Interstitial Flow in a 3D Microenvironment Increases Glioma Invasion by a CXCR4-Dependent Mechanism

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Abstract

Brain tumor invasion leads to recurrence and resistance to treatment. Glioma cells invade in distinct patterns, possibly determined by microenvironmental cues including chemokines, structural heterogeneity, and fluid flow. We hypothesized that flow originating from pressure differentials between the brain and tumor is active in glioma invasion. Using in vitro models, we show that interstitial flow promotes cell invasion in multiple glioma cell lines. Flow effects were CXCR4-dependent, because they were abrogated by CXCR4 inhibition. Furthermore, CXCR4 was activated in response to flow, which could be responsible for enhanced cell motility. Flow was seen to enhance cell polarization in the flow direction, and this flow-induced polarization could be blocked by CXCR4 inhibition or CXCL12 oversaturation in the matrix. Furthermore, using live imaging techniques in a three-dimensional flow chamber, there were more cells migrating and more cells migrating in the direction of flow. This study shows that interstitial flow is an active regulator of glioma invasion. The new mechanisms of glioma invasion that we identify here—namely, interstitial flow-enhanced motility, activation of CXCR4, and CXCL12-driven autologous chemotaxis—are significant in therapy to prevent or treat brain cancer invasion. Current treatment strategies can lead to edema and altered flow in the brain, and one popular experimental treatment in clinical trials, convection enhanced delivery, involves enhancement of flow in and around the tumor. A better understanding of how interstitial flow at the tumor margin can alter chemokine distributions, cell motility, and directed invasion offers a better understanding of treatment failure. Cancer Res; 73(5); 1536–46. ©2012 AACR.

Introduction

Glioma invasion is a clinical obstacle to therapy, occurring at multiple stages of cancer progression. Glioma cell invasion into the brain shows distinctive patterns known as Scherer’s structures that include white matter tract, perivascular, and parenchymal spread (1, 2). Mechanisms underlying these patterns are unclear, although hypotheses exist including following tissue paths of least resistance (1), fluid flow (3), structural cues (4), and protein interactions (5).

Although many mechanisms are involved in the invasion of glioma cells into the healthy brain, chemokines often guide this invasion. In particular, CXCL12 and its receptor CXCR4 are involved in brain cancer invasion and progression (6). Invasive tumors express high levels of CXCL12 and CXCR4 (7, 8) (9), and CXCR4 phosphorylation is seen at the leading edge of high-grade glioma (10). CXCL12 and its receptor are mediators of chemotaxis in the brain in development and disease and have been the most well studied in brain cancer (11). CXCL12 is found along white matter tracts, blood vessels, and subpial regions, possibly providing chemotactic guidance cues for invasion (2).

Fluid flow in the brain also follows these pathways (3). Such interstitial flow can theoretically redistribute chemokine gradients (12) that can guide cell migration. We previously showed in vitro that slow interstitial flow enhanced the invasion of CCR7-expressing human cancer cells that secreted CCR7 ligands (13) possibly due to pericellular ligand gradient formation around individual cells. The resulting "autologous chemotaxis" could be an important mechanism of tumor cell homing to lymphatics.

Although lymphatics are not present in the brain, fluid flow is important. Careful regulation of cerebrospinal fluid (CSF) flow patterns in the brain maintains the neuronal microenvironment and function. Recently, Iliff and colleagues delineated mechanisms of fluid drainage from the brain indicating that fluid flow pathways in perivascular spaces act like a brain lymphatic system clearing the brain interstitium (14). When a brain tumor develops, the flow patterns are disrupted because of tumor fluid drainage, cyst development, and edema-associated heightened interstitial pressures in the tumor as compared with the surrounding brain leading to flow from the...
tumor bulk (15, 16). Tumors originating further from white matter tracts (conduits for fluid flow) have a better prognosis (17). Other tracts such as blood vessels and meningeal linings are hotspots for invasion and correlate with flow pathways and chemokine secretion in the brain (6, 18). Therefore, it stands to reason that fluid flow, CXCL12 gradients, and white matter tracts are interrelated.

Here, we show that flow and CXCR4–CXCL12 signaling are indeed correlated, both in vivo and in vitro, and propose a new mechanism for flow-directed tumor cell invasion in the brain. We show that physiologically relevant fluid flow can substantially enhance glioma cell invasion in a CXCR4-dependent manner. Two mechanisms were identified: CXCL12-mediated autologous chemotaxis and flow-enhanced CXCR4 phosphorylation. Our data therefore introduce fluid flow as an active modulator of CXCR4-mediated glioma invasion.

