Cancer Research

The Chemokine Receptor CXCR7 Is Highly Expressed in Human Glioma Cells and Mediates Antiapoptotic Effects

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Abstract

The chemokine CXCL12/stromal cell–derived factor-1 and its receptor CXCR4 play a major role in tumor invasion, proliferation, and metastasis. Recently, CXCR7 was identified as a novel, alternate receptor for CXCL12 and CXCL11/I-TAC. Because both chemokines are expressed abundantly in human astrocytomas and glioblastomas, we investigated the occurrence and function of both receptors in astroglial tumors. *In situ*, CXCR7 is highly expressed on tumor endothelial, microglial, and glioma cells whereas CXCR4 has a much more restricted localization; CXCL12 is often colocalized with CXCR7. CXCR7 transcription in tumor homogenates increased with malignancy. *In vitro*, CXCR7 was highly expressed in all glioma cell lines investigated whereas CXCR4 was only scarcely transcribed on one of eight lines. In contrast, a tumor stem-like cell line preferentially expressed CXCR4 which diminished upon differentiation, whereas CXCR7 increased drastically. Stimulation of CXCR7-positive glioma cells (CXCR4- and CXCR3-negative) by CXCL12 induced transient phosphorylation of extracellular signal-regulated kinases Erk1/2, indicating that the receptor is functionally active. The phosphoinositide-specific phospholipase C inhibitor U73122 effectively inhibited Erk activation and suggests that the mitogen-activated protein kinase pathway is activated indirectly. Whereas proliferation and migration were little influenced, chemokine stimulation prevented camptothecin- and temozolomide-induced apoptosis. The selective CXCR7 antagonist CCX733 reduced the antiapoptotic effects of CXCL12 as shown by nuclear (Nicoletti) staining, caspase-3/7 activity assays, and cleavage of poly(ADP-ribose) polymerase-1. Thus, CXCR7 is a functional receptor for CXCL12 in astrocytomas/glioblastomas and mediates resistance to drug-induced apoptosis. Whereas CXCR7 is found on "differentiated" glioma cells, the alternate receptor CXCR4 is also localized on glioma stem-like cells. *Cancer Res; 70(8); 3299–308. ©2010 AACR.*

Introduction

Chemokines, a group of small (8–15 kDa) chemotactic cytokines, and their receptors have been discovered as essential mediators of leukocyte migration in the immune system. However, they also play a critical role in tumor initiation, promotion, progression, and metastasis (1). The chemokine CXCL12 (or stromal cell–derived factor-1) seems to be of particular importance in tumor biology, especially in tumor metastasis. Interaction of CXCL12 with its receptor CXCR4 that is expressed on some tumor cells directs them to peripheral tissues like lung, liver, lymph nodes, or bone marrow in which the ligand is constitutively expressed (2). Furthermore, the CXCL12/stromal cell–derived factor-1–CXCR4 axis promotes paracrine tumor growth, tumor cell invasiveness, and tumor angiogenesis (3–6). Recently, a novel receptor for CXCL12 has been identified, termed CXCR7/RDC1 (7, 8). Aside from CXCL12, CXCR7 binds with 10-fold lower affinity CXCL11/I-TAC (IFN-inducible T cell α chemoattractant) which is also a ligand for CXCR3 (that is also targeted by CXCL9/Mig and CXCL10/IP-10).

In healthy mouse, CXCR7 is expressed in cardiomyocytes, osteocytes, and brain cells (9–11). CXCR7-deficient mice die prenatally with severe heart defects that are also reported for CXCL12 and CXCR4 knockout animals (10). In humans, CXCR7 is—as far as we know—expressed on tumor-associated blood vessels and on distinct malignant cells including breast, lung, and prostate cancer cells (8, 12–14). The biological importance of CXCR7, its signal transduction, and further effects apparently depend on the cell types investigated. In breast cancer cells, CXCR7 activation fails to cause Ca^{2+} mobilization or cell migration, but provides growth advantage (8), whereas in prostate cancer cells, CXCR7 expression is associated with enhanced adhesive and invasive activities in addition to increased survival (13).

Because CXCL12 is essential for brain development and adult brain homeostasis by regulating neuronal migration and neurogenesis (15), and is highly expressed in the embryonic

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and adult brain, we investigated whether its newly discovered receptor CXCR7 is also expressed in normal or malignant human brain tissues. Previously, CXCL12 and CXCR4 have been detected in human gliomas (3–5), but CXCR4 expression seemed to be limited only to a very small subset of glioma cells (6, 16, 17). Here, we show that CXCR7 is much higher and broader expressed in astrocytoma and glioblastoma cells than in normal brain tissue. CXCR4 is found on glioma stem-like cells and is diminished upon differentiation, whereas CXCR7 is increased. Moreover, CXCR7 is functionally active in glioma cell lines and inhibits apoptosis induced by camptothecin and temozolomide.

Materials and Methods

Peptides and inhibitors. Recombinant human cytokines/factors were from Immunotools, Tebu-bio, or Chemicon. U0126 and camptothecin were from Biomol, U73122 and KT5720 were from Calbiochem and were dissolved in DMSO, whereas CCX733 was from ChemoCentryx. Temozolomide (Sigma-Aldrich) was adjusted to stocks of 20 mg/mL in DMSO; the final concentration was 400 μg/mL.

Cell culture and tissue samples. Surgically dissected human malignant and normal brain samples were obtained in accordance with the Helsinki Declaration of 1975, with approval from the ethics committee. Human glioma cell lines were from Deutsches Krebsforschungszentrum (Heidelberg, Germany) or cultivated from tumors in DMEM (Invitrogen) plus 10% FCS (18); other cell types were obtained as described (19). Short-term glioma cell cultures were cultivated in DMEM plus 10% FCS (primary cultures) or in neurosphere medium (20) plus 10 ng/mL of basic fibroblast growth factor (bFGF), and 20 ng/mL epidermal growth factors (EGF; ref. 21; glioma spheres). The glioma stem-like cell lines 25/07 (with/without leukemia-inhibitory factor, LIF) were cultured over 1 y to enrich cells with self-renewal capacities. These cell lines were characterized by the formation of neurospheres, the ability to survive and proliferate under stem cell conditions and to differentiate into cells of different lineages (astroglial, neuronal, and oligodendroglial). For differentiation, cells were dissociated, recovered for 3 d with neurosphere medium containing bFGF and EGF (−/+LIF) and changed to neurosphere medium with 10% FCS.

Quantitative reverse transcription-PCR. RNA was isolated with Trizol (Invitrogen), digested by DNase, cDNA was synthesized and real-time reverse transcription-PCR (RT-PCR) was performed (19) using TaqMan primer probes (Applied Biosystems): *hGAPDH* (Hs99999905_m1), *hCXCL11* (Hs00171138_m1), *hCXCL12* (Hs00171022_m1), *hCXCR3* (Hs00171041_m1), *hCXCR4* (Hs00607978_s1), *hCXCR7* (Hs00664172_s1), *hGFAP* (Hs00157674_m1), *hSox2* (Hs 00602736_s1), *hMusashi1* (Hs 00237052_m1). Five nonmalignant brain specimens, seven astrocytomas (grade 1), six astrocytomas (grade 2), seven astrocytomas (grade 3), and up to 14 glioblastomas (WHO grade 4) were analyzed. Cycles of thresholds (C_T) were determined with an ABI PRISM 7000 sequence detection system and ΔC_T values = C_T gene of interest − C_T GAPDH calculated. ΔC_T = 3.33 corresponds to one magnitude lower gene expression compared with GAPDH. For each gene, logarithmic linear dependence of C_T values from copy numbers was verified by using different amounts of cDNA.

