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

Citation for published version (APA):
Chapter 6

The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases

The Chemokine Receptor CXCR4 Is Required for Outgrowth of Colon Carcinoma Micrometastases

Ingrid S. Zeelenberg, Lisette Ruuls-Van Stalle, and Ed Roos

Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, the Netherlands

ABSTRACT

CXCR4, the receptor for the chemokine stromal-cell-derived factor (SDF)-1 (CXCL12), is involved in lymphocyte trafficking. We have demonstrated previously that it is required for invasion of lymphoma cells into tissues and therefore essential for lymphoma metastasis. CXCR4 is also expressed by carcinoma cells, and CXCR4 antibodies were recently shown to reduce metastases of a mammary carcinoma cell line. This was also ascribed to impaired invasion. We have blocked CXCR4 function in CT-26 colon carcinoma cells by transfection of SDF-1, extended with a KDEL sequence. The SDF-KDEL protein is retained in the endoplasmic reticulum by the KDEL-receptor and binds CXCR4, which is thus prevented from reaching the cell surface. We found that metastases of these cells to liver and lungs was greatly reduced and often completely blocked. Surprisingly, however, our observations indicate that this was not attributable to inhibition of invasion but rather to impairment of outgrowth of micrometastases: (a) in contrast to the lymphoma cells, metastasis was not affected by the transfected S1 subunit of pertussis toxin. S1 completely inhibited GI protein signaling, which is required for SDF-1-induced invasion; (b) CXCR4 levels were very low in CT-26 cells grown in vitro but strongly up-regulated in vivo. Strong up-regulation was not seen in the lungs until 7 days after tail vein injection. CXCR4 can thus have no role in initial invasion in the lungs; and (c) CXCR4-deficient cells did colonize the lungs to the same extent as control cells and survived. However, they did not expand, whereas control cells proliferated rapidly after a lag period of ≥7 days. We conclude that CXCR4 is up-regulated by the microenvironment and that isolated metastatic cells are likely to require CXCR4 signals to initiate proliferation. Our results suggest that CXCR4 inhibitors have potential as anticancer agents to suppress outgrowth of micrometastases.

INTRODUCTION

Chemokines are small proteins that regulate leukocyte trafficking. They are found predominantly in inflamed tissues, but some are expressed constitutively (1). Prominent among the latter is CXCL12, also known as SDF-1, which is present in virtually all tissues (2,3). It is a potent chemotractant for many leukocyte subtypes (4,5). Its receptor is CXCR4 (6), and migration signaling depends on GI protein, which can be blocked by pertussis toxin (7). The influx of leukocytes into tissues has similarities with the invasion of tissues by metastasizing tumor cells, suggesting a role for chemokine receptors in metastasis, in particular of hematopoietic tumors.

To investigate the role of CXCR4 in the dissemination of lymphomas, we have used previously an "intrakinase approach" (3). This method was originally proposed as gene therapy for AIDS (8), because certain HIV strains use CXCR4 as an essential coreceptor for infection (6, 9, 10). The ligand of CXCR4, SDF-1 (CXCL12), is extended with a KDEL sequence, which results in binding to the KDEL-receptor. The function of this receptor is to retain resident ER proteins in the endoplasmic reticulum. Transfected SDF-KDEL, retained in the ER, binds newly synthesized CXCR4, which is therefore prevented from reaching the surface, so that it cannot relay signals by extracellular SDF-1. Using a T-cell hybridoma as a model lymphoma, we thus showed that CXCR4 is essential for metastasis (3). This was in line with the effect of pertussis toxin, which inactivates GI proteins. Cells transfected with the S1 catalytic subunit of this toxin also lost metastatic capacity (11, 12). In vitro, pertussis toxin blocked CXCR4-dependent invasion as well as chemotaxis toward SDF-1. This indicates that the main role of SDF-1 (CXCL12) and CXCR4 in lymphoma metastasis is to induce invasion into the tissues.

