FUNCTIONS AND THERAPEUTIC TARGETING OF CHEMOKINES AND CHEMOKINE RECEPTORS IN GLIOBLASTOMA PROGRESSION

By

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To my warm family, dear friends, and everyone I love
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ 4

LIST OF TABLE .................................................................................................................. 8

LIST OF FIGURES .............................................................................................................. 9

LIST OF ABBREVIATIONS ............................................................................................... 10

ABSTRACT .......................................................................................................................... 13

CHAPTER

1 INTRODUCTION .............................................................................................................. 16

   Glioblastoma ...................................................................................................................... 16
      Overview of Disease .................................................................................................. 16
      Treatments ............................................................................................................... 16
      Therapeutic Resistance .......................................................................................... 17
      Subclasses ............................................................................................................... 18
   Microglial Infiltration in GBM .................................................................................. 21
   Glioma Stem-Like Cancer Cells .............................................................................. 22
   Angiogenesis and Anti-Angiogenic Therapy in GBM ....... .............................. 24
   Chemokines and Chemokine Receptors ............................................................. 26
   Chemokine Systems in Cancers .......................................................................... 27
      CCL5/CCR1/CCR3/CCR5 .................................................................................. 29
      CXCL11/CXCL12 and CXCR4/CXCR7 ........................................................... 29
   GBM Models ............................................................................................................... 32
      Murine Glioma 261 (GL261) Model ................................................................. 32
      Human Gliomasphere Model ........................................................................... 33
   Significance and Specific Aims ............................................................................. 34

2 MATERIALS AND METHODS .................................................................................. 37

   Animals ....................................................................................................................... 37
   Cell Culture ............................................................................................................... 37
   Primary Microglia Isolation .................................................................................. 38
   Reverse Transcription Polymerase Chain Reaction ....................................... 39
   Cytokine Protein Array .......................................................................................... 39
   In Situ Hybridization ............................................................................................. 40
   Immunocytochemistry ............................................................................................. 40
   Immunohistochemistry ............................................................................................ 41
   Flow Cytometry ....................................................................................................... 42
   Migration Assay ....................................................................................................... 42
   Short-Term Proliferation Assay ............................................................................ 43
Sphere Formation Assay ................................................................. 43
Apoptosis Assay ........................................................................ 43
Tube Formation Assay................................................................. 44
Intracranial Injection of Glioma Cells ............................................ 44
AMD3100 and Cediranib Treatments.............................................. 45
Kaplan-Meier Survival Analysis ................................................... 45
Statistical Analysis ..................................................................... 45

3 ROLE OF CCL5 IN MICROGLIA/MACROPHAGES-GBM CROSSTALK .... 47

Results .......................................................................................... 48
  CCL5 Is Expressed by Murine GL261 and Human GBM Cell Lines .... 48
  CCL5, CCR1, and CCR5 Are Present in Murine GL261 GBM .......... 49
  Neither CCR1 nor CCR5 Deficiency Contributed to the Infiltration of
  Immune Cells into GBM or Impacted the Survival of Tumor Bearing
  Mice ............................................................................................. 50
  CCL5 Interacted with Its Microglia/Macrophages-Expressed Receptors,
  CCR1 and CCR5, in a Redundant Manner ................................. 51
Discussion ...................................................................................... 52

4 HETEROGENEITY OF CXCR4 AND CXCR7 IN GBM............................ 60

Results .......................................................................................... 61
  CXCR4, CXCR7, CXCL11, and CXCL12 Were Expressed in Primary
  Patient-Derived GBM Cell Lines and/or Tumors .......................... 61
  Both CXCR4+ and CXCR7+ Cells Were Capable of Generating Tumors In
  Vivo ............................................................................................. 61
  CXCL12 Stimulated Tube Formation of L0 Cells through CXCR4 ...... 62
  CXCL12 Had No Effect on TMZ-Induced Apoptosis in CXCR4+ and
  CXCR7+ Cells ........................................................................... 62
  CXCL11 Contributed No Impact on In Vitro Functions of GBM Cell Lines .. 63
Discussion ...................................................................................... 64

5 EXPRESSION AND FUNCTION OF CXCR4 IN GBM AFTER ANTI-
ANGIOGENIC THERAPY .................................................................. 73

Results .......................................................................................... 74
  Heterogeneous Expression of VEGF Receptors by Different Primary
  Patient-Derived GBM Cell Lines ................................................ 74
  Anti-VEGF/VEGFR Agents Increased the Expression of CXCR4 in
  Primary Patient-Derived GBM Cell Lines and Xenograft Tumors that
  Were Positive for VEGFRs ......................................................... 75
  Anti-VEGFR Drugs Enhanced the Migratory Effect of VEGFR(s)-
  Expressing GBM Cell Lines toward CXCL12 ............................ 76
  The Combination of a CXCR4 Antagonist and a VEGFR Inhibitor Provided
  a Greater Beneficial Effect on the Survival of GBM Bearing Animals .... 76
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>46</td>
</tr>
<tr>
<td>List of primers used for RT-PCR analyses</td>
<td>46</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3-1</td>
<td>Expression of CCL5 by murine GL261 and human GBM cell lines</td>
</tr>
<tr>
<td>3-2</td>
<td>Expression of CCL5 and its receptors CCR1 and CCR5 in murine GL261 glioblastomas</td>
</tr>
<tr>
<td>3-3</td>
<td>Effect of CCR1 and CCR5 deficiency on infiltration of immune cells into glioblastoma and survival of tumor bearing mice</td>
</tr>
<tr>
<td>3-4</td>
<td>In vitro expression of CCR1 and CCR5 by primary microglia cultures and the effect of Met-CCL5 on CCL5-stimulated migration of microglia</td>
</tr>
<tr>
<td>4-1</td>
<td>CXCR4, CXCR7, CXCL11, and CXCL12 were expressed in primary patient-derived GBM cell lines and/or tumors</td>
</tr>
<tr>
<td>4-2</td>
<td>CXCR4⁺, CXCR7⁺, and CXCR4⁺/CXCR7⁺ cells generated tumors in vivo</td>
</tr>
<tr>
<td>4-3</td>
<td>CXCL12 stimulation of L0 tube formation in vitro was mediated by CXCR4</td>
</tr>
<tr>
<td>4-4</td>
<td>Differential impact of TMZ on the distribution of CXCR4- and CXCR7-expressing apoptotic and non-apoptotic subpopulations</td>
</tr>
<tr>
<td>4-5</td>
<td>CXCL11 had no effect on in vitro functions of GBM cell lines</td>
</tr>
<tr>
<td>5-1</td>
<td>VEGFRs were heterogeneously expressed by different primary Patient-derived GBM cell lines</td>
</tr>
<tr>
<td>5-2</td>
<td>VEGFR inhibitors upregulated CXCR4 in VEGFR-expressing GBM cell lines</td>
</tr>
<tr>
<td>5-3</td>
<td>VEGFR inhibitors enhanced the migratory effect of CXCL12 on S2 and S3 cells</td>
</tr>
<tr>
<td>5-4</td>
<td>Combination of CXCR4 and VEGFR inhibitors prolonged survival of S3 tumor bearing mice</td>
</tr>
<tr>
<td>5-5</td>
<td>Upregulation of CXCR4 expression by anti-angiogenic drugs was independent of HGF/MET signaling</td>
</tr>
<tr>
<td>5-6</td>
<td>Increased CXCR4 expression by anti-angiogenic drugs was regulated by TGFβ/TGFβRs signaling</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
</tr>
<tr>
<td>AMD3100</td>
<td>1,1'-[1,4-Phenylenebis(methylene)]bis [1,4,8,11-tetraazacyclotetradecane]</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>CCDN2</td>
<td>Cyclin D2 gene</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulated factor 1</td>
</tr>
<tr>
<td>CSLC</td>
<td>Cancer stem-like cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBRT</td>
<td>External beam radiotherapy</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ERK/MAPK</td>
<td>Extracellular signal-regulated kinase/Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GABRA1</td>
<td>Gamma-aminobutyric acid A receptor alpha 1</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma</td>
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<tr>
<td>G-CSF</td>
<td>Granular colony stimulated factor</td>
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<td>GIM</td>
<td>Glioma infiltrated microglia/macrophages</td>
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<tr>
<td>GPCR</td>
<td>G protein couple receptor</td>
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<tr>
<td>GSLC</td>
<td>Glioma stem-like cell</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia induced factor 1 alpha</td>
</tr>
<tr>
<td>IDH1</td>
<td>Iso citrate dehydrogenase 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity modulated radiotherapy</td>
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<tr>
<td>ISH</td>
<td><em>In situ</em> hybridization</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/Signal transducer and activator of transcription</td>
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<tr>
<td>MET</td>
<td>Hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanine-DNA methyltransferase</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>NBI 74330</td>
<td>N-[1-[3-(4-ethoxyphenyl)-4-oxopyrido[2,3-d]pyrimidin-2-yl]ethyl]-2-[4-fluoro-3-(trifluoromethyl)phenyl]-N-(pyridin-3-ylmethyl)acetamide</td>
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<tr>
<td>NEFL</td>
<td>Neurofilament, light polypeptide</td>
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<td>NES</td>
<td>Nestin</td>
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<td>NF1</td>
<td>Neurofibromin 1</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells protein complex</td>
</tr>
<tr>
<td>NKX2-2</td>
<td>NK2 homeobox 2</td>
</tr>
<tr>
<td>NOTCH3</td>
<td>Neurogenic locus notch homolog protein 3</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD-scid IL2Rγnull</td>
</tr>
<tr>
<td>OLIG2</td>
<td>Oligodendrocyte lineage transcription factor 2</td>
</tr>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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</table>
PFS  Progression free survival
PI-3K  Phosphatidylinositol 3- and 4-kinase
PTEN  Phosphatase and tensin homolog
PTP1B  Protein tyrosine phosphatase 1B
RANTES  Regulated on activation, normal T cell expressed and secreted
RB  Retinoblastoma protein
RELB  V-rel reticuloendotheliosis viral oncogene homolog B
RPMI  Roswell Park Memorial Institute medium
RT  Radiotherapy
RT-PCR  Reverse transcription-polymerase chain reaction
SLC12A5  Solute carrier family 12 (potassium/chloride transporter), member 5
SMO  Smoothened protein
SOX-2  SRY (sex determining region Y)-box 2
SYT1  Synaptotagmin I
TCGA  The cancer genome atlas
TGFβ  Transforming growth factor beta
TGFβR  Transforming growth factor beta receptor
TMZ  Temozolomide
TNFRSF1A  Tumor necrosis factor receptor superfamily, member 1A
TP53  Tumor protein 53
TRADD  Tumor necrosis factor receptor type 1-associated DEATH domain protein
VEGF  Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
WHO  World Health Organization
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By

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August 2013

Chair: Jeffrey K. Harrison
Major: Medical Sciences – Physiology and Pharmacology

WHO grade IV glioblastoma (GBM) is one of the most aggressive forms of primary brain cancer, due to its lethality, mortality, and high resistance to intensive therapies. Median survival for patients with GBM is under 15 months following standard of care therapy with surgery, radiation, and Temozolomide (TMZ). Moreover, there is no effective therapy following recurrence. Heterogeneity within the tumor microenvironment, the presence of a stem-like subpopulation, a prominent tumor vascularization, and limited drug delivery through the blood brain barrier (BBB) are major impediments to chemotherapy directly targeting GBM cells. Hence, therapeutic strategies that indirectly attack tumor cells by targeting vital components in tumorigenic regulation receive major attention. Having diverse functions in many aspects of cancer biology, chemokine systems are potential candidates for this new approach. In this project, we were interested in the roles of chemokine systems within GBM microenvironment. The research objectives were addressed from several angles including how chemokine systems contribute to GBM progression through recruitment of immune cells to the tumor, how they regulate stem-like properties of tumor cells; and
how changes in tumor microenvironment, as a result of anti-vascular endothelial growth factor (VEGF) therapy, impact the expression and function of chemokine systems.

In the first part of this project, we elucidated the role chemokine CCL5 and its receptors CCR1, CCR3, and CCR5 in the recruitment of microglia into GBM microenvironment, using an immune-competent murine GBM model. We reported that CCL5, CCR1, and CCR5 were expressed in GBM. Individual deletion of CCR1 or CCR5 had little to no effect on the survival of tumor bearing mice, as well as the numbers of GBM-infiltrated microglia and lymphocytes. CCL5 promoted *in vitro* migration of wild type, CCR1- or CCR5-deficient microglia that was blocked by the CCR1/CCR5 dual antagonist, Met-CCL5. These data suggest that CCL5 may direct the infiltration of microglia into GBM microenvironment through CCR1 and CCR5 in a redundant manner.

The study reported in the second phase of this project followed up on results previously generated in this laboratory that focused on the heterogeneity of chemokine receptors CXCR4 and CXCR7 in regulating cancer stem-like properties of GBM cells. Using different primary patient-derived GBM cell lines, we showed that these two chemokine receptors were expressed by all of the examined GBM cell lines and that subpopulations containing either CXCR4+/CXCR7-, CXCR4+/CXCR7+, CXCR4+/CXCR7-, or CXCR4+/CXCR7+ cells were able to generate tumors *in vivo*. In addition to regulating other *in vitro* functions, CXCL12 also stimulated the *in vitro* tube formation of one of the GBM lines. CXCL12 had no effect on the distribution of CXCR4- and CXCR7-expressing cells in either the apoptotic and non-apoptotic subpopulations evident after treatment with TMZ. Moreover, we showed that CXCL11, another ligand for CXCR7, did not have an effect on CXCR7-mediated functions of GBM cells. This study provides
additional evidence to support functional heterogeneity of CXCR4 and CXCR7 in the regulation of cancer stem-like properties.

The final part of this project focused on investigating the effect of anti-VEGF therapy on the expression and function of chemokine receptor CXCR4 in primary patient-derived GBM cell lines and tumors. Data from *in vitro* and *in vivo* studies strongly indicated that VEGF/vascular endothelial growth factor receptor (VEGFR) inhibition could increase the expression of CXCR4 in VEGFR-positive cells lines, but not in VEGFR-negative lines. The increase in CXCR4 by anti-VEGFR therapies also enhanced the migratory effect of CXCL12 in the VEGFR-positive cells. Kaplan-Meier survival analysis indicated a statistically significant difference in the survival of tumor bearing mice that received combined treatment of AMD3100 (a CXCR4 antagonist) and Cediranib (a VEGFR inhibitor), when compared to control, AMD3100, or Cediranib treated cohorts. Further analysis pointed to a mechanism for the increase in CXCR4 expression stimulated by VEGFR inhibitors that was dependent on transforming growth factor β (TGFβ)/transforming growth factor β receptor (TGFβR), but not the hepatocyte growth factor (HGF)/hepatocyte growth factor receptor (MET) signaling pathway. This study elucidates a *de novo* mechanism that leads to an enhanced invasive phenotype post-anti-VEGF therapy and suggests new therapeutic approaches to target GBM.
Overview of Disease

GBM accounts for 52% of all primary brain tumor cases and 20% of all intracranial tumors. According to the World Health Organization (WHO) classification for brain cancers, GBM was categorized as grade IV, based on WHO grading system, due to its morbidity and mortality. Two features that guide pathologists to distinguish this grade IV GBM from other lower grade gliomas are the presence of necrotic areas surrounded by anaplastic cells within the tumor and extremely marked presence of tumor blood vessel networks (1, 2). GBM is one of the most malignant and aggressive forms of primary brain tumor since it is characterized with poor prognosis and high rate of therapeutic resistance, that together lead to a short median survival time. Despite standard treatment giving to patients with GBM, the median survival is usually less than 15 months after diagnosis. Less than 2% of patients suffering with GBM will survive longer than 5 years, which frequently occurs in the younger age group. GBM has received extensive attention from researchers and thus significant advances in understanding the molecular biology of this type of tumor and in therapeutic strategies targeting GBM have been achieved within the past 23 years.

