Human Melanoma Metastases Express Functional CXCR4

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Abstract
Purpose: The chemokine receptor CXCR4 was identified as an independent predictor of poor prognosis in primary melanoma. The aim of the study was to investigate the role of CXCR4 in human melanoma metastases.

Experimental Design: CXCR4 expression was evaluated in melanoma metastases and in metastatic cell lines through immunohistochemistry, immunoblotting, immunofluorescence, and reverse transcription-PCR. The function of CXCR4 was tested in the presence of the ligand, CXCL12, through induction of extracellular signal-regulated kinase-1 and -2 (Erk-1 and -2) phosphorylation, proliferation, apoptosis, and migration capabilities.

Results: CXCR4 expression was detected in 33 out of 63 (52.4%) metastases from cutaneous melanomas. Metastatic melanoma cell lines expressed cell surface CXCR4; PES 43, Alo 40, and COPA cell lines showed the highest levels of CXCR4 (>90% of positive cells); PES 41, Alo 39, PES 47, POAG, and CIMA cell lines showed low to moderate degrees of expression (5-65% of positive cells). Other chemokine receptors, CCR7 and CCR10, were detected on the melanoma cell lines; CXCL12 activated Erk-1 and Erk-2, whose induction was specifically inhibited by AMD3100 treatment. CXCL12 increased the growth in PES 41, PES 43, and PES 47 cells under suboptimal (1% serum) and serum-free culture conditions; AMD3100 (1 μmol/L) inhibited the spontaneous and CXCL12-induced proliferation. No rescue from apoptosis was shown but PES 41, PES 43, and PES 47 cells migrate toward CXCL12.

Conclusions: These findings indicate that CXCR4 is expressed and active in human melanoma metastases, suggesting that active inhibitors such as AMD3100 may be experienced in human melanoma.

The incidence and mortality rate of melanoma have increased in the last 30 years. The National Cancer Institute Surveillance, Epidemiology, and End Results database documents increases of 61% in annual diagnoses of cutaneous melanoma and of 165% in annual mortality from 1950 to 2000 (1). Metastatic spread may arise from very small tumor masses and in about two-thirds of all cases of malignant melanoma, spreading develop primarily as locoregional metastases. In about one-third of the cases, primary development of distant metastases is observed (2). The metastatic potential of primary melanoma is considerably higher than that of other primary solid tumors when comparing the size of primary lesion. The usual outcome for patients with distant metastases remains bleak, with median survival of 6 to 10 months and ≤5% of patients surviving for >5 years (1). Except for high-dose IFN as adjuvant therapy in stage III disease, little success has emerged over the last 20 years for metastatic melanoma (3). The underlying molecular events that explain malignant melanoma genesis and progression have been only partially characterized, and only a small number of genes have been identified as playing key roles in melanoma. Among these, some cell cycle regulators, apoptotic, signal transduction, cell adhesion, and matrix digestion genes have been shown to be deregulated in this neoplasm (4). Chemokines and their receptors have emerged as attractive targets regulating the migration of tumor cells in vivo. There is evidence that antigen-presenting cells such as dendritic cells, Langerhans cells, T cells, and natural killer cells bearing chemokine receptors migrate from the skin to the draining lymph node in response to specific chemotactic factors referred to as chemokines. Chemokines have been hypothesized to recruit solid tumor cells to lymph nodes. Expression of chemokine receptors on tumor cells and their involvement in metastases revealed that these receptors could indeed provide migratory directions to tumor cells (5-8). The most widely expressed chemokine receptor among cancers is likely to be CXCR4, described in breast cancer, glioblastoma, pancreatic, prostatic, etc.

