The Chemokine Receptor CXCR6 and Its Ligand CXCL16 Are Expressed in Carcinomas and Inhibit Proliferation

Joost Meijer,[1] Janneke Ogink,[1] Bas Kreike,[2,3] Dimitry Nuyten,[2] Karin E. de Visser,[4] and Ed Roos[1]

Divisions of [1]Cell Biology, [2]Radiotherapy, [3]Experimental Therapy, and [4]Molecular Biology, The Netherlands Cancer Institute, Amsterdam, the Netherlands

Abstract

The chemokine receptor CXCR6 and its ligand CXCL16 are involved in inflammation. Thus far, they were known to be expressed mainly by T cells and macrophages, respectively. However, we detected both in all of 170 human primary mammary carcinomas and at similar levels in all 8 human mammary carcinoma cell lines tested by microarray analysis. Expression was confirmed by reverse transcription-PCR and for the cell lines also by fluorescence-activated cell sorting analysis. CXCR6 and CXCL16 were also detected in several mouse and human mammary, colon, and pancreatic carcinoma cell lines. CXCL16 is a transmembrane protein from which the soluble chemokine can be cleaved off. The transmembrane form is present on the surface of the carcinoma cells. Surprisingly, suppression of either CXCR6 or CXCL16 led to greatly enhanced proliferation *in vitro* as well as *in vivo*, indicating that their interaction inhibits proliferation. This notion was verified using inhibitory antibodies and by introduction of CXCL16 into a rare CXCL16-negative cell line. The effect was mediated by the G protein–coupled receptor CXCR6 because it was blocked by the G_i protein inhibitor pertussis toxin. In contrast, the soluble CXCL16 chemokine enhanced proliferation, and this was also mediated by CXCR6 but not via G_i protein. It is remarkable that both CXCR6 and CXCL16 are expressed by all mammary carcinomas because cells that lose either acquire a growth advantage and should be selected during tumor progression. This suggests an unknown important role in tumor formation. Proteases, possibly macrophage derived, might convert inhibitory transmembrane CXCL16 into the stimulatory chemokine. [Cancer Res 2008;68(12):4701–8]

Introduction

Chemokines are small proteins that are mainly produced in inflamed tissues and control the migration of blood cells. Some are also constitutively expressed, mainly in lymphoid but also in other tissues. Chemokines bind to receptors that trigger adhesion and motility on binding of their ligands. Surprisingly, several chemokine receptors are expressed by tumors that are not derived from blood cells (1–3). The best-studied example is CXCR4 that was detected in many tumors, including carcinomas (4–9), melanomas (10), and brain tumors (11), and its presence often correlated with malignancy (6–10). Indeed, CXCR4 was shown to be required for metastasis of a mammary and a colon carcinoma (4, 5). This was initially ascribed to an essential role in invasion (4). For the CT26 colon carcinoma, we showed, however, that CXCR4 was not involved in colonization of tissues but required for outgrowth of micrometastases (5). Furthermore, we detected CXCR5 in pancreatic carcinomas and several colon and pancreatic carcinoma cell lines (12). This receptor was thus far only known for its effect on migration of B lymphocytes. Its ligand CXCL13 promoted proliferation of the CXCR5-positive carcinoma cells, and this was shown to be relevant for metastasis to the liver where CXCL13 is present (12). More recently, CXCR7 was identified as a second receptor for CXCL12, in addition to CXCR4 (13). It was found in many tumors and on binding of ligand, it enhanced survival of the tumor cells. We observed that CXCL12 promoted proliferation of carcinoma cells, mediated by either CXCR7 or CXCR4 or both.[5] Together, the data indicate that chemokines can act as growth and survival factors for carcinoma cells that express the appropriate receptors.

While analyzing data from microarray analysis of mammary carcinomas, we noted substantial expression of CXCR6 as well as its ligand, CXCL16. The chemokine receptor CXCR6, previously known as Bonzo or STRL33 (14), is expressed in subsets of T lymphocytes and involved in migration into inflamed tissues in, for example, arthritis (15, 16) or the inflamed liver (17). It is also present on natural killer (NK) T cells in liver capillaries and required for their association with liver sinusoidal endothelium (18). In CXCR6 knock-in mice, in which green fluorescent protein (GFP) replaced the CXCR6 coding sequence, GFP was not detected in normal healthy tissues, except for the T and NK T cells (18), indicating that CXCR6 is expressed in other cells only in inflamed tissues. Expression may be induced by inflammation mediators as shown, for example, for lipopolysaccharide acting on aortic smooth muscle cells (19).

The CXCR6 ligand CXCL16 (20, 21) was independently identified as the SR-PSOX scavenger receptor that binds phosphatidylserine and oxidized lipoproteins and is involved in phagocytosis of bacteria by macrophages (22). In addition to macrophages (16) and epithelium in lymph nodes (23), it is expressed by different cell types in inflamed tissues [e.g., inflamed areas in the liver (17)]. It differs from most other chemokines in that it is a transmembrane protein from which the soluble chemokine has to be cleaved off by proteases such as ADAM-10 and ADAM-17 (24, 25) that are produced by activated macrophages (16). We show here that both CXCR6 and CXCL16 are consistently expressed by mammary carcinomas and by colon and pancreatic carcinoma cell lines. Remarkably, their interaction leads to a reduction in proliferation.

Requests for reprints: Ed Roos, Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066CX Amsterdam, the Netherlands. Phone: 31-20-5121931; Fax: 31-20-5121944; E-mail: e.roos@nki.nl.

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[5] J. Meijer et al., submitted for publication.

