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CXCL12 mediates apoptosis resistance in rat C6 glioma cells

KIRSTEN HATTERMANN[1], ROLF MENTLEIN[1] and JANKA HELD-FEINDT[2]

[1]Department of Anatomy, University of Kiel, D-24098 Kiel, Germany; [2]Department of Neurosurgery, University Medical Center Schleswig-Holstein, D-24105 Kiel, Germany

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Abstract. The chemokine CXCL12/stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 regulate migration and patterning processes during brain development, but also contribute to proliferation and expansion of gliomas, the most malignant brain tumors. Recently, a previous orphan-receptor CXCR7/RDC-1 was discovered to be a second receptor for CXCL12. CXCR7 has been detected in normal brain parenchyma, but in particular in human brain tumors. However, little is known about the functional relevance of CXCR7. Since the well-characterized rat C6 glioma cell line is commonly used as a glioma model *in vitro* and *in vivo*, we investigated the expression, regulation and function of CXCL12 and its receptors in these tumor cells. Whereas CXCL12 and CXCR7 were transcribed at notable levels, CXCR4 was quite low. By sublethal doses of temozolomide, an alkylating drug commonly used in adjuvant glioma therapy, transcription of CXCL12 and its receptors were significantly induced. Decreased proliferation resulting from this sublethal treatment with temozolomide could be completely restored to normal proliferation rates by simultaneous stimulation with CXCL12. Similarly, CXCL12 protected C6 cells from apoptosis under treatment with higher temozolomide doses. Thus, the CXCL12-CXCR7 axis promotes glioma progression, and the rat C6 glioma cell line may be a useful model to further investigate these mechanisms *in vitro* and *in vivo*.

Introduction

Gliomas are the most common type of primary brain tumors, and *glioblastoma multiforme* (GBM) is the most malignant form. Current standard therapy is surgical resection followed by adjuvant radiotherapy combined with chemotherapy. Despite recent advances in surgical techniques and adjuvant therapy, prognosis for GBM patients is still poor, with a median survival time of approximately 12-15 months (1). This is mainly due to rapid recurrence of intracranial tumor foci which is caused by diffusive infiltrative proliferation and resistance to adjuvant therapy of remaining glioma cells after surgery. Therefore, knowledge of proliferative, chemotactic and invasive factors is of crucial importance to improve therapies.

Chemokines are a family of small peptides (8-15 kDa) that were first discovered as cytokines with chemotactic properties (2). Interacting with their G-protein coupled seven-transmembrane receptors, they are responsible for leukocyte trafficking and intercellular communications (3). In the central nervous system, chemokines and their receptors are involved in various processes in health and disease, including neuronal patterning (4), migration and proliferation of glial precursor cells (5) as well as neuroinflammation (6). Furthermore, they play decisive roles in tumor initiation, promotion, progression and metastasis (7,8). Especially the chemokine CXCL12/SDF-1 (stromal cell-derived factor-1) has attracted great interest as it directs CXCR4-expressing tumor cells to peripheral tissues like the lung, liver, lymph nodes and bone marrow (9).

In human glioblastomas, CXCL12 and its receptor CXCR4 are localized in regions of necrosis and angiogenesis (10), and mediate proliferation of glioblastoma progenitor cells (11). Recently, a second receptor for CXCL12 was deorphanized and named CXCR7 (12) which, in addition to CXCL12, binds CXCL11/I-TAC (interferone-inducible T cell α chemoattractant) with a 10-fold lower affinity.

In the CNS, CXCR7 can be found in the developing and adult rat brain (13), in primary rat astrocytes and Schwann cells (14), and it is upregulated *in vivo* and *in vitro* during maturation of oligodendrocytes (15). In human glioblastomas CXCR7 is located on tumor-associated capillaries and on the mass of tumor cells (16).

Although CXCR7 was at first supposed to be merely a decoy receptor (17), recent reports show that CXCR7 directly mediates cellular effects beside its function in controlling extracellular CXCL12 levels, facilitating and regulating CXCR4-mediated effects like migration (18) and reduction of temozolomide-induced apoptosis (16).

