Expression of CXC Chemokine Receptor-4 Enhances the Pulmonary Metastatic Potential of Murine B16 Melanoma Cells

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ABSTRACT

The chemokine receptors CC chemokine receptor (CCR) 7 and CXC chemokine receptor (CXCR) 4 have been implicated in cancer metastasis. To evaluate whether CXCR4 is sufficient to increase tumor metastasis in an organ-specific manner, we transduced murine B16 melanoma cells with CXCR4-CXCR4-B16 and followed the metastatic fate of the transduced cells in both i.v. and s.c. inoculation models of metastasis. CXCR4-B16 cells demonstrated marked increases (>10-fold) in pulmonary metastasis compared with vector (pLNCX2)-B16 after i.v. and s.c. inoculation of tumor cells. The increase in metastasis could be completely inhibited by T22, a small peptide antagonist of CXCR4. As early as 24 and 48 h after i.v. injection, CXCR4-B16 cells were significantly increased in the lung compared with control B16 cells by 5- and 10-fold (P < 0.05), respectively. CXCR4-B16 cells adhered better to both dermal and pulmonary microvascular endothelial cells relative to control B16 cells. Moreover, CXCL12 promoted the growth of CXCR4-CXCR4-B16 cells in vitro. Whereas expression of CXCR4 in B16 cells dramatically enhanced pulmonary metastasis, metastasis to the lymph nodes, liver, and kidney was rare. Immunohistochemical analysis of the lung revealed human cutaneous melanoma and pulmonary metastases revealed CXCR4 expression. Thus, CXCR4 plays a potentially important role in promoting organ-selective metastasis, possibly by stimulating tumor adhesion to microvascular endothelial cells and by enhancing the growth of tumor cells under stress.

INTRODUCTION

The metastasis of tumor cells represents the primary source of clinical morbidity and mortality in the large majority of solid tumors. Metastasis of cancer cells may result from entry of tumor cells into either lymphatic or blood vessels. Invasion of lymphatic vessels results in metastasis to regional draining LNs. For many tumors, including melanoma, the presence of regional LN metastases is the single best predictor of patient survival. Invasion of veins or capillaries may result in the distribution of tumor cells to many body sites, but it is well known that some locations are much more likely to be sites of tumor metastasis than others. Melanoma, for example, tends to metastasize to the lung, liver, and brain. Although several proteins such as RhoC and vascular endothelial growth factor C and D, have recently been shown to enhance metastasis and/or tumor invasion, the mechanisms underlying organ-selective metastasis are still under intense investigation.

Metastasis is a complex event with many factors that can potentially affect tumor distribution, including properties of tumor cells as well as the microenvironment where the tumor cells ultimately localize. There is emerging data suggesting that chemokines and their receptors may play key roles in determining the site of metastasis. In a screen of 12 melanoma cell lines, Müller et al. determined that only three chemokine receptors (CCR7, CCR10, and CXCR4) were consistently expressed. Known to be critical for the migration of antigen-presenting cells from the periphery to draining LNs via afferent lymphatics, CCR7 was demonstrated to dramatically increase metastasis of B16 murine melanoma to regional LNs, suggesting that tumor cells can use this receptor in a manner analogous to that of antigen-presenting cells for transit to the LNs. Müller et al. demonstrated that primary tumors of breast cancer expressed CXCR4 at the protein level and that many breast cancer and melanoma cell lines expressed CXCR4 (as well as CCR7) at the mRNA level. Moreover, exposure of breast cancer cells to a function-blocking anti-CXCR4 mAb inhibited metastasis to the lungs and LNs, suggesting that CXCR4 might be involved in the selective metastasis of cancer cells to body sites that show high expression of CXCL12 (also known as stromal-derived factor-1/SDF-1), the only known ligand of CXCR4. Others, based on in vitro adhesion and chemotaxis experiments, have also suggested a role for CXCL12/CXCR4 in the metastasis of neuroblastoma, melanoma, and prostate cancer cells.

