CXCL12 Does Not Attract CXCR4\(^+\) Human Metastatic Neuroblastoma Cells: Clinical Implications

Iрма Airoldi,\(^1\) Лиззия Раффагельло,\(^1\) Ерх Тиоован,\(^2\) Клаудия Коко,\(^1\) Барбара Карлини,\(^1\) Альберто Амадори,\(^2\) Мария Валерия Корриас,\(^1\) и Вито Пистоиа\(^1\)

**Abstract**

**Purpose:** The role of CXCR4 in bone marrow localization of neuroblastoma cells has been recently proposed. The aim of this study was to investigate the expression and chemotactic functionality of CXCR4 in human metastatic neuroblastoma cells isolated from the bone marrow and, for comparison, in a panel of neuroblastoma cell lines.

**Experimental Design:** CXCR4 expression and chemotactic functionality were investigated in metastatic neuroblastoma cells isolated from patient bone marrow and in neuroblastoma cell lines. The former cells were isolated as CD45\(^-\) or GD2\(^+\) cells by immunomagnetic bead manipulation. Chemotactic assays were done in a transwell system. Regulator of G protein signaling expression was investigated by reverse transcription-PCR.

**Results:** Metastatic neuroblastoma cells consistently expressed CXCR4, which was also detected in 5 of 10 neuroblastoma cell lines. CXCL12 did not stimulate the chemotaxis of primary tumor cells or cell lines in either normoxia or hypoxia, irrespective of CXCR4 up-regulation detected under the latter condition. Accordingly, neuroblastoma cells failed to modulate filamentous actin and to activate mitogen-activated protein kinase upon treatment with CXCL12. RGS16 mRNA was consistently expressed in primary tumor cells and cell lines, but its down-regulation by RNA interference did not restore CXCR4 chemotactic functionality.

**Conclusions:** These results show unambiguously that CXCR4 expressed in human metastatic neuroblastoma cells is not functional and do not support the clinical use of CXCR4 antagonists to prevent neuroblastoma metastasis.

Primary tumor cells of different origin or cell lines thereof express CXCR4, migrate in vitro to its ligand CXCL12 and metastasize in vivo to anatomic sites where CXCL12 is expressed (1–10). Accordingly, CXCR4 up-regulation has been shown in some human metastatic versus primary tumors (11–13). CXCR4 belongs to the wide family of G protein-coupled receptors. Regulator of G protein signaling (RGS) proteins terminate G protein-coupled receptor-mediated signaling by shortening the lifetime of Gα-GTP (14) and dampening mitogen-activated protein kinase (MAPK) phosphorylation (15–18). RGS1, RGS3, RGS13, RGS16, and RGS18 have been shown to turn off CXCR4 signaling in different experimental systems (19–21).

Preclinical studies indicate that metastatic spreading of CXCR4\(^+\) tumor cells can be inhibited by anti-CXCR4 antibodies or CXCR4 antagonists (1, 3, 4, 22). These results delineate a novel strategy for prevention and/or treatment of metastases that will certainly have an effect on the clinical practice of oncology. Neuroblastoma is a pediatric tumor of neuroectodermal origin whose overall 5 year survival is 45% according to a recent European study (23). Therefore, novel therapeutic approaches for neuroblastomas with poor prognoses are needed. Among them, inhibition of dissemination of tumor cells from primary to metastatic sites may represent an attractive strategy.

The bone marrow is the major site of neuroblastoma metastasis, both at diagnosis and at relapse. A previous study done using neuroblastoma cell lines and a recent report on primary neuroblastoma tumors supported the potential involvement of CXCR4 in bone marrow localization of metastatic neuroblastoma cells (5, 9).

Here, we have investigated CXCR4 expression and function in primary metastatic neuroblasts isolated from the bone marrow of patients with neuroblastoma in comparison with a panel of neuroblastoma cell lines.

