Pivotal Role of CXCR3 in Melanoma Cell Metastasis to Lymph Nodes

Kenji Kawada, 1,3 Masahiro Sonoshita, 1 Hiromi Sakashita, 2 Arimichi Takabayashi, 4 Yoshio Yamaoka, 3 Toshiaki Manabe, 2 Kayo Inaba, 5 Nagahiro Minato, 6 Masanobu Oshima, 1 and Makoto Mark Taketo 1

Departments of 1Pharmacology, 2Clinical Pathology, and 5Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, Kyoto; 3Kitano Hospital Medical Institute, Osaka; and Departments of 4Animal Development and Physiology, and 6Immunology and Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

ABSTRACT

Chemokines and their receptors play key roles in leukocyte trafficking and are also implicated in cancer metastasis to specific organs. Here we show that mouse B16F10 melanoma cells constitutively express chemokine receptor CXCR3, and that its ligands CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC induce cellular responses in vitro, such as actin polymerization, migration, invasion, and cell survival. To determine whether CXCR3 could play a role in metastasis to lymph nodes (LN), we constructed B16F10 cells with reduced CXCR3 expression by antisense RNA and investigated their metastatic activities after s.c. inoculations to syngeneic hosts, C57BL/6 mice. The metastatic frequency of these cells to LNs was markedly reduced to ~15% (P < 0.05) compared with the parental or empty vector-transduced cells. On the other hand, pretreatment of mice with complete Freund’s adjuvant increased the levels of CXCL9 and CXCL10 in the draining LNs, which caused 2.5–3.0-fold increase (P < 0.05) in the metastatic frequency of B16F10 cells to the nodes with much greater efficiency. Importantly, such a stimulation of metastasis was largely suppressed when CXCR3 expression in B16F10 cells was reduced by antisense RNA or when mice were treated with specific antibodies against CXCL9 and CXCL10. We also demonstrate that CXCR3 is expressed on several human melanoma cell lines as well as primary human melanoma tissues (5 of 9 samples tested). These results suggest that CXCR3 inhibitors may be promising therapeutic agents for treatment of LN metastasis, including that of melanoma.

INTRODUCTION

Lymph node (LN) metastasis is one of the earliest features of tumor cell dissemination in most human cancers, and ~60% of metastasis is found in regional LNs in malignant melanoma (1). It has been proposed that lymphogenous and hematogenous metastases occur rather simultaneously, because lymphatic and lymphaticovenous shunts often bypass regional LNs and allow dissemination of malignant cells at an early stage (2). Thus, metastasis of cancer cells to the regional LN appears to be a reflection of the biological aggressiveness of the primary tumors (3), and its assessment is critical for predicting prognosis and setting up therapeutic strategies (4).

Chemokines are structurally related, small-polypeptide signaling molecules that bind to and activate a family of G-protein-coupled receptors. Chemokines are divided into four families, CXC, CC, C, and CX3C, based on the positions of four conserved-cysteine residues. Important roles of chemokines and their receptors have been demonstrated in inflammation, infection, tissue injury, allergy, and cardiovascular diseases, as well as in malignant tumors (5). The role of chemokines in malignant tumors appears complex. Whereas many chemokines show antitumor activity by stimulating immune cells or by inhibiting tumor neovascularization, other chemokines may promote tumor growth and metastasis by direct growth stimulation, enhancing cell motility, or angiogenesis (6). Regarding the direct role of chemokines in tumor metastasis, recent reports suggested that chemokine receptors CXCR4 and CCR7 play significant roles in metastasis of melanoma, breast, and ovarian cancers to specific tissues (7–9). In contrast, the role of CXCR3 in metastasis has not been elucidated, although it is expressed on some human cancer cells including melanoma and malignant B lymphocytes and mediates chemotaxis in these cancer cells (10, 11).

In this article, we demonstrate that CXCR3 plays a critical role in B16F10 melanoma cell metastasis to LNs, and that increased expression of CXCL9 and CXCL10 in the LNs by complete Freund’s adjuvant (CFA) treatment facilitates melanoma cell metastasis through CXCR3. These results suggest that CXCR3 can be a novel therapeutic target to suppress LN metastasis in some cancers that express it.