Materials and Methods

In vivo glioma analysis

Fisher 344 (Harlan) male rats were inoculated with 250,000 enhanced GFP-expressing RT2 glioma cells 3 mm lateral and 1 mm anterior to bregma at a depth of 3.3 mm. Ten days after injection, Evans blue (50 mg/kg in saline) was injected intravenously 12 hours before intracardial perfusion with saline. Brains were collected, soaked in sucrose, cryosectioned, and sectioned at 16 μm. Sections were stained for CXCL12 (AbCam), white matter tracts (fluoromyelin, Invitrogen), and cell nuclei [4',6-diamidino-2-phenylindole (DAPI), Invitrogen]. Overlap coefficients and gradients were determined using ImageJ. Drainage was determined by measuring the distance of Evans blue staining into the brain at the top and bottom of the tumor. All animal procedures were conducted in accordance with Georgia Institute of Technology Institutional Animal Care and Use Committee (Atlanta, GA).

Cell lines and culture

RT2 and eGFP-RT2 cells were a kind gift from Helen Fillmore (Virginia Commonwealth University, Richmond, VA), U87MG and C6 cells were from American Type Culture Collection, and 9L cells were provided by the Neurosurgery Tissue Bank at University of California San Francisco (San Francisco, CA). All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Mediatech) with 10% FBS and 1% penicillin/streptomycin.

Three-dimensional cell assays

Experiments were carried out with 12 mm diameter, 8-μm pore cell culture inserts (Millipore). Tumor cells (10^5) were seeded in 100 μL gel (0.1% hyaluronan; Glycosan Biosystems) and 0.12% rat tail collagen I (BD Biosciences). For chemotactic (100 nmol/L), the chemical was added to the gel and media. The above setup was modified to examine the effects of interstitial flow on tumor cell migration. After the gel was cast and set, a pressure head of 1 cm media was established leading to an average velocity of 0.7 μm/s through the cell/gel compartment. For static conditions, media were held level inside and outside of the insert.

Western blot analysis, immunostaining, flow cytometry, and ELISA

Protein was harvested from cells after 16-hour flow in gels. To account for matrix-bound ligands, 3 compartments were analyzed: medium, matrix-associated protein (by digestion with collagenase D; BD Biosciences), and cells [by lysis using radioimmunoprecipitation assay (RIPA) buffer; (Sigma)]. Protein secretion was quantified using a CXCL12 ELISA (R&D). Western blotting was conducted using chicken anti-CXCR4 (Sigma), rabbit anti-p-CXCR4 (S339; AbCam), horseradish peroxidase-conjugated goat anti-chicken IgG and anti-rabbit IgG (BioRad), and a Western Pico ECL substrate kit (Pierce). The following antibodies/stains were used for immunostaining: CXCL12 (AbCam), fluorescein isothiocyanate (FITC)-labeled anti-GFP (Invitrogen). For cultured cells and flow cytometry, anti-rat CXCR4 (Sigma), p-CXCR4 (AbCam), and p-Akt (AbCam) were used. AlexaFluor-labeled secondary antibodies, AF594 phallolidin, and rhodamine-fluoromycin were obtained from Invitrogen.

Polarization assays and CXCR4 image analysis

A total of 5 × 10^5 RT2 cells in hyaluronan/collagen matrix were added to radial flow chambers and exposed to pressure-driven flow for 16 hours. Chambers were fixed in 4% paraformaldehyde, permeabilized with 0.05% Triton, and immunostained using the appropriate antibodies. High-resolution images were taken using confocal microscopy, and widefield microscopy was used for assessment of polarized cells in randomized all regions of the chamber. Imaging parameters were held constant for all pictures, and intensities were measured in ImageJ and normalized to static conditions.