Since rat C6 glioma cells are an often used *in vitro* and *in vivo* model for gliomas, we investigated the expression of

Correspondence to: Dr Kirsten Hattermann, Department of Anatomy, University of Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany
E-mail: k.hattermann@anat.uni-kiel.de

Abbreviations: CXCL11/I-TAC, interferon-inducible T cell α chemoattractant; CXCL12/SDF-1, stromal cell-derived factor-1; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcription polymerase chain reaction

Key words: brain tumor, chemokines, receptors, apoptosis resistance

CXCL12, CXCR4 and CXCR7 in this cell line, their regulation upon temozolomide treatment, and the CXCL12-mediated effects on proliferation and apoptosis caused by temozolomide treatment.

Materials and methods

Peptides and inhibitors. Recombinant rat CXCL12 was obtained from PeproTech (Rocky Hill, NJ, USA). Temozolomide was purchased from Sigma-Aldrich/Fluka (St. Louis, MO, USA) and dissolved in DMSO (Sigma-Aldrich; stock concentration 20 mg/ml). The specific CXCR7 antagonist CCX733 was a kind gift from M.E.T. Penfold and T.J. Schall from ChemoCentryx, (Mountain View, CA, USA).

Cultivation and stimulation of glioma cells. The rat glioma cell line C6 was obtained from Deutsches Krebsforschungszentrum (DKFZ; Heidelberg, Germany). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) plus 10% fetal calf serum (FCS; Invitrogen) and routinely checked for purity by immunostaining (19) on glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark) and for Mycoplasma contamination by staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Quantitative RT-PCR (qRT-PCR). For mRNA expression analysis, cells were harvested using the QIAzol lysis reagent (Qiagen, Hilden, Germany) and total RNA was isolated following the manufacturer's instructions. Genomic DNA was digested by RNase-free DNase (1 U/μl, Promega, Madison, WI, USA), and cDNA was synthesized using RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 U/μl, Fermentas, Vilnius, Lithuania). Real-time RT-PCR was performed as previously described (20) using TaqMan primer probes (Applied Biosystems, Foster City, CA, USA): rGAPDH (Rn99999916_s1), rCXCL12 (Rn00573260_m1), rCXCR4 (Rn00573522_s1), rCXCR7 (Rn00584358_m1), rGFAP (Rn00566603_m1), rSox2 (Rn01286286_g1), rMusashi1 (Rn00596059_m1), rCD133 (Rn00572720_m1), rNestin (Rn00564394_m1). Samples were analyzed in duplicates. Reactions were performed and the Cycle of Threshold values (C_T) were measured with the MyiQ™ Single Color Real-time PCR Detection System (Bio-Rad, Munich, Germany), and ΔC_T values = C_T(gene of interest) - C_T(GAPDH) were calculated. Due to a logarithmic reaction process, a ΔC_T value of 3.33 corresponds to one magnitude lower gene expression compared to GAPDH. For stimuli-induced mRNA regulation $\Delta\Delta C_T$ values were calculated: $\Delta\Delta C_T = 2^{-[\Delta CT(stimulus) - \Delta CT(control)]}$.

Immunocytochemistry. Rat C6 glioma cells were seeded onto poly-D-lysine-coated cover slips and grown for 24 h. Then, cells were washed with phosphate-buffered saline (PBS) and pre-incubated with serum-free medium or serum-containing growth medium for 1 h. Cells were fixed with ice-cold acetone/methanol 1:1 (10 min) or Zamboni's fixative (4% paraformaldehyde + picrinic acid, 30 min), blocked with 0.5% bovine serum albumin/0.5% glycine in PBS (60 min) and incubated with mouse anti-CXCR7 (clone 11G8, a kind gift from M.E.T. Penfold and T.J. Schall from ChemoCentryx, dilution 1:200). The secondary antibody was goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, dilution 1:1000), and nuclei were counterstained with DAPI. Slides were viewed with an Axiovert 200 microscope (Carl Zeiss, Jena, Germany).

Proliferation assays. For proliferation assays, 50,000 cells were seeded onto 35-mm culture dishes (Greiner Bio-One, Frickenhausen, Germany) and pre-cultivated for 24 h in DMEM with 10% FCS. The medium was changed to DMEM + 0.5% FCS and stimuli were added (3 individual dishes for each stimulus). The medium supplied with stimuli was renewed after 3 days, and the cells were harvested 2 days later. DNA was measured fluorimetrically as previously described (21) using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen). Measured DNA contents were normalized to unstimulated controls (= 100%).