MATERIALS AND METHODS

Cell Lines, Reagents, and Tissue Samples. B16F10 mouse melanoma cells and C32TG, G361, HMV-I and SK-Mel 28 human melanoma cells were obtained from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). Female C37BL/6j mice (6–8 weeks old) were purchased fromCLEA Japan (Osaka, Japan). Recombinant chemokines were from Peprotech (Rocky Hill, NJ). Primary melanoma tissue samples were collected, with informed consent, from either diagnostic biopsies or upon surgery at Kyoto University Hospital and Kitano Hospital. Histopathological diagnosis was confirmed for each specimen.

Reverse Transcription-PCR (RT-PCR) Analysis. Total RNA from cultured cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer’s protocol. Two μg of each RNA sample were reverse-transcribed and subjected to PCR under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, for 30 cycles. The primers for CXCR3 were 5‘-GGCGGACACGCAGCCAT-3’ and 5‘-AAGTGGACAGAGGTTGTC-3’; and for CCR10, 5‘-CTGAAATCTAGAAGTACCAC-3’ and 5‘-CAAAAAGGACATAGGACCCG-3’. The control RT-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed to normalize the sample amounts.

Immunohistochemistry and Immunofluorescence Microscopy. Formalin-fixed, paraffin-embedded sections were stained with antimonoc ContrastCXCR3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or antihuman CXCR3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) by the avidin-biotin immunoperoxidase method. In primary human melanoma tissues, microwave antigen retrieval was performed. For immunofluorescence staining of cytokines, cells are incubated in 0.5% fetal bovine serum for 4 h, treated with CXCL9 (100 ng/ml) for 5 min, fixed, and stained with rhodamine phalloidin (Molecular Probes, Eugene, OR). Frozen, OCT compound-embedded LNs were sectioned at 4 μm and stained with antibodies for mouse CXCL9 (R&D Systems) or CXCL10 (Santa Cruz Biotechnology) simultaneously with CD11b (BD Pharmingen, San Jose, CA), followed by biotinylated secondary antibody and Fluorescein Avidin DCS (Vector Laboratories, Burlingame, CA) or antibiotex A594 antibody (Molecular Probes).

Chemotaxis and Chemoinvasion Assays. Migration and invasion were assayed in 24-well Transwell cell culture chambers (8 μm-pore membranes; Coster, Cambridge, MA) as described (12). Membranes were precoated with fibronectin (10–20 μg/ml) for chemotaxis or with Matrigel (30 μg/ml) for invasion studies. After B16F10 cells (5×105 or 2.5×106 cells/ml for chemotaxis or invasion studies) were added to the upper chamber and incubated for 6 h for chemotaxis, or 24 h for invasion studies, cells attached on the
lower surface of the membrane were counted in at least five different fields (original magnification, ×200). Chemotaxis and invasion indices were defined as the ratios of migrating cell numbers in the experimental groups divided by those in the controls. At least three experiments were performed for each set. Chemokinesis was tested in checkerboard assays and was negative for all of the chemokines. Proteins from normal and inflamed LNs were extracted in Tris-HCl with protease inhibitor as described (13). For neutralizing studies, protein extracts were preincubated with various concentrations of anti-CXCL9, anti-CXCL11 (R&D Systems), anti-CXCL10, and anti-CCL21 (Peprotech) antibodies, respectively.

**Phosphorylation of Focal Adhesion Kinase (FAK) and Paxillin.** B16F10 cells (4 × 10^5 cells) were incubated for 2 h without serum on collagen-coated 6-cm dishes, treated with CXCL9 (100 ng/ml), and lysed with 1 ml of lysis buffer [50 mM Tris, 150 mM NaCl, 1 mM EGTA, 2 mM NaN_3, 50 mM NaF, 1% NP-40, 4 mM Na_2PO_4, and protease inhibitors (pH 7.4)]. Each 0.45-ml lysate was precipitated with anti-FAK or anti-paxillin antibody (Upstate Biotechnology, Lake Placid, NY) using protein G-Sepharose. One half of the precipitate was blotted and analyzed using enhanced chemiluminescence phosphorylation detection kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), whereas the other half was with anti-FAK or antipaxillin antibody.

**Cell Viability Assay.** B16F10 cells (1 × 10^6 cells) were incubated in triplicate for 48 h in serum-containing (0.1 and 10% FCS) or serum-free medium with or without CXCL9 (100 ng/ml). Viable cells were counted by the trypan blue dye exclusion method. At least three sets of experiments were performed for each set.