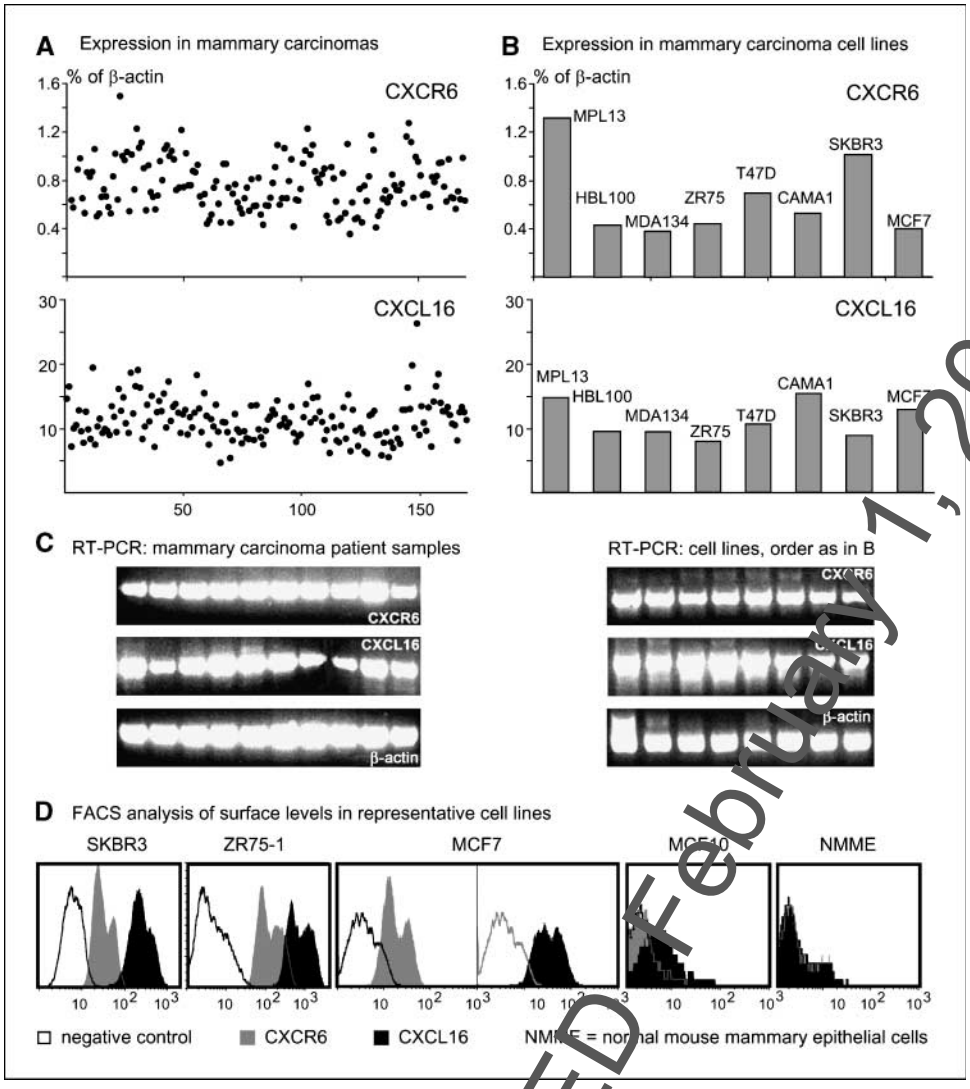

Figure 1. CXCR6 and CXCL16 expression in mammary carcinoma: signal intensity on microarrays in 170 patient tumor samples (*A*) and 8 cell lines (*B*), normalized to that of β-actin. *C,* RT-PCR of selected tumor samples and the cell lines. *D,* FACS analysis of three representative mammary carcinoma cell lines, the MCF10 nontransformed mammary cell line, and normal mammary epithelial cells (*NMME*), isolated from mouse mammary glands.

Materials and Methods

Cell culture. Cell lines were derived from the Netherlands Cancer Institute cryobank or from sources described previously (5, 12), except for KEP1 cells that were kindly provided by Dr. J. Jonkers (Netherlands Cancer Institute). All carcinoma cell lines were cultured in DMEM with 10% FCS. The Phoenix virus packaging cell line[6] was cultured in DMEM with 10% FCS and 0.584 g/L L-glutamine. Mouse mammary epithelial cells were isolated from 2-mo-old FVBn mice by digestion of glands with 3 mg/mL collagenase A and 1.5 mg/mL trypsin for 1 h at 37°C. After 1 h in DMEM + 10% FCS, suspended organoids were separated from attached fibroblasts and incubated in DMEM:F12 with 10% FCS, penicillin-streptomycin, 5 μg/mL insulin, 5 ng/mL epidermal growth factor, and 5 ng/mL cholera toxin. After 1 wk of culture, cells were harvested by trypsinization. Medium, FCS, and supplements were from Life Technologies, Ltd.

Microarray analysis. Tissue samples of the primary tumors were snap frozen in liquid nitrogen within 1 h after surgery. RNA was isolated from frozen sections. In the first and the last section, the percentage of tumor cells was assessed after H&E staining; we used only tumors containing on average >50% tumor cells. Total RNA was isolated with RNAzol B (Campro Scientific) and from breast cancer cell lines using Trizol (Invitrogen). RNA was amplified and hybridized to the Human Genome Oligo Set version 3.0 arrays. Details and protocols can be found at the Netherlands Cancer Institute microarray Web site.[7] Expression was confirmed in randomly selected samples by reverse transcription-PCR (RT-PCR) using the one-step RT-PCR kit (Qiagen) for 35 cycles with specific primers for CXCR6, CXCL16, and β-actin.

Generation and transduction of DNA constructs. Luciferase was introduced as described (12). The CXCL16-KDEL intrakine, full-length transmembrane CXCL16 (TM-CXCL16), and soluble CXCL16 (sCXCL16) constructs were generated using a one-step RT-PCR kit on human lymph node–derived mRNA and cloned into the pLZRS-IRES-hygroEGFP vector (26). The constructs were verified by sequencing. Vectors were transfected into the virus packaging Phoenix cells, and the supernatant was used to infect tumor cells. Transduced cells were selected with 0.05 mg/mL (CT26), 0.1 mg/mL (KEP1 and Panc02), or 0.2 mg/mL (HCT116) hygromycin (Calbiochem) and cells with high GFP levels were isolated by fluorescence-activated cell sorting (FACS). Control cells were transduced with the empty vector and similarly sorted. The RNA interference (RNAi) sequences GAAACTCCCTGGTTCTGAT (CXCR6) and CTGAGCTCACTGTTTCTCA (CXCL16) were incorporated into the micro-RNA-30 sequence (22) and cloned into the LMP (MSCV/LTRmiR30-PIG)

[6] http://www.stanford.edu/group/nolan

[7] http://microarrays.nki.nl

vector (Open Biosystems) that also contains the GFP cDNA under the control of the phosphoglycerate kinase promoter.[8] The chosen sequences were derived from the RNAi Codex.[9] Constructs were transduced using Phoenix cells as above and selected with 1.25 μg/mL (KEP1 and Panc02) or 2.5 μg/mL (CT26) puromycin (Sigma-Aldrich), and cells with high GFP levels were FACS sorted.