Immunohistochemistry/cytochemistry. Immunohistochemistry was performed (22) with cryostat sections fixed with ice-cold acetone/methanol (1:1), blocking with Sudan black and with 0.5% glycine/0.5% bovine serum albumin, and incubation with primary (overnight, 4°C) and secondary antibodies (1 h, 37°C). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Primary antibodies were anti-CXCL12 (rabbit, 1:100; Abcam), anti-CXCR4 (rabbit, 1:100; Imgenex, or goat, 1:500; Abcam), anti-CXCR7 (rabbit; 1:50 or 1:100, Abcam: ab12780 and ab 72100), anti-glial fibrillary acidic protein (GFAP; mouse monoclonal, 1:100; DAKO), anti–Iba-1 (goat, 1:100; Santa Cruz Biotechnology, Inc.), anti–MIB-1 (mouse monoclonal, 1:100; DAKO; MIB = made in Borstel, corresponding to Ki-67), anti-Pecam (goat, 1:100; Santa Cruz Biotechnology). Secondary antibodies were goat anti-rabbit IgG Alexa Fluor 555-labeled, donkey anti-rabbit IgG Alexa fluor 488-labeled, goat anti-mouse IgG Alexa Fluor 488-labeled, and donkey anti-goat IgG Alexa Fluor 488-labeled and donkey anti-goat IgG Alexa Fluor 555-labeled (Invitrogen; diluted 1:1,000). For costainings, mixtures of primary and secondary antibodies of different species were applied simultaneously. Costainings with two rabbit antibodies had an additional blocking step with donkey anti-rabbit F(ab)′ fragments (Dianova) following the first primary and secondary antibody incubations. Glioma stem-like cells were fixed with acetone and stained as above with anti-βIII tubulin (mouse monoclonal, 1:500; AbD Serotec) and anti-GFAP (mouse monoclonal, 1:100; DAKO); goat anti-mouse IgG Alexa Fluor 488 served as secondary antibody.

Stimulation of cells. For kinase phosphorylation analysis, A764 and U343 glioma cells were washed serum-free and stimulated in DMEM with 1% bovine serum albumin. 25/07 glioma stem-like cells were kept in neurosphere medium without bFGF and EGF for 36 h prior to stimulation and stimulated in DMEM/F12 with 0.5% BSA. Kinase inhibitors were applied and preincubated for 1 h before adding the stimulators. CCX733 was preincubated for 30 min (for caspase-3/7 activity assay, 10 nmol/L) or applied simultaneously with other stimuli (Nicoletti staining, 100 nmol/L). All inhibitor concentrations were maintained during experiments. Camptothecin and temozolomide stimulations were performed in DMEM plus 0.2% FCS (A764 and U343) or in neurosphere medium with bFGF and EGF.

Western blotting. Western blot experiments were performed as described (23). Lysates from 25/07 glioma stem-like cells were adjusted to ~5,000 cells/lane; otherwise, 5 μg of protein was applied. Blots were incubated with anti-CXCR7 (rabbit, 1:500; Abcam), reblotting with anti–caveolin-1 (N-20, rabbit, 1:200; Santa Cruz Biotechnology), anti-pErk1/2 (Thr^{202}/Tyr^{204}; 1:500), anti-pAkt (1:350), or anti-pp38 (1:250; Cell Signaling Technology), reprobed after stripping with methanol and 0.1 mol/L glycine/HCl buffer (pH 2.5) with anti-Erk2 (1:500; Santa Cruz Biotechnology) or anti-Akt (1:300; Cell Signaling Technology). For analysis of poly(ADP-ribose) polymerase (PARP) cleavage, blots were incubated

