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Annexin A2 and Microglia/Macrophages in Glioma Progression

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Abstract of the Dissertation

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Gliomas are highly invasive brain tumors with the occurrence of numerous microglia/macrophages (MG/MP) in and around the tumor. Annexin A2 is overexpressed in many cancers and correlates with increased plasmin activity on the tumor cell surface. Plasmin mediates degradation of extracellular matrix and angiogenesis to facilitate tumor growth. In this study, we used a mouse glioma cell line, GL261-EGFP, and stable clones transfected with an annexin A2 knockdown (annA2KD) construct, GL261-EGFP-annA2KD. We found that the annA2KD decreased glioma cell migration *in vitro* and decreased membrane-bound plasmin activity. *In vivo* we injected GL261-EGFP cells into the mouse brain and the glioma progression was followed. Knockdown of annexin A2 in glioma cells decreased tumor size and slowed down tumor progression, characterized by decreased invasion, angiogenesis and proliferation, as well as increased apoptosis in tumor tissue of the annA2KD group. We also investigated the interaction between glioma

and MG/MPs, and the contribution of MG/MPs to glioma progression. We used a glioma-microglia *in culture* system to establish the effects the tumor and microglial cells have on each other. We then assessed glioma progression *in vivo* after MG/MP ablation or in the setting of exaggerated MG/MP activation. We show that glioma cells activate microglia but inhibit their phagocytic activities. Local ablation of MG/MP *in vivo* significantly decreased tumor size and improved survival curves. Conversely, pharmacological activation of MG/MP increased glioma size through stimulating tumor proliferation and inhibiting apoptosis. In agreement with recent reports we found that expression of the chemokine CCL21 was enhanced after MG/MP activation and correlates with tumor growth. However the macrophage/microglial inhibitory factor (MIF) had the opposite effects on glioma progression. We expect that these experiments will provide a better insight into the role of annexin A2 and MG/MPs in glioma progression, and will hopefully allow us to design potential ways to interfere with the glioma-microglia interaction and affect tumor growth and survival.

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List of Abbreviations

ACM	astrocyte-conditioned medium
Aβ	beta-amyloid
annA2KD	annexin A2 knockdown
annA2 KO	annexin A2 knockout
APC	antigen presenting cell
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CNS	central nervous system
CNTF	cilliary neurotrophic growth factor
CSF-1	colony stimulating factor-1
DC	detergent-compatible
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
GBM	glioblastoma multiforme
GCM	glioma-conditioned medium
GCV	ganciclovir
GFAP	glial fibrillary acidic protein
GIM	glioma-infiltrating microglia/macrophages
GM-CSF	granulocyte-macrophage stimulating factor
H&E	hematoxylin and eosin
HGF/SF	hepatocyte growth factor/scatter factor
HRP	horseradish peroxidase
HSVTK	herpes simplex thymidine kinase
IACUC	Institutional Animal Care and Use Committee
ICH	intracerebral hemorrhage
IFN	Interferon

Ig	immunoglobulin
IL	interleukin
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MG/MP	microglia/macrophages
MHC	major histocompatibility complex
MIF	macrophage/microglial inhibitory factor
MMP	matrix metalloproteinase
MVD	micro-vessel density
OSM	oncostatin-M
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
p-histone H3	phospho-histone H3
PKC	protein kinase C
plg	plasminogen
PVDF	polyvinylidene difluoride
Rb	retinoblastoma
SEM	standard error of the mean
TGF	tumor growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase type plasminogen activator
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WT	wild type

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Chapter 1

Introduction

Gliomas

Gliomas are the most common primary brain tumors, which account for 1.4% of all cancers and 2.4% of all cancer deaths in the United States. There are approximately 20,500 newly diagnosed cases and 12,500 deaths due to primary malignant brain tumors each year (Gladson et al., 2010). The symptoms depend on the anatomical site that gliomas affect. A brain glioma can lead to increased intracranial pressure, which causes headaches, nausea and vomiting, seizures or convulsions, memory deficits, mood and personality alterations, and cranial nerve disorders including changes in speech, vision and hearing. Spinal cord gliomas can cause pain, weakness or numbness in the extremities (Gladson et al., 2010). The incidence rate of gliomas is high in the 0-8-year and 50-70-year age groups (Pollack, 1994; Wen and Kesari, 2008), and there is a slight male predominance (Ohgaki and Kleihues, 2005). The risk factors for glioma development are not clear, but several occupations, environmental carcinogens, and diet (*N*-nitroso compounds) have been reported to act as predisposing factors (Ohgaki and Kleihues, 2005).