Computation of extracellular CXCL12 distribution

The velocity profile through the extracellular matrix (ECM) around the cell used a value for permeability of K = 10^{-12} cm^2 (determined from the literature) and range of v = 0, 0.02, 0.2, and 2 μm/s (19). Three species were modeled: P, the cell-released protease; CXCL12, the cell-released CXCL12; and HS-CXCL12, the matrix-bound CXCL12. Constant flux boundary conditions were assumed for both P and CXCL12 at cell surfaces, with CXCL12 fluxes measured experimentally (Supplementary Fig. S3), along with zero flux inlet boundary conditions. The diffusion coefficients were assumed to be 140 μm^2/s for CXCL12 and 80 μm^2/s for P (12). k_in and k_off were assumed to be 9.3 × 10^4 (mol/L)^{-1} s^{-1} and 0.116 × 10^{-14} (mol/L)^{-1} s^{-1}, respectively, based on our own measurements (k_d = 12.4 nmol/L; Supplementary Fig. S3), and k rel was assumed to be 1 × 10^10 (mol/L)^{-1} s^{-1}. HS was calculated to be 2.6 mmol/L with 0.1% HA concentration in the gel. Binding constant of CXCL12 to the matrix was determined through a modified ELISA-based assay as described in

Published OnlineFirst December 27, 2012; DOI: 10.1158/0008-5472.CAN-12-2838
Supplementary Methods with data points fit to single site-specific binding kinetics curve ($R^2 = 0.97$) for determination of $k_p$. The calculations were conducted using COMSOL Multiphysics modeling software on a personal computer.

**Live imaging and analysis of cells under flow**

RT2 cells were seeded at $3 \times 10^5$ cells/mL in a 3-dimensional (3D) flow chamber (20) in 3D gels as described for static flow assays, but with L-15 medium in place of DMEM. Images were taken every 15 minutes for 16 hours at four locations in each chamber (4 chambers total: 2 static, 2 flow). Cells in videos were tracked using ImageJ and analyzed using MATLAB for migration parameters. After live imaging, FITC-dextran was flowed through the system to determine flow rates by fluorescence recovery after photobleaching analysis and assess any fluid channeling.

**Statistical analysis**

Data were analyzed using one-way ANOVA with Tukey post $t$ tests for pairs of data, and Bennett test for unequal variance was used to adjust significance. Results are reported with $n =$ number of experiments carried out (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Averages of replicates in experiments were used to determine statistical significance. Data are shown as SEM. Graphs were generated using GraphPad Prism Software.

**Results**

**In vivo. RT2 glioma invasion correlates with fluid flow and CXCL12**

To determine correlation of invasion pathways with fluid flow patterns and CXCL12 distribution, we inoculated male Fisher 344 rats with eGFP-expressing RT2 glioma, a highly invasive model that recapitulates many attributes of human glioma invasion (21). After 9 days, intravenous Evans blue was administered to visualize fluid leak from tumor vasculature and subsequent drainage from the tumor into the surrounding brain. Although not a direct measure of all flow patterns in the brain, the drainage seen from leaking blood vessels is the primary contributor to altered fluid drainage, CXCL12 gradients, and tumor invasion (22) and indicates the flow seen by peripheral glioma cells in the tumor. Serial sections were compared for localization of white matter tracts and nuclei (Fig. 1A and Supplementary Fig. S1), cancer cells (GFP, green), flow pathways (Evans blue, red) and CXCL12 (cyan; Fig. 1B). Colocalization between fluid drainage, CXCL12 gradients, and cell invasion beyond the tumor bulk was confirmed by image analysis and Manders Overlap R calculations (Fig. 1C). An area of high fluid drainage (high) and low fluid drainage (low) as assessed by distance of Evans blue drainage beyond the tumor bulk were selected for analysis. It was seen that CXCL12 extended beyond the invasion of cancer cells indicating the possible movement of the chemokine by flow or expression by other cells in the area.

There were high levels of CXCL12 seen in the tumor bulk (within dotted lines, Fig. 1B). Measurement of CXCL12 from the border of the tumor bulk outward into the brain indicated distinct macroscopic gradients of CXCL12, which were higher in the high drainage region than in the low drainage region (Supplementary Fig. S2). The gradients would indicate that the tumor cells should be migrating toward the tumor bulk where there is a sink of CXCL12; however, we know that brain cancer cells do the opposite (23) while being highly chemotactic toward CXCL12. On a microscopic level, we were...
interested in determining whether there is a more complex link between this chemokine, interstitial flow, and invasion of glioma.

