natural killer (NK) cell depletion, mice were given an i.p. injection of rabbit asialo-GM1 ganglioside antibody (Wako Bioproducts) in saline. Anti-NK cell treatments were administered on days −1 and +3 relative to tumor cell injection. This protocol depletes 50% to 70% of NK cells from the spleen. All mice were housed, cared for, and used in strict accordance with the U.S. Department of Agriculture regulations and the NIH Health Guide for the Care and Use of Laboratory Animals. The University of Maryland School of Medicine Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Immunohistochemistry

A tissue microarray was prepared from formalin-fixed, paraffin-embedded tissue specimens acquired through the Breast Cancer Prognostic Study of the Karmanos Cancer Institute, Detroit, MI. This was a prospective study of 1,306 women with newly diagnosed breast cancer from 1975 to 1983. Clinical and pathologic data were collected from all study subjects. Follow-up data collection from the women continued until 1998. A subset of 75 sequential tissue blocks were used for the current study. For women who died of breast cancer, the mean time to death was 6.1 y, and for those alive at last follow-up, the mean follow-up time was 12.4 y. To prepare the tissue array, H&E-stained sections were reviewed under a light microscope to verify the presence of tumor tissue. From these defined areas, core biopsies 0.6 mm in diameter were taken and arrayed in the recipient paraffin block. Whenever possible, multiple samples were obtained from the same donor block to accommodate heterogeneity. The array also contained normal breast specimens and other normal tissues that serve as positive controls and to assist in aligning the array. Five-micron-thick sections were stained with H&E and immunohistochemistry was done using the bench-MARKXT (Ventana, Inc.) protocol with cell conditioner No. 1 (pH 8.0–9.0) and mouse anti-human CXCR3 monoclonal antibody (R&D Systems) or isotype control antibody (R&D Systems). Staining intensity was scored semiquantitatively using a scale from 0 to 3: 0, no staining; 1, equivocally positive; 2, definitely positive; 3, intensely positive. Specimens were scored in a blinded fashion by two investigators (K.N. and W.H.R.).

Statistical Methods

For studies in mice, the distribution of the number of metastases was compared between the prespecified treatment groups using the nonparametric exact Wilcoxon rank-sum test for groups with a relatively small sample size (fewer than 10 mice). The general linear model approach was applied to estimate and compare average number of lung metastases across larger treatment groups. The square root transformation of the metastasis data was used to decrease variability and to ensure approximate normality. All tests were two-sided and done at the 0.05 level of significance. No adjustments for multiple comparisons were made. In the retrospective cohort study, survival time was defined to be the time from study entry to death, and disease-free interval was defined to be the time from study entry to first metastasis. Women who became lost to follow-up or who had not had the event at the time of last follow-up were censored at that time. CXCR3 expression for each patient was defined to be the maximum CXCR3 score of the patient’s multiple tissue microarray cores (1, 2, and 3 for 1+, 2+, or 3+, respectively). Kaplan-Meier survival curves were computed. Unadjusted and adjusted hazard ratios were estimated using Cox proportional hazard models.

Results

The prognostic significance of CXCR3 expression in breast cancers has not been evaluated. We examined the expression of CXCR3 by immunohistochemistry in 75 breast tumors from women who had participated in the Breast Cancer Prognostic Study, a prospective study of 1,306 women newly diagnosed with breast cancer from 1975 to 1983 carried out at the Karmanos Cancer Institute, Detroit,
MI. A tissue microarray was prepared that contained one to three tumor samples from each specimen. The tissue array was stained with antibody to human CXCR3 or isotype-control antibody. Two blinded investigators evaluated the degree of epithelial CXCR3+ staining on a scale of 0 (no positive cells) to 3+ (>75% positive cells). Figure 1 shows representative examples of invasive and in situ carcinomas of the breast showing strong CXCR3 staining of the cytoplasm and cytoplasmic membrane in malignant epithelial cells. Figure 1G shows a normal duct, negative for CXCR3, which was adjacent to a CXCR3-positive invasive ductal carcinoma. Normal ducts were negative or weakly positive (1+) in other specimens.