Treatments

The standard treatment for patients with GBM consists of surgical resection, followed by radiotherapy (RT) and chemotherapy (3). Surgery is the first stage of GBM treatment in the effort of removing the burden of the tumor mass. Because the percentage of tumor removal is positively correlated with patient's quality of life, novel
method has been examined in which a fluorescent dye, named 5-aminolevulinic acid was used as a guide for surgeon to significantly increase the higher rate of complete resection (4, 5). After surgery, radiation is the mainstay of treatment for GBM patients. The traditional method used for standard RT is focal, fractionated external beam radiotherapy (EBRT). However, intensity modulated radiotherapy (IMRT), in which the radiation beam can be broken up into many “beamlets” with adjustable intensity, becomes widely used over traditional EBRT (6) because this advanced technology allows radiation to precisely target tumor with minimal effect on healthy tissue around tumor site. Because GBM contains the presence of necrotic areas, which are highly resistant to radiation, the use of chemotherapy, in combination with radiation, improves survival rates of patients. TMZ is one of the most popular alkylating agents used to treat GBM (7). Besides these standard treatments, therapeutic molecular-targeted agents have been widely explored for investigation due to its promising effectiveness on specific targeting aberrant signaling pathways that regulate angiogenesis or tumor progression such as epithelial growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), phosphatidylinositol 3- and 4-kinase (PI-3K). Abnormalities in these signaling pathways were reported in GBM. These defects include mutations or dysregulation of single or multiple factors in these pathways causing constitutive activation of downstream events, resulting in tumor development and progression (8-15).

**Therapeutic Resistance**

Despite advances in aggressive treatment for this lethal tumor, GBM still holds a significantly high rate of resistance due to various factors within the tumor
microenvironment. In chemotherapy for instance, the common mechanism for resistance to alkylating agents, such as TMZ, is through $O^6$-methylguanine-DNA methyltransferase (MGMT) gene (16). Methylation of the promoter region of this gene silences it, leading to greater sensitivity to TMZ (17). Thus, GBM patients that have methylated MGMT promoter are more susceptible to chemotherapy, when compared to patients with un-methylated MGMT promoter. Recently, increasing evidence suggests a crucial role of cancer stem-like cells (CSLCs) in therapeutic resistance in GBM. In a study of radioresistance in GBM using cell cultures derived from primary human tumor specimens and xenograft tumors, Bao et al., showed that the population of cells enriched for glioma CSLCs was dramatically increased by irradiation and that irradiated CSLCs have survival advantages relative to the non-CSLC population. Radioresistant tumors displayed an increased percentage of CD133$^+$ cells compared with the parental cell population. Furthermore, radiation had little effect on the ability of CSLCs to regrow tumors (18). The resistance to radiation of the cell population enriched in CSLCs may be the result of activation of deoxyribonucleic acid (DNA) damage repair mechanisms.

**Subclasses**

The heterogeneity at different levels within the tumor microenvironment contributes to ineffectiveness in treatment for GBM. This molecular heterogeneity suggests the existence of multiple molecular subtypes among different GBM cases. Integrated genomic analyses have identified 4 different molecular subtypes of GBM, based on profiles of gene expression from the cancer genome atlas (TCGA) Research Network database (19-21). These classifications include classical, mesenchymal, proneural, and neural subtypes.
**Classical:** chromosome 7 amplification paired with chromosome 10 loss and high level of epithelial growth factor receptor (EGFR) expression are 2 hallmarks of this subtype. The abnormality in EGFR amplification, which is caused by a point or constitutively active *vIII* EGFR mutation (a EGFR class III variant that lacks 267 amino acids from its extracellular domains) in most of the cases, was observed in 97% of the classical and infrequently in other subtypes. Moreover, while tumor protein 53 (TP53) is the most frequently mutated gene in GBM, there is a lack of this mutation in a subset of this subclass. Besides EGFR, aberrant expression of retinoblastoma protein (RB) pathway components, such as RB1, cyclin-dependent kinase 4 (CDK4), and cyclin D2 (CCDN2), as well as neural precursors and stem cell markers nestin (NES), neurogenic locus notch homolog protein 3 (NOTCH3), smoothened protein (SMO) were also evident in the classical subtype. Using data from the brain transcriptome database to define gene sets associated with different cell types (22), they also found that the genetic profile of the classical group was strongly associated with the murine astrocytic signature.

**Mesenchymal:** lower expression or the loss of neurofibromin 1 (NF1) due to focal hemizygous deletions of a region at 17q11.2 is the feature of this subclass. The loss of NF1 function is not due to gene silencing as no methylation probes were detected in or adjacent to the NF1 locus. The mutation of NF1 gene frequently correlates to that of phosphatase and tensin homolog (PTEN). The higher necrosis and inflammatory infiltration in the mesenchymal subtype may be the consequence of high expression of genes in the tumor necrosis factor superfamily and nuclear factor kappa-light-chain-enhancer of activated B cells protein complex (NF-κB) pathways, such as
tumor necrosis factor receptor type 1 associated death domain protein (TRADD), v-rel reticuloendotheliosis viral oncogene homolog B (RELB), tumor necrosis factor receptor superfamily member 1A (TNFRSF1A). Regarding neural cell types, the mesenchymal class was strongly associated with the microglial and cultured astroglial signatures (23).

**Proneural**: this subclass was characterized with the alteration of platelet-derived growth factor receptor (PDGFRA) and point mutation in iso citrate dehydrogenase 1 (IDH1), which contribute to abnormal cell growth. TP53 mutations and loss of heterozygosity were frequent events in this subtype. In addition, one of the features of classical group, chromosome 7 amplification paired with chromosome 10 loss, was sometimes occurred but only in 54% of proneural samples. This class was enriched in the oligodendrocytic, but not astrocytic signature. This can be explained by the high expression of oligodendrocytic development genes such as PDGFRA, NK2 homeobox 2 (NKX2-2), and oligodendrocyte lineage transcription factor 2 (OLIG2). Besides the difference in gene expression profile, this subtype is also distinct from other groups with respect to clinical correlates. GBM patients classified as the proneural group were significantly younger, and thus tended to have longer survival times. However, TCGA data analysis revealed that aggressive treatment did not alter survival of patients in the proneural group while other subtypes responded well to treatment (24).

**Neural**: this subclass was identified by the expression of neuron markers such as neurofilament light polypeptide (NEFL), gamma-aminobutyric acid A receptor α1 (GABRA1), synaptotagmin 1 (SYT1), and solute carrier family 12 member 5 (SLC12A5). While this group also shared mutations in many of the same gene, it did not stand out from the others as it did not have significantly lower or higher rates of mutations. The
neural class showed association with oligodendrocytic and astrocytic signatures, in addition to strong enrichment for neuronal expressing genes. This subtype also contained relatively older patients.

Understanding the gene expression-based molecular classification of GBM brings vital implications for therapeutic strategies targeting GBM. The identification of molecular signatures among different GBM subtypes could lead to diagnostic tests to classify a patient to a specific subclass. These findings also suggest a rationale for developing personalized therapies in order to provide better beneficial effect in treatment of GBM patients.

**Microglial Infiltration in GBM**

A large proportion of microglia/macrophages cells are found within this type of malignant human glioma as well as mouse models of the disease (25-27). Generally, these myeloid-derived, macrophage-like microglial cells function as the main form of immune defense in the central nervous system (28-32). When activated by pathological changes in the brain, they transform from a resting state to a pro-inflammatory phenotype that is capable of phagocytosis, cytotoxicity, and antigen presentation through the high expression of major histocompatibility complex (MHC) class II molecules (28, 33-36).

Opposite to their normal function in the immune system, glioma infiltrated microglia/macrophages show an anti-inflammatory phenotype, in which the expression of MHC class II molecules is down-regulated, especially in brain tumors with high malignancy (37-44), possibly by glioma-secreted factors, including interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), TGFβ, and prostaglandin E2, (27). A positive
correlation between the number of infiltrating microglia/macrophages and the proliferation rate of the tumor is also evident (25, 45). Thus, these immune cells are believed to contribute to the local immunosuppressive milieu of glioma, as well as promote tumor progression (46-48). Despite the potential importance of microglia/macrophages in glioma tumorigenesis, the mechanism by which microglia/macrophages infiltrate into the tumor is still unknown. Increasing evidence supports the hypothesis that glioma regulates this mechanism through different factors, including HGF, colony stimulated factors 1 (CSF-1), granular colony stimulated factor (G-CSF) (49-51), or possibly matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9), which are found abundantly within glioma (52). Current studies strongly suggest this localization may require the presence of chemokines, a family of chemoattractant cytokines, in the tumor microenvironment (53-56).

**Glioma Stem-Like Cancer Cells**

Within the microenvironment of GBM, CSLCs lay at the pinnacle of the cellular hierarchy. The evidence for their existence was first reported in acute myeloid leukemia by fluorescent activated cell sorting (FACS) based on the expression of cell surface markers associated with normal hematopoietic stem cells (57). Since then, increasing studies demonstrated the presence of CSLCs in other solid tumors including breast, bone, liver, colon, prostate, pancreas, head and neck squamous cell carcinoma, and melanoma (58-64). The presence of CSLCs in gliomas, called glioma stem-like cells (GSLCs), was also documented (65, 66). In accordance with the similarity in cell surface markers to normal neural stem cells, GSLCs should have an ability to maintain its own subpopulation via self-renewal while recapitulating the phenotype of the parental tumor.
Although the concept for the existence of GSCLCs is widely accepted, the standard for identifying this population both *in vitro* and *in vivo* with cell markers remains a point of debate. CD133 (Prominin 1) is the first cell surface neural stem cell marker to be identified in human specimens (67) and is now widely used as a GSCLC enriching marker. Other markers include CD44, CD24, CD15, Nestin, and sex determining region Y box 2 (SOX2). It is reported that CD133 positive tumor cells share a similar gene expression profile with that of embryonic stem cells (68). Moreover, CD133 positive GSCs isolated from human GBM were capable of tumor initiation while the CD133 negative cells from the same patients were not able to form solid tumors (58, 59, 65, 69, 70). Several studies have also reported the tumorigenic capacity of CD133 negative cells (69, 71, 72). This population with apparent stem cell-like properties but distinct molecular profiles and growth characteristics *in vitro* and *in vivo* (69). It is likely that these CD133 negative GSCLCs express other stage-specific embryonic antigen, such as AB5 and CD44, which were reported to enrich for GSCs independent of CD133 (72-74). Relating to a clinical aspect, CD133 positive cells showed more resistance to radiation or chemotherapy, when compared to the CD133 negative population (18). These data suggest that CD133 expression may correlate to poor prognosis, tumor recurrence, or fatal malignancy of GBM.

Presented as a rare subpopulation within the heterogenous tumor microenvironment, GSCLCs have attracted considerable attention. Increasing evidence suggests that the GSCLC population tends to locate to specific tumor niches that associate with either perivascular domains or hypoxic regions. Using different cancer stem cell markers, a number of studies have shown that GSCLCs are co-localized with
vascular endothelial cell markers such as CD34 and CD31 (75, 76). It is believed that residing in perivascular niches is necessary for GSLCs to maintain their cancer stem-like properties, and to induce tumor growth in vivo (77, 78). The bidirectional interplay between GSLCs and tumor vascular niches was elucidated when in vivo studies showed that GSCs can trans-differentiate into endothelial cells (79-81) or into vascular pericytes to support vascular functions and tumor growth (82). In addition to the perivascular niche, the prevalence of a hypoxic niche in maintaining GSLCs properties was also evident (83, 84). It has been shown that hypoxia inducible factor 1α (HIF1α), a critical indicator of hypoxia, could raise the stem-like population over five-fold, determined by the percentage of CD133-expressing cells, in neurospheres. This enhancement paralleled the increase in clonogenicity by two-fold (85), as well as the expansion of GSLCs (86).

**Angiogenesis and Anti-Angiogenic Therapy in GBM**

Prominent tumor vascularization is one of the two hallmarks of GBM that are used to distinguish it from the lower grade III glioma. In contrast to highly organized normal brain vasculature, tumor vessels in GBM are disorganized, highly permeable, have abnormal endothelial cell walls, pericyte coverage, and basement membrane structure (87). The presence of this abnormal tumor vascular network impairs BBB which results in brain edema, a serious life-threatening symptom of patients with GBM. The formation of tumor blood vessels occurs through angiogenesis, which is mediated by the proliferation and migration of local endothelial cells, and vasculogenesis, which originates from bone marrow derived cells migrating to the tumor site (88, 89). The angiogenic process is orchestrated by the simultaneous increase in gene expression of
VEGF, FGF, IL-6, IL-8, HIF-1α, and angiopoietins. Among these factors, VEGF is the most common denominator required for angiogenesis through the VEGF receptor signaling pathway in GBM. Thus, it becomes a potential therapeutic target for GBM treatment.

Targeting tumor angiogenesis has received significant attention because of its pivotal role in tumor progression. Therapeutic strategies that target angiogenesis have been proposed as an alternative treatment and have received great attention due to its beneficial effectiveness, particularly in highly vascularized tumors, such as GBM. Tumor angiogenesis is strongly regulated by the VEGF/VEGFR system, of which both ligands and receptors have been found in malignant GBM (90-92). Thus, targeting this signaling pathway may exert a detrimental effect on angiogenesis and improve survival benefit to patients with GBM. Data from clinical trials suggested that, in some patients with recurrent GBM treated with either anti-VEGF antibodies (Bevacizumab, Aflibercept, etc.) or small molecular VEGF receptor tyrosine kinase inhibitors (e.g. Cediranib or Vandetanib), these agents could enhance 6-month progression-free survival (PFS), as well as radio-graphic responses (93-97). These promising clinical results prompt extensive studies on other potential VEGF/VEGFR signaling inhibitors for anti-angiogenic therapy. Despite these achievements in prolonging the PFS, they failed to maintain an enduring overall survival benefit to patients. Under VEGF pathway inhibition with Bevacizumab, for instance, a statistically significant increase in infiltrative tumor progression was identified in Bevacizumab responders (98-100). Several underlying mechanisms for the tumor recurrence after anti-VEGF therapy have been proposed (99). First, blocking VEGF signaling may stimulate other pro-angiogenic factors to re-
establish tumor neovascularization, such as FGFs and angiopoietins (96, 101). Second, the depletion in oxygen and nutrient, as a result of vessel regression during anti-angiogenic therapy, could recruit various bone marrow-derived vascular progenitor cells to the tumor site in order to elicit new blood vessel networks (102-108). Third, the impairment of neo-vascular network by anti-angiogenic agents can result in an increase in pericyte coverage to enhance tumor blood vessel integrity and protect them from disruption (88, 109-111). Lastly, the suppression of neo-vascularization after anti-angiogenic therapy can induce novel invasive mechanism(s) to assist tumor cells to escape oxygen and nutrient deprivation (103, 112-114). The enhanced infiltrative relapse following Bevacizumab was observed when this agent was evaluated as either first- or second-line treatment (98, 100, 115). Even though the evidence suggests that enhanced tumor invasion may be a direct consequence of anti-angiogenic therapy, the underlying mechanism that controls this phenomenon is still not completely understood. A recent published study proposed that the HGF/c-MET signaling pathway may be involved in this enhanced invasive phenotype post anti-angiogenic therapy targeting VEGFRs (116). In the presence of anti-angiogenic agents, VEGFR2 forms heterodimers with MET, and this interaction causes the dissociation of protein tyrosine phosphatase 1B (PTP1B) from MET, thereby unmasking MET activity stimulating invasion of GBM cells. These data suggest that integrating VEGFR and MET inhibitors will prevent tumor recurrence after anti-angiogenic therapy.