Chemokine receptors are not only expressed by leukocytes but also by epithelial cells (13) and several types of carcinomas (14-17). Recently, CXCR4 antibodies were shown to reduce metastasis of a breast carcinoma cell line, suggesting that also for carcinomas, CXCR4 is essential for invasion into tissues (15). We have studied the involvement of CXCR4 in metastasis for a colon carcinoma cell line using the same approaches as applied to the lymphoma cells. Surface expression of CXCR4 was blocked using the intrakine approach, and GI protein function was inhibited by transfecting the catalytic subunit S1 of pertussis toxin. Our results indicate that CXCR4 is indeed essential for metastasis formation but, interestingly, not for invasion into the tissues. Rather, CXCR4 appears to be crucial for the outgrowth of single cells or small micrometastases. This is in line with accumulating evidence that SDF-1 (CXCL12) is a survival factor for many cell types (18-24). Our results suggest that inhibition of CXCR4 may be used therapeutically to suppress outgrowth of micrometastases.

MATERIALS and METHODS

Cell Culture. CT-26 colon carcinoma cells (25) were kindly provided by Dr. I. J. Fidler and cultured in DMEM (Life Technologies, Ltd., Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies, Ltd.). CT-26 transfectants were cultured in the same medium supplemented with either zoein (Invitrogen, Carlsbad, CA) or hygromycin (Calbiochem-Novabiochem Corp., La Jolla, CA).

Generation and Transduction of DNA Constructs. The S1 construct encoding the catalytic subunit of pertussis toxin was cloned into the retroviral vector pLZR5-ires-Zeo as described (12). The SDF-KDEL construct was generated by PCR and cloned into the retroviral vector pLZR-Hyg-EFGP as described (3).

Western Blotting. SDS-PAGE-separated cell lysates were blooted to nitrocellulose, which was then blocked with 1% BSA and 3% nonfat dried milk. The membrane was incubated for 1 h with the mouse 151C1 monoclonal antibody against S1 (26), followed by sheep antihorseparadish peroxidase-coupled immunoglobulin (Amersham Life Sciences, Little Chalfont, England). Stained proteins were visualized by enhanced chemiluminescence (ECL kit; Amersham).

ADP-Ribosylation Assay. To determine whether the GI protein had been ADP-ribosylated by the endogenously produced S1 protein, membranes of the CT-26 cells and transfectants were obtained, and an ADP-ribosylation assay was performed as described (11, 27).

Metastasis. CT-26 cells or transfectants were washed, and 5 × 10^7 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 liver. The animals were sacrificed 7 days after injection, and lung and liver were excised and fixed in formaldehyde to examine for metastases.

Received 10/1/11, accepted 4/8/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supporting information available online at www.jem.org (12 pages).

1. The abbreviations used are: SDF, stromal-cell-derived factor; FACS, fluorescence-activated cell sorter; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescence protein; RT-PCR, reverse transcription-PCR.

2. To whom requests for reprints should be addressed. Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX Amsterdam, the Netherlands. Phone: 31-20-5121931; Fax: 31-20-5121944. E-mail: e.roos@nki.nl.

3. The abbreviations used are: SDF, stromal-cell-derived factor; FACS, fluorescence-activated cell sorter; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescence protein; RT-PCR, reverse transcription-PCR.
spleen. Autopsies were performed when animals were moribund or after 6 weeks and examined macroscopically and microscopically for the presence of metastases. These experiments were approved by the institute's Animal Welfare Committee.

Flow Cytometry, CXCR4 on cells was stained with the phycoerythrin-labeled 12G5 monoclonal antibody (PharMingen, San Jose, CA) and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

RT-PCR. Control and SDF-KDEL-transduced CT-26 cells (10^6) were injected into a tail vein, and two mice were killed after 24 h and at 3-day intervals thereafter. The lungs of both mice were divided into two parts, which were separated by homogenized into RNAsol (Tel-Test, Inc., Friendswood, TX), and total RNA was extracted as described by the manufacturers. The RT-PCR was performed with 4% of the total RNA (i.e., 2% of the RNA from the complete lungs), using the One Step RT-PCR kit (Qiagen, Hilden, Germany) and primers for GFP and actin. The PCR products obtained from three of the four samples were resolved by electrophoresis on 1.7% agarose gels and viewed after ethidium bromide staining.