Human Cancer Biology
col, thyroid, and non–small cell lung cancers (6–20). In melanoma cells, several chemokine receptors have been described. CXCR4 and CXCR3 were originally reported in human melanoma cell lines (21). Further insight into the mechanism of CXCL12/SDF1-α-mediated cell invasion was described. SDF1-α promotes invasion across basement membranes through the induction of membrane type 1-matrix metalloproteinase. Moreover, SDF1-α triggered the activation of small GTPases such as RhoA and Rac1 (22). Other chemokine receptors were described on melanoma cells; expression of CCR7 (23) and CXCR3 (24) were mainly correlated to lymph node metastases, whereas the expression of CCR10 was mainly detected in skin metastases (25). The role of the CXCR4-CXCL12 axis was also validated in in vivo studies. B16 melanoma cells transfected with CXCR4 produced an increased number of pulmonary nodules compared with the lung metastases induced by B16 cells transfected with mock vector (26). CXCR4-specific ligand, CXCL12, is frequently produced at sites of melanoma metastases. Cardones et al. showed that CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via β1 integrin through CXCL12; in vivo, metastases of CXCR4-B16 cells to murine lungs was strongly inhibited by anti-CXCL12 and two different anti-β1 integrin monoclonal antibodies (27).

Recently, it was shown that CXCR4 expression predicted prognosis in human primary melanoma (28). The aim of the study was to evaluate the role of CXCR4 in human melanoma metastases. CXCR4 expression was analyzed in a panel of 63 melanoma metastases and 8 metastases-derived human melanoma cell lines. CXCR4 was expressed on 52.4% of metastases; the CXCR4 receptor was active in the melanoma cell lines and was inhibited by the specific antagonist, AMD3100. Thus, strategies targeting the CXCR4 receptor may be of benefit in melanoma patients.

**Materials and Methods**

**Immunohistochemistry.** Two serial 5-μm sections of formalin-fixed, paraffin-embedded cutaneous melanomas were stained; one for H&E and the other immunostained using the biotin-streptavidin-peroxidase method (YLEM). Deparaffinized sections were microwaved in 1 mmol/L EDTA (pH 8.0) for two cycles of 5 minutes each to unmask epitopes. After treatment with 1% hydrogen peroxide for 30 minutes to block endogenous peroxide, the sections were subsequently incubated with monoclonal antibodies (anti-CXCR4, MAB 172; R&D Systems, Minneapolis, MN) for 2 hours at room temperature. The sections were then incubated with biotin-labeled secondary antibody (1:30) for 30 minutes and with streptavidin-peroxidase (1:30) for 10 minutes. Slides were stained for 10 minutes with AEC chromogen (DAKO Cytomation, Milan, Italy) and then counterstained with hematoxylin, washed and mounted in water fluid. The dilutions of the monoclonal antibody, biotin-labeled secondary antibody, and streptavidin-peroxidase were made with PBS (pH 7.4) containing 5% bovine serum albumin. CXCR4 staining was defined as positive when expression involved >80% of neoplastic cells. Moreover, membrane positivity, cytoplasmic positivity, or both membrane and cytoplasmic positivity and absolute percentage of positivity were scored. All series included positive controls of well-characterized sections (melanomas and breast cancer). Negative controls were obtained by substituting the primary antibody with a mouse myeloma protein of the same subclass, at the same concentration as the monoclonal antibody. All controls gave satisfactory results. Macrophage positivity was used as an adequate internal positive control for each case, in order to validate the technical procedures. Slides were evaluated by two blinded observers (G. Botti and R. Franco); discordant cases were discussed and concordance was then achieved.

**Cell lines.** Melanoma metastases were obtained from patients undergoing surgery with curative intent. PES 41, PES 43, PES 47, Colo 38, Alo 39, Alo 40, COPA, and CIMA cells were cultivated in Iscove’s modified Dulbecco’s medium (Cambrex Bioscience, Verviers, Belgium) supplemented with heat-inactivated 10% fetal bovine serum, penicillin, and streptomycin (100 units/ml each). The cell lines PES 41, PES 43, and PES 47 were isolated from different metastases of the same patient (PES 41 and PES 47 from two s.c. metastases and PES 43 from a lung metastases). Also, Alo 39 and Alo 40 were derived from two lymph node metastases of the same patient.

**Flow cytometry.** To evaluate the expression of CXCR4, adherent cancer cells at subconfluence (50-70% confluent) were detached with 2 mmol/L EDTA in PBS, washed, resuspended in ice-cold PBS, and incubated for 30 minutes at 4°C with anti-CXCR4 antibody PE conjugated (FAB 173P, clone 44717; R&D Systems) or mouse IgG2a PE conjugated as negative control. After three washes in PBS, the cells were analyzed by FACSscan cytometer (Becton Dickinson Immunocytometry Systems; Becton Dickinson, Mountain View, CA).