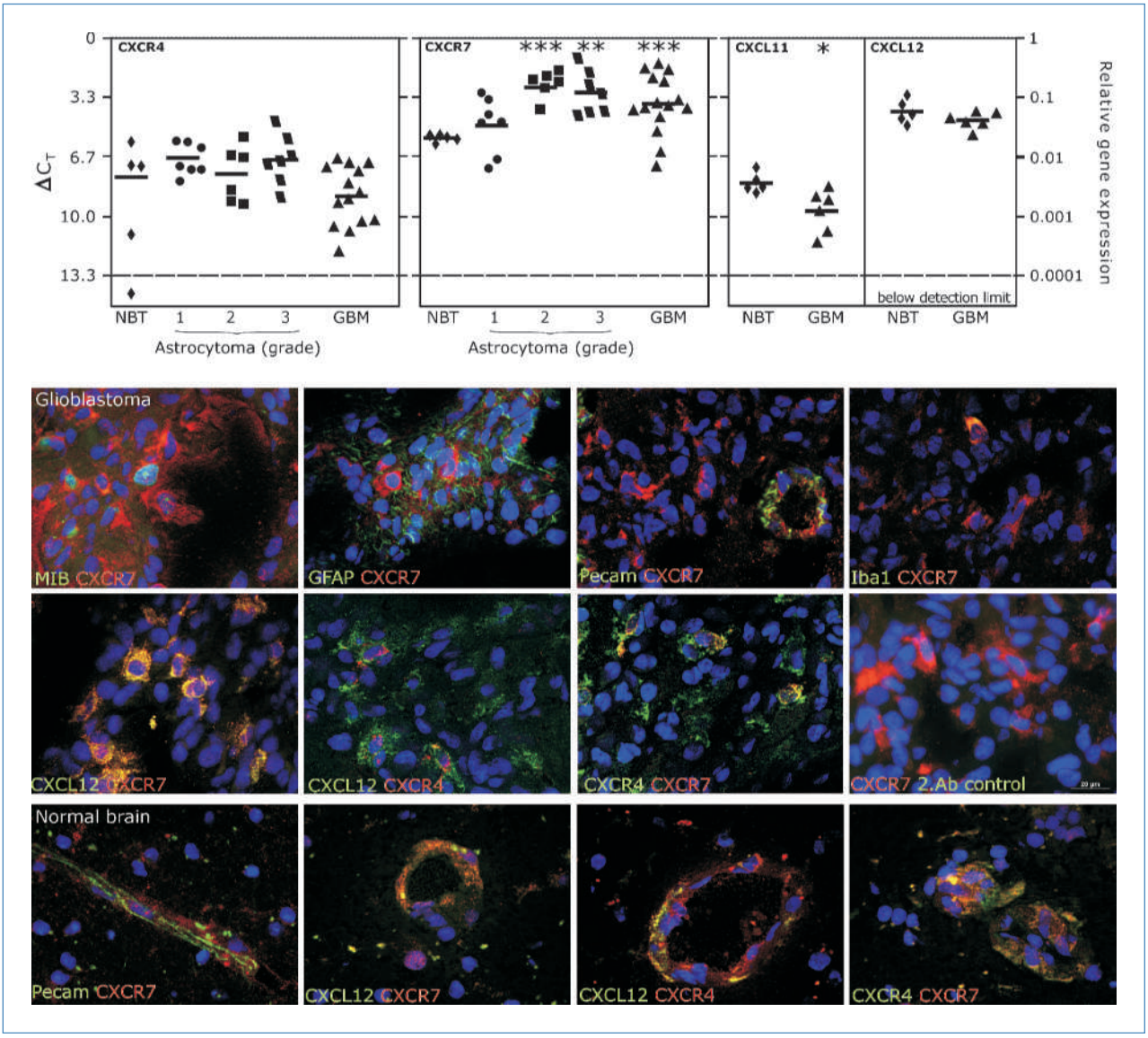

Figure 1. Transcription and fluorescence-immunohistochemistry of chemokine receptors, their ligands, and cellular markers in human astroglial tumors and normal brain. Top, solid astrocytomas of different WHO grades [7 WHO grades 1 and 3, 6 grade 2, and 14 glioblastoma multiforme (GBM)] and five normal brain tissue samples were analyzed for mRNA expression by quantitative RT-PCR. ΔC_T values to GAPDH are given; $\Delta C_T = 3.33$ corresponds to one magnitude. CXCR4 expression shows great variations in homogenates. CXCR7 is particularly elevated in high-grade astrocytomas and glioblastomas. Bottom, costainings (examples) of CXCR7 (red/blue, nuclear counterstain) with CXCR4 and CXCL12 or cell type–specific markers (green) in tissue sections. In normal brain, CXCR7 and CXCR4 expression are restricted to Pecam-positive endothelial cells/capillaries. Here, the receptors are often colocalized. Their ligand, CXCL12, is often found bound to the capillaries and its fluorescence signals often merge with that of CXCR7, less than with CXCR4. In glioblastomas, CXCR7 expression is found throughout samples with varying intensities. CXCR7 is localized on endothelial cells, but in particular on tumor cells positive for the proliferation marker MIB/Ki67 and in GFAP-positive regions; also, Iba1-positive microglial cells/monocytes are positive. CXCL12 fluorescence frequently merged with that of CXCR7, indicating that the receptor is often occupied by its ligand. CXCR4 was only detected on a few cells and is rarely colocalized with CXCR7. Antibody control is shown for costaining with antibodies of the same species; other controls were negative.

with anti-cleaved PARP (Asp124, 1:500; Cell Signaling) and reprobed with GAPDH (1:250; Santa Cruz Biotechnology).

Migration and proliferation assays. Migration was analyzed in wound-healing assays (Scratch assay) in DMEM + 0.2% FCS (23). Proliferation was determined with subconfluent cells after 24 h in DMEM containing 0.2% FCS BSA by the measurement of DNA with CyQuant reagent (18) and normalized to absolute cell number (three individual dishes for each stimulus).

Apoptosis and caspase assays. For modified Nicoletti staining (24), cells were seeded on poly-D-lysine–coated coverslips, grown overnight, and stimulated in DMEM + 0.2% FCS (24 or 48 h). After rinsing with PBS, cells were fixed with ice-cold acetone/methanol (1:1, for 10 min), washed, and

nuclei stained with DAPI. Apoptotic nuclei were evaluated and counted by a person without bias. For caspase activity assays, cells were seeded on 35 mm six-well plates, precultivated for 48 h, and stimulated in DMEM + 0.2% FCS for 48 h. Caspase-3/7 activity was measured with a homogenous luminescent assay (Caspase-Glo, Promega). The luminescence signal was normalized to absolute cell numbers that were counted in parallel with a flow cytometer (Galaxy Argon Plus, DAKO).

Statistical analysis. Values are given as means ± SD. Statistical significance was analyzed by a two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

***CXCR7 is highly expressed in astrocytomas and glioblastomas* in situ.** In an initial step, we investigated the expression of the chemokine receptors, CXCR7 and CXCR4, as well as their ligands, CXCL12 and CXCL11, in malignant and nonmalignant brain samples by quantitative RT-PCR and immunohistochemistry (Fig. 1). CXCR4 transcription showed great variations in normal brain and astrocytoma tissues, but did not significantly change with malignancy. In contrast, CXCR7 transcription significantly increased in high-grade astrocytomas as compared with normal brain tissues. Transcription of the ligands was comparable or even a bit lower in tumor samples than in normal brain.

To identify cells that express chemokine receptors in astroglial tumors, we performed fluorescence coimmunostainings of CXCR7 together with nuclei, CXCR4, CXCL12, proliferation, and cell type–specific markers (Fig. 1, bottom). In normal brain, CXCR4 and CXCR7 were localized mainly on endothelial cells positive for their marker Pecam (platelet/endothelial cell adhesion molecule-1, CD31). On capillaries, both receptors were frequently colocalized. The ligand CXCL12 was found near the endothelial receptors (and its immunofluorescence often merged with that of the receptors, in particular with that of CXCR7), but also at other sites

