Widespread CXCR4 Activation in Astrocytomas Revealed by Phospho-CXCR4-Specific Antibodies

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Abstract
The chemokine receptor CXCR4 is expressed in many cancers where it may regulate tumor cell growth and migration. The role of CXCR4 in cancer will depend on it being in an activated, signaling state. To better define the significance of CXCR4 expression in cancer, we developed an antibody that can distinguish CXCR4 phosphorylated on serine 339, a residue previously identified as a site for ligand-induced phosphorylation. With this antibody, we investigated the mechanisms of CXCR4 phosphorylation and evaluated the phosphorylation status of CXCR4 in human astrocytomas. In vitro, phosphorylation of serine 339 occurred in response to CXCL12 or epidermal growth factor (EGF) treatment and was increased by protein kinase C activation. In all grades of astrocytomas, CXCR4 was expressed in tumor cells and some endothelial cells, whereas CXCL12 was present in endothelial cells and infiltrating microglia. We found that CXCR4 phosphorylated on serine 339 was present in tumor cells and vascular endothelial cells in all grades of astrocytoma. These data indicate that CXCR4 is expressed and activated in astrocytomas and that phosphorylation of CXCR4 can occur through ligand activation or transactivation via the EGF receptor. These studies extend the potential roles of CXCR4 in cancer to include functions associated with benign (grade 1) tumors. The ability to distinguish phosphorylated CXCR4 will be invaluable for the continued analysis of the role of CXCR4 in cancer and the development of CXCR4 antagonist therapy for patients suffering with primary tumors of the brain and other sites. (Cancer Res 2005; 65(24): 11392-9)

Introduction
Identification of novel therapeutic targets is critical to the advancement of cancer treatment. The chemokine receptor CXCR4, which is expressed in as many as 23 different tumor types, is a particularly exciting new target (1). Most models of CXCR4 function in cancer focus on its potential role as a mediator of motility, invasiveness, and metastatic behavior (2). CXCR4 activation, however, is also necessary for growth in intracranial models of primary brain tumors (3) as well as in models of primary breast cancer (4). The growth effects of CXCR4 activation are reminiscent of its role in normal hematopoiesis (5-7) and in cerebellar (8), hippocampal (9, 10), and retinal development (11).

Although two mutations of CXCR4 have been identified in cancer (12), the effect of these on CXCR4 function has not yet been determined. CXCR4 activation is thought to be primarily ligand dependent and this view is supported by the antitumor efficacy of AMD 3100 (3, 4), an antagonist of CXCL12 binding (13-16). CXCR4 is phosphorylated in response to ligand binding in a G-protein-coupled receptor kinase 2-dependent fashion (17-19). Receptor phosphorylation stimulates the interaction of β-arrestin with the carboxy terminus (18, 20). This terminates CXCR4-mediated activation of Gαi but promotes dynamin-dependent, clathrin-mediated receptor endocytosis and enhances CXCR4-RAF-dependent signaling (17, 20, 21). Phosphorylation of CXCR4 can also occur in response to the activation of other receptors and involve additional kinases, such as protein kinase C (PKC; refs. 17–19) or tyrosine kinases (22, 23). Thus, the regulation of phosphorylation and internalization has significant effect on CXCR4-mediated cellular responses. For this reason, descriptions of CXCR4 expression alone may not be adequate evidence to postulate a functional role for this receptor in a given cancer. Localization of phosphorylated, activated forms of CXCR4 would more definitively indicate that CXCR4 is contributing to tumor biology.

Orsini et al. (18) examined the relationship between CXCL12-induced CXCR4 phosphorylation and internalization. In this study, both serine 338 (S338) and 339 (S339) were simultaneously mutated to prevent phosphorylation and this significantly inhibited ligand-induced CXCR4 internalization. As both serines were mutated together, it was not possible to ascribe a functional consequence to phosphorylation of either residue alone. To clarify the relationship between CXCL12 binding and CXCR4 phosphorylation, we generated an antibody that specifically recognizes phosphoserine 339-CXCR4 (P339-CXCR4) and used it to examine the regulation of CXCR4 phosphorylation in vitro and the phosphorylation status of CXCR4 in all grades of astrocytomas. The widespread expression of CXCR4 in cancer and the clinical availability of CXCR4 antagonists highlight the importance of studying CXCR4 in astrocytomas where current therapeutic options are limited.