Gliomas arise from glial cells including astrocytes, oligodendrocytes, or ependymal cells, and are classified as astrocytomas, oligodendrogliomas, mixed

oligoastrocytomas or ependymomas based on light microscopic similarities between host cells and tumor cells, as well as similarities in protein expression pattern by immunohistochemistry such as glial fibrillary acidic protein (GFAP) normally expressed by astrocytes (Louis, 2006). Histologically, gliomas are graded from I to IV according to the World Health Organization (WHO) criteria (Louis et al., 2007). Grade I tumors (benign gliomas, also known as pilocytic astrocytoma) occur more frequently in children, and patients usually have a good prognosis (Pollack, 1994; Wen and Kesari, 2008). Grade II to IV gliomas are considered as malignant tumors and have much worse prognosis (Louis, 2006). Grade II tumors are characterized by hypercellularity, and the median survival of patients with Grade II tumors is 5 – 8 years (Louis, 2006). Anaplastic astrocytomas belong to Grade III tumors with a 3-year median survival, and are characterized by hypercellularity, nuclear atypia and mitotic features (Louis et al., 2007). Glioblastoma multiforme (GBM) belongs to Grade IV tumors and is the most malignant form of astrocytoma with extremely poor prognosis of approximately 1 year. Histologically they are characterized by hypercellularity, nuclear atypia, mitotic figures, and evidence of angiogenesis and/or necrosis (Louis, 2006). Oligodendrogliomas are graded as II or III based on their histological characteristics, and usually have better prognosis than astrocytomas (Louis, 2006).

Several abnormalities of chromosomes or gene expression are found in gliomas, some of which are reported to correlate with clinical grade (Holland, 2001). In the lower grade gliomas, overexpression of certain growth factors and their receptors are commonly found, such as platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF2) and cilliary neurotrophic growth factor (CNTF) (Holland, 2001; Shih and Holland, 2006;

Stiver, 2004; Weis et al., 1999). Moreover, p53 is often found mutated in glioma tissue (Ohgaki and Kleihues, 2005). In addition to the above abnormalities, the anaplastic gliomas are characterized by deletion of INK4A, amplification of cyclin-dependent kinase 4 (CDK4), or loss of retinoblastoma (Rb), which lead to disrupted cell cycle (Soni et al., 2005). The GBMs often have additional chromosomal abnormalities including loss of 10q22–25, which is a coding region for several tumor suppressors, such as PTEN (Holland, 2001; Wiencke et al., 2007). The sum effect of these alterations contributes to the biology of these tumors.

The current standard treatment for brain gliomas is an approach combining surgical resection, followed by radiation and chemotherapy with temozolomide (Clarke et al., 2010). Although many therapeutic approaches have been explored, there has been no major improvement in survival over the last 30 years (DeAngelis, 2001; Legler et al., 1999). Gliomas are highly invasive tumors, and the microscopic infiltrative loci outside of tumor mass contribute to most of the tumor relapse following surgery (Tsatas and Kaye, 2003).

tPA/plasminogen proteolytic system and tumor invasion

Invasion is a complex process that includes detachment of tumor cells from the the tumor invasive front to peripheral tissue, adhesion to extracelluar matrix (ECM) proteins, and subsequent degradation and remodeling of ECM by tumor-released proteases. Through this mechanism, tumor cells create intercellular spaces, where they can migrate by an active process requiring membrane synthesis and rearrangement of

cytoskeleton (Levicar et al., 2003b). It is well documented that tumor-released proteases, such as plasminogen activator (PA), matrix metalloproteinases (MMPs) and lysosomal cathepsins, play critical roles in tumor invasion (Rao, 2003).

