The CXCR5 Chemokine Receptor Is Expressed by Carcinoma Cells and Promotes Growth of Colon Carcinoma in the Liver

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Abstract

The chemokine receptor CXCR5 is expressed by B cells and certain T cells and controls their migration into and within lymph nodes. Its ligand BCA-1/CXCL13 is present in lymph nodes and spleen and also in the liver. Surprisingly, we detected CXCR5 in several mouse and human carcinoma cell lines. CXCR5 was particularly prominent in pancreatic carcinoma cell lines and was also detected by immunohistochemistry in 7 of 18 human pancreatic carcinoma tissues. Expression in CT26 colon carcinoma was low in vitro, upregulated in vivo, and rapidly lost when cells were explanted in vitro. CXCL13 strongly promoted proliferation of CXCR5-transfected CT26 cells in vitro. In the liver, after intrasplenic injection, these CXCR5 transfectants initially grew faster than controls, but the growth rate of control tumors accelerated later to become similar to the transfectants, likely due to the up-regulation of CXCR5. Inhibition of CXCR5 function, by trapping CXCR5 in the endoplasmic reticulum using a CXCL13-KDEL “intrakine,” had no effect on initial growth of liver foci but later caused a prolonged growth arrest. In contrast, s.c. and lung tumors of CXCR5- and intrakine-transfected cells grew at similar rates as controls. We conclude that expression of CXCR5 on tumor cells promotes the growth of tumor cells in the liver and, at least for CT26 cells, seems to be required for outgrowth to large liver tumors. Given the limited expression on normal cells, CXCR5 may constitute an attractive target for therapy, particularly for pancreatic carcinoma. (Cancer Res 2006; 66(19): 9576-82)

Introduction

Chemokines are small polypeptides that direct the migration of leukocytes (1). They are mainly active in inflamed tissues but some are produced constitutively, such as SDF-1/CXCL12, which is present in virtually all tissues (2). Because of their role in migration, it was a surprise when chemokine receptors were also detected on nonmigratory cells. An example is the SDF-1 receptor CXCR4 that is often present on normal epithelial and, especially, carcinoma cells (3–6) and in other tumor types, including neuronal tumors (7) and melanomas (8).

Less surprisingly, CXCR4 is also present on hematopoietic tumors. Similarly as in normal lymphocytes, we found CXCR4 to be involved in invasion of lymphoma cells into tissues and therefore required for metastasis formation (2). This was shown using an “intrakine” approach (9). The intrakine is a chemokine, extended with a KDEL sequence. This binds to the KDEL receptor that retains resident endoplasmic reticulum proteins in the endoplasmic reticulum. The CXCL12-KDEL intrakine binds to CXCR4, which is thereby trapped in the endoplasmic reticulum. We prefer this method because suppression of receptor function can be virtually complete and stable for many months, as required for in vivo experiments. Thus far, we have not been able to achieve this with alternative methods, such as RNA interference.

Expression of CXCR4 on carcinoma cells suggested a role in invasion and metastasis of carcinomas similar to the lymphomas. We used the intrakine approach on colon carcinoma cells and found that, virtually, no liver and lung metastases were formed when CXCR4 function was completely suppressed (6). Remarkably, however, this was not due to reduced invasion but rather to inhibition of the outgrowth of established micrometastases. Indeed, the CT26 colon carcinoma cells expressed very little CXCR4 in vitro, and the injected cells therefore did not have CXCR4 to use for invasion of tissues. CXCR4 was strongly up-regulated in vivo but only after 7 to 9 days and could therefore only then exert its influence.

In addition to CXCL12, the liver contains other constitutively expressed chemokines (e.g., BCA-1/CXCL13; refs. 10, 11). This chemokine binds to CXCR5/BLR1, is mainly expressed in lymph nodes and spleen, and is essential for influx and localization within lymph nodes of mature B lymphocytes and a subset of helper T lymphocytes that express CXCR5 (10–13).