Isolation and Analysis of Carcinoma Cells from the Lungs. To isolate cells from the lungs in early stages of metastasis formation, we injected a much larger number of CT-26 cells into the tail vein than in other experiments, i.e., 2 × 10^6 instead of 10^6 cells. The mice were killed after 2, 7, or 9 days. The lungs were cut into small pieces, incubated for 45 min at 37°C in DMEM containing 0.2% collagenase (Worthington Biochemical Co., Lakewood, NJ) and 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO), and gently teased apart to obtain a cell suspension. Tumor cells were isolated by FACScan sorting, based on forward and side scatter and on GFP fluorescence. The sorted CT-26 cells were incubated with a phycoerythrin-labeled anti-CXCR4 antibody and analyzed by FACScan, similarly as described above.

RESULTS

CXCR4 Is Required for Colon Carcinoma Metastasis. Using RT-PCR, we found that CXCR4 is expressed by CT-26 murine colon carcinoma cells (data not shown). To investigate whether CXCR4 plays a role in metastasis, we transduced the CT-26 cells with the pLZRS-SDF-KDEL-ires-hyg-EGFP vector (Fig. 1A), as described previously (3). As explained above, SDF-1 (CXCL12) with the COOH-terminal KDEL extension is retained in the ER, binds to CXCR4, and prevents this receptor from reaching the surface (Fig. 1B). SDF-KDEL was expressed from a bicistronic mRNA together with a hygromycin-EGFP fusion protein, so that expression of both proteins should correlate. After selection for hygromycin resistance, cells with high EGFP expression, and therefore high SDF-KDEL levels, were isolated by FACScan sorting (Fig. 1C). Thus, we obtained cells with EGFP levels that in T-cell hybridoma cells were sufficient to completely block CXCR4 transport to the cell surface (3). As described below (see Fig. 7), this was also true for the SDF-KDEL-transduced CT-26 cells. Similarly as for the T-cell hybridoma cells, in vitro proliferation of the CT-26 cells was not affected by the high levels of SDF-KDEL or hygromycin-EGFP fusion protein (data not shown).

When CT-26 cells are injected into the spleen, part of the cells form local tumors in that organ, whereas others are transported via the portal circulation to the liver where they form metastases. SDF-KDEL-expressing cells formed intraplastic tumors to the same extent as control CT-26 cells (Fig. 2A), showing that these cells were capable of growing in vivo. However, no metastases were formed in the liver, in strong contrast to the control cells (Fig. 2B). Formation of lung metastases was induced by injection of cells into a tail vein and was also greatly reduced for SDF-KDEL-transduced cells (Fig. 2C). Although all mice injected with control cells became moribund within 30 days, 50% of the mice injected with the SDF-KDEL-transduced cells survived for 6 weeks with no detectable micrometastases. The other 50% of the mice became moribund later than the control mice and had one or two metastases, in strong contrast to the numerous metastases in the lungs of control mice (Fig. 2D).

FACS analysis of cells isolated from control lung metastases and the few lung tumors formed by the SDF-KDEL-transduced cells showed that GFP levels were somewhat reduced as compared with the injected cells, to a similar extent in the two populations (Fig. 3A). CXCR4 expression was high in most control cells, independent of GFP levels. In contrast, CXCR4 levels were low in SDF-KDEL transfectants that had retained substantial GFP expression, as expected if the intrakine approach is effective. In cells that had lost GFP expression, however, CXCR4 was present on the cell surface. So clearly, the few metastases that did develop contained many cells that expressed CXCR4. This result may explain why these metastases developed at all. More than 90% of the analyzed cells were carcinoma cells, based on their nondiploid DNA content (Fig. 3C), and <5% were leukocytes as shown by staining the pan-leukocyte marker CD45 (Fig. 3C). This result indicates that CXCR4 plays an important role in metastasis formation by CT-26 colon carcinoma cells.