Chemokines and Chemokine Receptors

Chemokines are small chemo-attractant cytokines that contain over 50 members (117, 118). They are classified into four subgroups C, CC, CXC, and CX3C, based on
the location of the first two cysteine residues at the N-terminal region of the sequence. These chemokines function through their seven transmembrane G protein couple receptors (GPCRs). While the CC and CXC families have multiple members, the C family has only two chemokines (XCL1, 2) and one receptor (XCR1), and the CX3C family has one chemokine (CX3CR1) and one receptor (CX3CL1). In the CXC family, the ligands are further divided into two groups, according to the presence or absence of a Glu-Leu-Arg (ELR) motif (119). This ELR motif is located at the N-terminus adjacent to the first cysteine amino acid residue. ELR+ CXC chemokines have opposite functions to ELR- CXC chemokines regarding angiogenesis (120). The ELR+ chemokines promote angiogenesis by regulating neutrophil migration (117, 120, 121). On the other hand, ELR- chemokines are angiostatic peptides (120, 122, 123). Most chemokines are secreted with the exception of CX3CL1 and CXCL16 (124). The relationship between chemokines and their receptors are promiscuous as some chemokines bind to multiple receptors and some receptors in turn bind multiple chemokines, whereas certain chemokines interact with single receptor and some receptors bind only one chemokine. As a result, there is a high degree of redundancy in their binding specificity and in the activation of downstream signaling pathways regulated by chemokine systems. First discovered as critical factors that regulate leukocyte homing, chemokines and their receptors are commonly known for their functions not only in homeostasis but also in inflammatory responses during pathological processes (121, 122, 125-135).

**Chemokine Systems in Cancers**

Because of their contribution in inflammation, it is not surprising that chemokine systems have roles in cancer biology. Due to their chemoattractant property,
chemokines and chemokine receptors are well-known for guiding the migration of cells (118, 136-140) and thus regulating metastasis. In this pivotal characteristic of cancer, chemokine receptors may potentially facilitate tumor dissemination at each of the key steps of metastasis, including adherence of tumor cells to endothelium, extravasation from blood vessels, metastatic colonization, and protection from the host response via activation of key survival pathways such as extracellular signal-regulated kinase (ERK)/motigen-activated protein kinase (MAPK), phosphatidylinositol 3-and 4-kinase (PI-3K)/(v-akt murine thymoma viral oncogene homolog 1(AKT)/mammalian target of rapamycin (mTOR), or Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (141, 142). Moreover, during tumorigenesis, chemokine networks play important roles in many processes required for tumor development, such as tumor growth, proliferation, invasion, angiogenesis, and recruitment of immune cells to tumor microenvironment (143-147). In the case of GBM, treatment with the CXCR4 antagonist, AMD 3100, inhibits growth of murine intracranial GBM and reduces the proliferation of tumor cells (148). We have determined that the CXCR3 antagonist, NBI-74330, decreases tumor growth and prolongs survival in glioma-bearing mice (149).

With regard to the interaction between microglia/macrophages and glioma, increasing evidence supports the participation of chemokines in glioma-microglia/macrophages crosstalk (150, 151). For example, CCL2 is a well described candidate for this function as an anti-CCL2 antibody prevented the migration of microglia/macrophages in response to CCL2 (152, 153). In contrast, recent evidence indicated that CCL7 (monocyte chemo-attractant protein-3), but not CCL2, is indeed the chemokine responsible for this function since CCL7 was predominantly expressed in glioma cell
lines and the expression level of CCL7 is correlated to the percentage of glioma infiltrated microglia/macrophages (GBM) in glioma tissues (154).

**CCL5/CCR1/CCR3/CCR5**

Another member of the CC family, CCL5 (alternatively named regulated on activation normal T cell expressed and secreted, or RANTES) also promotes macrophage and lymphocyte infiltration in various types of human cancers (155, 156). In a transplantable model of breast carcinoma, CCL5 was determined to be expressed by tumor cells, while its receptor, CCR5, was localized to infiltrating macrophages and lymphocytes. Furthermore, the dual CCR1/CCR5 antagonist, Met-CCL5, was able to reduce tumor growth and inhibit the migration of macrophages and lymphocytes into 410.4 tumors, suggesting a potential role of CCL5 in tumor-promoting macrophage/lymphocyte infiltration (155). A query of the NCI REMBRANDT (National Cancer Institute REpository for Molecular BRAin Neoplasia DaTa) database using CCL5 as the search term, revealed an association between CCL5 expression and survival outcomes in “All Glioma” patients. A significantly shorter mean survival time was evident in tumors having high CCL5 as compared to those characterized by intermediate levels of CCL5; insufficient numbers of patients with low CCL5 limited analysis of this patient population. The observations suggest a significant role of CCL5 within glioma, as well as a potential mechanism of immune and tumor cells interaction, through this ligand.

**CXCL11/CXCL12 and CXCR4/CXCR7**

CXCR4 and its ligand CXCL12 (SDF-1α) is one of the common chemokine receptor/chemokine pairs studied in the tumorigenesis. Present in different types of
cancer, including but not limited to pancreatic cancer (127), colon cancer (157), ovarian cancer (158), lymphoma (159), medulloblastoma and glioma (134, 141, 148, 160), CXCL12 and CXCR4 are best known for their roles in metastasis, angiogenesis, and tumor growth (103, 127, 138, 141, 142, 148, 157, 158, 161-175). Evidence for CXCL12/CXCR4 function in metastasis has been described (138). These investigators showed that CXCR4 was expressed by primary breast cancer cells while the ligand CXCL12 was highly detected in the lymph nodes, lungs, liver, and bone, all of which are frequent metastatic sites of breast cancer. Moreover, blocking CXCL12/CXCR4 interactions with a neutralizing anti-CXCR4 antibody impaired the metastasis of breast cancer cells to regional lymph nodes and lung in a xenograft model. In the context of glioma, CXCR4 is elevated in GBM and grade III glioma compared with grade II glioma (176). Like other cancers, the CXCL12/CXCR4 axis also contributes diverse roles in the malignancy of gliomas. CXCR4 is overexpressed in GBM-derived sphere cultures when compared with the differentiated tumor cells and the coexpression of CXCR4 with the progenitor-cell marker CD133 was detected within cancerous populations in specimens of human GBM (173). Moreover, studies from our group demonstrated the expression of CXCR4 was increased in the slow cycling population of primary patient-derived GBM cells, which are enriched in GSLC markers (177). The presence of both CXCL12 and CXCR4 in the tumor regions characterized by necrosis and angiogenesis suggests the possibility for their involvement in angiogenic regulation (161, 178). CXCR4 expression is primarily under the control of HIF-1 in hypoxic pseudopalisading cells in and around areas of necrosis, whereas VEGF released by these cells is responsible for CXCR4 upregulation in microvessels (169, 179). The activation of CXCR4 by CXCL12 also
promoted mobilization of bone marrow cells, which were then recruited to form new vessels (103). The fact that CXCL12 is strongly expressed in neurons, blood vessels, subpial regions, and white-matter tracts that form the basis of Scherer’s secondary structures, and that CXCR4 is dominant in invading glioma cells implies additional function of CXCL12/CXCR4 in tumor invasion and growth, possibly through the activation of metalloproteinases MMP-2 and MMP-9 (179). Although no direct in vivo studies for CXCL12-regulated glioma invasion were reported, evidence for promoting in vitro migration of glioma cells by CXCL12 are well established (146, 162, 177). In addition, targeting CXCR4 with AMD3100, a CXCR4 antagonist, inhibited the growth of murine intracranial GBM and the proliferation of tumor cells (180).

Besides CXCR4, CXCL12 was recently found to have additional interactions with another chemokine receptor, named CXCR7. The presence of this receptor was discovered when murine fetal liver cells from CXCR4 knockout mice could still bind CXCL12 and discrepancies were observed between CXCR4 expression and CXCL12 binding affinity in several human cancer cell lines (165). The expression of CXCR7 was reported in many tumor cell lines, in tumor associated endothelial cells, as well as in vascular endothelium (165, 181, 182). These characteristics suggest that CXCR7 plays a role in regulating cancer biology. Unlike CXCR4, the property of CXCR7 in GPCR mediated signaling transduction is not understood. Several mechanisms underlying CXCR7 function have been proposed. One role that CXCR7 may play is to scavenge or sequester CXCL12, thereby generating gradients of CXCL12 that lead to differential signaling by CXCR4 (183). Another role for the receptor is that it may serve as a co-receptor for CXCR4 and enhancing CXCL12-mediated G_{i/o} protein signaling via CXCR4,
as the two receptors form heterodimers in the context of overexpression in transiently transfected cells. These observations suggest that ligand binding to CXCR7 results in crosstalk with CXCR4 mediated by intracellular signaling molecules (184, 185). More recently, it has been demonstrated that CXCR7 interacts with β-arrestin in a ligand dependent manner. CXCR7 can signal through β-arrestin and act as an endogenous β-arrestin-biased receptor (184). In GBM, a recent study has reported that while CXCR4 is involved in regulating cancer stem-like functions of tumor cells, CXCR7 has a distinct role in controlling apoptosis of bulk tumor population of cells (186). However, our results show that CXCR4 and CXCR7 have overlapping functions in the regulation of stem-like properties of GBM cells (177). Therefore, the CXCL12-CXCR4-CXCR7 axis in cancers could be more complicated and the direct and indirect activities of CXCR7 may play critical roles in tumor progression.

**GBM Models**

**Murine Glioma 261 (GL261) Model**

The GL261 model was originally developed by Seligman and Shear through intracranial implantation of 20 methylcholanthrene pellets into the brains of C57BL/6 mice (187). This syngeneic model mimics closely the growth properties and immune response of human GBM. Pathological analyses showed that GL261 tumors represent diffusely infiltrating and invasive characteristics. The invading cells in this model display secondary structure of Scherer. Similar to human GBM, GL261 tumors also have highly vascular and necrotic area, which express CXCR4, CD31, HIF-1, and VEGF (71). Based on the similar characteristics with human GBM and the immune-sufficient property (188), GL261 is a strong model for our study that focuses on the role of
chemokine CCL5 and its receptors CCR1/CCR3/CCR5 in crosstalk mechanisms between tumors and host immune cells within the tumor microenvironment.

**Human Gliomasphere Model**

With the appreciation of the concept of cancer stem-like cells in glioma biology, the utility of neurospheres as a model to study GBM has received extensive attention from researchers (189). Fine and colleagues have proposed that GBM stem cells from the gliomasphere culture model have a considerable advantage over traditional serum-cultured models in which tumor cell lines were cultured as an adherent monolayer (190). When cultured in stem cell-enriched gliomasphere conditions (NBE, serum free medium supplemented with B27, human recombinant EGF, and bFGF), tumor cells derived from primary GBMs have remarkable similarity to normal neural stem cells, including the ability to form neurospheres *in vitro*, potential for indefinite self-renewal, ability for differentiation into glial and neuronal lineages, the resemblance of gene expression profile to NSCs, and genetic stability through serial *in vitro* passage. More importantly, tumor cells cultured in NBE condition harbor all genetic aberrations and *in vivo* phenotype of the parental primary GBM from which they were derived. Taking advantage of this model, we have established our studies on neurospheres from five different primary patient-derived GBM cell lines, which have been confirmed to have genotypic and phenotypic similarity to parental tumors. Moreover, these gliomaspheres expressed chemokine and chemokine receptor patterns of interest. The diversity in different GBM cell lines and the unique characteristic of gliomaspheres provided us a powerful tool to explore the role of chemokine systems in regulating GBM progression.
Significance and Specific Aims

Chemokines and chemokine receptors have significant contributions in many physiological processes required for normal development, as well as tumorigenesis. Chemokine systems control many different tumor events, including but not limited to tumor growth, migration, angiogenesis, metastasis, and recruitment of immunosuppressive cells into the tumor microenvironment. Thus targeting chemokine systems are an emerging therapeutic strategy to treat cancer. Indeed, a vast number of studies have been conducted in many different types of cancer. However, the roles of chemokine systems in gliomagenesis and progression remain uncharacterized. Studying chemokine and chemokine receptor functions in the scheme of GBM is a challenging task due to the heterogeneity of GBM microenvironment and the promiscuous relationship between chemokines and their receptors. In this project, we elucidated the roles of various chemokine systems in different aspects of glioma biology. Focusing on the functions of chemokine systems in GBM will not only provide more understanding of basic mechanisms of tumor biology but also suggest potential therapeutic strategy for future treatment of GBM.

The Specific Aims of this project included:

1. Detecting the expression of chemokines and chemokine receptors in murine and human GBM cell lines and in xenograft tumors.

Understanding the expression profile of chemokines and chemokine receptors is crucial for further analysis of their functions in GBM. Expression of different chemokines and receptors in murine GL261, traditionally used human GBM cells, and primary patient-derived GBM cell lines was characterized. Reverse transcription-polymerase chain reaction (RT-PCR), flow cytometry,
immunocytochemistry, and protein array analyses were used to detect *in vitro* mRNA and protein levels of various chemokines and chemokine receptors under study. Their *in vivo* expression was also examined using in situ hybridization and immunohistochemical methods.

2. **Evaluating the impact of chemokine receptor deficiency in the recruitment of microglia and other immune cells into GBM.**

Chemokine receptors were hypothesized to mediate the migration of microglia and lymphocytes into tumors. The chemokine expression profile determined in aim 1 provided us candidate chemokine receptors to study the recruitment of tumor-infiltrating immune cells. Using multiple chemokine receptor deficient mice, we examined the number of microglia and lymphocytes within GBM tumors from wild type and chemokine receptor-deficient animals.

3. **Determining the role of chemokine systems in regulating cancer stem-like cell properties of GBM.**

The role of chemokine system in cancer stem-like properties of glioma cells was not well defined. In this project, we elucidated the role of chemokines and their receptors in regulating the stem-like functions of primary patient-derived GBM cell lines using different *in vitro* functional assays, including migration, sphere formation, short-term proliferation, and tube formation. The ability of these cells to initiate tumors *in vivo* was also evaluated through the implantation of these cells to the brains of immune-deficient mice.

4. **Studying the impact of chemokine receptor deficiency on the survival of GBM bearing mice.**

A goal related to Aim 3 involved evaluating inhibition of chemokine receptors using pharmacological approaches in order to test the possibility of using chemokine
receptor antagonists as chemotherapy drugs. Murine and human GBM cells were co-incubated with chemokines and paired receptor inhibitors to determine the tumor cell growth rates in vitro. In addition, glioma-bearing animals were treated with a specific chemokine receptor antagonist and Kaplan-Meier survival analysis was performed to address this aim.

5. **Addressing underlying mechanisms that regulate chemokine receptor post anti-VEGF/VEGFR inhibition.**

It is crucial to understand the underlying mechanism that regulates a biological event. Taking the complexity of signaling network in cancer biology, the crosstalk between signaling pathways to mediate tumor cell functions is not uncommon. In this study we studied the underlying mechanism that regulated the VEGFR-dependent expression of CXCR4. The impact of HGF/Met and TGFβ/TGFβR signaling pathways on the regulation of CXCR4 expression by VEGFR inhibitors was evaluated with pharmacological approaches, including exogenous ligands and inhibitors targeting these pathways. The effect of these molecular targeted agents on CXCR4 expression was measured with flow cytometry.
CHAPTER 2
MATERIALS AND METHODS

Animals

Wild type (WT) C57BL/6 mice were obtained from either Jackson Laboratories (Bar Harbor, ME) or Taconic Inc. (Hudson, NY). CCR1- and CCR5- deficient (−/−) mice, backcrossed to the C57BL/6 background for greater than 10 generations, were obtained from Taconic Inc. and Jackson Laboratories, respectively. NOD-scid IL2Rγnull (NSG) mice were from Jackson Laboratories. All procedures involving mice were carried out in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee (IACUC).