**Generation of CXCR3 Antisense Transfectants.** A 544-bp segment at the 5′ end of CXCR3 cDNA was amplified by RT-PCR, subcloned into TA cloning site of pcRII (Invitrogen, Carlsbad, CA), and then inserted, as a BamHI/XbaI fragment, into pcDNA3.1/Hygro (Invitrogen) in the antisense orientation. The following primers were used: 5′-AAGCCATGTACCTT-GAGGTTA-3′ and 5′-CAGACAGAGCCCCCATAACAQG-3′. The orientation of the subcloned gene was verified by restriction analysis with several enzymes and sequencing. With calcium phosphate precipitates, stable transfectants were selected with hygromycin B (200 μg/ml) for 3 weeks. CXCR3 protein was determined by Western blotting for 40 μg of cell lysate. For calcium mobilization assay, the relative fluorescence was monitored after addition of CXCL9 (300 ng/ml) in a Fluoroskan Ascent FL (Labsystems, Helsinki, Finland), after preincubation with 20 μM Fluo-3 a.m. (Molecular Probes). For proliferation analysis, cells (1 × 10^5 cells) were incubated for 48 h in medium supplemented with 5% fetal bovine serum and counted in a hemocytometer.

**Quantitative RT-PCR Analyses.** Total RNA from homogenized LNs was extracted using ISOLate, treated with DNase I to eliminate possible genomic DNA contamination, and then reverse-transcribed. Thereafter, cDNA was amplified and measured using an ABI-7700 DNA Sequence Detector (PerkinElmer Corp., Foster City, CA). The following primers and probes were used: tyrosinase-related protein-1 (TRP-1), 5′-CCTAGCTAGTTCCTGTGGA-CATGA-3′, 5′-TGGACGCCCCTCTAGATAAGA-3′, and 5′-CAGAACAGCTTGCCAGAGGT-3′; CXCL9, 5′-AGAATCTCAGCTGCTCAAGGCTTCCTTATGTTC-3′, and 5′-CCAATGCTGCAGAGAGGCTTCCTTATGTTC-3′; CXCL10, 5′-AGAATCTCAGCTGCTCAAGGCTTCCTTATGTTC-3′, and 5′-CCAATGCTGCAGAGAGGCTTCCTTATGTTC-3′; CXCL11, 5′-AGAATCTCAGCTGCTCAAGGCTTCCTTATGTTC-3′, and 5′-CCAATGCTGCAGAGAGGCTTCCTTATGTTC-3′; and CXCL12, 5′-AGAATCTCAGCTGCTCAAGGCTTCCTTATGTTC-3′, and 5′-CCAATGCTGCAGAGAGGCTTCCTTATGTTC-3′. The glyceraldehyde-3-phosphate dehydrogenase mRNA levels were used for normalization. Serial dilution studies demonstrated that the TRP-1 signal could be detected above background in the cDNA samples derived from as few as 50 melanoma cells mixed with 5 × 10^6 murine LN cells.

**Flow Cytometry.** CCR3 on melanoma cells was stained with anti-human CXCR3 antibody and analyzed in a FACSscan (Becton Dickinson, Mountain View, CA) as described (10).

**In Vivo Metastasis Studies.** For spontaneous metastasis experiments, B16F10 parental, EV1, AS1, and AS2 transfectant cells (5 × 10^5 cells in 30 μl of PBS) were injected into the right hind footpad at day 0. At day 7, right popliteal LNs were dissected and pooled for each group of 5 mice to quantify the tumor burden by quantitative RT-PCR as described (8). Note that there is only one popliteal LN on each side in the mouse. At day 21, mice were euthanized and necropsied for gross metastasis in the right popliteal LNs. In the CFA-treated metastasis experiments, 30 μl of CFA or PBS were injected into the right ankle of C57BL/6 females at day −3. For neutralization studies, mice preincjected with CFA were injected with 1 μg each of goat anti-CXCL9 and anti-CXCL10 antibodies or 2 μg of control goat IgG in 20 μl of PBS into the popliteal region, daily for the next 7 days, followed by three times per week for 2 more weeks. All of the animal experiments were approved by the Animal Care and Use Committee of Kyoto University.