Expression of CXCR5 in carcinomas seemed highly unlikely, so we were surprised to detect it by reverse transcription-PCR (RT-PCR) in CT26 colon carcinoma cells. By fluorescence-activated cell sorting (FACS) analysis, we observed very low levels (RT-PCR) in CT26 colon carcinoma cells. By fluorescence-activated cell sorting (FACS) analysis, we observed very low levels in vitro. Similarly as for CXCR4, however, it was strongly up-regulated in vivo. Furthermore, CXCR5 was detected on several other carcinoma cell lines in vitro and/or in vivo. We show here that CXCL13 strongly promotes proliferation of CXCR5-expressing CT26 cells and that expression of CXCR5 leads to enhanced growth of CT26 cells in the liver. Suppression of CXCR5 function using a CXCL13-KDEL intrakine had no effect on initial growth in the liver but eventually led to a prolonged growth arrest. For the CT26 carcinoma, CXCR5 thus seems to be required for outgrowth of liver lesions to macroscopic tumors.

Materials and Methods

Cell culture. CT26 mouse colon carcinoma cells were kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX); MCA38 mouse colon carcinoma cells were provided by Dr. M.J. Mäurer (Johannes Gutenberg University, Mainz, Germany); MB49 mouse bladder carcinoma cells were provided by Dr. P.J. Selby (St. James University, Leeds, United Kingdom); and Panc02 mouse pancreatic

Note: J. Meijer and I.S. Zeelenberg contributed equally to this work.

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carcinoma cells were provided by Dr. T. Sauerbruch (Rheinische Friedrichs- Wilhelm University, Bonn, Germany). MDA-MB-231 human mammary carcinoma cells were obtained from Dr. J. Massague (The Netherlands Cancer Institute, Amsterdam, the Netherlands). The origin of the additional pancreatic carcinoma cell lines AB18-4, AsPC1, BxPC3, Colo357, HPAF2, MiaPaCa2, Panc1, Panc86, PancTu-1, and PTF-SEP was described before (14). All cells were cultured in DMEM (Life Technologies Ltd., Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Life Technologies). The virus packaging cell line Phoenix was cultured in DMEM with 10% FCS, penicillin, streptomycin, and 0.584 g/L L-glutamine.3

RT-PCR. The organs of a BALB/c mouse or tumors dissected from livers or spleens were frozen in liquid nitrogen and RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RT-PCR was done on the resulting cDNAs with CXCL13-specific primers and β-actin primers as control using a one-step RT-PCR kit (Qiagen).

Immunohistochemistry. Formalin-fixed pancreatic ductal adenocarcinoma specimens from the archive of the Department of Pathology, University Hospital Schleswig-Holstien, Campus Kiel (Kiel, Germany) were processed for paraffin sectioning. Sections were incubated with undiluted RF8B2 anti-CXCR5 monoclonal antibody (15) supernatant (generous gift from R. Förster, Hannover Medical School, Hannover, Germany) overnight and blocked with nonimmune serum. Sections were stained with Vectastain avidin-biotin complex method Elite kit (Vector Laboratories, Burlingame, CA). For negative control, the primary antibody was omitted. Staining was evaluated by a scoring method, based on a value for the percentage of stained tumor cells (1-10%, 1; 10-50%, 2; 50-80%, 3; and >80%, 4), multiplied with a value for the staining intensity (weak, 1; moderate, 2; and strong, 3). The final score (0-12) was then subdivided into weak (1-4), moderate (5-8), and strong (9-12) categories.

Generation and transduction of DNA constructs. The CXCR5 construct was generated by RT-PCR using RNA isolated from a human B-cell line, verified by sequencing, and cloned into the retroviral vector pLZRS-RES-zeo (16). This was transfected into the virus-packaging Phoenix cells, and the supernatant was used to infect the CT26 cells. Transduced cells were selected with 50 μg/mL zeocin (Invitrogen, Carlsbad, CA). We used RT-PCR on mouse spleen RNA to generate a CXCL13-HA-KDEL construct, encoding mouse CXCL13 extended with a HA tag and a KDEL sequence. To obtain cells with high CXCL13-KDEL levels, we used the retroviral vector pLZRS-IRES-puroEGFP, generated from pLZRS-IRES-zeo, by replacing the cDNA for zeocin resistance with that for a mouse CXCL13 extended with a HA tag and a KDEL sequence, encoding mouse CXCL13 extended with a HA tag and a KDEL sequence. This construct was used for expression of CXCL13-HA-KDEL by transduction of CT26 carcinoma cells (data not shown). FACS analysis revealed, however, that CXCR5 surface levels were low on it in mouse CT26 colon carcinoma cells (data not shown). FACS