**Selection of matrix for in vitro interstitial flow studies**

The ECM of the brain is largely composed of hyaluronan, proteoglycans, and various basement membrane proteins such as laminin (5). *In vitro* several matrices have been used to examine the behavior of glioma cells including basement membrane extract (24), hyaluronan (24), laminin (25), and collagen I (26, 27). We also measured the flow velocities through matrices of the same height in the tissue culture insert to determine whether we could achieve comparable average interstitial flows to that reported *in vivo* in a reproducible manner (Supplementary Fig. S4A). Of the combinations that were tried including 1.8 mg/mL Collagen I (BD Biosciences), 0.5 mg/mL basement membrane extract (Matrigel, BD Biosciences), 1.0 mg/mL hyaluronan (Glycosan), 1.0 mg/mL hyaluronan + 1.2 mg/mL Collagen I, the combination of collagen I and hyaluronan yielded the flow velocity (0.72 ± 0.02 μm/s) most similar to that *in vivo* with consistent cell morphology while recapitulating the most abundant matrix component of the brain (hyaluronan). Furthermore, as stiffness is known to alter invasion of cancer cells (28), we measured the stiffness of the gels to ensure that addition of the collagen would not affect invasion in this manner. Stiffness was not significantly different in the hyaluronan + collagen gel as compared with the hyaluronan gel alone (Supplementary Fig. S4A). These Young moduli are within range of those in the brain, which are between 300 and 1,000 Pa (29). To determine the best matrix for our experiments, we embedded glioma cells (RT2) and examined their response to serum in 1-mm high gels atop 8-μm pore tissue culture inserts and examined their morphology in the gel (Supplementary Fig. S4B).

**Glioma invasion is enhanced by interstitial flow and CXCL12 gradients**

We compared 4 glioma cell lines *in vitro* for response to interstitial flow: RT2 (invasive rat astrocytoma), U87MG (invasive *in vitro*, human glioblastoma), C6 (invasive rat astrocytoma), and 9L (noninvasive rat gliosarcoma; baseline invasion without any stimulus Fig. 2B). Using 3D cultures of cells in the hyaluronan/collagen I matrix atop 8-μm porous inserts, with interstitial flow rates of 0 or 0.72 ± 0.02 μm/s (Fig. 2A), we found that the 3 invasive cell lines were about 2-fold more invasive under flow (Fig. 2C). Noninvasive 9L cells showed no change with flow.

We next compared the chemotactic response of the 4 cell lines with gradients of CXCL12 (Fig. 2D). Cells were cultured in the same 3D gels as before and allowed to invade toward CXCL12 in the lower chamber (100 nmol/L) under static conditions. Interestingly, cell response to CXCL12 gradients mirrored their responses to flow and ranged from nearly no response (9L) to a strong response (RT2). Indeed, correlations were positive between CXCL12 chemoinvasion, invasive potential of the cell lines, and flow response (Supplementary Fig. S5).

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Published OnlineFirst December 27, 2012; DOI: 10.1158/0008-5472.CAN-12-2838

www.aacjrournals.org Cancer Res; 73(5) March 1, 2013 1539

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Flow-enhanced invasion is CXCR4-dependent

As mentioned, the CXCL12–CXCR4 chemotactic axis has been highly implicated in glioma progression and invasion. As there was a correlation between CXCL12 chemotactic response and the CXCL12–CXCR4 axis in general and response to interstitial flow, we wanted to determine whether the flow-enhanced invasion of glioma cells was related to CXCL12 gradients. We examined cell invasion under flow with either AMD3100, a CXCR4 antagonist (30), or 100 nmol/L uniformly distributed CXCL12 to overpower any endogenous CXCL12 gradients. Under both conditions, the flow response was abrogated in RT2, C6, and U87MG cells (Fig. 3A and Supplementary Fig. S6). The secretion of CXCL12 was not affected by flow in any cells (Fig. 3B and Supplementary Fig. S7). Similarly, the percentage of cells expressing CXCR4 (Supplementary Fig. S7) and the overall mean value of CXCR4 did not significantly increase (Fig. 3D and E), indicating that the enhancement was not due to a change in CXCR4 or CXCL12 expression. Although the expression levels of CXCL12 and CXCR4 did not change, baseline levels of these 2 proteins may have an effect on their ability to respond to flow. RT2, the most flow-responsive cell line, has higher secretion levels of CXCL12 than the other cell lines. Furthermore, 9L, which is completely non–flow-responsive, had relatively zero expression of both CXCR4 and CXCL12. This also indicates that although there is no change, there may be a relation between the baseline expression levels and ability to respond to flow as shown by the weak