Flow cytometry. Cultures were trypsinized and 10^6 cells were incubated for 60 min with CXCR6 mAb2154 or CXCL16 mAb503 for mouse cells and CXCR6 mAb699 or CXCL16 mAb976 (R&D Systems) for human cells, and next with phycoerythrin-conjugated antibodies against mouse IgG, and analyzed using a Becton Dickinson FACScan using CellQuest software.

Proliferation assay. CT26 cells (5×10^3 or 5×10^4 per well) or Panc02 or KEP1 cells (5×10^4 per well) were seeded in several 96-well plates in DMEM with 10% FCS. Luciferase activity was measured every 24 h after addition of 10 μL of 15 mg/mL D-luciferin using a Xenogen IVIS imaging system (see below). A different plate was assessed for each of the time points. The data were normalized by setting the value at day 0 at 1. Because this assay did not involve any washing steps, it was quite reproducible, with SDs of triplicates of ~0.02%. To some wells, we added 100 ng/mL recombinant sCXCL16 (PeproTech) or 1 μg/mL CXCR6 mAb2154 or CXCL16 mAb503 (R&D Systems) and this was repeated every 48 h. After the last measurement, cells from triplicate wells were trypsinized and manually counted.

Tumor growth *in vivo*. All procedures involving animals were approved by the Animal Welfare Committee. For CT26 cells, we used syngeneic BALB/c mice, and for KEP1 and HCT116 cells, we used nude mice, both 6 to 8 wk old. To generate s.c. tumors, either 10^5 cells were s.c. injected in 0.2 mL PBS or 10^3 cells were dispersed in 0.5 mL Matrigel (Becton Dickinson) at 0°C and injected s.c. in mice anesthetized with 3% isoflurane (1-chloro-2,2,2-triflouroethyl-diflouromethyl-ether). For generation of liver metastases, 0.1 mL PBS containing 10^6 HCT116 cells was injected into the spleen, which was removed 5 min later.

***In vivo* bioluminescence imaging.** D-Luciferin (Xenogen) was dissolved at 15 mg/mL in sterile PBS and stored at −20°C. Animals were anesthetized with 3% isoflurane. Luciferin solution was injected i.p. (0.01 mL/g body weight). Light emission was measured 5 min later using a cooled charge-coupled device camera (IVIS, Xenogen) coupled to Living Image acquisition and analysis software. Signal intensity was quantified as the total counts measured over the region of interest.

Results

CXCR6 and CXCL16 are expressed in human mammary carcinoma. Microarray analysis of the human MDA-MD-431 mammary carcinoma cell line revealed substantial expression of the chemokine receptor CXCR6 and its ligand CXCL16. To determine whether this is generally true for mammary carcinomas, we used data from an ongoing study of 170 human primary breast carcinomas that will be published elsewhere. In brief, 61 of the 170 tumors were from patients who developed an ipsilateral breast recurrence after breast conserving therapy (i.e., breast conserving surgery followed by radiotherapy), and 37 of 143 patients developed distant metastasis during clinical follow-up. The metastatic status of the remaining 27 patients is unknown. All tumor samples contained >50% tumor cells. The hybridization signals were normalized to those of β-actin. As shown in Fig. 1*A*, CXCR6 was detected in all tumors with an average of 0.8% of β-actin (range, 0.4–1.5; SD, 0.2). CXCL16 was also expressed in all tumors, with an average of 11% of β-actin (range, 5–26; SD, 3). No difference was observed between nonmetastatic and metastatic tumors. These numbers do not accurately reflect mRNA levels because signal intensity differs between individual oligonucleotides, but they are suitable for comparison between the tumors and provide a rough indication of expression levels.

Next, we obtained similar data from microarray analysis of eight human mammary carcinoma cell lines, as shown in Fig. 1*B*. CXCR6 and CXCL16 were detected in all cells at levels comparable with the tumors: CXCR6 at an average of 0.7% of β-actin (range, 0.4–1.3; SD, 0.3) and CXCL16 at an average of 11% (range, 8–15; SD, 3). To confirm expression, we performed RT-PCR on mRNA from all cell lines and 10 randomly selected tumors, 50% of which from patients who developed metastasis during clinical follow-up. As shown in Fig. 1*C*, both CXCR6 and CXCL16 were detected in all samples. FACS analysis revealed protein on the cell surface. Three representative examples (SKBR3, ZR75-1, and MCF7) are shown in Fig. 1*D*. Both CXCR6 and CXCL16 were detected at different but always substantial levels. Because CXCL16 was at the surface, at least part of the expressed protein was maintained as the uncleaved transmembrane form. Similarly as the microarray data, the FACS analysis suggested higher levels of CXCL16 than of CXCR6, but this is not certain because FACS data obtained with different antibodies cannot be readily compared. Because microarray data of cell lines and tumors were similar, the protein levels in the carcinoma cells in the primary human breast cancers are likely to be comparable with those of the cell lines.

FACS analysis of mouse and human mammary, colon, and pancreatic carcinoma cell lines revealed expression of CXCR6 and CXCL16 with one notable exception, the KEP1 mouse mammary