Cell Culture

The GL261 glioma cell line was maintained in Roswell Park Memorial Institute medium (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin, 4 mM L-glutamine. The U118 glioma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, 1% penicillin–streptomycin, 2 mM L-glutamine. The T98G, and U87 glioma cell lines were maintained in Eagle’s minimum essential medium (EMEM) supplemented with 10% heat-inactivated FBS, 1% penicillin–streptomycin, 1% sodium pyruvate and 2 mM L-glutamine. All gliomaspheres (GS) were cultured in DMEM/F12 medium supplemented with 2% B27, 20 ng/mL of epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF), 5 μg/mL of heparin and 1% penicillin–streptomycin. All the cells were grown in a humidified incubator at 37 °C with 5% CO2.

DMEM, EMEM, RPMI-1640, DMEM/F12 medium, B27, EGF, bFGF, L-glutamine and
antibiotics were obtained from Gibco-BRL (Invitrogen). Sodium pyruvate and heparin were purchased from Sigma–Aldrich. FBS was from HyClone (Thermo Scientific).

**Primary Microglia Isolation**

Primary microglia were harvested from postnatal one day old mouse pups using a previously published protocol (191). Briefly, brain tissue was removed, mechanically and enzymatically dissociated, and kept in medium A containing 0.585% of glucose (Sigma-Aldrich), 15 mM 2-[4-(2hydroxyethyl)piperazin-1-yl] ethane sulfonic acid (HEPES), 100 u/mL penicillin, and 100 µg/mL streptomycin in Hank’s balanced salt solution (HBSS)(Gibco). The finely minced brain tissue was incubated in 0.25% trypsin medium (Gibco) for 30-45 min at 37°C. The medium was aspirated and replaced with trypsin inhibitor medium (Invitrogen). After incubation for 4 min at room temperature, the tissue was triturated with a fire-polished glass pipette and then centrifuged for 15 min at 100xg. The supernatant was aspirated and the cell suspension was plated in T75 flasks with DMEM/F12 medium supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin and streptomycin. Culture medium was changed every 3-4 days. After 15 days, cultures were treated with 0.0625% trypsin-ethylenediaminetetraacetic acid (EDTA) (diluted in DMEM/F12) for 1 h at 37°C to lift astrocytes and neurons from the flasks, leaving an essentially pure culture of primary microglia. The cultures were checked for purity and found to be greater than 97% microglia as measured by cell-type specific expression of CD11b. Purified primary microglia were collected using 2.5% trypsin with EDTA for RT-PCR and migration analyses.
Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from glioma cells with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA contamination was removed by RQ1 RNase-free DNase treatment (Promega). Total RNA was then quantified and stored at -80°C. RNA (1 μg) was retrotranscribed with iScript complementary DNA (cDNA) synthesis kit (Bio-Rad). Synthesized cDNA was subjected to polymerase chain reaction analysis. Polymerase chain reaction (PCR) was performed by heating for 96 °C for 2 min, followed by amplification for 35 cycles: 96°C for 30s, 56°C for 1 min and 72°C for 1 min. Touchdown PCR was utilized in some cases. For touchdown PCR, the annealing temperature started at 65°C and was decreased by 1°C every cycle for 15 cycles and reached 50°C. 50°C was then used for the remaining number of cycles. The parameters of denaturing and elongating temperatures were the same as regular PCR protocol. All primers used in the studies and predicted PCR product sizes are listed in Table 2-1.

Cytokine Protein Array

The presence of cytokines in GL261 cell conditioned medium was determined by mouse cytokine antibody array (R&D System, Minneapolis, MN). Membranes were treated with blocking buffer and incubated for 30 min at room temperature. The membrane was then exposed to an aliquot (1 mL) of GL261 conditioned medium and incubated for 1 h at room temperature. After this incubation period, the membranes were washed five times with a washing buffer and incubated for 1 h at room temperature with biotin-conjugated antibodies against murine cytokines (dilution: 1:250 dilution). Thereafter, the membranes were washed five times, incubated for 1 h at room
temperature with horseradish peroxidase (HRP)-conjugated streptavidin (dilution: 1:1000) and washed five times again. Finally, the reaction was developed in a mixture of SuperSignal West Pico luminol/enhancer and stable peroxide solutions (Thermo Scientific, Rockford, IL) and exposed to X-ray film. The densities of signals on films were then analyzed with ImageJ software (NIH). This experiment was performed in duplicate.

**In Situ Hybridization**

In situ hybridization (ISH) was performed as described previously (Harrison et al., 2003). Briefly, ISH probes were generated by PCR using cDNA synthesized from total RNA extracted from GL261 glioma cells. DNA fragments were cloned into pGEM-7 (Promega). To generate the antisense and sense (c)RNA hybridization probes, plasmids were linearized and then subjected to *in vitro* transcription using either T7 or SP6 RNA polymerase in the presence of [³³P]UTP. Brain sections were hybridized separately with antisense and sense probes. In all cases, no signals were detected in sections probed with “sense” riboprobes. After ISH, sections were apposed to film and subsequently dipped in LM-1 emulsion and stored at 4 °C. Slides were developed (after exposure for 1–4 weeks), fixed, and counterstained with hematoxylin and eosin. Multiple sections from tumor bearing animals were analyzed and included the following number of mice: 11 wild type (JAX Laboratory); 9 wild type (Taconic Inc); 7 CCR1-/-; 2 CCR5-/-.

**Immunocytochemistry**

Gliomaspheres were harvested and gently dissociated with diluted Accumax solution in PBS (1:500). Cells were then blocked with 2% BSA in PBS for 1 hr, incubated with mouse anti-human CXCR4 antibody (R&D Systems) for 1 hr, and with
goat anti-mouse IgG Alexa 488 for 1 hr. Washing steps were performed with PBS (3 times, 5 min each) between the staining steps. Finally, the sections were counterstained with DAPI, mounted with aqueous mounting solution and subjected to photography with Zeiss fluorescent microscope at 20X and 40X objectives.

**Immunohistochemistry**

For immunohistochemistry, brain sections were heated for 30 min at 70°C, deparaffinized in Xylene (3x5 min), and then rehydrated by stepwise immersion in 100% EtOH (2x5 min), 95% EtOH (2x5 min), and 70% EtOH (1x3 min). After deparaffinization, the samples were rinsed with deionized water and processed to antigen retrieval with sodium citrate buffer, pH 6, for 30 min at 98°C. After cooled down to room temperature, the samples were washed with deionized water for 5 min, quenched with 3% H₂O₂ for 10 min at room temperature to block endogenous peroxidase activities, and processed to standard immunohistochemistry staining. Briefly, the sections were initially blocked with 5% BSA in TBS-T for 1 hr, incubated with primary antibodies overnight at 4°C, and then with secondary antibodies for 1 hr at the room temperature. Washing steps were performed with TBS-T (3 times, 5 min each). Finally, the sections were counterstained with DAPI for imaging with fluorescent microscope or developed with 3,3′-diaminobenzidine (DAB) kit as manufacturer's instruction. The following primary antibodies were used: rat anti-CD4 (dilution 1:50, BD Pharmingen), rat anti-CD8 (dilution 1:50, Serotec), rat anti-CD11b (dilution 1:50, Serotec), mouse anti-human Nestin (dilution 1:100, Thermo Fisher Scientific, MA), rabbit anti-human Nestin (1:100, Abcam), mouse anti-human CXCR4 (dilution 1:50, R&D Systems). The secondary antibodies were used: goat anti-rat IgG Alexa 594, goat anti-rabbit IgG Alexa 594, goat
anti-mouse IgG Alexa 594, goat anti-mouse IgG Alexa 488 (1:500, Invitrogen), goat anti-mouse HRP conjugated IgG (1:3000, Perkin Elmer). For quantification of CD4+, CD8+, and CD11b+ cells, the number of cells in three high-powered fields (the visible area under 20X magnification) in three sections from multiple animals were calculated to determine the mean and standard error of mean. The data were subjected to statistical analysis (one tail T-test). The numbers of animals used in each group are indicated in the graphs.

Flow Cytometry

Gliomaspheres were dissociated with Accumax solution (Innovative Cell Technologies, Inc.), washed with ice cold PBS and subsequently incubated in blocking solution containing 5 μg/mL of mouse and rat IgG in 5% BSA diluted in PBS for 15 min at room temperature. Cells were then incubated with specific antibody for 30 min on ice. Mouse anti-human CXCR4-allophycocyanin (APC), mouse anti-CXCR7-phycoerythrin (PE), mouse anti-CXCR3-PE (dilution 1:10, R&D Systems). Samples were then washed and analyzed with BD LSR II system (BD Biosciences). Dead cells were excluded by DAPI staining. All data were analyzed by FlowJo software version 7.6 (Tree Star). Each experiment was repeated at least three times with different cell preparations.

Migration Assay

In vitro migration assays were performed using 24-well transwell units with 8-μm polycarbonate filters (BD Falcon, Franklin Lakes, NJ). After trypsinization with 2.5% trypsin, microglia (2x10^4 cells) were placed in the top compartment in 500μl of serum-free DMEM/F12 with or without 50nM of Met-CCL5. The bottom wells contained 500μl of serum-free DMEM/F12 with or without 20nM of CCL5. The plates were incubated for
24 hours at 37°C. Non-migrating cells were removed by wiping the upper side of the insert with a cotton swab. Migrating cells were fixed with 4% paraformaldehyde for 20 min at room temperature, stained with hematoxylin, and quantified by counting the number of cells on 4 random areas on the lower side of the membrane. Each experiment was performed in triplicate and repeated three times using different microglial preparations.

**Short-Term Proliferation Assay**

Cells were plated in 48 well-plates at a density of 10 cells per µl and treated with multiple concentration of CXCL11 (0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM). After 7 days with L0 and 9 days with L1 line, spheres were dissociated with Accumax solution and counted. Cells cultured in medium without CXCL11 served as the control. Each experiment was performed in triplicate and three independent experiments were repeated with different batches of cell preparations.

**Sphere Formation Assay**

Primary sphere formation assays were performed to quantify stem-like cell frequency within primary GBM cells. Cells were plated in 384-well plates at a density of 500 cells per well per 50 µl medium containing multiple concentrations of CXCL11 (0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM). Sphere numbers were quantitated after 9 days of incubation. Each experiment was performed in triplicate and three independent experiments were established.

**Apoptosis Assay**

Cells were plated in 6 well plates at a density of 5x10^4 cells per mL at day 0 in culture medium. To induce apoptosis, TMZ at a concentration of 500 µM (Sigma-
Aldrich, MO) was added to the sphere cultures at day 4, 5, and 6, in the presence and absence of CXCL12 (5 nM). At day 7, cells were collected, stained with a cell-permeable, FITC-conjugated irreversible pan-caspase inhibitor (ApoStat, R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions, and subjected to flow cytometry analysis to quantitate caspase activity of cells undergoing apoptosis. Unstained samples were included as controls to determine nonspecific staining in each treated group. The experiment was performed at least three times independently.

**Tube Formation Assay**

Cell were dispersed into single cells and cultured in Matrigel (BD Biosciences, CA) coated 48 well plates, at a density of 45,000 cells per well, with serum-free M131 medium supplemented with 5% of microvascular growth supplement (Invitrogen, CA). The cells were treated with 20 nM of CXCL12, with or without 1 µM of AMD3100, or 100 nM of CCX733, CCX771, or CCX704. After 48 hr, cells were stained with 2 µg/mL of AM Calcein fluorescent dye (BD Biosciences, CA) and photographed with Zeiss inverted microscope. Total tube length, tube area, and branch points were measured using Metamorph software. Three experiments were repeated independently.

**Intracranial Injection of Glioma Cells**

GL261 glioma cells or primary patient-derived GBM cells (10^5 cells/µL/brain) were injected 3 mm deep into the right cerebral hemisphere (1 mm posterior and 2 mm lateral from Bregma) of wild type C57/B6, CCR1- and CCR5- deficient mice or NSG mice. Tumor-bearing mice were enthanized using sodium pentobarbital (32 mg/kg) and subsequently perfused with 0.9% saline followed by buffered 4% paraformaldehyde (PFA). Brains were surgically removed and post-fixed with 4% PFA. After fixation,
brains were processed to paraffin embedment, sectioned using a microtome, and subjected to H&E staining, in situ hybridization, or immunohistochemical analysis.

**AMD3100 and Cediranib Treatments**

AMD3100 was purchased from Tocris Bioscience (UK) and Cediranib (AZD 2171) was generously provided by Dr. Dietmar Siemann. Cediranib was delivered with oral gavage at the dose of 6 mg/kg, once a day, in 10% Tween 80 diluted in PBS after 2 week of surgery, for 4 weeks. Animals also received AMD3100 treatment alone or in combination with Cediranib, at the dose of 5 mg/kg, twice a day in sterile PBS, injected subcutaneously, beginning from the 4th week after implantation, for 2 weeks. A control group of mice was treated with vehicle only. Numbers of mice in each treated group are indicated on the survival curve.

**Kaplan-Meier Survival Analysis**

For Kaplan–Meier survival analysis, percentages of surviving mice in each group of animals were recorded daily after GBM cells implantation. The endpoint was defined by a lack of physical activity and a body weight reduction of greater than 15 %. The data were subjected to Log-rank analysis in order to determine if significant differences existed in survival between the experimental groups.

**Statistical Analysis**

All data were presented as mean and standard error of mean. The difference in means between groups were subjected to analysis using student’s t-test with one-tailed distribution and alpha 0.05. Survival data were subjected to log-rank test to determine statistically significant differences between groups. Result with a p-value <0.05 was considered significant and was indicated in the figures or figure legends.
Table 2-1. List of primers used for RT-PCR analyses.

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CHAPTER 3
ROLE OF CCL5 IN MICROGLIA/MACROPHAGES-GBM CROSSTALK

The presence of microglia/macrophages cells is documented within tumor environment of human glioma as well as mouse models of the disease (25-27, 192). Opposite to their normal function in the immune system, glioma infiltrated microglia/macrophages show an anti-inflammatory phenotype, in which the expression of MHC class II molecules is down-regulated, especially in brain tumors with high malignancy (37, 40, 41, 193, 194). A positive correlation between the number of infiltrating microglia/macrophages and the proliferation rate of the tumor is also evident (25, 45). Thus, these immune cells are believed to contribute to the local immunosuppressive milieu of glioma, as well as promote tumor progression (195-197). Despite the potential importance of microglia/macrophages in glioma tumorigenesis, the mechanism by which microglia/macrophages infiltrate into the tumor is still unknown. Current studies strongly suggest this localization may require the presence of chemokines, a family of chemoattractant cytokines, in the tumor microenvironment (56, 152, 153, 198).

A query of the NCI REMBRANDT (National Cancer Institution REpository for Molecular BRAin Neoplasia DaTa) database using CCL5 as the search term, revealed an association between CCL5 expression and survival outcomes in “All Glioma” patients. A significantly shorter mean survival time was evident in tumors having high CCL5 as compared to those characterized by intermediate levels of CCL5; insufficient numbers of patients with low CCL5 limited analysis of this patient population. The observations suggest a significant role of CCL5 within glioma, as well as a potential mechanism of immune and tumor cells interaction, through this ligand.
Although the expression of CCL5 and its receptors, CCR1 and CCR5, is documented in different cancer models, no study on the role of the CCL5/CCR1/CCR5 axis in GBM has been reported. Herein, we establish that CCL5 is highly expressed in murine and human GBM cell lines, and found abundantly in tumors of GBM bearing mice where CCR1 and CCR5 are also expressed. Individual deletion of either CCR1 or CCR5 had little to no impact on survival rates of GBM-bearing or the numbers of tumor-infiltrated immune CD11b⁺, CD4⁺, and CD8⁺ cells. An in vitro analysis showed that CCR1 and CCR5 were expressed by primary microglia suggesting functional redundancy in this system. Indeed, the dual CCR1/CCR5 antagonist, Met-CCL5, was able to inhibit CCL5-dependent migration of wild type, CCR1- and CCR5-deficient microglia. Collectively, the results indicate that the infiltration of microglia/macrophages into GBM, as well as the survival of tumor bearing mice, does not solely depend on either CCR1 or CCR5 but suggests a potential mechanism of redundancy, where CCL5 directs the infiltration of microglia/macrophages into GBM through both CCR1 and CCR5.

