[CANCER RESEARCH 63, 6751–6757, October 15, 2003]

CXCR4 Enhances Adhesion of B16 Tumor Cells to Endothelial Cells *in Vitro* and *in Vivo* via β_1 Integrin

Adela R. Cardones, Takashi Murakami,[1] and Sam T. Hwang[2]

Dermatology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

The chemokine receptor, CXCR4, is expressed by human melanomas, and its ligand, CXCL12, is frequently produced at sites of melanoma metastasis. Herein, we examine CXCR4-enhanced binding of B16 murine melanoma cells to endothelial cells (ECs) and recombinant adhesion molecules *in vitro* to determine the role of tumor- and EC-derived adhesion molecules in tumor metastasis. By flow cytometry, unstimulated primary lung ECs showed constitutive expression of vascular cellular adhesion molecule 1 (VCAM-1), whereas skin-derived ECs did not. All B16 cell lines tested showed constitutive expression of α_4 and β_1 integrin chains but showed no expression of β_2 integrins. CXCR4-B16 arrest on VCAM-1/immunoglobulin-coated plates and tumor necrosis factor α-stimulated ECs under physiological shear stress conditions (1.5 dynes/cm^2) was rapid, resistant to shear stress of 10 dynes/cm^2, and showed no evidence of rolling before arrest. *In vitro*, CXCR4-B16 cell binding to ECs was blocked by anti-β_1 and anti-CXCL12 monoclonal antibodies. *In vivo*, metastasis of CXCR4-B16 cells to murine lungs was strongly inhibited by anti-CXCL12 and two different anti-β_1 monoclonal antibodies. Finally, CXCR4-B16 exposed to CXCL12 rapidly increased binding affinity for soluble VCAM-1/immunoglobulin as detected by a flow cytometric assay. Thus, β_1 integrins play a critical role in CXCR4-mediated B16 tumor cell metastasis *in vivo* and may be a potential target for inhibition of tumor metastasis, particularly to the lung.

INTRODUCTION

Metastasis of tumor cells to vital organs represents the major source of mortality in cancer. In melanoma, metastasis occurs in approximately 10% of patients. Half of melanoma metastases go to distant organs, leading to serious complications, including death. Whereas the entire process of metastasis is complex (1) and consists of multiple steps including detachment of tumor cells from the primary tumor and invasion of blood vessels, interaction of circulating tumor cells with vascular endothelium is a crucial step that precedes tumor cell extravasation into and invasion of the target organ. Therefore, our studies focus on the factors involved in the attachment of tumor cells to vascular endothelium at the site of distant metastasis.

The involvement of adhesion molecules in tumor migration has been intensively investigated. Integrins, especially the β_1 integrins, play important roles in melanoma invasion and metastasis through enhancement of motility and migration (2). Expression of α_1, α_3, α_4, α_5, and α_6 integrins by melanoma cells lines correlated with metastatic potential (3), and in the first study to demonstrate adhesion of tumor cells under shear stress conditions, the A375M human melanoma line bound to recombinant VCAM-1,[3] presumably through a β_1 integrin (4).

Because integrins constitutively exhibit low affinity for their ligands, other cell surface receptors, such as chemokine receptors, must usually be engaged first to increase the affinity as well as avidity of integrins for their ligands (5, 6). Chemokine receptors comprise four homologous families of seven-transmembrane-spanning, G protein-coupled receptors that activate key intracellular signaling pathways controlling cell shape, migration (chemotaxis), and proliferation (7). One of their functions is to increase leukocyte integrin affinity and avidity (5), leading to firm adhesion of leukocytes to vascular endothelium at sites of inflammation (8).

Recent work has shown that several chemokine receptors play critical roles in organ-selective cancer metastasis (9, 10). CCR7 is expressed by several cancers, including melanoma, and mediates enhanced metastasis to regional draining LNs (11, 12). Another receptor, CXCR4, is widely expressed in breast, prostate, and lung cancers as well as in melanoma and appears to promote metastasis of tumor cells to distant organs such as the lung (13, 14). CXCL12, the only known CXCR4 ligand, is expressed by stromal cells, including fibroblasts, and ECs (15) and is abundantly expressed in several tissues that are frequent sites of melanoma metastasis (13).

The mechanism by which CXCR4 expression enhances tumor metastasis is still unclear. In static assays, activation of CXCR4 by its ligand, CXCL12, appears to be able to trigger adhesion of a variety of tumor cell lines to extracellular matrix substrates such as fibronectin (16–18) and to vascular ECs (14). *In vivo*, tumor cells must be able to adhere to ECs despite the shear force exerted by vascular blood flow. To date, the ability of CXCR4 to trigger adhesion of cancer cells to ECs under shear stress conditions has not been investigated.

Because a CXCR4 inhibitor (T22 peptide) dramatically reduced pulmonary metastasis of CXCR4-expressing B16 melanoma cells *in vivo* (14), we sought to address two related questions in this study. First, when is CXCR4 function required in the metastatic process? Second, is CXCR4 activation sufficient to provide the necessary signal to convert integrins from a resting to active conformation, leading to tumor cell arrest under shear stress conditions? We now demonstrate that CXCR4 is required at an early step in metastasis and that, indeed, CXCR4 activation stimulates firm adhesion of B16 melanoma cells to both recombinant VCAM-1 and ECs under shear stress in a β_1 integrin-dependent manner. These studies provide a mechanistic link between recent findings that indicate that chemokine receptors facilitate organ-selective metastasis and earlier findings that demonstrated a role for β_1 integrins in cancer dissemination.

MATERIALS AND METHODS

Animals, Cell Lines, and Reagents. Female B57BL/6 mice (8–12 weeks old), as described previously (14), were used in experiments that were approved by the National Cancer Institute Animal Use and Care Committee. As

Received 4/23/03; revised 7/29/03; accepted 8/1/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] T. M. was supported, in part, by the Yasuda Medical Research Foundation.

[2] To whom requests for reprints should be addressed, at Dermatology Branch, Center for Cancer Research, National Cancer Institute, Building 10, Room 12N246, 10 Center Drive, Bethesda, MD 20892. Phone: (301) 496-8724; Fax: (301) 496-5370, E-mail: hwangs@mail.nih.gov.

