A dialog between glioma and microglia that promotes tumor invasiveness through the CCL2/CCR2/interleukin-6 axis

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Abbreviations: FGF, fibroblast growth factor; GDNF, glial-derived neurotrophic factor; IL, interleukin; KGF, keratinocyte growth factor; MMP, matrix metalloproteinase.

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Introduction

Malignant gliomas are highly proliferative and invasive tumors within the central nervous system. Several factors account for their tumorigenicity, including genetic mutations and amplification of signaling pathways, and their exploitation of surrounding non-transformed brain cells for molecules essential for glioma growth and invasiveness (1,2). Among the latter molecules, chemokines and chemokine receptors expressed by glioma cells have been shown to modulate glioma cell invasiveness, survival, proliferation and angiogenesis (3,10–13). For example, we reported that among the CXCRs examined, CXCR4 was expressed prominently on most glioma lines and resected glioma specimens and that the addition of the CXCR4 ligand, CXCL12, increased the activity of protein kinase B/Akt and promoted the survival (14) and invasiveness (15) of glioma cells. Although a number of chemokines have been described on glioma cells, the function of these proteins in glioma biology is far from complete, particularly their roles in the glioma microenvironment.

The microenvironment around glioma cells in situ comprises not only of stromal central nervous system-intrinsic cell types, such as astrocytes and microglia, but also of inflammatory cells that have infiltrated into the tumor from the circulation, including lymphocytes, macrophages and neutrophils (16,17). Although these infiltrated immune cells may seek to curb tumor growth initially, much evidence point to the tumor subsequently neutralizing the immune cells (16,18). Furthermore, glioma cells exploit immune and neural cells in their microenvironment for increased growth, invasiveness and for angiogenesis (2,19).

To address the mechanisms of glioma growth and its exploitation of the microenvironment, we have sought to identify chemokines that are commonly expressed across a variety of human glioma cell lines, with the expectation that such chemokines could be important regulators of autocrine and paracrine growth. Our results reveal that glioma cells produce CCL2 in particular but that this was not utilized for autocrine growth. Instead, we determined that CCL2 stimulated microglia, which in turn provides IL-6 to stimulate glioma invasiveness. These results highlight both the importance of chemokines in glioma biology and the glioma exploitation of its microenvironment.

Material and methods

Glioma cell lines and microglia

The human glioma cell lines U251N, U373 and U87 were obtained from American Type Culture Collection (Manassas, VA). Lines LN18, LN71, LN215, LN229, LN308, LN340, LN405, LN427, LN428, LN443, LN464, LN827 and LN992 were previously obtained from Dr Erwin Van Meir (Emory University, Atlanta, GA). The use of these lines has been reported elsewhere (14,20). Cells were maintained in feeding medium of minimum essential medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 0.1% L-glutamine and 1 mM sodium pyruvate. Unless otherwise stated, all experiments were conducted in this medium.

Non-transformed microglia of >95% purity were isolated from the brains of adult human undergoing resection to treat intractable epilepsy as described previously (21). Feeding medium was identical to that for glioma cells.

RNase protection assay

Glioma cells at 80% confluence in 100 mm dishes were harvested by applying 1 ml of Trizol (Invitrogen, Burlington, Ontario, Canada) per dish. Total RNA was extracted and 15 μg was used for each lane. A CC chemokine (hCK-5; Pharmingen, Mississauga, Ontario, Canada) and CC receptor (hCR-5) multiprobe template sets were used and RNase protection assay was performed as detailed by the manufacturer. L32 and Glycerinaldehyde-3 phosphate dehydrogenase were loading controls present in these multiprobe sets.

Flow cytometry

Glioma cells at 80% confluence were scrapped off and were stained with a monoclonal anti-human CCR2-phycocerythrin antibody (R&D, Minneapolis, MN) for 30 min at 4°C. Cells were then washed, fixed and analyzed by flow cytometry. Human adult microglia were similarly processed.