**RESULTS**

**Expression of Chemokine Receptors in Metastatic Mouse Melanoma Cell Lines.** Mouse melanoma cell line B16F10 provides a convenient transplantable model that is metastatic to LNs in the syngeneic host, C57BL/6 (14). We first determined expression of various chemokine receptors (CCR1-CR10, CXCR2-CXCR5, XCR1, and CX(CR1) in B16F10 cells by RT-PCR. Among them, only CXCR3 and CCR10 were expressed with no detectable mRNA for other chemokine receptors (Fig. 1A). Expression was not found for any ligands for CXCR3 (CXCL9, CXCL10, or CXCL11) or CCR10 (CCL27/ESkine/CTACK/ALP/ILC or CCL28/MEC; data not shown). We then confirmed expression of the CXCR3 protein by immunohistochemistry in transplanted tumors. B16F10 expressed CXCR3 at the

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**Fig. 1.** Expression of chemokine receptor CXCR3. A, detection of mRNAs for chemokine receptors CXCR3 and CCR10 in B16F10 melanoma cells by reverse transcription-PCR. RNA from spleen was used as a positive control, whereas B16F10 mRNA without reverse transcription was a negative control. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control. B–G, expression of CXCR3 protein in B16F10 melanoma sections determined by immunohistochemistry (3 weeks postinoculation). B and C, a primary transplanted tumor. D and E, a metastatic lung nodule. F and G, a metastatic lung nodule stained with the IgG isotype. Note that dark spots in the controls show melanin pigments produced by melanoma cells. C, E, and G, stained with antimonouse CXCR3 antibody (Hematoxylin counterstaining). Scale bars, 100 μm.
primary inoculation site as well as in the metastatic foci of LNs and lungs (Fig. 1, B–G).

Chemokine-Mediated Migration and Invasion. In addition to CXCL9, CXCL10, and CXCL11, a CC chemokine CCL21/SLC can also bind to CXCR3 in mice although with a lower affinity (15). To determine whether these chemokines could induce migration and invasion, we then performed in vitro assays (Fig. 2, A and B). Among them, CXCL9, CXCL10, and CXCL11 caused directional migration and invasion of B16F10 cells in a dose-dependent manner. In contrast, CCL21 did not induce either response even at 500 ng/ml. The strongest responses of B16F10 cells to these chemokines were obtained at concentrations similar to those for leukocytes (16).

CXCL9-Induced Rearrangements of Actin Cytoskeleton, Focal Adhesions, and Cell Survival. Binding of a chemokine to its receptor triggers intracellular actin polymerization, a prerequisite for cell motility and migration, in cultured leukocytes (17). As expected, B16F10 cells also showed a polarized pattern of F-actin staining in higher proportions of cells when incubated with CXCL9, a ligand for CXCR3. Addition of CXCL9 at 100 ng/ml increased the proportion of F-actin polarized cells approximately twice (Fig. 2C). Their phosphorylation took place shortly after addition of CXCL9, lasted for ~10 min, and was reduced to the baseline level after 30 min. In human melanoma cell line BLM, CXCL9 up-regulates \( \beta_1 \) integrin-dependent cell adhesion to fibronectin (10). Thus, it is conceivable that CXCL9 induces a rapid and transient up-regulation of \( \beta_1 \) integrin-mediated adhesion of B16F10 cells to the extracellular matrix in the LN. In addition to induction of chemotaxis, activation of CXCR4 by its ligand CXCL12/SDF-1α stimulates cell growth and survival in some cell types (20, 21). Therefore, we analyzed the effect of CXCL9 on cell viability in B16F10 cells (Fig. 2F). CXCL9 did not show any effects on cell proliferation under either normal (10% FCS) or low (0.1% FCS) serum condition. Without serum, on the other hand, CXCL9 significantly enhanced cell survival compared with the untreated control (\( P < 0.05 \)).