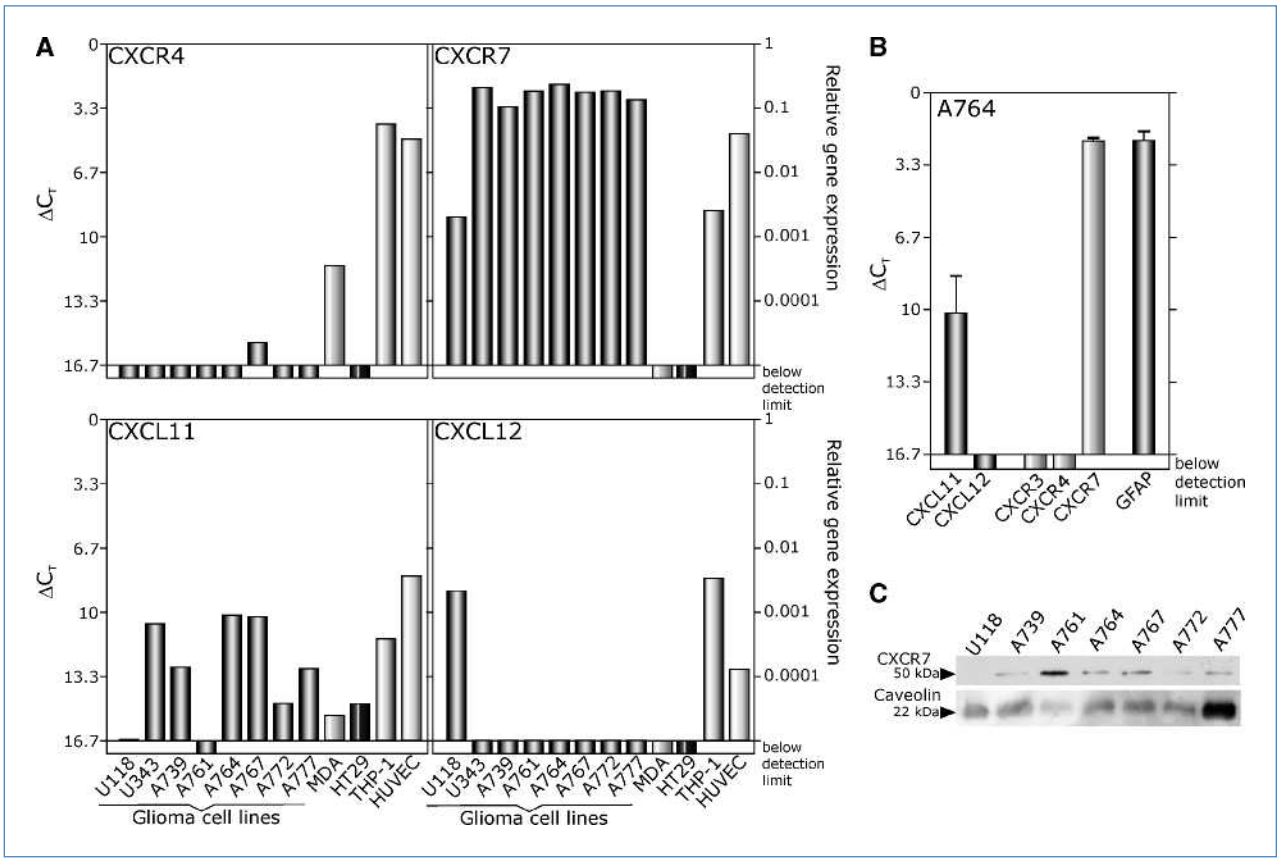

Figure 2. Expression of chemokine receptors and ligands in cultivated human glioma and other cells as measured by quantitative RT-PCR. A, CXCR4 transcription was absent or very low (one of eight) in glioma cell lines, whereas CXCR7 was highly transcribed in all glioma cells. Both receptors were highly transcribed in human endothelial cells (HUVEC) and moderately in the THP-1 monocyte cell line. CXCR7 is absent in breast (MDA-MB 231) and colon (HT29) carcinoma cells, CXCR4 only in breast tumor cells (of the malignant cells tested). The ligands CXCL11 and CXCL12 are transcribed in some glioma cells, in monocytes and endothelial cells at moderate levels. B, the A764 glioma cell line chosen for functional experiments transcribes CXCR7, but not CXCR4 or CXCR3; CXCL11 is transcribed at moderate levels, but not CXCL12. A764 cells are GFAP positive. Means ± SD are given from $n = 3$ independent RNA isolations from different subcultures. C, Western blots corroborate the results of CXCR7 transcription on glioma cells (protein bands at 50 kDa), loading was controlled by reblotting with caveolin-1 ($n = 2$).

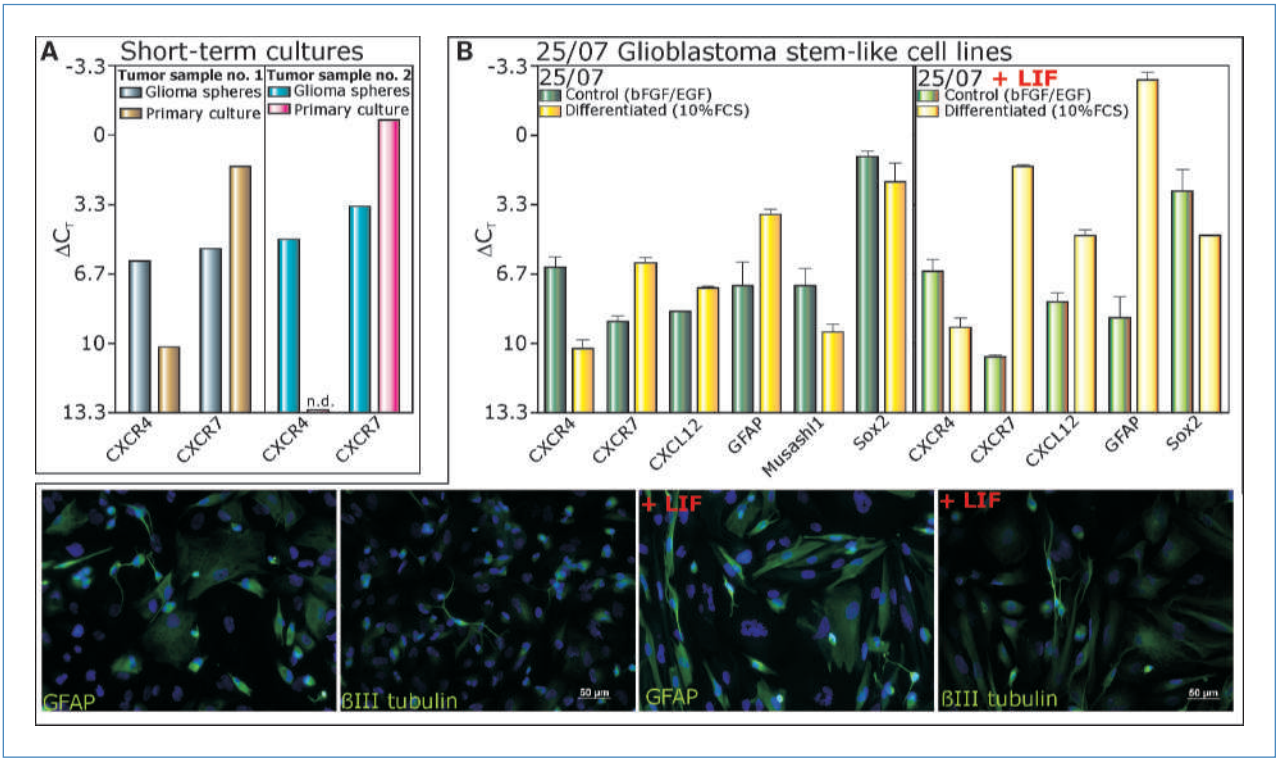

Figure 3. A, CXCR4 and CXCR7 expression in glioma spheres and corresponding primary cultures of two individual glioblastoma samples. Cells were grown separately in neurosphere medium or in DMEM + 10% FCS, and RNA isolated after 10 to 15 d. CXCR4 was not detectable or was transcribed 10-fold lower in primary compared with sphere cultures, whereas CXCR7 expression was up to one magnitude higher in primary compared with corresponding glioma sphere cultures. B, expression patterns of CXCR4, CXCR7, and CXCL12 in undifferentiated and differentiated 25/07 glioma stem-like cells (generated without/with 10 ng/mL LIF over 1 y). Differentiation was induced by 10% FCS, and cells compared by quantitative RT-PCR and immunocytochemistry. Differentiated cells expressed glial (GFAP) and neuronal (βIII tubulin) markers, whereas stem cell markers (Musashi1 and Sox2) were decreased. In both independent experiments, differentiation decreased CXCR4 expression by about one magnitude and increased CXCR7 expression by one to several magnitudes. CXCL12 was moderately upregulated (n = 2 individual subcultures).

in the parenchyma. In glioblastomas, tumor vessels were also CXCR4- and CXCR7-positive. However, most CXCR7 immunoreactivity was found in GFAP-positive (glial fibrillary acidic protein, astroglial-marker) regions and on proliferating cells (proliferation marker MIB = Ki67). Iba1-positive cells were also positive for CXCR7 (Iba-1: macrophage/microglial-marker). Fluorescence of CXCL12-staining typically merged with that of CXCR7 (green and red fluorescence yielding yellow), indicating that CXCL12 is frequently bound to CXCR7. The alternate receptor CXCR4 was detected on only few cells often clustered in groups, within the tumor and rarely colocalized with CXCR7 (except capillaries). On some tumor cells, CXCL12 was also detected in the vicinity of CXCR4, but fluorescence merged less (as compared with CXCR7).

In normal brain, CXCR7 and CXCR4 are mainly localized only on endothelial cells. In glioblastomas, CXCR7 is particularly expressed on the tumor cells and CXCR4 on a very small subpopulation of them; tumor endothelial cells and macrophages are also CXCR7-immunopositive.