All carcinoma revealed some CXCR3 expression in the malignant cells. Twelve percent were considered to be 1+, 64% were 2+, and 24% were highly positive (3+) for CXCR3 (Table 1). Tumor size, estrogen receptor status, and number of involved lymph nodes were not significantly different among the three groups defined by CXCR3 expression; however, both mean tumor size and mean number of positive lymph nodes increased as CXCR3 expression increased. Kaplan-Meier curves for overall survival for the three groups of patients defined by CXCR3 are presented in Fig. 1. High CXCR3 was associated with a poor prognosis (Table 2A, univariate model; hazard ratio, 1.56). This trend was marginally significant (P = 0.071). Mortality in women with tumors low in CXCR3 was a relatively late event occurring many months after diagnosis, in contrast to those with tumors high in CXCR3, among whom mortality was a continual event occurring at all times after diagnosis. As expected, the presence of positive lymph nodes was associated with poor survival (Table 2A; hazard ratio, 1.88; P = 0.043). Neither CXCR3 or positive lymph node status was significant in a linear multivariate Cox regression model (see Table 2B, model 1). The addition of an interaction between these two variables to the model provided evidence that the relationship between CXCR3 and survival was different between women with and without positive lymph nodes at the time of diagnosis. For those who had no positive lymph nodes at diagnosis, the
The hazard ratio for CXCR3 was 2.62 (Table 2B, model 2; \( P = 0.020 \)). In contrast, for those who had at least one positive lymph node, the hazard ratio for CXCR3 was not significant (Table 2B, model 2; adjusted hazard ratio, 0.97; \( P = 0.929 \)). Kaplan-Meier curves for overall survival for the three groups defined by CXCR3 are presented in Fig. 1 for those with and without positive lymph nodes at the time of diagnosis. Similar results were obtained for disease-free interval (Supplementary Table S1; Supplementary Fig. S1). Thus, high CXCR3 was highly predictive of poor survival in women without positive lymph nodes at diagnosis.

To examine the role of tumor CXCR3 in breast cancer behavior, we used a murine model of metastatic breast cancer. Using CXCR3 shRNA in a MSCV-retroviral vector, we transduced murine mammary tumor cell lines 410.4 and 66.1 to express reduced levels of CXCR3. Multiple clones were derived and CXCR3 mRNA and protein expression was evaluated. Metastatic murine mammary tumor cell 66.1 expresses CXCR3 (Supplementary Fig. 2) that is reduced by shRNA. In comparison with 66.1 tumor cells transduced with nonsilencing shRNA (vector), CXCR3 expression was reduced by 41% to 51% in two CXCR3 shRNA–expressing clones (66.1shCXCR3A and 66.1shCXCR3B) chosen for further study. To determine if CXCR3 was functionally inhibited, we compared the ability of vector-control and CXCR3-silenced cells to migrate to the specific CXCR3 ligand, CXCL10 (200 ng/mL). 66.1-vector–expressing cells migrated to CXCL10, but migration of 66.1shCXCR3A cells was reduced by 40% and CXCL10–specific migration of 66.1shCXCR3B cells was completely ablated by CXCR3 gene silencing (data not shown).

These 66.1shCXCR3 clones were evaluated for lung colonizing ability following i.v. injection into syngeneic BALB/cByJ female mice. 66.1 parental, 66.1-vector, 66.1shCXCR3A, or 66.1shCXCR3B cells (1 \( \times 10^5 \)) were injected into groups of five mice, and 21 days later, mice were euthanized and lung tumor colonies were enumerated.

Figure 2A shows, in two independent shRNA clones, that reduced expression of CXCR3 compromises the lung colonizing ability of mammary tumor cells. The number of lung tumor colonies was reduced by 54% to 60% in comparison with vector-control cells. Using a second CXCR3 shRNA and a different (Trc) vector, we derived three additional cell lines stably expressing reduced levels of CXCR3 (Fig. 2B). When these cells were injected i.v. into...
BALB/cByJ mice, lung colonization was reduced by 71%, 52%, and 73%, confirming that CXCR3 genesilencing, rather than off-target effects of shRNA vectors, is responsible for the reduction of lung colonizing ability.

To determine if local tumor growth was affected by decreased levels of CXCR3, \( \frac{3}{10^5} \) vector-control or shCXCR3-expressing tumor cells were implanted s.c. proximal to the mammary gland in BALB/cByJ female mice. Tumor growth was evaluated twice weekly by caliper measurement. CXCR3 gene silencing did not affect the local growth rate of 66.1 cells (Fig. 3A). When individual tumors achieved an average diameter of 18 mm, mice were euthanized and surface lung tumor colonies were enumerated. Spontaneous metastases from the mammary gland tumor were markedly inhibited by CXCR3 gene silencing (Fig. 3B). Animals implanted with 66.1-vector cells had an average of 34.1 \pm 6.1 lung metastases; mice bearing 66.1shCXCR3A had 9.5 \pm 1.3 lung metastases. Likewise, implantation of 66.1shCXCR3B cells reduced spontaneous metastatic capacity (2.2 \pm 1.0 lung colonies). Similar results were obtained using 410.4 cells with reduced levels of CXCR3; changes in CXCR3 expression level did not affect local tumor growth but significantly inhibited lung colonizing capacity (data not shown). Thus, these data