**Immunofluorescence.** Cells were grown onto 15 mm glass coverslips and fixed with 4% paraformaldehyde but not permeabilized. The primary antibody anti-CXCR4 (10 μg/ml, MAB 172; R&D Systems) was incubated overnight in 3% bovine serum albumin in PBS. TRITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at 1:1000 dilution in 3% bovine serum albumin in PBS for 45 minutes. After washing in PBS, the images were analyzed with a confocal microscope.

**RNA isolation and reverse transcriptase-PCR.** Total cellular RNA from melanoma cell lines was extracted using TriPure reagent (Roche Diagnostics Corporation, Indianapolis, IN). Briefly, cells were homogenized and RNA was extracted using a monophosphoric solution of guanidine thiocyanate and phenol. The RNA was quantified and assessed for purity by UV spectrophotometry. The expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase was assessed on all RNA samples as an internal control. Chemokine and chemokine receptors mRNA were detected by reverse transcriptase-PCR. DNase-treated RNA (2 μg) was reversed transcribed with Superscript II RNase H-reverse transcriptase according to the manufacturer’s instructions (Invitrogen-Life Technologies, Carlsbad, CA). Reverse transcriptase-PCR was carried out using 2 μl of cDNA in a 20 μl final reaction mixture. A Robocycler gradient 96 (Stratagene, La Jolla, CA) was used for the amplification. Cycling conditions of the respective PCR were as follows: initial denaturation (4 minutes at 94°C) followed by 32 cycles of denaturation (1 minute at 94°C), annealing (75 seconds at 56°C, CXCR4; 58°C, CXCL12; 60°C, CCR10; 62°C, CCR2), and elongation (3 minutes at 72°C). Ten microliters of the products were run on a 2% agarose gel and analyzed under UV light. The gene-specific primers used for the amplification were as follows: CXCR4, 5’-GGGGCTTCTACTTTTGTGGCTTCCT-3’ (forward) and 5’-TGGGATGGTGCACGCTTGGAG-3’ (reverse); CXCL12, 5’-GGGGCTTCTACTTTTGTGGCTTCCT-3’ (forward) and 5’-TGGGATGGTGCACGCTTGGAG-3’ (reverse); CCR7, 5’-TCTCCTCTTACGCAACACTGTCCTCAGCG-3’ (forward) and 5’-GAGGACGAGGCAGGCTTGGAG-3’ (reverse); CCR10, 5’-GGGGCTTCTACTTTTGTGGCTTCCT-3’ (forward) and 5’-TATTCCCACTACCTCTTGTG-3’ (reverse).

**Western blot.** Cells were homogenized in lysis buffer (40 mmol/L Heps [pH 7.5], 120 mmol/L NaCl, 5 mmol/L MgCl2, 1 mmol/L EDTA, 0.5 mmol/L EDTA, 1% Triton X-100) containing protease (Complete Tablets, EDTA-free; Roche) and phosphatase (20 mmol/L β-glycerophosphate, 2.5 mmol/L Na-Pi) inhibitors. Total lysate was cleared by centrifugation at 15,000 × g for 20 minutes. The protein concentration of the supernatant was measured using Bio-Rad protein assay. Extracts from melanoma cell lines were separated on 12% SDS-PAGE, transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany) by electroblotting at 250 mA for 2 hours. The following primary proteins were detected using specific antibodies: RhoA (dil 1:1000; Stressgen, Victoria, Canada), RhoC (dil 1:1000; Stressgen, Victoria, Canada), and RhoD (dil 1:1000; Stressgen, Victoria, Canada) with 5% bovine serum albumin in PBS for 1 hour at room temperature. Membranes were washed in PBS and incubated with horseradish peroxidase-conjugated secondary antibody (dil 1:1000; Life Technologies, Carlsbad, CA), and visualized with ECL (Amersham Biosciences, Freiburg, Germany).
antibodies were used: anti-CXCR4 (ProSci, Inc., Poway, CA), anti-P-ERK (sc 7383, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-ERK2 (sc 154G, Santa Cruz Biotechnology). Anti-mouse, anti-rabbit, and anti-goat IgG coupled to peroxidase were used as secondary antibodies (Sigma-Aldrich, Corp., St. Louis, MO) and the signal was revealed through chemiluminescence detection kit (ECL detection kit, Amersham Biosciences).