Construction and Analysis of B16F10 Transfectants with Reduced CXCR3 Expression. To evaluate the role of CXCR3, we isolated B16F10 transfectant clonal cell lines in which expression of CXCR3 was reduced by an antisense RNA construct (“Materials and Methods”). Three antisense transfectants (AS1, AS2, and AS3) and two empty vector transfectants (EV1 and EV2) were established. The presence of antisense transcripts was verified by RT-PCR (data not shown). A Western blot analysis showed that the CXCR3 protein levels in the antisense clones were decreased to 20–25% of those in the parental or empty vector-transfected clones (Fig. 3A). To determine their responses to the CXCR3 ligands, we performed calcium mobilization and chemotaxis assays. Intracellular calcium flux is one of the earliest biochemical events that takes place in response to...
chemokines (22). Addition of CXCL9 to B16F10 cells induced 15–20% increase in the intracellular Ca\(^{2+}\) concentration. Although the B16F10 transfectants with an empty vector showed similar increases in the Ca\(^{2+}\) concentration, those with the antisense construct showed only modest increases of ∼5% (Fig. 3B). Consistent with the result, the CXCL9-induced migratory responses were virtually eliminated in the antisense-transfectant clones (Fig. 3C). To rule out possible effects of CXCR3 suppression on cell proliferation, we determined the growth rates of the parental and transfectant B16F10 clones, without significant difference among the clones (Fig. 3D).

**Suppressed Metastasis of Antisense-CXCR3 Cells to LNs.** To assess the effect of CXCR3 on LN metastasis, we inoculated B16F10 melanoma cells into the hind footpads of syngeneic mice. One week later, we collected popliteal LNs and quantified the mRNA levels for TRP-1, a melanocyte-specific marker, by real-time RT-PCR. This assay gives a sensitive quantification of LN micrometastasis and has been used clinically to stage human melanoma (23). In a week, the primary tumors in the footpads became grossly visible, and macroscopic metastasis was found in LN of some mice. Quantitatively, TRP-1 mRNA level from the mice inoculated with the antisense-transfectant clones (AS1 and AS2) was reduced to only 12.5%–25% of that with the empty vector-transfectant clones (EV1; \(P < 0.05\)), whereas that with the parental cells was essentially the same as with the EV1 clone (Fig. 4A). The expression levels of TRP-1 mRNA in cultured cells were virtually the same among the parental, empty vector- and antisense-transfectant clones (8–9 pg/ng cDNA).

To evaluate the tumor metastasis in a later stage, we dissected popliteal LNs and examined metastatic foci 3 weeks after inoculations (Fig. 4B), when the primary tumors in the footpads reached ∼1 cm in diameter. Although the parental and EV1 clones formed foci in 40% (10 of 25) of the LNs in total, the AS1- and AS2-transfected clones metastasized to only 6.3% (1 of 16) of LNs (Table 1; \(P < 0.05\)). In more detail, only 12.5% (1 of 8) and 0% (0 of 8) of the mice inoculated with AS1- and AS2-transfected clones, respectively, showed metastatic foci, whereas 37.5% (6 of 16) and 44.1% (4 of 9) of the mice formed foci with the parental and EV1-transfectant clones, respectively. Regarding the transplanted primary tumors, there was no significant difference in size among mice inoculated with the parental, empty vector- and antisense-transfectant clones (Table 1; Student’s \(t\) test). We also examined lung metastasis and found no significant difference among these clones. Protein extracts from the primary tumors were analyzed for CXCR3 expression by Western blotting to confirm that reduced CXCR3 expression was maintained in the antisense-transfectant clones in vivo (data not shown). These results demonstrate that expression of CXCR3 plays a pivotal role in B16F10 melanoma cell metastasis to the draining LNs.

**Enhanced Migration of B16F10 by CFA-induced CXCL9 and CXCL10.** Both CXCR3 ligands, CXCL9 and CXCL10, are expressed constitutively at high levels within lymphoid organs, but not in the lung, liver, or brain (24, 25). Moreover, localized inflammation induced by CFA up-regulates CXCL9 and CXCL10 in the draining LNs by IFN-γ produced from Th1(+) lymphocytes (26, 27). To identify the CXCR3 ligands in inflamed LNs, we first determined by quantitative RT-PCR the mRNA levels for CXCL9, CXCL10, CXCL11, and CCL21 in the LNs 3 days after injection of CFA or PBS (Fig. 5A). Upon inflammation, there were 2.0- and 6.7-fold increases in the mRNA levels for CXCL9 and CXCL10, respectively, whereas little CXCL11 mRNA was detected. Interestingly, CCL21 mRNA decreased to ∼20% upon inflammation. The mRNAs for CCR10 ligands CCL27 and CCL28 were undetectable in the LNs (data not shown), although CCL27 is a skin-specific homeostatic chemokine (28). We then examined expression and localization of CXCL9 and CXCL10 proteins within the LN by immunohistochemistry (Fig. 5B). Both CXCL9 and CXCL10 were markedly up-regulated in the in-
flamed LN compared with the control LNs, and they were found mainly in the subcapsular and cortical sinuses, where CD11b (H11001) macrophages were abundant (data for CXCL10 not shown). Because tumor cells initially arrive at the subcapsular sinus in LNs as tumor emboli via afferent lymphatics (29), the locale of CXCL9 and CXCL10 expression coincides with the initial arrest site of the tumor cells.