**Materials and Methods**

**Patient samples.** This investigation was done following approval by a local institutional review board. The criteria for diagnosis and evaluation of disease extension have been reported elsewhere (24). An aliquot of...
bone marrow aspirates done for diagnostic purposes was obtained from six patients with stage 4 disease at diagnosis. The median age was 3.3 years (range, 8 months to 5.9 years) and all of the patients had consistent bone marrow infiltration (2+/3+), as assessed by morphologic analysis. At present (8-10 months after diagnosis) one of the patients has died of disease and four, including an infant, have relapsed.

**Cell separation and culture.** Bone marrow aspirates were depleted of erythrocytes by osmotic lysis and washed in PBS. CD45−neuroblasts were isolated from bone marrow cell suspensions by incubation first with CD45 (Caltag, Burlingame, CA) and anti-CXCR4, CD45, and 10% FCS (Sigma). After washing with PBS, the slides were incubated with rabbit anti-goat serum for 1 hour and subsequently with immunomagnetic beads coated with anti-mouse immunoglobulins, according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, neuroblasts were positively selected as GD2+ cells using the same technique.

**(Monoclonal antibodies and flow cytometry.** Surface staining was done as previously described (26). The source of anti-GD2 mAb (IgG2a) was the supernatant of the ME361-S2a murine hybridoma, purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

**Actin polymerization.** Actin polymerization was investigated as previously described (27). Briefly, neuroblastoma or BRG cells were serum-starved for 18 hours before being incubated for 5 minutes with human CXCL12 (100 or 500 ng/mL). Subsequently, cells were fixed in 1% paraformaldehyde and permeabilized with ice-cold methanol (Sigma). Cells (1 × 10⁶) were then incubated with 20 μL of FITC-labeled rabbit anti-MAPK (pTpY185/187) in 2% PBS, 0.1% saponin (Sigma), FCS for 30 minutes. After extensive washing, cells were resuspended in 500 μL of PBS before being analyzed by flow cytometry.

**Reverse transcription-PCR.** RNA was extracted from primary neuroblasts or neuroblastoma cell lines using RNeasy mini kit from Qiagen GmbH (Hilden, Germany) and subjected to RT-PCR. Primer sequences and amplification profiles were the following: G3PDH, 5′ ATACGTGGCAGAACCACCTATGG and 3′ GGGTCTACATGGCAACTGTGAG; CXCR4, 5′ AACATTGCTTTTCTTTTCTC and 3′ TGAGGTGGGCTTGAATTGAC; RGS16, 5′ TCTCTGTCTAACCCAAAGG and 3′ ACAAAGCCAGCGCAACTAC; G3S, 5′ CAGAAGGACGCGGCAAGC and 3′ TGGATGTGGGCTTGAGT; RGS13, 5′ AACATGTCCITTCTTGTG and 3′ TGGTGAAGCAGTTGATG; RGS15, 5′ GGGACACACGCGTGGAG and 3′ AACAATGCCAACAACACCT; RGS18, 5′ TGCGAATTGCAATAAGCC and 3′ TGATTTGATTACAGCA. Amplification profile was 94°C for 1 minute, annealing at 60°C (G3PDH, 55°C (CXCR4), 49°C (RGS13, and RGS16), 51°C (RGS16), 58°C (RGS18) for 1 minute and extension at 72°C for 1 minute. Each cycle of amplification was repeated 35 times. Ten microliters of each sample were electrophoresed through a 1% agarose gel containing ethidium bromide. The specificity of amplification products was checked by confirming the known base-pair sequence length.