Apoptosis assay. Apoptosis was analyzed by a modified Nicoletti staining (22). Cells were seeded onto poly-D-lysine-coated coverslips and pre-cultivated for 24 h in DMEM + 10% FCS. Stimulations were performed in DMEM + 0.5% FCS for 48 h. After rinsing with PBS cells were fixed with ice-cold acetone/methanol (1:1; 10 min), washed 3x with PBS, nuclei were stained with DAPI and slides were embedded with Immu-Mount (Shandon, Pittsburgh, PA, USA). Samples were viewed and pictures were taken with an Axiovert 200 M fluorescence microscope (Zeiss, Oberkochem, Germany). Apoptotic nuclei were evaluated and counted in a blinded manner.

Statistical analysis. Values are expressed as means ± standard deviations of the mean (SD). Statistical significance was analyzed by a two-tailed Student's t-test. $^{*}P<0.05$ and $^{**}P<0.01$.

Results

Transcription of chemokine/receptors and cell markers in rat C6 glioma cells. Initially, we analyzed the rat glioma cell line C6 by qRT-PCR for expression of the chemokine CXCL12 and its receptors CXCR4 and CXCR7 and for cell markers which can typically be found in gliomas (Fig. 1A). The glioma cells showed considerable mRNA amounts of the chemokine CXCL12 (ΔC_T=9.9±1.1) and its receptor CXCR7 (ΔC_T=9.1±0.2) while expression of the receptor CXCR4 was less prominent (ΔC_T=14.6±1.2).

Furthermore, the cells were characterized by moderate levels of GFAP (glial fibrillary acidic protein; ΔC_T 9.5±1.8), a marker for astrocytes which is also mostly expressed by glioma cells, and nestin (ΔC_T=4.7±0.6), a marker for astrocyte progenitors as well as for reactive astrocytes. Furthermore, we tested for Musashi-1, Sox-2 and CD133 (prominin-1), which are markers for (neural) stem and progenitor cells, and are discussed as markers for the stem cell character of glioma cells regarding the tumor stem cell hypothesis (23). Transcription of CD133, a transmembrane glycoprotein is very low (ΔC_T=17.5±0.5), just above the detection limit, while the RNA-binding protein Musashi-1 is expressed at moderate level (ΔC_T=12.6±0.3) and the transcription factor Sox-2 at a comparably high level (ΔC_T=7.0±0.7).

CXCL12 and its receptors CXCR4 and CXCR7 are induced by temozolomide. To investigate the influence of temozolomide