Results

CXCR5 is expressed on CT26 carcinoma cells in vivo. CXCL13/BCA-1 has been reported to be expressed in the liver, and we confirmed this by RT-PCR (Fig. 1A). To study possible effects of this chemokine on carcinoma cells in the liver, we assessed expression of its receptor CXCR5 by RT-PCR and detected it in mouse CT26 colon carcinoma cells (data not shown), FACS analysis revealed, however, that CXCR5 surface levels were low on the in vitro–cultured cells (Fig. 1B). In contrast, surface levels were high on cells isolated from CT26 tumors, growing in either the spleen, liver, lungs, or s.c. (Fig. 1B and D). To assess the kinetics of CXCR5 induction, we injected a larger cell dose (2 × 106) into a lateral tail vein and isolated cells from the lungs shortly thereafter. Cells were still mainly CXCR5+ after 2 days, but most cells were CXCR5− after 7 days (Fig. 1C). On ex vivo culture of tumor cells

3 http://www.stanford.edu/group/nolan/,
isolated from the lungs, CXCR5 levels dropped rapidly (Fig. 1D), indicating that expression in vivo was not due to selective outgrowth of a minor variant with stable high expression.

**CXCR5 is expressed by several different carcinoma cell lines.** Next, we assessed CXCR5 expression on other carcinoma cell lines. Mouse MCA38 colon and MC49 bladder carcinoma (Fig. 2A) and human CAPAN-1 and CAPAN-2 and mouse Panc02 pancreatic carcinoma (Fig. 2B) expressed CXCR5 in vitro, and this was increased in MCA38 and MB49 cells derived from tumors in the spleen (not tested for the human CAPAN cell lines). In the Panc02 cells, a minority acquired high expression in vivo, whereas expression on the majority appeared to decrease somewhat. MDA-MB-231 mammary carcinoma cells (Fig. 2C) did not express CXCR5 in vitro but it was strongly up-regulated on MDA-MB-231 cells in vivo. For comparison, we also assessed CXCR4 levels. In vitro, CXCR4 was not detected on CXCR5⁺ cell lines but CXCR4 was present on all CXCR5⁺ cell lines, although the levels differed. For instance, CXCR4 levels were relatively low on the pancreatic carcinomas (Fig. 2B).

Conversely, a MDA-MB-231 variant clone with high stable CXCR4 expression in vitro, which we isolated by FACS sorting from a population that contained a small proportion of such cells, also expressed CXCR5 but much less (Fig. 2D). Both CXCR4 and CXCR5 were up-regulated in vivo to roughly similar levels (Fig. 2D).

**CXCR5 in human pancreatic carcinoma.** We next determined whether CXCR5 is expressed in human tumors. Given the high expression in the CAPAN cell lines, even in vitro, we concentrated on ductal pancreatic carcinoma. First, we established by RT-PCR that CXCR5 is expressed by all of 10 pancreatic carcinoma cell lines (A818-4, AsPC1, BxPc3, Colo357, HPAF2, MiaPaCa2, Panc1, Panc89, PancTu-1, and PT45P1), in addition to CAPAN-1 and CAPAN-2 (data not shown). Next, we did immunohistochemical staining on 18 human pancreatic carcinoma specimens. In 7 of 18 tissues, CXCR5 staining was moderate to strong in tumor cells (Fig. 3A-C). A positive control, a reactive lymph node, showed intense reaction in a characteristic B-lymphocyte pattern (Fig. 3D).