Growth curve viability and cell growth assay. Cells (20-50 × 10^3) were seeded in a six-multiwell system in a medium culture containing 10% fetal bovine serum. After 24 hours, the medium was replaced as indicated and SDF1-α (20 ng/ml, from Upstate, Lake Placid, NY) was added in the presence or absence of AMD3100 (Sigma-Aldrich). Cells were trypsinized each day and counted by using hemocytometer.

Results

**CXCR4 is expressed in human melanoma metastases.** Recently, CXCR4 was identified as an independent predictor of poor prognosis in primary melanoma (28). To evaluate the role of CXCR4 in human melanoma metastases, CXCR4 expression was analyzed in a panel of 63 melanoma metastases from melanoma patients who had undergone curative intent surgery (29 s.c., 22 lymph node, and 12 visceral metastases). CXCR4 expression was detected in 33 out of 63 (52.4%) metastases from cutaneous melanomas (15 out of 29 s.c., 12 out of 22 lymph node, 6 out of 12 visceral metastases). Figure 1 shows some examples of CXCR4 staining of melanoma on a dermal infiltration by epidermotropic melanoma (A), in liver metastases with normal hepatocytes weakly expressing CXCR4 (B), in nodal metastases (C), and lung metastases (D). The staining was mostly cytoplasmic and few cases showed focal membrane positivity.

**CXCR4 detection on human metastatic melanoma cell lines.** The CXCR4 cell surface was evaluated on cell lines derived from melanoma metastases. Figure 2 showed CXCR4 protein through flow cytometry, immunoblotting, and immunofluorescence (A, B, and C, respectively). Metastatic melanoma cell lines expressed cell surface CXCR4 (PES 43, Alo 40, and COPA cell lines showed the highest levels of CXCR4 (>90% of positive cells); PES 41, Alo 39, PES 47, POAG, and CIMa cell lines showed low to moderate degrees of expression (5-65% of positive cells). In Fig. 2B, the metastatic melanoma cell lines expressed CXCR4 protein compared with Colo 38, human melanoma cell line, and to human primary melanocytes (MPR1). In Fig. 2C, immunofluorescent staining of CXCR4 was detectable in PES cells. PES 43 showed the most intense staining compared with PES 47 and PES 41 cells. In addition to the cell surface punctated staining, there is a clear cytoplasmic CXCR4 staining concentrated perinuclearly in all three lines tested. It might represent a functional status of the receptor because the binding to the specific ligand induces receptor internalization (29).

**Human metastatic melanoma cell lines expressed CXCR4 and other chemokine receptors.** CXCR4 expression was then evaluated in eight metastatic melanoma cell lines by reverse transcription-PCR. CXCR4 expression was detected in PES 41, PES 43, PES 47, Colo 38, Alo 39, and Alo 40 cells with different amount of CXCR4 transcripts; in particular, PES 43 and PES 47 showed high CXCR4 expression compared with normal peripheral blood mononuclear cells, used as positive controls (Fig. 3). Consistent with previous reports (23, 24, 26), the expression of CCR7 and CCR10 was detected on melanoma cell lines, CCR7 was expressed mainly in PES 47 cells, whereas CCR10 was detectable in all tested cell lines (Fig. 3).

**CXCL12 increased the growth of human melanoma cells.** CXCL12 production was described in human cancer cells such as ovary (18) and colon cancers (30). CXCL12 was not detectable in the human melanoma cell lines analyzed through ELISA and cytofluorometric evaluation (data not shown). mRNA for CXCL12 was detected in Alo 40, PES 41, and PES 47 cells (Fig. 3).