***CXCR7 is highly expressed in glioma cells* in vitro.** Next, we determined CXCR7 in cultivated glioma cells (Fig. 2). All eight glioma cell lines investigated highly transcribed CXCR7 mRNA, whereas CXCR4 was hardly detectable in only one of them (Fig. 2A). CXCR7 transcription in glioma cells was typically 20- to 100-fold higher than in endothelial cells (human umbilical vein endothelial cells) or the monocytic cell line THP-1; a breast cancer (MDA-MB-231) and colon carcinoma (HT29) cell line proved to be CXCR7-negative. CXCR7-expression was confirmed at the protein level by Western blot experiments yielding specific bands at ~50 kDa in all cells with high mRNA transcription (Fig. 2C). CXCR4 mRNA was detected at high levels in cultivated endothelial as well as monocytic cells, at moderate levels in the CXCR7-negative breast cancer cells. The CXCR7 ligands, CXCL11 and CXCL12, were transcribed by some glioma cell lines and also by the monocytic cell line as well as by endothelial cells (Fig. 2A). CXCR7 expression could not be regulated by hypoxia (5% O_2) nor by stimulation with its ligands, cytokines (tumor necrosis factor-α, IFNγ, interleukin-1β, interleukin-6), or growth factors (EGF, bFGF, VEGF) in A764 glioma cells (data not shown).

Functional studies were then performed with the glioma cell lines A764 and U343 that express high levels of CXCR7, but not CXCR4 nor CXCR3; they do not transcribe the ligand CXCL12, but CXCL11 at low to moderate levels (Fig. 2B and C).

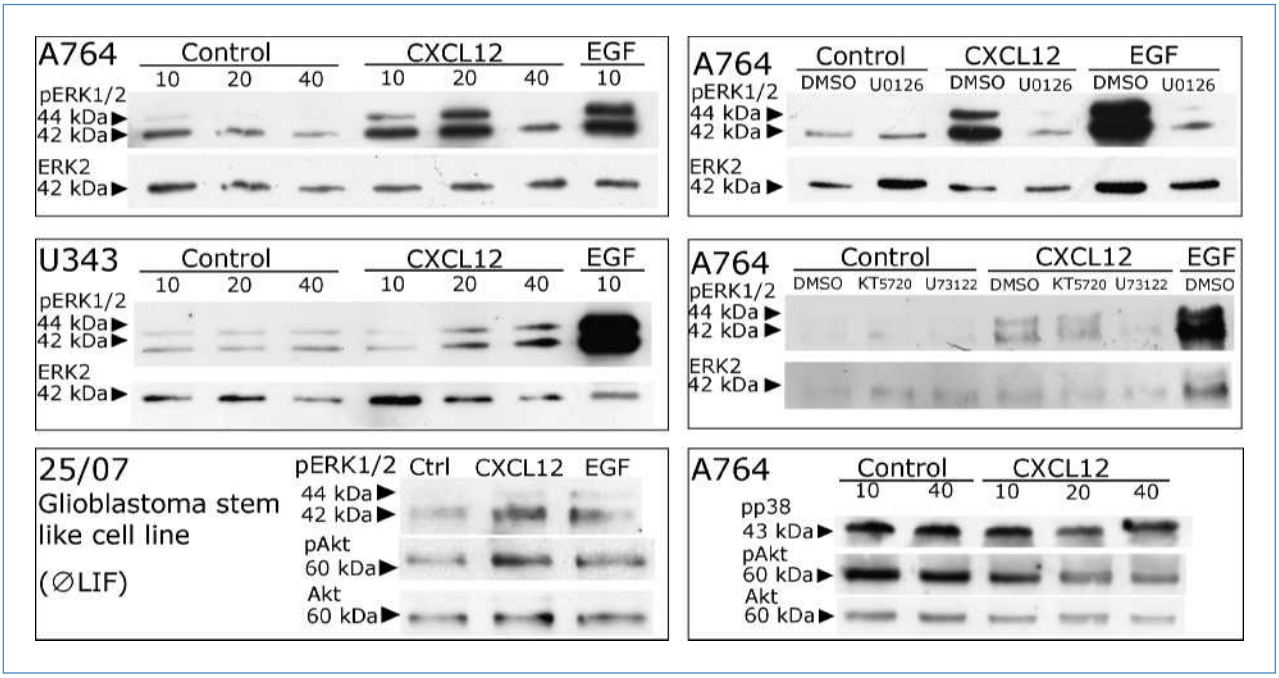

Figure 4. Signal transduction of CXCR7 in human A764/U343 glioma and glioma stem-like cells. Left, stimulation with 1 nmol/L of CXCL12 in serum-free medium induces transient phosphorylation of extracellular signal-regulated kinases Erk1/2 in A764 and U343 glioma cells and of Erk1/2 as well as Akt in glioma stem-like cells (15 min). Reblots with antibodies to nonphosphorylated kinases ensures equal loading; EGF was used as a positive control. Right, in A764 cells, Erk phosphorylation after 20 min could be inhibited by preincubation with U0126 (100 μmol/L, 1 h), a selective inhibitor of the upstream kinase to Erk, MEK; controls with vehicle DMSO. Erk phosphorylation after 20 min could also be inhibited by preincubation with 10 μmol/L of U73122, an inhibitor of phosphoinositide-specific phospholipase C, but only slightly with 2 μmol/L of KT5720, an inhibitor of protein kinase A. Stimulation with 1 nmol/L of CXCL12 does not induce activation of p38 or Akt. Blots are representative examples of at least three individual stimulations, each with different chemoluminescence systems and exposure times that could account for the different relative intensities between individual blots.

Short-term glioma stem-like cell cultures show higher CXCR4 and lower CXCR7 expression levels than corresponding primary cultures. Short-term cultures were obtained in parallel from glioblastoma samples in neurosphere and conventional medium. In neurosphere medium, cells formed free-floating aggregates within a few days and were analyzed after 10 to 15 days. In conventional cultures in medium plus 10% FCS, cells grew adherent and were harvested after the first subcultivation. Whereas glioma sphere cultures expressed moderate levels of CXCR4 in two independent

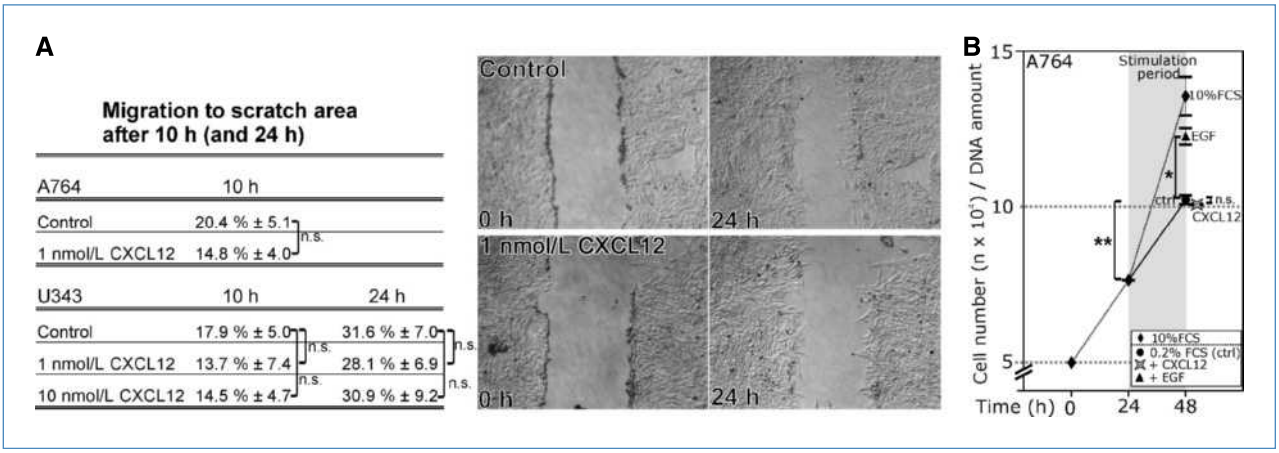

Figure 5. Influence of CXCL12 on proliferation and migration of human glioma cells. A, migration of A764 and U343 glioma cells in a wound-healing assay is not significantly affected by 1 nmol/L of CXCL12 (DMEM + 0.2% FCS). B, proliferation is not significantly increased by 1 nmol/L of CXCL12 (serum-free medium), but by 10 ng/mL of EGF or 10% FCS.