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Figure 3. CXCR4 dependence of interstitial flow enhanced invasion. A, invasion of RT2 glioma with CXCR4 blocking (10 μmol/L AMD3100) or uniformly distributed CXCL12 (100 nmol/L) under static and flow conditions (n = 6, P < 0.001). B, CXCL12 protein levels from RT2 cells in tissue culture inserts as measured by ELISA (n = 4, n.s.). C, schematic of radial flow chamber used for imaging studies of cells in vitro. D, representative images of single RT2 cells (nucleus, blue), stained for CXCR4 (red) and phosphorylated CXCR4 (p–CXCR4, green) after 16 hours of flow or static conditions. Scale bar, 5 μm; arrow indicates direction of flow. E, quantification of signal intensity (normalized to static for each experiment) from immunostained cells; 55 cells from 3 experiments (t test for each protein; ***, P < 0.001). F, representative Western blots of total and p–CXCR4 after 16 hours of static or flow conditions through tissue culture insert 3D gels. Data are mean ± SEM.
correlation between expression levels and flow response (Supplementary Fig. S5). To examine the uniqueness of this phenomenon to the CXCR4–CXCL12 axis, we looked at blocking other receptors known to be involved in chemotaxis of glioma, namely TGFβR, EGF receptor (EGFR), and platelet-derived growth factor receptor (PDGFR). Blocking of these receptors did not abrogate the flow-enhanced invasion (Supplementary Fig. S7).

**Flow increases CXCR4 phosphorylation**

For direct imaging of cell invasion under flow conditions, cells were seeded in radial flow chambers, where migration is horizontal, and in which cells can be fixed, stained, and imaged consistently. Cells were exposed to radial flow for 16 hours and stained for both total CXCR4 and p-CXCR4. Expression of p-CXCR4 increased under flow (Fig. 3C and D), which was confirmed by Western blotting (Fig. 3E).

**Cells polarize in the flow direction in a CXCR4-dependent manner**

Using the radial flow chamber, we analyzed the degree to which cells were polarized in the flow direction by staining for p-AKT (Fig. 4A). When flow was applied, there was a significant increase in the percentage of cells generally polarized in any direction (Fig. 4B), which was blocked by AMD3100 and enhanced by exogenous CXCL12. For each polarized cell, the direction of polarization relative to the flow direction was measured according to this staining and binned into 1 of 4 quartiles (Fig. 4C). Under static conditions, cells were approximately equally polarized in all directions. Under flow conditions, there was an increase in the percentage of polarized cells that were polarized in the direction of flow. This polarization was blocked by adding exogenous CXCL12 or by blocking CXCR4. Therefore, both an increase in the number of
polarized cells and in their direction (toward flow) was induced by fluid flow in a CXCR4-dependent manner.

The change from general polarization to polarization in the direction of flow accounted for 12.8% of the cell population. Through immunocytochemistry, the number of cells expressing both CXCL12 and CXCR4 was determined (Fig. 4D) yielding a similar number (15.6%). There may be a subpopulation of cells that are candidates for autologous chemotaxis, whereas cells expressing just CXCR4 (>40%) have generally enhanced motility.

**Autologous chemotaxis as a mechanism for cell polarization**

Using an experimentally determined value for CXCL12 matrix binding ($k_d = 12.6$ mmol/L, Supplementary Fig. S8), CXCL12 secretion rates (0.16 pg/h/1,000 cells), and literature-reported values for matrix diffusion (31), we modeled hypothetical pericellular CXCL12 gradients under varying flow rates (Fig. 4E with graphs of CXCL12 levels around cells). We predicted that pericellular gradients of 1.4% could form under flow rates of 0.2 µm/s and became more pronounced at 2 µm/s (9.2%), which is relevant in brain tumor edema conditions (3, 15). This gradient forms from the upstream side toward the downstream side leading to a higher concentration of CXCL12 felt by the front of a cell as opposed to the back (Fig. 4E graph).