CXCR4 Levels on CT-26 Colon Carcinoma Are Low In Vivo but Strongly Up-Regulated In Vivo. Although we readily detected CXCR4 expression by RT-PCR in CT-26 cells from in vitro cultures, the FACS analysis showed that CXCR4 surface levels were exceedingly low, and most cells were essentially CXCR4 negative (Fig. 3B). However, CXCR4 was strongly up-regulated in vivo, in the liver and lung metastases and also in the tumors formed in the spleen (Fig. 3B). Virtually all analyzed cells were carcinoma cells, again based on nondiploid DNA content and lack of CD45, similarly as shown for the lung metastases in Fig. 3C. The high levels on the metastasized cells were not attributable to selection of a minor subpopulation with high constitutive CXCR4 expression, because CXCR4 expression was lost within 2-4 days on ex vivo culture (Fig. 3A). Thus, it seems likely that CXCR4 expression is induced and maintained by the in vivo microenvironment, possibly by factors produced by stromal cells. If so, it would seem unlikely that CXCR4 is involved in the initial coloniza-
The liver. Survival curve (and representative photographs have been injected into a tail vein with either wild-type, mock-transduced and weights of livers of these mice, showing that cells without CXCR4 do not metastasize to the liver. Survival curve (C) and representative photographs (D) of lungs of mice, which had been injected into a tail vein with either wild-type, mock-transduced (CV), or intrakine-transduced (SDF-KDEL) CT-26 cells, showing that lung metastasis of the latter is strongly reduced.

Next, cells were injected into a tail vein or into the spleen to generate lung and liver metastases, respectively. We compared the S1-transduced cells with control nontransduced cells and cells that express CXCR4. A similar up-regulation of CXCR4 in vivo was observed for TA3/St mammary carcinoma cells, which also have very low CXCR4 surface levels in vitro (data not shown), suggesting that this is a more general phenomenon.

GI Proteins Are Not Involved in Colon Carcinoma Metastasis. To further investigate whether CXCR4 is involved in invasion, we blocked Gi protein activity, which is required for migration signals induced by G protein-coupled receptors, including chemokine receptors, such as CXCR4. The S1 catalytic subunit of pertussis toxin was introduced into the cells using the retroviral vector pLZR-S1-IRES-Zeo (Fig. 4A), similarly as described previously (12). The S1 subunit was expressed at high levels (Fig. 4B). To demonstrate that Gi proteins were completely inactivated, we performed an ADP-ribosylation assay. Isolated membranes of both control and S1-transduced cells were treated with 32P-NAD and pertussis toxin. The toxin labels all Gi proteins, except those that have already been ADP-ribosylated by the S1 subunit in the transduced cells. Label was readily incorporated into control membranes but not in those derived from S1-transduced cells (Fig. 4B). This shows that in the latter, all Gi proteins had been ADP-ribosylated and thus inactivated by the S1 subunit protein.

Fig. 2. Metastasis of intrakine-transduced CT-26 cells. A, photographs and weights of spleens from mice, in which the spleen was injected with either mock-transduced (CV) or intrakine-transduced (SDF-KDEL) CT-26 cells. No difference was seen. B, photographs and weights of livers of those mice, showing that cells without CXCR4 do not metastasize to the liver. Survival curve (C) and representative photographs (D) of lungs of mice, which had been injected into a tail vein with either wild-type, mock-transduced (CV), or intrakine-transduced (SDF-KDEL) CT-26 cells, showing that lung metastasis of the latter is strongly reduced.

![Diagram](image-url)
Chapter 6

Fig. 4. Expression of the S1 catalytic subunit of pertussis toxin. A, schematic representation of the expression vector. B, Western blot showing the expression of the S1 protein in the transduced, but not nontransduced, CT-26 cells. In C, ADP-ribosylation of Gi proteins by pertussis toxin is only seen in membranes of the wild-type and not the S1-transduced cells, showing that in the latter, all Gi proteins have been ADP-ribosylated and are consequently all inactive. WT, wild-type nontransduced CT-26 cells; S1, S1-transduced CT-26 cells.

were mock transduced (with the empty pLZRS-IRES-Zeo vector). In contrast to lymphoma cells, the complete inactivation of Gi proteins had no effect at all on metastasis formation. All mice were moribund at the same time with extensive metastasis (Fig. 5). Clearly, Gi protein signaling is not important for colon carcinoma metastasis, and because Gi has been shown to be essential for migration induced by SDF-1 in all cell types tested, including carcinoma cells (7, 28), CXCR4 therefore does not appear to be required for induction of invasion. Similar results were obtained with TA3/S1 mammary carcinoma cells: the S1 subunit, expressed at levels that led to complete inhibition of Gi function, did not have an effect on metastasis (data not shown).

