Inhibition of CXCR4 by CTCE-9908 inhibits breast cancer metastasis to lung and bone

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Abstract. Metastasis occurs, in part, due to tumor cell responses to chemokine secretion by ectopic organs or tissues. SDF-1 is constitutively expressed in tissues where metastases frequently develop while breast carcinoma cells express the receptor for SDF-1, CXCR4, which is correlated with increased bone metastasis and poor overall survival. We hypothesized that treatment with a CXCR4 antagonist, CTCE-9908, would decrease incidence of bone and lung metastasis. Treatment with CTCE-9908 (25 mg/kg) began the day prior to or the day of intravenous or intracardiac tumor cell inoculation of MDA-MB-231 human breast carcinoma cells expressing enhanced green fluorescent protein (GFP) into athymic mice. After 5 or 8 weeks (i.c. and i.v. injections, respectively), the presence of fluorescent foci at metastatic sites was assessed. Somewhat surprisingly, CTCE-9908 treatment did not decrease incidence of metastasis as hypothesized. However, CTCE-9908 did decrease metastatic burden (i.e., size of metastases) in all organs examined (lungs, bone, heart, liver, kidneys, pancreas and spleen). Based upon this and other studies, the use of CTCE-9908 is promising as an adjuvant therapy for metastatic disease.

Introduction

Chemokines are chemoattractant molecules that regulate cellular trafficking (1-4). Secretion of chemokines by tumor cells has been correlated with angiogenesis, tumor growth and metastasis in a variety of experimental and human tumors (reviewed in refs. 1, 5). The organotropism of cancer metastasis is believed to occur, at least in part, through secretion of chemokines by distant organs/tissues that direct the migration of tumor cells that come into proximity to the tissue (1, 5). SDF-1 (stromal cell derived factor-1) is a chemoattractant for hematopoietic cells to the bone marrow. It is secreted by osteoblasts in marrow stroma (6, 7). Secretion of SDF-1 from other organs and at sites of injury facilitates the migration of tissue-committed stem cells that are used for organ regeneration and repair (7, 8). Similarly, stem cells are recruited in response to hypoxia-induced HIF1α promotion of SDF-1 expression (9).

CXCR4 is the major SDF-1/CXCL12 receptor. CXCR4 is highly expressed on hematopoietic cells in peripheral blood as well as early hematopoietic progenitor cells (10). CXCR4 appears to regulate maintenance of hematopoietic progenitor cells in the bone marrow and recruitment to other sites (11). CXCR4 is not expressed to an appreciable level in the normal breast (5). Yet, in atypical ductal hyperplasia, 90% of samples are positive for CXCR4 expression and it increases as breast cancer progresses to invasive disease (5, 12). Increased expression in breast cancer is associated with poor overall survival and more specifically with the development of metastatic disease (reviewed in ref. 13). Correspondingly, SDF-1 is constitutively expressed in tissues where metastases develop, specifically lung, liver and bone (5, 12, 14). Directed migration of breast cancer cells has been associated with CXCR4 signaling in response to SDF-1 and neutralizing antibodies to CXCR4 can inhibit metastasis of breast cancer to lymph nodes and lung. CXCR4 was identified as one of a small number of genes that were enriched in breast cancer cells selected for increased bone metastasis in an in vivo model (15). Overexpression of CXCR4 alone caused a small, but significant increase in the number of bone metastases, but when combined with expression of IL-11 and osteopontin, CXCR4 expression caused a dramatic increase in incidence and number of bone metastasis. Similarly, expression of CXCR4 in breast cancer patients has been correlated with higher risk of bone...
metastasis (16-18). We note that SDF-1 also binds CXCR7, but the role of CXCR7 in cancer metastasis has not been as well studied; therefore, the studies reported here focus exclusively on CXCR4.

It has been indicated that SDF-1 may affect the tumor microenvironment. It was demonstrated that carcinoma-associated fibroblasts secrete SDF-1 in response to hypoxia which can induce motility and invasiveness in CXCR4 expressing tumor cells (19,20). Also, secretion of SDF-1 by the stroma may promote tumor angiogenesis through the recruitment of hematopoietic endothelial progenitor cells (19). All of these mechanisms may contribute to selective retention of tumor cells at sites of high SDF-1 secretion, such as the bone marrow resulting in the development of metastatic disease.