The functions of PA/plasminogen system in tumor growth, invasion and angiogenesis have been widely studied (McMahon and Kwaan, 2008). Two distinct types of PA have been identified: urokinase type-PA (uPA) and tissue PA (tPA), which are responsible for the conversion of the enzymatically inactive zymogen, plasminogen, into the active serine protease, plasmin. Plasminogen, has 5 consecutive kringle domans, and is cleaved by PAs at the Arg^{561}-Val^{562} peptide bond, an event which generates the two-chain serine protease, plasmin (Collen, 1999). Plasmin is a potent protease capable of direct breakdown of many ECM components, including fibronectin, laminin and proteoglycans, and its overproduction has been reported to facilitate tumor invasion and angiogenesis (Dano et al., 1985; DeClerck et al., 1997; Mignatti and Rifkin, 1993). Plasmin can also activate MMPs and latent growth factors, which contribute to ECM degradation and tumor expansion (Carmeliet et al., 1997; McColl et al., 2003).

tPA, the enzyme generating plasmin, is a serine protease and its overexpression has been reported in melanoma, neuroblastoma, acute leukemia and pancreatic cancer (Herlyn et al., 1990; Paciucci et al., 1996; Reiter et al., 1997; Sugiura et al., 1999). In pancreatic cancer, tPA promotes tumor invasion, growth and angiogenesis via its proteolytic activity, as well as tumor cell proliferation through a cytokine-like mechanism (Aguilar et al., 2004; Diaz et al., 2002; Ortiz-Zapater et al., 2007; Paciucci et al., 1998). However, little is known for the role of tPA in glioma invasion. Salmaggi et al. report

that tPA is expressed in glioma specimens and mainly localized in tumor cells as well as in the ECM. The expression level of tPA in glioma is significantly higher than in patients' blood, which suggested that glioma cells are the major source of tPA (Salmaggi et al., 2006).

Annexin A2

Annexin A2 (annexin II, p36, calpactin I, lipocortin II), an abundant 36 kDa protein, belongs to the annexin family which consists of approximately 20 proteins, which bind to acidic phospholipids in a Ca^{2+}-dependent manner (Kaetzel et al., 1990). At its C-terminus, there are four 70-amino-acid repeated sequences, consisting five alpha helices wound into a right-handed superhelix, which is responsible for the Ca^{2+}-dependent binding to cell membrane (Kang et al., 1999). The N-terminus of annexin A2 contains a high affinity binding site for S100A10 (p11), as well as tyrosine and serine residues that are substrates of protein kinase C (PKC) and v-Src phosphorylation (Kang et al., 1999). Usually annexin A2 exists either as a monomer in the cytoplasm, or as a component of a heterotetrameric complex with S100A10 localized underneath the cell membrane (Zobiack et al., 2001).

The functions of annexin A2 in physiological and pathological conditions have been widely investigated (Kim and Hajjar, 2002). Annexin A2 tetramer binds to actin filaments, which modulate membrane organization and trafficking events (Hayes et al., 2004; Kang et al., 1999). Moreover annexin A2 is one of the major substrates of v-Src, which regulates cell motility and transformation including actin dynamics, cytoskeleton

rearrangement and focal adhesion turnover (Boschek et al., 1981; Meijne et al., 1997). It has been reported that cell transformation is dependent on annexin A2 expression (Hayes and Moss, 2009). In addition, intracellular annexin A2 has been proposed to regulate endocytosis and exocytosis, as well as vesicle trafficking and fusion (Creutz, 1992; Jost et al., 1997; Mayran et al., 2003). Furthermore, intracellular annexin A2 can regulate cell proliferation and differentiation as a result of PKC and v-Src phosphorylation following insulin or PDGF receptor activation (Kim and Hajjar, 2002).