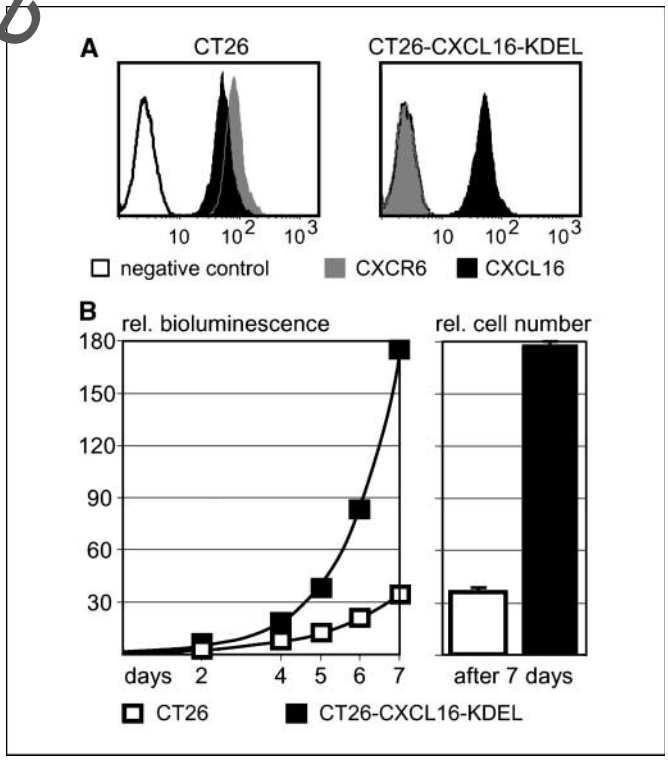

Figure 2. Suppression of CXCR6 leads to increased proliferation of CT26 colon carcinoma cells. *A,* FACS analysis showing that trapping of CXCR6 in the ER by the CXCL16-KDEL intrakine results in cells completely devoid of surface CXCR6. *B,* proliferation in 10% FCS. *Left,* bioluminescence (with value at day 0 set to 1; SE values of ~0.02%); *right,* manually counted cells after 7 d. *Columns,* mean of triplicates; *bars,* SE. $P < 0.0001$, *t* test of difference. Shown is one experiment representative of three with similar results.

[8] http://www.openbiosystems.com/RNAi/RetroviralCloningVectors
[9] http://codex.cshl.edu/scripts/newmain.pl

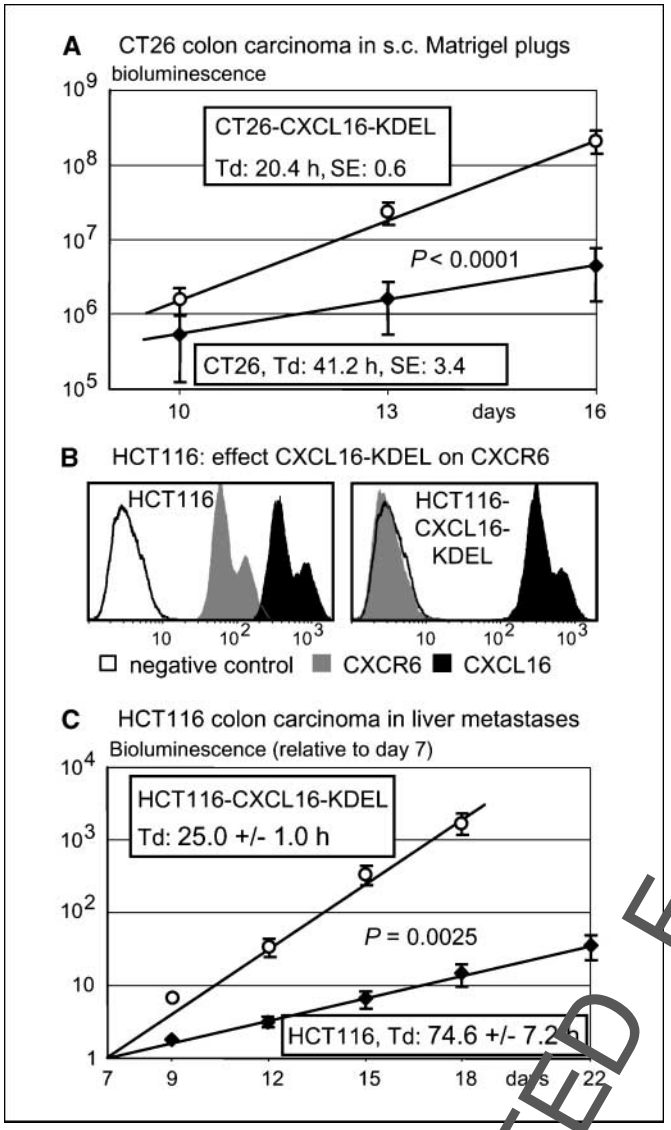

Figure 3. Effect of CXCR6 suppression on colon carcinoma growth *in vivo*, measured by bioluminescence. *A,* CT26 cells dispersed in s.c. Matrigel plugs. Values are averages of four plugs. *B,* complete suppression of surface CXCR6 by the CXCL16-KDEL intrakine, shown by FACS analysis. *C,* growth of HCT116 liver metastases. Values are averages of three mice, normalized to 1 at day 7. For CT26 and HCT116 tumors, the Td was calculated for individual plugs or mice, respectively. *Points,* average Td; *bars,* SE. *P* value of the difference was assessed with Student's *t* test.

carcinoma cell line that expressed CXCR6 but not CXCL16 (see below; Fig. 5*C*). In contrast, the human normal mammary epithelial cell line MCF10 and normal mouse mammary epithelial cells, isolated from mammary glands, had no CXCR6 and very little or no CXCL16 on their surface (Fig. 1*D*), indicating that expression is specific for tumor cells. To study potential effects of CXCR6 and CXCL16, we selected four cell lines: CT26 mouse and HCT116 human colon carcinoma, Panc02 mouse pancreatic carcinoma, and KEP1 mouse mammary carcinoma.

Proliferation of colon carcinoma cells is enhanced on suppression of CXCR6. To suppress CXCR6, we used the intrakine approach. An intrakine is a chemokine, extended with a COOH-terminal KDEL sequence. It binds to the KDEL receptor that retains resident endoplasmic reticulum (ER) proteins, and is therefore trapped in the ER where it binds its receptor that is also trapped. Previously, we have thus generated cells completely devoid of surface CXCR4 (5), CXCR5 (12), and CXCR7.[5] The intrakine used here was the sCXCL16 (i.e., only the chemokine domain) with the KDEL extension. As shown in Fig. 2*A*, CT26 cells expressing this intrakine were completely devoid of surface CXCR6, whereas CXCL16 was not affected. (To avoid confusion: please note that the CXCL16 detected by FACS in the CXCL16-KDEL cells is the endogenous transmembrane protein, not the intrakine that remains in the ER.) Apparently, the endogenous CXCL16 does not compete with the intrakine, quite probably because the chemokine domain sits on the rod-like mucin stalk that extends far from the membrane, so that it cannot bind to the membrane-embedded CXCR6.

