Role of CXCL13-CXCR5 Crosstalk Between Malignant Neuroblastoma Cells and Schwannian Stromal Cells in Neuroblastic Tumors

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Abstract

Neuroblastoma is a stroma-poor (SP) aggressive pediatric cancer belonging to neuroblastic tumors, also including ganglioneuroblastoma and ganglioneuroma, two stroma-rich (SR) less aggressive tumors. Our previous gene-expression profiling analysis showed a different CXCL13 mRNA expression between SP and SR tumors. Therefore, we studied 13 SP and 13 SR tumors by reverse transcription quantitative real-time PCR (RT-qPCR) and we found that CXCR5b was more expressed in SP than in SR and CXCL13 was predominantly expressed in SR tumors. Then, we isolated neuroblastic and Schwannian stromal cells by laser capture microdissection and we found that malignant neuroblasts express CXCR5b mRNA, whereas Schwannian stromal cells express CXCL13. Immunohistochemistry confirmed that stroma expresses CXCL13 but not CXCR5. To better understand the role of CXCL13 and CXCR5 in neuroblastic tumors we studied 11 neuroblastoma cell lines and we detected a heterogeneous expression of CXCL13 and CXCR5b. Interestingly, we found that only CXCR5b splice variant was expressed in both tumors and neuroblastoma lines, whereas CXCR5a was never detected. Moreover, we found that neuroblastoma cells expressing CXCR5 receptor migrate toward a source of recombinant CXCL13. Lastly, neuroblastoma cells induced to glial cell differentiation expressed CXCL13 mRNA and protein. The chemokine released in the culture medium was able to stimulate chemotaxis of LA1–5S neuroblastoma cells. Collectively, our data suggest that CXCL13 produced by stromal cells may contribute to the generation of an environment in which the malignant neuroblasts are retained, thus limiting the possible development of metastases in patients with SR tumor. Mol Cancer Res; 9(7); 1–9. ©2011 AACR.

Introduction

Neuroblastic tumors are a group of pediatric cancers that onset as localized or disseminated disease (1). Patients with localized tumor have good prognosis with a 3-years overall survival (OS) of 90% to 95%, whereas patients older than one year with a disseminated disease have a worse prognosis with a 3-years OS ranging between 30% and 35%. This dramatic behavior of the metastatic disease is mainly due to the capacity of neuroblastoma cells to metastasize at bone, bone marrow, liver, and lymph nodes. Particularly, in patients with disseminated disease, the normal bone marrow cell population is almost completely substituted by malignant neuroblasts that find a favorable environment for their proliferation.

Neuroblastic tumors show a quite heterogeneous histology with different tumor histotypes including: neuroblastoma predominantly composed of small round undifferentiated or poorly differentiated neuroblastic cells, with few or absent Schwannian stromal cells, ganglioneuroblastoma intermixed (GNB), and ganglioneuroma (GN) mostly composed of Schwannian stromal cells in which some nests or very few neuroblastic cells are present (2). The absence of stromal cells makes the tumor more aggressive and patients have a worse outcome. On the contrary, the abundance of Schwannian stromal cells, as observed in GNB and GN, is associated with a less aggressive tumor and a localized disease with a more favorable prognosis (1, 3).

Several evidences support a nonmalignant origin of Schwannian stromal cells (4, 5), which are supposed to
control tumor growth by secreting soluble factors that influence cell proliferation, differentiation, and angiogenesis. Indeed, various molecules have been found expressed by Schwannian stromal cells (6, 7), although their role in controlling neuroblastic cells growth and dissemination is not completely understood. In several tumors apart neuroblastic tumors, the presence of stromal cells is associated with a better disease outcome and the contiguity between stroma and tumor cells has an important role in cancer growth and invasion.

Recently, we studied a series of neuroblastic tumors by gene-expression profiling analysis and we observed different levels of CXCL13 transcripts in microdissected neuroblastic cells with respect to Schwannian stromal cells (5, 8). Our results showed that this chemokine was more expressed in stromal cells than in malignant neuroblasts, suggesting a functional role of CXCL13 in the interaction between stroma and neuroblastic cells.