In addition to these interesting findings, extracellular annexin A2 has been found to play an important role in cell surface proteolysis. Extracellular/cell surface associated annexin A2 was first identified as a cell surface receptor for both tPA and plasminogen on endothelial cells, and functions to bring these proteins into close spatial proximity and also protects them from their inhibitors, α_2-antiplasmin and PA inhibitor type 1 (PAI-1). Such protection significantly increases the catalytic efficiency of tPA on plasminogen (Hajjar et al., 1994). It has been proposed that plasminogen binds to annexin A2 C-terminus via its kringle domains (Hajjar et al., 1994), and tPA binds to annexin A2 N-terminus via its fibronectin finger domain (Hajjar et al., 1998). In agreement with these findings, annexin A2 knockout (annA2KO) mice has been shown to have defects in neoangiogenesis as a result of deficient cell surface proteolysis (Ling et al., 2004). Finally, in human peripheral monocytes, annexin A2 heterotetramer was shown to be the surface receptor for the plasmin-induced signaling, including the release of tumor necrosis factor-α (TNF-α) (Laumonnier et al., 2006). In a similar pattern, annexin A2 also acts as a surface receptor for another protease, cathepsin B, regulating cell surface proteolysis (Mai et al., 2000; Roshy et al., 2003).

These emerging data suggest that annexin A2 is an interesting molecular step in regulating proteolysis on tumor cell surface, which is essential for tumor invasion, metastasis and angiogenesis.

Annexin A2 and tumors

Annexin A2 is overexpressed in many tumors including breast cancer, pancreatic cancer and gliomas (Bao et al., 2009; Diaz et al., 2004; Ohno et al., 2009; Reeves et al., 1992; Sharma et al., 2006), and regulates tumor progression in several aspects. First it is correlated to the increased plasmin generation on the tumor cell surface, which facilitates the invasiveness and metastatic potential of solid tumors (Diaz et al., 2004; Sharma et al., 2006). Second, annexin A2 also serves as a receptor for cathepsin B on the surface of cancer cells including breast cancer and gliomas, which contributes to cell surface proteolysis (Mai et al., 2000; Roshy et al., 2003). Finally, the interaction of annexin A2 with tenascin C, an ECM protein, has also been suggested to contribute to the migration of cancer cells (Chung et al., 1996).

Annexin A2 has been shown to trigger angiogenesis *in vivo* (Ling et al., 2004) and its overexpression and subsequent overproduction of plasmin in cancer correlated to the formation of neovasculature (Sharma et al., 2010). It has been well established that plasmin is a key component regulating angiogenesis through degradation of ECM proteins as well as activation of angiogenic growth factors and cytokines (Lijnen, 2001; Pepper, 2001).

Several studies have shown that annexin A2 can induce cancer cell proliferation *in vitro* (Bao et al., 2009; Huang et al., 2008; Zhang et al., 2009). Moreover, it was reported that annexin A2 is involved in cell cycle regulation with peak expression during G1 – S and S – G2 phases (Chiang et al., 1993), and its downregulation was observed after p53 overexpression in lung cancer cells, which induced cancer apoptosis (Huang et al., 2008).

Taking these data together, annexin A2 promotes cancer progression through modulation of ECM remodeling, enhancement of angiogenesis and tumor proliferation. A recent proteomic study showing the localization of annexin A2 in the pseudopodia of invasive glioma cells (Beckner et al., 2005) and the decreased migration ability of human glioma cells by annexin A2 knockdown (Tatenhorst et al., 2006) suggest that this protein is an promising candidate for targeted therapy for glioma patients.

Microglia

Microglia are abundant in CNS and comprise 5% to 20% of the total glial cell population (Lawson et al., 1990). It has been established that microglia derive from a monocytic origin. Microglia precursors from mesodermal hematopoietic cells enter the developing brain during perinatal stages and differentiate there into mature microglia (Ling and Wong, 1993). Mature microglia express some macrophage-specific markers including Toll-like receptors (TLR), CD11b and F4/80, while the expression level of leukocyte common antigen, CD45, is lower than that of macrophages (Akiyama and McGeer, 1990; Becher and Antel, 1996; Bsibsi et al., 2002; Dick et al., 1995).

CNS is an immune-priviledged organ without any lymphatic system and is shielded from the peripheral circulatory system by the blood-brain barrier (BBB). Therefore it is isolated from most of the peripheral immune cells, soluble factors and plasma proteins under normal conditions (Kreutzberg, 1996). However, under pathological conditions, the T lymphocytes and antigen presenting cells (APC) infiltrate into CNS and regulate immunosurveillance coordinating with systemic immune response (Kreutzberg, 1996). As brain resident macrophages, microglia play an important role in patrolling the active immune response against CNS diseases (Streit et al., 1999).

