Expression of Functional Chemokine Receptors CXCR3 and CXCR4 on Human Melanoma Cells*

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Chemokines are secreted into the tumor microenvironment by tumor-infiltrating inflammatory cells as well as by tumor cells. Chemokine receptors mediate agonist-dependent cell responses, including migration and activation of several signaling pathways. In the present study we show that several human melanoma cell lines and melanoma cells on macroscopically infiltrated lymph nodes express the chemokine receptors CXCR3 and CXCR4. Using the highly invasive melanoma cell line BLM, we demonstrate that the chemokine Mig, a ligand for CXCR3, activates the small GTPases RhoA and Rac1, induces a reorganization of the actin cytoskeleton, and triggers cell chemotaxis and modulation of integrin VLA-5- and VLA-4-dependent cell adhesion to fibronectin. Furthermore, the chemokine SDF-1α, the ligand of CXCR4, triggered modulation of β1 integrin-dependent melanoma cell adhesion to fibronectin. Additionally, Mig and SDF-1α activated MAPKs p44/42 and p38 on melanoma cells. Expression of functional CXCR3 and CXCR4 receptors on melanoma cells indicates that they might contribute to cell motility during invasion as well as to regulation of cell proliferation and survival.

The chemokines are a family of low molecular weight cytokines that elicit cell migration responses and activation of several signaling pathways (1–4). The chemokines have been classified into four families, CXC, CC, C, and CX3C, which differ in the position of four conserved cysteines. The two major families are the CXC and CC chemokines, which interact with seven-transmembrane G-protein-linked CXCR and CCR receptors, respectively (1–4). Important roles for chemokines have been demonstrated in inflammatory diseases, hematopoiesis, angiogenesis, tumor rejection, and human immunodeficiency virus, type 1 infection (4, 5).

Binding of chemokines to their receptors triggers the activation of several molecules and signaling pathways, including the activation of the Janus tyrosine kinase/signal transducers and activators of transcription pathway, activation of small GTPases of the Rho family, and activation of phosphatidylinositol 3-kinase and MAP1 kinase pathways (3–6). Cells respond to chemokines by altering their morphology during migration in which cell polarization with redistribution of several membrane receptors and rearrangement of actin cytoskeleton takes place (7). Cell migration also involves sequential adhesion and detachment steps, and transient up-regulation of the activity of cell adhesion receptors mediated by chemokines is likely to play an important role during migration. Hence, several chemokines including the CXC chemokine SDF-1α (CXCL12) and the CC chemokines monocyte chemoattractant protein-1 (CCL2) and RANTES (regulated on activation normal T cell expressed and secreted) (CCL5) modulate the adhesive activity of integrins VLA-4 and VLA-5 in lymphocytes and in bone marrow hematopoietic progenitor and myeloma cells (8–12).

In addition to tumor cells, the tumor microenvironment includes inflammatory cells such as macrophages and T lymphocytes, which are attracted by cytokines and chemokines secreted by tumor cells (13). Moreover, activation of macrophages and T lymphocytes results in secretion of additional chemokines. IL-8 (CXCL8), MGS/GROα (melanoma growth-stimulatory activity/growth-regulated oncogene α) (CXCL1), Mig (CXCL9), and IP-10 (CXCL10) are found in the tumor microenvironment in melanoma (14–16), and therefore they potentially could bind to their receptors on cells present in the tumor. Most of the work on chemokines in melanoma has centered on IL-8, which is produced by melanoma cells and contributes to their growth (14, 15, 17). Mig (monokine induced by interferon-γ) is produced mainly by activated macrophages and binds to CXCR3, which is functionally expressed on activated T and B cells and on endothelial cells (18–21). On the other hand, SDF-1α is expressed on most tissues and exerts its chemotactic and activating functions upon interaction with its receptor CXCR4 (22).