CXCR4-deficient Cells Colonize the Lungs but Do Not Grow into Macrometastases. To follow the development of metastases, we made use of the similarly high GFP levels in control and SDF-KDEL-transduced cells, to detect cells in the lungs by RT-PCR. After 35 cycles, RT-PCR of GFP was able to detect one tumor cell (Fig. 6). Cells were i.v. injected, and lungs were obtained after different intervals. For analysis, we used ~2% of the RNA isolated from the lungs. The results suggest that after 24 h, between 1 and 10 cells were present in the samples, i.e., between ~50 and ~500 in the complete lungs (Fig. 6). This was found for both control cells and SDF-KDEL transfectants. This number did not change for 6 days, showing that initially, the CT-26 cells did not proliferate. After 9 days, the number of control cells was increased to ~5000. When only 25 cycles of PCR were performed, the detection limit was 1000 cells, i.e., 500,000 in the complete lungs. In control lungs, this number was reached between 13 and 20 days after injection and continued to increase thereafter (Fig. 5). In contrast, no GFP was detected in any of the lungs containing SDF-KDEL transfectants, ~27 days, showing that the total number remained <50,000. However, the cells were still present, as detected after 35 cycles. Their number also increased somewhat, but it remained <50,000 in any of the lungs in this experiment, and no macrometastases were seen in the lungs obtained after 27 days.

To confirm these results, we analyzed CT-26 carcinoma cells isolated from the lungs at early stages of metastasis formation. To be able to do so, we injected many more cells (2 × 10^9 instead of 10^6) and excised the lungs after 2, 7, or 9 days. The lungs were digested with collagenase and hyaluronidase, and the small number of carcinoma cells was isolated from the resulting cell population by FACScan sorting, based on forward and side scatter characteristics and GFP fluorescence. FACScan analysis of CXCR4 levels showed that CXCR4 was hardly up-regulated after 2 days (Fig. 7). After 7 days, however, ~40% had become CXCR4 positive. Strikingly, CXCR4 levels then rapidly increased; after 9 days, all cells were CXCR4 positive, and the CXCR4 levels were much higher. In contrast, all SDF-KDEL-transduced cells were still CXCR4 negative after 9 days, indicating that the SDF-KDEL approach was in fact effective and that the SDF-KDEL levels were sufficiently high to completely prevent the transport of CXCR4 to the surface.

Fig. 5. Metastasis of the wild-type and S1-transduced CT-26 cells. Mice injected either into the spleen or a tail vein with nontransduced, mock-transduced, or S1-transduced CT-26 cells all developed extensive liver or lung metastasis, respectively. Shown are survival curves that are similar for both injection routes. For spleen injection, photographs of liver and spleen are shown and both spleen and liver weights. No differences were seen. WT, wild-type nontransduced CT-26 cells; control vector (CV), mock-transduced CT-26 cells; S1, S1-transduced CT-26 cells.

Fig. 6. Detection of CT-26 cells in the lungs by GFP-specific RT-PCR. After 35 cycles, one cell can be detected in the sample (2% of lungs), i.e., ~50 cells in the complete lungs. The results show that both mock- and SDF-KDEL-transduced cells get established in the lungs. The number of cells remains low and constant for 6 days, and then the control cells expand rapidly, in contrast to the SDF-KDEL cells, which remain present but proliferate slowly. RT-PCR was performed in triplicate for each time point after injection. After 25 cycles, 1000 cells can be detected (~500,000 in the complete lungs). After 13–20 days, control cells are detected, but even after 4 weeks, no visible band is amplified from the lungs containing the SDF-KDEL-transduced cells. Bottom, actin controls for all samples. Bottom right, water controls and actin and devoid of GFP in lungs of noninjected mice. CV, lungs from mice injected with mock-transduced cells; SDF-KDEL, lungs from mice injected with SDF-KDEL-transduced cells, which retain CXCR4 in the ER.
It is difficult to quantitate the number of CT-26 cells present in the lungs in this way, given the loss of cells that is likely to occur during the procedure. Nevertheless, the results were in agreement with those of the RT-PCR analysis. Because we injected ~20 times more cells, we expected a yield between 1,000 and 10,000, and we obtained between 10,000 and 20,000 cells after both 2 and 7 days. The yield increased to ~60,000 after 9 days. This confirms the conclusions from the RT-PCR analysis that there is a lag period of ≥6 days and that cells start to proliferate after that period. In contrast, only ~25,000 cells were isolated after 9 days from the lungs of mice injected with the SDF-KDEL-transduced cells, again in agreement with the RT-PCR data.