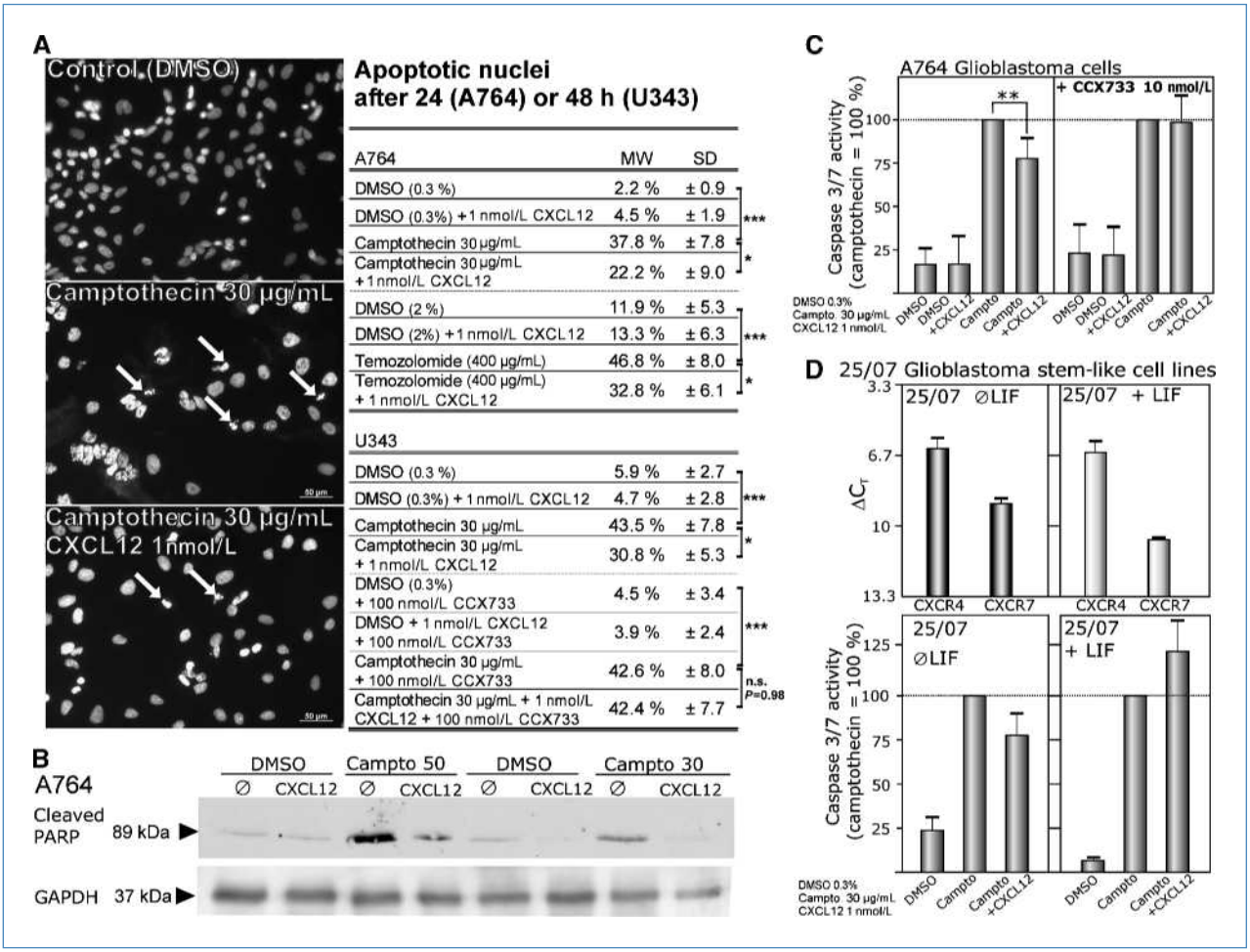

Figure 6. CXCL12-mediated effects on apoptosis in human glioma and glioma stem-like cells. A, apoptosis induced by camptothecin (30 µg/mL) and temozolomide (400 µg/mL) is significantly reduced by 1 nmol/L of CXCL12; cells were stimulated in DMEM + 0.2% FCS for 24 h (A764) or 48 h (U343) and apoptotic nuclei (arrows) were counted. This effect could be blocked by simultaneous incubation with the CXCR7 antagonist CCX733 (100 nmol/L; $n = 4$). *, $P < 0.05$; ***, $P < 0.001$; B, camptothecin-induced (50 and 30 µg/mL) cleavage of PARP is rigorously reduced by costimulation with 1 nmol/L of CXCL12 (24 h, DMEM + 0.2% FCS); camptothecin solvent DMSO serves as control. C, caspase-3/7 activity in A764 cells (with high CXCR7 transcription) induced by camptothecin (30 µg/mL) is reduced by coincubation with 1 nmol/L of CXCL12; the CXCR7 antagonist CCX733 completely abolishes this antiapoptotic effect (cells stimulated in DMEM + 0.2% FCS for 24 h). **, $P < 0.01$. D, camptothecin-induced caspase-3/7 activity in glioma stem-like cells is not significantly reduced by CXCL12 (24 h). In particular, apoptosis cannot be reduced in cells with very low transcription (qRT-PCR) of CXCR7 but high transcription of CXCR4 (those precultivated with 10 ng/mL **, of LIF).

preparations, in conventional primary cultures, CXCR4 transcription was reduced to the tenth part or were not even detectable (Fig. 3A). In contrast, CXCR7 expression was more than one magnitude higher in conventional primary than in corresponding sphere cultures. Thus, stem cell–favoring conditions (neurosphere medium) preserved CXCR4 expression whereas CXCR7 was markedly higher under conventional culture conditions.

CXCR4 on glioma stem-like cells diminishes upon differentiation whereas CXCR7 increases. The influence of differentiation was investigated with the glioma stem-like cell line 25/07, which was established by long-term culture from a glioblastoma in the absence or presence of LIF. Like glioma spheres in short-term culture, these cells showed high CXCR4 and low CXCR7 transcription. Upon differentiation with 10% FCS, CXCR4 diminished and CXCR7 increased, in particular, in LIF-precultivated cells; here, CXCR7 transcription reached similar values as found in glioma cell lines (Fig. 3B). Differentiation also strongly increased the astroglial marker GFAP; the neuronal marker βIII-tubulin was detectable on a small number of cells. On the contrary, the stem cell markers Musashi1 and Sox2 decreased with differentiation. Thus, CXCR4 is found more on glioma stem-like cells whereas CXCR7 is mainly expressed on differentiated tumor cells.