Chemokines are a large family of molecules that, together with their receptors, are involved in the regulation of chemotaxis, cell proliferation, cell migration, and several other crucial cell–cell interactions (9). Moreover, chemokines play a major role in tumor growth, angiogenesis, and invasion (10, 11) in several cancers and neoplastic cells may express chemokine receptors (12). Over the last years, CCR1, CCR5, CCR6, CCR9, CXCR1, CXCR2, CXCR4, CXCR5, and CXCR6 were found expressed in neuroblastoma and it has been shown that CXCR4 is virtually expressed in all human neuroblastoma cell lines (13–16).

Starting from our observation we further investigated the expression of CXCL13 and its receptor CXCR5 in neuroblastic primary tumors and human neuroblastoma cell lines. CXCL13 and CXCR5 mRNAs were differently expressed between Schwannian stromal cells and neuroblastic cells suggesting a cell–cell crosstalk via CXCL13-CXCR5 axis. This crosstalk may contribute to retain neuroblastic cells within stroma-rich tumors and possibly to inhibit the malignant cell dissemination. Our results add new information about the CXCL13–CXCR5 axis in neuroblastic tumors and in the crosstalk between neuroblastic and stromal cells.

Materials and Methods

Tumor samples, neuroblastoma cell lines, and total RNA isolation

Tumor specimens were collected from 26 patients at onset of disease, who were diagnosed with a primary neuroblastic tumor and referred to the Gaslini Children’s Hospital. The study was approved by Ethics committee of the Gaslini Children’s Hospital and informed consent was obtained by all children’s legal guardians. According to the International Neuroblastoma Pathology Committee (2), 13 samples were from SP tumors with at least 80% of neuroblastic cells, and 13 were from SR with Schwannian stromal cells ranging from 80% to 90% (Supplementary Table 1). Eleven neuroblastoma cell lines (ACN, GI-CA-N, GI-LI-N, GI-ME-N, IMR-5, IMR-32, LA1–5S, SH-SY5Y, SK-N-BE(2), SK-N-BE(2)c, SK-N-SH) and the Raji B-lymphocytic cell line were cultured at 37°C and 5% CO₂ in RPMI 1640, supplemented with L-glutamine, penicilline/streptomycin, nonessential amino acids, and 10% FBS (Lonza). Cells were removed from substrate with PBS/EDTA. All cell lines were tested for mycoplasma contamination and authenticated (Supplementary Table 2). Total RNA was isolated from neuroblastoma cell lines by PerfectPure RNA Cell Kit (5Prime), and from tumor tissues by PerfectPure Tissue RNA Cell Kit (5Prime), and treated with RNase-free DNase I. RNA integrity and quantification were checked by RNA 6000 Nano LabChip kit and 2100 BioAnalyzer instrument (Agilent Technologies).

Isolation of neuroblastic and Schwannian stromal cells by laser capture microdissection and total RNA isolation

About 800 cells were laser microdissected from 3 SP and 3 SR frozen tumors to obtain pure cell populations of neuroblastic cells and Schwannian stromal cells, as previously described (8). Total RNA from laser capture microdissection-derived material was extracted by PicoPure RNA isolation kit (Arcturus Engineering), including a DNase treatment, RNA quality control and quantification whereas conducted by RNA 6000 Pico LabChip kit and the 2100 BioAnalyzer instrument (Agilent Technologies).

Reverse-transcription quantitative real-time PCR

RNA (1 μg) from neuroblastoma cell lines and tumors was reverse transcribed using 20 pmols of random hexamers (Eppendorf) and 200 U of SuperScript II enzyme (Invitrogen Life Technologies). RNA (50 ng) from microdissected cells was amplified and reverse transcribed by WT-Ovation RNA Amplification System kit (NuGEN Technologies). CXCL13, CXCR5, CXCR5a, S100A6, and 18S rRNA transcripts were quantified by TaqMan Gene Expression Assays (Applied Biosystems). An assay specific for CXCR5b isoform was designed by PrimerDesign Ltd. 18S rRNA was used as reference gene. Any amplified product with a quantification cycle (Cq) higher than 36 cycle was considered undetectable. According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (17, 18), a checklist including technical details is submitted as Supplementary data (MIQE checklist). The relative amount of each transcript was determined using the equation 2\(^{-\Delta\Delta Cq}\), where \(\Delta Cq = \left(Cq_{target gene} - Cq_{18S rRNA}\right)\).