During invasion and dissemination, tumor cells migrate through tissues involving dynamic regulation of cell adhesion associated with morphological alterations that must include changes in the organization of the actin cytoskeleton. Chemokines are potential candidates to induce such cellular re...
sponses upon binding to their receptors. In the present work we found that human melanoma cells express the chemokine receptors CXCR3 and CXCR4, which mediate agonist-dependent cell migration and activation, raising the possibility that these receptors could play a relevant role during tumor cell invasion and growth.

**Experimental Procedures**

**Cells and Antibodies**—The human melanoma cell line BLM was cultured in DMEM medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium) and antibiotics. The melanoma cell lines MeWo, SK-Mel 37, and A375 were obtained from patients undergoing surgery at the Department of Immuno-Oncology and Surgery, Hospital General Universitario Gregorio Marañón, Madrid. Histopathological diagnosis was confirmed for each specimen. A homogeneous cell suspension was obtained by mechanic disruption of tumoral tissue fragments in RPMI 1640 medium. Monoclonal antibodies to CXCR3, CXCR4, CXCR1, and CXCR2 were purchased from R&D Systems (Minneapolis, MN). The integrin anti-αv, α5, β4, and anti-β3 Lia 1/2, 1/2, anti-VE-cadherin TEA 1/1, and control antibody F3X63 mAb were gifts of Dr. Francisco Sánchez-Madrid (Hospital de la Princesa, Madrid). The anti-αvβ3 mAb was purchased from Neurobiotec (France). Anti-CD45, a monoclonal antibody against S-100, was obtained from Dako (Glostrup, Denmark) and normal rabbit serum (Zymed Laboratories Inc. San Francisco, CA) was used as its negative control. Anti-fibroblast AS02-elon was purchased from Dianova (Hamburg, Germany), and anti-gp100 HMB-45 mAb was from Signet Laboratories Inc. (Dedham, MA).

**Flow Cytometry**—Melanoma cells at subconfluency (50–70%) were detached with 2 mM EDTA in PBS, washed, resuspended in ice-cold PBS, and incubated on ice for 30 min with primary antibodies. After washing, cells were incubated with FITC-conjugated anti-mouse secondary antibodies (DAKO A/S, Copenhagen, Denmark) and analyzed on a Coulter Epics XL flow cytometer. The levels of chemokine receptor expression were quantified as we reported previously (23) based on a calibration curve.

**Western Blotting**—For p44/42 and p38 MAP kinase analysis, melanoma cells in subconfluent monolayers were first starved in DMEM without serum, detached, and resuspended in DMEM, 0.4% BSA. After incubation with chemokines at 37 °C, cells were lysed in 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 30 mM sodium pyrophosphate, 10 μM sodium vanadate, 1 μM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophotically transferred onto polyvinylidenefluoride membranes that were incubated with anti-phospho MAP kinase antibodies (New England Biolabs, Beverly, MA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Dako). Protein bands were visualized using ECL detection (Amersham Pharmacia Biotech). After stripping and blocking, the same blots were reprobed with anti-MAP kinase antibodies (New England Biolabs) to test for total protein content.

**Results**

**Expression of CXCR3 and CXCR4 on Human Melanoma Cells**—Analysis by flow cytometry of several melanoma cell lines indicated that CXCR3, a receptor for chemokines Mig, IP-10, and interferon-inducible T cell o-chemoattractant, was moderately expressed mainly on the highly invasive BLM cell line and to a lesser degree on SK-Mel 37 cells, whereas low or no expression was detected on MeWo and A375 cells (Fig. 1A).
The SDF-1α receptor, CXCR4, was mainly expressed on MeWo and A375 cells and at lower levels on BLM cells. Quantitative analysis of CXCR3 and CXCR4 expression using BLM and MeWo cells showed 47,179/110061,554 receptor sites/cell for CXCR3 and 111,354/1100611,503 receptor sites/cell for CXCR4. Expression of CXCR1 and CXCR2, receptors for IL-8, was barely detected on these melanoma cell lines (not shown). Immunofluorescence microscopy analysis of permeabilized melanoma cells confirmed the expression of CXCR3 and CXCR4 on BLM and MeWo cells, respectively (Fig. 1B).