Materials and Methods

Chemicals, reagents, and antibodies. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. All tissue culture reagents and media were obtained from Invitrogen (Carlsbad, CA) unless otherwise indicated. CXCR4 antibodies included monoclonal antibody (R&D, Minneapolis, MN) and polyclonal antibodies (Leinco, St. Louis, MO). CXCL12 rabbit polyclonal antibody was from Peprotech (Rocky Hills, NJ), and actin antibodies were from Sigma. Normal goat and rabbit sera and IgG isotype control antibodies were from Jackson ImmunoResearch (West Grove, PA).
Antibody production and purification. Peptides P338, P339, and the nonphosphorylated form of this sequence (peptide 332-46, GKRGGHSSVTESES human CXCR4, g5-F030175) were synthesized at the Tufts University Core Facility (Boston, MA) with an amino terminal cysteine to facilitate conjugation to keyhole limpet hemocyanin (KLH). P339 phosphopeptide was conjugated to KLH according to published procedures (24). Conjugated peptide was supplied to Covance Research Products (Denver, PA) where rabbits were immunized according to their protocol and with approval from the Washington University School of Medicine Animal Studies Committee. Sera was screened by dot blot for reactivity against the immunizing peptide. Sera with a positive screen were subjected to affinity purification by sequential column chromatography. Sera was first applied to a protein A-Sepharose column and eluted with 100 mmol/L glycine (pH 3.0). Column fractions were immediately brought to pH 7.4 with 1 mol/L Tris. Protein A eluates were next applied to an affinity resin composed of the nonphosphorylated peptide conjugated to Affi-Gel Hydrazide (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. The flow-through from this column represented IgG depleted of antibodies that recognized the nonphosphorylated form of the peptide. This flow-through fraction was next applied to P339 Affi-Gel Hydrazide resin. Phosphopeptide-specific antibodies were eluted with 100 mmol/L glycine (pH 3) and immediately brought to pH 7.4 with 1 mol/L Tris.

Cell culture, transfections, and stimulation of CXCR4 phosphorylation. The U87 glioblastoma multiforme (GBM, grade 4) cell line was obtained from the American Type Culture Collection (Manassas, VA). The LN428 GBM cell line was a kind gift of Dr. Erwin van Meir (Walter Reed Army Institute of Research, Emory University, Atlanta, GA). U87 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS; Biomedical, Foster City, CA). LN428 cells were grown in DMEM (Fisher, Pittsburgh, PA) supplemented with 10% horse serum. All cells were grown in the presence of penicillin-streptomycin. The coding sequence for human CXCR4 was obtained with high-fidelity reverse transcription-PCR (Superscript III Platinum, Invitrogen) and cloned into the expression vector pMSCVneo (Clontech, Palo Alto, CA) according to the instructions of the manufacturer. The flow-through from this column represented IgG depleted of antibodies that recognized the nonphosphorylated form of the peptide. This flow-through fraction was next applied to P339 Affi-Gel Hydrazide resin. Phosphopeptide-specific antibodies were eluted with 100 mmol/L glycine (pH 3) and immediately brought to pH 7.4 with 1 mol/L Tris.

Human brain and tumor tissue acquisition. Four brain autopsy and 28 astrocytoma, formalin-fixed, paraffin-embedded archival specimens were retrieved from the pathology files at Washington University School of Medicine. Samples were obtained in accordance with an Institutional Review Board–approved protocol for human research. Autopsy specimens were from children who died without a known primary central nervous system cause. Astrocytomas were classified and graded according to the WHO guidelines (25), consisting of four sporadic, optic pathway pilocytic astrocytomas (grade 1), three sporadic pilocytic astrocytomas from other locations (grade 1), five diffuse astrocytomas (grade 2), eight anaplastic astrocytomas (grade 3), and eight GBMs (grade 4).

Tissue sections and immunohistochemistry. Sections (5 μm) were deparaffinized in xylene and rehydrated in descending alcohols. Endogenous peroxidase was blocked with 3% H2O2 in TBST [10 mmol/L Tris (pH 8.0), 0.15 mol/L NaCl, 0.05 Tween] and nonspecific avidin/biotin binding sites were blocked with Vector Blocking kit (Vector Laboratories, Burlingame, CA). Sections were additionally blocked with 10% serum from Burlingame, CA). Sections were additionally blocked with 10% serum from

Activity of the antipeptide antibody was evaluated by Western blot–based assays using lysates from the glioblastoma cell line LN428, which is known to express high levels of CXCR4 (26). LN428 cells were treated with 1 μg/mL of CXCL12 to promote CXCR4 phosphorylation. Western blot analysis with the P339-CXCR4–directed antibody resulted in the labeling of several bands, including a predominant band at 47 kDa (Fig. 1A). Reprobing of stripped blots with an antibody directed against the nonphosphorylated amino terminus of CXCR4 indicated that the band at 47 kDa was CXCR4. To further show antibody specificity, we immunoprecipitated CXCR4 from LN428 cultures treated with CXCL12. Immunoprecipitated CXCR4 was detected using a rabbit polyclonal antibody (1:66 dilution). Peptide and phosphopeptide treatment were done as described above.