We conclude that SDF-KDEL-transduced cells, that are CXCR4 deficient, survive in the lungs for a prolonged period but do not grow out and form macrometastases. We also conclude that normal CT-26 cells do not expand during a lag period of ≥6 days, suggesting that local changes have to occur before the cells can proliferate.

**DISCUSSION**

We showed previously that the chemokine receptor CXCR4 is required for metastasis of a T-cell lymphoma cell line (3). We proposed that CXCR4 is involved in invasion of this lymphoma into tissues, because metastasis is also dependent on G protein in the lungs and fibroblast monolayers. Here, we present evidence strongly indicating that CXCR4 also plays an important role in metastasis formation by CT-26 colon carcinoma cells. Surprisingly, however, in contrast to the lymphoma data, our results indicate that CXCR4 does not play a role in invasion but rather in the outgrowth of established single tumor cells or micrometastases. This conclusion is based on the following observations: (a) we introduced an intrakine into the cells to prevent CXCR4 from reaching the surface. This greatly reduced the metastatic capacity, and in most experiments, the cells did not form macrometastases at all, indicating that CXCR4 is essential for metastasis formation; (b) the CT-26 cells hardly express CXCR4 in vitro, so that CXCR4 is not present on most of the injected cells. In fact, the cells do not migrate toward SDF-1 in vitro (data not shown). Therefore, CXCR4 cannot be involved in invasion, at least not initially, and in particular not in invasion of the lungs by i.v. injected cells. In contrast, CXCR4 levels were high on cells isolated from tumors and metastases. This is not caused by selection of a few cells with high constitutive expression because the cells rapidly lose CXCR4 on ex vivo culture. CXCR4 is apparently induced by the in vivo microenvironment. A substantial increase in CXCR4 levels is not seen until at least 7 days after colonization of the lungs. Therefore, CXCR4 plays its pivotal role after the cells have invaded; (c) metastasis was not prevented on complete inhibition of G protein activity. CXCR4-induced cell migration, which absolutely requires G in all cells in which it was tested, including carcinoma cells (7, 28), is therefore not relevant; and (d) similar numbers of CXCR4-deficient and control cells colonize the lungs. However, the control cells proliferate rapidly after an initial lag period, whereas CXCR4-deficient cells expand very slowly if at all. Thus, CXCR4-deficient cells are able to invade and survive, but they hardly proliferate.

Recently, CXCR4 antibodies were shown to reduce metastasis of a breast carcinoma cell line (15). It was proposed that CXCR4 was involved in invasion of tissues by the tumor cells. This seems an obvious explanation because the breast carcinoma cell line that was used had high constitutive CXCR4 expression and showed migratory responses to the CXCR4 ligand CXCL12 (SDF-1) in vitro. However, the antibodies were administered continuously during the metastasis assay period and were therefore present at the time that established micrometastases expanded. It is quite conceivable that, in addition to the possible inhibition of invasion, the antibodies also affected the outgrowth of micrometastases, in line with our results. In this study, we used the TA3/St mammary carcinoma cell line, in addition to the CT-26 colon carcinoma. Unfortunately, we did not achieve sufficiently high levels of SDF-KDEL protein to block CXCR4, and we could therefore not test the role of CXCR4 in metastasis. However, similarly as for CT-26 cells, CXCR4 levels were low on the TA3/St cells in vitro but greatly up-regulated in vivo. Furthermore, metastasis was not blocked by complete inhibition of G protein function (data not shown). If CXCR4 is as essential for this mammary carcinoma as for the cell line described by Miller et al. (15), these results would indicate that CXCR4 is not involved in invasion of mammary carcinoma cells but more likely required for outgrowth.

The "intrakine" approach that we used here to block CXCR4 function was validated previously in a T-cell lymphoma (3). The effect was highly specific, because functional responses to another chemokine were not affected. Most experiments with the lymphoma cells (and all presently described experiments with CT-26 cells) were performed with KDEL-conjugated wild-type SDF-1. However, to exclude that the CXCL12 (SDF-1) in the ER influenced cellular behavior by signals possibly elicited from the ER-retained CXCR4, we also used a mutant SDF-KDEL construct for the lymphoma studies. This mutant can still bind CXCR4 but does not trigger signals. The mutant SDF-KDEL inhibited CXCR4-mediated invasion and metastasis in the T-cell lymphoma, similarly as the control nontumoral SDF-KDEL, showing that the SDF-KDEL effects were not attributable to signaling from the ER (3).