Collectively, abundant evidence supports multiple roles for CXCR4 and SDF-1 in various steps of cancer metastasis (2,6). The purpose of this study was to test the efficacy of CTCE-9908, a CXCR4 antagonist (21), as an anti-metastatic agent for breast cancer. Previous studies have indicated efficacy of CTCE-9908 as an anti-metastatic to lung (21); however, its efficacy in preventing metastasis to other organs has not yet been evaluated. Since SDF-1 is produced by cells in bone marrow and CXCR4 is aberrantly expressed on metastatic breast cancer cells, we hypothesized that treatment of mice with CTCE-9908 would interfere with ‘homing’ of breast cancer cells to bone and, therefore, decrease the incidence of bone metastasis. Moreover, since many organs express SDF-1, it was also hypothesized that the incidence of metastasis to other organs would be decreased by treatment with CTCE-9908.

Materials and methods

Cell lines and culture. Metastatic human breast carcinoma cell line, MDA-MB-231 (MDA-231) was stably transduced with an HIV type 1-based, lentiviral vector system constitutively expressing enhanced GFP (22). For the lentivirus, the GFP coding sequence was inserted into the vector 5' of the internal ribosome entry site and puromycin sequences, each of which were under control of the early cytomegalovirus promoter. Infectious stocks were prepared by transfection of 293T cells and used at a multiplicity of infection of ~10. Cells were cultured in a mixture (1:1 vol/vol) of DMEM and Ham's F12 media (DMEM/F12; Invitrogen, Carlsbad, CA) supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 0.02 mmol/l nonessential amino acids, 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), without antibiotics or antifungotics (cdME/F12). All cultures were confirmed to be negative for Mycoplasma spp. infection using a PCR-based test (Takara, Shiga, Japan). The brightest 15% fluorescing cells were sorted using either Coulter EPICS V cell sorter (Beckman-Coulter, Fullerton, CA) or a BD FACSaria cell sorter (BD Biosciences Immunocytometry Systems, San Jose, CA). The selected cells have remained stable in the absence of selective pressure for more than two years.

Intravenous injections. Cells at 80-90% confluence were detached using a mixture of 0.5 mmol/l EDTA and 0.05% trypsin in CaCl2- and MgCl2- and NaHCO3-free HBSS. Cells were not used unless viability was >95%, but was usually >98%. Viable cells were counted using a hemacytometer and resuspended at a final concentration of 1.0x106 cells/ml in ice-cold HBSS. Female athymic mice (3-4 weeks; Harlan Sprague-Dawley, Indianapolis, IN) were briefly restrained while cells (2x106 in 0.2 ml) were injected into the lateral tail vein. Ten mice were injected per treatment group.
Intracardiac injections. Viable cells were detached and counted as above using a hemacytometer and resuspended at a final concentration of $1.5 \times 10^6$ cells/ml in ice-cold HBSS. Female athymic mice (5-6 weeks) were anesthetized by i.m. administration of a mixture of ketamine-HCl (129 mg/kg), and xylazine (4 mg/kg). Cells ($3 \times 10^5$ in 0.2 ml) were injected into the left ventricle of the heart in the third/fourth or fourth/fifth intracostal space. The presence of bright red- as opposed to burgundy-colored blood prior to and at the end of each inoculation confirmed injection of the entire volume into the arterial system. Twenty mice were injected per treatment group. Intracardiac injections are routinely performed with >95% efficiency and <5% mortality (23).

Treatments. Mice were injected subcutaneously daily with either 100 μl water or 25 mg/kg CTCE-9908 in 100 μl water. Injections were rotated among four to six sites on the dorsolateral flank. This dose was based upon prior in vivo studies and recommended pharmacokinetic studies (21). Treatment was initiated either the day of (D0) or the day prior to tumor cell injection (D-1). These times were chosen because CXCR4 was thought to impact mostly early retention and arrest stages of cancer metastasis.

Mice were weighed weekly to determine the proper amount of CTCE-9908 to administer. Mice were necropsied 5 weeks post-inoculation (for i.c. injected mice) or 8 weeks post-inoculation (for i.v. injected mice) following anesthesia with ketamine/xylazine and euthanasia by cervical dislocation.