**Short interfering RNA and RNA interference.** LAN-5, GI-LI-N, IMR-32, and SH-SY-5Y neuroblastoma cell lines were transfected with RGS16 or unrelated short interfering RNA (siRNA) purchased from Ambion (Austin, TX). Three different short interfering RGS16 sequences were used with similar results in terms of down-regulation of RGS16 transcript. The sequences are the following: RGS16 A, 5′ GGAAUCCCUAAGAUAUUCCUC and 3′ GACUUAUUUCCUAGGAUCCCCtt; RGS16 B, 5′ GGAAGUGUUCUAGUGUGGUtt and 3′ ACCACCAACAAGGCGTCCG; RGS16 C, 5′ GGAGAUGCAGAGAAGG and 3′ UUCUUGAGAUAAGUUCUGUCUGC. Transfection was carried out using 3 × 10⁴ cells of the siPORT NeoX transfection agent (Ambion), 150 nmol/L of siRNA and following the guidelines of the manufacturer.
Quantitative RT-PCR. The cell lines were subjected to RNA extraction using the RNeasy micro kit (Qiagen), 24, 48 and 72 hours after transfection with the RGS16 or unrelated siRNA. One microgram of total RNA was reverse-transcribed in a total volume of 30 μL. Five microliters of cDNA were amplified in triplicate samples using primers and probes specific for β2-microglobulin or for RGS16 (Applied Biosystems, Foster City, CA). To estimate the relative amount of RGS16 transcript, the comparative threshold method (28) was employed using β2-microglobulin as endogenous reference and the amount of RGS16 transcript in each untransfected cell line as calibrator.

Results

CXCR4 expression and chemotaxis of primary neuroblastoma cells in response to CXCL12. Bone marrow aspirates from six patients with stage IV neuroblastoma were found to contain GD2 neuroblasts in the following proportions: BM1, 34.3%; BM2, 31.6%; BM3, 50.8%; BM4, 25.6%; BM5, 65.7%; and BM6, 44.4%. GD2 neuroblasts coexpressing CXCR4 were detected in all bone marrow samples and ranged from 25% to 85% (mean, 55%). Two representative experiments carried out with BM1 and BM5 are shown in Fig. 1A.

The CD45+ cell fraction isolated from bone marrow samples contained >90% neuroblasts, as assessed by staining with anti-GD2 mAb; in contrast, <5% GD2 neuroblasts were consistently detected in purified CD45+ cells.

Next, chemotaxis of paired CD45+ and CD45− bone marrow cell fractions to CXCL12 was investigated. CD45+ neuroblasts did not migrate to a wide range of CXCL12 concentrations (30, 100, 300, 500, and 1,000 ng/mL). In contrast, the CD45+ cell fractions showed a sustained chemotactic response to 100 ng/mL CXCL12 (Fig. 1B).

CXCR4 expression and chemotaxis of neuroblastoma cell lines in response to CXCL12. As shown in Fig. 2A, CXCR4 mRNA was found to be expressed in all neuroblastoma cell lines. Surface CXCR4 was detected on GI-LI-N (34% positive cells), LAN-5 (30% positive cells), IMR-32 (23% positive cells), HTLA-230 (16% positive cells), and SH-SY-5Y (5.5% positive cells), but not in ACN, GI-ME-N, GI-CA-N, LAN-1, SK-N-SH, or SK-N-BE-2c, neuroblastoma cell lines (Fig. 2B). These figures were stable over time, as shown by repeated testing of cell lines at 2-month intervals.

The seemingly low proportions of CXCR4+ SH-SY-5Y cells here detected in comparison with previous data [see Sanders et al. in ref. (9)] prompted additional experiments in which CXCR4 expression was investigated in this cell line by immunocytochemistry. Indeed, CXCR4 was found to be expressed at very low intensities in the majority of cells and at higher intensities in the minority of cells (5-10%). A likely explanation for these apparently discrepant findings is the usage in this study of cells that have somewhat down-regulated CXCR4 due to considerable time in culture.

Following culture of neuroblastoma cells in 1% oxygen tension, which mimics the hypoxic conditions found in the tumor microenvironment, a strong CXCR4 up-regulation was observed (Fig. 2B). The proportions of CXCR4+ cells raised to 92% in GI-LI-N cells, 97% in LAN-5 cells, 93% in IMR-32 cells, 85% in HTLA-230 cells, and 40% in SH-SY-5Y cells.