Results

Characterization of the anti-phospho-CXCR4 antibody. Phosphopeptide corresponding to amino acids 332 to 346 (332-46) of human CXCR4 phosphorylated on S339 (P339) was injected into rabbits and phosphospecific antibody was isolated from serum by sequential column chromatography.

The activity of the antipeptide antibody was evaluated by Western blot–based assays using lysates from the glioblastoma cell line LN428, which is known to express high levels of CXCR4 (26). LN428 cells were treated with 1 μg/mL of CXCL12 to promote CXCR4 phosphorylation. Western blot analysis with the P339-CXCR4–directed antibody resulted in the labeling of several bands, including a predominant band at 47 kDa (Fig. 1A). Reprobing of stripped blots with an antibody directed against the nonphosphorylated amino terminus of CXCR4 indicated that the band at 47 kDa was CXCR4. To further show antibody specificity, we immunoprecipitated CXCR4 from LN428 cultures treated with CXCL12. Immunoprecipitated CXCR4 was detected using a rabbit polyclonal antibody (1:66 dilution). Immunoaustron reactions were detected using the corresponding secondary biotin-conjugated antibodies augmented by streptavidin–horseradish peroxidase and visualized by 3,3'-diaminobenzidine supplied by DAKO (Carpinteria, CA). Slides were then counterstained with hematoxylin, dehydrated through a series of alcohols and xylene, and coveredslipped in 50:50 xylene/Permount. Control sections were incubated with antisera in the presence of a 100 μmol/L excess of peptide or with isotype-matched IgG. Scoring of immunoreactivity was done by three independent observers according to the following scale: CXCR4, phospho-CXCR4 score: 0 = no staining; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; and 4 = 76-100% of tumor cells stained. The CXCL12 score was based on the percentage of blood vessels that stained positively for CXCL12. For each antigen, a mean score for each individual tumor specimen was determined from the three independent scores. Statistical analysis was done on the differences between grade 1 and grades 2 to 4 staining scores by two-tailed t test. Specificity of phosphospecific antibody staining was ascertained through two separate methods. In the first method, antibodies were preincubated for 1 hour at room temperature with PBS, 100 μmol/L nonphosphorylated peptide 332 to 346 or 100 μmol/L of P338 or P339 before application to tissue sections. In the second method, tissue sections were preincubated with 8,000 units/ml: A phosphatase for 30 minutes at 25°C before antibody incubation.
treating immobilized protein blots with phosphorylation for antibody recognition was further shown by CXCR4 when phosphorylated on P339. The requirement for CXCR4 was CXCR4 and that the phosphospecific antibody only recognized the use of this antibody for evaluating the relationship between ligand binding and CXCR4 function is dependent on its level of total CXCR4 but dramatically reduced that component antibody incubation. Phosphatase treatment did not decrease the phosphospecific antibody recognizes CXCR4 only when it is recognized by the phosphospecific antibody. Together, these data indicate that this antibody recognizes CXCR4 only when it is phosphorylated on P339-CXCR4 antibody was inhibited by preincubation with the unphosphorylated peptide or an alternate phosphopeptide. This strongly suggested that only the 47 kDa band represents specific CXCR4 labeling. Additional support for the specificity of the peptide antibody was obtained by Western blot analysis of U87 GBM cells transfected with CXCR4. U87 GBM cells express less CXCR4 than CXCR4-transfected U87 cells (T) were probed with P339-CXCR4–directed antibodies as indicated. Actin is provided as loading control. The P339-CXCR4–directed antibody recognizes an immunoprecipitated protein of 47 kDa that was also recognized by the anti-CXCR4 antibody, suggesting that only this band represents specific CXCR4 labeling. Additional support for the specificity of the peptide antibody was obtained by Western blot analysis of U87 GBM cells transfected with CXCR4. U87 GBM cells express less CXCR4 than CXCR4-transfected U87 cells (T) were probed with P339-CXCR4–directed antibodies as indicated. Actin is provided as loading control. The P339-CXCR4–directed antibody recognizes a phosphorylated form of CXCR4. A, Western blot analysis of LN428 GBM cells treated with CXCL12. Lysates were probed with P339-CXCR4 antibody, stripped, and reprobed with CXCR4-directed antibodies. Molecular weight markers are indicated. B, Western blot analysis of CXCR4 immunoprecipitated from LN428 GBM treated with CXCL12. Blots were first probed with the P339-CXCR4 antibody as indicated, then stripped and reprobed with CXCR4 antibody. Calculated molecular weight of the labeled band was 47 kDa. C, lysates from untransfected U87 cells (U) and CXCR4-transfected U87 cells (T) were probed with P339-CXCR4–directed antibodies as indicated. Actin is provided as loading control. D, effect of peptide competition and phosphatase treatment on P339-CXCR4 antibody labeling; absence of competing peptide (con), preincubation with peptide P332-46 (332-46), peptide P338 or peptide P339, absence of λ phosphatase (−lambda) or presence of λ phosphatase (+lambda). Total CXCR4 labeling serves as loading control.