Whereas the data of Müller et al. suggested that CXCR4 was necessary for optimal metastasis of breast cancer cells after i.v. inoculation, the question remained whether CXCR4 was sufficient for such metastasis to occur because at least two other chemokines, including CCR7, appeared to be coexpressed in the majority of human breast and melanoma cancer lines. Moreover, the mechanism by which CXCR4 confers enhanced metastasis was not directly addressed. Although homing effects analogous to the rapid adhesion of lymphocytes at sites of inflammation could possibly be mediated by CXCR4 expression in tumor cells, there is evidence that CXCR4 ligation may also provide prosurvival and proliferative signals for cells such as hematopoietic stem cells. In combination with our previous data regarding the role of CCR7 in regional LN metastasis, we propose that chemokine receptors may play divergent yet critical roles in the site-specific dissemination of tumor cells.

MATERIALS AND METHODS

Animals, Cell Lines, and Reagents. Female B57BL/6 mice (8–12 weeks old) from the NCI-Frederick Cancer Research and Development Center (Frederick, MD) were used in all experiments, which have been approved by the NCI Animal Use and Care Committee. Syngeneic B16/F1 melanoma cells were provided by the NCI-Frederick Cancer Research and Development Center and grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 10% heat-inactivated FCS and supplements (13). Human DMECs were derived from neonatal foreskin and cultured as described previously (22). Human LMECs (Clonetics-Cambrex, East Rutherford, NJ) were grown in EGM-2 MV as recommended by the supplier. The CXCR4 function-blocking peptide, T22 (NH2-RRWCRYRKCYGKYCRYKR-COOH with disulfide bridges between the first and last as well as the second and third cysteine residues; Ref. 23), and a control peptide of similar amino acid composition, ALA (NH2-RRWCYRKCRYKCYRKR-COOH, Ref. 23), were synthesized by Synpep (Dublin,
CA) and high-performance liquid chromatography-purified to >92% purity. In control experiments, T22 (500 ng/ml) was shown to specifically block in vitro chemotaxis of activated bone marrow-derived DCs to 200 ng/ml CXCL12, whereas ALA had no inhibitory effect (data not shown).

**Immunohistochemical Analysis.** Samples of primary cutaneous human melanoma and pulmonary metastases were obtained with institutional approval, frozen in liquid nitrogen, sectioned (5 μm), and fixed in ice-cold acetone. Endogenous peroxidase activity was blocked by hydrogen peroxide (0.3%) for 30 min at room temperature. Sections were stained with anti-human CXCR4 mAb (4 μg/ml; R&D Systems, Minneapolis, MN) overnight at 4°C, a biotinylated antimonouse secondary antibody, and a streptavidin-horseradish peroxidase complex (Vectastain ABC; Vector Laboratories, Burlingame, CA). 3-Amino-9-ethylcarbazole (Vector Laboratories) was used for color (red) visualization. Sections were counterstained with hematoxylin.

**Retroviral Transduction of B16/F1 Melanoma Cells.** Human CXCR4 cDNA (Ref. 24; a gift from Dr. E. Berger, National Institute of Allergy and Infectious Diseases) was subcloned into the pLNCX2 retroviral vector (Clontech, Palo Alto, CA). Using this vector, B16/F1 melanoma cells were transduced using a method previously described (13) to yield CXCR4-B16 cells. Transduction efficiency varied from 40–80% (Fig. 1A).

Both the chemokine receptor-transduced and vector-transduced tumor lines were subsequently retrovirally transduced (using a puromycin-based selection system) with firefly (Photinus pyralis) luciferase cDNA that was originally PCR-amplified from a luciferase-containing expression vector (pGL3-luciferase; Promega, Madison, WI). For convenience, the CXCR4-luciferase B16 cell line is referred to hereafter as CXCR4-B16, whereas the control vector-luciferase-transduced cell line is called pLNCX2-B16.