Figure 2. A, parental 66.1 (66.1P), 66.1-vector (66.1Ve), or 66.1shCXCR3A or 66.1shCXCR3B cells \( \frac{1}{10^5} \) were injected i.v. in the tail vein of syngeneic female BALB/cByJ mice, and 21 d later, mice were euthanized and surface lung tumor colonies were quantified (five mice per group). B, 66.1Vtrc or three independent clones expressing shCXCR3 \( \frac{1}{10^5} \) were injected into the tail vein of BALB/cByJ mice, and lung tumor colonies quantified at day +21 (10 mice per group).

Figure 3. A, 66.1-vector or 66.1shCXCR3A or 66.1shCXCR3B cells \( \frac{3}{10^5} \) were injected s.c. proximal to the mammary gland of BALB/cByJ mice and tumor growth was monitored by caliper measurement. Tumor size was expressed as the average of the longest diameter and the perpendicular diameter; bars, SE. B, when tumors in mice shown in A achieved an average diameter of 18 mm, mice were euthanized on an individual basis and surface lung tumor colonies were counted.
confirm the data derived from i.v. introduction of tumor cells and show that CXCR3 gene silencing compromises the ability of mammary gland–implanted tumors to metastasize to the lungs. Inhibition of CXCR3 expression limits metastasis without affecting the size of the locally growing tumor.

Gene silencing could affect many properties of tumor cells. Other laboratories have reported that CXCR3 ligands can promote, inhibit, or have no effect on proliferation of cultured CXCR3-positive tumor cells (4–6). Proliferation of 66.1 cells expressing shCXCR3 is comparable to that of vector-control cells. We also determined the effect of exogenous ligand on proliferation of mammary tumor cells. Proliferation of tumor cells in vitro was marginally affected by the addition of CXCR3 ligands in concentrations ranging from 10 to 200 ng/mL. At the highest concentration used, proliferation of 66.1 tumor cells was inhibited by 0% to 13%. Thus, the inhibitory effects of CXCR3 silencing on metastasis are unlikely to be related to the direct inhibitory effects of ligand on cell proliferation because reduced receptor expression would allow tumor cells to escape this mechanism of inhibition.

We have previously shown that control of tumor metastasis in this model is highly dependent on functioning NK cells and, in some cases, on IFN-γ (7, 8). To determine if NK cells play a role in the reduced metastasis by CXCR3 knockdown, we compared the lung colonizing abilities of 66.1-vector and 66.1shCXCR3 cells in normal BALB/cByJ mice, BALB/cByJ mice depleted of NK cells, and mice mutant for IFN-γ (Fig. 4A and B). Lung colonization of shCXCR3 cells was reduced by 33% versus 66.1-vector cells in normal mice, but this protective effect was completely negated in asialo-GM1 antibody-treated mice.

Discussion

There are 18 to 22 chemokine receptors that bind more than 40 ligands (2). In the adult organism, the physiologic role of these receptor/chemokine pairs is to direct migration, chiefly of inflammatory and immune cells, to sites of tissue injury or inflammation. It has become increasingly clear that expression of some chemokine receptors on transformed cells contributes to malignant behavior (1, 2, 9). In particular, the chemokine receptor CXCR4, which recognizes the ligand CXCL12 (SDF1α), seems to contribute to the aggressive behavior of a very broad range of malignancies including those of the breast, ovary, and melanocytes (10–12). A CXCL12 gradient may promote the translocation of CXCR4-positive tumor cells to sites of ligand expression. The induction of CXCR4 expression by hypoxia may also facilitate escape of tumor cells from low-oxygen environments (13). Pharmacologic antagonism or gene silencing of CXCR4 reduces metastatic potential in several models (14, 15).