Under normal physiological condition, microglia assume a resting status with a ramified morphology characterized by small cell bodies with multiple long and thin branched processes (Kreutzberg, 1996). They express low level of major histocompatibility complex (MHC) class II proteins and co-stimulatory molecules, which suppress their ability to present antigen to T cells (Carson et al., 1998). They monitor their microenvironment through pinocytosis and interaction with neurons (Nimmerjahn et al., 2005), and remain quiescent until stimuli from injury, infection, neurodegenerative process or tumors activate them, such as lipopolysaccharide (LPS), beta-amyloid (Aβ), thrombin, IFN-γ, and other pro-inflammatory cytokines (Dheen et al., 2007; Kreutzberg, 1996).

Upon activation, microglia undergo a series of stereotypic changes in morphology, gene expression, phagocytosis, proliferation and migration (Kreutzberg, 1996; Streit et al., 1999). Morphologically, microglia retract their processes and their cell bodies become enlarged with numerous phagocytic vesicles. They turn into an ameboid

like shape when fully activated (Kreutzberg, 1996). The expression level of opsonic receptors, such as complement receptors (CR1, CR3, CR4) and Fc gamma receptors (I, II, III), are increased after activation. These receptors bind to complement components and immunoglobulin fragments respectively and in turn enhance microglial phagocytic activity (Barnum, 1999; Peress et al., 1993). The phagocytosed material is degraded by the immediate induction of reactive radicals (Kreutzberg, 1996). After activation microglia increase the expression of cell adhesion molecules, MHC class II and co-stimulatory factors for antigen presentation to T cells. Costimulatory molecules CD80 and CD86 on microglia bind to CD28 on T cells, and induce T cells to secret cytokines and proliferate (Slavik et al., 1999). Subsequently CD40L on T cells binds to CD40 on microglia, which increases the expression of CD80, CD86, and MHC class II protein as well as increases the release of nitric synthase by microglia (van Kooten, 2000). Meanwhile microglia secrete complement, glutamate analogs, cytokines, growth factors, reactive oxygen species and chemokines, which modulate the immune reaction and recruit other microglia from distant brain regions and inflammatory cells to the site of injury including neutrophils, lymphocytes and monocytes (Hanisch, 2002).

Since microglia are closely related to peripheral macrophages in regard to their origins, phagocytic activity, and surface markers, they play a crucial role in the innate and adaptive immune reactions in response to gliomas (Frei et al., 1994; Graeber et al., 2002; Hussain et al., 2006).

Microglia/Macrophages (MG/MP) and tumors

The tumor microenvironment contains excessively proliferating tumor cells and tumor stromal cells, including resident fibroblasts, adipocytes, blood and lymph vessels as well as infiltrating immune cells (Condeelis and Pollard, 2006). Among the infiltrating inflammatory cells, tumor-associated macrophages have been shown to have dual roles, both tumor-rejecting and tumor-promoting (Allavena et al., 2008; Solinas et al., 2009). Macrophages can secrete anti-tumor cytokines and interact with T cells to destroy tumor cells (Galani et al., 2009). On the other hand they can be recruited by tumor-released chemokines and switch their immune response to a tumor-supporting function by the influence of the tumor microenvironment, characterized by regulating matrix remodeling and angiogenesis, and enhancing tumor invasion and intravasation (Demuth and Berens, 2004; Pollard, 2004). Many studies have shown that high density of tumor-associated macrophages correlates with poor prognosis (Bingle et al., 2002).