To confirm the biological activities of chemokines induced in the inflamed LNs, we additionally determined the chemotactic activities of protein extracts from the CFA-induced inflamed LNs (Fig. 5). The extracts showed migratory responses twice as much as those of the normal LNs (sample 3). As expected, anti-CXCL9 or anti-CXCL10 antibodies significantly suppressed the migratory response in a dose-dependent manner (samples 4–7), but anti-CXCL11 or anti-CCL21 antibodies showed no effects (samples 10–13). Migration of B16F10 cells was additionally suppressed when anti-CXCL9 and anti-CXCL10 antibodies were combined (samples 8 and 9).

Enhanced Metastasis to Draining LNs by CFA-Induced CXCL9 and CXCL10. To further confirm that CXCL9 and CXCL10 within the draining LN stimulate B16F10 metastasis, we treated host

Table 1 Metastasis frequency of B16F10 clones to LNs

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>No. of metastatic LNs</th>
<th>Primary tumor volume (mm³)</th>
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<tr>
<td>B16F10 (n = 16)</td>
<td>6/16 (37.5)</td>
<td>364 ± 115</td>
</tr>
<tr>
<td>EV1 (n = 9)</td>
<td>4/9 (44.1)</td>
<td>322 ± 77</td>
</tr>
<tr>
<td>Total (n = 25)</td>
<td>10/25 (40.0%)</td>
<td>337 ± 83</td>
</tr>
<tr>
<td>AS1 (n = 8)</td>
<td>1/8 (12.5)</td>
<td>339 ± 83</td>
</tr>
<tr>
<td>AS2 (n = 8)</td>
<td>0/8 (0.0)</td>
<td>307 ± 90</td>
</tr>
<tr>
<td>Total (n = 16)</td>
<td>1/16 (6.3%)</td>
<td>337 ± 83</td>
</tr>
</tbody>
</table>

* LN, lymph node.

a Primary tumor size on day 21. Values are mean ± SD.

* Note that the difference between the value here (40%) and that in Table 2 control (30%) is not statistically significant (P = 0.49).

b p < 0.05 compared with “total of B16F10 and EV1” (Fisher’s exact test).

flamed LN compared with the control LNs, and they were found mainly in the subcapsular and cortical sinuses, where CD11b (+) macrophages were abundant (data for CXCL10 not shown). Because tumor cells initially arrive at the subcapsular sinus in LNs as tumor emboli via afferent lymphatics (29), the locale of CXCL9 and CXCL10 expression coincides with the initial arrest site of the tumor cells.

To confirm the biological activities of chemokines induced in the inflamed LNs, we additionally determined the chemotactic activities of protein extracts from the CFA-induced inflamed LNs (Fig. 5C). The extracts showed migratory responses twice as much as those of the normal LNs (sample 3). As expected, anti-CXCL9 or anti-CXCL10 antibodies significantly suppressed the migratory response in a dose-dependent manner (samples 4–7), but anti-CXCL11 or anti-CCL21 antibodies showed no effects (samples 10–13). Migration of B16F10 cells was additionally suppressed when anti-CXCL9 and anti-CXCL10 antibodies were combined (samples 8 and 9).

Enhanced Metastasis to Draining LNs by CFA-Induced CXCL9 and CXCL10. To further confirm that CXCL9 and CXCL10 within the draining LN stimulate B16F10 metastasis, we treated host

Fig. 4. Suppression of B16F10 metastasis to lymph node (LN) by inhibition of CXCR3 expression. Experimental schedules are shown above each set. A, quantification of melanoma tumor marker tyrosinase-related protein-1 (TRP-1) mRNA in LN by real-time reverse transcription-PCR 1 week after tumor inoculations. Mean, bars, ± SD. (Student’s t test; *, P < 0.05). Each circle represents a set of 5 mouse samples pooled. B, representative photographs of the popliteal LNs from mice 3 weeks after inoculations with B16F10, EV1, AS1, and AS2, respectively. Black metastatic foci are visible because B16F10 produces melanin pigments. Note that only one popliteal LN was found on each side. Scale in mm.