CXCR5 detection in neuroblastoma cells by flow cytometry

Neuroblastoma cell suspensions (3 × 10⁵ cells/tube) and Raji cell line suspension (1.5 × 10⁵ cells/tube) were washed with PBS and incubated with 2.5 μg/ml monoclonal mouse anti-human CXCR5/Blr-1 primary antibody (R&D Systems) in 2% BSA/PBS for 30 minutes on ice. After incubation, cells were washed twice with 2% BSA/PBS and 50 μl of goat anti-mouse IgG2b-PE secondary antibody (Becton Dickinson) was added. Cell suspensions were incubated for 30 minutes on ice. Percentage of positive
cells and mean fluorescence intensity of stained cells were analyzed using a FACScan (Becton Dickinson). The background levels were set using cells stained with secondary antibody alone and viable cells were discriminated by evaluating the level of propidium incorporation.

**CXCR5 and CXCL13 detection in neuroblastoma cells by immunofluorescence**

Neuroblastoma cells were directly fixed in a methanol fixing solution and were dropped on microscope glass slides and dried at room temperature (RT). Slides were incubated 30 minutes with 10% goat serum in PBS. Primary monoclonal anti-human CXCR5/Blr-1 mouse antibody was diluted 1:40 with PBS 1.5% normal goat serum, applied on slides at a concentration of 12 μg/ml and incubated 1 hour at RT in a humidified chamber. After washing, slides were incubated 40 minutes at RT in a humidified chamber with goat anti-mouse-IgG2b-FITC conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted to a concentration of 1.5 μg/ml with PBS 3% goat normal serum. To identify the expression of CXCL13, acetone permeabilized cells were incubated 1 hour at room temperature in a humidified chamber with primary anti-human CXCL13 goat antibody diluted 1:50 with PBS 1.5% normal donkey serum, applied on slides at 2 μg/ml. After washing, slides were incubated 45 minutes at RT in a humidified chamber with donkey anti-goat-IgG(H+L) TRITC conjugated secondary antibody (Jackson ImmunoResearch Europe Ltd) diluted 1:50 with PBS 3% donkey normal serum. Staining without primary antibodies was carried out as negative control. Slides were mounted with mounting medium and inspected under the microscope with a fluorescence lamp.

**CXCR5 detection in primary tumor by immunofluorescence**

Frozen sections of 4 μm from 5 SP and 5 SR tumors were fixed in 4% paraformaldehyde for 2 minutes. Sections were incubated with Ultra V Block (Thermo Fisher Scientific, LabVision Inc.) for 8 minutes at RT to block unspecific binding of the antibody. Primary monoclonal anti-human CXCR5/Blr-1 mouse antibody was diluted 1:50 with PBS and incubated 1 hour at RT in a humidified chamber. After washing, slides were incubated 45 minutes at RT in a humidified chamber with donkey anti-goat-IgG(H+L) TRITC conjugated secondary antibody (Jackson ImmunoResearch Europe Ltd) diluted 1:50 with PBS 3% donkey normal serum. Staining without primary antibodies was carried out as negative control. Slides were mounted with mounting medium and inspected under the microscope with a fluorescence lamp.

**CXCR5 and CXCL13 detection in primary tumors and neuroblastoma cells by immunohistochemistry**

Immunohistochemistry analysis was conducted using polymeric colorimetric technique and automated immunostainer Benchmark XT (Ventana Medical System). Sections of 5 μm from the same tumor samples were air dried and fixed (10 minutes with acetone and 5–10 seconds in methanol at RT) and methanol fixed neuroblastoma cell lines (SH-SY5Y and LA1–5S) were incubated for 30 minutes at 37°C with monoclonal anti-human CXCR5/BLR1 mouse antibody (20 μg/ml for neuroblastoma cell lines; 10 μg/ml for tumor sections) and anti-human CXCL13/BLC/BCA-1 goat antibody (0.8 μg/ml for tumor sections) (R&D Systems, Inc) and anti-human GFAP (Glial fibrillary acidic protein) polyclonal rabbit antibody (Ventana Medical System). Anti-CXCR5 antibody was detected by UltraViewRed detection kit (Ventana Medical System) based on polymeric alkaline phosphatase detection system. Staining with anti-CXCL13 antibody was detected by DAKO Cytomation, Glostrup, Denmark). Fixed cells and sections were counterstained with Gill modified hematoxylin (Ventana Medical System), dehydrated, and mounted. Staining without primary antibody was carried out as negative control. Staining of Raji cells and frozen or paraffin-embedded from lymph nodes and spleen samples was carried out as positive control.