Figure 1. P339-CXCR4–directed antibody recognizes a phosphorylated form of CXCR4. A, Western blot analysis of LN428 GBM cells treated with CXCL12. Lysates were probed with P339-CXCR4 antibody, stripped, and reprobed with CXCR4-directed antibodies. Molecular weight markers are indicated. B, Western blot analysis of CXCR4 immunoprecipitated from LN428 GBM treated with CXCL12. Blots were first probed with the P339-CXCR4 antibody as indicated, then stripped and reprobed with CXCR4 antibody. Calculated molecular weight of the labeled band was 47 kDa. C, lysates from untransfected U87 cells (U) and CXCR4-transfected U87 cells (T) were probed with P339-CXCR4–directed antibodies as indicated. Actin is provided as loading control. D, effect of peptide competition and λ phosphatase treatment on P339-CXCR4 antibody labeling; absence of competing peptide (con), preincubation with peptide P332-46 (332-46), peptide P338 or peptide P339, absence of λ phosphatase (−lambda) or presence of λ phosphatase (+lambda). Total CXCR4 labeling serves as loading control.

Figure 2. P339-CXCR4 antibody recognizes a CXCL12-induced form of CXCR4. A, Western blot analysis with P339-CXCR4–directed antibodies of LN428 GBM cells treated with CXCL12 for 0 or 10 minutes as indicated. Blots were stripped and reprobed with CXCR4 antibody as loading control. B, LN428 GBM cells were exposed to 1 μg/mL CXCL12 for 0 or 10 minutes. Samples were fixed at t = 0, t = 10, and t = 30 minutes. The latter sample was incubated for an additional 20 minutes after CXCL12 washout. Fixed cells were labeled with P339-CXCR4 antibody. Bar, 10 μm. C, effect of 10-minute exposure to 0.1 μg/mL CXCL12 (L12), 4 μM EGF, or 10 nM PMA on P339-CXCR4 immunoreactivity. Untreated cells serve as a control. Total CXCR4 immunoreactivity serves as loading control.

Figure 3. P339-CXCR4 antibody recognizes a CXCL12-induced form of CXCR4. The use of this antibody for evaluating the relationship between ligand binding and CXCR4 function is dependent on its ability to distinguish a ligand-activated form of the receptor. Serum starvation increased CXCL12 responsiveness (data not shown), and for all of these experiments LN428 GBM cells were serum starved for 24 hours before CXCL12 treatment. Serum-starved LN428 cells were treated with 1 μg/mL CXCL12 for 0 or 10 minutes and the abundance of phospho-CXCR4 was determined by Western blot and immunolocalization in fixed cells. In the absence of CXCL12, there was little P339-CXCR4 immunoreactivity but this was significantly increased by treatment with CXCL12 (Fig. 2A). The magnitude of the increase was highly dependent on the baseline level and ranged from 2- to >10-fold (data not shown). A similar CXCL12-induced increase in P339-CXCR4 immunoreactivity was seen by immunofluorescent analysis of paraformaldehyde-fixed cells (Fig. 2B). After 10 minutes of exposure to CXCL12, P339-CXCR4 could be seen localized to the surface of LN428 cells. CXCL12 was then washed away and cells were incubated in its absence for an additional 20 minutes. Surface labeling declined as a function of time and P339-CXCR4 was seen accumulating intracellularly. These observations suggest that the P339-CXCR4 antibody recognizes a CXCL12-induced change in CXCR4 and that this change is functionally relevant to receptor trafficking. To evaluate whether other stimuli might promote S339 phosphorylation, cells were treated with CXCL12, EGF, or the activator of protein kinase C, PMA. All three agents stimulated P339 phosphorylation (Fig. 2C). We routinely observed greater P339-CXCR4 immunoreactivity in response to PMA or EGF than in response to CXCL12.