The five CXCR4+ neuroblastoma cell lines did not migrate in a transwell assay to CXCL12 when tested at different concentrations (30, 100, 300, 500, and 1,000 ng/mL) or at different times (2, 4, 6, and 16 hours; Fig. 2C). Likewise, no chemotaxis was detected when the pore size of the filter was increased from 5 to 8 or 12 μm. In contrast, in the same experiments, the Tanque cell line (control) was attracted by CXCL12.

Next, these experiments were repeated in the anaerobic work station following overnight preincubation of cell lines in 1% O2. Again no chemotaxis of CXCR4+ neuroblastoma cell lines were observed at any CXCL12 concentration, whereas Tanque cells migrated to CXCL12 (Fig. 2C). In addition, CD45− bone marrow neuroblasts failed to migrate to CXCL12 in the same experimental conditions, whereas paired CD45+ bone marrow cell fractions were attracted by the chemokine (data not shown).

Redistribution of actin filaments (F-actin) on the cell surface is an early event associated with chemotaxis. We therefore investigated F-actin modulation in the HTLA-230 cell line following incubation with increasing concentrations of CXCL12. As shown in Fig. 2D (left), 20 seconds of incubation of neuroblastoma cells with CXCL12 did not modify actin polymerization at any concentration tested, as assessed by flow cytometry. The same result was obtained when F-actin modulation was investigated at different time points. Figure 2D (right) shows time-dependent F-actin modulation detected in BRG cells upon incubation with CXCL12.

Fig. 1. CXCR4 expression and chemotaxis of primary neuroblastoma cells in response to CXCL12. A, representative CXCR4 surface staining of GD2 neuroblasts from two bone marrow aspirates (BM1 and BM5). Dark profiles, cells stained with an IgG2a antibody of irrelevant specificity (anti-GD2 control), followed by phycoerythrin-conjugated goat anti-mouse IgG2a antibodies. After washing, cells were incubated with an IgG1 antibody of irrelevant specificity (anti-CXCR4 control), followed by FITC-conjugated goat anti-mouse IgG2a antibodies. Open profiles, staining obtained using anti-GD2 mAb, followed by phycoerythrin-conjugated goat anti-mouse IgG2a antibodies, an anti-CXCR4 mAb and, subsequently, FITC-conjugated goat anti-mouse IgG1 antibodies. The profiles were obtained by setting the gate on GD2+ cells. B, chemotaxis of neuroblasts isolated from bone marrow aspirates in response to CXCL12. CD45+ neuroblasts and CD45− cell fractions were incubated with 300 ng/mL of CXCL12 or medium for 2 hours in transwell plates. Results are means from experiments done using six different bone marrow fractions.
Expression of RGS in bone marrow neuroblasts and in neuroblastoma cell lines. To gain insight into the potential mechanism(s) underlying the chemotactic unresponsiveness of neuroblasts to CXCL12, expression of a panel of RGS proteins involved in the negative control of CXCR4 signaling (RGS1, RGS3, RGS13, RGS16, and RGS18) was next investigated by RT-PCR. These experiments were carried out with primary neuroblasts isolated from four bone marrow samples and with four CXCR4+ neuroblastoma cell lines. Figure 3A shows the results obtained with these cell lines and two representative bone marrow samples.

RGS1, RGS3, RGS13, RGS16, and RGS18 transcripts were consistently expressed in primary CD45+ neuroblasts (Fig. 3A). RGS16 mRNA was detected in all neuroblastoma cell lines, RGS3 transcript was found in HTLA-230, LAN-5, and SK-SV-5Y, but not in IMR-32 cells, and RGS13 mRNA was expressed in LAN-5 cells only. RGS1 and RGS18 mRNAs were never detected in any cell line (Fig. 3A).

RGS3 and RGS16 interfere with CXCR4 signal transduction by reducing the duration and magnitude of G protein signaling, and thus at least in part inhibiting MAPK phosphorylation (15–18). Therefore, using flow cytometry, we investigated...
MAPK phosphorylation in the HTLA-230 neuroblastoma cell line following treatment with CXCL12. As shown in Fig. 3B (left), phosphorylated MAPK was not detected under these conditions, suggesting that CXCR4-dependent MAPK activation was impaired. In contrast, BRG cells incubated with CXCL12 displayed evident MAPK activation (Fig. 3B, right).