**RESULTS**

To obtain consistent levels of CXCR4 expression, CXCR4-B16 cells were stained with PE-conjugated antihuman CXCR4 mAb (clone 12G5; PharMingen, San Diego, CA), treated with anti-PE MicroBeads (Miltenyi Biotec Inc., Auburn, CA), washed, and loaded onto a MACS MS column (Miltenyi Biotec Inc.) for positive magnet-based selection. The positive fraction was then cultured with G418 and puromycin to maintain expression of CXCR4 and luciferase, respectively.

s.c. and i.v. Inoculation of Transduced Cell Lines. CXCR4-B16 and pLNCX2-B16 cells in exponential growth phase were harvested by trypsinization and washed twice in PBS before injection. Cell viability was >95% as determined by trypan blue dye exclusion. For the footpad (i.e., s.c.) injection, B16 cells (4 × 10^5 cells in 20 μl of PBS) were s.c. injected into the left footpads of C57BL/6 mice. Tumor growth was monitored 3 times/week by measurement of two maximum perpendicular tumor diameters. When tumors reached 5–7 mm in size, animals were euthanized before harvesting of lung and LNs. Pooled LNs were removed by gently pulling skin (including the dermis and s.c. fat) above the popliteal fossa and carefully exposing the LNs resting in the adipose tissue of the fossa with a pair of forceps. For i.v. injection, 4 × 10^5 CXCR4-B16 or pLNCX2-B16 cells in 200 μl were injected into the tail veins of mice. T22 and ALA peptides in sterile PBS were administered via an i.p. route using 4 μg peptide/mouse daily for the indicated duration of treatment. Experiments were performed 2–4 times each with similar trends; representative experiments are shown.

**Luciferase Assay.** Luciferase activity was measured using a luciferase reporter assay system (Promega). Cultured cells (Fig. 1C) were lysed with 100 μl of undiluted lysis buffer and then assayed in the presence of luciferin using a Thermo Labsystems MLX 96-well luminometer (Helsinki, Finland). Units are in arbitrary light units. For tissue assays, organs from each animal were homogenized in 1 ml of lysis buffer, of which 25 μl aliquots were then assayed in duplicate. Means of duplicates were used to represent the luciferase activity for a given tissue from a particular animal. Five to ten animals per experimental treatment group were used for typical experiments.

**Cell Adhesion Assay.** Before interaction with tumor cells, resting confluent DMECs or LMECs were exposed to 100 ng/ml recombinant CXCL12 or CCL21 for 15 min at 20°C as described previously (25). After unbound chemokine was washed away, calcein-acetoxymethyl-labeled (Molecular Probes, Eugene, OR) CXCR4-B16 or pLNCX2-B16 cells (2.5 × 10^5/ml in DMEM containing 10% FCS) were injected at 1.5 dynes/cm^2 into a parallel plate flow chamber (Glycotech, Gaithersburg, MD) containing confluent DMECs or LMECs. In some cases, tumor cells were treated with pertussis toxin (Calbiochem, San Diego, CA) at 100 ng/ml for 2 h at 37°C to block Gi protein-coupled signaling. After 2 min, flow was stopped, which enabled B16 cells to interact with MECs in the absence of shear stress. After 5 min, shear was reestablished at 1.5 dynes/cm^2 for up to 2 min to detach unbound cells; adherent cells were digitally photographed in 5–6 random fields (each field = 1.18 mm^2) with excitation at 488 nm and emission at 513 nm using sufficient exposure time (~1 s) to allow distinction between nonmoving and moving cells (which appeared as dim streaks; Ref. 22). Cells were then counted with the software program IPlab (Scanalytics, Fairfax, VA).

**Cell Proliferation Assay.** CXCR4-B16 and pLNCX2-B16 cells (2 × 10^4 cells/well) were cultured in triplicate for 24 h in 24-well plates containing B16 growth medium (DMEM and 10% FCS). After rinsing the cells in serum-free DMEM, the B16 cells were incubated for 40–48 h at 37°C in DMEM containing low serum (0.5% FCS). At this point, CXCL12 (500 ng/ml) was added to the cells by itself or with either T22 or control ALA peptide at 1 μg/ml for the indicated times. Cells were then harvested 24 or 48 h later and counted by a hemacytometer.

**Statistical Calculation.** P values were based on two-sided, parametric Student’s t tests using the program Instat (Graphpad, San Diego, CA) with statistical significance set at P < 0.05.