CXCR7 activation induces phosphorylation of Erk1/2. To assess that CXCR7 is a functional receptor on glioma cells, A764 as well as U343 cells were stimulated with CXCL12, and the phosphorylation of kinases was investigated by Western blot experiments. In both cell lines, stimulation of CXCR7 resulted in a transient phosphorylation of Erk1/2

(extracellular signal-regulated kinase; Fig. 4, left), but neither of the kinases Akt nor p38 (Fig. 4, bottom right). This phosphorylation was successfully blocked by the highly selective inhibitor U0126 of the upstream ERK kinase (MEK; Fig. 4, top right), indicating that CXCR7 transduces signals via MEK/ERK signal transduction pathway. Activation of the mitogen-activated protein kinase pathway occurred not directly, but apparently via phosphoinositide-specific phospholipase C because the selective inhibitor U73122 abolished Erk1/2 phosphorylation; the protein kinase A inhibitor KT5720 exhibited fewer inhibitory effects (Fig. 4, middle right; both inhibitors showed no effects of EGF-induced phosphorylation that occurs directly via receptor adapter proteins; data not shown). As possibly activated transcription factors, we investigated the phosphorylation of cyclic AMP response element-binding protein, activating transcription factor 2, Elk-1, and the nuclear localization of NF-κB; however, none were measurably induced/activated (see Supplementary Material, Supplementary Fig. S1). Stimulation of glioma stem-like cells (line 25/07 without LIF) with CXCL12 yielded weak phosphorylation of Akt as well as of Erk1/2 (Fig. 4, bottom left). These results show that CXCR7 in glioma cells signals preferentially via protein kinase C, which subsequently activates the mitogen-activated protein kinase/ERK kinase pathway. In glioma stem-like cells, alternative pathways may also contribute to signaling.

CXCR7 activation inhibits apoptosis. As biological responses, chemokine receptor activation often induces chemotaxis and/or proliferation. When A764 and U343 glioma cells were exposed to 1 nmol/L of CXCL12 in a wound-healing assay, no significant migratory effects were observed (Fig. 5A). Also, CXCL12 did not significantly increase proliferation as did EGF or FCS, neither in A764 (Fig. 5B) nor in U343 cells (data not shown).

However, CXCL12 inhibited the apoptosis of glioma cells after exposure to camptothecin and temozolomide as investigated with three different methods: as determined by modified Nicoletti staining experiments, CXCL12 diminished camptothecin- and temozolomide-induced apoptosis significantly to about one third or one fourth (depending on time/agent) in A764 and U343 cells (Fig. 6A). The receptor specificity could be proven by simultaneous stimulation with a specific, nonpeptide antagonist for CXCR7, CCX733 (25), which completely restored the apoptotic response to camptothecin (Fig. 6A). Furthermore, cleavage of PARP was efficiently reduced by CXCL12 in A764 cells (Fig. 6B). Moreover, camptothecin-induced activation of caspase-3/-7 was significantly inhibited when cells were coincubated with CXCL12; again, the CXCR7 antagonist CCX733 completely abolished the antiapoptotic effect (Fig. 6C).

In contrast, CXCL12 had no significant antiapoptotic effect in glioblastoma stem-like cells (Fig. 6D). CXCL12 did not (or insignificantly) alter camptothecin-induced caspase-3/-7 activation. In summary, CXCR7 is a functionally active CXCL12 receptor on glioma cells and mediates antiapoptotic effects whereas receptor expression in glioma stem-like cells is low and therefore lacks pronounced CXCR7 effects.

Discussion

Among chemokine receptors, CXCR4 has been found to be most widely expressed in multiple types of tumors and has been shown to be implicated in tumor cell invasion, metastasis, as well as in survival and proliferation (2, 5). Less information is available on the occurrence and biological role of the newly discovered second receptor for the CXCR4-ligand CXCL12, i.e., CXCR7 (8, 12–14). Reportedly, human gliomas and astrocytomas produce several chemokines like CCL2/MCP-1, CXCL8/interleukin-8, CXCL10, CXCL11/I-TAC, CXCL12/stromal cell–derived factor-1, and CXCL16 (19, 26). Their corresponding receptors have been identified either on astrocytoma/glioma cells (or subsets), on tumor-infiltrating microglial cells/macrophages, on tumor endothelial cells, or on tumor-infiltrating neural stem cells (17, 27).

CXCR4 has been originally described as a major widespread receptor on glioma cells (3, 4), and its occurrence and localization were addressed in several previous studies. WHO grade 3 and 4 glial tumors robustly express CXCR4, and the expression levels correlate with the magnetic resonance imaging–based finding of a diffuse and more extensive disease process in the brain (28). Whereas CXCL12 is often produced in the pseudopalisading cells and the proliferating microvessels (29), CXCR4 expression is found on tumor endothelial and tumor cells, often in areas of necrosis and angiogenesis (3, 29); probably also due to CXCR4 induction by hypoxia (29). However, CXCL12 and CXCR4 are also colocalized on tumor cells (5). Furthermore, CXCR4 was found on glioma tumor stem cells and on neural stem cells that invade this tumor (6, 16, 17, 30). In fact, neural stem cells display extensive tropism for gliomas (31), which seems to be mediated by CXCL12 production of the tumor cells and the corresponding CXCR4 receptors on stem cells (16). In our own investigation (which was not focused on CXCR4), we could sustain some of these findings (compare Fig. 1 and Results), but find a bit more restricted localization of CXCR4 *in situ*. The alternate CXCL12 receptor, CXCR7, occurs on tumor endothelial cells and tumor cells, here only rarely together with CXCR4. Taken together, both receptors occur in astrocytomas/glioblastomas *in situ* on tumor endothelial cells; CXCR4 is found on certain subsets of glioma cells and on stem-like cells whereas CXCR7 has a more extensive distribution on (mostly) other parts of glioma cells and on tumor-infiltrating microglial cells.

In vitro, we detected low CXCR4 expression on only one of eight glioma cell lines tested. In contrast, CXCR7 was transcribed on all glioma cell lines screened. Glioma cell lines are generated by several passages in medium containing FCS, conditions that are unfavorable for tumor stem cells. These can be cultivated under serum-free conditions (neurosphere medium) in the presence of bFGF and EGF (20, 21). In short-term cultures comparing these stem cell–favoring and conventional culture conditions, glioma spheres showed markedly higher CXCR4 and lower CXCR7 expression than primary cultures in FCS-containing medium. In accordance, a glioma stem-like cell line showed high CXCR4 and low CXCR7 expression. FCS-induced differentiation drastically

diminished CXCR4 expression and induced CXCR7. Effects were particularly high in glioma stem-like cells precultivated with LIF. It seems that the addition of this cytokine improves the preservation of the stem cell character compared with bFGF and EGF alone. Thus, *in situ* and *in vitro* findings suggest that CXCR7 is expressed on "differentiated" glioma/astrocytoma cells, whereas CXCR4 is found more on glioma stem cells.