[3] The abbreviations used are: VCAM-1, vascular cellular adhesion molecule 1; DMEC, dermal microvascular endothelial cell; LMEC, lung microvascular endothelial cell; mAb, monoclonal antibody; PTX, pertussis toxin; TNF, tumor necrosis factor; EC, endothelial cell; LN, lymph node; cDMEM, DMEM with 10% heat-inactivated FCS; Ig, immunoglobulin.

described previously (14), murine B16 cells were sequentially transduced with cDNA encoding either human CXCR4 (a gift from Dr. E. Berger; National Institute of Allergy and Infectious Diseases) in the pLNCX2 retroviral vector (Clontech, Palo Alto, CA) or pLNCX2 alone (empty vector) and then with cDNA encoding firefly (*Photinus puralis*) luciferase. For convenience, the CXCR4-luciferase B16 cell line is hereafter called CXCR4-B16, whereas the control vector-luciferase-transduced cell line is called pLNCX2-B16. CXCR4- and pLNCX2-B16 cells (14) were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 10% heat-inactivated FCS (cDMEM) and supplements, including puromycin and G418. Human DMECs (Emory University Department of Dermatology, Atlanta, GA) were derived from neonatal foreskin and cultured as described previously (19). Human LMECs (Clonetics-Cambrex, East Rutherford, NJ) were grown in EGM-2 MV (Clonetics-Cambrex). Mouse antihuman CXCR4 mAb (clone 44716.111), anti-human CXCL12 mAb (clone 77014.111), human TNF-α, VCAM-1/Ig, E-selectin, and P-selectin/Ig chimeras were purchased from R&D Systems (Minneapolis, MN). Purified rat antimouse mAbs against β_1, β_2, β_7, α_4, α_L, and α_V integrin and FITC-conjugated mouse antihuman CD106 (VCAM-1) were purchased from BD-PharMingen (San Diego, CA). The CXCR4 antagonist peptide T22 [NH_2-RRWCYRKCYKGYCYRKCR-COOH, (14, 20)] and an inactive control peptide (ALA, described in Ref. 20) were synthesized by Synpep (Dublin, CA). Chemokines were purchased from Peprotech (Rocky Hill, NJ). A mAb (B16MAB-1) against an uncharacterized cell surface antigen found on B16 cells was raised by immunizing hamsters with lysates of B16 cells. Hybridoma culture supernatants (Rockland Immunochemicals, Gilbertsville, PA) were screened for cell surface binding activity against B16 cells by flow cytometry. One clone (B16MAB-1) showed uniform binding to B16 cells but lacked binding to NIH 3T3 cells and murine LN immunocytes.

Flow Cytometry. B16 cells or ECs were first incubated with specific mAb (10 μg/ml) for 30–60 min at 4°C in 0.1% BSA/PBS, washed twice, and then incubated with FITC-conjugated secondary antibody (1:100) for 30 min at 4°C. Flow cytometric analysis was performed using FloJo software (TreeStar Inc., San Carlos, CA).

Soluble VCAM-1 Binding Assay. CXCR4-B16 or pLNCX2-B16 cells were placed in suspension (cDMEM, warmed to 37°C) and exposed to chemokine (500 ng/ml) in the presence of VCAM-1/Ig (or P-selectin/Ig as a control). As a positive control, Mn^{2+} (2 mM) was added to the suspension to increase integrin affinity for VCAM-1. VCAM-1/IgG or P-selectin/IgG chimera (2 μg/ml) was then added to the suspension. After 45 s, cells were fixed for 10 min with 4% paraformaldehyde at room temperature. The cells were washed and resuspended in 0.1% BSA/PBS and incubated with a FITC-conjugated antihuman IgG antibody at 4°C for 30 min before flow cytometric analysis.

Dynamic Flow Assay. Cell suspension plates (35 mm; Model # 430588; Corning Inc.) were coated with human VCAM-1/IgG chimera (1 μg/ml) overnight at 4°C in Tris-buffered saline (6). Plates were then briefly rinsed with PBS, co-coated with 1 μg/ml CXCL12 or CCL19 or no chemokine in PBS for 2 h at 4°C, and blocked with 1% BSA/PBS for 1 h at 4°C. Calcein-acetomethyl-labeled (Molecular Probes, Eugene, OR) CXCR4-B16 or pLNCX2-B16 cells (2.5×10^5 cells/ml in cDMEM) were injected at 1.5 dynes/cm^2 into a parallel plate flow chamber (Glycotech, Gaithersburg, MD) that had been placed over the adhesion molecule- and/or chemokine-coated suspension plate.

For dynamic flow assays using ECs, LMECs at passage 5–6 were cultured to confluence in 35-mm tissue culture plates (Model # 430165; Corning Inc.) and treated with TNF-α (10 ng/ml for 4 h at 37°C) before introduction of tumor cells. In some cases, ECs were exposed to 500 ng/ml recombinant CXCL12 or CCL19 for 15 min at 20°C as described previously (21), and unbound chemokine was washed away. Where indicated, LMECs were treated with anti-CXCL12 antibody or isotype control (100 μg/ml for 30 min at 37°C). All unbound antibody was washed off before infusion of B16 cells. When used, PTX (Calbiochem, San Diego, CA) was incubated with B16 cells at a concentration of 100 ng/ml for 2 h at 37°C to block G_i protein-coupled signaling. Where indicated, tumor cells were also treated with anti-CXCR4 mAb, hamster anti-mouse/rat β_1 integrin mAb (Ha2/5; BD-PharMingen), rat anti-mouse β_1 integrin (HMb1-1; BD-PharMingen), anti-B16 mAb, or isotype at 10 μg/ml for 30 min at 37°C and washed once before resuspension and infusion.

For dynamic assays on recombinant molecule-coated plates as well as on LMECs, tumor cells were introduced into the chamber under a constant shear stress of 1.5 dynes/cm^2 without allowing cells to settle at any time during the assay. Ten min after flow was initiated, arrested cells were digitally photographed (without flow being decreased) in 4–6 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 moving cells, which appeared as dim streaks, and stationary cells. Cells were then enumerated with the software program IPlab (Scanalytics, Fairfax, VA).

***In Vivo* Metastasis Assays.** 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. CXCR4-B16 or pLNCX2-B16 cells (4×10^5 cells in 200 μl of PBS) were then 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 as described previously (14). Mice were euthanized after 14 days for gross inspection of lungs and luciferase quantification of metastasis by luciferase activity (14). In experiments shown in Fig. 6, CXCR4-B16 cells were resuspended in 0.1% BSA/PBS and treated with a hamster antirat β_1 integrin mAb (Ha2/5; BD-PharMingen), a rat antimouse β_1 integrin mAb (9EG7; BD-PharMingen), or, as negative control, a hamster anti-B16 mAb (B16MAB-1) or rat IgG (10 μg/ml) for 30 min at 37°C before injection into the parallel plate flow chamber.