CCL2 construct and stable transfection

Full-length CCL2 cDNA (Invivogen) was subcloned to the BamHI site of pcDNA3.1 expression vector. For transfection, 200,000 U87 cells were

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seeded in each well of six-well plate. Two microgram of pcDNA3.1-CCL2 or vector was transfected with Fugene 6 (Roche, Mississauga, Ontario, Canada) according to the manufacturer’s recommended protocol. Four hundred micrograms per milliliter of G418 (Calbiochem, Mississauga, Ontario, Canada) was added to the cells 48 h after transfection to select for positive clones.

**Enzyme-linked immunosorbent assay**

Five thousand U87 vector-transfected or CCL2 clones were seeded into each well of a 96-well plate. The amount of CCL2 secreted into the medium after 24 h of culture was analyzed using 50 μl of cell-conditioned medium. CCL2 enzyme-linked immunosorbent assay was analyzed as described by the manufacturer (R&D system).

For microgel production of IL-6, 10 000 microgelia were seeded per well of a 96-well plate. CCL2 was applied for 24 h and culture medium was then used in IL-6 enzyme-linked immunosorbent assays as per manufacturer (R&D system) descriptions.

**Matrigel invasion assay**

Invasiveness of cells across a mixture of extracellular matrix proteins, matrigel, was examined as described previously (12). In brief, invasion chambers (BD Biosciences, Bedford, MA) containing membrane with 8 μm pore size coated with growth factor-reduced Matrigel were rehydrated with 0.5 ml warm culture medium in a 37°C incubator for 2 h. Glioma cells were pre-stained with carboxyfluorescein succinimidyl ester (Molecular Probes, Burlington, Ontario, Canada), so that they could be identified by green fluorescence later. Fifty thousand U87 vector or CCL2 cells, with or without 10 000 human adult microgelia, were suspended in 0.5 ml serum-free medium (AIM-V). The cell mixture was added to the top compartment of each invasion chamber with or without treatment (see below). A total of 0.75 ml of 10% fetal bovine serum-containing medium was added to the bottom chamber, so that the serum acted as a chemoattractant. Reconstituent human keratinocyte growth factor (KGF) protein (Abcam, Cambridge, MA) or IL-6 protein (R&D system) or anti-human IL-6 neutralizing antibody (R&D systems) at 10 μg/ml or anti-CD4 at 10 μg/ml was added to both the top and the bottom compartments of each Boyden chambers when cells were seeded. The entire chamber set was incubated for 24 h at 37°C. The cells that remained in the upper wells were then swabbed off, whereas cells that invaded across the matrigel barrier and adhered onto the underside of the membrane were fixed in 95% ethanol/5% acetic acid for 15 min. The membrane was then cut off and invaded glioma cells were visualized by green fluorescence. Four fields per membrane were counted.

**Gene array**

U87 vector or CCL2-overexpressing clones were cocultured with human microgelia for 24 h. Three microgram of RNA was used for probe synthesis, and then cDNA GEArray kit for 96 human growth factors/cytokines (SuperArray Bioscience Corporation, Frederick, MD) was applied to measure the change of the expression level of molecules after glioma–microgelia co-culture. The growth factors/cytokines included members of the epidermal growth factor family (Amphiregulin, HB-epidermal growth factor, epidermal growth factor receptor, HB-EGF, NEKGF, NGF, GDNF, HB-EGF, NEKGF, NGF, GDNF, HB-EGF, NEKGF, NGF), transforming growth factor family (TGFα and TGFβ1–3), neuronal growth factors (BDNF, CNTF, glial-derived neurotrophic factor (BDNF), NGFA and B, midkine, neuropilin 1 and 2, NTG6, NTG3, NTG5, cytokines (M-CSF, GM-CSF, G-CSF, EPO, IL-1α and IL-1β, IL-2 to IL-11, IL-12α, IL-12β, IL-13 to IL-16, IL-17α-F, IL-18 to IL-20, IL-22, SCF and tumor necrosis factor-α and β) and other related growth factors (CTGF, CXCL1, EGF1, GH1, GH2, hepatocyte growth factor, insulin-like growth factor 1 and 2, INS, PLGF and PTN).