![Figure 4A](image1)
![Figure 4B](image2)

**Figure 4.** A, 66.1-vector or 66.1shCXCR3B tumor cells (1 × 10⁵) were injected i.v. into either syngeneic BALB/cByJ or BALB/c IFN-γ−/− mice, and lung tumor colonies were enumerated at day +21. B, 66.1-vector or 66.1shCXCR3B cells (1 × 10⁵) were injected i.v. into BALB/cByJ mice or BALB/cByJ mice treated on day −1 or +3 with 100 μL of asialo-GM1 antibody, and lung tumor colonies enumerated at day +21.
Considerably less is known about the role of the CXCR3 receptor, which binds the ligands CXCL9, CXCL10, and CXCL11. Human and murine breast cancer cell lines express CXCR3 mRNA and protein (3, 6, 16). CXCR3 has also been detected on melanoma, ovarian and renal cell carcinoma, B-cell leukemia, prostate, and colorectal cell lines (17–22). CXCR3 mediates migration of some tumor cells and variously affects ligand-induced proliferation, supporting or inhibiting proliferation of some cells but having no effect on others (4–6). Limited data are available on the expression of CXCR3 in primary human malignancies. Kawada et al. (17) first reported CXCR3 in primary human melanoma specimens; five of nine specimens were positive for CXCR3. Another laboratory has also shown that CXCR3 was detected in 32% of human melanomas examined and was associated with tumor thickness, absent lymphocyte infiltration, and the presence of distant metastasis (23). CXCR3 has been identified in biopsies of ovarian and renal cell carcinomas (18, 19). Two laboratories have described the expression of CXCR3 in colon cancer (22, 24) and observed CXCR3 both in the cytoplasm and plasma membrane of malignant, but not normal, colonic epithelium. CXCR3 expression was strongly correlated with lymph node metastasis. Lymph nodes were positive for tumor cells in 23% of cases in which the primary tumor was negative for CXCR3; 77% of CXCR3-positive tumors were associated with lymphatic involvement. Furthermore, CXCR3-positive tumors had a significantly worse prognosis. Notably, the CXCR3 ligand, CXCL10, was detected in liver, lungs, and lymph nodes, preferred sites of colon cancer metastasis. In colon cancer cell lines, CXCL10 promoted expression of metalloproteinase 9, tumor cell migration, and adhesion to laminin.

We had previously reported that human and murine breast carcinoma cell lines express CXCR3 and that pharmacologic antagonism of CXCR3 reduces metastatic potential of murine breast tumor cells (3). Others have reported that CXCR3 was detected in a small series of breast malignancies (6). Nothing was known, however, about the relationship of CXCR3 in breast cancer to clinical behavior. We have now examined CXCR3 expression in early-stage breast cancer in a series of women for whom long-term follow-up data were available. We detected CXCR3 in malignant epithelium of all samples examined. Eighty-eight percent of the samples were highly positive with either 2+ or 3+ staining. As observed in colon cancer, positive staining was detected in both the cytoplasm and plasma membrane of malignant cells and rarely in stromal cells. Like melanoma and colon cancer, the degree of epithelial staining is positively associated with poor overall survival. The higher the CXCR3 expression, the worse survival was observed, and this association was most notable in women diagnosed without evidence of nodal metastasis. A trend toward larger tumor size and number of involved lymph nodes and higher CXCR3 detection was also observed. Although CXCR3 expression had been reported previously in human breast cancer cell lines (16), the current report is the first to show a relationship between CXCR3 and behavior of clinical breast cancers. Thus, this data adds to the literature that like CXCR3 expression in melanoma and colon cancer, CXCR3 is an indicator of a poor prognosis in breast cancer.

Interestingly, although a positive correlation is observed for CXCR3 expression and poor prognosis in melanoma, colon cancer, and now breast cancer, the converse relationship is observed in chronic B-cell lymphocytic leukemia and clear cell renal cell carcinoma (20, 25). In these two disease settings, low CXCR3 expression, rather than abundant CXCR3, is associated with shorter survival. These contradictory findings indicate that CXCR3 is likely to play many roles beyond mediation of tumor cell migration.