RESULTS

Early, but not Late, Treatment with T22 Blocks CXCR4-Mediated Metastasis. We demonstrated previously that the CXCR4 inhibitory peptide, T22, blocked pulmonary metastasis of CXCR4-B16 cells when this CXCR4 inhibitor was used daily throughout the course of the 14-day metastasis assay (14). To determine whether short-term treatment with T22 was also effective in blocking metas-

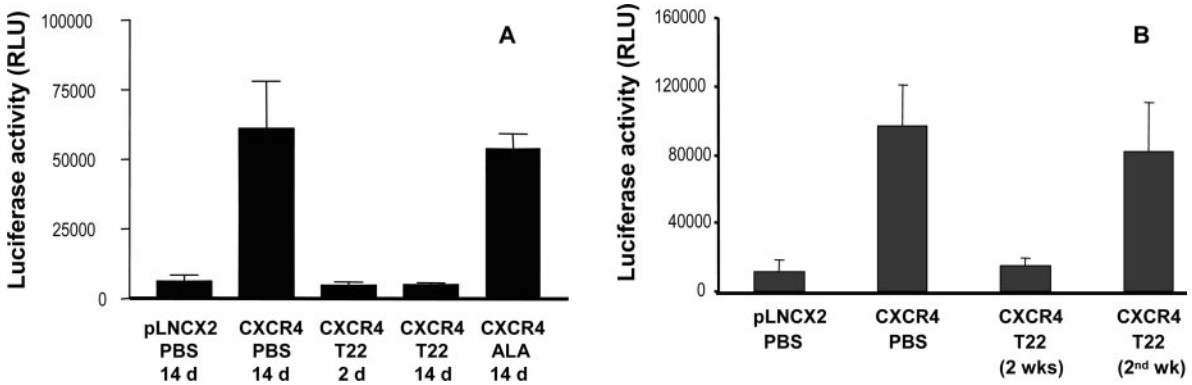

Fig. 1. Early treatment of mice with T22 blocks pulmonary metastasis. *A* and *B*, groups of mice (n = 5 mice/group) were injected with CXCR4-B16 melanoma cells and treated with either PBS, the CXCR4 inhibitor (T22), or a control peptide (ALA) for the indicated number of days. After 14 days, the mice were euthanized, and the lungs were harvested for luciferase-based quantification of metastasis (*B*). *RLU*, relative light units.

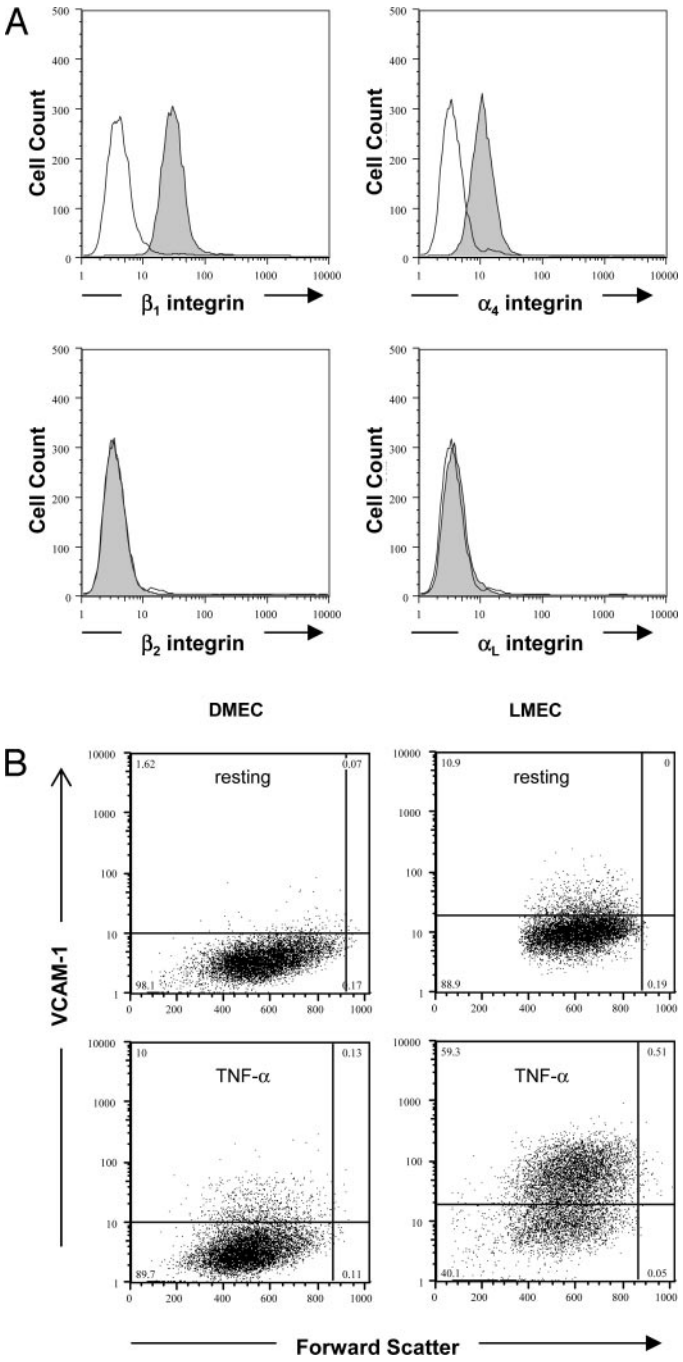

Fig. 2. Adhesion molecule expression profile of B16 cells and LMECs. *A*, CXCR4-B16 cells were stained with anti-β_1, α_4, β_7, β_2, and α_L mAb or with human E-selectin/Ig chimera (R&D Systems) and FITC-labeled anti-human IgG. *B*, untreated and TNF-α-treated (10 ng/ml for 4 h at 37°C) DMECs and LMECs were removed from culture plates with trypsin and EDTA, stained with anti-VCAM-1 mAb, and analyzed by flow cytometry.

tasis, we modified our *in vivo* assay and gave the drug only for the first 2 days of the 14-day waiting period after i.v. injection of CXCR4-B16 and pLNCX2-B16 cells. In one group of animals, the CXCR4 antagonist peptide T22 was administered i.p. shortly after tumor inoculation and again 24 h later. In another group of CXCR4-B16-injected mice, T22 was given daily for 14 days. Administration of T22 for the first 2 days was clearly as effective as giving T22 daily for the entire 2-week course with regard to protection against CXCR4-mediated pulmonary metastasis (Fig. 1*A*).

To determine whether T22 treatment during the last 7 days of the metastasis assay could reduce tumor burden, T22 was given daily either during the entire 2-week treatment period or just for the second week (Fig. 1*B*). Treatment during the second week yielded the same metastatic burden as PBS (mock) treatment; both were ineffective compared with daily T22 treatment in reducing metastasis. Thus, early treatment of CXCR-B16 cells with T22 is essential for protecting mice against lung metastasis, suggesting that CXCR4 promotes metastasis at a relatively early time point after i.v. inoculation.