Fig. 5. Chemokine expression in lymph node (LN) and migration of B16F10 cells toward the protein extracts of LN. A, quantitative reverse transcription-PCR analysis of chemokines in the inflamed LN, 3 days after complete Freund’s adjuvant (CFA) injection. Control mice were injected with PBS. Mean, bars, ± SD. (Student’s t test; *, P < 0.01). B, expression of CXCL9 protein (green) in the LN, 3 days after CFA or PBS injection, shown simultaneously with localizations of CD11b⁺ cells (red) and nuclei of cells within LN (blue). SS, subcapsular sinus; BF, B cell follicle. Scale bars, 20 μm. C, chemotaxis of B16F10 cells by LN extracts (1 mg of protein/sample). *, P < 0.01; **, P < 0.01 (sample 3 versus 4–9), and †, P < 0.05 (sample 4 or 6 versus 8).

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mice with CFA 3 days before the melanoma cell inoculations. One week later, the LNs from the mice preinjected with CFA contained 11 times more TRP-1 mRNA than those from the mice with PBS (Fig. 6A; P < 0.01). This increase in the TRP-1 mRNA level by injection of CFA was comparable between the mice inoculated with the parental and EV1 clones. In contrast, the mRNA level in the LN was only 17%–36% in mice inoculated with the AS1 or AS2 clones compared with that of EV1-injected mice (Fig. 6A; P < 0.01).

Three weeks after inoculations, the parental B16F10 cells formed foci in 91% (21 of 23) of the LNs in the host mice that had been injected with CFA, although the melanoma cells metastasized to only 30% (6 of 20) of LNs in mice preinjected with PBS (Table 2; P < 0.01). Importantly, this stimulation of LN metastasis by the CFA preinjection was suppressed significantly when the CXCR3 antisense-transfectant clones were inoculated (Fig. 6B; Table 2; P < 0.01). Only 46% (7 of 15) and 33% (5 of 15) of the mice inoculated with AS1- and AS2-transfected clones, respectively, showed metastatic foci of relatively small size. Regarding the primary tumor transplants, there was no significant difference in size among mice inoculated with the parental, empty vector- and antisense-transfectant clones (Table 2; Student’s t test). We also examined lung metastasis, but found no significant difference among these clones.

To additionally verify the roles of CXCR3 and its ligands in B16F10 metastasis to LNs, we attempted to suppress metastasis with antibodies against CXCL9 and CXCL10 induced by prior injection of CFA. Namely, we repeatedly injected neutralizing antibodies into the popliteal region around the LN and compared the effects with the control IgG isotype (Fig. 6C). Three weeks after the inoculation of parental B16F10 cells, only 3 of 7 antibody-treated mice contained macroscopic metastatic foci, although all 7 of the IgG-treated controls had LN metastasis. Furthermore, only 1 LN (i.e., 1 mouse) contained foci >1 mm among 7 mice treated with the antibodies, although all 7 of the nodes from the IgG controls contained much larger foci of 2–4 mm. Thus, CXCL9 and CXCL10 induced by CFA were responsible for the stimulation of B16F10 metastasis to the draining LNs. Inhibition of CXCR3 activation, either by reduced receptor expression or by neutralized ligand chemokines, can suppress LN metastasis at both incidence and size of the foci.

Expression of CXCR3 on Human Melanoma Cells. Analysis by flow cytometry of several human melanoma cell lines showed that CXCR3 was expressed moderately on C32TG, G361, and HMV-I cell lines and at low levels on SK-Mel 28 (Fig. 7A). Immunofluorescence microscopy of permeabilized melanoma cells confirmed the expression of CXCR3 on C32TG, G361, and HMV-I cell lines (Fig. 7B; data for G361 or HMV-I not shown). We additionally found in vivo expression of CXCR3 protein in primary human melanoma samples by immunohistochemistry. Samples from 5 of 9 patients expressed CXCR3 (Fig. 7C).