**Immunofluorescence detection of IL-6 in glioma specimens**

We used paraffin-embedded sections from the University of Calgary pathology bank collected from patients who died with glioblastoma, with diagnosis confirmed at autopsy. Sections were deparaffinized, subjected to endogenous peroxidase inactivation using 1% H2O2 in methanol and blocked for non-specific binding for 1 h at room temperature. Ten micrograms per milliliter of mouse anti-human CD68 (Ventana Medical System, Tucson, AZ) and rabbit anti-human IL-6 (Abcam) were then applied, followed by secondary antibodies conjugated to Alexa Fluor 488 (1:750; Invitrogen) or Alexa Fluor 564 (1:750; Invitrogen). Stainings were then visualized using an immunofluorescence microscope.

**Statistical analyses**

The one-way analysis of variance with post hoc Tukey’s comparisons was used to analyze multiple groups, whereas the unpaired t-test was used for comparisons of two groups. The histograms displayed in the figures of tissue culture results typically are mean ± standard error of the mean of quadruplicate analyses, and the results were repeated at least twice.
transcripts were analyzed by enzyme-linked immunosorbent assay (data not shown). CXCL8 (IL-8) messenger RNA was also detected in several lines (9 of 16) but since CXCL8 has been examined extensively in several studies (22–24), we chose to focus further on CCL2 rather than CXCL8 for the remainder of this study.

The CCL2 receptor, CCR2, is expressed on microglia but not by glioma cell lines

In a previous study (14), we analyzed glioma lines for their expression of receptors for the CXC family of chemokines and reported that CXCR4 was expressed by a majority of lines. In the current study, we focused on the receptors for CC chemokines since CCR2 is the receptor for CCL2. Figure 2A shows that with the exception of faint expression of CCR1, CCRs including CCR2 were undetectable in the glioma lines examined. Moreover, flow cytometry analyses for CCR2 failed to detect the CCR2 receptor on glioma cells (Figure 2B). Instead, we found CCR2 expression on microglia (Figure 2C). These results suggest that glioma-derived CCL2 could not act in an autocrine manner but could exert its paracrine effect on CCR2-expressing cells, such as microglia.

CCL2 overexpression does not confer growth advantage when glioma cells are cultured in isolation

To test the hypothesis that CCL2 produced by glioma cells is not an autocrine factor since these cells lack CCR2, we overexpressed CCL2 in glioma cells. We selected the U87 glioma line as this has low intrinsic level of CCL2 (Figure 1). The cells were either stably transfected with pcDNA.1-CCL2 construct or with vector control and selected using G418. Two clones (clones 1 and 2) that stably overexpressed 10- to 12-fold of CCL2 above vector control or parental line (Figure 3A) were tested for invasive properties through the three-dimensional matrix of matrigel. Notably, overexpressing CCL2 did not promote the invasive capacity of U87 cells compared with vector-transfected controls (Figure 3B). Moreover, the proliferation rate determined over 96 h of culture was not altered in the CCL2-overexpressing clones compared with vector controls (data not shown).

Coculture of glioma cells with microglia enhances invasiveness of glioma cells

Next, we cocultured human microglia with glioma cells. Figure 3C is an example of such cocultures, where microglia were identified immunohistochemically using the Iba1 antibody and where glioma cells were detected by green fluorescence as they had been previously exposed to carboxyfluorescein succinimidyl ester. When the cocultures were seeded onto matrigel-coated Boyden chambers, the number of green fluorescing glioma cells that had invaded across the matrigel after 24 h was evaluated. We found that the invasiveness of vector-transfected cells was increased in coculture with microglia when compared with their plating onto non-microglia chambers; significantly, the invasiveness of CCL2-overexpressing clones in the presence of microglia was increased even further (Figure 3D). Thus, microglia promote the invasiveness of glioma cells particularly when the transformed cells had elevated levels of CCL2.