Remarkably, the CT26-CXCL16-KDEL cells proliferated much faster than control cells, as shown in Fig. 2*B*. This was not only seen in suboptimal conditions (1% FCS; data not shown) but was in fact more prominent in 10% FCS, with a >5-fold increase in cell number after 7 days compared with <2-fold in 1% FCS. The cells expressed luciferase and quantification of cell numbers was based on bioluminescence after addition of luciferin to the wells. The reliability of this method was confirmed by manually counting of cells at the end of the experiment (see Fig. 2*B*).

Suppression of CXCR6 accelerates tumor growth *in vivo*. To test the relevance for growth *in vivo*, we s.c. injected cells in Matrigel. Because the Matrigel solidified instantly at 37°C, cells remained dispersed and grew out as separate tumors. After i.p. injection of luciferin, tumor growth was assessed by bioluminescence, which could be reliably measured from day 10. Also *in vivo*, the CT26-CXCL16-KDEL cells proliferated faster (Fig. 3*A*). The doubling time (Td) was reduced by ~50% and this difference was highly significant. To show that this effect was not specific for mouse tumors or for CT26 cells, we repeated the experiment with human HCT116 colon carcinoma cells. Figure 3*B* shows the CXCR6 and CXCL16 surface levels and the complete suppression of CXCR6 by the CXCL16-KDEL intrakine in HCT116 cells. Again, the intrakine caused the cells to grow much faster in culture (data not shown). In s.c. Matrigel plugs, the Td was reduced from an average of 57 h to an average of 34 h (data not shown).

To confirm that the results of the Matrigel assay were relevant for tumors growing in tissues, we measured the growth of HCT116 liver metastases by bioluminescence in nude mice after intrasplenic injection and subsequent splenectomy. Because the tumor burden varied between animals, we normalized the data for each mouse to the values at day 7 after injection for proper comparison. As shown in Fig. 3*C*, the intrakine-expressing cells proliferated much faster with a 3-fold reduced Td. These results show that the CXCR6-induced reduction in proliferation is relevant for tumor growth *in vivo*. They were obtained in nude mice and, therefore, not due to effects of CXCR6 on antitumor immune responses.

Reduced proliferation is due to interaction between CXCR6 and CXCL16. To investigate whether reduced proliferation was due to interaction between CXCR6 and CXCL16, we used RNAi with constructs in which target sequences were incorporated into a microRNA sequence. This results in more efficient knockdown (27). In fact, we achieved complete and stable suppression of CXCR6 and CXCL16 in Panc02 pancreatic carcinoma cells (Fig. 4*A*) and CT26 cells (Fig. 4*B*). CXCR6 RNAi accelerated proliferation of the relatively slowly growing

Panc02 cells to a similar extent as the CXCL16-KDEL intrakine, with an ~3-fold increase in cell number after 7 days (Fig. 4*A*). Importantly, CXCL16 RNAi similarly enhanced proliferation, indicating that inhibition is due to interaction between CXCL16 and CXCR6. Proliferation of the faster growing CT26 colon carcinoma cells was also enhanced similarly as by the intrakine, with a >5-fold increase in cell number (Fig. 4*B*).

To further test the notion that CXCR6 and CXCL16 need to interact, we used antibodies that inhibit their binding. As shown for both CT26 and Panc02 cells in Fig. 4*C*, proliferation was accelerated by either CXCR6 or CXCL16 antibodies almost as much as by intrakine-induced suppression of CXCR6. This shows that the effect is due to interaction between CXCR6 and CXCL16.

The sCXCL16 chemokine enhances proliferation. The observed effect could be mediated by either the TM-CXCL16 or the sCXCL16 chemokine after cleavage by proteases. To test this, we used recombinant sCXCL16 chemokine on cells in which CXCL16 had been knocked down by RNAi and on KEP1 mammary carcinoma cells that do not express CXCL16. The soluble chemokine did not reduce proliferation but instead enhanced it by ~50% for Panc02-CXCL16-RNAi cells and >2-fold for CT26-CXCL16-RNAi cells (Fig. 5*A*). The combined effects of CXCL16 RNAi and the soluble chemokine led to a >10-fold increase in CT26 cell number after 7 days. Growth of CXCL16-negative KEP1 cells was similarly increased (Fig. 5*B*). Proliferation of wild-type CT26 cells, which express CXCL16, was also enhanced but to a much lesser extent (data not shown) probably due to competition with the TM-CXCL16 for binding to CXCR6. This will be confirmed below for KEP1 cells (see Fig. 5*B*). We conclude that the reduction of proliferation induced by the chemokine-receptor interaction cannot be due to sCXCL16 and should thus be mediated by the transmembrane form of CXCL16.

The TM-CXCL16 protein is responsible for reduced proliferation. To prove the effect of TM-CXCL16, we introduced it into KEP1 cells that do not express CXCL16. We expected the transduced CXCL16-positive cells to grow slower and therefore to be outcompeted by faster growing cells with no or less CXCL16. To prevent this, we added CXCL16 antibodies to the medium up to the moment when we had obtained a population with homogeneous expression levels (Fig. 5*B*). As a control, we also expressed the soluble chemokine. The TM-CXCL16 reduced proliferation by ~50%, whereas the cells expressing the sCXCL16 proliferated faster at a rate similar to or even somewhat faster than of control cells treated with recombinant soluble chemokine (Fig. 5*B*). Cotransfected or exogenously added sCXCL16 also enhanced growth of the TM-CXCL16 transfectants but to a lesser extent than wild-type KEP1 cells probably due to competition with the TM-CXCL16 for binding to CXCR6. sCXCL16 had no effect on