**Results**

**CCL5 Is Expressed by Murine GL261 and Human GBM Cell Lines**

Because the chemokine CCL5 has been found in various types of tumor, including prostate, cervical, and breast cancers (199, 200), we were interested in determining if CCL5 was also present in GBM. The expression of CCL5 in vitro was assessed by RT-PCR analysis in the murine GL261 GBM cell line. The results showed that CCL5 mRNA was present in cells cultured as adherent monolayers and under culture conditions that favored formation of gliomaspheres; increased levels of CCL5
mRNA were noted in the gliomaspheres. In addition, CCL5 peptide was detected in the GL261 conditioned medium, using a cytokine protein array (Fig. 3-1A), indicating that GL261 cells are capable of both producing and releasing CCL5 protein. The expression of this chemokine was also identified in several different human GBM cells. CCL5 mRNA, detected by RT-PCR analysis, was found in primary patient-derived lines L0, L1, and L2 that were cultured under serum-free condition in the presence of EGF and bFGF, as well as the commonly used GBM lines T98G, U87, and U118 cultured in the presence of serum (Fig. 3-1B). None of the lines expressed CCR1 and CCR5 (data not shown).

**CCL5, CCR1, and CCR5 Are Present in Murine GL261 GBM**

The *in vivo* expression of CCL5 in the tissues taken from GL261 GBM-bearing wild type mice was evaluated by RT-PCR and *in situ* hybridization analyses. In both cases, mRNA levels of CCL5 were significantly elevated in the tumor tissue, when compared to the normal brain area (Fig. 3-2A, B). Together with the *in vitro* data above, these results suggested that tumor cells are the primary source for CCL5 production within GBM. In addition, the two major receptors of this chemokine, CCR1 and CCR5, were strongly detected in the tumor tissues (Fig. 3-2A, C). We also evaluated the expression of CCR3, another low affinity CCL5 receptor. The lack of signal of this receptor from *in situ* hybridization analysis indicated that CCR3 was not expressed in intracranial GL261 GBMs (Fig. 3-2C). Immunohistochemical localization of CCR1 and CCR5 within the tumors was evaluated but the lack of availability of suitable antibodies precluded definitive identification of these receptors.
The expression of CCL5 and its receptors, CCR1 and CCR5, was also analyzed in the tissues collected from tumor bearing CCR1-/- and CCR5-/- mice. *In situ* hybridization analysis showed that CCL5 was expressed at high levels within tumors of these animals, suggesting that CCR1 and CCR5 deficiency did not affect the expression of this chemokine within the tumor. In contrast, while both CCR1 and CCR5 were present in the tumors from wild type mice, the signals for CCR1 and CCR5 were obviously diminished in GBMs from CCR1-/- and CCR5-/- mice, respectively (Fig. 3-2C). This observation indicates that intratumoral CCR1- and CCR5-expressing cells are derived from the recipient mice, and not from the culture-derived GL261 GBM cells that were implanted into the animals.

**Neither CCR1 nor CCR5 Deficiency Contributed to the Infiltration of Immune Cells into GBM or Impacted the Survival of Tumor Bearing Mice**

To evaluate the hypothesis that CCL5 directs the localization of microglia/macrophages into GBM in a CCR1 or CCR5 dependent manner, we characterized immune cell infiltration and animal survival in tumor bearing CCR1- and CCR5-deficient mice. Immunohistochemical analysis was performed to investigate if CCR1 and CCR5 regulated the infiltration of immune cells, including CD11b+ microglia/macrophages, CD4+ and CD8+ T cells, into GBM. Quantitative analysis did not show any statistically significant differences in the number of these cells located within the GBMs of tumor bearing CCR1-/- and CCR5-/- mice, as compared to the wild type animals (Fig. 3-3A). The effect of CCR1 and CCR5 deficiency on the survival of GBM bearing mice among wild type, CCR1-/-, and CCR5-/- groups was also assessed using Kaplan-Meier survival analysis. No significant difference in the survival rate between tumor bearing wild type and CCR5-/- groups were evident (p value = 0.77). The life
span of GBM bearing CCR1-/- mice, however, was slightly shorter than the life span of the wild type (p value = 0.0007). The median survival time of tumor bearing CCR1-/- mice after GL261 cell implantation was 19 days, while that of GBM bearing wild type mice was 22 days (Fig. 3-3B). Together, these results suggest that CCR1 and CCR5 deficiency had no impact on the infiltration of CD11\(^+\), CD4\(^+\), and CD8\(^+\) immune cells into GBM, and at best, only a modest effect on the survival rate of tumor bearing mice.

**CCL5 Interacted with Its Microglia/Macrophages-Expressed Receptors, CCR1 and CCR5, in a Redundant Manner**

The *in vitro* expression of CCR1 and CCR5 was determined on primary cultures of microglia derived from wild type, CCR1-/-, and CCR5-/- mice. Both CCR1 and CCR5 mRNAs were detected in microglia using RT-PCR analysis, suggesting that these CCL5 receptors are co-expressed by these cells *in vitro* (Fig. 3-4A). The absence of CCR1 and CCR5 mRNA signals in CCR1-/- and CCR5-/- mice, respectively, confirmed that these gene products were deleted in the microglia derived from the CCR1- and CCR5-deficient animals. CX3CR1, a microglia/macrophages-expressed chemokine receptor, was detected in microglial cultures from all of the animals. We also pursued the expression of CCR1 and CCR5 protein by microglia *in vitro*, but similar to our attempts to detect these receptors *in vivo*, the lack of quality antibodies reactive towards murine CCR1 and CCR5 posed significant challenges to identifying these receptors. The presence of CCL5 receptors CCR1 and CCR5 in microglia encouraged us to ask the question if CCL5 could direct the migration of microglia *in vitro*. Using modified Boyden chamber assay, we showed that microglia migrated toward CCL5 gradient in a dose dependent manner (Fig. 3-4B). Moreover, this migratory effect may be regulated by MMP2, MMP9, and MMP14, as mRNA levels were upregulated by CCL5 (Fig. 3-4C).
These matrix metalloproteinases are well known for their function in migratory process of tumor cells. Opposite to these *in vitro* data, our *in vivo* data reported above showed that neither CCR1 nor CCR5 contribute individually to the action of CCL5 on the localization of microglia/macrophages into GBM, as well as the survival of tumor bearing mice. We next examined the possibility that CCL5 may interact with CCR1 and CCR5 in a redundant manner. Indeed, migration of wild type, as well as CCR1-/- and CCR5-/- microglia was stimulated by CCL5. Met-CCL5, a modified form of CCL5 that has antagonist activity at both CCR1 and CCR5, completely blocked the migration by CCL5 in all groups (Fig. 3-4D).

**Discussion**

The presence of a high number of infiltrated microglia/macrophages within glioma, confirmed by various studies both in human glioma and rodent models of the disease (25, 26, 192), has prompted further investigations into the role of these immune competent cells within the tumor microenvironment. Accumulating evidence suggests that microglia/macrophages facilitate glioma growth by contributing to the immunosuppressive environment and directly assisting in tumor growth and invasion (45, 150, 197, 201, 202). Understanding the mechanisms by which microglia/macrophages cells are localized to glioma will provide novel therapeutic targets for intervention. Here we report on the expression of CCL5 and its receptors, CCR1 and CCR5, in GBM. Our data demonstrate that CCL5 is highly expressed by human GBM and murine GL261 GBM cells *in vitro*, as well within intracranial GBMs *in vivo*. Moreover, CCL5 is upregulated in GL261 cells cultured under conditions that favors growth of the cells as spheres; cells cultured under these conditions exhibit a
more malignant phenotype (188, 190). Its receptors CCR1 and CCR5, on the other hand, are expressed by cultured microglia, but not by GBM cells, and are also highly expressed within intracranial tumors. The loss of CCR1 and CCR5 in situ hybridization signals from tumor sections from CCR1- and CCR5-deficient mice also indicates that the GBM cells do not express either of these receptors. *In vitro*, CCL5 could mediate the migratory function of microglia in a dose dependent manner through the induction of MMP2, MMP9, MMP14. These data support the hypothesis that GBM-expressed CCL5 may be a key regulator in the crosstalk between GBM and microglia/macrophages, through an interaction with microglia/macrophages-expressed CCR1 or CCR5, and that blocking either CCR1 or CCR5 could prevent the GBM localization of microglia/macrophages.

GBM growth and numbers of intra-tumoral microglia/macrophages cells, together with CD4+ and CD8+ T cells, were evaluated in mice individually deficient in either CCR1 or CCR5. Our data showed that microglia/macrophages infiltration was not attenuated in either CCR1- or CCR5-deficient tumor-bearing animals. CD4+ and CD8+ cells were also similar in tumors from receptor deficient and wild type mice. Moreover, there was only a modest, in the case of CCR1-deficiency, or no difference, with CCR5-deficiency, in survival rates between GBM-bearing wild type mice and the chemokine receptor-deficient tumor-bearing animals. Our explanation for these observations is that CCL5 has a high affinity for both CCR1 and CCR5, and blocking either one of them individually is not sufficient to inhibit the actions of this chemokine. Due to the promiscuous interactions of chemokines and chemokine receptors, this redundant mechanism needs to be considered. Indeed, this issue has been addressed in several
studies (130, 194, 203, 204). In a recent study that characterized the role of these receptors in a periodontal disease model, Repeke and colleagues found that CCR1- and CCR5-deficient mice present a lower leukocyte infiltration and alveolar bone loss than wild type mice, yet inhibiting both of these receptors by Met-CCL5 (a dual CCR1/CCR5 antagonist) severely attenuated the inflammatory bone resorption (204). A redundancy mechanism in GBM is consistent with our in vitro investigation which indicates that CCR1 and CCR5 are co-expressed by primary microglia in vitro, and that the dual antagonist Met-CCL5 could completely block the migratory effect of CCL5 on these cells regardless of whether the cells expressed CCR1 or CCR5. Nonetheless, our data does not exclude the possibility that CCL5 may act through another unidentified receptor(s).

While the evidence for a cooperative role of CCR1 and CCR5 is suggested from the in vitro study, a redundancy of these chemokine receptors in this murine GBM model has not yet been evaluated in vivo. A combined CCR1- and CCR5-deficient mouse would offer a model system to test the redundancy hypothesis. However, the CCR1 and CCR5 genes are located adjacent to each other on murine chromosome 9, providing a major limitation in generating the double knockout mice by simply breeding CCR1- and CCR5-deficient lines. Alternative pharmacologically-based approaches are worth considering but these also have limitations. Although Met-CCL5 has been used as a dual antagonist for both CCR1 and CCR5 in in vitro studies (155, 205), effectively targeting these receptors located within the brain would require the peptide to penetrate the blood brain barrier, one of the most important obstacles that restricted the delivery of molecularly targeted therapy to glioma (206). A combined pharmacological and
genetic approach is an alternative strategy worthy of consideration. In particular, CCR1-deficient mice could be treated with a CCR5 antagonist, e.g. TAK-779, while CCR5-deficient mice would be treated with the CCR1 antagonist, BX-741. Both of these drugs have been tested for their effective delivery to the CNS, when administered systemically (207, 208). However, as BX-741 and TAK-779 were specifically designed for human CCR1 and CCR5 respectively, the low binding affinity for mouse CCR1 and CCR5 is a primary concern. These obstacles together provide significant challenges for addressing the redundancy hypothesis through in vivo experimentation.

The interaction between microglia/macrophages and GBM is complex and likely to involve bi-directional signaling. After recruitment into the tumor milieu by GBM cells, microglia/macrophages lose control of their immune surveillance property and, in turn, help facilitate tumor progression. Since the recruitment of microglia/macrophages likely depends on their interaction with numerous GBM-secreted factors or by other components in the tumor microenvironment, it is not surprising that this process is regulated in a redundant manner. Our findings support the notion that the function of CCL5 in GBM is not dependent on individual interactions with either CCR1 or CCR5. To the contrary, the CCL5/CCR1/CCR5 network likely involves a redundant mechanism, adding more complexity to GBM-microglia/macrophages crosstalk and signaling a need to block both receptors to prevent the actions of this chemokine in this highly malignant form of brain cancer.
Figure 3-1. Expression of CCL5 by murine GL261 and human GBM cell lines. A) Left: RT-PCR analysis detecting CCL5 mRNA in murine GL261 cells, cultured as either adherent monolayers (A) or gliomaspheres (S). GAPDH served as a control. Right: CCL5 protein detected in GL261 conditioned medium, using cytokine protein array. B) RT-PCR analysis of CCL5 mRNA in different human GBM cell lines, L0, L1, and L2 as well as commonly used standard GBM cell lines, T98G, U87, and U118. GAPDH served as a control.
Figure 3-2. Expression of CCL5 and its receptors CCR1 and CCR5 in murine GL261 GBMs. A) RT-PCR analysis in normal (N) and tumor (T) tissues. In situ hybridization (ISH) analysis depicting the expression of B) CCL5 in normal (N) and tumor (T) sections from a glioma-bearing wild type (WT) C57BL/6 mouse, and C) CCL5, CCR1, CCR3, and CCR5 in WT, CCR1 and CCR5-deficient mice.
Figure 3-3. Effect of CCR1 and CCR5 deficiency on infiltration of immune cells into GBM and survival of tumor bearing mice. A) Numbers of intra-GBM CD11b+, CD4+, and CD8+ in CCR1 (left, black) and CCR5 (right, black)-deficient mice, compared to WT (white) mice. B) Kaplan-Meier survival plots of GBM bearing CCR1 (left, square) and CCR5 (right, square)-deficient mice, compared to WT (circle) mice. N is the number of animal that was used in each group.
Figure 3-4. *In vitro* expression of CCR1 and CCR5 by primary microglia cultures and the effect of Met-CCL5 on CCL5-stimulated migration of microglia. A) RT-PCR analysis of CCR1 and CCR5 expressed by WT, CCR1- (CCR1−/−), and CCR5- (CCR5−/−) deficient microglia. CX3CR1 and GAPDH served as controls. B) WT microglia migrated toward CCL5 in a concentration dependent manner. C) CCL5 stimulated the mRNA expression of MMP2, MMP9, and MMP14 in WT microglia, detected by RT-PCR analysis. D) Migration of WT, CCR1−, and CCR5-deficient microglia treated with CCL5 (black), or with CCL5 and Met-CCL5 (stripe), compared to control (white). *p < 0.05, **p<0.001, ***p<0.0001.
CHAPTER 4
HETEROGENEITY OF CXCR4 AND CXCR7 IN GBM

Increasing evidence suggests that the poor prognosis and the failure of current advanced treatments targeting GBM are sustained by a distinct group of glioma cancer stem-like cells. This rare subpopulation displays stem-like properties, including proliferation, self-renewal, multipotency, and expression of different stem-cell markers. The current therapies are thought to focus only on tumor mass but show minimal impact on this stem-like subpopulation, which is capable of driving tumor recurrence and regrowth. CXCR4, a well-known chemokine receptor for its diverse roles in tumor biology, and a related receptor, CXCR7, are found in human GBM. Although the presence of these receptors in GBM is well documented, their function(s) in modulating glioma stem cell-like subpopulations is not thoroughly understood. A current hypothesis states that CXCR4 regulates the stem-like features of glioma cells while CXCR7 is responsible for inhibition of apoptosis of the bulk population of tumor cells (186). From our perspective, the roles of these receptors in GBM are more complicated. Our previous study illustrated a high degree of heterogeneity in surface expression patterns of CXCR4 and CXCR7 among different primary patient-derived GBM cell lines. CXCR4 and CXCR7 have variable activities in in vitro functions, including sphere formation, migration, short-term proliferation, under the stimulation of CXCL12 (177). Following up on these findings, we expanded our investigation on the mRNA/protein expression of CXCR4/CXCR7/CXCL11/CXCL12 by primary patient-derived GBM cell lines and/or tumors. The ability of CXCR4 and CXCR7 subpopulations to initiate in vivo tumor formation of GBM cell lines was also examined. The impact of CXCL11 on CXCR7-
stimulated *in vitro* functions and the effect of CXCL12 on tube formation and apoptosis of these cell lines are also described in studies presented herein.