Specificity of P339-CXCR4 staining in tissue sections. Phosphorylation of S339 was regulated by CXCL12 and EGF and associated with changes in subcellular localization of CXCR4.
Therefore, S339 seems to be an important regulatory site for CXCR4 function and we sought to define the specificity of P339-CXCR4 staining in tumor sections. To show that P339-CXCR4 was able to specifically recognize the appropriate phosphorylated form of CXCR4 in tissue sections, we did peptide competitions and phosphatase treatment before labeling of human astrocytoma specimens. As described below, tumor gemistocytes, which appeared as large cells with eccentric nuclei, were labeled strongly by the P339-CXCR4-directed antibody and this provided a convenient experimental subject for assays of antibody specificity. Gemistocyte staining with P339-CXCR4 (Fig. 3A) displayed a characteristic diffuse cytoplasmic and membranous pattern. Staining was not diminished by preincubation with the non-phosphorylated form of the immunizing peptide (332-46) or the P338 peptide but was abolished by preincubation with the P339 peptide. Incubation of tissue sections with λ phosphatase abolished P339-CXCR4 antibody labeling without diminishing CXCR4 antibody staining (Fig. 3B). Therefore, P339-CXCR4 specifically recognizes CXCR4 phosphorylated on S339 in Western blot and immunohistochemical analyses.

To determine what relationship P339-CXCR4 immunoreactivity had to CXCL12 expression, we examined human brain specimens for CXCL12, CXCR4, and P339-CXCR4 immunolabeling. The hypothalamus of human infants (<1 year old) contained areas with high levels of neuronal CXCL12 expression (Fig. 3B, HPT a) and other areas with little to no CXCL12 expression (Fig. 3B, HPT b). Both areas exhibited neuronal and astrocytic CXCR4 expression, but P339-CXCR4 staining was limited to neurons in the region of the hypothalamus that also expressed CXCL12. These data indicate that in normal human brain, P339-CXCR4, but not total CXCR4, immunoreactivity is highly correlated with CXCL12 expression.

**Human astrocytomas of all grades exhibit phospho-CXCR4 labeling.** In previous studies, we found that GBM and medulloblastoma expressed CXCR4 and that the endothelium of tumor-associated blood vessels expressed CXCL12 (3). The importance of this potential paracrine relationship was highlighted by the significant antitumor effect exerted by the specific CXCR4 antagonist AMD 3100 against intracranial xenografts of GBM and medulloblastoma. As this drug is reported to antagonize CXCL12 binding, we concluded that the paracrine relationship between CXCR4 and CXCL12 was critical for ligand activation of CXCR4 and subsequent survival signaling (13–16). We examined eight grade 1, five grade 2, eight grade 3, and eight grade 4 astrocytomas to determine whether this paracrine relationship was evident regardless of histologic grade and whether the activation of CXCR4 as determined by P339-CXCR4 staining was dependent on proximity to CXCL12. All grades of astrocytoma expressed CXCL12, CXCR4, and P339-CXCR4 (Supplemental Fig. S1). Similar patterns of staining were observed in all grades of astrocytoma and are highlighted in representative images from a grade 2 (Fig. 4A) and a grade 4 (Fig. 4B) tumor. In agreement with our prior studies, CXCL12 expression was localized to the endothelium of tumor-associated blood vessels in all grades of astrocytoma (Fig. 4A, CXCL12, arrowhead), including the endothelial cells of glomeruloid tufts in grade 4 tumors (Fig. 4B, CXCL12, arrowhead). In several examples, CXCL12 was also localized to scattered cells possessing microglial morphology (Fig. 4A, CXCL12 inset). In addition to vascular endothelium and infiltrating microglia, occasional tumor cells expressed CXCL12 (data not shown).

CXCR4 was primarily localized to tumor cells in all grades of astrocytoma. The activation state of expressed CXCR4 was evident in the diffuse phospho-CXCR4 (pCXCR4) immunoreactivity that was present in all grades of astrocytomas. All tumor specimens exhibited P339-CXCR4 staining in a punctate, cytoplasmic, and membranous distribution similar to what we have described for total CXCR4 staining (Fig. 4; Supplemental Fig. S1). One specimen with no evident CXCR4 expression exhibited a small degree of P339-CXCR4 immunoreactivity. The pan-CXCR4 antibody is directed against the amino terminus of the molecule whereas P339-CXCR4 is directed against the carboxy tail. Thus, this tumor may possess a mutation in the amino terminus of CXCR4 that interferes with the pan-CXCR4 antibody binding but not that of P339-CXCR4.

The endothelium of tumor-associated blood vessels was also frequently labeled by anti-CXCR4 and anti-P339-CXCR4 antibodies (Fig. 4, CXCR4 and P339-CXCR4, arrowheads), suggesting a possible autocrine relationship for endothelial cell CXCR4 activation. This may reflect the involvement of CXCR4 in the migration and proliferation of vascular endothelial cells during tumor angiogenesis (27, 28). In addition, there was a consistently high degree of pCXCR4 content in gemistocytes (Fig. 4A, P339-CXCR4 inset). The significance of this finding remains to be evaluated although it is intriguing that high numbers of gemistocytes in low-grade lesions have been associated with more rapid progression and a poorer prognosis. This observation could be consistent with a role for CXCR4 in glioma progression (29–31).