Identification of factors elevated in glioma–microglia cocultures through CCL2

We sought to identify molecules when glioma cells were exposed to microglia, particularly in a CCL2-dependent context. U87 vector-transfected cells with undetectable levels of CCL2 secretion were cocultured with microglia for 24 h and the gene array profiles were contrasted with those from CCL2-overexpressing U87 cells that were in coculture with microglia. Figure 4A depicts results of such arrays that were then normalized for Glyceraldehyde-3 phosphate dehydrogenase signals within each membrane. The top molecules elevated in the CCL2-overexpressing glioma/microglia coculture compared with the vector/microglia coculture were then recorded (Figure 4B). When the results of three separate experiments were compared, we found that KGF, also called FGF-7, and GDNF were elevated in all three cocultures of
CCL2-overexpressing glioma cells with microglia; IL-6 was in the top 10 gene hits in two cocultures (Figure 4B) but ranked in the top 15 in the third. We thus focused on KGF, GDNF and IL-6. We validated the elevation of KGF and IL-6 by real-time polymerase chain reaction (Figure 4C and D), but the elevation of GDNF could not be duplicated through polymerase chain reaction (data not shown).

Thus, gene array studies and confirmatory polymerase chain reactions identified KGF and IL-6 as possible candidates in CCL2-dependent microglia-stimulated glioma invasiveness.

**KGF does not promote glioma invasiveness whereas IL-6 can: blocking IL-6 impedes microglia-stimulated invasiveness of CCL2-expressing glioma cells**

KGF has cancer-promoting potential in epithelial cells (25,26) because it is a known promoter of cell motility (27,28). We first assessed the expression of KGF on resected human glioma specimens but found that no immunohistochemical signal could be detected (data not shown). In addition, recombinant KGF (20–100 ng/ml) did not promote the invasiveness of U87 cells in Boyden chamber assays (Figure 5A). These results do not support a role for KGF in microglia-stimulated glioma invasiveness, even though KGF transcripts are significantly elevated by CCL2-expressing glioma cells in coculture with microglia.

We investigated IL-6 next by incubating the U87 parental cell line with recombinant cytokine. Figure 5B shows that 10 and 100 ng/ml IL-6 significantly promoted invasiveness of glioma cells across a matrigel barrier, highlighting its potential as a molecule elevated in glioma–microglia coculture (Figure 4) to promote glioma invasiveness. To support this, the CCL2-overexpressing U87 clones were cocultured with microglia in matrigel Boyden chambers with or without a neutralizing antibody to IL-6. We found that the addition of the IL-6 blocking antibody reduced glioma invasiveness in glioma–microglia coculture, under conditions in which a non-specific anti-CD4 control antibody, used as an isotype control, was employed (Figure 5C and D).

Finally, we addressed whether CCL2 could increase the production of IL-6 by microglia. In basal culture condition, microglia already produced significant amounts of IL-6 (3446 ± 22 pg/ml) (mean ± standard deviation, n of 4) and CCL2 (10 ng/ml) alone could not regulate IL-6 further (3078 ± 26 pg/ml). However, when microglia were primed by IL-1β (10 ng/ml), which by itself did not elevate IL-6 (3645 ± 24 pg/ml), the addition of CCL2 led to an increase of IL-6 (5026 ± 26 pg/ml; P < 0.05 compared with all groups, analysis of variance).

**Detection of IL-6 in glioma specimens**

The above results highlight a CCL2–CCR2 engagement that leads to the production of IL-6 by microglia, with the IL-6 in turn acting on glioma cells to promote their invasiveness. To determine whether this axis could be applicable to gliomas in situ, we stained glioma specimens for IL-6. Figure 6A shows a tumor specimen where IL-6 was detectable on CD68+ profiles. As microglia are immunoreactive for CD68 (29), these data suggest that glioma–microglia coculture, under conditions in which a non-specific anti-CD4 control antibody, used as an isotype control, was employed (Figure 5C and D).