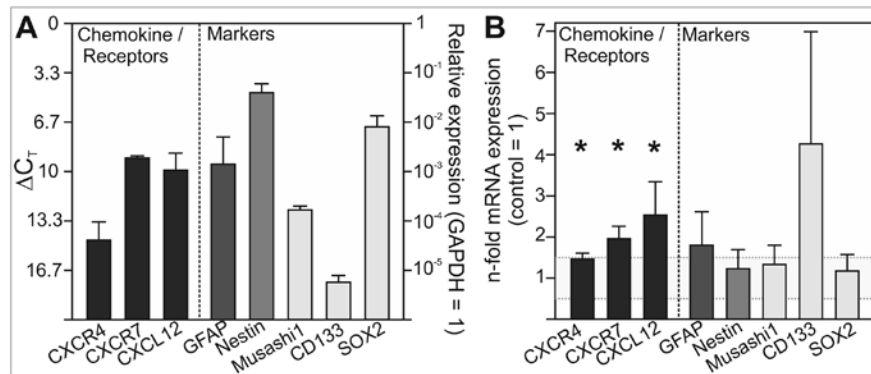

Figure 1. (A) Transcription and (B) regulation of CXCL12 and its receptors CXCR4 and CXCR7 as well as cellular markers in C6 glioma cells. (A) Rat C6 glioma cells were analyzed for mRNA expression by qRT-PCR. ΔC_T values to GAPDH are shown; ΔC_T = 3.33 corresponds to 10-fold lower expression. Comparatively, a low level expression of CXCR4 is found in C6 glioma cells, whereas CXCL12 and its second receptor CXCR7 are expressed at a medium level. Nestin and Sox-2 were found to be highly expressed, while GFAP and Musashi-1 were expressed at medium and CD133 at a low level; n=3 individual cultures. (B) C6 glioma cells were exposed to 20 μg/ml temozolomide for 24 h and analyzed for induction of mRNA transcription in comparison to DMSO-treated controls. $\Delta\Delta C_T$ values corresponding to n-fold expression change are shown. While expression of cellular markers is not influenced by temozolomide treatment, CXCL12 and its receptors CXCR4 and CXCR7 are significantly upregulated (paired Student's t-test); n=3 individual stimulations. $^*P<0.05$

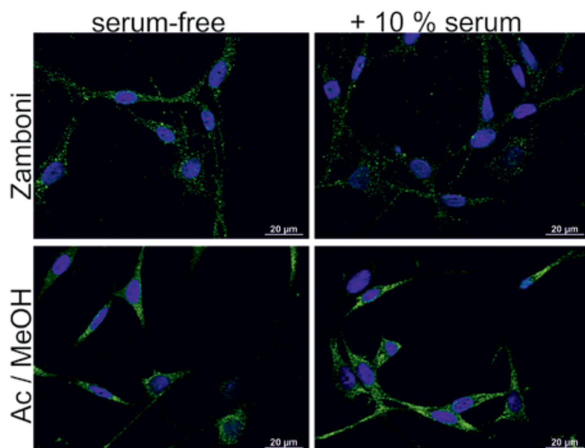

Figure 2. Surface and intracellular expression of CXCR7 in rat C6 glioma cells. C6 glioma cells were pre-incubated with serum-free medium or growth medium containing 10% fetal calf serum, fixed with either Zamboni's fixative which conserves membrane integrity or with ice-cold acetone/ methanol which allows intracellular detection due to membrane permeabilization. After Zamboni fixation, serum-deprived cells show stronger surface expression of CXCR7 compared to non-starved cells (upper row), whereas intracellular immunoreactivity (acetone/methanol fixation, lower row) is stronger in non-starved cells.

on the transcription of CXCL12 and its receptors we stimulated C6 cells with 20 μg/ml temozolomide (or 0.1% DMSO as solvent control) for 24 h and analyzed mRNA expression by qRT-PCR (Fig. 1B). The chemokine CXCL12 was clearly upregulated (2.5±0.8-fold), and also its receptors CXCR4 (1.5±0.3-fold) and CXCR7 (2.0±0.3-fold) were significantly induced by temozolomide (paired Student's t-test). The transcription of the cell markers GFAP, Nestin, Musashi-1, Sox-2 and CD133 was not significantly influenced by temozolomide treatment.

Surface and intracellular expression of CXCR7. Chemokine receptors are usually internalized upon binding of their appropriate ligand and are subjected to intracellular recycling processes (24) which is also reported for CXCR7 (25). As surface expression of CXCR7 is crucial for cellular responses elicited by binding of its ligands, we next analyzed surface and intracellular expression of CXCR7 by fluorescence immunocytochemistry. C6 glioma cells were pre-incubated with serum-free medium for 1 h to allow for re-expression of CXCR7 at the cell surface, or with normal growth medium containing fetal calf serum. After fixation with Zamboni's fixative which does not permeabilize the cells and therefore allows only surface detection of antigens, serum-free pre-incubated cells showed stronger immunoreactivity for CXCR7 than cells pre-incubated with serum, although these had also detectable portions of surface CXCR7 (Fig. 2). Vice versa, serum pre-incubated cells showed stronger immunoreactivity for CXCR7 when a mixture of ice-cold acetone/methanol was used for fixation which permeabilizes and partially lyses the membrane revealing intracellular antigens. Thus, CXCR7 is expressed on the surface of C6 glioma cells which is a requirement for the following investigations on functional effects upon CXCL12 stimulation. In addition, serum-deprivation can enhance surface expression of CXCR7 probably due to re-expression of internalized receptors.