**Stimulation of CXCR5 promotes proliferation of carcinoma cells.** To test effects of CXCL13 on the CT26 cells in vitro,
we transfected (human) CXCR5 cDNA and sorted the cells to achieve homogeneous expression. The CXCR5 level was within the range observed in vivo (compare Fig. 1B and Fig. 5A). The CXCR5 was functional as shown by chemotaxis toward the CXCR5 ligand CXCL13 (Fig. 4A). CXCR5-induced chemotaxis is unique in that it is not sensitive to pertussis toxin, as we confirm here (Fig. 4A). Next, we tested effects of CXCL13 on proliferation of these CXCR5-CT26 cells in either minimal or optimal conditions. In keratinocyte medium with 1% FCS, the cells survived (as determined by propidium iodide staining) but proliferated very slowly. This growth was 3-fold increase by CXCL13 (Fig. 4B), which had no effect on control CT26 cells. In DMEM with 10% FCS, the cells proliferated well but, remarkably, CXCL13 still increased growth at ~3-fold (Fig. 4B). We detected few, if any, apoptotic cells by propidium iodide staining in any of these cultures, so that the effects are due to enhanced proliferation rather than survival. We conclude that CXCL13 is a potent growth factor for CXCR5+ carcinoma cells.

CXCR5 promotes tumor growth in liver and spleen but not s.c. or in lungs. The effects of CXCL13 in vitro suggested that proliferation of the colon carcinoma cells in the liver and spleen could be promoted by the CXCL13 present in these organs, once CXCR5 is up-regulated. To study this, we used an “intrakine” approach shown previously to be quite effective for the generation of CXCR4-deficient cells (6, 9). To suppress CXCR5 function, we transfected CXCL13 extended with a KDEL sequence (CXCL13-KDEL) into CT26 cells. For this, we used a bicistronic vector that also encoded GFP and sorted the cells for high GFP and therefore high CXCL13-KDEL levels. The cells were injected into the spleen and isolated from the resulting spleen tumors. The up-regulation of CXCR5 in the CT26-CXCL13-KDEL cells was almost completely prevented (Fig. 5A), showing that the intrakine approach was effective. The levels of (mouse) CXCR5 mRNA are low in vitro in all CT26 cell lines in vivo but were strongly induced in vivo as also shown in Fig. 5A. Mouse CXCR5 mRNA is also up-regulated in CXCR5-CT26 cells (transfected with human CXCR5 not detected with the primers used) and in the CXCL13-KDEL-CT26 cells that have no CXCR5 on the surface. CXCR5 protein is absent in vitro but present in all cells in vivo, as well as in the CXCL13-KDEL-CT26 cells. This shows that CXCR5 is retained inside the cells by the CXCL13-KDEL intrakine and not degraded.

Next, we injected $10^6$ CXCL13-KDEL-CT26, CXCR5-CT26, or control CT26 cells into the spleen. This led to the formation of a spleen tumor and multiple liver foci. In 50% of the mice, the spleen was removed after a few minutes. This did not reduce tumor formation in the liver, indicating that the foci were mainly formed by CXCL13-induced migration and proliferation.
from cells that moved immediately to the liver rather than from cells derived later from the spleen tumor. All mice were sacrificed when the first animal(s) showed signs of distress after 14 days. If CXCL13 affects growth in these organs, the CXCR5-CT26 tumors should be larger than the CT26 controls because the former would be stimulated from day 1, whereas the latter would first have to up-regulate CXCR5. Conversely, the CXCL13-KDEL-CT26 tumors should be smaller than the controls. This was in fact observed. In the liver, the effect was the most evident (Fig. 5B), with highly significant differences in tumor weight (liver weight — the average weight of control livers). Moreover, four of nine livers of mice injected with CXCL13-KDEL-CT26 cells did not contain macroscopically visible foci, and in the others, only small yellowish patches were seen. In all mice from which the spleen had not been removed, large spleen tumors developed. Although less striking than the liver foci, the sizes of spleen tumors also differed significantly (Fig. 5C). The difference between CT26 control and CXCL13-KDEL-CT26 cells was confirmed in a second experiment (liver weight, 5.7 ± 0.4 versus 2.3 ± 0.8; P < 0.0001; spleen, 1.7 ± 0.1 versus 1.1 ± 0.1; P < 0.0001). If CXCL13 in spleen and liver is responsible for these effects, tumor growth should not be affected at sites that do not contain CXCL13. Indeed, we did not observe differences in s.c. (Fig. 5D) or lung tumors (data not shown).