Adhesion Molecule Profile of B16 Cells and Microvascular ECs. Chemokine receptor-triggered adherence of leukocytes involves cell surface integrins and their cognate receptors on ECs. Thus, we characterized the adhesion molecule profile of B16 cells and two types of microvascular ECs. Both pLNCX2-B16 and CXCR4-B16 cells expressed the β_1 and α_4 integrin chains but not the β_2, β_7, or α_L (CD11a) chains (Fig. 2*A*). E-selectin ligands were also not detectable after exposure of B16 cells to an E-selectin/Ig chimeric protein (data not shown). Cultured LMECs expressed more VCAM-1 [a major Ig

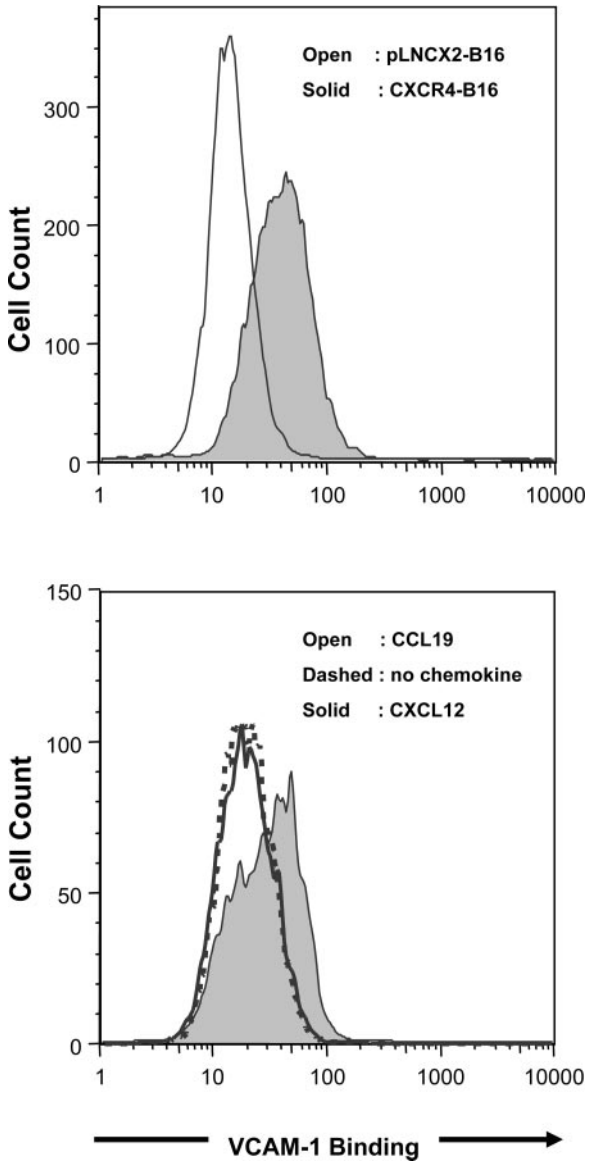

Fig. 3. Rapid binding of soluble VCAM-1/Ig to CXCL12-stimulated CXCR4-B16 cells. *Top panel*, CXCR4-B16 and pLNCX2-B16 cells were exposed to CXCL12 and soluble VCAM-1/Ig for 45 s, fixed, and stained with FITC-labeled antihuman IgG antibody. *Bottom panel*, CXCR4-B16 cells were exposed to soluble VCAM-1/Ig in the presence of the indicated chemokine (500 ng/ml) or no chemokine (PBS). After 45 s, cells were fixed and stained with antihuman IgG antibody to detect bound VCAM-1/Ig.

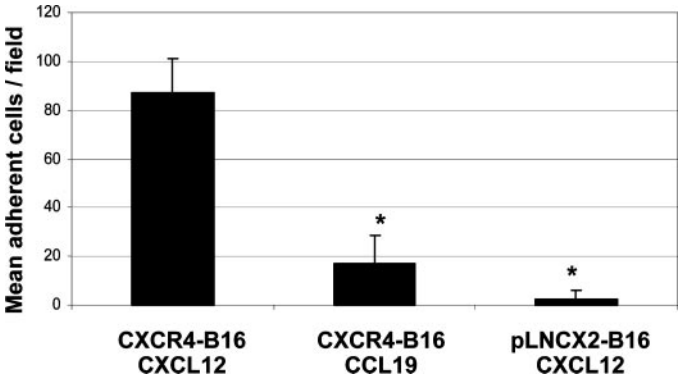

Fig. 4. CXCR4-B16 cells arrest efficiently to VCAM-1/Ig-coated plates under shear stress conditions. CXCR4-B16 and pLNCX2-B16 cells were injected at 1.5 dynes/cm^2 into a parallel plate flow chamber in which the bottom plate had been coated with VCAM-1/Ig and the indicated chemokines. After 10 min, adherent cells were photographed (6 low-power fields/condition) and counted (mean ± SD). *, $P < 0.001$ *versus* CXCR4-B16 binding on CXCL12-/VCAM-1-Ig-coated plates.

superfamily receptor for $\alpha_4\beta_1$ integrin (very late antigen 4)], compared with DMECs under both resting and TNF-stimulated conditions (Fig. 2*B*). Thus, β_1, but not β_2, integrins are expressed by B16 melanoma cells, and a major β_1 integrin ligand, VCAM-1, is readily expressed by cytokine-stimulated lung-derived ECs.

CXCL12 Enhances Binding of CXCR4-B16 Cells to Soluble VCAM-1. To determine whether CXCL12 directly enhanced the binding of β_1 integrin to VCAM-1, CXCR4-B16 and pLNCX2-B16 cells were exposed to soluble VCAM-1-human IgG chimera (VCAM-1/Ig) or a P-selectin/Ig fusion protein (as a negative control) in the presence and absence of chemokines. CXCR4-B16 cells clearly demonstrated increased binding of soluble VCAM-1/IgG chimera compared with CXCL12-stimulated pLNCX2-B16 (Fig. 3, *top histogram*) within 45 s of the addition of CXCL12. The addition of CXCL12, but not CCL19 (an unrelated chemokine), to CXCR4-B16 cells stimulated enhanced binding of VCAM-1/Ig chimera (Fig. 3, *bottom histogram*), whereas the addition of CXCL12 did not affect binding of VCAM-1/Ig to pLNCX2-B16 cells (data not shown). Mn^{2+}-treated B16 cells uniformly increased binding of VCAM-1/Ig (data not shown). Therefore, CXCL12 rapidly enhances β_1 integrin affinity for VCAM-1.