CXCL12 abrogates the antiproliferative effects of temozolomide. Due to the induction of CXCL12 and its receptors in C6 glioma cells we hypothesized that CXCL12 could contribute to rescue mechanisms against the cytotoxicity of temozolomide. Thus, we investigated the effects of CXCL12 on proliferation in long-term stimulation with sublethal concentrations of temozolomide. The alkylating agent temozolomide which is commonly used in glioma therapy was applied to C6 glioma cells for 5 days (serum-low medium and stimuli renewed after 3 days) with or without simultaneous stimulation with 1 nM CXCL12, and DNA content was measured to analyze effects on proliferation. In Fig. 3, one representative experiment of the 3 is shown. Temozolomide treatment reduced proliferation to 85.9±9.6% (20 μg/ml) or 66.1±1.6% (100 μg/ml), whereas the solvent control (1% DMSO) did not significantly reduce the proliferation rate (93.1±5.8%). Simultaneous stimulation with 1 nM CXCL12 could restore normal proliferation in

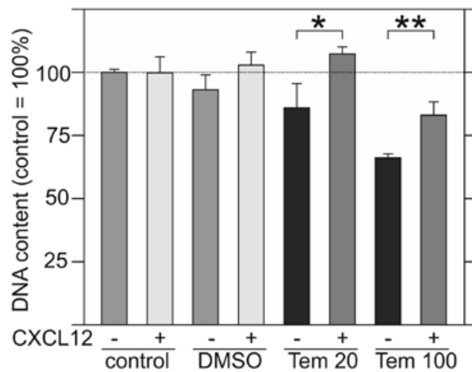

Figure 3. CXCL12-mediated effects on proliferation. C6 glioma cells were incubated with or without 1 nM CXCL12 and different concentration of the cytotoxic agent temozolomide or its solvent DMSO (1%) for 5 days (medium and stimuli renewed after 3 days). DNA content was measured and normalized to unstimulated control (100%). CXCL12 does not influence proliferation of unchallenged cells (control and DMSO), but when cell proliferation is decreased due to treatment with temozolomide, simultaneous stimulation with 1 nM CXCL12 can completely restore normal proliferation rate (20 μg/ml temozolomide) or at least alleviate the anti-proliferative effects of 100 μg/ml temozolomide. Mean values of three individual dishes are shown; a representative experiment out of the three individual experiments. $^{*}P<0.05$, $^{**}P<0.01$.

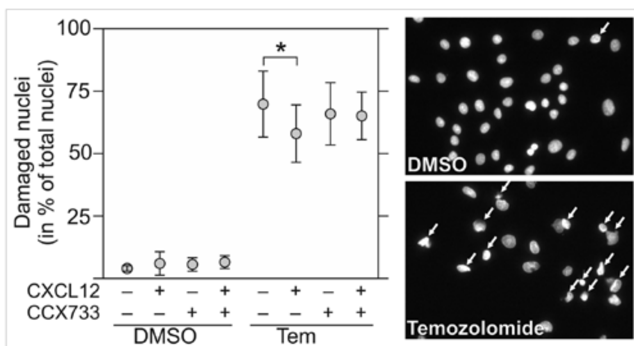

Figure 4. CXCL12-mediated effects on apoptosis. C6 glioma cells were seeded onto cover slips and incubated with or without 1 nM CXCL12, 200 μg/ml temozolomide (Tem), 1% DMSO (solvent control) and 100 nM CCX733 (selective CXCR7 inhibitor) for 48 h and fixed. Nuclei were stained with DAPI. Six pictures per sample were captured (examples: right panel) and apoptosis was determined as percentage of damaged nuclei (arrows) to the total number of nuclei. In DMSO-treated controls, percentage of damaged nuclei was low (3-5%) and not influenced by CXCL12 or by CCX733. In contrast, temozolomide-treated samples showed about 70% damaged nuclei which could be significantly reduced by simultaneous stimulation with CXCL12 to about 58%. The selective CXCR7 inhibitor CCX733 could diminish this protective effect (65% damaged nuclei). Representative data from two individual experiments are shown. $^{*}P<0.05$.

combination with 20 μg/ml temozolomide (107.2±2.8%) or at least minimize the antiproliferative effects of 100 μg/ml temozolomide (83.0±5.6%). Results were significant (two-tailed Student's t-test).