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The tumor microenvironment is an important regulator of transformed properties as many tumor types and their surrounding stromal cells interact and influence one another. For brain tumors, we reported that glioma cells exploit astrocytes in their microenvironment to activate matrix metalloproteinase (MMP)-2, a protease that remodels the matrix and allows glioma cells to invade (19). In another inquiry, we investigated chemokines and found that across a number of glioma lines, there was an enrichment of the chemokine receptor CXCR4, but its ligand stromal cell-derived factor-1 (SDF-1α and CXCL12) was produced by astrocytes. We determined that astroglial CXCL12 increased PI-3K/Akt activity within differentiated glioma cells and improved their survival and invasiveness (14,15), adding to the concept that adjacent glia are exploited for glioma tumorigenicity.

Besides neural cells of the central nervous system, resident microglia cells or immune cells that infiltrate from the circulation into gliomas are significant contributors to transformed growth. These cells produce a variety of inflammatory cytokines, chemokines and growth factors, which can facilitate malignant progression (30). We have focused on chemokines in this study as they can facilitate tumor growth, invasion and metastasis through several mechanisms including the degradation of the extracellular matrix for invasiveness and metastasis. Several chemokines are elevated in gliomas, and there is good evidence that these chemokines and their receptors may be important intercellular mediators of communication between glioma and its surroundings (5–7,30–32). To identify key chemokines produced by gliomas that may be utilized to exploit their microenvironment, we used herein a multiprobe RNAse protection assay to address which chemokine was commonly produced by human glioma lines. Our results highlight CCL2 to be commonly expressed by gliomas, whereas its receptor CCR2 was on microglia. Moreover, we found no evidence for CCL2 as an autocrine growth factor for gliomas. Instead, we determined that CCL2 stimulated microglia to produce IL-6 that in turn promotes invasiveness in glioma cells. Other growth factors were also elevated in the glioma–microglia coculture in a CCL2-dependent manner, including GDNF, KGF and a variety of FGFs. We found no evidence for KGF or GDNF (data not shown) to promote glioma invasiveness but the roles of other FGFs will have to be evaluated in future experiments.

There are limitations to our study. First, there are 48 human chemokines, so our analyses cover only a spectrum of potential chemokines that may have roles in gliomas. As well, by using a gene array of 96 human growth factors/chemokines to investigate molecules altered in glioma–microglia interactions, a significant limitation is the confinement of analyses to a relatively minor number of factors. The discovery of IL-6 is thus fortuitous and does not negate the possibility that other molecules may be more critical in glioma–microglia

![Fig. 4. Gene array analyses highlight specific growth factors that were elevated by CCL2 in glioma–microglia cocultures. (A) An example of a gene array probe of RNA from a coculture of vector control with microglia or CCL2-overexpressing clone with microglia. The Glyceraldehyde-3 phosphate dehydrogenase signals used for normalization across membranes are indicated by the boxed area. (B) Top 10 factors (in descending order) elevated in three separate experiments, when comparing Glyceraldehyde-3 phosphate dehydrogenase-normalized signals of coculture of CCL2-overexpressing clone with microglia, to coculture of vector-transfected cells with microglia; IL-6 was ranked in the 11–15 positions in the third experiment. The elevation of KGF (C) and IL-6 (D) was verified by real-time polymerase chain reaction. *P < 0.05, **P < 0.01.](https://academic.oup.com/carcin/article-abstract/33/2/312/2464075)
Fig. 5. IL-6 promotes the invasiveness of glioma cells in glioma–microglia coculture. Although recombinant KGF (A) did not promote invasiveness of glioma cells when these were cultured alone, IL-6 (B) was effective. **P < 0.05, ***P < 0.001. (C) In coculture of CCL2-overexpressing clone 1 with microglia, the invasiveness of glioma cells was reduced by a function-blocking antibody to IL-6. An isotype antibody control raised to CD4 did not affect invasiveness. ***P < 0.001 and reproduced in another experiment. (D) Representation of transmigrated cells after 24 h in clone 1/microglia coculture under control conditions or when exposed to anti-IL-6 or anti-CD4. Cells were fluorescing green by being pre-labeled with carboxyfluorescein succinimidyl ester prior to experiment.
and other growth factors to stimulate glioma cells (4) to regulate microglia. (3) and other growth factors to stimulate glioma cells (4) to regulate microglia. Moreover, microglia-derived ties (46–51). In support, murine microglia cells in culture promote the in vitro motility of mouse glioma cell line (52).