CXCL12 Enhances Arrest of B16-CXCR4 Cells on VCAM-1/Ig-Coated Plates. Like leukocytes, tumor cells that adhere to vascular ECs are subject to shear forces in vascular channels. To determine whether CXCR4-CXCL12 interaction mediates the arrest of CXCR4-B16 cells on VCAM-1 under physiological flow conditions, CXCR4-B16 or pLNCX2-B16 cells were infused over plates co-coated with VCAM-1/Ig and either CXCL12 or a control chemokine, CCL19. CXCR4-B16 cells arrested 5 times more efficiently on plates coated with VCAM-1 and CXCL12 than on plates coated with VCAM-1/Ig and CCL19 ($P = 0.001$; Fig. 4). CXCR4-B16 cells also arrested more efficiently than pLNCX2-B16 cells on VCAM-1/CXCL12-coated plates ($P < 0.001$). Interestingly, CXCR4-B16 cells demonstrated no appreciable rolling before rapid binding to VCAM-1/Ig in real-time recordings (data not shown). Thus, under dynamic adhesion conditions, CXCR4 expression enabled more efficient arrest of tumor cells to VCAM-1 in the presence of CXCL12.

CXCL12 Mediates Adhesion of B16-CXCR4 Cells to Stimulated LMECs under Physiological Shear Stress. We used a dynamic, *in vitro* model of B16 tumor cell adhesion to LMECs under shear stress conditions to model the shear forces faced by tumor cells when they adhere to vascular ECs during the metastatic process. CXCR4-B16 cells (*versus* pLNCX2-B16 cells) arrested ~3 times more efficiently to LMECs, even in the absence of exogenously added CXCL12 (Fig. 5*A*). With exogenously added CXCL12, adhesion of CXCR4-B16 cells was ~5-fold greater than that of pLNCX2-B16 cells. In both cases, adherent cells were resistant to washing at shear stress up to 10 dynes/cm^2 (data not shown). To determine whether

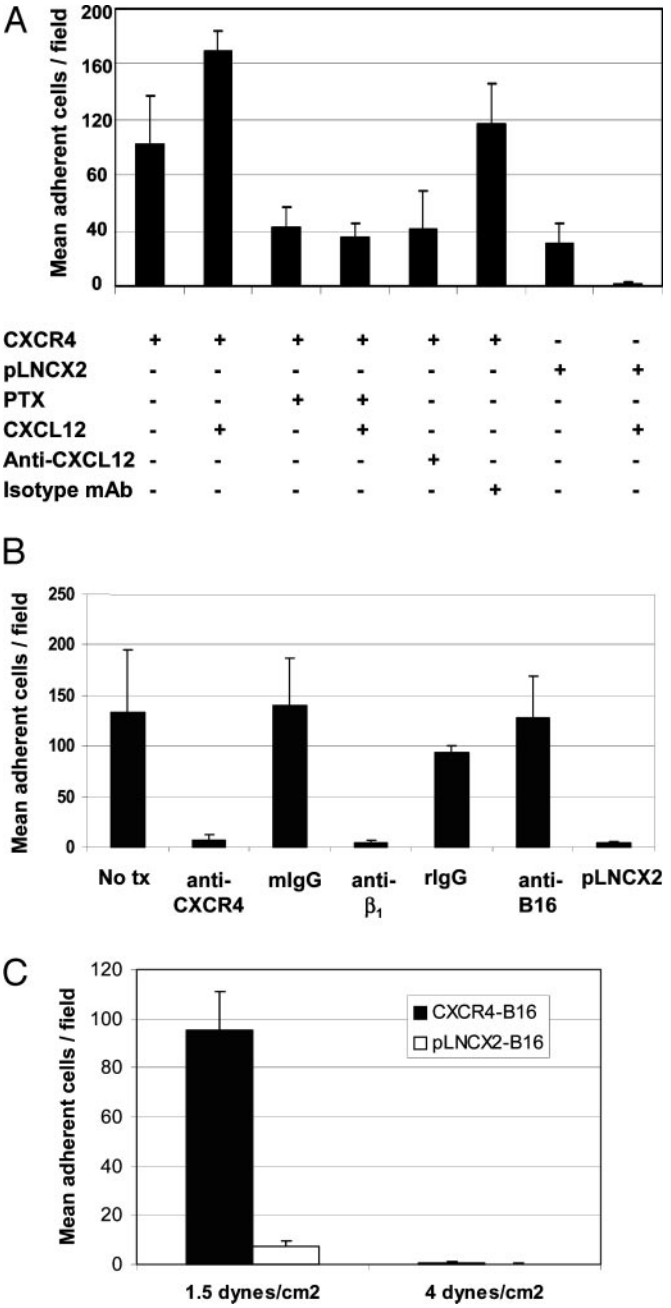

Fig. 5. Adherence of B16 cells to TNF-α-stimulated LMECs under shear stress conditions. *A*, calcein-labeled CXCR4-B16 and pLNCX2-B16 cells were injected at 1.5 dynes/cm^2 in the flow chamber containing a monolayer of TNF-α-activated LMECs. In two cases, tumor cells were pretreated with PTX before introduction into the flow chamber. Where indicated with a + symbol, LMEC monolayers were treated with anti-CXCL12 or isotype control mAb before introduction of B16 cells. Exogenous exposure of LMECs to recombinant CXCL12 before introduction of B16 cells is indicated by the + sign in the row labeled *CXCL12*. *B*, CXCR4-B16 cells (*left six columns*) or pLNCX2-B16 (*far right column*) cells were injected into the parallel plate flow chamber as described in *A*. Before injection into the flow chamber, CXCR4-B16 cells were treated with isotype control mouse IgG (*mIgG*) and rat IgG (*rIgG*), anti-CXCR4 mAb, rat antimouse β_1 mAb (clone 9EG7; BD-PharMingen), or anti-B16 mAb (B16MAB-1) for 30 min at 37°C. The *first column* on the *left* indicates nontreated CXCR4-B16 cells (*No tx*). In both *A* and *B*, adherent cells were counted ($n = 6$ random fields/condition; mean ± SD) 10 min after initiation of flow. *C*, CXCR4-B16 and pLNCX2-B16 cells were injected into the flow chamber containing LMECs (as described above) at the indicated shear stresses for 10 min. The mean ± SD of adherent cells is shown.