CXCL12 mediates anti-apoptotic effects via CXCR7. Additionally, we analyzed the protective effect of CXCL12 when apoptosis was induced by treatment with higher temozolomide concentration (200 μg/ml) for 48 h and subsequent staining of the nuclei (modified Nicoletti staining). A representative experiment of two individual experiments is shown in Fig. 4. While DMSO-treated controls had only 3-5% damaged nuclei (of the total counted nuclei), temozolomide treatment resulted in 69.8±13.2% damaged nuclei. This percentage of apoptotic cells was reduced significantly to 58.0±11.5% in samples co-stimulated with 1 nM CXCL12. In samples that were additionally supplied with the specific antagonist of CXCR7, CCX733, the percentage of damaged nuclei was elevated again to 65.1±9.5%. Thus, CXCL12 could reduce the proportion of apoptotic and dead cells and this effect could be almost completely abolished by CCX733, indicating that the anti-apoptotic effect of CXCL12 is mainly mediated via CXCR7.

Discussion

The rat C6 glioma cell line was generated by isolation of S100-expressing cells from a rat glioma induced by N-methylnitrosurea (26). It is characterized by moderate expression of stem cell markers and the astroglial marker GFAP which is comparable to human glioblastoma cell lines isolated from solid glioblastoma tissue samples. However, further differentiation by activation of IL-6 pathways is possible (27). C6 cells transcribe the chemokine CXCL12 and its receptors CXCR7 in considerable amounts, while the alternative receptor CXCR4 is expressed to a lower extent. The chemotherapeutic agent temozolomide induces transcription of CXCL12 as well as its receptors CXCR4 and CXCR7 suggesting a functional role in the cellular response to this cytotoxic challenge. Indeed, simultaneous stimulation with CXCL12 and sublethal temozolomide doses could restore normal proliferation rates, and could reduce the percentage of apoptotic cells in high-dose treatment.

The expression balance of CXCL12 and its receptors CXCR4 and CXCR7 seems to be linked to differentiation and maturation, especially in the CNS. While CXCR4 is reported to be more important during brain development, especially in migration and survival of neuronal and oligodendroglial precursor cells (28), CXCR7 is induced during maturation of glial cells (15), and is the predominant receptor for CXCL12 in the adult rodent brain (13). This seems to be reproduced during glioblastoma pathogenesis where glioblastoma stem-like cells show high CXCR4 and low CXCR7 levels and more mature glioblastoma cells switch to a predominant CXCR7 expression (16).

When CXCR7 was discovered, it was supposed to be a non-signaling decoy or scavenger receptor for CXCL12 as it fails to couple to G-proteins due to alteration in a conserved DRYLAIV-motif (17), and does not cause Ca^{2+} mobilization upon activation as is known for most chemokine receptors (29). However, recent investigations have shown that CXCR7 can signal via β-arrestin (30) resulting for example in CXCL12-dependent activation of MAP-kinase in interneurons *in vivo* (31), and MAP-kinase and Akt pathways in primary rat astrocytes and Schwann cells (14) and human glioblastoma cell lines (16). Furthermore, the second ligand for CXCR7, CXCL11 can also induce activation of MAP-kinase and Akt signaling pathways (32) and has chemotactic properties on vascular smooth muscle cells (30). Examples of the reported effects of CXCL12 via CXCR7 are chemotaxis in T lymphocytes (12), angiogenesis and invasion in hepatocellular carcinomas, while metastasis

was not affected (33), and increased tumor growth of various tumor types *in vivo* (29,34). The latter might be explained by CXCL12-CXCR7-mediated advantage in survival of injected tumor cells and escape from the host's immune defense. However, in human glioblastoma cells, the CXCL12-CXCR7-axis promotes a rescue from chemically-induced apoptosis (16) which is corroborated by our new results in rat C6 glioma cells.

In summary, rat C6 glioma cells may serve as a glioblastoma model *in vitro* and *in vivo* as they show characteristics of human glioblastoma cells, and, like in human glioblastoma cell lines, CXCL12 mediates anti-apoptotic effects via its recently discovered receptor CXCR7.

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