**Glial differentiation assays**

N-type (SH-SY5Y), I-type (SK-N-BE(2)c) and S-type (LA1–5S) neuroblastoma cell lines were grown by adding 10 mmol/L 5′-Bromo-2′-deoxyuridine (BrdU) (Sigma Aldrich, Inc.) to cell culture medium to induce glial differentiation (19). Treatment was maintained for 3 weeks by renewing media three times per week. During the first and the second week of treatment, cell proliferation was not affected by the treatment and cells were split twice. Culture media of treated cells were collected every three days for 3 weeks and then concentrated from 40 ml to 200 μl with centrifugal filter devices Centricron Plus-70 (Millipore, Inc.). Total RNA was extracted from the cells after 1, 2, and 3 weeks of treatment with BrdU. Pictures of differentiated cells have been taken after 3 weeks of treatment.

**CXCL13 detection in neuroblastoma cell line conditioned medium by ELISA**

CXCL13 protein concentration was detected in neuroblastoma cell line conditioned medium by Quantikine Human CXCL13 Immunoassay (R&D Systems, Inc.), following the manufacturer’s recommendations. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody for CXCL13 was precoated onto a microplate. Human recombinant CXCL13 (hrCXCL13) standards, saliva, untreated, and BrdU differentiated neuroblastoma cells conditioned media were pipetted into the wells and CXCL13 was bound by the immobilized antibody. After washing, an enzyme-linked monoclonal antibody specific for CXCL13 was added. After incubation and extensive washing, a substrate solution was added and the amount of CXCL13 was quantified by a colorimetric measurement on a standard curve of serial dilution of hrCXCL13.

**Neuroblastoma cell migration assay**

Cell migration assays were conducted on SH-SY5Y, ACN, and LA1–5S cells in microchambers (NeuroProbes Gaithersburg) as previously described (20). Cells were extensively
washed with PBS, resuspended in serum-free medium, and placed in the upper compartment. The two compartments of the chamber were separated by 5 μm pore size polycarbonate filters coated with 0.01% gelatine. The hrCXCL13 (100 or 200 ng/ml) and concentrated serum-free conditioned medium from BrdU-differentiated neuroblastoma cells were used as chemoattractants in the lower chamber. To obtain specific neutralization of hrCXCL13 bioactivity a blocking antibody to human CXCL13/BLC/BCA-1 (R&D Systems, Inc) was added to hrCXCL13 and to BrdU-differentiated neuroblastoma cells conditioned medium in the lower chamber at 3.5 μg/ml and 0.5 μg/ml, respectively. To specifically neutralize CXCR5 receptor on neuroblastoma cell surface, cells were previously incubated 30 minutes with 15 μg/ml blocking monoclonal antihuman CXCR5/BLR1 mouse antibody (R&D Systems, Inc). After 6 hours of incubation at 37°C in 5% CO₂, the filters were recovered, the cells on the upper surface were mechanically removed, and the cells on the lower surface were fixed and stained. The migration was measured by counting the remaining cells on the filter lower surface. Experiments were done in six replicates and repeated three times.

**Results**

**CXCL13 and CXCR5b mRNA expression in whole tumor, isolated neuroblastic and Schwannian stromal cells, and neuroblastoma cell lines**

We carried out RT-qPCR of 26 tumor samples to assess the expression of CXCL13 and CXCR5b mRNA in neuroblastic tumors. We firstly observed that SP tumors showed higher levels of CXCR5b mRNA than SR tumors. Conversely, CXCL13 was more expressed in SR tumors (Fig. 1). Noteworthy, RT-qPCR analysis showed CXCR5b transcript variant (accession number 032966; ref. 21) in neuroblastic tumors. In our analysis we used primers for both CXCR5a and CXCR5b variants but only the CXCR5b transcript was detected whereas CXCR5a was not detectable in any samples (data not shown).