Assessment of metastases. For mice injected i.v. with tumor cells, lungs were removed and fixed in Bouin’s fixative for 5 weeks. Surface metastases were counted by microscopy using a Leica MZFLIII dissecting microscope with 10x objective. The average number of visible metastases per lung was quantified for each treatment group. Incidence of metastasis was reported as the percentage of mice that had ≥1 lung focus. For mice injected i.v. with tumor cells, femurs, ribs, heart, lung, liver, pancreas, spleen, brain, kidneys, uterine horn and ovaries were removed and examined by low magnification fluorescence stereomicroscopy.

Although all tissues were examined, the focus of the i.c. experiments was bone metastasis. Intact, dissected bones and organs from individual mice were placed in 25 ml glass scintillation vials containing freshly prepared, ice-cold (0-4°C) 4% paraformaldehyde in Ca$^{2+}$- and Mg$^{2+}$-free Dulbecco’s

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Table I. Effect of CTCE-9908 on experimental breast cancer lung and bone metastases.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route of injection</th>
<th>Incidence</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>i.v.</td>
<td>18/20</td>
<td>11±3</td>
</tr>
<tr>
<td>D-1</td>
<td>i.v.</td>
<td>20/20</td>
<td>11±3</td>
</tr>
<tr>
<td>D0</td>
<td>i.v.</td>
<td>18/20</td>
<td>7±2</td>
</tr>
<tr>
<td>Control</td>
<td>i.c.</td>
<td>16/17</td>
<td>39±5</td>
</tr>
<tr>
<td>D-1</td>
<td>i.c.</td>
<td>16/16</td>
<td>19±3</td>
</tr>
<tr>
<td>D0</td>
<td>i.c.</td>
<td>12/12</td>
<td>25±4</td>
</tr>
</tbody>
</table>

Femur foci

| Control   | i.c.               | 39/40     | 5±1        |
| D-1       | i.c.               | 37/37     | 7±1        |
| D0        | i.c.               | 25/25     | 7±1        |

Athymic mice were injected with $2 \times 10^5$ cells (i.v.) or $3 \times 10^5$ cells (i.c.) and CTCE-9908 (25 mg/kg s.c.) treatment was initiated the same day (D0) or one day preinjection (D-1) and continued until mice were euthanized and necropsied. *P<0.05.

Figure 2. Athymic mice were injected into the left cardiac ventricle with $3.0 \times 10^6$ GFP-expressing MDA-MB-231 cells in 200 μl of HBSS. The mice were necropsied 5 weeks later. (A) Fluorescent photographs were taken of both the dorsal and ventral sides of each femur from all treatment groups at magnification, x1.25. Representative photos are shown. (B) The green fluorescent foci in each femur in each treatment group was counted. The average number of foci per hindlimb was determined. (C) The location of each green fluorescent foci was mapped using BoneMetMap (25). (A) The maps for each mouse in each treatment group were merged to show the overall green fluorescence in each treatment group indicating the overall metastatic burden and pattern of metastatic foci for each treatment group.
cells were visualized using a fluorescent stereomicroscope. Fluorescent foci were counted 5 weeks post-injection following organ removal.

In the mice injected i.v., the incidence of macroscopic lung metastasis decreased size, but not incidence, of lung foci. In the mice injected i.v., the incidence of macroscopic lung metastasis was 90% in control and D0 treated mice and 100% in the D-1 treated mice (Table I). The number of metastases per lung decreased from 11 in control and D-1 treated mice to 7 in D0 treated mice (Table I; Fig. 1A and B). This change was not statistically significant. In i.c. injected animals, green fluorescent foci [Note: A focus is not necessarily a metastasis. A focus is defined as a green spot greater than or equal to a tumor cell diameter (i.e., 25 μm)] were found in all CTCE-9908 treated animals (Table I), while the control group had a 94% incidence in of foci in the lung. The difference was not statistically significant. However, the number of bona fide metastases per lung significantly decreased from 39 in the untreated, control group to 25 (P=0.022) and 19 (P=0.001) in the D0 and D-1 treated groups, respectively (Table I; Fig. 1C). The size of the metastases (>50 cells) also decreased in the CTCE-9908 treated animals (data were not quantified, but representative images illustrate the point).