Tumor staining scores reveal several interesting patterns of CXCL12, CXCR4, and P339-CXCR4 expression (Fig. 5; Table 1).
Greater than 50% of all tumor cells, regardless of grade, expressed CXCR4 (mean score for all grades of astrocytoma is 2.59 ± 0.25) but <50% of all endothelial cells express CXCL12 (mean score 1.74 ± 0.12). Grades 2 to 4 tumors that exist as a spectrum of disease called diffusely infiltrating astrocytomas (32) exhibited similar overall staining for CXCR4 and pCXCR4 regardless of grade. Grade 1 tumors are pilocytic astrocytomas that typically exhibit a more circumscribed growth pattern and whose pathogenesis differs from the diffuse astrocytomas, grades 2 through 4 (33). CXCR4 expression (Fig. 5) was greatest in grade 1 tumors (3.29 ± 0.29) and exhibited a statistically significant difference compared with grades 2 to 4 disease (2.25 ± 0.16, *P* < 0.005). However, the importance of activated CXCR4 to increasingly aggressive tumor behavior was reflected in the increased proportion of CXCR4 that was present in the activated state in diffuse astrocytomas. One hundred percent of samples (3) and now report CXCR4 expression in lower-grade astrocytomas as well.

**Discussion**

To evaluate the role of CXCR4 in malignant brain tumor growth, we sought to correlate CXCR4 phosphorylation and function. CXCR4 is a seven-transmembrane spanning G protein coupled receptor (GPCR) with no intrinsic kinase activity. Multiple serines and threonines, as well as tyrosines, can be phosphorylated in response to both ligand binding or activity in parallel signaling pathways (18). Many of these sites can affect receptor trafficking and signaling, making the definition of an activated, phosphorylated form of the receptor complex and the development of a phosphospecific antibody directed at an activated state of a GPCR difficult. We were most interested in a CXCL12-activated form of CXCR4 as we suspected this would be critical to the role of CXCR4 in cancer and the target for CXCR4 antagonists, such as AMD 3100 (14, 15). Previous work on CXCR4 phosphorylation, internalization, and signaling has identified the carboxy tail as a critical domain for CXCL12-induced receptor internalization (17, 19, 20, 34, 35). Within the carboxy terminus, there are at least two motifs necessary for CXCL12-induced phosphorylation and internalization. These are the dileucine motif L328 and L329 and the serines at positions 338 and 339 (18). We generated an antibody directed against the peptide corresponding to amino acids 332 to 346 of human CXCR4 phosphorylated at position 339. This antibody clearly recognized a phosphorylated form of CXCR4 as evidenced by the immunoreactivity of CXCR4 immunoprecipitates and transfections and the loss of immunoreactivity on phosphatase treatment. The P339-CXCR4 antibody also recognized a CXCL12-induced change in CXCR4 on Western blot analysis as well as CXCL12-induced changes in the subcellular localization of CXCR4, indicating that this is a ligand-activated form of the receptor.

In all grades of astrocytomas, CXCR4 was present and activated. These findings suggest that CXCR4 plays a role in tumor biology regardless of the degree of malignancy and broaden the scope of potential CXCR4 functions in cancer. At present, the focus of most cancer-related CXCR4 research is on the relationship between CXCR4 and features of malignancy, such as tumor cell motility and metastatic behavior (2), or, more recently, angiogenesis (36). Rempel et al. (37) described CXCL12 and CXCR4 expression in GBM and suggested that the expression was related to necrosis and angiogenesis. The regulation of CXCL12 and CXCR4 expression by hypoxia (38) or vascular endothelial growth factor (39, 40) is consistent with this hypothesis. However, we observed a more diffuse pattern of CXCR4 staining in GBM and medulloblastoma samples (3) and now report CXCR4 expression in lower-grade astrocytomas as well.

**Figure 4.** CXCL12, CXCR4, and P339-CXCR4 expression in grade 2 and grade 4 astrocytoma. A, representative sections from a grade 2 astrocytoma were stained for CXCL12, CXCR4, and P339-CXCR4 as indicated. Arrowheads, vascular endothelium. CXCL12 inset, microglial cell. Two images are shown for CXCR4 staining to better highlight endothelial and tumor cell staining. P339-CXCR4 inset, gemistocyte. Bar for panels, 20 μm. Bar for insets, 10 μm. B, representative sections from a grade 4 astrocytoma were stained for CXCL12, CXCR4, and P339-CXCR4 as indicated. Arrowheads, vascular endothelium. Bar, 20 μm.