**Effect of CXCR5 on growth in the liver, assessed with bioluminescence.** To assess growth rates *in vivo*, we generated CT26 cells that express luciferase, so that growth could be monitored by measuring bioluminescence. We transfected CXCR5 or CXCL13-KDEL into these CT26-luc cells. The cells were injected into the spleen that was subsequently removed. The dose was reduced to 5 × 10⁴ cells to extend the observation period. For comparison, we measured the growth rate in the lungs after tail vein injection. With this new set of cell lines, the differences in liver tumor growth were similar as with the original transfectants (data not shown). Bioluminescence data from two experiments are shown in Fig. 6 for a total of 3 to 4 each of mice injected with either CXCR5-CT26-luc, control CT-26-luc, or CXCL13-KDEL-CT26-luc cells. Initial values differed between mice, reflecting variation in the number of foci, without systematic differences between the three cell lines. Two general conclusions can be drawn. First, the CXCR5-CT26-luc liver metastases grew at a constant high rate with a T₉₀ of 27 ± 3 hours. In contrast, the CT26-luc metastases initially grew much slower (T₉₀ 59 ± 9 hours; P = 0.0007, for difference with CXCR5-CT26) but later accelerated to a growth rate similar to that of CXCR5-CT26 (T₉₀ 26 ± 3 hours; P = 0.0002, for difference with initial rates). Growth of CXCL13-KDEL-CT26-luc tumors was initially comparable with those of CT26-luc (T₉₀ 51 ± 8 hours). Remarkably, however, growth stopped completely after 16 to 20 days and the bioluminescence remained constant thereafter. At this time point, tumors were still so small (also in control mice) that they were difficult to detect. In the two mice injected with the CXCL13-KDEL-CT26-luc cells that are shown in Fig. 6B, the signal eventually increased but this was more localized. On autopsy, no foci were found in the liver, but a tumor was present in the abdomen, again showing that these cells grow well outside but not within the liver. Indeed, lung tumors of the three cell lines grew at a constant and similar rate (data not shown).

**Discussion**

Here, we have shown that CXCR5, until recently thought to be specific for hematopoietic cells, is expressed by several different carcinoma cell lines. In some cases, expression is very low or absent *in vitro*, but it is greatly enhanced *in vivo*, independent of the site where the tumor is growing. Recently, CXCR5 was described to be expressed on the tumor cells in ~60% of 100
human colon carcinoma specimens as detected by immunohistochemistry (15), confirming that many colon carcinomas express CXCR5. In preliminary tests, we have detected CXCR5 mRNA by quantitative RT-PCR in 6 of 10 colon carcinoma specimens tested (data not shown) in line with these results. Because of the relatively high expression in vitro on pancreatic carcinoma cell lines, we decided to focus on this tumor type for immunohistochemical analysis of patient material. We found that CXCR5 is moderately to strongly expressed in 7 of 18 specimens tested. Together with the abovementioned data on colon cancer, this clearly shows that CXCR5 expression is not a peculiarity of cell lines but occurs in human colon and pancreatic carcinoma. The CXCR5 on CT26 colon carcinoma cells had a major effect on tumor growth in the spleen and especially in the liver, two tissues in which the CXCR5 ligand CXCL13 is present. In contrast, no effect was seen in the skin and the lungs that do not contain CXCL13. We conclude that CXCR5 expression promotes tumor growth at sites where CXCL13 is produced.