CTCE-9908 decreases the size of bone and other extra-pulmonary foci. The incidence of green fluorescent foci in the femur (98-100%) did not change significantly when mice were treated with CTCE-9908 (25 mg/kg s.c.) treatment was initiated the same day (D0) or one day pre-injection (D-1) and continued until mice were euthanized and necropsied. GFP-expressing MDA-MB-231 cells were visualized using a fluorescent stereomicroscope. Fluorescent foci were counted 5 weeks post-injection following organ removal.

Table II. Effect of CTCE-9908 on the incidence and frequency of metastases in multiple organs following injection of MDA-MB-231 cells.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Incidence</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>D-1</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>10/17</td>
<td>13/15</td>
</tr>
<tr>
<td>Heart</td>
<td>16/17</td>
<td>14/16</td>
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<tr>
<td>Liver</td>
<td>15/18</td>
<td>13/16</td>
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<tr>
<td>Spleen</td>
<td>15/17</td>
<td>14/16</td>
</tr>
<tr>
<td>Pancreas</td>
<td>12/16</td>
<td>13/16</td>
</tr>
<tr>
<td>Uterine horn</td>
<td>8/16</td>
<td>8/13</td>
</tr>
<tr>
<td>Ribs</td>
<td>16/17</td>
<td>16/16</td>
</tr>
<tr>
<td>Kidney</td>
<td>33/36</td>
<td>31/32</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>D-1</td>
</tr>
<tr>
<td></td>
<td>4±1</td>
<td>8±3</td>
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<tr>
<td></td>
<td>89±18</td>
<td>48±10</td>
</tr>
<tr>
<td></td>
<td>14±2</td>
<td>7±1</td>
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</tbody>
</table>

Athymic mice were injected with 3x10⁵ cells into the left ventricle of the heart and CTCE-9908 (25 mg/kg s.c.) treatment was initiated the same day (D0) or one day pre-injection (D-1) and continued until mice were euthanized and necropsied. GFP-expressing MDA-MB-231 cells were visualized using a fluorescent stereomicroscope. Fluorescent foci were counted 5 weeks post-injection following organ removal.
tumor cells was significantly lower in CTCE-9908 treated mice (Fig. 2C), especially when treatment began contemporaneously with tumor cell inoculation (i.e., D0).

The distinction between fluorescent foci, which can represent single cells to macroscopic metastatic foci, is critical. It is well known that solid tumors can release millions of tumor cells daily, even from small masses. However, the formation of clinically relevant metastasis is infrequent. The initial seeding of various organs is much less relevant from a clinical perspective than the formation of macroscopic lesions (13,26). Interpretation of the data presented in this study is impacted greatly by these considerations. We have advocated previously that a metastasis be defined as a mass of ≥50 cells (26), which is used consistently in this study. While that definition may be open for discussion, the principles guide our presentation and interpretation of data presented herein.

Previous studies show efficacy of CTCE-9908 in decreasing formation of lung metastases from osteosarcoma and melanoma (21). However, in contrast to the previous study, pretreatment of breast cancer cells or mice with CTCE-9908 was not required. Importantly, data with MDA-MB-231 cells demonstrate that inhibition of CXCR4 substantially decreased metastatic burden in multiple organs, but most significantly in the bone.

Based upon the known roles of CXCR4 in hematopoiesis, inhibition of CXCR4 signaling would be predicted to alter adhesion, invasion, migration, ‘homing’ and/or growth rate of neoplastic cells. Impairment of tumor cells to adhere, invade and grow was observed previously (21). However, the data using the MDA-MB-231 cells suggest that the role(s) of CXCR4 in breast cancer metastasis may be more complex. Based upon the modest changes in the incidence of metastases (and individual tumor cell foci), antagonism of CXCR4 does not appear to abolish mechanical arrest of cancer cells. The decrease in focus number and numbers of metastases may be explained, in part, by CTCE-9908 induction of anoikis in the circulation or apoptosis subsequent to arrival at some secondary sites (21,27,28). The hypothesis that SDF-1 and CXCR4 are involved in tumor cell ‘homing’ is not borne out by the data obtained in this study. This conclusion is based upon known differences in SDF-1 expression in various tissues (5), but a relatively uniform retention of fluorescent foci in virtually every site.