Clinical studies have shown that local macrophage density correlates with the level of angiogenesis by quantifying micro-vessel density, indicating a role of macrophages in this process (Leek and Harris, 2002). When macrophages are activated by stimuli released from tumor cells such as growth factors and cytokines, macrophages can be induced to exhibit angiogenic capacity (Tanaka et al., 2002; Ueno et al., 2000; White et al., 2001). It has been reported that tumor cells can release acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (GM-CSF), insulin-like growth factor-1, PDGF, tumor growth factor (TGF)-β, TNF-α, interleukin(IL)-1, IL-6, IL-8, prostaglandins, interferons (IFN) and thrombospondin, which are able to activate macrophages and influence angiogenesis (Siveen and Kuttan, 2009). Moreover tumor-

associated macrophages can release proteases including MMPs and plasmin, which mediate ECM proteolysis and in turn regulate new blood vessel formation and tumor invasion (Siveen and Kuttan, 2009). Tumor-associated macrophages have been shown to be a major source of MMP-9 (gelatinase B) and MT1-MMP, which promote ECM degradation (Giraudo et al., 2004; Markovic et al., 2009). In addition, uPA is overexpressed by tumor-associated macrophages in various human cancers, which is results to increased level of plasmin (Hildenbrand et al., 1999). Thus tumor-associated macrophages can affect ECM degradation, and endothelial cell proliferation and migration, which are essential steps of angiogenesis.

It has been shown that tumor metastasis is inhibited in experimental tumor models with macrophage depletion (Lin et al., 2002). Possible mechanism is the paracrine loop of colony stimulating factor-1 (CSF-1) and EGF between tumor cells and tumor associated macrophages. Cancer cells secret CSF-1, which attract macrophages and induces expression of EGF in macrophages. EGF secreted by macrophages associated with blood vessels induces chemotaxis of cancer cells, which contributes to cancer metastasis and intravasation (Condeelis and Pollard, 2006; Wyckoff et al., 2004).

One characteristic of gliomas is that the tumors are infiltrated by large numbers of activated and proliferating MG/MP, and the extent of MG/MP infiltration correlates positively with the degree of malignancy (Morimura et al., 1990; Morris and Esiri, 1991; Roggendorf et al., 1996). Glioma cells release chemokines and growth factors such as CSF-1, GM-CSF, monocyte chemoattractant protein-1 (MCP-1), and hepatocyte growth factor/scatter factor (HGF/SF), which recruit and promote the growth of tumor-

infiltrating MG/MP (Alterman and Stanley, 1994; Badie et al., 1999; Kielian et al., 2002; Leung et al., 1997; Nitta et al., 1992). In addition, glioma cells release Th2/Th3 cytokines, such as IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M (OSM), and TGF-β, which are able to inhibit cytotoxic T-cell activation and MG/MP activation (Constam et al., 1992; Goswami et al., 1998; Halfter et al., 1998; Hao et al., 2002; Murphy et al., 1995). The studies on the innate immune response of MG/MP isolated from glioma tissue demonstrated that they expressed TLRs and mediated phagocytosis, but lacked production of IL-1β and TNF-α, which are important cytokines for tumor rejection (Frei et al., 1994; Hussain et al., 2006). Moreover it has been shown that the antigen-presenting function of microglia is compromised in gliomas, since the MHC class II protein and co-stimulatory factors exhibited decreased expression levels (Badie et al., 2002; Flugel et al., 1999). In addition MG/MP have been proposed to promote glioma growth by secreting growth factors, such as TGF- β , immune-suppressive cytokines (IL-10) and angiogenic factors (VEGF) (Alterman and Stanley, 1994; Demuth et al., 2007; Galasso et al., 2000; Lafuente et al., 1999; Wagner et al., 1999; Wesolowska et al., 2008).

Therefore tumor-associated MG/MP regulate the tumor microenvironment, which in turn modulates the tumor progression. Studying the interactions between MG/MP and tumor cells will provide insight into the supporting role of microenvironment to tumor invasion and metastasis, which may provide new targets for designing anticancer therapies.

In this study, we focused on the modulation of microenvironment by glioma cells. In the first part, we investigated how the expression of annexin A2 affected glioma progression, which suggested the favorable modulation of tumor-released protease activity for glioma patients. In the second part, we investigated the interactions between glioma cells and microglia/macrophages (MG/MP), as well as the consequences of modulating MG/MP activity in the glioma-harboring microenvironment, which suggested that manipulation of the activation state of MG/MP appears to be a potentially promising novel interventional approach for the treatment of gliomas.