The CXCR4-deficient cells readily formed tumors when injected into the spleen and also s.c. (the latter not shown). However, in those cases, a large number of cells (5 × 10⁴) was introduced, and, inevi-
ably, a wound was made. Growth and survival factors secreted by the tumor cells and/or wound factors produced by inflammatory cells may have allowed the cells to proliferate in the absence of CXCR4 signals. The requirements for metastasis formation are much more stringent, in that single cells or small cell clusters have to proliferate in normal, non-inflamed tissues. This proliferation apparently depends on a suitable microenvironment that stimulates the carcinoma cells to express CXCR4. The SDF-1 that is available in almost all tissues (2, 3) then apparently serves as one of the essential factors that promote proliferation of these cells.

The SDF-KDEL transfectants usually did not form metastases at all. However, in some experiments, a few metastases did form in the lungs after tail vein injection (Fig. 2C). These metastases contained CXCR4-positive cells (Fig. 3B) and may thus have arisen from cells with low SDF-KDEL levels in which not all CXCR4 was retained in the ER. However, the metastases also contained a large proportion of CXCR4-negative cells, showing that not all cells need to be CXCR4 positive. This result is not necessarily inconsistent with the essential role for CXCR4 that we propose here, because CXCR4 may be lost from cells at later stages of metastatic growth. Indeed, all control CT-26 cells are uniformly highly CXCR4 positive after 9 days (Fig. 7), whereas many cells isolated from the macrophages have low or no CXCR4 (Fig. 3B). As discussed above, CXCR4 is apparently induced by interactions with lung cells. When the metastases become larger, the tumor cells will outgrow the lung cells, and the interactions will often be lost. Many tumor cells will therefore rapidly lose CXCR4, similarly as ex vivo. This effect will be stronger in cells that have residual SDF-KDEL and in the single larger metastases formed by the SDF-KDEL cells compared with the multiple small metastases formed by control CT-26 cells in a shorter time period.

Our results are in line with many observations showing that CXCR4 is essential for survival and proliferation of multiple cell types, including early hematopoietic cells of different lineages (18–24). In fact, SDF-1 was originally identified as a B-cell growth factor (24), and both SDF-1 and CXCR4 knockout mice show severe defects in hematopoiesis (19, 30). Mitogen-activated protein-kinase signaling pathways have been proposed to be involved in this survival and proliferation effect. Although the extracellular signal-regulated kinase 1/2 signals triggered by SDF-1 were found to be sensitive to pertussis toxin (31), SDF-1-stimulated p38 and c-Jun-NH2-terminal kinase activity was reported to be insensitive to the toxin (32). Many chemokine receptors couple to other G proteins, in addition to Gi, e.g., we have shown that Gq plays a role in CXCR4 signaling (33). In addition, the Janus kinase-signal transducers and activators of transcription pathway has been implicated and is apparently not dependent on Gi (34). Because pertussis toxin does not affect metastasis, such Gi-independent signaling pathways should be involved in the in vivo stimulation of carcinoma proliferation by SDF-1.

The demonstrated role of CXCR4 in metastasis development has important therapeutic implications. Metastasized carcinoma cells often remain in a dormant state as single cells (35), as is apparently true for CT-26 cells in the 1st week after colonization of the lungs. Our results suggest that inhibition of CXCR4 function can retard their outgrowth and may thus prolong survival. CXCR4 is a co-receptor for certain HIV strains (6, 9, 10), and for AIDS therapy, specific inhibitors have therefore been developed (36–38), which are being tested in clinical trials (39, 40). Such inhibitors may be of benefit in cancer treatment.

ACKNOWLEDGMENTS

We thank Ton Schrauwer for excellent technical assistance in animal experiments. We also thank O. Van Tellingen for providing us with the CT-26 cells with approval of Dr. J. J. Fidler. Finally, we thank G. P. Nolan (Stanford University, Stanford, CA) for the PL2ZRS vector and 9N3CE cells and G. A. Michiels, J. G. Collard, and R. D. Soede for modifications of the PL2ZRS vector.

REFERENCES