To study whether CXCR3 and CXCR4 were expressed by melanoma cells in tumoral tissues we performed flow cytometry and histochemical analysis using a combination of quite selective melanoma markers, S-100 and gp100/pmel 17 (29–31). For flow cytometry determinations, we obtained single cell suspensions from metastatic melanoma surgical specimens and immediately determined the expression of these chemokine receptors. Analysis showed that CXCR3 and CXCR4 were expressed on the surface of metastatic melanoma cells from macroscopically infiltrated lymph nodes (n = 5) (Fig. 2A). CXCR1 was expressed at low levels, whereas no expression of CXCR2 was detected. S-100 was expressed by 99% of the cells, whereas less than 1% reacted with antibodies to fibroblast and endothelial markers (not shown), and analysis was performed excluding CD45+ cells. Immunohistochemical analysis of seven cases with lymph node metastases and three cases with subcutaneous metastases revealed in all cases low to moderate expression of CXCR3 and CXCR4 on melanoma cells (Fig. 2B) with some...
heterogeneity in the level of expression of both molecules. In most instances, expression of CXCR4 was less intense than that of CXCR3. Tumor cells were identified by their expression of the melanocyte-specific antigen gp100 with the HMB-45 mAb. Altogether these data indicate that human melanoma cells express the chemokine receptors CXCR3 and CXCR4, raising the possibility that they could mediate agonist-dependent cell responses.

**Effect of Mig on RhoA and Rac1 Activation and in the Actin Cytoskeleton on BLM Melanoma Cells**—Small GTPases of the Rho family, like RhoA and Rac1, are key regulators of the organization of the actin cytoskeleton, and changes in their activation state influence cell morphology and migration (32, 33). Upstream events leading to exchange of GDP for GTP binding to Rho GTPases activate these proteins, which can then interact with downstream targets to produce different biological responses. We used the BLM melanoma cell line to study whether Mig could influence the activation of RhoA and Rac1 by using “pull-down” assays with GST fusion proteins containing domains derived from Rho GTPase targets. Untreated BLM cells exhibited very low levels of active RhoA, but after a short (1-min) incubation with Mig there was a 15–20-fold increase in the amount of active RhoA as detected with the GST-C21 fusion protein (Fig. 3). Although active Rac1 levels were high in untreated BLM cells, there was an increase (2-fold) in active Rac1 after a 1-min exposure to Mig as detected with the GST-PAK-CD fusion protein. Activation of RhoA and Rac1 by Mig on BLM cells was transient as longer times of incubation with the chemokine resulted in a decrease in the activation of these GTPases (Fig. 3).

Investigation of the capability of Mig to alter the organization of the actin cytoskeleton on BLM melanoma cells was followed by FITC-phalloidin using a laser scanning cytofluorometer. Whereas no changes in total fluorescence values were obtained comparing samples incubated with or without Mig, we observed that in samples incubated with this chemokine (Fig. 4, b and d) there was a higher proportion of BLM cells showing a polarized pattern of F-actin staining than in samples incubated in the absence of Mig (Fig. 4, a and c). The percentages of cells showing a homogeneous F-actin staining were 23 ± 8% for cells incubated with Mig and 51 ± 7% of cells incubated in medium alone (n = 3). These differences were maintained for up to 7 min of incubation (not shown).

**Mig Triggers Chemotaxis on Melanoma BLM Cells**—To determine whether Mig could induce migration on BLM cells, we...
carried out chemotaxis assays through filters coated with extracellular matrix proteins. Mig was capable of triggering a chemotactic response on BLM cells across fibronectin and, to a lesser extent, across collagen in the range of 50–200 ng/ml of the chemokine (Fig. 5). As expected due to the fact that Mig signals through a receptor coupled to Gi (19, 34), pertussis toxin, an inhibitor of the Gi subunit of G-proteins, completely abolished the Mig-induced BLM chemotaxis. Taken together these results indicate that Mig promotes migration of BLM melanoma cells and affects their cytoskeleton.