Because neuroblastic tumors show tissue heterogeneity and nonmalignant cells may infiltrate the tumor, we isolated either neuroblastic or Schwannian stromal cells from 6 neuroblastic tumors (3 SP and 3 SR) by laser capture microdissection. Next, we extracted total RNA from both purified cell populations and we carried out RT-qPCR for

![Figure 1. CXCL13 and CXCR5b expression in tumors. CXCL13 and CXCR5b expression by RT-qPCR in 26 Neuroblastic tumors: 13 SP and 13 SR tumors. Left, the level of CXCL13 and CXCR5 in SP and SR are shown, whereas right, shows the box plot representation; P value is reported. Most of SR tumors express CXCL13 and very few express CXCR5; on the contrary, SP tumors express CXCR5 at various amount. CXCL13 is very low or undetectable expressed in SP tumors. (MNE, Mean normalized expression).](image)

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CXCL13 and CXCR5b. We observed expression of CXCL13 in the stromal cell components, as opposed to very low or undetectable levels observed in isolated neuroblastic cells (Fig. 2), supporting our results obtained by whole tumors analysis. On the contrary, we definitely detected CXCR5b expression in the isolated neuroblastic cells but not in stromal cells (Fig. 2). Afterward, we investigated the expression of CXCL13 and CXCR5b mRNA in 11 neuroblastoma cell lines and we detected a variable expression of CXCL13 and CXCR5b. Among neuroblastoma cell lines, 3/11 (27%) express CXCL13 (GI-LI-N, IMR5, SK-N-SH) and 9/11 (82%) express CXCR5b (GI-LI-N, GI-ME-N, IMR-32, IMR5, LA-1–5S, SH-SY5Y, SK-N-BE(2), SK-N-BE(2)c, SK-N-SH). GI-LI-N, IMR5, SK-N-SH cells express both CXCL13 and CXCR5b and ACN and GI-CA-N cells do not express either CXCL13 or CXCR5b (data not shown).

CXCR5 and CXCL13 protein expression in neuroblastic tumors and neuroblastoma cell lines

To show that Schwannian stromal cells of SR tumors express CXCL13 but not CXCR5 we carried out immunohistochemistry analysis on tumor tissue. As shown in Fig. 3C, Schwannian stromal cells strongly express CXCL13, whereas CXCR5 is not expressed in the same tissue (Fig. 3D). Because CXCR5b mRNA was found variable expressed we employed immunofluorescence technique to have a better sensibility to detect CXCR5 expression. CXCR5 protein was observed in neuroblastic cells (Fig. 3E and 3F), but not in SR tumors (data not shown).

Next, we analyzed SH-SY5Y and LA1–5S neuroblastoma cell lines by either immunofluorescence (Fig. 3G and 3H) or immunohistochemistry (Fig. 3I and Fig. 3J) and we observed a mild surface expression of CXCR5 receptor in about 20% of SH-SY5Y and 30% of LA1–5S neuroblastoma cells, respectively (Fig. 3G and Fig. 3H). Immunofluorescence analysis also showed some cytoplasmic staining, possibly related to a CXCR5 internalization. Immunofluorescence analysis was also carried out in the other neuroblastoma cell lines with similar results and we confirmed CXCR5 expression in SK-N-BE(2), SK-N-BE(2)c and SK-N-SH cells by FACS analysis (data not shown). Although IMR-5, SK-N-SH and GI-LI-N, expressed CXCL13 mRNA, only IMR-5 showed CXCL13 cytoplasmatic expression but the secreted protein was not detected (data not shown).

Induction of neuroblastoma cells to glial differentiation and CXCL13 production

Human neuroblastoma cell lines retain a bilineage potential and may differentiate toward a neuroblast or stromal/glial phenotype on different conditions (19). Because secreted CXCL13 was not detectable by ELISA in the conditioned medium of any neuroblastoma cell line (data not shown), we treated SH-SY5Y, SK-N-BE(2)c and LA1–5S cells with BrdU for 3 weeks to evaluate whether neuroblastoma cells produce CXCL13 after glial differentiation induction. Following BrdU treatment most of SH-SY5Y and SK-N-BE(2)c cells acquired an evident flat, large and more adherent cell morphology resembling Schwannian stromal cells (Fig. 4A). A morphologic change was also observed in LA1–5S cells at a less extent. The induction towards glial cell lineage was shown by increase of S100A6 mRNA expression in all 3 cell lines (Fig. 4A). In addition, a variable de novo CXCL13 mRNA induction was detected in neuroblastoma cell lines reaching a maximum at 3 weeks in BrdU-differentiated SK-N-BE(2)c cells (Fig. 4B). The secretion of CXCL13 protein in the culture medium was remarkable in differentiated SK-N-BE(2)c and LA1–5S (Fig. 4C).