**Figure 5.** Comparison of grade 1 versus grades 2 to 4 astrocytoma staining scores. Scatter plots of grade 1 or grades 2 to 4 tumor CXCL12, CXCR4, and P339-CXCR4 staining scores and the ratio of P339-CXCR4 to CXCR4 (pCXCR4/CXCR4). Horizontal lines, mean. Ratio of pCXCR4 to CXCR4 was calculated for each tumor. Scatter plot of individual tumor values. *, *P* < 0.05, two-tailed t test.
Invasiveness is not a feature of grade 1 astrocytomas and necrosis is not a feature of astrocytomas grades 1 to 3 (25). Although angiogenesis may be necessary for all tumor growth, microvascular proliferation is not encountered in astrocytomas below grade 4 (41). Therefore, the expression of CXCL12 and CXCR4 cannot be strictly related to invasion, hypoxia, necrosis, or angiogenesis, and the functions of CXCR4 cannot be confined to mediating malignant behavior alone. Rather, the diffuse expression of CXCR4, together with the effects of AMD 3100 on intracranial xenograft growth (3), suggests that CXCR4 regulates tumor cell growth regardless of grade.

This does not necessarily mean that CXCR4 has equivalent function in all grades of astrocytoma or other cancers. The EGF and platelet-derived growth factor (PDGF) receptors are similarly present in both low- and high-grade astrocytomas but increased tumor grade is associated with increased expression of either receptor (42–45). The level of receptor expression may affect the strength of receptor signals and determine the pattern of receptor–receptor (42–45). The level of receptor expression may affect the strength of receptor signals and determine the pattern of receptor–receptor (42–45). The level of receptor expression may affect the strength of receptor signals and determine the pattern of receptor–receptor (42–45). Therefore, if the activity of other receptors affects S339 phosphorylation, it cannot be strictly related to invasion, hypoxia, necrosis, or angiogenesis, and the functions of CXCR4 cannot be confined to mediating malignant behavior alone. Rather, the diffuse expression of CXCR4, together with the effects of AMD 3100 on intracranial xenograft growth (3), suggests that CXCR4 regulates tumor cell growth regardless of grade.

In normal human brains, as well as in all tumor cases, the phosphorylation of CXCR4 on serine 339 was only observed in association with CXCL12. This supports the hypothesis that ligand activation of CXCR4 is the predominant mechanism for regulating its activity. In vitro studies indicate that EGF receptor activation can also induce CXCR4 phosphorylation. EGF receptor activation is a prominent feature in glioblastomas (grade 4) and, thus, would be expected to frequently occur in proximity to CXCR4 (46, 47). The ability of EGF to stimulate CXCR4 phosphorylation and potentially modulate its signaling raises the possibility that the role of CXCR4 in astrocytomas, or other EGF receptor-expressing cancers, may also be linked to EGF receptor function. It remains to be determined whether other receptor tyrosine kinases that are frequently activated in astrocytomas, such as PDGF receptor (42, 48–50) or insulin-like growth factor-I receptor (51), can also promote CXCR4 phosphorylation.

Multiple kinases are known to phosphorylate GPCRs, including GPCR kinases, PKC, and Akt (52). We showed that CXCL12 and EGF treatment, as well as PKC activation, could induce CXCR4 phosphorylation. PKC phosphorylation of CXCR4 can regulate internalization; however, in previously published studies, it seemed to involve a mechanism distinct from CXCL12-induced endocytosis (17–19). Data presented here suggest that S339 may serve as an integration point for multiple intracellular pathways in the regulation of CXCR4 signaling and internalization. It is interesting to consider that the regulation of CXCR4 internalization and signaling through the activation of other receptors could alter its functions in malignancy. CXCR4 activation of the heterotrimeric G protein Gi is stimulated by ligand and inhibited by β-arrestin binding (20, 21). β-Arrestin bound to CXCR4 can serve as a scaffold for the binding of RAP and mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinase and, therefore, this does not terminate the activation of the MAP kinase pathway (53, 54). S339 phosphorylation is a regulatory step in arrestin binding. Therefore, if the activity of other receptors affects S339 phosphorylation and β-arrestin binding, they could alter the balance of heterotrimeric G protein–dependent and G protein–independent signals downstream of CXCR4 activation. In this light, it will be interesting to examine other tumor types for CXCL12 and P339-CXCR4 expression. Particularly enlightening would be the comparison of tumors in which CXCR4 is suspected to play primarily a role in regulating motility compared with one for which it contributes to primary tumor growth. CXCR4-mediated chemotaxis is enhanced by β-arrestin binding (21), whereas primary tumor growth may require Gi-dependent signals such as calcium flux or the suppression of cyclic AMP. The coactivation of kinases either through genetic change or downstream of receptor activation could result in a change in P339 phosphorylation, β-arrestin binding, and the consequences of CXCR4 activation.