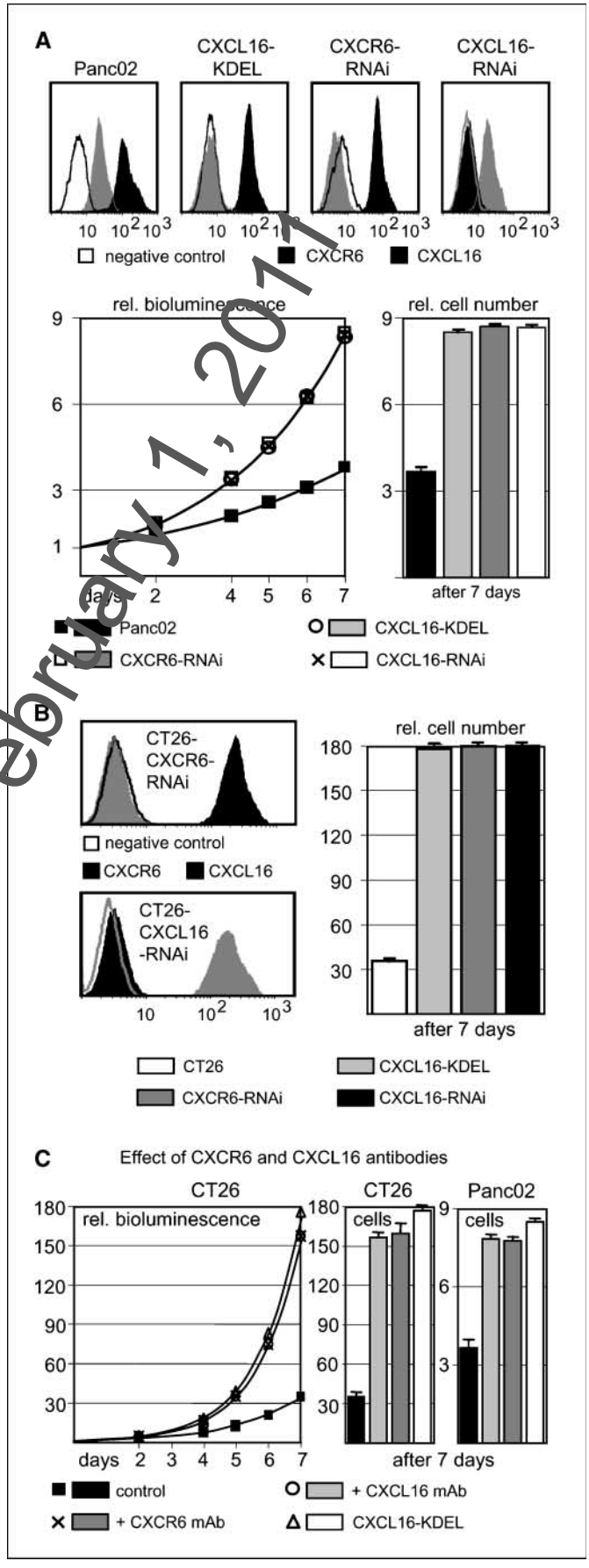

Figure 4. Effects of CXCR6 and CXCL16 RNAi on proliferation. *A,* FACS analysis, showing complete knockdown of CXCL16 by RNAi and of CXCR6 by RNAi or intrakine in Panc02 pancreatic carcinoma cells, and proliferation of these cells in 10% FCS, as measured by bioluminescence (with value at day 0 set to 1; *left*) and by counting cells after 7 d (*right*). *Columns,* mean of triplicates; *bars,* SE. Difference between CXCR6- or CXCL16-negative cells and controls: $P < 0.0001$. For bioluminescence values, SE was ~0.02%. Shown is one experiment representative of two with similar results. *B,* FACS analysis showing complete suppression of CXCR6 and CXCL16 by RNAi in CT26 cells, and proliferation of CT26 cells, measured and shown similarly as in *A. C,* effect of CXCR6 and CXCL16 antibodies and, for comparison, proliferation of CXCL16-KDEL intrakine-transfected cells, measured and shown as in *A* and *B. mAb,* monoclonal antibody. Results are from one experiment representative of two with similar results.