Triggering of neuroblastoma cells migration by CXCL13

We tested whether the expression of CXCR5 could be sufficient to trigger neuroblastoma cell migration in response to CXCL13 stimulation by chemotaxis assays. Figure 5A shows that the CXCR5-positive SH-SY5Y and LA1–5S cells significantly migrated in presence of hrCXCL13 chemokine, whereas the CXRC5-negative ACN cells failed to migrate (P = not significant, data not shown). LA1–5S cell migration was inhibited by either blocking the receptor or the chemokine activity, following addition of specific antibodies against CXCR5 or hrCXCL13 (Fig. 5B). LA1–5S cell migration was also observed when the conditioned medium of neuroblastoma cells differentiated to the glial cell lineage were used as chemotaxants, thus further substantiating the possibility that cell-released CXCL13 induces neuroblastoma cell migration (Fig. 5C). This result shows that neuroblastoma cells induced to glial cell lineage and acquiring a Schwannian-like morphology secrete CXCL13 that stimulates chemotaxis

Figure 2. CXCL13 and CXCR5b expression in isolated neuroblastic and Schwannian stromal cells. CXCL13 and CXCR5b expression by RT-qPCR in neuroblastic (Nb) and Schwannian stromal (SS) cell populations isolated by Laser Capture Microdissection. Isolated SS express CXCL13 that is almost undetectable in Nb cells. The receptor was found expressed in isolated neuroblastic cells of 2750_Nb sample and at very low levels in the remaining Nb samples but not detected in SS cells. (MNE, Mean normalized expression).
in neuroblasts expressing CXCR5. Moreover, migration of LA1–5S neuroblastoma cells was significantly suppressed by the addition of a neutralizing anti-CXCL13 antibody. On the contrary, CXCR5-negative ACN cells showed a more limited migration than LA1–5S in response to the conditioned medium of Schwannian-like cells and their migration was not inhibited by anti-CXCL13 antibody (Fig. 5C).
Chemokines play several roles in both physiologic and pathologic conditions. Hereby, we show that CXCL13 and its receptor CXCR5 are involved in the relation between neuroblastic and Schwannian stromal cells of neuroblastic tumors, a heterogeneous group of pediatric cancers. Several chemokines have been found expressed in neuroblastoma; in particular, the role of the CXCR4-CXCL12 axis in neuroblastoma cells proliferation, survival, and bone marrow dissemination is still controversial (13, 14, 22). Airoldi and colleagues (15) showed that neuroblastoma cells express CXCR5 and they hypothesized that malignant cells are able to migrate even in the bone marrow in response to CXCL13 stimulation.

We confirm that neuroblastoma cells express CXCR5 receptor and we add a detailed analysis of CXCL13 and CXCR5 expression in neuroblastic tumors. Our results show that only malignant neuroblasts express CXCR5 protein and we show, for the first time, that CXCL13 is also secreted by Schwannian stromal cells of neuroblastic tumors.

Neuroblastic tumors stroma-rich tumors are usually composed of abundant stroma and less amount of neuroblastic cells; these tumors onset as a localized mass and rarely show metastatic disease. Patients with stroma-rich tumor are at low risk of relapse after complete surgical tumor resection and they usually have a good outcome. Because we observed that CXCL13 is expressed by Schwannian stromal cells, and neuroblastic cells express CXCR5 we hypothesize that CXCL13-CXCR5 axis may contribute to retain malignant CXCR5-positive neuroblasts in stroma-rich tumors limiting their metastatic spreading. Such hypothesis is strongly supported by the ability of neuroblastoma cells to migrate toward a source of hrCXCL13. Moreover, our data indicate that the CXCR5b mRNA variant encodes for a functionally active CXCR5b protein, which is capable to specifically respond to the CXCL13 stimulus.