The effect of CXCL12 on tumor cell proliferation was assessed. Under optimal culture conditions (in the presence of 10% serum), the addition of CXCL12 did not affect the cell growth of PES 41, PES 43, and PES 47, neither did AMD3100, a specific CXCR4 inhibitor (Fig. 4).

Under suboptimal culture conditions (1% serum), CXCL12 increased the growth in PES 41, PES 43, and PES 47 cells. Treatment with AMD3100 (1 μmol/L) inhibited the spontaneous and CXCL12-induced proliferation in PES 43 and PES 41 human melanoma cells, less in PES 47 human melanoma cells (Fig. 4). Interestingly, 200 ng/ml of CXCL12 was weaker than 20 ng/ml in promoting cell growth, mainly in PES 43 cells in which CXCR4 receptor is overexpressed. Furthermore, in serum-free conditions, CXCL12 (20 ng/ml) induced cell growth and AMD3100 specifically inhibited the spontaneous and CXCL12-induced growth in PES 41, PES 43, and PES 47 cells (Fig. 4). Moreover, CXCL12 (20 or 100 ng/ml) was not able to rescue PES 43, PES 47, and Colo 38 cells from apoptosis (data not shown).

**AMD3100 inhibited the CXCL12-induced activation of extracellular signal-regulated kinases 1 and 2 in human melanoma cells.** To study whether CXCL12 could activate a downstream pathway, we focused our attention on the activation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1 and 2 (ERK1/2) in human melanoma cells. PES 43 and PES 41 cells were serum-starved for 48 hours and then incubated with CXCL12 (20 ng/ml). Changes in the phosphorylation of p44/42 (Erk1/2) kinases were analyzed by Western blotting. In PES 43 and PES 41 cells, human melanoma cell lines derived, respectively, from a lung and s.c. metastases, CXCL12 induced rapid (2-minute) ERK1/2 activation. Notably, no Erk activation...
was induced in normal human melanocytes. Moreover, the addition of AMD3100 (1 μmol/L) inhibited the CXCL12-induced activation of Erk (Fig. 5, top). ERK1/2 activation was also inhibited in PES 43 cells (Fig. 5, bottom) and in PES 41 human melanoma cells (data not shown) by T22, a specific peptide inhibiting CXCR4 receptor. The analysis of cell lysates for the total expression of mitogen-activated protein kinases ensured the equal loading of proteins in different lanes (Fig. 5, top and bottom). AKT phosphorylation was not induced in PES 43 and PES 41 cells following treatment with CXCL12 (20 and 200 ng/mL; data not shown).

The CXCR4 receptor also induced the migration of PES cells toward CXCL12. PES 41, PES 43, and PES 47 migrated toward CXCL12 (20 ng/mL) and the migration was specifically inhibited by AMD3100 (data not shown).

Discussion

The role of CXCR4 in human melanoma metastases was investigated. CXCR4 expression was evaluated on 63 melanoma metastases and on 8 cell lines isolated from melanoma metastases. CXCR4 was detectable in 33 out of 63 melanoma metastases by immunohistochemistry (52.4%; 15 of 29 s.c., 12 of 22 lymph node, 6 of 12 visceral metastases). In cell lines isolated from melanoma metastases, CXCR4 receptor was expressed and functional because CXCL12, the specific CXCR4 ligand induced Erk1-2 activation, cell migration, and cell growth.

Several chemokine receptors have been described on human melanoma cells such as CXCR4 (21, 31), CCR7 (23), CXCR3 (24), CCR10 (25), and CXCR2 (32). In this article, CXCR4 expression was described in eight metastatic melanoma cell lines with a wide range in the receptor level. Three cell lines derived from the same patient (PES 43, PES 41, and PES 47) expressed different levels and activity of the receptor. In agreement with Murakami et al. (26), PES 43 cell line derived from lung metastasis expressed high levels of CXCR4 receptor. PES 43 cells also expressed CCR10, a chemokine receptor mainly described in s.c. melanoma metastasis (25). The two cell lines, PES 41 and PES 47, originated from s.c. nodules, expressed the CCR7 chemokine receptor in addition to CXCR4. PES 47 cells expressed the mRNA for the CXCL12, the specific CXCR4 ligand, which is not otherwise detectable by ELISA and cytofluorimetric detection. These evidences suggest that other chemokine receptors and the production of ligand at the metastasization site may affect the efficacy of the process.