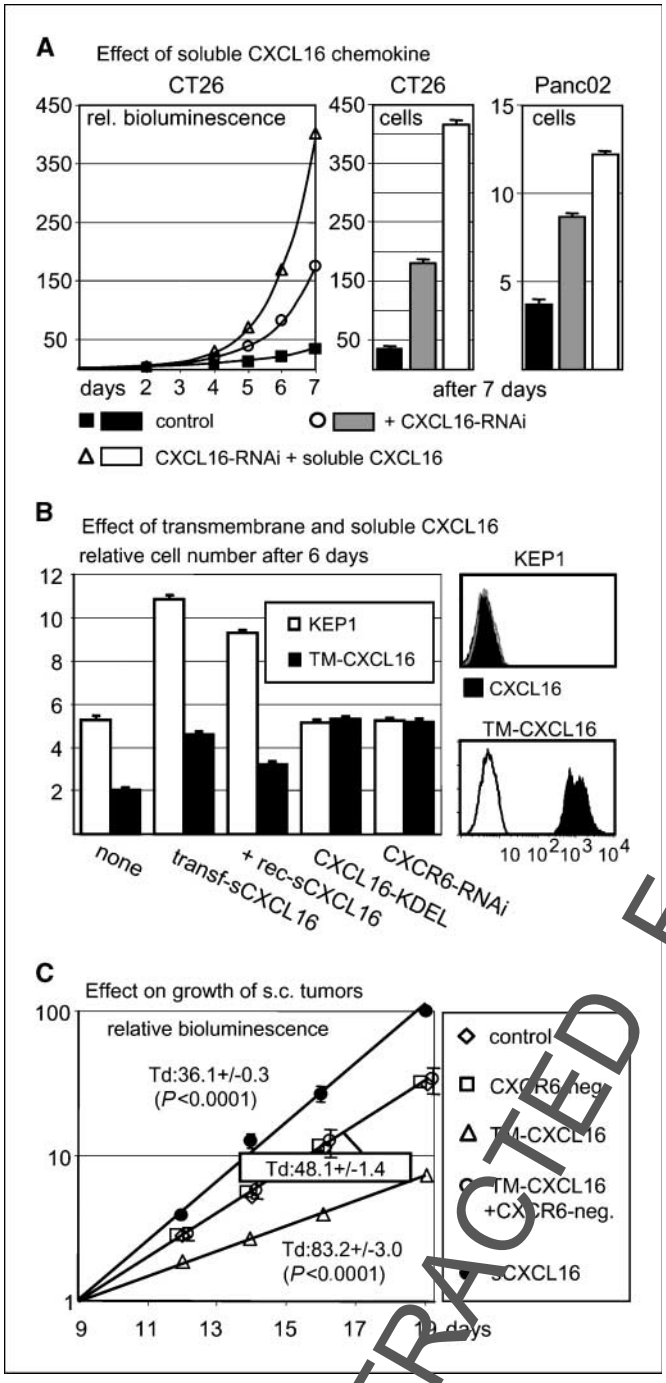

Figure 5. Effect of sCXCL16 and TM-CXCL16. *A,* effects of exogenously added recombinant sCXCL16 on proliferation of CXCL16-negative (CXCL16 RNAi) CT26 and Panc02 cells, shown as in Fig. 4*A* and *B*. *B,* effect of transfected TM-CXCL16 on proliferation of CXCL16-negative KEP1 mammary carcinoma cells. FACS analysis shows TM-CXCL16 expression levels. Proliferation is shown as relative cell number after 6 d (set to 1 on day 0). *Columns,* mean; *bars,* SE. Effects of exogenously added sCXCL16 (*rec-sCXCL16*) or cotransfected sCXCL16 (*transf-sCXCL16*), and CXCR6 suppression by cotransfected CXCR6 RNAi or CXCL16-KDEL intrakine are shown. *C,* growth of s.c. tumors of KEP1 transfectants, measured by bioluminescence, normalized to 1 at day 9 after injection. Values are averages of four tumors. Error bars (SE) are only shown when larger than the symbols. Tds were calculated separately for each tumor. Shown are averages for controls (nontransfected, CXCR6 negative: 50% of tumors CXCR6 RNAi, 50% CXCL16-KDEL, and cotransfected TM-CXCL16 + CXCR6 RNAi) as well as for TM-CXCL16-KEP1 and sCXCL16-KEP1 transfectants. Differences between Tds of the latter and controls were highly significant ($P < 0.0001$, Student's t test).

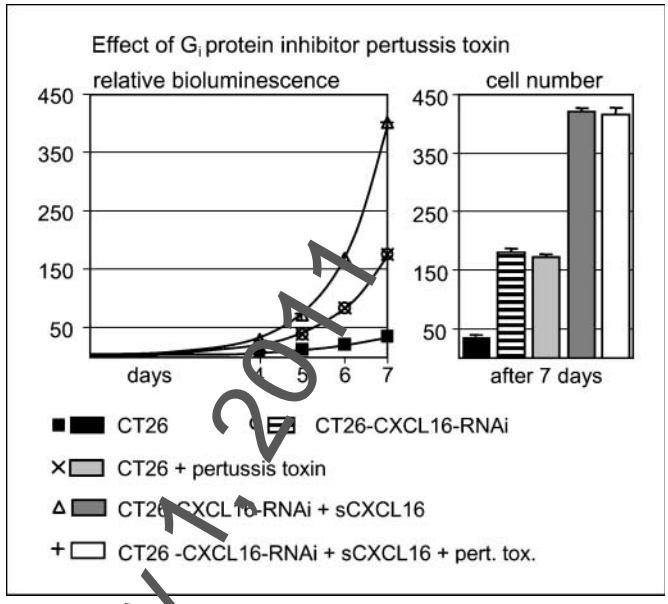

Figure 6. Effect of pertussis toxin on proliferation of CT26 cells, measured by bioluminescence (*left*) and by counting cells after 7 d (*right*). *Columns,* mean; *bars,* SE. An experiment with Panc02 cells yielded similar results.

CXCR6-negative cells (CXCL16-KDEL or CXCR6-RNAi), showing that its effect is mediated by CXCR6. These results clearly show that growth is reduced by TM-CXCL16 but enhanced by sCXCL16. To show the relevance for growth *in vivo*, we s.c. injected the KEP1 transfectants. The TM-CXCL16-KEP1 tumors grew slower (Td = 83 versus 48 h; $P < 0.0001$) but not when CXCR6 was suppressed by RNAi (Fig. 5*C*). In contrast, the sCXCL16-KEP1 tumors grew faster (Td = 36 versus 48 h; $P < 0.0001$), similarly as cells *in vitro*. The growth rate of TM-CXCL16-KEP1 tumors remained constant and did not increase later, as would have been expected if substantial amounts of the inhibitory TM-CXCL16 would be cleaved in the tumor and converted to the stimulatory sCXCL16.

Reduction of proliferation is due to G_i protein–transmitted signals. The signals responsible for reduced proliferation could be transmitted by either CXCR6 or CXCL16. Because CXCR6 belongs to the superfamily of G protein–coupled receptors, we tested this using pertussis toxin that ADP-ribosylates G_i proteins and impedes their function. Strikingly, pertussis toxin enhanced proliferation to the same extent as suppression of either CXCR6 or CXCL16, as illustrated using CXCL16 RNAi-suppressed CT26 cells in Fig. 6. The toxin did not further enhance proliferation of cells in which CXCR6-CXCL16 interaction was blocked (Fig. 6), indicating that the effect of the toxin is entirely due to inhibition of signals triggered by this interaction. This strongly indicates that the relevant signals are triggered by CXCR6. In contrast, the increase in proliferation induced by the soluble chemokine was not affected by pertussis toxin (Fig. 6), although it was certainly mediated by CXCR6 because it was not observed when CXCR6 was suppressed (data not shown for CT26 cells, but see Fig. 5*B* for KEP1 cells). Clearly, G_i protein is not involved in stimulation of proliferation by sCXCL16.

Discussion

We have shown here that the chemokine receptor CXCR6 as well as its ligand CXCL16 are expressed by mammary, colon, and pancreatic carcinoma cell lines and in all human primary breast

carcinomas examined. Previously, we reported that binding of ligands to other receptors of this subfamily, CXCR5 (12), CXCR4, and CXCR7[5] enhanced proliferation of carcinoma cells. We now show that the same is true for CXCR6 (i.e., when activated by the soluble chemokine). However, CXCL16 is exceptional in that it is synthesized as a transmembrane protein from which the soluble chemokine has to be cleaved off. This transmembrane form of the protein was found at substantial levels on the carcinoma cell surface. Surprisingly, and in contrast to the soluble chemokine, it caused a reduction in proliferation on binding to CXCR6.