**Modulation of Melanoma Cell Adhesion to Fibronectin by Mig and SDF-1**—To investigate whether Mig and SDF-1 were capable of influencing melanoma cell adhesion, we performed adhesion assays to both whole plasma fibronectin, which contains interaction sites for integrins VLA-5 and VLA-4, and to FN-H89, a fragment of fibronectin that contains the CS-1 site for interaction with VLA-4 but lacks the RGD central binding domain, a binding site for VLA-5. Mig up-regulated by 1.7–2.9-fold (n = 5) the adhesion of BLM cells to whole fibronectin with an optimal increase at 50 ng/ml of the chemokine and an adhesion time of 3.5 min, whereas anti-α5 mAb substantially, but not totally, inhibited the up-regulated BLM adhesion (Fig. 6A). Mig also augmented BLM adhesion to FN-H89 (1.3–2.2-fold increase, n = 5), which was blocked by anti-α4 mAb, and anti-α4 mAb partially inhibited the enhanced adhesion to whole FN (Fig. 6A). Together these data suggest that both integrins, but preferentially VLA-5, are used by BLM cells to attach to fibronectin. As several chemokines, including SDF-1α and SLC (CXCL21), can be found attached on endothelium (10, 35), we investigated whether Mig coimmobilized with whole FN could also modulate melanoma cell adhesion. We obtained an average of a 2.2-fold increase of BLM adhesion to...
FN triggered by coimmobilized Mig at an optimal coating concentration of 200 ng/ml of the chemokine (Fig. 6A). Anti-α4 mAb completely blocked the up-regulated adhesion, suggesting that VLA-5 and/or VLA-4 were involved in this adhesion. Instead, IL-1β coimmobilized with FN did not influence BLM adhesion, and Mig alone did not support adhesion (Fig. 6A).

**FIG. 5.** Mig triggers chemotaxis on BLM melanoma cells. Subconfluent cultures of BLM cells were incubated for 16 h in the absence (−) or presence (+) of pertussis toxin (PTX). Cells were detached, resuspended in migration buffer, and allowed to migrate in response to different concentrations of Mig across fibronectin- or collagen-coated filters. Cell migration was quantified as described under “Experimental Procedures,” and data (mean ± S.D. values of quadruplicate samples) represent one of two independent experiments.

**FIG. 6.** Effect of Mig and SDF-1α on melanoma cell adhesion to fibronectin. BCECF-AM-labeled BLM and MeWo melanoma cells were detached, resuspended in ice-cold adhesion medium without (Control) or with the indicated concentrations of Mig (A, Soluble Mig) or SDF-1α (B, Soluble SDF-1α), and added to 96-well plates coated with fibronectin or FN-H89. Plates were kept for 15 min on ice to place cells in contact with ligands and subsequently placed at 37 °C for 3.5 min. Unbound cells were washed, and adhered cells were quantified in a fluorescence analyzer. Some samples were preincubated with anti-αi mAb before adding cells to wells. For adhesion to fibronectin coimmobilized with Mig (B), BCECF-AM-labeled BLM cells were added to wells coated either with fibronectin alone (Control), with Mig alone, with fibronectin and the indicated concentrations of Mig, or with fibronectin and IL-1β. Plates were incubated at 37 °C for 6 min, and after washing, bound cells were quantified as above. Some samples were incubated with anti-β1 mAb before adding cells to wells. Panels in A show data representing the mean ± S.D. of triplicate samples from one representative result of five (for soluble Mig) and three (for coimmobilized Mig) experiments, while panels in B show data representing the mean ± S.D. of triplicate samples from one representative result of three independent experiments.
SDF-1α was also capable of increasing MeWo cell adhesion to whole FN and FN-H89, involving both VLA-5 and VLA-4 (Fig. 6B).