**DISCUSSION**

Metastasis appears to be an inefficient process because only a few metastatic foci are formed even when millions of cancer cells are injected into a mouse (30). Recent studies revealed that the least efficient steps in metastasis are the growth of the micrometastatic foci and their persistence (31), which strongly suggests the importance of interactions between cancer cells and the tissue environment of the specific metastatic sites. Accumulating evidence indicates that organ-specific metastasis can be aided by interactions between chemokine receptors on cancer cells and ligand chemokines in target organs. For example, breast cancer cells express CXCR4 and preferentially metastasize to lung and bone that are abundant in CXCR4-specific ligand CXCL12 (7, 32). It has been demonstrated that CXCR3 and CXCR4 are expressed in human melanoma cell lines as well as melanoma foci

![Image](https://example.com/image.png)
in infiltrated LNs and that expression of CXCR3 is often higher than that of CXCR4 (10). Notably, forced expression of CXCR4 in B16 melanoma cells enhances pulmonary metastasis, but metastasis to LNs, liver, or kidneys is not affected (33). Here, we have demonstrated that CXCR3 signaling is responsible for LN metastasis of B16F10 cells, without affecting metastasis to the lung. These results collectively indicate that distinct chemokine receptors control metastasis of melanoma cells to specific organs, CXCR3 to LNs and CXCR4 to lungs. We also found that CXCR3 is expressed in both B16F10 (high metastatic potential) and B16F1 (low metastatic potential) at similar levels (data not shown). Because B16F10 cells were selected for their ability to colonize the lung after cycles of i.v. injection and isolation from metastatic lung foci (34), expression of CXCR3 was not affected by the selection, probably.

Sentinel LNs in tumor-bearing hosts are the primary site where specific immune responses to the tumor antigens can be initiated to develop systemic tumor immunity (3, 35, 36). In addition to the intrinsic immunogenicity of tumor cells, the modes and amounts of tumor cells migrating into the sentinel LN significantly affect whether efficient immune responses can be elicited against them (36). LNs often show reactive histopathology with tumors, as follicular hyperplasia, sinus histiocytosis, lymphoid cell depletion, fibrosis, angiogenesis, sarcoideal reaction, and so forth (37, 38), although their precise roles in tumor dissemination remain unknown. Thus, migration of tumor cells from the primary sites into sentinel LNs is an important process for both possible host antitumor responses and establishment of eventual tumor metastasis. In the present study, we have demonstrated that CXCL9 and CXCL10 within LNs facilitate B16F10 melanoma cell metastasis through CXCR3, whereas it remains to be investigated whether accelerated migration of B16F10 cells into LNs helps initiate specific antitumor immunity or not.

CXCR3 is induced on Th1-type lymphocytes upon activation by IFN-γ (39), and its ligands, CXCL9, CXCL10, and CXCL11 are also up-regulated by IFN-γ, attracting effector Th1 cells to the sites of local inflammation (40–42). It has been demonstrated that CXCL9 and CXCL10 are responsible, at least in part, for the antitumor effect of IL-12 that is mediated by IFN-γ (43). In fact, CXCL9 gene therapy combined with an antibody–IL-2 fusion protein suppresses growth and lung metastasis of mouse colon carcinoma cells (44). CXCL9 also promotes tumor necrosis when injected directly into the tumor tissue (45). Notably, CXCL9 and CXCL10 play additional roles in tumor microenvironment. For example, CXCL9 activates RhoA and Rac1, induces actin reorganization, and triggers migration and invasion of human melanoma cells (10). Here we have demonstrated that CXCL9 and CXCL10 within LNs facilitate B16F10 metastasis to LNs through CXCR3 signaling, which causes such diverse cellular effects as cytoskeletal reorganization, migration, invasion, and enhanced cell survival. In the tumor microenvironment, abundant host leukocytes are often found in both the tumor tissue and stroma (46). It is suggested that inflammatory cells, cytokines, and chemokines found in tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host antitumor response (47). For example, tumor-associated macrophages, a major component of the inflammatory infiltrates, play dual roles. Although they may kill tumor cells after activation by IL-2, IL-12, and IFN (48), tumor-associated macrophages produce a number of potent angiogenic and lymphangiogenic growth factors, cytokines, and proteases that can enhance tumor progression. Thus, our present results suggest that inhibition of CXCR3 receptor may be a potential therapeutic target against LN metastasis of melanomas and other CXCR3-expressing tumor cells.

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