Because RGS16 was always detected in primary neuroblasts and neuroblastoma cell lines, we next addressed its potential role in dampening CXCR4 signaling. RGS16 protein expression, as assessed by immunofluorescence was found to be low in LAN-5, GI-LI-N, IMR-32, and SH-SY-5Y cells (range, 3-10%) and was absent in HTLA-230 cells.

Next, LAN-5, GI-LI-N, IMR-32, and SH-SY-5Y cells were transfected with RGS16-specific or unrelated siRNA, and inhibition of RGS16 transcription was investigated by quantitative RT-PCR. As shown in Fig. 3C, RGS16 mRNA levels were strongly down-regulated 24 hours after transfection with RGS16-specific, but not in unrelated, siRNA. This effect was no longer detectable after 48 or 72 hours from transfection (data not shown). The low constitutive expression of the RGS16 protein did not allow us to establish whether the latter had been down-regulated by siRNA transfection.

Finally, LAN-5, GI-LI-N, IMR-32, and SH-SY-5Y cells were subjected to chemotactic assays in the presence or absence of CXCL12 at 24, 48, and 72 hours after transfection. These cell lines were not attracted by CXCL12 at any time tested, suggesting that RGS16 was not involved in CXCR4 functional inactivation (data not shown).

Discussion

Various in vivo studies have shown that CXCR4 inhibition by antibodies or small antagonists counteracts the metastatic potential of tumor cells (13, 29). Thus, a new promising area of clinical investigation aimed at targeting metastatic tumor cells is emerging: examples of malignancies potentially amenable to such treatment are breast, ovarian, pancreatic, and prostate cancers, gliomas, as well as different leukemias and lymphomas (13).
Previously, it was shown that a panel of neuroblastoma cell lines expressed CXCR4 at low levels and that SH-SY5Y did not migrate to CXCL12 (9). However, following transfection of the CXCR4 gene and consequent up-regulation of surface CXCR4, the latter cells were attracted by CXCL12 and invaded in vitro CXCL12 producing bone marrow stromal cells (9), suggesting CXCR4 involvement in bone marrow metastasis formation by primary neuroblastoma cells.

In this study, we show unambiguously that the majority of human metastatic neuroblastoma cells isolated from the bone marrow express CXCR4 but are not attracted by the CXCR4-specific ligand, CXCL12. Similar results were obtained with five CXCR4+ neuroblastoma cell lines. Because the tumor microenvironment is hypoxic, we investigated whether exposure of neuroblastoma cells to low oxygen tension influenced CXCR4 expression and functionality. Although CXCR4 was found in the majority of primary tumors, it was not identified in any of the cultured neuroblastoma cells. Although CXCR4 expression and functionality were not detected in the majority of cultured neuroblastoma cells, we did not rule out the possibility that CXCR4 inhibitors could be used in the clinical setting to interfere with neuroblastoma cell growth rather than with metastatic spreading.

All primary neuroblastoma cell samples and neuroblastoma cell lines were found to express RGS16 transcript, pointing to the potential involvement of the RGS16 protein in CXCR4 inactivation. This hypothesis was tested by down-regulating RGS16 mRNA in CXCR4+ cell lines through RNA interference. However, no CXCR4-driven chemotaxis of neuroblastoma cells was observed under these conditions. These results suggest that other still undefined mechanisms are responsible for chemotactic inactivation of CXCR4 in human neuroblastoma cells.

Acknowledgments

We thank Chiara Bernardini for excellent secretarial assistance.

References

CXCL12 Does Not Attract CXCR4\(^+\) Human Metastatic Neuroblastoma Cells: Clinical Implications

Irma Airoldi, Lizzia Raffaghello, Erich Piovan, et al.


**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/1/77

**Cited articles**
This article cites 32 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/1/77.full.html#ref-list-1

**Citing articles**
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/12/1/77.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.