These data extend previous findings in which we described CXCR4 and CXCL12 expression in a neural tumor, medulloblastoma, as well as in GBM (3). These data suggest that stromal factors, such as CXCL12, may play critically important roles in tumor formation and growth. If this hypothesis is correct, CXCR4 antagonist therapy may be broadly applicable in patients with benign and malignant brain tumors. Based on data presented here, this could include children suffering with grade 1 astrocytomas. Pilocytic astrocytomas are the most common glioma of childhood (33). Although many of these tumors are adequately treated with surgery and radiation therapy, many, such as those that occur in the region of the hypothalamus, or adjacent to other eloquent areas, are unresectable and frequently cannot be radiated due to the young age of the patients. For these children, chemotherapy is indicated but associated with only an ~60% response rate (55, 56). Thus, there remains, even for these biologically benign tumors, a pressing need for additional, effective therapies. These studies provide a rationale for considering CXCR4 antagonist therapy in children and adults with low-grade tumors. It remains to be shown whether CXCR4 in other tumor types is similarly activated and amenable to small molecule inhibition.

The P339-CXCR4–directed antibody is a novel reagent that could be useful for stratifying patients in preparation for CXCR4 antagonist therapy and following response to targeted treatment.

### Table 1. Mean scores for astrocytoma staining

<table>
<thead>
<tr>
<th>Grade</th>
<th>CXCL12*</th>
<th>CXCR4†</th>
<th>pCXCR4‡</th>
<th>pCXCR4/ CXCR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=7)</td>
<td>1-3</td>
<td>2-4</td>
<td>1-3</td>
<td>0.25-1.5</td>
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<tr>
<td>Mean ± SE</td>
<td>1.57 ± 0.3</td>
<td>3.29 ± 0.29</td>
<td>2.29 ± 0.29</td>
<td>0.76 ± 0.15</td>
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<td>2 (n=5)</td>
<td>1-4</td>
<td>0-4</td>
<td>1-4</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>2 ± 0.55</td>
<td>2.2 ± 0.8</td>
<td>2.2 ± 0.49</td>
<td>1.13 ± 0.28</td>
</tr>
<tr>
<td>3 (n=8)</td>
<td>1-3</td>
<td>1-4</td>
<td>1-4</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.88 ± 0.35</td>
<td>2.63 ± 0.38</td>
<td>2.63 ± 0.38</td>
<td>0.18 ± 0.17</td>
</tr>
<tr>
<td>4 (n=8)</td>
<td>1-2</td>
<td>2-3</td>
<td>1-3</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.5 ± 0.19</td>
<td>2.25 ± 0.16</td>
<td>2.14 ± 0.24</td>
<td>1.0 ± 0.13</td>
</tr>
</tbody>
</table>

*Scores were determined as the mean score from three independent observers using the following scale: 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of blood vessels positive.
†Scores were determined as the mean score from three independent observers using the following scale: 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of tumor cells positive.
‡Mean = 50% and therefore cannot be strictly related to invasion, hypoxia, necrosis, or angiogenesis, and the functions of CXCR4 cannot be confined to mediating malignant behavior alone. Rather, the diffuse expression of CXCR4, together with the effects of AMD 3100 on intracranial xenograft growth (3), suggests that CXCR4 regulates tumor cell growth regardless of grade.

This does not necessarily mean that CXCR4 has equivalent function in all grades of astrocytoma or other cancers. The EGF and platelet-derived growth factor (PDGF) receptors are similarly present in both low- and high-grade astrocytomas but increased tumor grade is associated with increased expression of either receptor (42–45). The level of receptor expression may affect the strength of receptor signals and determine the pattern of receptor-mediated effects. We found that the fraction of CXCR4 that was present in a phosphorylated state was increased in the diffuse astrocytomas compared with the grade 1 tumors. It will be important to ascertain whether the level of CXCR4 activation correlates with increasing tumor grade in a variety of tumor types.

In normal human brains, as well as in all tumor cases, the phosphorylation of CXCR4 on serine 339 was only observed in association with CXCL12. This supports the hypothesis that ligand activation of CXCR4 is the predominant mechanism for regulating its activity. In vitro studies indicate that EGF receptor activation can also induce CXCR4 phosphorylation. EGF receptor activation is a prominent feature in glioblastomas (grade 4) and, thus, would be expected to frequently occur in proximity to CXCR4 (46, 47). The ability of EGF to stimulate CXCR4 phosphorylation and potentially modulate its signaling raises the possibility that the role of CXCR4 in astrocytomas, or other EGF receptor-expressing cancers, may also be linked to EGF receptor function. It remains to be
References


Widespread CXCR4 Activation in Astrocytomas Revealed by Phospho-CXCR4-Specific Antibodies

B. Mark Woerner, Nicole M. Warrington, Andrew L. Kung, et al.