To examine the functional role of CXCR3 in breast tumor metastasis, we expressed a CXCR3 shRNA in highly metastatic murine mammary tumor cells and compared the metastatic potentials of tumor cells with high or low CXCR3 expression. Reduced CXCR3 gene expression resulted in a corresponding reduced ability to form lung colonies after i.v. administration or to metastasize spontaneously from mammary gland–implanted tumors. These data confirm our previous report using a small molecular weight antagonist of CXCR3 (3) and show that tumor CXCR3 is the relevant target and mediates breast tumor metastasis. These data are in agreement with a demonstrated role for CXCR3 in mediating metastasis of melanoma and colon carcinoma (17, 22). It is interesting, however, that tumor CXCR3, in those disease settings, mediates metastasis to the lymph nodes but not the lungs. In the current report, receptor antagonism prevents metastasis to the lungs. This different tissue tropism for the same receptor may reflect differences in the tumor type or other complex factors including the local tissue environment required to support tumors of different histologic origins. Different tumor models may also be optimized to examine different disease properties. For example, CXCR3 gene transduction increased the metastatic ability of colon cancers from intrarectal implantation; however, this model may not support the formation of pulmonary metastases. A common conclusion reached by each study is that neither increased expression of CXCR3 levels (22) nor reduced expression by gene silencing (current report) or pharmacologic antagonism (3) of breast tumors affects the growth of the primary tumor. Thus, based on studies from several laboratories and in several tumor types, it seems that the CXCR3 receptor plays a more important role in tumor metastasis than in the primary expansion of the tumor.

The antimetastatic activity of CXCR3 gene silencing reported here is unlikely to be related to the direct effects of CXCR3 on tumor cell proliferation for several reasons. First, CXCR3 shRNA–expressing mammary tumor cells proliferate at the same rate as vector-transduced cells. Second, addition of CXCR3 ligands (CXCL9, CXCL10, or CXCL11) to parental or vector-control tumor cells marginally inhibits proliferation. Thus, in a progressively growing tumor, CXCR3 silencing would render tumor cells relatively resistant to any growth inhibitory activities of CXCR3 ligands.
The data on the effects of CXCR3 ligands on tumor cell proliferation are contradictory: In some human tumor cells, ligand induces tumor cell proliferation (6, 17, 22), and in other cells, ligand has no effect on cell growth (24). Some of these differences are likely attributable to the expression of CXCR3 splice variants in different cells (24, 26). In some cells, CXCR3a supports cell proliferation (6) whereas CXCR3b delivers an antiproliferative signal. If CXCR3b is inhibited with siRNA, the proliferative response to CXCL10 is enhanced, supporting the hypothesis that CXCR3b is antiproliferative, whereas CXCR3a supports tumor cell proliferation. Different experimental conditions, including the presence or absence of serum in cell growth assays, may also affect these different conclusions. In the current study, CXCR3 may functionally resemble the CXCR3b splice variant expressed in humans because ligands modestly inhibit tumor cell proliferation.

The most compelling evidence that the antimetastatic effect of CXCR3 gene silencing is not a direct effect on tumor cell proliferation is our novel observation that this therapeutic effect is compromised in the absence of functioning NK cells or the cytokine IFN-γ. These data suggest that a model whereby CXCR3 simply mediates tumor cell migration may not reflect the complex role of this receptor. Although many laboratories have shown that tumor cells will migrate in vitro in response to CXCR3 ligands, we propose that to inhibit metastasis, not only must directed migration be disrupted but NK cells are also required to execute the coup de grâce to kill these remaining (randomly?) circulating tumor cells. IFN-γ may be necessary to adequately activate the NK cells to carry out these functions.

The role of endogenous CXCR3 ligands acting on CXCR3-positive cells is considerably more complex with evidence for a beneficial effect mediated by direct inhibitory effects on tumor cell proliferation (5) and/or on the infiltration of protective T and NK cells (4, 7) versus a tumor-growth promoting effect (6). The mammary tumor cells used for the current studies produce little, if any, CXCR3 ligand except when stimulated in vitro with IFN-γ. We have shown, however, that forced overexpression of CXCL9 leads to marked inhibition of both local and metastatic tumor growth (7). This therapeutic effect is mediated by host T-cell and NK cell infiltration into the tumor. We have proposed that in the setting of forced, local, and very high ligand expression, the tumor-expressed CXCR3 responds to this reverse gradient by “staying home” where the ligand expression is now higher than in the lungs or other sites of ligand expression.

Taken together, these data represent growing evidence that CXCR3 is a determinant of malignant behavior in several epithelial malignancies. The association observed between CXCR3 expression and poor survival, which was strongest in node-negative disease in which survival would otherwise be favorable, suggests that CXCR3 may be prognostically important. The preclinical data showing that suppression of CXCR3 expression or receptor signaling limits metastasis support the hypothesis that CXCR3 contributes to metastatic success. Our previous report showing that a small molecular weight pharmacologic antagonist of CXCR3 limits metastatic disease provides a potential approach to target this receptor clinically (3). These studies support the continued examination of this receptor as a potential therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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