**Activation of p44/42 and p38 MAP Kinases by Mig and SDF-1α on Melanoma Cells**—To study whether Mig and SDF-1α could activate MAP kinases on melanoma cells, we incubated BLM and MeWo cells for different times with these chemokines, and changes in the phosphorylation of p44/42 (Erk1/2) and p38 MAP kinases were analyzed by Western blotting using antibodies recognizing the phosphorylated form of these kinases. Mig rapidly (0.5 min) up-regulated the phosphorylation of p44/42 MAP kinase on BLM cells; this increase was still detected after 7 min of exposure to the chemokine although to a lesser degree (Fig. 7A). Untreated BLM cells displayed significant phosphorylation of p38 MAP kinase, but exposure to Mig triggered an increase in this phosphorylation, but slower and to a lesser extent compared with p44/42 (Fig. 7A). No detectable basal phosphorylation of p38 MAP kinase was observed in MeWo melanoma cells in the absence of chemokines (Fig. 7B). Incubation with SDF-1α notably up-regulated the phosphorylation of p38 MAP kinase, which was already detected at 0.5 min, peaked at 1 min, and remained highly phosphorylated at 10 min of incubation (Fig. 7B).

**DISCUSSION**

During tumorigenesis, melanoma cells might encounter different chemokines produced in the tumor microenvironment by infiltrating inflammatory cells, such as macrophages and activated T lymphocytes, as well as by tumor cells (13) or displayed on the endothelium at metastasis sites. In the present study we show that several melanoma cell lines, including the highly invasive melanoma cell line BLM, and melanoma cells from macroscopically infiltrated lymph nodes express CXCR3, the receptor for chemokines Mig, IP-10, and interferon-inducible T cell α-chemoattractant, and/or CXCR4, the receptor for SDF-1α. For the analysis of CXCR3 and CXCR4 expression on melanoma cells in metastatic tissues, we used as markers for melanoma staining a combination of two antibodies, HMB-45 and anti-S-100, which are widely used for melanoma diagnoses and which recognize the gp100 and S-100 antigens, respectively (29–31). Expression of CXCR3 and CXCR4 on metastatic melanoma cells was found in all samples as determined by flow cytometry and immunohistochemistry analysis, but expression followed a heterogeneous pattern from low to moderate expression levels. Whether expression of CXCR3 and CXCR4 on melanoma cells in tumoral tissues reflects a specific activation status or an adaptation of melanoma cells to local microenvironment signals is not known yet and represents an important issue of study.

Mig is produced by activated tumor-infiltrating macrophages in the tumor microenvironment in malignant human melanoma (16), and it has been proposed that interferon-γ secreted by tumor-infiltrating T cells might be the activation signal for Mig production. The chemokine IP-10, an additional agonist for CXCR3, is also present in the tumor microenvironment in human melanoma (16), thus representing another candidate to trigger CXCR3-mediated cell responses. On the other hand, SDF-1α is expressed by most cell types (22), and it is likely produced by tumor stromal cells. Therefore, the expression of CXCR3 and CXCR4 by melanoma cells that we report here makes them susceptible to Mig and SDF-1α effects.

Indeed, Mig and SDF-1α triggered several responses in BLM and MeWo melanoma cells, respectively. First, Mig rapidly activated the small GTPases RhoA and Rac1, key regulatory molecules of the organization of actin cytoskeleton (32, 33). Mig also induced a rearrangement of F-actin, resulting in polarization of actin at cell edges, and triggered migration of BLM cells across fibronectin- and collagen-coated filters. There is increasing evidence linking activation of Rho family GTPases with an increase in tumor cell migration and invasion (36). Expression of activated forms of RhoA, Rac1, and several of their downstream effectors in different tumor cell types, including melanoma, hepatoma, T lymphoma, and breast carcinoma, has been reported to play important roles in tumor cell motility and invasion (37–42). Therefore, activation of RhoA and Rac1 by Mig, and perhaps by SDF-1α, together with a reorganization of the actin cytoskeleton that we show here on melanoma cells could represent a mechanism contributing to their migration and invasion, which will constitute the object of future studies.