**Results**

**CXCR4, CXCR7, CXCL11, and CXCL12 Were Expressed in Primary Patient-Derived GBM Cell Lines and/or Tumors**

It has been reported that CXCR4 and CXCR7, together with their shared ligand CXCL12 were expressed in human GBM and normal brain (186). In this study, we were interested in determining if these chemokine receptors and their ligands, CXCL11 and CXCL12, were also expressed in various primary patient-derived GBM cell lines and xenograft model, using RT-PCR and IHC analyses. Our results showed that, at a mRNA level, CXCR4, CXCR7, and CXCL11 were present while CXCL12 was absent or at undetectable levels, in all GBM cell lines (Fig. 4-1A). These chemokines and chemokine receptors were also evaluated in the tumors derived from immune-deficient mice implanted with primary patient-derived GBM cell lines using IHC analysis. CXCR4, CXCR7, CXCL11, and CXCL12 were all found in the tumor sections of xenografts (Fig. 4-1B).

**Both CXCR4⁺ and CXCR7⁺ Cells Were Capable of Generating Tumors In Vivo**

In a previous study from the laboratory, we found that CXCR4⁺ and CXCR7⁺ cells were more abundant in the slow-cycling subpopulation, which is enriched with cancer stem cell markers. We hypothesized that CXCR4 and/or CXCR7 might regulate cancer stem cell phenotypes. To test this, we evaluated the ability of *in vivo* tumor formation of CXCR4⁻ and CXCR7-expressing cells. Subpopulations containing either CXCR4⁺, CXCR7⁺, CXCR4⁺/CXCR7⁺ (double positive), or CXCR4⁻/CXCR7⁻ (double negative) were isolated from GBM L0 line using FACS and implanted intracranially in immune-
deficient NSG mice. This line was chosen as the model system since it was the only one that showed effects of CXCL12 on \textit{in vitro} sphere formation, a property often associated with a tumor initiating subpopulation. Our result showed that all subpopulations successfully initiated tumors \textit{in vivo} (Fig. 4-2). These data suggest that neither CXCR4 nor CXCR7 are directly involved in regulating phenotypes of tumor initiating cells.

**CXCL12 Stimulated Tube Formation of L0 Cells through CXCR4**

Transdifferentiation of GBM tumor-derived endothelial cells has been documented by multiple groups (79-81) and CXCL12 has been reported to promote \textit{in vitro} tube formation and VEGF production of endothelial cells (169, 209). Therefore we tested the hypothesis that CXCL12 might be involved in phenotypic changes associated with the differentiation of GBM cells into endothelial cells. CXCL12 promoted tube formation of GBM L0 cells (Fig. 4-3A). Total tube area, total tube length, and total branch points were significantly increased by CXCL12 when compared to control treated L0 cells (Fig. 4-3B). The effects of CXCL12 were blocked by AMD3100 but not CCX733 or CCX771, indicating that CXCR4, but not CXCR7, is involved in the CXCL12 stimulated-tube formation of L0 cells. CXCL12 had no effect on tube formation of the L1, L2, and S2 lines (data not shown).

**CXCL12 Had No Effect on TMZ-Induced Apoptosis in CXCR4\(^+\) and CXCR7\(^+\) Cells**

An anti-apoptotic effect mediated by stimulation of CXCR7, but not CXCR4, has been reported in GBM cell lines (186). We evaluated the impact of CXCL12 on TMZ-induced apoptosis in various primary GBM cell lines. TMZ induced apoptosis in all four lines (Fig. 4-4A). A differential effect of TMZ treatment on the relative percentages of
CXCR4- and CXCR7-expressing subpopulations was evident (Fig. 4-4B). The percentage of CXCR4+ cells was increased in lines L0, L1, and L2 after TMZ treatment. TMZ did not impact the percentage of CXCR4-expressing subpopulation in the S2 line. CXCR7+ subpopulations were increased in all lines after TMZ treatment. Moreover, heterogeneous expression of CXCR4 and CXCR7 was observed between apoptotic and non-apoptotic subpopulations among the various cell lines after TMZ treatment. The percentage of CXCR4-expressing cells in the non-apoptotic subpopulation remained unchanged in L0 cells, increased in the L1 and L2 cells, and decreased in the S2 line. The percentage of CXCR7-expressing cells undergoing apoptosis increased in all lines. Increases in the CXCR7+ cells in the non-apoptotic subpopulation occurred in the L0, L1, and L2 cells but not in the S2 line after TMZ treatment. In all lines, CXCL12 had no effect on the distribution of CXCR4- and CXCR7-expressing cells in either the apoptotic and non-apoptotic subpopulations.

**CXCL11 Contributed No Impact on In Vitro Functions of GBM Cell Lines**

In previous study, our group demonstrated that CXCR4 and CXCR7 heterogeneously regulated *in vitro* functions of different primary patient-derived GBM cell lines. In the presence of CXCL12, both CXCR4 and CXCR7 stimulated the migration in L0 and S2 lines and short-term proliferation in L0 and L1 cells, while only CXCR7, but not CXCR4, initiated sphere formation of L0; involvement of these specific receptors was determined using selective antagonists for CXCR4 and CXCR7. Because CXCL11 is an additional ligand of CXCR7, we evaluated the impact of this chemokine on the *in vitro* functions that were regulated by CXCR7 in GBM cell lines, as reported previously. Our data showed that CXCL11 contributed no impact on sphere formation.
(L0, Figure 4-5A), proliferation (L0 and L1, Figure 4-5B), and migration (L0 and S2, Figure 4-5C) of the GBM lines tested. These results suggest that CXCR7 regulation of in vitro functions in primary patient-derived GBM cell lines is solely dependent on CXCL12, but not CXCL11.

Discussion

CXCR4, with its sole ligand CXCL12, were reported to be present in human GBM (166, 169, 173-175, 177, 209-214) and their expression correlated with survival rate of GBM patients. CXCR4 and CXCL12 have been well documented for their various roles in GBM development and progression, including promoting tumor growth, facilitating tumor cell invasion toward a CXCL12 source, and initiating angiogenesis (173, 174, 177, 209, 211-213). CXCR7, an additional receptor for CXCL12, was also present in human GBM tissue. Unlike CXCR4, the role of CXCR7 in GBM is poorly understood. A previous study has shown that CXCR4 is upregulated in stem-like GBM cells while CXCR7 is elevated in differentiated GBM cells (186). The proposed function of CXCR7 is to prevent GBM cells from apoptosis, which may contribute to the resistance to therapy. Although the mechanism regulated by CXCR7 was unclear, there is evidence suggesting that CXCR7 may serve as a CXCR4 signaling regulator by 1) depleting extracellular CXCL12 or 2) dimerizing with CXCR4 to either block or facilitate CXCR4-regulated signal transduction. Based on this complex interaction, it is not surprising that CXCR4 and CXCR7 have overlapping functions in controlling GBM progression.

Indeed, our previous studies have demonstrated a high degree of heterogeneity in surface expression of CXCR4 and CXCR7 in different primary patient-derived GBM cell lines. Variable percentages of CXCR4$^+$, CXCR7$^+$, and CXCR4$^+$/CXCR7$^+$ (double
positive) populations were evident in different GBM cell lines: GBM L0 had a relatively higher percentage of CXCR4\(^+\) cells than CXCR7\(^+\) cells while GBM L1 and L2 contained more CXCR7\(^+\) cells, GBM S2 meanwhile showed comparable percentages of CXCR4\(^+\) and CXCR7\(^+\) cells. In contrast, chemokine receptors CCR3 and CXCR3 were co-expressed on a small but similar percentage of cells in all cell lines. Sub-population of cells that have both CXCR4 and CXCR7 were observed in all GBM lines. A slow cycling sub-population of cells, which express stem-like cell markers and exhibit high tumorigenic frequencies, were enriched for CXCR3, CXCR4, and CXCR7. *In vitro* functional studies showed that both CXCR4 and CXCR7 played multiple roles in promoting tumor cell growth, migration, and sphere formation, depending on specific GBM cell lines.

The fact that both CXCR4 and CXCR7 were enriched in slow cycling sub-population that contained stem-like markers and that both CXCR4\(^+\) and CXCR7\(^+\) cells were able to initiate spheres *in vitro* suggested that these receptors may regulate cancer stem-like phenotypes of these cells. To confirm the role of these chemokine receptors in this context, we asked the question if cells expressing CXCR4, CXCR7, or both were able to generate tumors *in vivo*. L0 was chosen for this study because it is the only line that displayed CXCL12 regulated *in vitro* sphere formation, among the four examined lines. The data reported in this chapter confirmed that both CXCR4- and CXCR7-expressing cells were capable of forming tumors in immune-deficient mice. Together with *in vitro* data, this direct evidence suggested that tumor formation is independent of CXCR4 or CXCR7.
Based on the results of Hattermann et al. (186), we also anticipated that stimulation of CXCR7 would have protected GBM cells from apoptosis induced by TMZ. However, while all lines were sensitive to TMZ-induced apoptosis, none were protected from apoptosis by CXCL12. Moreover, CXCR4 and CXCR7 presented in a heterogeneous expression pattern between apoptotic and non-apoptotic sub-populations among different GBM cell lines. There was no evidence for the correlation between CXCR4 or CXCR7 expression and apoptotic phenomenon. This lack of a CXCR7 effect to inhibit apoptosis is likely related to the nature of the cell culture system used to evaluate this phenomenon. The CXCR7 phenotype reported previously was found in adherent GBM cells grown in serum, which is distinct from the study reported here that were performed on sphere cultures, i.e. non-adherent serum-free conditions. In fact, data from Hattermann and colleagues indicated that CXCR7 stimulation had little to no effect on Camptothecin-induced apoptosis in a GBM stem-like cell line (186). These authors attributed the lack of effect as a result of low level expression of CXCR7 in the stem-like cells.

In cancer biology, unlike CXCR4/CXCL12 axis that has earned considerable attention on its role, the involvement of recently discovered CXCR7 as another receptor for CXCL12 remains less well understood. In our studies reported previously and herein, we showed that, under the stimulation of CXCL12, CXCR7 regulated proliferation, sphere formation, and migration of GBM cells. Besides CXCL12, CXCR7 can also bind to CXCL11, an established ligand for CXCR3. Thus, we expanded our study to address if CXCL11 could stimulate CXCR7-regulated functions of GBM cells. The results, however, did not support this possibility. Consistent with these data,
previous published study showed that GBM cells did not migrate toward a CXCL11 gradient alone. However, at the low dose, CXCL11 could enhance the migration of tumor cells toward a CXCL12 gradient (215). This observation suggests that CXCL11 may work in a cooperative manner with CXCL12 to direct CXCR7 activities.
CXCR4, CXCR7, CXCL11, and CXCL12 were expressed in primary patient-derived GBM cell lines and/or tumors. A) Expression of CXCL11, CXCR4, and CXCR7 mRNAs in GBM cell lines, using RT-PCR analysis. Note that all cell lines express CXCL11, CXCR4, and CXCR7 but none are positive for CXCL12. Actin served as control. B) Expression of CXCR4, CXCR7, CXCL11, and CXCL12 in xenografts, detected by immunohistochemistry. Representative fluorescent micrographs depict the expression of CXCR4 and CXCL12 (left), CXCR7 and CXCL11 (middle), CXCL11 and Nestin (right) within high power fields in the tumors derived from immuno-deficient mice implanted with primary patient-derived GBM cells.
Figure 4-2. CXCR4⁺, CXCR7⁺, and CXCR4⁺/CXCR7⁺ cells generated tumors in vivo. Representative sections from tumors derived from L0 sub-populations. The various sub-populations indicated in the figures were implanted intracranially into NSG mice. Shown are representative sections subjected to anti-human Nestin immunohistochemistry. Note that all sub-populations are capable of forming tumors in vivo.
Figure 4-3. CXCL12 stimulation of L0 tube formation in vitro was mediated by CXCR4. A) Representative images of control and CXCL12 (20 nM) treated GBM L0 cells. B) CXCL12 (20 nM) significantly increased total tube length, total tube area, and total branch points of L0 cells. AMD3100 (1 µM) inhibited CXCL12 stimulation of tube formation of L0 cells. CXCR7 inhibitors did not block the stimulation of CXCL12. All conditions contained 0.1% DMSO. *p<0.05; **p<0.01. Representative results of three individual experiments performed in triplicate are shown.
Figure 4-4. Differential impact of TMZ on the distribution of CXCR4- and CXCR7-expressing apoptotic and non-apoptotic subpopulations. A) GBM lines were treated with TMZ as described in Methods. Apoptotic subpopulations, determined by positive ApoStat staining, were detected in TMZ treated samples in all cell lines. Histograms are representative from three independent experiments. B) Heterogeneity in CXCR4- and CXCR7-expressing subpopulations in the apoptotic and non-apoptotic subpopulations after TMZ treatment, as determined by flow cytometry analysis. Bar graphs represent the average of three independent experiments.
CXCL11 had no effect on in vitro functions of GBM cell lines. CXCL11 concentration-response assessment indicated that CXCL11 contributed no effect on A) sphere formation of L0 cells, B) short-term proliferation of L0 and L1, C) migration of L0 and S2.
CHAPTER 5
EXPRESSION AND FUNCTION OF CXCR4 IN GBM AFTER ANTI-ANGIOGENIC THERAPY

Patients diagnosed with GBM receive standard treatments including surgical removal of the tumor, followed by radiation in combination with chemotherapy. These interventions, however, only provide a modest impact on patient survival. Because GBM is one of the most vascularized solid tumors, anti-angiogenic agents, which target VEGF signaling pathways and block the formation of new tumor blood vessels, have been evaluated as an alternative treatment strategy for GBM patients. Data from clinical trials suggested that, in some patients with recurrent GBM treated with either anti-VEGF antibodies (Bevacizumab, Aflibercept, etc.) or small molecular VEGF receptor tyrosine kinase inhibitors (e.g. Cediranib or Vandetanib), these agents could enhance 6-month progression free survival rate, as well as radio-graphic response. In spite of these promising achievements, they failed to maintain an enduring survival benefit to patients. Under VEGF pathway inhibition, however, a significant fraction of tumors begin growing back in a more invasive manner. A recent published study (116) proposed that the HGF/MET signaling pathway may be involved in this enhanced invasive phenotype post anti-angiogenic therapy targeting VEGFRs. In the presence of anti-angiogenic agents, VEGFR2 forms heterodimers with MET, and this interaction causes the dissociation of PTP1B from MET, thereby unmasking MET activity in GBM cells. These data suggest that integrating VEGFR and MET inhibitors will prevent tumor recurrence after anti-angiogenic therapy. Herein, we propose an additional mechanism in which anti-angiogenic therapies might regulate the enhanced invasive phenotype through a pathway involving chemokine receptor CXCR4. We provide evidence that anti-
angiogenic agents elevate the expression of CXCR4 in primary patient-derived GBM cell lines expressing VEGFRs, and in tumors derived from one of these cell lines. This upregulation is independent of HGF/MET signaling, but dependent on TGFβ receptor serine/threonine kinase activity. Moreover, the combination of an anti-angiogenic agent (Cediranib) and a CXCR4 antagonist (AMD3100) provided the most survival benefit to tumor-bearing animals, compared to either agent used as a single treatment. These data suggest that the CXCL12/CXCR4 axis, together with TGFβ/TGFβR may contribute to the invasive phenotype of recurrent tumors after anti-angiogenic therapy. Thus, the combination of VEGFR pathway inhibitors, CXCR4 antagonists, and TGFβR inhibitors may provide an alternative strategy to halt tumor progression and provide more substantial benefit to patients with GBM.