Microglia cells may initially exert host defense mechanisms against glioma specimen is a macrophage or microglia (39). Although these tumor types besides gliomas.

Invasiveness of glioma cells, IL-6 has been found to increase the glioma aggressiveness including invasiveness (36). Recently, IL-6 was identified as a growth factor for glioma stem cells (37). Although we did not examine the mechanisms by which IL-6 may drive the invasiveness of glioma cells, IL-6 has been found to increase the glioma production of MMP-2 (38), a protease implicated in glioma invasiveness.

Fig. 6. Expression of IL-6 on CD68 cells in vivo. (A) Sample from a high-grade glioma was analyzed within the tumor mass. IL-6 signal was detected and these overlapped with CD68+ cells that were either macrophages or microglia. (B) A proposed glioma–microglia dialog where CCL2 is produced by glioma (1) to stimulate CCR2 on microglia (2) that in turn provides IL-6 (3) and other growth factors to stimulate glioma cells (4) to regulate properties, such as invasiveness.

Interactions. A future plan is to employ genome-wide arrays to extend analyses beyond IL-6 in glioma–microglia interactions.

The discovery of IL-6 as an important microglia-derived invasive factor for glioma cells in this study is in line with other observations reported for IL-6 in gliomas. Notably, IL-6 levels are elevated in high-grade glioblastoma specimens that are richly infiltrated with microglia and macrophages (33–35), and IL-6 expression is correlated with glioma aggressiveness including invasiveness (36). Recently, IL-6 was identified as a growth factor for glioma stem cells (37). Although we did not examine the mechanisms by which IL-6 may drive the invasiveness of glioma cells, IL-6 has been found to increase the glioma production of MMP-2 (38), a protease implicated in glioma motility (19). Finally, we note that IL-6 is implicated in many aspects of tumorigenesis beyond invasiveness, and it is associated with many tumor types besides gliomas.

The glioma–microglia dialog consisting of the CCL2/CCR2/IL-6 axis adds to a significant literature on the interactions between these two cell types. Indeed, it has been estimated that every third cell in glioma specimen is a macrophage or microglia (39). Although these microglia cells may initially exert host defense mechanisms against gliomas (40–45), it is long been appreciated that adaptive and innate immunity are deficient in gliomas (16,18). Indeed, microglia in the tumor microenvironment may be co-opted to enhance glioma properties (46–51). In support, murine microglia cells in culture promote the motility of mouse glioma cell line (52). Moreover, microglia-derived soluble factors increase glioma invasiveness in matrigel assay in vitro and that is associated with activation of the PI-3K/Akt signaling pathway (53). Platten et al. (48) showed that glioma lines that recruited more microglia into intracerebral implants in rats resulted in larger tumor size and they attributed this to an effect on CCL2. In cultured brain slices where microglia were previously depleted with clodronate-filled liposomes, injected glioma cells had decreased infiltrative capacity compared with glioma cells injected into control brain slices; the presence of microglia and glioma invasiveness was correlated with MMP-2 expression (54). Furthermore, we determined that microglia are very rich sources of MMPs (55), so there is the potential of these microglial MMPs to be recruited for matrix turnover in the glioma microenvironment. Another study has shown that microglia can stimulate glioma cell invasiveness by increased activity of Membrane type1–MMP (56). These results emphasize the dialog between gliomas and microglia; however, as in the context of the current findings, the microglia activity appears to be co-opted by gliomas.

In summary, our findings have highlighted the important role of the glioma microenvironment in regulating its tumorigenicity. Specifically, we implicate the CCL2/CCR2/IL-6 axis in glioma–microglia dialog and have highlighted it for therapeutic intervention to reduce the glioma exploitation of its environment.

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