Chemokine receptor signals: role in migration, invasion and cancer metastasis
Zeelenberg, I.S.

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Chapter 7

A putative role for the chemokine receptor CXCR5 in tumor growth of colon carcinoma cells.

Preliminary report
A putative role for the chemokine receptor CXCR5 in tumor growth of colon carcinoma cells

I.S. Zeelenberg, J. Meijer, E. Roos

Chemokines regulate leukocyte trafficking in inflammation and lymphoid organs. The chemokine receptor CXCR5 and its ligand CXCL13 (BCA-1/BLC) are involved in B cell trafficking and function. Recently, chemokine receptors have been shown to play a role in metastasis of lymphomas and carcinomas. CXCR5 is expressed on certain lymphomas, but expression on carcinomas has not been described. We show here that the mouse colon carcinoma cell line CT-26 expresses CXCR5. Expression is very low in vitro, but is markedly upregulated in vivo. We show that its ligand, CXCL13, can enhance proliferation of CXCR5 expressing CT-26 cells. Moreover, CXCL13 is expressed at the sites of metastasis. This study suggests a role for CXCR5 in the outgrowth of colon carcinoma metastases.

Introduction
Chemokines are small proteins that regulate leukocyte trafficking. They are found predominantly in inflamed tissues, but some are expressed constitutively. The chemokine CXCL13 (BCA-1/BLC), the only known ligand for the receptor CXCR5, is constitutively expressed in secondary lymphoid organs and in the liver. CXCR5 is mainly expressed on peripheral blood B cells, but also on a small subset of memory T cells. CXCL13 is expressed in the B cell areas in secondary lymphoid organs to attract B cells to this site where they mature. The CXCR5-positive T-helper cells are also attracted to this area to provide B-cell help. Mice lacking CXCR5 have a severe defect in normal B cell migration and localization. The mice have no inguinal lymph nodes and they lack germinal centers in the spleen.

Recently, chemokine receptors were shown to play an important role in the metastasis of tumors. CXCR4 is essential for the metastasis of breast carcinomas, colon carcinomas and lymphomas, and has been implicated in metastasis of melanomas and many other tumors. CCR7 has been implicated in the metastasis to lymph nodes. Also other chemokine receptors were shown to be expressed on tumor cells, but their role is less clear. CXCR5 is mainly expressed on certain lymphomas, such as Burkitt's lymphoma, cutaneous B cell lymphoma, gastric lymphoma and classical Hodgkin disease. However, CXCR5 was so far not described to be expressed by carcinomas.

It was proposed that chemokine receptors are involved in invasion of tissues by the tumor cells. This seemed an obvious explanation since the tumor cells often express high levels of chemokine receptors and migrate in response to chemokines. We showed for a T cell lymphoma that this was indeed the case, since blocking of CXCR4 resulted in greatly reduced invasion as well as a virtually complete inhibition of metastasis. Next, we showed that CXCR4 is also essential for metastasis of the mouse CT-26 colon carcinoma cell line. However, in that tumor CXCR4 cannot play a role in invasion. Firstly, metastasis was not blocked by stable expression of the catalytic subunit of pertussis toxin, which completely blocked G proteins, necessary for CXCR4-induced migration. Secondly, CXCR4 levels were low in vitro, but strongly upregulated in vivo and only several days after lung colonization. Finally, we showed that the CXCR4-deficient cells did colonize the lungs, but did not grow out, suggesting a role in growth and survival, rather than in invasion.

CXCR5 has only been implicated in metastasis of lymphomas, which is expected since CXCR5 is primarily expressed on B and T lymphocytes from which these malignancies originate. Remarkably, however, we found that CXCR5 is also expressed by the CT-26 cells. Similarly as we found for CXCR4, the CXCR5 levels were low in vitro, but strongly upregulated in vivo. Furthermore, the ligand CXCL13 is abundantly present in the metastases. We show that CXCL13 can promote proliferation of CXCR5-expressing colon carcinoma cells. Whether CXCR5 plays an important role in the metastasis of the colon carcinoma cell line remains to be established, but these results suggest that, similarly as CXCR4, it may promote the outgrowth of the metastases.
Materials and methods

Cell Culture. CT-26 colon carcinoma cells19 were kindly provided by Dr. I.J. Fidler (MD Anderson Cancer Center, University of Texas, Houston, TX, USA) and cultured in DMEM (Life Technologies, Ltd., Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies, Ltd.), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies, Ltd.). CT-26 transfectants were cultured in the same medium supplemented with zeocin (Invitrogen, Carlsbad, CA) or puromycin (Sigma-Aldrich, St. Louis, MO, USA). The virus-packaging cell line FNXE20 was cultured in DMEM medium (Life Technologies, Ltd.) supplemented with 100 IU/ml of penicillin, 100 μg/ml of streptomycin (Life Technologies, Ltd.), 10% FCS (Life Technologies, Ltd.) and 0.584 g/l L-glutamine (Life Technologies, Ltd.).