**Results**

**Heterogeneous Expression of VEGF Receptors by Different Primary Patient-Derived GBM Cell Lines**

GBM is one of the most vascularized tumors. VEGFR2, a well-studied VEGF receptor responsible for formation of physiological vasculogenesis and pathological angiogenesis, has been found in both initial and recurrent high graded gliomas (90, 91). The mRNA expression pattern of VEGF and its three receptor isoforms, VEGFR1, VEGFR2, and VEGFR3, were evaluated in various primary patient-derived GBM cell lines. While VEGF was expressed by all five examined GBM cell lines, RT-PCR analysis showed that expression of VEGFRs varied amongst these lines: all three VEGFR mRNAs were found in S2 and S3 lines. However, none of them were detected in the L0, L1, and L2 lines (Fig. 5-1A). Consistent with *in vitro* mRNA expression data, immunohistochemistry analysis showed that VEGFR2 was detected in the tumor
derived from S3, but not L0, cell line. The co-localization of VEGFR2 and human Nestin indicated that VEGFR2 was expressed by human S3 GBM cells (Fig. 5-1B). These data indicate heterogeneity in the expression of VEGFRs in different primary patient-derived GBM cell lines.

**Anti-VEGF/VEGFR Agents Increased the Expression of CXCR4 in Primary Patient-Derived GBM Cell Lines and Xenograft Tumors that Were Positive for VEGFRs**

Most, if not all, GBM patients receiving anti-angiogenic agents were reported to have tumor relapse with an associated enhanced invasive phenotype after initial response to therapy (98-100, 115). The factors and mechanisms regulating this phenomenon are currently ill-defined. As a chemokine receptor that is widely found in GBM cell lines and tumor tissues and well known for its role in stimulating tumor invasion, CXCR4 may be potentially involved in the tumor invasiveness initiated by anti-angiogenic therapy. To test this hypothesis, we first examined the impact of anti-angiogenic agents on the surface expression of CXCR4 in our different primary patient-derived GBM lines. Both flow cytometry and immunocytochemical analyses showed that CXCR4 expression was significantly increased in VEGFR(s)-positive S2 and S3 cells but not in the VEGFR(s)-negative L0 cell line, after the lines were treated with two different small molecule VEGFR protein kinase inhibitors, i.e. Cediranib or Vandetanib (Fig. 5-2A, B). CXCR4 was also increased in the S3 line with Bevacizumab treatment. The VEGFR kinase inhibitors had no impact on the expression of CXCR7, another receptor for CXCL12, in either VEGFR-positive or VEGFR-negative GBM cell lines (data not shown). Consistent with the *in vitro* data, CXCR4 was also highly elevated in VEGFR-positive S3 derived tumors from NSG mice treated with Cediranib, while fewer CXCR4-expressing cells were detected in tumors from vehicle-treated animals.
Moreover, our data demonstrated that the increase in CXCR4 expression was evident in both normoxic and hypoxic zones, as determined by proximity to cells expressing HIF1α. This observation indicates that the elevation of CXCR4 by Cediranib in S3 GBM is not solely regulated by hypoxia induced by an anti-angiogenic agent (Fig. 5-2C). These in vitro and in vivo results suggest that anti-angiogenic drugs can enhance the expression of CXCR4 in GBM cell lines and VEGFR(s)-expressing tumors.

Anti-VEGFR Drugs Enhanced the Migratory Effect of VEGFR(s)-Expressing GBM Cell Lines toward CXCL12

Our published data showed that CXCR4 regulated the in vitro migration of S2, but not S3 GBM cells, toward a CXCL12 gradient (177). Because CXCR4 was elevated by anti-angiogenic drugs, we then determined if these agents could enhance the migratory effect of CXCL12 on these cell lines. Consistent with our previous results, in the absence of Cediranib or Vandetanib pre-treatment, CXCL12 induced the migration of S2, but not S3 cells. However, Cediranib or Vandetanib pre-treatment enhanced the CXCL12 directed migration in S2 and induced a migratory effect of S3 cells (Fig. 5-3). Based on the findings described above, this enhanced migratory effect of CXCL12 in these cells may be due to the increase in CXCR4 expression stimulated by Cediranib or Vandetanib.

The Combination of a CXCR4 Antagonist and a VEGFR Inhibitor Provided a Greater Beneficial Effect on the Survival of GBM Bearing Animals

Based on the upregulation of CXCR4 by anti-angiogenic agents reported herein, we sought to determine whether antagonism of CXCR4 after anti-angiogenic therapy could provide an enhanced beneficial effect on the survival of tumor bearing mice. To address this question, VEGFR-positive S3 cells were intracranially implanted to NSG
mice and tumors were allowed to initiate in this xenograft model for 2 weeks. After this period, tumor bearing animals were sequentially treated with or without Cediranib. Following an additional two weeks, control and Cediranib-treated mice were further subdivided into vehicle and AMD3100 (CXCR4 antagonist) treatment groups, (treatment scheme is depicted in Fig. 5-4A. Kaplan-Meier survival analysis showed that combined treatment of Cediranib and AMD3100 provided the greatest survival advantage to tumor bearing mice, when compared to control (p<0.0001), AMD3100 (p<0.0001), or Cediranib (p=0.0085) treated groups. Although single treatment with Cediranib also contributed statistically significant benefit to animal survival (p=0.0003), no effect of monotherapy with AMD3100 on survival on tumor bearing mice was evident (p=0.1027), when compared to the control-treated cohort (Fig. 5-4B). These results strongly suggest that an alternative therapy scheme that targets both VEGFR and CXCR4 signaling might render an advantage over single treatment with agents that target either of these receptor pathways.

Anti-Angiogenic Agents Stimulated CXCR4 Expression in a HGF/MET Independent and TGFβ/TGFβRs Dependent Manner

Lu and colleagues reported that anti-VEGF agents regulated an enhanced invasiveness phenotype in GBM by unmasking MET signaling inhibition (116). Because MET was shown to be an upstream regulator of CXCR4 (175), we initially hypothesized that the increase in CXCR4 expression seen after VEGF/VEGFR inhibition is directly regulated by the HGF/MET signaling pathway. The effect of HGF on CXCR4 expression was then evaluated in the absence or presence of Vandetanib. Flow cytometry analysis showed that exogenous HGF did not impact CXCR4 levels (Fig. 5-5B). The lack of an exogenous HGF effect could be due to the presence of endogenous HGF generated by
these cell lines, as evident by RT-PCR analysis (Fig. 5-5A). However, a MET inhibitor, BMS777607, was unable to inhibit the Vandetanib effect on CXCR4 expression (Fig. 5-5C). Hence, the lack of effect of HGF or MET inhibitors indicates that the anti-angiogenic regulation of CXCR4 expression is independent of the HGF/MET signaling pathway.

TGFβ is known for its roles in both pro- and anti-inflammatory regulation and in tumor progression (216-220). In GBM, previously published data from a xenograft model indicated that TGFβ was highly expressed in the tumor receiving anti-angiogenic treatment (221). Thus, we then hypothesized that TGFβ is a factor involved in the anti-angiogenic stimulation of CXCR4 expression. RT-PCR analysis determined that mRNA levels of at least two forms of TGFβ (TGFβ1, TGFβ2, TGFβ3) and all TGFβRs (TGFβR1, TGFβR2, TGFβR3) were found in all of the cell lines under study (Fig. 5-6A). Moreover, stimulation of GBM cells with exogenous TGFβ in the absence or presence of Cediranib or Vandetanib, indicated that TGFβ could only synergize with Cediranib, but not Vandetanib, to provide prominent effects on CXCR4 expression in VEGFR-positive S3, but not the VEGFR-negative L0, cell line. Despite the presence of VEGFR, TGFβ did not contribute significant impact on Cediranib enhanced CXCR4 expression in S2 line (Fig. 5-6B and data not shown). We also used a TGFβR inhibitor as a pharmacological approach to test the dependence of this pathway on the Cediranib or Vandetanib regulation of CXCR4. The TGFβR inhibitor suppressed the Cediranib, but not Vandetanib, effect on the expression of CXCR4 in S3, and little in S2, lines (Fig. 5-6C). These findings strongly support a mechanism in which the enhancement of CXCR4 expression by anti-angiogenic agents is controlled by TGFβ/TGFβR signaling.
Discussion

As the most common and aggressive primary brain tumor in adults with extremely poor prognosis, GBM has received major attention for therapeutic strategy, in order to improve survival rate of patients with this disease. In an effort to seek advanced intervention to halt GBM progression, anti-angiogenic therapy obtained an accelerated approval from US Food and Drug Administration for treatment of recurrent GBM, because of its favorable outcomes during phase I and II clinical trials. While drugs targeting VEGF and/or VEGFR signaling pathways are now evaluated for frontline regimens for GBM, increasing evidence is raising concern since tumor relapse post anti-angiogenic treatment occurs in most, if not all, GBM patients. Data from both laboratory and clinical studies indicate that after initial adaptive response to anti-angiogenic drugs, tumors tend to grow back in a more invasive and uncontrollable manner (95, 98-100, 221). The underlying mechanism responsible for this tumor resistance remains elusive. While searching for an involvement of chemokine receptor CXCR4 in post anti-angiogenic phenomenon, we found that surface expression of CXCR4 was up-regulated by either VEGF- or VEGFRs-targeting inhibitors (Bevacizumab, Cediranib, and Vandetanib) in VEGFR-positive GBM cell lines. The lack of Cediranib or Vandetanib effect on CXCR4 expression in VEGFR-negative GBM cell lines suggested that these small molecule kinase inhibitors targeted VEGFRs, but not other receptor kinases, as these VEGFR inhibitors did impact CXCR4 in L0, L1, and L2 cells. These lines were categorized in the classical (or proliferative) subclass in which high expression of EGFR is evident. In addition, this effect seemed to be specific to the expression of CXCR4 as no stimulation was observed with CXCR7 level when examined in the same study (data...
not shown). Consistent with these data, our in vivo observation reported an increase in CXCR4 expression in the tumors derived from VEGFR-positive GBM cells implanted animals after receiving Cediranib treatment. Moreover, the greatest survival rate was documented in the cohort of tumor bearing animals treated with a combination of Cediranib and AMD3100, a CXCR4 inhibitor, when compared to vehicle or single agent treated groups. Using pharmacological approaches, we demonstrated that the mechanism for up-regulation of CXCR4 by anti-angiogenic agents is dependent on TGFβ/TGFβRs, but not HGF/MET signaling transduction.

One of the remarkable features of GBM that contribute to poor prognosis and therapy resistance is the great heterogeneity within and across tumors. Because of this, it is not surprising to see diverse mRNA expression of VEGFRs among our different primary patient-derived GBM cell lines. While VEGF was observed in all lines, it was not the case with VEGFRs. All VEGFRs (VEGFR1, VEGFR2, and VEGFR3) were only expressed by selective GBM cell lines, namely S2 and S3. The absence of VEGFRs in some GBM cell lines may be due to the down regulation of these receptors to undetectable levels. Indeed, when comparing fold change (>=-0.5: down-regulated, <=0.5: up-regulated) in the expression of VEGF, VEGFR1, and VEGFR2, based on tumor/normal ratio using TCGA database, we found that VEGF was prominently up-regulated in 390 samples (99.2%) and only 3 samples were shown to be down-regulated (0.8%), in a total of 393 samples. In contrast, the change in gene expression of VEGFR1 and VEGFR2 was more heterogeneous among GBM patient samples: 197 up-regulated (77.3%) and 58 down-regulated (22.7%) with VEGFR1, 174 up-regulated
and 53 down-regulated (23.3%) with VEGFR2 (data not shown). The gene expression of VEGFR3 was not analyzed due to the lack of information in the database.

GBM progression involves multiple processes, including, but not limited to, tumor cell proliferation, infiltration, and angiogenesis. The formation of new blood vessels from pre-existing ones appears to be the critical step in tumor malignancy, as it will provide nutrient supply for tumor cells in order to perform other activities. Hence, it was theoretically assumed that blocking angiogenesis might arrest overall activities of tumor progression and thus sustain survival benefit to patients with GBM. Laboratory and clinical studies, however, have questioned this approach. The lack of effectiveness of anti-angiogenic therapy on GBM progression can be explained by two possibilities. First, inhibition of angiogenic formation may preclude the expansion of tumor growth, due to the lack of nutrient supply, but contribute little to no effect on the infiltrative component of tumor cells, which is angiogenesis dependent. This hypothesis is based on the observation in anaplastic astrocytoma, in which no vascular proliferation was evident. The infiltration of tumor cells in this grade III glioma relied on a pre-established vascular network in the brain. Second, angiogenic blockage has direct impact on the infiltrative nature of tumor cells and thus promotes an enhanced invasive phenotype. A previous report suggests that the introduction of anti-VEGF antibody could unmask the signaling activity of MET by removing inhibition due to protein tyrosine phosphatase 1B, thus promoting the invasive phenotype of tumor cells through this receptor (116).

Evidence from our study indicates at an additional possibility. CXCR4, together with its ligand CXCL12, are well known for its migrating property in many different types of cancer, including GBM (127, 166, 214, 222). The fact that CXCR4 was up-regulated by
anti-angiogenic agents in primary patient-derived GBM cell lines and in Cediranib treated tumors, points to a potential mechanism in which anti-angiogenic therapy promotes invasion of tumor cells by elevating the expression of CXCR4. This proposal is supported by our *in vitro* data, which showed that VEGFRs inhibitors were able to induce or enhance migration toward CXCL12 of VEGFRs-positive cell lines. Despite the promising observation from this preliminary *in vitro* data, further *in vivo* analysis focusing on combined effects of anti-angiogenic therapy and CXCR4 in tumor invasion will be needed to support this hypothesis.

In the context of the heterogeneity and complexity within the tumor microenvironment, it is inevitable that an event is regulated by multiple factors or signaling pathways. While previous studies support a relationship between anti-angiogenesis and HGF/MET signaling pathway in GBM (116), our data provide a possibility for an additional mechanism in which the enhanced invasiveness phenotype post anti-angiogenic therapy is regulated through CXCR4 signaling pathway. Of additional interest, our data suggest an involvement of TGFβ/TGFβRs signaling, as an intermediate, in this crosstalk. Indeed, in the U87 GBM xenograft model, Piao Y et al. showed that the expression of TGFβ was markedly increased in the tumors treated with either Bevacizumab, or Sunitinib, or combined Bevacizumab and Sunitinib, when compared with tumors in the control group (221). The TGFβ-dependent-upregulation of CXCR4 was also evident in a different study. Hardee et al. showed that LY364947, a small-molecule inhibitor of TGFβ type 1 receptor kinase was able to inhibit the increase in CXCR4 expression stimulated by radiation in murine GL261 neurospheres (89). Together with these observations, our data support a potential interaction among three
signaling pathways VEGF/VEGFRs, TGFβ/TGFβRs, and CXCL12/CXCR4: inhibition of anti-angiogenesis by blocking VEGF/VEGFRs signaling will induce TGFβ/TGFβR activity, resulting in its stimulation of CXCL12/CXCR4 pathways, which in turn promotes tumor invasiveness. Our study demonstrated that the up-regulation of CXCR4 by anti-angiogenic agents is dependent on TGFβ/TGFβRs. However, a link for the direct effect of these drugs on TGFβ/TGFβRs is still missing. It will be interesting to see if anti-angiogenic agents are able to induce the activation of TGFβ/TGFβRs in our VEGFRs-positive cell lines and tumors. This information will provide more convincing evidence for the possibility of mutual crosstalk among these three signaling pathways.