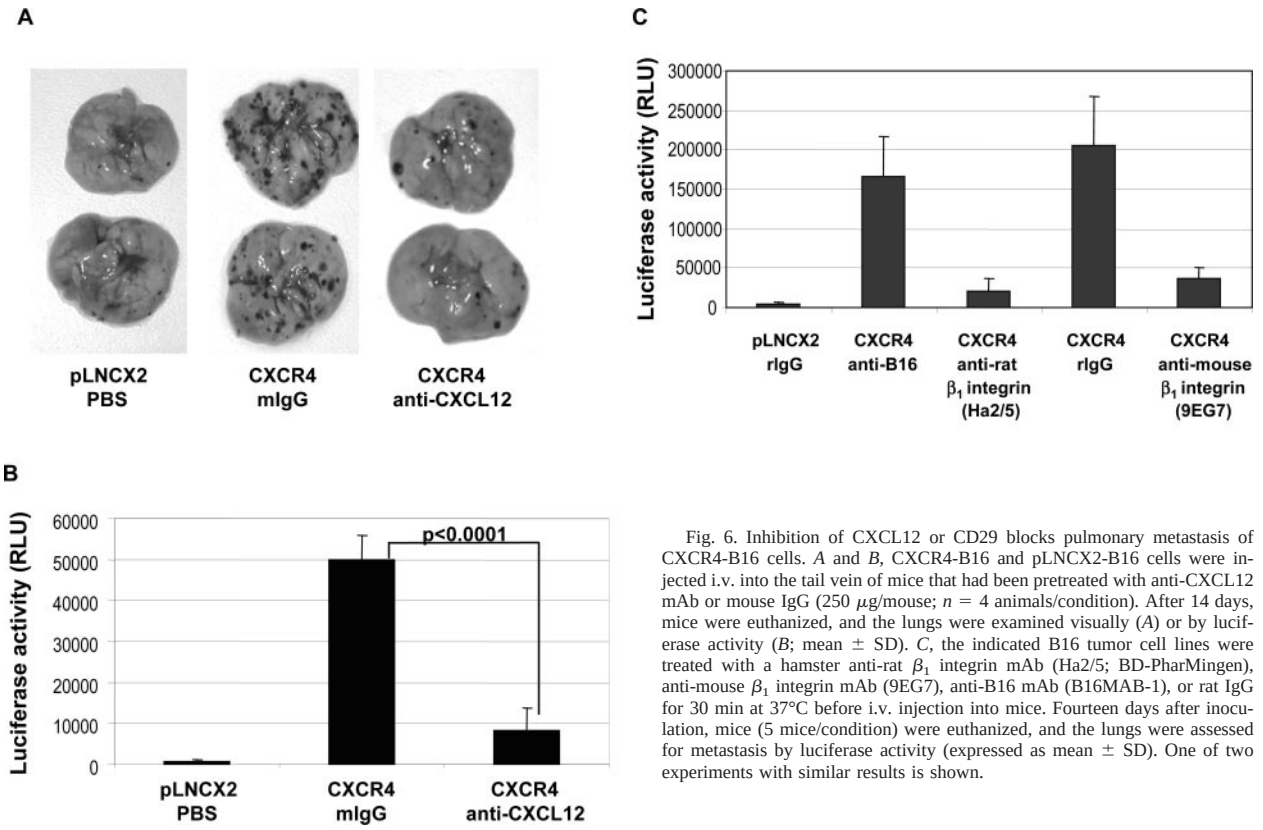

Fig. 6. Inhibition of CXCL12 or CD29 blocks pulmonary metastasis of CXCR4-B16 cells. *A* and *B*, CXCR4-B16 and pLNCX2-B16 cells were injected i.v. into the tail vein of mice that had been pretreated with anti-CXCL12 mAb or mouse IgG (250 μg/mouse; $n = 4$ animals/condition). After 14 days, mice were euthanized, and the lungs were examined visually (*A*) or by luciferase activity (*B*; mean ± SD). *C*, the indicated B16 tumor cell lines were treated with a hamster anti-rat β_1 integrin mAb (Ha2/5; BD-PharMingen), anti-mouse β_1 integrin mAb (9EG7), anti-B16 mAb (B16MAB-1), or rat IgG for 30 min at 37°C before i.v. injection into mice. Fourteen days after inoculation, mice (5 mice/condition) were euthanized, and the lungs were assessed for metastasis by luciferase activity (expressed as mean ± SD). One of two experiments with similar results is shown.

LMEC-derived CXCL12 was essential for CXCR4-B16 arrest, LMECs were pretreated with a neutralizing anti-CXCL12 mAb, which resulted in significant inhibition of CXCR4-B16 cell arrest. An isotype control mAb did not block CXCR4-B16 binding (Fig. 5*A*). As expected, pretreatment of CXCR4-B16 cells with PTX blocked binding, indicating that a G_i-coupled receptor was involved in the arrest of CXCR4-B16 cells.

To determine whether β_1 integrins were critical for the arrest of B16 cells under flow conditions, CXCR4-B16 cells were pretreated with anti-β_1 integrin antibody and then injected into the flow chamber containing TNF-α-stimulated LMECs. Adherence of the tumor cells was subsequently blocked by >90% (Fig. 5*B*), which was similar to the decrease in binding observed when the B16 cells were treated with anti-CXCR4 mAb (Fig. 5*B*). CXCR4-B16 cells treated with a surface-binding antibody (B16MAB-1) showed no significant decrease in adhesion to LMECs (Fig. 5*B*), suggesting that the inhibitory effect of the anti-β_1 mAb was specific. Although the epitope for B16MAB-1 binding has not yet been characterized, it is unlikely to be β_1 integrin because LN immunocytes, the majority of which express β_1 integrins, did not express the antigen recognized by B16MAB-1 (data not shown). Thus, CXCR4-mediated arrest of B16 cells on activated ECs requires the participation of β_1 integrin.

Adhesion mediated by CXCR4 was dependent on shear stress because far fewer cells adhered at 4 *versus* 1.5 dynes/cm^2 (Fig. 5*C*), and no rolling or adherence of CXCR4-B16 cells on LMECs was observed at 10 dynes/cm^2 (data not shown), consistent with other studies demonstrating optimal adherence of tumor cells and leukocytes at shear stresses less than 3 dynes/cm^2 (4, 22, 23). Prior to adhesion, rolling behavior of tumor cells was not observed at shear stresses ranging from 1 to 10 dynes/cm^2. In static assays, CXCR4-B16 arrested 5 times more efficiently compared with pLNCX2-B16 on LMECs (data not shown), demonstrating that without shear stress, CXCR4 activation also results in enhanced binding of tumor cells to LMECs.

Neutralizing Anti-CXCL12 or Anti-β_1 Integrin mAb Prevents CXCR4-Mediated Pulmonary Metastasis. Based on the *in vitro* results above, we postulated that EC-derived CXCL12 is required for activation CXCR4, leading to downstream activation of β_1 integrin and subsequent firm binding of tumor cells to EC adhesion molecules such as VCAM-1. To determine whether blockade of CXCL12 can suppress metastasis of B16 cells, mice were given a single dose of either neutralizing anti-CXCL12 mAb or an isotype-matched mouse IgG isotype (i.p.) 20 min before i.v. injection of tumor cells. As shown in Fig. 6*A*, anti-CXCL12 mAb blocked the formation of lung metastasis compared with isotype, as evidenced by the gross appearance of the lungs (Fig. 6*A*) and by luciferase quantification ($P < 0.001$; Fig. 6*B*). No gross metastasis in other organs (brain and liver) was observed. Therefore, neutralization of CXCL12 is effective in preventing CXCR4-mediated pulmonary metastasis.