Second, Mig up-regulated integrin VLA-5 and VLA-4-dependent BLM adhesion to fibronectin both when the chemokine was used soluble or coimmobilized with fibronectin. Moreover, increased adhesion to fibronectin mediated by these integrins was also detected when we used MeWo melanoma cells incubated with SDF-1α. Tumor cells must attach to a variety of extracellular matrix proteins during invasion and metastasis, and it has been reported that VLA-5 and VLA-4 mediate melanoma cell adhesion to fibronectin (27, 43). An increase in melanoma cell attachment by these chemokines was only detected at short times (<5 min), suggesting that a dynamic regulation of melanoma cell adhesion by chemokines might be used to move in a series of adhesion and detachment steps during their migration and/or invasion. Clustering of integrins, which might depend on the activation of Rho GTPases (44), and/or alterations in integrin affinity due to chemokines could contribute to increased attachment of melanoma VLA-5 and VLA-4 for fibronectin. Modulation of VLA-5- and VLA-4-dependent cell adhesion by chemokines, including Mig and SDF-1α, has also been shown for hematopoietic progenitor cells, lymphocytes, and leukemic cells (8–11, 45), suggesting the presence of common pathways for integrin activation in different cell types.

Third, phosphorylation of MAP kinases p44/42 and p38 was up-regulated on melanoma cells by Mig and SDF-1α. Activation of these kinases by SDF-1α has also been described in hematopoietic progenitor and myeloma cells (11, 12, 46). The p44/42 MAP kinase can also be activated by cell adhesion (47, 48),...
Although we have performed our assays in solution and without serum, therefore in the absence of cell adhesion, suggesting that Mig can directly trigger activation of this kinase independently of adhesion. Exposure of melanoma cells to Mig and SDF-1α could modulate the activation state of p44/42 and p38 MAP kinases in response to mitogens and cell adhesion, and hence this modulation could contribute to the control of melanoma cell proliferation, survival, and apoptosis linked to the activation of these kinases (49, 50).

Matrix metalloproteinases and their tissue inhibitors are involved in degradation of basement membranes and extracellular matrix, an essential step in melanoma cell invasion (for review, see Ref. 51). IL-8 up-regulates MMP-2 activity in human melanoma cells (17), and recent data demonstrated that SDF-1α induced secretion of matrix metalloproteinases and their tissue inhibitors by bone marrow cells (52, 53). Although it has not been addressed in the present study, the control of metalloproteinase secretion might represent an additional melanoma cell response to Mig and SDF-1α that could contribute to tissue invasion.

An angiostatic role for Mig and IP-10 has been reported (39–41) that Mig can directly trigger activation of this kinase independently of adhesion. Exposure of melanoma cells to Mig and SDF-1α might present opposing functions during tumor development: on one side it could inhibit endothelial cell proliferation and promote tumor rejection, and on the other side, according to the present results on activation of melanoma cell adhesion and migration, it could contribute to tumor invasion. It is tempting to hypothesize that an imbalance between these Mig- and IP-10-induced effects might represent a mechanism participating in the control of tumorigenesis.

In a recent report focused on CXCR4 expression and its involvement in the metastasis of human breast cancer cells, it was also shown that several melanoma cell lines expressed CXCR4 (57). Using reverse transcription-polymerase chain reaction analyses, expression of CXCR3 was not found on melanoma BLM cells. It is likely that different variants of this cell line exist due to different culture conditions, and in addition, we found a higher expression of CXCR3 on subconfluent BLM cultures than in confluent ones (not shown); this might help to explain these different results.

In summary, the results from this study indicate that CXCR3 and CXCR4 are expressed on melanoma cells and that agonists for these chemokine receptors are capable of activating several pathways associated with cell motility and modulation of cell proliferation. Altogether these data raise the possibility that CXCR3 and CXCR4 could be involved in melanoma tumorigenesis.

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