This conclusion is based on the following evidence. First, suppression of CXCR6 by an intrakine that traps it in the ER (26, 28), as well as knockdown of either CXCR6 or CXCL16 by RNAi, led to greatly enhanced proliferation of the three cell lines tested (CT26 and HCT116 human colon carcinoma and Panc02 mouse pancreatic carcinoma). Second, it was due to direct interaction between the two proteins because blocking antibodies against either CXCL16 or CXCR6 enhanced proliferation to the same extent as by CXCR6 or CXCL16 suppression. Third, introduction of TM-CXCL16 into CXCL16-negative KEP1 cells also led to reduced proliferation but not in cells in which CXCR6 was suppressed, whereas expression of the sCXCL16 instead enhanced growth similarly as externally applied chemokine.

An obvious difference between the two forms of CXCL16 is that the transmembrane protein cannot be taken up by CXCR6 on an adjacent cell, whereas the soluble chemokine is readily internalized. Signals triggered by the TM-CXCL16 are therefore likely persistent, in contrast to transient signals induced by the soluble chemokine. This might explain the opposite effects because growth inhibition by persistent signaling has been described for several G protein–coupled receptors. Examples are gonadotrophin-releasing hormone (GRHR), somatotrophin (sst2), and M3 muscarinic acetylcholine (29–31) receptors that cause persistent activation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2, p38, and c-Jun NH_2-terminal kinase, respectively. Whereas the growth-promoting M2 and sst3 receptors are rapidly degraded after internalization, the M3 and sst2 receptors are rapidly recycled to the surface (32, 33), which is a likely explanation for the persistence of the signals. Furthermore, the growth-promoting AT1 angiotensin receptor is rapidly internalized, whereas the inhibitory AT2 receptor remains at the cell surface (34, 35), and also the inhibitory GRHR is not internalized (36). These observations indicate that opposite effects of closely related G protein–coupled receptors on proliferation are due to transient versus persistent signaling. Our results suggest that this may even be true for the same receptor, CXCR6, binding to ligands that can either be internalized or not. Alternative explanations can, however, not be excluded. It is, for example, conceivable that proliferation signals are delivered from the endosome (37) and would therefore require internalization of receptor with the ligand, which is not possible for the transmembrane form of CXCL16. However, internalization of the receptor seems to be less important for G protein–coupled receptors compared with receptor tyrosine kinases (38). The relevant signal pathways for CXCR6 are also qualitatively different because growth reduction was impaired by pertussis toxin, similarly as described for GRHR and sst2 receptors (29, 30), and therefore dependent on G_i proteins. In contrast, G_i is not involved in growth enhancement by sCXCL16, as we have previously also observed for CXCR5 (12).

We found that both CXCR6 and CXCL16 were expressed by all 170 examined mammary carcinomas at similar levels comparable with those of eight mammary carcinoma cell lines tested. Because the latter all had substantial amounts of both proteins on the cell surface, and because the carcinoma specimens contained at least 50% carcinoma cells, the microarray data indicate that the carcinoma cells in the tumors had surface levels comparable with the cell lines. CXCR6 has also been detected in nasopharyngeal carcinomas (39), gliomas (40), melanomas (41), and part of colon carcinomas (42). We observed neither receptor nor ligand in normal mouse mammary epithelial cells and, except for very low levels of CXCL16, in nontransformed MCF10 human mammary carcinoma cells. This suggests that expression is tumor specific, at least in the mammary gland. In the colon, normal epithelial cells have been reported to express CXCR6, whereas carcinoma cells sometimes did not (42), so it remains to be determined whether our data on colon carcinoma cell lines are representative for primary colon tumors. However, the reported expression in normal colon differs from what has been reported for mice (18) and is also not in line with available expression data for human tissues,[10] so this remains to be established.

CXCL16 in tumors has thus far only been reported for colon carcinomas, ~15% of which showed high CXCL16 expression that was associated with a better prognosis (43). The suggested reason for this was an enhanced immune response due to influx of T lymphocytes triggered by CXCL16 chemokine. Our results suggests the alternative explanation that the tumors had reduced growth rates due to CXCR6-CXCL16 interaction.

Expression of the two proteins in all human primary breast cancers suggests an essential function. This is, however, clearly not true for advanced stages of tumorigenesis because CXCR6-suppressed tumors grew well *in vivo* and in fact grew much faster, also in metastases. Furthermore, KEP1 cells did not express CXCL16. They were derived from a mammary carcinoma that arose in p53 knockout mice with conditional E-cadherin deficiency in skin and mammary gland (44). These tumors closely mimic human invasive lobular carcinomas, including high metastatic capacity. KEP1 tumors arose from cells that already contained two oncogenic mutations, perhaps allowing the cells to skip early steps in tumorigenesis. It is therefore possible that CXCR6 and CXCL16 play a role in those early stages. Their expression may be induced by inflammation mediators that are essential for tumor formation (45). In this context, it is noteworthy that CXCL16 deficiency increases the severity of lesions in atherosclerosis-prone mice (46), quite in contrast to the expected reduction. This suggests that the CXCL16-CXCR6 interaction does not promote but rather attenuates inflammation. Perhaps CXCL16 has a similar protective role during tumor formation.

If the two proteins are not essential, the question arises why expression is maintained because any cell that loses either CXCR6 or CXCL16 has a growth advantage and should be selected during tumor progression and overgrow the tumors. One possibility is that sCXCL16 is cleaved off in the tumors and then actually increases proliferation. The microarray data of the primary breast cancers indicate that ADAM-10 and ADAM-17, two proteases that can catalyze this cleavage (24, 25), are not or marginally expressed. Several other members of the ADAM family and other proteases are present, however, and it remains to be

[10] http://symatlas.gnf.org

determined whether any of these can cleave CXCL16 as well. Cleavage would be expected to lead to accelerated growth later during tumor formation but this was not observed (see for example Fig. 5*C*) in the model tumors we used. This may be different in spontaneous mammary tumors that often contain macrophages that are known to stimulate tumor growth (47). ADAM-10 is produced by activated macrophages (16). This may not be readily detected in microarray analysis of whole tumors but locally, near macrophages, the levels may be sufficient and CXCL16 cleavage may thus contribute to stimulation of proliferation by macrophages. These notions will be addressed in our future research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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