CXCR4 may affect survival and/or proliferation of tumor cells once they arrive at the secondary site(s) (27-33). This possibility is supported by a consistent decrease in the size of fluorescent foci in every tissue when mice were treated with CTCE-9908. This interpretation is consistent with prior studies showing that the proliferation of tumor cells in vivo was affected by CXCR4 expression (27,34,35). Also, CTCE-9908 has been shown to decrease the growth of osteosarcoma and melanoma cells both in vitro and in vivo (21). The design of the studies reported here preclude assessment of whether individual seeded cells grew to form macroscopic colonies vs. whether individual cells coalesced for form larger foci. Another intriguing explanation for diminished growth of disseminated cells is based upon recent findings that CXCR4 is involved in the recruitment of hematopoietic endothelial progenitor cells to tumor microenvironments (19). Since tumors cannot grow beyond a few hundred micrometers

kidney, pancreas and spleen, while the size was decreased in the liver, but not in other organs (Fig. 3). There was no effect of CTCE-9908 on brain metastasis (Table II; Fig. 3).

Discussion

Based upon differential tissue expression and high expression in bone, we hypothesized that the incidence of metastasis to bone, and perhaps other organs, would decrease upon treatment with the CXCR4 antagonist, CTCE-9908. Unexpectedly, with the exception of the uterine horn and the ovary (Fig. 3), treatment did not affect the incidence of metastasis. Instead, in most cases, the number of foci per organ decreased and there was a dramatic decrease in the size of the foci in mice treated with CTCE-9908.

While the number of lung metastases after i.v. injection did not change significantly, occasional lung metastases following i.c. injection were significantly lower. The frequency of lung metastases is generally low following i.c. injection; so, the higher-than-expected frequency of foci observed in this study were likely due, in part, to leakage into the pleural cavity during the injection process (25). Regardless of the mechanism of seeding, it appears that CTCE-9908 was able to decrease the tumor burden in the lungs.

Bone metastases are reported for the femur because, in previous studies, the development of bone metastases elsewhere in the body was parallel to the size and incidence of osteolytic lesions in femurs (22,23). Although the number of fluorescent foci was slightly higher in the CTCE-9908 treated mice (Table I; Fig. 2A), visualization of individual cells was likely due to the decreased macroscopic lesions, thereby allowing easier visualization of single cells. Combinatorial analyses of the bone lesions (25) revealed that occupancy by tumor cells was significantly lower in CTCE-9908 treated mice.
without establishment of a robust blood supply (36-38). treatment with CTCE-9908 may prevent angiogenesis of micrometastases, preventing conversion to overt metastases (i.e., colonization).

The data presented herein support important roles for CXCR4 in breast cancer metastasis. Moreover, the data strongly support further development of CTCE-9908 as an anti-metastatic agent. Since metastasis is the most lethal and most morbidity-inducing attribute of cancer cells, control of metastasis offers great potential for improving survival and patient quality of life (13).

Arguments against metastasis as a rational therapeutic target have centered upon the probability that most antecedent steps of the metastatic cascade have occurred by the time of primary tumor discovery (13). The data presented herein implicate CXCR4 in the last step of metastasis, colonization. In support of this notion is accumulating data related to several metastasis suppressors, including BRMS1 and KISS1. Both metastasis suppressors significantly reduce (or prohibit) tumor cell proliferation in ectopic sites (22.39). Intriguingly, BRMS1 and KISS1 both regulate CXCR4 signaling (40.41). In non-small cell lung cancer, BRMS1 reduces CXCR4 expression through inhibition of NFkB resulting in an inhibition of SDF-1 induced migration (40). BRMS1 and CXCR4 are also inversely correlated in non-small cell lung cancer cases indicating that one mechanism of BRMS1 suppression of metastasis is downregulation of CXCR4. KISS1, and protease cleavage-derived kisspeptins, prevent SDF-1 induced CXCR4 chemotaxis (41). While further studies will be required to establish definitive connections between CXCR4, KISS1/BRMS1 and metastasis suppression, the implications provide testable hypotheses regarding mechanisms of metastasis suppression.

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