Generation and transduction of DNA constructs. The CXCR5 construct was generated by RT-PCR using RNA isolated from a human B cell line and sequenced to verify that no mutations had been introduced by the PCR. The construct was cloned into the retroviral vector pLZRS-IRES-Zeo.20 The vector was transfected by calcium-phosphate precipitation into the virus-packaging cell line FNXE. After 8 h, the medium was refreshed, and 48 h later the virus supernatant was collected and used to infect the CT-26 cells. Three days later, zeocin was added to select cells that had incorporated the cDNA.

To obtain cells with high EGFP levels, a retroviral vector pLZRS-IRES-puroEGFP was used. This vector was based on the pLZRS-IRES-zeo vector, in which the zeocin resistance cDNA was replaced by a cDNA encoding a puromycin resistance-enhanced green fluorescence protein (EGFP) fusion protein.20 CT-26 cells were transduced as described above and FACS-sorted to select a bulk population with high EGFP expression.

Flow cytometry. To assess CXCR5 levels, cultures were trypsinized and the resulting single-cell suspension was incubated for 45 min with 20 μl of 1:100 diluted anti-CXCR5 antibody (R&D systems, Minneapolis, MN, USA) or undiluted phycoerythrin (PE)- conjugated CXCR5 antibody (R&D systems). The cells were washed three times and either used directly (PE-anti-CXCR5) or incubated for another 45 min with phycoerythrin (PE)- conjugated anti-rat antibodies. The cells were washed again and resuspended in PBS. The analysis was performed on a Becton Dickinson FACSCalibur using CellQuest software.

Migration. Transwells with 8 μm pores were coated overnight with 1 μg/ml of fibronectin (Invitrogen, Paisley, United Kingdom) and then treated for 1 h with 1% BSA at room temperature. The lower chamber was filled with 250 μl DMEM supplemented with 1% FCS, 0.1% BSA and different concentrations of CXCL13 ( PeproTech Inc., Rocky Hill, NJ, USA). Cells were trypsinized, washed and 1 x10^6 cells were added in DMEM with 1% FCS and 0.1% BSA to the upper chamber of the transwell. After incubation for 6 h at 37°C and 5% CO2, the Transwell was removed and the top of the filter was wiped clean with a cotton tip, wetted in PBS. The cells on the bottom of the filter were fixed with 75% methanol/25% acidic acid for 20 min, stained with 0.25% Coomassie blue in 45% methanol/10% acidic acid for 30 min and counted. In some cases, cells were pretreated overnight with 200 ng/ml of the G inhibitory pertussis toxin (List Biological Laboratory, Campbell, CA, USA) prior to using them in the migration assay.

Proliferation assay. CT-26 cells expressing CXCR5 (5 x 10^5) were seeded in several 96-well plates in normal DMEM medium supplemented with 10% FCS or keratinocyte medium (Life Technologies, Ltd.) with 1% FCS, in both cases with or without 100 ng/ml CXCL13. Medium and chemokine were refreshed daily. Every day one plate was used to determine the number of cells by assaying hexosaminidase activity, using known numbers of cells as standard.22 In some cases, 200 ng/ml of the G inhibitory pertussis toxin (List Biological Laboratory) was added to the medium during the assay and refreshed daily.

Metastasis assay and isolation of tumor cells. CT-26 cells were washed, and 5 x 10^5 cells were suspended in 1 ml of PBS. Syngeneic 3-4 month-old BALB/c mice were injected with 0.2 ml of the cell suspension into a tail vein or 0.1 ml into the spleen. The mice were killed when moribund. The spleen, liver and lungs were isolated and cut into small pieces. The pieces were incubated for 45 min at 37°C in DMEM containing 0.25% collagenase ( Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.1% hyaluronidase (Sigma-Aldrich), and gently teased apart to obtain a single-cell suspension. The CXCR5 surface levels on the tumor cells were
measured by FACS analysis using a PE-labeled anti-CXCR5 antibody, as described above. To isolate cells in early stages of metastasis formation, a larger number of CT-26 cells expressing GFP was injected into a tail vein of the mice, i.e. $2 \times 10^6$ instead of $1 \times 10^5$ cells. A single cell suspension was made from the metastases, as described above. Tumor cells were isolated by FACS sorting, based on forward and side scatter and on GFP fluorescence. The sorted CT-26 cells were incubated with a PE-labeled CXCR5 antibody and analyzed by FACS analysis, as described above.