Thus, CXCR7 is a frequent receptor in glioblastomas *in situ* and in particular on glioma cells *in vitro*. Thus far, it is unclear to what extent CXCR7 could be considered as a signaling receptor when expressed alone or in combination with CXCR4 (8, 32). Recent studies with hCXCR7-transfected HEK293 cells and rat vascular smooth muscle cells that endogenously express CXCR7 (33) show a "nonclassical positive signaling" of CXCR7 through β-arrestin–mediated (processes that last for >10 minutes), but not through Gαi-mediated mechanisms (although other G protein–mediated ones were not excluded). In glioma cells that express only CXCR7, we could detect limited but pronounced effects upon CXCL12 stimulation: a transient phosphorylation of Erk 1/2 and an inhibition of camptothecin- and temozolomide-induced apoptosis were observed, but no effects on cyclic AMP production, proliferation, or chemotaxis as often reported for CXCR4 (5, 34). Erk phosphorylation could be reduced by the phosphoinositide-specific phospholipase C inhibitor, U73122, but not by the protein kinase A inhibitor, KT5720. The extent of the involvement of β-arrestin–mediated mechanisms and other mechanisms (which might explain the different activation kinetics) should be investigated in further detailed studies. It is tempting to speculate that some of the previously reported effects of CXCL12 on glioma cells, such as phosphorylation of kinases, induction of gene expression, or prevention of apoptosis, were at least in part mediated through CXCR7 (4, 35, 36).

Thus, tumor (or neural) stem cells seem to be attracted and nourished by glioma-produced CXCL12 via CXCR4, whereas the bulk of differentiated glioma cells is protected from apoptosis via CXCR7, which may also have a CXCL12-binding function to a certain extent. The interplay of CXCR4 and CXCR7 in tumor endothelial cells and CXCR7 function on tumor-invading microglial cells shall be elucidated in future experiments.

Disclosure of Potential Conflicts of Interest

M. Penfold and T.J. Schall own a patent on the inhibitor used in this study, though not for clinical use. The other authors disclosed no potential conflicts of interest.

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References

1. Vandercappellen J, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. Cancer Lett 2008;267:226–44.
2. Müller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50–6.
3. Rempel SA, Dudas S, Ge S, Gutierrez JA. Identification and localization of the cytokine SDF-1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin Cancer Res 2000;6:102–11.
4. Zhou Y, Larsen PH, Hao C, Yong VW. CXCR4 is a mayor chemokine receptor on glioma cells and mediates their survival. J Biol Chem 2002;277:49481–7.
5. Bajetto A, Barbieri F, Dorcaratto A, et al. Expression of CXC chemokine receptors 1-5 and their ligands in human glioma tissue: role of CXCR4 and SDF-1 in glioma cell proliferation and migration. Neurochem Int 2006;49:423–32.
6. Ehtesham M, Winston JA, Kabos P, Thompson RC. CXCR4 expression mediates glioma cell invasiveness. Oncogene 2006;25:2801–6.
7. Balabanian K, Lagane B, Infantino S, et al. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J Biol Chem 2005;280:35760–6.
8. Burns JM, Summers BC, Wang Y, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 2006;203:2201–13.
9. Sierro F, Biben C, Martínez-Muñoz L, et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proc Natl Acad Sci U S A 2007;104:14759–64.
10. Gerrits H, van Ingen Schenau DS, Bakker NE, et al. Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. Genesis 2008;46:235–45.
11. Schönemeier B, Kolodziej A, Schulz S, Jacobs S, Hoellt V, Stumm R. Regional and cellular localization of the CXCl12/SDF-1 chemokine receptor CXCR7 in the developing and adult rat brain. J Comp Neurol 2008;510:207–20.
12. Miao Z, Luker KE, Summers BC, et al. CXCR7 (RDC1) promotes breast and lung tumor growth *in vivo* and is expressed on tumor-associated vasculature. Proc Natl Acad Sci U S A 2007;104:15735–40.
13. Wang J, Shiozawa Y, Wang J, et al. The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. J Biol Chem 2008;283:4283–94.
14. Meijer J, Ogink J, Roos E. Effect of the chemokine receptor CXCR7 on proliferation of carcinoma cells *in vitro* and *in vivo*. Br J Cancer 2008;99:1493–501.
15. Reiss K, Mentlein R, Sievers J, Hartmann D. Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar external granular layer. Neuroscience 2002;115:295–305.
16. Ehtesham M, Yuan X, Kabos P, et al. Glioma tropic neural stem cells consist of astrocytic precursors and their migratory capacity is mediated by CXCR4. Neoplasia 2004;6:287–93.
17. Ma YH, Mentlein R, Knerlich F, Kruse ML, Mehdorn HM, Held-Feindt J. Expression of stem cell markers in human astrocytomas of different WHO grades. J Neurooncol 2008;86:31–45.

18. Mentlein R, Eichler O, Forstreuter F, Held-Feindt J. Somatostatin inhibits the production of vascular endothelial growth factor (VEGF) in glioma cells. Int J Cancer 2001;92:545–50.
19. Ludwig A, Schulte A, Schnack C, et al. Enhanced expression and shedding of the transmembrane chemokine CXCL16 by reactive astrocytes and glioma cells. J Neurochem 2005;93:1293–303.
20. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–10.
21. Yuan X, Curtin J, Xiong Y, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 2004;24:9392–400.
22. Held-Feindt J, Döner L, Sahan G, Mehdorn HM, Mentlein R. Cannabionoid receptors in human astroglial tumors. J Neurochem 2006;98: 886–93.
23. Hattermann K, Ludwig A, Gieselmann V, Held-Feindt J, Mentlein R. The chemokine CXCL16 induces migration and invasion of glial precursor cells via its receptor CXCR6. Mol Cell Neurosci 2008; 39:133–41.
24. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139: 271–9.
25. Hartmann TN, Grabovsky V, Pasvolsky R, et al. A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. J Leukoc Biol 2008;84:1130–40.
26. Maru SV, Holloway KA, Flynn G, et al. Chemokine production and chemokine receptor expression by human glioma cells: role of CXCL10 in tumour cell proliferation. J Neuroimmunol 2008;199:35–45.
27. Platten M, Kretz A, Naumann U, et al. Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas. Ann Neurol 2003;54:388–92.
28. Stevenson CB, Ehtesham M, McMillan KM, et al. CXCR4 expression is elevated in glioblastoma multiforme and correlates with an increase in intensity and extent of peritumoral T2-weighted magnetic resonance imaging signal abnormalities. Neurosurgery 2008;63: 560–9.
29. Komatani H, Sugita Y, Arakawa F, Ohshima K, Shigemori M. Expression of CXCL12 on pseudopalisading cells and proliferating microvessels in glioblastomas: an accelerated growth factor in glioblastomas. Int J Oncol 2009;34:665–72.
30. Salmaggi A, Boiardi A, Gelati M, et al. Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia 2006;54:850–60.
31. Aboody KS, Brown A, Rainov NG, et al. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. Proc Natl Acad Sci U S A 2001;97:12846–51.
32. Ehtesham M, Mapara KJ, Stevenson CB, Thompson RC. CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett 2009;274:305–12.
33. Rajagopal S, Kim J, Ahn S, et al. β-Arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. Proc Natl Acad Sci U S A 2010;107:628–32.
34. Yang L, Jackson E, Woerner BM, Perry A, Piwnica-Worms D, Rubin JB. Blocking CXCR4-mediated cyclic AMP suppression inhibits brain tumor growth *in vivo*. Cancer Res 2007;67:651–8.
35. Barbero S, Bonavia R, Bajetto A, et al. Stromal cell-derived factor 1α stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res 2003;63:1969–74.
36. Zhang J, Sarkar S, Yong VW. The chemokine stromal cell derived factor-1 (CXCL12) promotes glioma invasiveness through MT2-matrix metalloproteinase. Carcinogenesis 2005;26:2069–77.