**Live imaging reveals both increases in chemokinesis and chemotaxis of glioma under flow**

To determine whether autologous chemotaxis might be a mechanism for increased glioma migration as hypothesized, RT2 glioma cells were seeded in pillar chambers (Fig. 5A) in the same 3D matrix (20). Flow was applied at 0.2 to 0.8µm/s and imaged for 16 hours (Supplementary Video). Cell paths were tracked (polar plots Fig. 5B) and analyzed to determine differences in migration behavior (amount, directionality, speed, etc.). Total 3 experiments were run. Total cells migrating significantly increased under flow ($P < 0.05$; Fig. 5C). Persistence (Fig. 5D) and speed of cells (Fig. 5E) did not vary between conditions, however, there was a notable shift in velocity to the flow direction under that condition (Fig. 5F). To account for individual cell differences in velocity, we normalized individual cell velocities in the flow direction ($V_f$) to their individual cell speeds ($V$) to yield a standardized velocity measure over individual experiments and cells. A histogram of this $V_f/V$ indicates a shift in movement of the cell population toward the flow direction (Fig. 5G). Taking the area under the curve in the flow direction ($V_f/V > 0$) shows a significant increase in the percentage of cells moving in the flow direction ($P < 0.05$; Fig. 5H). These results indicate that there is a subpopulation of cancer cells that respond to flow by moving in the direction of flow. In addition, we see a general increase in the number of cells migrating. Therefore, we posit that both chemokinesis and autologous chemotaxis account for the increased migration that we see with glioma cells in response to interstitial flow.

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**Figure 5.** Live imaging and analysis of glioma cells’ response to interstitial flow. A, schematic of flow chamber used for live imaging studies with zoomed in field of view for cell tracking. B, representative field-of-view polar plots of migration tracks under static and flow conditions. C, quantification of the fraction of cells that migrate more than 2 cell diameters in 16 hours. , $P < 0.05$. D, persistence of cells calculated as net distance/total distance. E, cell speed throughout the experiment ($V_f$, µm/s). F, average cell velocity in the flow direction ($V_f$, µm/s). Flow direction indicated by arrow. G, normalized per cell velocity in the flow direction ($V_f/V$) histogram of all cells across all experiments. Shaded regions indicate area under the curve of cells moving positively in the flow direction for static (gray) and flow (black dots). H, area-under-the-curve analysis showing cells moving in the direction of flow ($V_f/V > 0$), $P < 0.05$). Box graphs represent mean (line) with minimum/maximum. Total of 3 separate experiments were run and mean numbers used for t test statistical analysis shown in box diagrams. Total cells tracked were 338 (static) and 280 (flow) with all represented in the histogram.
Discussion

In this study, we propose a new mechanism to explain patterns of glioma invasion in the brain. It has been proposed that invasion relates to the heterogeneity of ECM as evidenced by migration along vascular basement membranes (5). It has also been suggested that cancer cells respond to structural cues and reduced resistance for invasion along white matter tracts (4, 17, 23). Fluid flow has been attributed to passive movement of cancer cells in CSF as evidenced by the presence of cancer cells in CSF and the coincidence of flow pathways with migration pathways including white matter tracts, perivascular spaces, and leptomeningeal linings (3, 32). We show that interstitial flow enhances invasion of glioma cells in line with previously reported values (13). To do this, we have used several in vitro models of tumor invasion to study a variety of aspects of tumor cell invasion (receptor activation, cell polarization, chemokinesis, and chemotaxis) showing that each has an important role in the CXCL12-dependent flow response of glioma cells.

Although current theories have considered chemokines and flow pathways to be independent, our results suggest that they interact to promote glioma invasion. On the basis of correlations between gradients in vivo and response to flow and expression levels of chemokine–receptor pairs in vitro, we hypothesized that these characteristics of glioma malignancy are interrelated. Interstitial flow has been implicated in modulating several cell functions including transport function in the lymphatic endothelium (33), fibroblast alignment, migration, and TGF-β activation (34). A recent report examined the effect of shear flow on glioma cells and found that invasion decreased (35). However, this study primed the cells with acute flow followed by exposure to stable chemokine gradients and was interested in only the cells migrating after this flow period (under static conditions). In our study, we examine what happens during the flow period, which would be physiologically relevant in late-stage brain tumors with higher interstitial pressures and thus increased pressure gradients and see an increase in invasion.