To determine whether β_1 integrin was necessary in the formation of CXCR4-mediated pulmonary metastasis *in vivo*, we pretreated B16-CXCR4 cells with two different blocking antibodies against β_1 integrin before tail vein injection. Both anti-β_1 mAbs blocked CXCR4-B16 metastasis, whereas rat IgG isotype control mAb did not. Pretreatment of tumor cells with the B16MAB-1 mAb also did not inhibit metastasis (Fig. 5*B*), suggesting that the inhibitory effect of the β_1 integrin mAbs on metastasis was not due to nonspecific effects (Fig. 6*C*). Thus, β_1 integrin is required for CXCR4-mediated pulmonary metastasis *in vivo*.

DISCUSSION

Herein, we demonstrate that the role of CXCR4 in promoting metastasis comes at an early period after intravascular dissemination

of tumor cells. Furthermore, we demonstrate that CXCR4 activation rapidly increases affinity of β_1 integrin for VCAM-1, stimulates adhesion of B16 cells to ECs at 1.5 dynes/cm^2, and promotes pulmonary metastasis in a β_1 integrin-dependent manner. Whereas substantial evidence already suggested that β_1 integrin expressed by melanoma cells contributes to melanoma adhesion, invasion, motility, and metastasis (4, 24–26), our data now provide a link between chemokine receptors, β_1 integrins, and their shared role in promoting adherence of tumor cells to vascular ECs.

β_1 integrin was, indeed, critical to adhesion of the CXCR4-B16 to stimulated LMECs as shown in Fig. 5*B*. Rapidly enhanced binding of soluble VCAM-1/Ig after addition of CXCL12 suggested that direct increases in the affinity of β_1 integrin for VCAM-1 may contribute to increased adhesion of the tumor cells to activated, CXCL12-expressing, LMECs. Under flow conditions, anti-α_4 integrin antibodies (data not shown) and anti-β_1 integrin antibodies were both highly effective in blocking CXCL12-mediated adhesion of CXCR4-B16 cells to immobilized recombinant VCAM-1 (Fig. 5), suggesting that $\alpha_4\beta_1$ is the primary β_1 integrin required for B16 cell adhesion. Although not directly addressed in this study, previous work demonstrated that activation via a chemokine receptor (CCR6) could alter the surface distribution of β_1 integrin on arresting T cells (6). Therefore, it is possible that activation through CXCR4 regulates both the affinity and avidity of β_1 integrin for VCAM-1.

The vascular adhesion molecule, VCAM-1, has previously been reported to play a significant role in B16 melanoma metastasis. At baseline, VCAM-1 expression is low but detectable in several organs of mice, including the lungs (27). Activation *in vivo* with TNF-α (28) or lipopolysaccharide (27) dramatically stimulates VCAM-1 expression and increases B16 melanoma pulmonary metastases (28). In experimental spontaneous metastasis of B16 melanoma cells after orthotopic implantation, VCAM-1 expression at various vascular beds correlated with the occurrence of metastases (29). Some tumor-derived cytokines (*e.g.*, interleukin 18) appear to up-regulate VCAM-1 expression by hepatic ECs (30), and treatment of mice with an interleukin 18-binding protein reduces VCAM-1 expression in the hepatic microvasculature as well as B16 murine melanoma metastases in the liver.

Based on the inhibition of CXCR4-B16 metastasis by anti-CXCL12 mAb (Fig. 6, *A* and *B*), we propose that CXCL12, produced either by the pulmonary ECs themselves or accumulated on the lumenal surface of pulmonary ECs via transendocytosis (31), is a critical modulator of tumor cell adhesion through its receptor, CXCR4, on β_1 integrin-expressing melanoma cells. The absence of selectins or selectin ligands by melanoma cells does not affect the capture of these cells from the bloodstream because very late antigen 4, in contrast to other integrins, can mediate both transient binding (tethering) as well as firm adhesion (32).

Because tumor cells are frequently larger and less deformable than RBCs or leukocytes, physical size constraints have been proposed to lead to the entrapment of metastatic cells in the smaller capillaries of organs such as the lung or liver (33). Unlike leukocytes that appear to roll, arrest, and spread on ECs according to the well-described multistep model of leukocyte recruitment (34), tumor cells may be forced to stop (in some cases) when they are physically restricted by the diameter of the capillary vessels. Our results in shear stress-free, static assays on LMECs, however, demonstrated that CXCR4-expressing B16 cells showed significantly increased β_1 integrin-dependent adhesion compared with control cells that lack CXCR4 (14),[4] despite the close contact of all tumor cells with the EC monolayer. Thus, whether adhering to ECs in the presence or absence of shear stress, B16 melanoma cells demonstrate higher adhesion to the ECs when they express CXCR4.

In summary, we demonstrate that expression of CXCR4 by B16 cells facilitates adhesion of these tumor cells to activated LMECs via β_1 integrin-dependent mechanisms under both static and shear stress conditions through endogenous CXCR4 ligand. Neutralization of CXCL12 or inhibition of CXCR4 early in the course of metastasis had dramatic effects in decreasing metastasis. Late treatment with CXCR4 inhibitors may not effectively block metastatic growth, raising the possibility that enlarging metastatic tumor cells may have already lost dependency on CXCR4 for growth. Thus, anti-CXCR4 therapy may have clinical utility in blocking metastasis but may not be useful for the treatment of established metastatic disease.

[4] A. R. Cardones, unpublished data.

ACKNOWLEDGMENTS

We thank Dr. Mark C. Udey (National Cancer Institute, NIH) for many helpful suggestions and Dr. Edward Berger (National Institute of Allergy and Infectious Diseases, NIH) for the kind gift of CXCR4 cDNA.