**RESULTS**

**Transduction of B16 Melanoma Cells with Human CXCR4 and Luciferase.** As shown in our previous work, B16/F1 melanoma cells show little or no expression of chemokine receptors, including CCR7 and CXCR4 (13). Because mAbs were available that recognized human CXCR4 and because human and murine CXCL12 have nearly
we injected either CXCR4-B16 or pLN CX2-B16 cells into the footpads of mice and waited 18 days for tumors to grow in size. Footpad tumor sizes were not statistically different between the two groups ($P > 0.05$, $n = 7–10$). However, pulmonary metastases were significantly enhanced (~10-fold; $P = 0.03$) in the mice that had been injected with CXCR4-B16 cells in their footpads (Fig. 2B). Whereas significant differences between these two groups were readily apparent after quantification by luciferase assay, the gross appearance of the lungs of the two groups of mice revealed no obvious macroscopic tumor metastasis. We also examined the draining popliteal LNs of both sets of mice by luciferase assay and found no statistically significant pattern of enhanced LN metastasis in CXCR4-B16-injected animals. Thus, CXCR4 significantly increased pulmonary metastasis, in both i.v. and s.c. injection models, without enhancing LN metastasis.

To determine the kinetics of tumor accumulation after i.v. inoculation, we injected tumor cells and assessed accumulation in the lungs at 2, 6, 24, and 48 h after injection. Large numbers of cells were present to similar degrees in the lungs 2 h after injection, but this was followed by a sharp decrease in tumor cells over the next 4 h (Fig. 3). At the 24 h time point, however, there were ~5-fold more cells in CXCR4-B16-injected animals ($P < 0.05$), and there were 10-fold more cells at the 48 h time point ($P = 0.01$; Fig. 3). Thus, although CXCR4 expression did not lead to an increase in the number of accumulated tumor cells 2 or 6 h after injection, significantly increased numbers of tumor cells were found in the lungs of mice injected with CXCR4-B16 cells 24 and 48 h after initial i.v. inoculation.

**T22 Inhibits CXCR4-mediated Increases in Metastasis.** T22 is an 18-amino acid peptide that was first described to antagonize HIV-1 activity in vitro (26) and then shown to specifically block CXCR4-mediated HIV-1 fusion (23). To determine whether T22 would inhibit the CXCR4-mediated increase in metastasis, we injected CXCR4-B16 and pLN CX2-B16 cells i.v. into mice. Subgroups of CXCR4-B16 mice were treated daily with 4 µg of either T22 or ALA control peptide. CXCR4-B16 cells accumulated in greater numbers in the lung compared with pLN CX2-B16 cells (Fig. 4A), confirming the results described in Fig. 2A. Treatment of the CXCR4-B16-injected mice with T22 reduced metastasis to levels seen in pLN CX2-B16 injected mice, whereas treatment with ALA had no effect (Fig. 4A).

Histological examination of the lungs (Fig. 4B) of CXCR4-B16-injected mice revealed that the majority of the lung tissue was occu-
pied by tumor, explaining the increased mass of the lungs (Fig. 4C).
In lungs of pLNCX2-B16-injected mice or T22-treated CXCR4-B16-injected mice, however, smaller tumor foci that left the pulmonary architecture largely intact were observed. T22 did not block metastasis in pLNCX2-B16-injected mice (data not shown). Minimal levels of metastasis were detected in the livers, LNs, and kidneys of pLNCX2-B16- and CXCR4-B16-injected mice in several experiments. In one experiment, the mean luciferase levels for liver, kidney, and four pooled LNs from pLNCX2-B16-injected mice were 26, 6, and 9 units, respectively, whereas activities in CXCR4-B16-injected mice were 10, 15, and 10 units, respectively. The background luciferase activity of lung tissue for noninjected, unmanipulated mice was 4 units. The low absolute value of luciferase activity (near the limits of detection) shown by organs other than the lungs render statistical calculations uninformative. Interestingly, T22 had no significant effect on the growth of CXCR4-B16 cells injected into the footpad of mice compared with either PBS or ALA (data not shown). Thus, the CXCR4 antagonist, T22, blocks pulmonary metastasis, but not primary tumor growth, of CXCR4-B16 melanoma.