We found that one mechanism for CXCR4-dependent flow response was CXCR4 phosphorylation, which is correlated with tumor invasiveness clinically (10). It has been shown that shear flows can activate integrins through phosphorylation, and it is possible a similar mechanism is occurring with glioma cells in response to flow (36). However, as the flow response was abrogated with uniformly distributed CXCL12, we suggest that autologous chemotaxis accounts for enhanced invasion in a subpopulation of cells (i.e., those expressing both CXCL12 and CXCR4). Analysis of the size of this subpopulation compared with those cells directionally polarized with flow indicates that this may be an interesting conclusion. Our computational model suggested that slow interstitial flow can cause pericellular CXCL12 gradients of at least 1% to form around a circular cell. Such gradients are sufficient to elicit directional migration in other cell types including neutrophils (37), fibroblasts (38), and dendritic cells (39). We, in fact, see both directional cell polarization and migration in response to flow through our studies possibly indicating a role for autologous chemotaxis complementary to enhanced chemokinesis. In vivo, we see correlation of fluid flow pathways with chemokine dispersion, indicating on a gross scale that fluid flow carries chemokines downstream of the tumor bulk and beyond invading tumor cells. However, the highest concentrations of CXCL12 remain in the tumor bulk. Therefore, we suggest that the flow may be responsible for microscopic pericellular gradients forming at the tumor border as well as activation of CXCR4, which is known to occur in glioma (10). This mechanism is summarized in Fig. 6 showing both a high concentration of CXCL12 in the tumor bulk where cell concentrations are high but a lower overall concentration at the periphery allowing for formation of these microscopic pericellular gradients. Furthermore, other cells that interact with the tumor, such as microglia and astrocytes, can contribute to CXCL12-mediated cell motility.

While our studies suggest these mechanisms, we offer there are limitations to the conclusions we have made using the in vitro model system. The use of a matrix that contains collagen I is not ideal for in vivo replication as the brain matrix does not contain inherent levels of collagen I. However, it has been shown that glioma cells produce significant amounts of collagen I at the leading edge of brain tumors and often this matrix takes on a more filamentous form leading to an ability of the glioma cells to invade in a more efficient manner (40). Furthermore, the addition of collagen to the matrix of hyaluronan, the primary component of the brain extracellular space (5, 41), did not significantly alter tumor cell morphology or invasion while allowing flow velocities and stabilities within physiologic conditions.

Other mediators of invasion include matrix metalloproteinases (MMP), TGFβ, and growth factors such as EGF and PDGF (6). CXCL12 and CXCR4 blocking in vivo reduces proliferation (42), angiogenesis (8), and invasion of implanted glioma (30). We tested a few of these molecules to determine whether they might be involved in flow-mediated invasion, but blocking of PDGFR, EGFR, and TGFβ did not abrogate the flow-enhanced invasion like blocking of CXCR4. Other chemokines have been implicated in malignancy of glioma, including CXCL10–CXCR3 involvement in proliferation and CCL2, CCL3, and CXCL13 involvement in immune cell homing, but only the CXCL12–CXCR4 axis has been implicated in glioma invasion.

Understanding glioma dissemination is important to clinical and experimental treatments for glioblastoma. Standard of care (radiotherapy, resection, chemotherapy) often leads to edema and increased flow (43). Convection-enhanced delivery (CED), an experimental therapy, involves distribution of therapeutics through the brain via flow through inserted catheters. Clinical trials have been unsuccessful at showing increased benefit of this treatment (44), potentially due to the fact that standard-of-care treatment had already occurred that is more successful at eliminating proliferative cells. More invasive cells are left behind after treatment (45). Cells at these invasive fronts in human samples have activated CXCR4 (p-CXCR4; refs. 7, 10, 46). Furthermore, cells in pseudopalisading foci of glioma as well as surrounding blood vessels show enhanced signaling of CXCR4 and CXCL12 clinically (8, 47, 48). Treatment
of glioblastoma xenografts with a combination of BCNU and AMD3100 shows enhanced efficacy over the chemotherapeutic alone (49), leading the push to think of the CXCR4 axis as a factor in chemoresistance (50). Hence, the mechanism we introduce here might give another reason why this phenotype is linked to glioma progression as well as a role in therapy.

Furthermore, flow-enhanced motility and directedness in CXCL12⁺ CXCR4⁺ glioma cells would suggest that CED, which aims to increase interstitial flow and traditional therapies that cause inflammation-associated edema, could drive increased invasion in a subpopulation of glioma cells.

In summary, interstitial flow is not simply a passive component in glioma spread but rather an active modulator (Fig. 6). Not only can it alter receptor phosphorylation, but also can it skew autologously secreted chemokines to form pericellular gradients that guide glioma cell migration. Our findings are relevant to microenvironmental 3D modeling of glioblastoma, therapeutic strategies involving local enhancement of flow (e.g., CED), and strategies to target invasive areas of the tumor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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