The up-regulation of CXCR5 on the CT26 cells is comparable with that of CXCR4, which we described previously (6). In addition, for the other cell lines tested here, the FACS profiles of CXCR4 and CXCR5 in vitro are strikingly similar. The two receptors are not necessarily coregulated in carcinomas, however, because CAPAN-1 and CAPAN-2 pancreatic carcinoma cells in vitro have more CXCR5 than CXCR4* and a CXCR4* variant of MDA-MB-231 mammary carcinoma expresses much less CXCR5 than CXCR4 in vitro (see Fig. 2). The similarity in vivo may be due to stimuli that act on both. Factors that induce up-regulation are not well known for CXCR5, but for CXCR4, they have been studied to some extent. CXCR4 is strongly induced by hypoxia (17, 18), but this is probably not responsible for up-regulation we have seen in the lungs, because virtually, all tumor cells expressed CXCR4 in this highly oxygenated environment after only 7 days when the lesions were still very small (6). The same is true for CXCR5, as shown here. Inflammatory growth factors, such as interleukin (IL)-1 and IL-6 up-regulate CXCR4 in some cell types (19, 20) but not in CT26 cells in vitro. Whether they have an effect on CXCR5 remains to be tested. It is noteworthy that CXCR4 was detected by some authors in ~100% of MDA-MB-231 cells in vitro (21) and by others in only a small subpopulation (22). From the latter cell line, we isolated CXCR4* clones, only few of which retained CXCR4 on prolonged culture (Fig. 2D). This suggests that CXCR4 (and possibly also CXCR5) expression can be influenced by culture conditions and can differ between variants of the same tumor cell line.

CXCL13 induced migration of CXCR5* CT26 cells, as expected of a chemokine. However, we show that it is also a potent inducer of proliferation that greatly enhances growth of CT26 cells, even in medium with 10% serum, adding to growing evidence that chemokine receptor signaling affects survival and growth. This is particularly true for CXCR4 (7, 23–26). We previously provided evidence that CXCR4 plays an essential role in outgrowth of CT26 colon carcinoma cells in liver and lungs and not in invasion (6). For CXCR5, functions in processes other than migration have hardly been studied, except for a possible antipapoptotic effect in lymphomas (27). Our data indicate that the major effect of CXCR5 in vivo is enhanced tumor growth. Because neither CT26 control nor CXCL13-KDEL-CT26 cells express CXCR5 in the first few days in vivo, differences in initial invasion cannot explain their greatly distinct extent of tumor formation in the liver. In contrast, the differences in growth rates, as measured by bioluminescence, support the notion that CXCR5 promotes proliferation. Liver tumors of the CXCR5-transfected cells that express CXCR5 from day 1 grew initially faster than control cells and the rate remained constant for a prolonged period. In contrast, the growth rate of control tumors was much lower in the beginning but increased after ~10 days, when most cells express CXCR5, to a similar rate as that of the CXCR5-CT26 metastases. Furthermore, the initial rate of control and CXCL13-KDEL lesions was similar, as expected because initially neither expresses CXCR5. Because growth rates were similar in s.c. and lung tumors (i.e., in tissues without CXCL13), the effects are likely due to the CXCL13 in the liver. The effects in the spleen were less pronounced, possibly because the spleen tumors grew from a large number of injected cells, whereas the liver tumors grew from dispersed single cells. Growth factors produced by the tumor cells or by inflammatory cells in the wound created at the injection site may promote growth in the spleen. Yet, the significant difference in spleen tumor weights in this organ that also contains CXCL13 indicates that this CXCL13 promotes growth of CXCR5* cells in the spleen as well.

A striking result from this study is that CXCR5 not only promotes growth but apparently allows cells to overcome a strong growth-inhibitory effect that becomes apparent ~14 days after injection. This is clearly not due to an intrinsic defect in these cells because they do grow normally in s.c. and lung tumors. The reasons for this
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Moser B, Loetscher P. Lymphocyte traffic control by regulated on plasma cell differentiation (31). Selective killing of mature cells and not on pre- and pro-B cells and it is down-regulated on plasma cell differentiation (31). Selective killing of CXCR5+ cells (e.g., by a toxin conjugated to an antibody or to BCA-1), may therefore have limited side effects. Given the expression of CXCR5 in a substantial proportion of pancreatic carcinomas, this option may be particularly interesting for this aggressive and incurable tumor. We therefore aim to focus on CXCR5 in pancreatic carcinoma in the near future.

Acknowledgments

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References

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