The effect of the CXCR4-CXCL12 axis activation on the cell growth of melanoma cells is not dramatic, although present in stressed cell conditions. This result is in agreement with the intrinsic resistance of melanoma cells to conventional antineoplastic therapies (33). As assessed by the specific Erk1-2 activation, the receptor is functional in metastatic cell lines mainly in encouraging the cells' motility. Erk phosphorylation induction, cell growth, and migration are successfully inhibited.
by AMD3100 and T22 inhibitory peptide; thus, inhibition of the pathway may be very helpful in an early step of metastasization.

Malignant melanoma represents a peculiar neoplasm in which disease explosion follows a silent behavior. Previous results showed that the expression of CXCR4 correlated with a worse prognosis in terms of disease-free survival and overall survival (28). Here, the analysis of the data through a Kaplan-Meier curve showed no statistically significant differences in terms of overall survival between patients with CXCR4+ and CXCR4- metastases (data not shown). This evidence may represent the inefficacy of a single factor to affect prognosis in

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**Fig. 4.** CXCL12 increases growth of human melanoma cells. The effect of CXCL12 on cell proliferation was assessed through hemocytometric cell count in different culture conditions as previously reported.

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**Fig. 5.** AMD3100 inhibited the CXCL12-induced activation of ERK1 and ERK2 phosphorylation in human melanoma cells. Changes in the phosphorylation of p44/42 (Erk1/2) kinases were analyzed by Western blotting. PES 43 and PES 41 cells were serum-starved and treated with CXCL12 (20 ng/mL) in the presence or absence of AMD3100 (1 μmol/L; top). ERK1/2 activation was also inhibited in PES 43 human melanoma cells by T22, a specific peptide inhibiting CXCR4 receptor (bottom). The analysis of cell lysates for the total expression of mitogen-activated protein kinases ensured the equal loading of proteins in the different lanes (top and bottom).
an advanced state of disease. Nevertheless, CXCR4 expression remains in 33 of 63 melanoma metastases, suggesting that the receptor is involved in the metastasization process and thus is a suitable target for therapy.

The identification of new prognostic markers, such as CXCR4, features a subgroup of patients with a high risk of relapse, and are thus suitable for a tight follow up. Recently, the relationship between lymphocytes which expressed CC or CXC chemokine receptor and soluble chemokines derived from the host tissues in proximity to the tumor or the tumor itself was determined. Differential chemokine receptor expression by activated tumor-specific CD8+ T cells could be associated with divergent clinical outcomes, suggesting that they may serve as a biomarker of potential clinical responsiveness to immunotherapy. Expression of CXCR3 by CD8+ T cells was reported to be associated with survival in melanoma patients with stage III disease (34). We are currently evaluating the peripheral blood lymphocytes of melanoma patients for the distribution of specific chemokine receptors in CD8+ T cell subgroups. Moreover, we are tracing CXCR4 expression during the metastatic process through the detection of CXCR4-positive melanoma cells in biopsies of suspicious lymph nodes.

The inhibition of CXCR4 has been previously described. AMD3100, the small-molecule bicyclam, is the better studied derivative, in human melanoma clinical trials. AMD3465, inhibited intracellular calcium signaling, chemotaxis, CXCR4 endocytosis, and mitogen-activated protein kinase phosphorylation induced by CXCL12.

T22 is one of the several described inhibitory peptides (39–41). Some antagonist peptides in microcapsules have been tested in vivo studies demonstrating antitumor activity in murine models (42). The identification of new peptides with stronger CXCR4 antagonist activities have recently been described (43, 44). These results indicate that the CXCR4 receptor is frequently expressed in melanoma metastases, and is part of an active transduction pathway that is efficiently inhibited by the antagonist. Taken together, these evidences strongly support the value of CXCR4 inhibition in melanoma patients encouraging the use of active inhibitors such as AMD3100, or its derivatives, in human melanoma clinical trials.

References


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