REFERENCES

1. Engers, R., and Gabbert, H. E. Mechanisms of tumor metastasis: cell biological aspects and clinical implications, J. Cancer Res. Clin. Oncol., *126:* 682–692, 2000.
2. Seftor, R. E., Seftor, E. A., and Hendrix, M. J. Molecular role(s) for integrins in human melanoma invasion. Cancer Metastasis Rev., *18:* 359–375, 1999.
3. Menter, D. G., Fitzgerald, L., Patton, J. T., McIntire, L. V., and Nicolson, G. L. Human melanoma integrins contribute to arrest and stabilization potential while flowing over extracellular matrix. Immunol. Cell Biol., *73:* 575–583, 1995.
4. Giavazzi, R., Foppolo, M., Dossi, R., and Remuzzi, A. Rolling and adhesion of human tumor cells on vascular endothelium under physiological flow conditions. J. Clin. Investig., *92:* 3038–3044, 1993.
5. Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C., and Laudanna, C. Chemokines trigger immediate β_2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity, *13:* 759–769, 2000.
6. Maki, W., Morales, R. E., Carroll, V. A., Telford, W. G., Knibbs, R. N., Stoolman, L. M., and Hwang, S. T. CCR6 colocalizes with CD18 and enhances adhesion to activated endothelial cells in CCR6-transduced Jurkat T cells. J. Immunol., *169:* 2346–2353, 2002.
7. Locati, M., and Murphy, P. M. Chemokines and chemokine receptors: biology and clinical relevance in inflammation and AIDS. Annu. Rev. Med., *50:* 425–440, 1999.
8. Wong, M. M., and Fish, E. N. Chemokines: attractive mediators of the immune response. Semin. Immunol., *15:* 5–14, 2003.
9. Murphy, P. M. Chemokines and the molecular basis of cancer metastasis. N. Engl. J. Med., *345:* 833–835, 2001.
10. Balkwill, F. Chemokine biology in cancer. Semin. Immunol., *15:* 49–55, 2003.
11. Wiley, H., Gonzalez, E. B., Maki, W., Wu, M., and Hwang, S. T. Expression of CC chemokine receptor-7 (CCR7) and regional lymph node metastasis of B16 murine melanoma. J. Natl. Cancer Inst. (Bethesda), *93:* 1638–1643, 2001.
12. Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H., and Mori, M. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. Cancer Res., *62:* 2937–2941, 2002.
13. Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verástegul, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond.), *410:* 50–56, 2001.
14. Murakami, T., Maki, W., Cardones, A. R., Fang, H., Kyi, A. T., Nestle, F., and Hwang, S. T. Expression of CXC chemokine receptor (CXCR)-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. Cancer Res., *62:* 7328–7334, 2002.
15. Salvucci, O., Yao, L., Villalba, S., Sajewicz, A., Pittaluga, S., and Tosato, G. Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. Blood, *99:* 2703–2711, 2002.
16. Robledo, M. M., Bartolome, R. A., Longo, N., Rodriguez-Frade, J. M., Mellado, M., Longo, I., van Muijen, G. N., Sanchez-Mateos, P., and Teixido, J. Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. J. Biol. Chem., *276:* 45098–45105, 2001.
17. Libura, J., Drukala, J., Majka, M., Tomescu, O., Navenot, J. M., Kucia, M., Marquez, L., Peiper, S. C., Barr, F. G., Janowska-Wieczorek, A., and Ratajczak, M. Z. CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. Blood, *100:* 2597–2606, 2002.
18. Kijima, T., Maulik, G., Ma, P. C., Tibaldi, E. V., Turner, R. E., Rollins, B., Sattler, M., Johnson, B. E., and Salgia, R. Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells. Cancer Res., *62:* 6304–6311, 2002.

19. Erbe, D. V., Wolitzky, B. A., Presta, L. G., Norton, C. R., Ramos, R. J., Burns, D. K., Rumberger, R. M., Rao, B. N. N., Foxall, C., Brandley, B. K., and Lasky, L. A. Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. J. Cell Biol., *119:* 215–227, 1992.
20. Murakami, T., Nakajima, T., Koyanagi, Y., Tachibana, K., Fujii, N., Tamamura, H., Yoshida, N., Waki, M., Matsumoto, A., Yoshie, O., Kishimoto, T., Yamamoto, N., and Nagasawa, T. A small molecule CXCR4 inhibitor that blocks T cell line-trophic HIV-1 infection. J. Exp. Med., *186:* 1389–1393, 1997.
21. Cinamon, G., Shinder, V., and Alon, R. Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. Nat. Immunol., *2:* 515–522, 2001.
22. Lawrence, M. B., McIntire, L. V., and Eskin, S. G. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood, *70:* 1284–1290, 1987.
23. Lawrence, M. B., and Springer, T. A. Neutrophils roll on E-selectin. J. Immunol., *151:* 6338–6346, 1993.
24. Garofalo, A., Chirivi, R. G., Foglieni, C., Pigott, R., Mortarini, R., Martin-Padura, I., Anichini, A., Gearing, A. J., Sanchez-Madrid, F., Dejana, E., *et al.* Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastases. Cancer Res., *55:* 414–419, 1995.
25. Melchiori, A., Mortarini, R., Carlone, S., Marchisio, P. C., Anichini, A., Noonan, D. M., and Albini, A. The $\alpha_3\beta_1$ integrin is involved in melanoma cell migration and invasion. Exp. Cell Res., *219:* 233–242, 1995.
26. Price, E. A., Coombe, D. R., and Murray, J. C. β-1 Integrins mediate tumour cell adhesion to quiescent endothelial cells *in vitro*. Br. J. Cancer, *74:* 1762–1766, 1996.
27. Fries, J. W., Williams, A. J., Atkins, R. C., Newman, W., Lipscomb, M. F., and Collins, T. Expression of VCAM-1 and E-selectin in an *in vivo* model of endothelial activation. Am. J. Pathol., *143:* 725–737, 1993.
28. Okahara, H., Yagita, H., Miyake, K., and Okumura, K. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor α enhancement of experimental metastasis. Cancer Res., *54:* 3233–3236, 1994.
29. Langley, R. R., Carlisle, R., Ma, L., Specian, R. D., Gerritsen, M. E., and Granger, D. N. Endothelial expression of vascular cell adhesion molecule-1 correlates with metastatic pattern in spontaneous melanoma. Microcirculation, *8:* 335–345, 2001.
30. Vidal-Vanaclocha, F., Fantuzzi, G., Mendoza, L., Fuentes, A. M., Anasagasti, M. J., Martin, J., Carrascal, T., Walsh, P., Reznikov, L. L., Kim, S. H., Novick, D., Rubinstein, M., and Dinarello, C. A. IL-18 regulates IL-1β-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. Proc. Natl. Acad. Sci. USA, *97:* 734–739, 2000.
31. Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., and Rot, A. Transcytosis and surface presentation of IL-8 by venular endothelial cells. Cell, *91:* 385–395, 1997.
32. Alon, R., Kassner, P. D., Carr, M. W., Finger, E. B., Hemler, M. E., and Springer, T. A. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J. Cell Biol., *128:* 1243–1253, 1995.
33. Chambers, A. F., Groom, A. C., and MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer, *2:* 563–572, 2002.
34. Springer, T. A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell, *76:* 301–314, 1994.

CXCR4 Enhances Adhesion of B16 Tumor Cells to Endothelial Cells *in Vitro* and *in Vivo* via β_1 Integrin

Adela R. Cardones, Takashi Murakami and Sam T. Hwang

Cancer Res 2003;63:6751-6757.