Chemokine receptor signals: role in migration, invasion and cancer metastasis
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Chapter 6

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

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

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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 metastasis 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 metastasis 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 subset of pertussis toxin S1 completely inhibited G1 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-1,2 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 G1 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 intrakinase 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. J. 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 aecin (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 pLZRS-ires-Zeo as described (12). The SDF-KDEL construct was generated by PCR and cloned into the retroviral vector pLZRS-Hyg-EGFP as described (3).

Western Blotting. SDS-PAGE-separated cell lysates were blotted 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 antinouse horseradish 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 × 105 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. 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. CXC4 on cells was stained with the phycoerythrin-
labeled 12G5 monoclonal antibody (PharMingen, San Jose, CA) and analyzed
on a FACSscan (Becton Dickinson, Mountain View, CA).

RT-PCR. Control and SDF-KDEL-transduced CT-26 cells (10⁶) 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
separately homogenized into RNAzol (Tel-Test, Inc., Friendswood, TX), and
total RNA was extracted as described by the manufacturer. 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 × 10⁸ instead of 10⁶ 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 0.25% collagenase (Worthington Biochemical Corp., Lakewood,
NJ), 1% NP-40 (Calbiochem, San Diego, CA), and 0.1% hyaluronidase (Sigma-Aldrich, St.
Louis, MO), and gently
trauspeared apart to obtain a cell suspension. 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 phycoerythrin-labeled anti-CXC4 antibody
and analyzed by FACSscan, similarly as described above.

RESULTS

CXCR4 Is Required for Colon Carcinoma Metastasis. Using
RT-PCR, we found that CXC4 is expressed by CT-26 murine colon
carcinoma cells (data not shown). To investigate whether CXC4 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
CXC4, 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 FACS sorting (Fig. 1C). Thus, we obtained
cells with EGFP levels that in T-cell hybridoma cells were sufficient
to completely block CXC4 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 intrasplenic 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 Vitro
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 exceed-
ingly 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 microen-
environment, 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) had 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.

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 pLZRS-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.

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.

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 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.

Fig. 3. CXCR4 levels in CT-26 cells in vitro and in vivo. A, cells from in vitro cultures or derived from lung tumors were analyzed for GFP fluorescence and CXCR4 expression. The percentages of cells in each quadrant are shown in each corner. CXCR4 is up-regulated in vivo but lost again after a few days of ex vivo culture. GFP-negative cells derived from one of the few lung metastases formed after tail vein injection with SDF-KDEL-transduced cells express CXCR4, but in the GFP-positive cells, CXCR4 levels are much lower than in comparable control cells. Control vector (CV), mock-transduced cells; SDF-KDEL, intrakine-transduced cells, in which CXCR4 is retained in the ER and cannot reach the surface. B, CT-26 cells in vitro hardly express CXCR4, whereas cells isolated from tumors show high CXCR4 levels. Filled histograms are from cells stained with the phycoerythrin-labeled CXCR4 antibody; open histograms are from unstained cells. In C, the tumor cell suspension is mainly aneuploid and CD45 negative, indicating that most cells (>90%) are tumor cells. Shown are cells isolated from the lungs. Right, DNA content of the cells is shown as the filled histogram, whereas the gray histogram is from normal diploid cells. Left, filled histogram is from cells stained with the FITC-labeled CD45 antibody, and the open histogram is from unstained cells.
were mock transduced (with the empty pLZRS-IRES-Zeo vector). In contrast to lymphoma cells, the complete inactivation of G\(i\) proteins had no effect at all on metastasis formation. All mice were moribund at the same time with extensive metastasis (Fig. 5). Clearly, G\(i\) protein signaling is not important for colon carcinoma metastasis, and because G\(i\) 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 G\(i\) 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 \(\sim 2\%\) 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 \(\sim 50\) and \(\sim 500\) 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 \(\sim 5000\). When only 25 cycles of PCR were performed, the detection limit was 1,000 cells, i.e., 50,000 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, \(\pm 27\) days, showing that the total number remained \(\leq 50,000\). However, the cells were still present, as detected after 35 cycles. Their number also increased somewhat, but it remained \(\leq 50,000\) 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 (\(\times 10^5\) instead of \(10^5\)) 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 FACS 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.
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 proteins (11, 12), which are generally required for chemokine-induced migration of different cell types, including carcinoma cells (7, 28). Indeed, both CXCR4 and G proteins are required for *in vitro* invasion of the lymphoma cells into 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, and 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 Gi 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 seemed 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 Gi protein function (data not shown). If CXCR4 is as essential for this mammary carcinoma as for the cell line described by Müller 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 nontagged 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, inev-
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, noninflamed 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 (3, 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 of the macrometastases 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 (34), 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-NH(2)-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.

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