CXCL12 Enhances Adhesion of CXCR4-B16 Cells to Resting Microvascular Endothelial Cells. To determine whether enhanced adhesion might account for enhanced metastasis in CXCR4-B16 cells, we tested the ability of recombinant CXCL12 to induce arrest of CXCR4-B16 cells to both resting human MECs (DMECs) derived from neonatal foreskin or to LMECs, which are more likely to be similar to pulmonary endothelial cells in vivo. Endothelial cells rapidly capture certain chemokines, including CXCL12, from exogenous sources (25). Furthermore, CXCL12 has been shown to be expressed in situ by MECs in the lung and skin (27). We exposed DMECs and LMECs to recombinant chemokines, allowed either pLNCX2-B16 or CXCR4-B16 cells to make contact with the MECs in the absence of shear forces, and then increased wall shear stress to remove unbound melanoma cells. Exogenously added CXCL12 was able to stimulate adhesion of CXCR4-B16 cells to DMECs by ~5-fold, whereas CCL21 had no effect (Fig. 5). By contrast, pLNCX2-B16 cells showed little appreciable adhesion to DMECs either with (Fig. 5) or without CXCL12 (data not shown). With LMECs, we observed that CXCR4-B16 cells remained associated to the endothelial cells despite vigorous washing (Fig. 6B) and bound ~2.5 times as efficiently to LMECs as did the control (pLNCX2-B16) cells (Fig. 6A). In contrast to DMEC binding, exogenous pretreatment of LMECs with CXCL12 did not further increase CXCR4-B16 binding (see “Discussion”). Pretreatment of the CXCR4-B16 cells with pertussis toxin to block chemokine-mediated signaling resulted in decreasing binding to the level observed with pLNCX2-B16 cells (Fig. 6A). Thus, the expres-
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CXCR4 Increased Adhesion of B16 Cells to Both DMECs and LMECs.

CXCL12 Enhances CXCR4-B16 Growth under Low Serum Conditions in Vitro.

CXCL12 promotes accumulation of CXCR4-B16 cells cultured in vitro. CXCR4-B16 cells (2 \times 10^4 cells/well in triplicate) were initially plated in vitro under low serum (0.5% FCS) and exposed to CXCL12 (500 ng/ml) in the presence or absence of T22 or ALA (1 \mu g/ml) or to T22/ALA alone (1 \mu g/ml) as indicated and then counted at the indicated time points. At 24 h: *, P = 0.06 versus PBS-treated cells. At 48 h: *, P = 0.001; #, P = 0.43; and **, P = 0.04 (all versus PBS-treated cells).

CXCR4 Is Expressed by Primary Human Cutaneous Melanoma Tumor Cells as well as by Melanoma Cells Metastatic to the Lung.

CXCR4 and B16 Lung Metastases

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Fig. 7. CXCL12 promotes accumulation of CXCR4-B16 cells cultured in vitro. CXCR4-B16 cells (2 \times 10^4 cells/well in triplicate) were initially plated in vitro under low serum (0.5% FCS) and exposed to CXCL12 (500 ng/ml) in the presence or absence of T22 or ALA (1 \mu g/ml) or to T22/ALA alone (1 \mu g/ml) as indicated and then counted at the indicated time points. At 24 h: *, P = 0.06 versus PBS-treated cells. At 48 h: *, P = 0.001; #, P = 0.43; and **, P = 0.04 (all versus PBS-treated cells).

Fig. 8. CXCR4 is expressed by primary melanoma and by melanoma metastatic to lung. Primary cutaneous human melanoma (A and B; \times 100 magnification) and cutaneous melanoma metastatic to lung (C–E; \times 200 magnification) from another patient was stained with anti-human CXCR4 (A, C, and E) or isotype control mAb (B and D) and visualized with 3-amino-9-ethylcarbazole (red) as described in “Materials and Methods.” The counterstain is hematoxylin. E, a close-up view of the rectangular inset depicted in C.
tases tested showed strong CXCR4 reactivity, whereas all three primary cutaneous melanomas tested showed variable but readily detectable CXCR4 expression.