RT-PCR. CT-26 cells ($10^6$) were injected into a tail vein, and mice were killed at different time points, several days thereafter. The lungs of the mice were homogenized in RNAzol (Tel-Test, Inc., Friendswood, TX), and total RNA was extracted as described by the manufacturers. The RT-PCR was performed with 2% of the total RNA using the One Step RT-PCR kit (Qiagen, Hilden, Germany) and primers for CXCL13. The PCR products obtained were resolved by electrophoresis on a 1.75% agarose gel and viewed after ethidium bromide staining.

**Results**

**CXCR5 levels are upregulated in vivo.** Using RT-PCR, we found that CT-26 murine colon carcinoma cells express CXCR5 (data not shown). However, FACS analysis showed that CXCR5 surface levels were exceedingly low, and most cells were essentially CXCR5-negative (Fig. 1A), similarly as described before for CXCR4. CT-26 cells were injected into a tail vein or into the spleen of syngeneic mice. Similarly as observed for CXCR4, CXCR5 was strongly upregulated in vivo in the liver and lung metastases and also in the tumors formed in the spleen (Fig.1A). The high level of CXCR5 was not due to selection of a minor subpopulation with high constitutive CXCR5 expression, because CXCR5 expression was lost on most cells after 1 day of ex vivo culture (Fig. 1B). Virtually all CXCR5 expression was lost after 4 days of ex vivo culture. Thus, it seems likely that CXCR5 expression is induced and maintained by the in vivo microenvironment, possibly by factors produced by stromal cells. To investigate when CXCR5 was upregulated, we generated CT-26 cells expressing GFP by transduction of the retroviral vector pLZR5-IRES-puro-EGFP. Cells with high GFP levels were selected by FACS-sorting. We injected $2 \times 10^6$ CT-26-GFP cells into a tail vein of syngeneic mice and excised the lungs after 2 or 7 days. The small number of carcinoma cells was isolated from the resulting cell population by FACS sorting, based on forward and side scatter characteristics and GFP fluorescence. FACS analysis showed that CXCR5 was already upregulated after 2 days (Fig. 1C). After 7 days, virtually all cells were CXCR5-positive. If CXCR5 is involved in tumor metastasis, it seems unlikely that CXCR5 is involved in the initial colonization of the lungs by the i.v. injected cells, because these cells do not express CXCR5.

![Fig. 1](image.png)
Generation of CXCR5-expressing CT-26 cells. To investigate a possible role of CXCR5 in relevant functions in vitro, we stably expressed CXCR5 in the CT-26 cells. CXCR5 was introduced into CT-26 cells using the retroviral vector pLZRS-IRES-Zeo. Zeocin-resistant cells were checked for CXCR5 surface expression by FACS analysis (Fig. 2A). All cells expressed high levels of CXCR5 comparable to the expression levels they attain in vivo. To test the functionality of this CXCR5 expression, a migration assay was performed. The CXCR5-expressing CT-26 cells migrated specifically towards the ligand CXCL13, showing that the transfected CXCR5 is intact and functional (Fig. 2B). Pertussis toxin blocks G_{i} proteins, which are necessary for the migration and invasion of chemokines. The possible exception is migration towards CXCL13 which has been described to be insensitive to pertussis toxin, at least in certain cell types.\textsuperscript{23} Indeed, pretreatment of pertussis toxin did not inhibit migration of the CXCR5 transfectants towards CXCL13 (Fig. 2B).

CXCL13 enhances proliferation of CXCR5-expressing CT-26 cells. Since many CXC chemokines were originally identified as growth factors, we wondered whether CXCL13 could also have an enhancing effect on proliferation. The effect of CXCL13 on growth of the CXCR5-expressing CT-26 cells was tested under normal culture conditions and in more restricted medium. Surprisingly, addition of CXCL13 markedly enhanced the growth of the transfectants even in normal culture medium containing 10\% FCS (D10F; Fig. 3A). This effect was even more evident in restricted growth medium, i.e. keratinocyte medium with 1\% FCS (K1F; Fig. 3B). To investigate the involvement of G_{i} proteins in proliferation,
pertussis toxin was added to the cells during the proliferation assay. Treatment of the cells with pertussis toxin did not affect the proliferation-enhancing effect of CXCL13 as shown for the normal culture medium in Fig. 3C. The effect in restricted growth medium was similar (data not shown). However, also in the absence of CXCL13, pertussis toxin treatment resulted in an increase in the proliferation of the CXCR5 transfectants showing that proliferation of CT-26 cells is attenuated by factors acting through a Gi-coupled receptor.