The role of Schwannian stromal cells in neuroblastic tumors has been widely debated. Ambros and colleagues (4) suggested that these cells secrete factors that limit the proliferation of malignant neuroblasts and Chienski and...
colleagues (7) indicated that Schwannian stromal cells release other factors that modulate angiogenesis. Here, we show that CXCL13 is produced by Schwannian stromal cells adding new information about the factors released by stroma of neuroblastic tumors and we suggest a relevant role of the CXCL13/CXCR5 axis in the crosstalk between neuroblasts and stromal cells. The novel discovery that Schwannian stromal cells express CXCL13 supports the previous findings that cells of neural origin are able to produce CXCL13 (23, 24).

As an in vitro model to test the possible involvement of CXCL13 in the crosstalk between malignant neuroblasts and Schwannian stromal cells we induced glial cell differentiation of neuroblastoma cells. Neuroblastoma cell lines treated with BrdU induce immature neuroblasts to a more mature phenotype with a large and subadherent Schwannian-like morphology (19). After BrdU treatment, neuroblastoma cells stop to proliferate and produce calcyclin (25), a glial, and Schwannian cells marker. We show for the first time, that neuroblastoma cells, which possess a bilineage potential (19), express CXCL13 only after induction to a glial phenotype. Furthermore, we showed that LA1–5S neuroblastoma cells are able to migrate under the effect of the conditioned medium of BrdU differentiated neuroblastoma cells and that this effect is blocked by CXCL13-neutralizing antibodies. Overall data indicate that differentiated neuroblasts phenotypically similar to Schwannian stromal cell are able to secrete CXCL13 and to exert a chemotactic effect on CXCR5-positive neuroblastoma cells, whereas CXCR5-negative neuroblastoma cells showed limited migration possible due to a slight effect of other chemotactic factors produced by Schwannian-like cells. Collectively, our results strongly indicate a close relation between Schwannian stromal cells and neuroblastic cells mediated via a CXCL13–CXCR5 interaction. This observation is enforced by the finding that anti-CXCL13 antibody significantly inhibits the migration of CXCR5-positive neuroblastoma cells in response to the conditioned medium. Hence, in addition to the other factors that exert their effects on neuroblast survival and differentiation, and on tumor angiogenesis (4, 6, 7), our finding that Schwannian-like cells are able to secrete a chemotactic factor acting on neuroblastic cell migration adds insight on a broad role of Schwannian stromal cells in stroma-rich neuroblastic tumors.

In conclusion, our model suggests that CXCL13 produced by Schwannian stroma may contribute to limit dissemination of CXCR5-expressing neuroblasts outside the SR

![Figure 5](attachment:image_url)

**Figure 5.** CXCL-13 mediates neuroblastoma cells migration. A shows the number of migrating SH-SY5Y and LA1–5S neuroblastoma cells after 6 hours of incubation at two different doses (100 ng/ml, 200 ng/ml) of hrCXCL13 added in the lower chamber. A low number of cells migrate in the presence of serum free medium (SFM). B shows that CXCR5 positive LA1–5S migration is specifically mediated by the CXCR5/CXCL13 interaction by inhibiting both the receptor and the chemokine with anti-CXCR5 (15 ng/ml) and anti-CXCL13 (3.5 ng/ml) blocking antibody, respectively. C shows CXCR5-positive LA1–5S and CXCR5-negative ACN neuroblastoma cells chemotaxis in the presence of concentrated serum-free conditioned-medium of BrdU-differentiated neuroblastoma cells secreting CXCL13. Migration of LA1–5S cells is significantly reduced by anti-CXCL13 (0.5 ng/ml) blocking antibody added to the conditioned medium. ACN cells showed limited migration that was not influenced by anti-CXCL13 antibody. Statistical analysis was carried out by unpaired two-tailed t-test. (X-axis shows the different chemoattractants, y-axis the number of cells migrated/field. ns: not significant).
tumors, whereas CXCR5-positive neuroblastic cells of stroma-poor tumors more easily disseminate to CXCL13-producing distal sites, such as the bone marrow. Although we cannot exclude the influence of CXCL13 released from distal bone marrow sites on neuroblastoma cells, this effect appears unlikely in the case of localized tumors. Indeed, very low concentrations of CXCL13 are usually present in the systemic circulation, whereas the Schwannian stromal cells and malignant neuroblasts are in close contact thus facilitating local CXCL13-CXCR5 interaction. Obviously, this model cannot be the only reason for the different biological and clinical behavior of SR and SP tumors, which is predominantly related to the different genetic and molecular characteristics observed in localized and metastatic tumors (26).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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