**DISCUSSION**

In our previous work, we found that expression of CCR7 enhanced the metastasis of B16 cells from the periphery to draining LNcs but that CCCR7 expression did not affect metastasis to the lung after i.v. injection (13). Besides showing that CXCR4 can be expressed by primary and metastatic melanoma cells in human cancer patients, we now demonstrate in an experimental metastasis model that CXCR4 expression increases pulmonary metastasis after both i.v. and s.c. inoculation without affecting metastasis to several other sites, including the LNs, liver, and kidney. Thus, taken together, our results suggest that chemokine receptors may play distinct roles in the metastasis of common cancers. Moreover, they may explain why cancers that show high rates of both LN and distant organ metastasis concurrently express CCR7 and CXCR4. Whereas Müller et al. (8) suggest that CXCR4 may be involved in nodal metastasis, this interpretation must be made cautiously because a binding control antibody was not used in their experiments (8). Moreover, if DC migration from the periphery to draining LNs is taken as a model for tumor cell metastasis to LNs, the current literature does not support an important role for CXCR4 despite its up-regulation by activated DCs and the availability of effective CXCR4 antagonists. Our results demonstrate that CXCR4 specifically enhances metastasis in the lungs while minimally affecting metastasis to certain other sites (i.e., the LNs) that express significant levels of CXCL12. The mechanisms that enable some organs such as the liver to resist metastasis (2), despite high expression of CXCL12 (8), certainly deserve further consideration.

We found that CXCR4 may promote metastasis by at least two mechanisms. First, CXCR4-B16 cells showed dramatic increases in adhesion to MECs with and without prior exposure to recombinant CXCL12, depending upon whether DMECs or LMECs were used as adhesion substrates. Indeed, CXCL12 has been shown to enhance adhesion of CXCR4-expressing melanoma and hematopoietic cells to extracellular matrix proteins such as fibronectin (16, 28, 29). The significant difference in adhesion of CXCR4-B16 and pLNCX2-B16 in the absence of added CXCL12 may reflect endogenous low-level expression of CXCL12 by endothelial cells as observed previously (27). In fact, CXCL12 was detected on DMECs by flow cytometric analysis (data not shown). It is possible that LMECs expressed sufficient CXCL12 such that CXCR4-B16 cells were stimulated to bind even in the absence of exogenously added CXCL12 (Fig. 6A). The higher background binding observed with LMECs compared with DMECs may be indicative of an adhesion profile that could explain the high degree of sequestration of B16 tumor cells in the lung after i.v. inoculation.

In contrast to lymphocyte binding to DMECs (22), activation of DMECs and LMECs with cytokines is not needed to achieve significant binding of CXCR4-B16 cells. Thus, this model parallels the in vivo metastasis of cancer cells, in which tumor cells bind to presumably normal endothelial cells before invasion and proliferation. Our kinetic data (Fig. 3), as well as those of Fidler (30), reveal a relatively rapid, nonspecific initial capture followed by differences in adhesion and proliferation. Other studies have shown that cancer cells of both high and low metastatic potential arrest on hepatic blood vessels because of size constraints and that subsequent adhesion and proliferative events determine differences in metastatic potential (31).

Interestingly, CXCL12 promoted CXCR4-B16 cell growth or survival under the stress of low serum conditions (Fig. 7). During the course of metastasis, unattached tumor cells likely encounter other stressful conditions, as suggested by the result that 99% of i.v. injected B16 melanoma cells die within 24 h of inoculation (30). We speculate that the role of CXCR4 in the vascular metastasis of tumor cells bears some analogy to the role of this receptor in hematopoietic stem cell engraftment, where CXCR4 may be crucial (18). In this regard, CXCL12 has been shown to enhance the proliferation of circulating CD34+ stem cells in synergy with stem cell factor and other cytokines (19) through a mechanism that includes the suppression of apoptosis and the promotion of G1/G0 transition (20).

In summary, we demonstrate that expression of CXCR4 is sufficient to dramatically affect the metastatic accumulation of tumor cells in the lungs of mice. In contrast to our prior work with CCR7, expression of CXCR4 does not lead to increased metastasis in the draining regional LNs, suggesting that chemokine receptors may play distinct roles in organ-selective metastasis. Finally, the successful use of CXCR4-specific inhibitors such as T22 to block pulmonary metastases in CXCR4-B16-injected mice raises the possibility that small molecule inhibitors of CXCR4 such as T22, ALX40-4C (32), and AMD3100 (33) may have a role in preventing or ameliorating the morbidity and mortality associated with tumor metastasis in humans.

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