Given the complexity and heterogeneity within and around tumor microenvironments, the idea of utilizing combined therapy for cancer treatment is gaining appreciation. In GBM, the combination of Bevacizumab and chemotherapy exhibited a trend in clinical effectiveness with selective cytotoxic agents, compared to monotherapy (115, 223-225). The combination of anti-angiogenic therapy and molecularly targeted agents has also received attention, yet the effectiveness of this approach has not be addressed in a rigorous manner. Our study, for the first time, provides evidence for a synergetic effect of AMD3100 and Cediranib on the survival of animals bearing GBMs. Although a modest effect was seen with single treatment of Cediranib, but not AMD3100, when compared to the control group, combined treatment of AMD3100 and Cediranib contributed greater benefit than mono-treatments. It is reported that, by controlling edema, Cediranib increased the survival of animals with GBM, despite persistent tumor growth (96). On the other hand, published studies showed that AMD3100 treatment significantly impaired intracranial growth of U87 GBM.
(180) or migration of U87 and LN308 cell lines in hypoxic condition (169). Based on these data, the beneficial effect on survival we observed in our study with combined Cediranib and AMD3100 may be due to the alleviation of edema, controlled by Cediranib, and the reduction in tumor growth, and possibly invasion, controlled by AMD3100. Future efforts studying the impact of this combined treatment on tumor burden at different time points during the treatment will be very useful.

With the advantage of using multiple primary patient-derived GBM models, various types of anti-angiogenic agent, and versatile pharmacological approaches, we established strong evidence for the up-regulation of CXCR4 stimulated by anti-angiogenesis in a TGFβ/TGFβRs dependent, but HGF/MET independent, manner. More importantly, our promising survival analysis represents a significant scientific rationale for clinical evaluation of a combined therapeutic approach that targets both VEGF/VEGFRs regulated-angiogenesis and CXCL12/CXCR4 controlled-tumor progression. This proposed regimen may provide a major therapeutic advantage over monotherapy for patients with GBM.
Figure 5-1. VEGFRs were heterogeneously expressed by different primary patient-derived GBM cell lines. RT-PCR analysis identified VEGFR1,2,3 mRNAs in S2 and S3, but not in L0, L1, and L2 cells. VEGF mRNA was expressed by all cell lines. Actin was used as a control.
Figure 5-2. VEGFR inhibitors upregulated CXCR4 in VEGFR-expressing GBM cell lines and tumors. A) Flow cytometry analysis determined that CXCR4 is increased in either Cediranib (500nM)- or Vandetanib (500nM)-treated S2 and S3 cells, but not in L0 cells; Bevacizumab (125µg/mL) also increased CXCR4 in S3 cells. Non-immune IgG was included for staining control. B) High expression of CXCR4 (green) was evident in S2 and S3 cells treated with Cediranib, compared to control-treated cells, as detected by immunofluorescent staining. C) Immunohistochemical analysis showing increased CXCR4 (green) in tumor section from S3 tumor bearing-NSG mice treated with Cediranib, compared to vehicle-treated tumor bearing mice, in both normoxic and hypoxic regions, as defined by the expression of HIF1α (red).
Figure 5-3. VEGFR inhibitor enhanced the migratory effect of CXCL12 on S2 and S3 cells. Migration assays were performed with S2 (left) and S3 (right) cell lines in the absence or presence of CXCL12 (3nM). Cells were treated with Vandetanib (500nM) for 16hr prior to the migration assay. *p<0.05, **p<0.01, ***p<0.005
Figure 5-4. Combination of CXCR4 and VEGFR inhibitors prolonged survival of S3 tumor bearing mice. A) Treatment scheme for survival analysis of S3 tumor-bearing NSG mice. B) Kaplan-Meier survival analysis showed that the longest survival was achieved with combined treatment (n=9, median=49 days, p<0.0001), compared to vehicle-treated (n=9, median=35 days), single treatment with AMD3100 (n=9, median=38 days, p=0.1, p'<0.0001), or Cediranib (n=9, median=44 days, p=0.0003, p'=0.02). p: compared to Control group, p': compared to AMD3100+Cediranib group
Figure 5-5. Upregulation of CXCR4 expression by anti-angiogenic drugs was independent of HGF/MET signaling. A) RT-PCR analysis detected HGF and MET in all GBM lines. B) Recombinant HGF (100ng/mL) contributed no effect on CXCR4 expression in the absence or presence of Cediranib or Vandetanib in S2 and S3 cells. C) MET kinase inhibitor (BMS777607, 1µM) did not block the effect of Cediranib or Vandetanib (500nM)-induced CXCR4 expression in both S2 and S3 cell lines.
Figure 5-6. The increase in CXCR4 expression by anti-angiogenic drugs was regulated by TGFβ/TGFβR signaling pathway. A) RT-PCR analysis detected TGFβ2, β3, and TGFβRs in all GBM lines. B) Recombinant TGFβ (5ng/mL) enhanced the effect of Cediranib (500nM) on CXCR4 expression in S3, but not in L0 cells. C) TGFβR kinase inhibitor (TBRI, 1µM) blocked the effect of Vandetanib (500nM)-induced CXCR4 expression in both S2 and S3 cell lines.
Among more than 120 types of brain cancer, GBM is the most aggressive form due to its rapid growth rate, high invasiveness to surrounding normal tissues, ability to escape the host immune system, great heterogeneity and presence of cancer stem-like population within the tumor microenvironment. These factors not only contribute to the mortality and lethality of this life-threatening disease but also preclude the effectiveness of current treatments through resistance mechanisms. Thus, it is urgent for scientists to accelerate the search of novel therapeutic strategies, in the process of halting tumor progression.

Well known for their roles in many physiological and pathological processes, chemokine systems also function in tumorigenesis, such as metastasis, tumor growth and invasion, angiogenesis, CSC regulation, as well as communication between tumor cells and the host. Hence, targeting chemokines and their receptors may provide an advantage to cancer treatment. In this project, we evaluated the functions and therapeutic potential of various chemokines/chemokine receptors in different aspects of GBM biology, including the recruitment of immune cells to GBM, glioma stem-like properties, and tumor resistance after anti-VEGF therapy.

Summary of Main Findings

The Role of Chemokines and Chemokine Receptors in Microglia and Tumor Crosstalk

Using murine GL261 and human GBM cell lines, we showed that CCL5 was highly expressed in these lines and in tumors of GBM bearing mice where CCR1 and CCR5, but not CCR3, were also expressed. Using a syngeneic model which is immuno-
competent, we demonstrated that the infiltration of CCR1 and CCR5-expressing microglia into CCL5-expressing glioma, as well as the survival of tumor bearing mice, are not solely dependent on individual CCR1 or CCR5 receptor, as numbers of glioma-infiltrated microglia and survival rates of tumor bearing CCR1 or CCR-deficient mice, are similar to that of tumor-bearing wild type animals. CCL5 promoted \textit{in vitro} migration of wild type, CCR1 or CCR5-deficient microglia. This migration of the genotypically distinct microglia was effectively blocked by the dual CCR1/CCR5 antagonist, Met-CCL5. These data suggest that CCL5 functions within the glioma microenvironment through neither CCR1 nor CCR5 individually, but in a redundant manner.

**The Heterogeneous Expression and Function of Chemokine Receptors in GBM**

According to our recently published data, Liu et al. showed a high degree of heterogeneity in surface expression patterns of CXCR4 and CXCR7 receptors among different primary patient-derived GBM cell lines. CXCR4 and CXCR7 have variable activities in \textit{in vitro} functions, including sphere formation, migration, short-term proliferation, under the stimulation of CXCL12 (177). To follow up this finding, we wanted to complete our understanding by addressing mRNA and protein expression of CXCR4, CXCR7, CXCL11, and CXCL12 by primary patient-derived GBM cell lines and tumors and elucidating other \textit{in vitro} functions. The ability of specific CXCR4 and CXCR7-expressing subpopulations to initiate \textit{in vivo} tumor formation was also a goal that needed addressing. Finally, the impact of CXCL11, another ligand for CXCR7, on CXCR7-stimulated \textit{in vitro} functions was also described in this extended study. RT-PCR analysis showed that CXCR4, CXCR7, and its ligand CXCL11 was expressed by all examined GBM cell lines, while no evidence for CXCL12 expression was observed. We
also demonstrated that any of these subpopulations derived from L0 cell lines, including CXCR4⁻/CXCR7⁻, CXCR4⁺/CXCR7⁻, CXCR4⁻/CXCR7⁺, and CXCR4⁺/CXCR7⁺, was able to initiate tumor formation \textit{in vivo}. In addition to other \textit{in vitro} functions, L0 also stimulated tube formation of GBM cells from L0, but not from other lines. CXCL12 had no effect on the distribution of CXCR4- and CXCR7-expressing cells in either the apoptotic and non-apoptotic subpopulations. Our study also indicated that CXCL11 did not contribute any impact on CXCR7 \textit{in vitro} activities, which had been shown to be regulated by CXCL12.

\textbf{Involvement of Chemokine Receptor in the GBM Enhanced Invasive Phenotype after Anti-VEGF Therapy}

Tumor recurrence with enhanced invasiveness after anti-VEGF therapy is well reported. However, the mechanism(s) that regulates this phenomenon is still not thoroughly understood. In this project, we elucidated the expression and function of CXCR4 in primary patient-derived GBM cell lines and tumors receiving anti-VEGF therapy. When characterizing several primary patient-derived GBM cell lines, we found heterogeneity in the level of expression of VEGFRs; some lines were VEGFR positive while others were VEGFR negative. The levels of CXCR4 were elevated, both \textit{in vitro} and \textit{in vivo}, after VEGF/VEGFR inhibitor treatment (Bevacizumab, Cediranib or Vandetanib) only in VEGFR-expressing GBM cells, while CXCR7 was not affected by VEGFR pathway inhibition. Based on a recently published finding describing a role of HGF in enhanced invasive phenotype after anti-VEGF therapy in GBM (116), we initially hypothesized that the upregulation of CXCR4 by VEGFR inhibitor was regulated by HGF/MET activation. Surprisingly, our data strongly suggested that this regulation is independent of the MET signaling pathway, but dependent on TGFβ receptor
serine/threonine kinase activity. Of interest, the combination of the CXCR4 antagonist AMD3100 and Cediranib enhanced the survival of tumor bearing mice implanted with VEGFR expressing GBM cells. These data provide supportive evidence for an additional therapeutic strategy involving blocking both CXCR4 and VEGFR signaling pathways.

**Clinical Impact and Future Directions of the Findings**

GBM exhibits a high resistance rate to various treatment strategies. The failure of monotherapy in GBM treatment prompted us to search for more effective interventions, among which is combined therapy. In terms of complexity of the tumor microenvironment, where tumor cells are directly impacted by heterogeneous factors, the concept of combined therapy is well appreciated because it targets multiple events at the same time and thus provides better beneficial effects on suppressing tumor growth. Taking advantage of versatile GBM models and pharmacological approaches, we provide more mechanistic understanding of the role of chemokines and their receptors in GBM progression, and also suggest the potential of using anti-chemokine system agents in combination with other therapies. In the study of CXCR4 regulation by anti-VEGF therapy, our Kaplan-Meier survival analysis that showed a synergistic effect of a CXCR4 antagonist and a VEGFR inhibitor on survival of tumor bearing animals, suggesting a promising combined therapeutic treatment approach for GBM patients.

Besides the concept of combined therapy to target multiple events during tumorigenesis, personalized regimens are another prospective approach to improve the effectiveness of cancer treatment. This idea is fueled by the heterogenous characteristics of cancer among different patients. With the ability of having multiple
primary GBM cell lines derived from various patients, we have demonstrated marked heterogeneity in chemokine receptor expression and function among different examined GBM cell lines, suggesting that selective strategies targeting these receptors may be considered, based on the expression pattern in each individual. Moreover, we also showed heterogeneity in VEGFR(s) expression among various primary human-derived GBM cell lines and tumors. This observation might explain, in part, the intrinsic non-responsive resistance that is evident in some patients where no discernible beneficial effect of anti-VEGF therapy was achieved (96, 99). Thus, identifying the expression profile of VEGFRs in GBM from individual patients could provide a better rationale for choosing anti-VEGF therapy.

The failure of cancer treatment can be the consequence of redundancy in regulating tumorigenic mechanisms. If an activity of tumor cells is mediated by multiple components, therapy targeting a specific factor will not likely completely suppress it. Indeed, the concept of redundancy has been demonstrated in several areas of this project. When determining the role of CCL5 in the recruitment of microglia into GBM microenvironment using CCR1- or CCR5-deficient microglia and a dual antagonist for CCR1 and CCR5, we provide evidence that tumor secreted CCL5 may direct the infiltration of microglia through both CCR1 and CCR5. The presence of more than one VEGFR and TGFβ isoforms within the same primary patient-derived cell lines also raises the questions for the role of these factors. Do these isoforms regulate similar activity in a redundant manner or do they serve distinct functions in tumor biology? Addressing this question will provide insights for designing novel therapeutic approaches.
The findings reported herein suggest versatile roles for chemokines and their receptors in many aspects of GBM, including directing the recruitment of microglia into tumor microenvironment, regulating the stem-like properties of glioma cells, and mediating the tumor response after anti-VEGF therapy. Despite the crucial impact of chemokine systems in tumor progression, molecular and/or cellular mechanisms controlling these events are still elusive. This can be addressed in further investigations that focus on the signaling communication between different chemokine receptors, such as CCR1 and CCR5 or CXCR4 and CXCR7. In addition, signaling networks that include chemokine receptors and other signaling pathways, e.g. CXCR4, VEGFR, and TGFβR are also worthy of future study. Elucidation of these crosstalk mechanisms will be crucial for not only providing insights into the critical roles of chemokine systems in GBM, but also for identifying new therapeutic targets for GBM treatment.

Evidence for the roles of chemokine systems in GBM progression were established not only with GBM cell lines but also with GBM mouse models, which were carefully chosen to address specific questions. In the first project, we used the GL261 murine GBM model to evaluate the role of CCL5 and its receptors in the infiltration of the host immune cells into GBM. The advantage of this chemically induced rodent cell lines grafted in syngeneic immunocompetent mice is that both the innate and acquired immune systems are present during the development of the tumor. One drawback, however, is in the histological characteristics of this mouse glioma. Although tumors developed in this model system display some level of invasion, they fail to show single cell infiltration to the hemisphere and microvascular abnormality, a hallmark of human GBM. In an effort to use a model system that more closely resembles human GBM
within the CNS, we generated an orthotropic xenograft GBM model in which primary patient-derived GBM cell lines containing stem-like properties when cultured in serum-free condition were intracranially transplanted into the brain of immune-deficient mice. This orthotopic xenograft model was utilized in several instances to test the effect of therapeutic approaches on GBM progression. The major advantage of this model is the ability of stem-like cancer cells to establish extensive infiltrative lesions when transplanted into CNS. Nonetheless, a significant pitfall associated with human xenograft GBM model, using immune-deficient mice, is the concern that the host immune system has been disrupted. This deficiency in immunity may alter the characteristics of human GBM during tumor development and progression, or impact the evaluation of therapeutic outcomes form this model system.
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BIOGRAPHICAL SKETCH

Kien Pham was born in 1982 in the highland central of Vietnam. She started her undergrad study at the College of Natural Science, National University of Vietnam in Ho Chi Minh City with a major in general biology, and finished with her bachelor degree in Molecular and Cellular Biology at the University of Arizona in 2006. Here, she continued to earn her Professional Science Master degree in Applied Biosciences in 2008. With the interest in cancer biology, she decided to achieve her higher education further and received her PhD degree from the University of Florida with major in Biomedical Sciences. During her PhD training in Dr. Jeffrey K. Harrison’s lab, Kien spent most of her time studying the role of various chemokine systems in different aspects of glioblastoma. Her works were well appreciated in several national scientific conferences and published in two peer-review journals. She was also the recipient of several awards from the University of Arizona and the University of Florida. With a passion for research in cancer biology, Kien hopes that her studies will contribute to the understanding of tumorigenesis, as well as to the discovery of new therapies for cancer treatment.