**CXCL13 is expressed at the sites of metastasis.** CXCL13 is known to be expressed in the liver, which is the major site of metastasis after spleen injection. However, in the lungs, the major site of metastasis after tail vein injection, CXCL13 is normally hardly expressed (Fig. 4). We investigated whether CXCL13 is upregulated in the lungs after invasion of the tumor cells. CT-26 cells were injected into a tail vein and the mice were killed at different time points, several days after injection. The lungs were isolated and RNA was extracted. An RT-PCR on the total RNA of the lungs revealed that CXCL13 levels were indeed upregulated at the site of metastasis even the first day after injection (Fig. 4). After 3 weeks the amount of CXCL13 is even higher. Previously, we found that between 50 and 500 CT-26 cells have survived in the lungs, 24 h after tail vein injection of 10⁵ cells. An unlikely possibility was that the CXCL13 detected in the lungs at this time point, was autocrine CXCL13, produced by these tumor cells. We therefore performed an RT-PCR using RNA isolated from 1000 CT-26 cells, but no CXCL13 expression was detected (Fig. 4). Thus, it is more likely that the CXCL13 is produced by other cells, apparently in reaction to the influx of the tumor cells.

**Discussion**

Using RT-PCR, we made the surprising discovery that CT-26 colon carcinoma cells express CXCR5, a chemokine receptor that was hitherto only known to be expressed by B cells and certain T cells. FACS-analysis then showed that the surface levels were exceedingly low and most cells did not express CXCR5 at all. Strikingly, however, CXCR5 was strongly upregulated in vivo. We found that CXCL13 can enhance proliferation in CXCR5-expressing CT-26 colon carcinoma cells. Moreover, we show that CXCL13 is expressed at the sites of metastasis. These results strongly suggest a role for CXCL13 and CXCR5 in the outgrowth of metastases.

Migration towards chemokines is normally blocked by pertussis toxin, an inhibitor of G proteins. Treatment of B cells with pertussis toxin, which blocks G proteins, inhibits the chemotaxis towards CXCL13. However, chemotaxis and Ca²⁺ signaling of CXCR5-transfected HEK293 cells appears to be pertussis toxin-insensitive, whereas Erk activation is G protein-dependent. Indeed, migration towards CXCL13 of the CXCR5-expressing CT-26 cells was also found to be pertussis toxin-insensitive. This indicates that G-protein coupling can differ between cell types. The metastasis of the CT-26 colon carcinoma cell line is not dependent on G proteins. Since migration and therefore probably also invasion towards CXCL13 is not dependent on G proteins, it was conceivable that CXCR5 plays a role in the invasion of the CT-26 cells into the target sites of metastasis. However, in vitro CT-26 cells do not express CXCR5 or only at very low levels, so that CXCR5 can not participate in invasion shortly after injection, as is particularly evident for lung metastasis after tail vein injection. CXCR5 is upregulated later in the process of metastasis, after two days and more extensively after seven days, suggesting a role after invasion. Another possibility was that variant cells with constitutive CXCR5 expression were selected during the process. If so, the cells should maintain expression in vitro. However, the cells rapidly lose CXCR5 after a few days in culture, which argues strongly against this possibility. The most likely explanation is, similarly as previously postulated for CXCR4, that signals from the microenvironment induce the expression of CXCR5 which is rapidly lost in the absence of these signals in vitro.

The effect of CXCL13 on proliferation is remarkable but perhaps not surprising, since
many chemokines can also act as growth factors. Four CXC chemokines, CXCL1, CXCL2, CXCL3 and CXCL12 were actually originally identified as growth factors.\(^{24,25}\) However, for CXCL13 this has not been described before. The effect was very strong since even in medium containing 10% FCS, CXCL13 enhanced the proliferation of the CT-26 cells. This effect can be very important for metastasis, since CXCL13 is present at the sites of metastasis. This is true for the liver, which is known to contain CXCL13\(^{34}\), but we found CXCL13 also in the lung metastases. It remains to be established whether CXCL13 is produced by the CT-26 cells, or provided by stromal cells. We previously estimated that 50 to 500 tumor cells are present in the lungs 1 day after i.v. injection of 10\(^5\) cells.\(^{11}\) The RT-PCR in Figure 4 shows that no CXCL13 is detectable in 1000 CT-26 cells. Therefore, it is more likely that the CXCL13 is produced by the stromal cells. It should be noted that growth factors are likely to be scarce in normal lung or liver, which is the environment in which the single metastasized tumor cell finds itself, after the initial invasion of the tissue. After induction of CXCR5, which occurs within two days, the autocrine or paracrine CXCL13 is likely to contribute to metastasis formation. This remains to be demonstrated.

Pertussis toxin did not inhibit the proliferation-enhancing effect of CXCL13, suggesting that proliferation of CXCL13 is not G-protein-dependent. This is important since CT-26 cells, stably expressing the catalytic subunit of pertussis toxin, can still metastasize.\(^{11}\) However, we found that pertussis toxin by itself enhanced the proliferation of CT-26 cells in vitro. This indicates that a growth-inhibitory factor is present in the medium, or perhaps produced by the CT-26 cells themselves, which acts, at least in part, via a G\(_i\) protein-coupled receptor. The factor needs to be identified before we can judge its possible role in vivo.

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References