Chapter 2

Overexpression of Annexin A2 by Glioma Cells Promotes Tumor Progression

Introduction

Malignant gliomas are primary CNS tumors arising from glial cells and are one of the deadliest cancers - median survival time is only one year even with aggressive surgical resection combined with irradiation and chemotherapy. Although many therapeutic approaches have been explored, there has been no major improvement in survival over the last 30 years (DeAngelis, 2001; Legler et al., 1999). Gliomas are highly invasive, and the microscopic infiltrative loci outside of tumor mass contribute to most of the tumor relapse following surgery (Tsatas and Kaye, 2003). It is well documented that glioma-released proteases, like plasminogen activator (PA), matrix metalloproteinases (MMPs) and lysosomal cathepsins, play critical roles in glioma invasion (Levicar et al., 2003a; Rao, 2003).

The role of PA/plasmin system in tumor growth, invasion and angiogenesis has been widely studied (McMahon and Kwaan, 2008). Two distinct types of PA have been identified: uPA and tPA, which are responsible for the conversion of the enzymatically inactive zymogen, plasminogen, into the active serine protease, plasmin (Collen, 1999).

Plasmin is a potent protease capable of the direct breakdown of many ECM components, including fibronectin, laminin and proteoglycans. It facilitates tumor invasion and angiogenesis (Dano et al., 1985; DeClerck et al., 1997; Mignatti and Rifkin, 1993). Plasmin can also activate MMPs and growth factors, which contribute to ECM degradation and tumor expansion (Carmeliet et al., 1997; McColl et al., 2003).

Extracellular/cell surface associated annexin A2 was first identified as a cell surface receptor for both tPA and plasminogen on endothelial cells, and functions to bring them into close spatial proximity, which increases the catalytic efficiency of tPA on plasminogen and also protects them from their inhibitors (Hajjar et al., 1994). Annexin A2 is overexpressed in many tumors including breast cancer, pancreatic cancer and gliomas (Bao et al., 2009; Diaz et al., 2004; Ohno et al., 2009; Reeves et al., 1992; Sharma et al., 2006). It subsequently emerges as an attractive potential receptor for the increased plasmin generation on the tumor cell surface (Diaz et al., 2004; Sharma et al., 2006). However, most of these studies have been carried out in cell culture system; little is known about the subsequent effects of annexin A2 knockdown (annA2KD) on tumor development *in vivo*.

A recent proteomic study showing the localization of annexin A2 in the pseudopodia of invasive glioma cells (Beckner et al., 2005) and the decreased migration ability of human glioma cells by annexin A2 knockdown (Tatenhorst et al., 2006) make this protein a candidate for targeted therapy for glioma patients. In this study, we used a mouse glioma cell line, GL261-EGFP, and annA2KD clones, GL261-EGFP-annA2KD to observe the effects of annexin A2 on glioma migration, proliferation, apoptosis, invasion

and angiogenesis *in vivo*. We showed that decrease of annexin A2 expression in glioma cells slowed down glioma progression with targeted inhibition of tumor invasion and angiogenesis, suggesting a potential glioma therapy target by modulating tumor-released protease activity.

Materials and methods

Cell Lines

GL261, a glioma cell line derived from C57BL/6 mice, was obtained from ATCC. The cell line was cultured in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS) and 1mM sodium pyruvate (Fisher Mediatech). The cell line was transfected with pEGFP-N1 using Lipofectamine (Invitrogen) and then selected with 500μg/ml neomycin (Geneticin, Invitrogen) to generate the stable cell lines GL261-EGFP.

The pSuper/blast-annA2i vector (made by Dr. Iordanis Gravanis), expressing shRNAs under the control of the RNA polymerase III H1 promoter, was used for annexin A2 knockdown. Sequences 148-166 and 692-710 of the mouse annexin A2 mRNA open reading frame were targeted for RNAi-mediated knockdown. GL261-EGFP cells were transfected with pSuper/blast-annA2i and then subjected to 10 μg/ml blasticidin (Invivogen) and 500μg/ml neomycin selection to generate the stable cell lines GL261-EGFP-annA2KD with decreased level of annexin A2 as confirmed by western blot.

N9, a mouse microglia cell line, is a kind gift from Dr. Steven Barger (University of Arkansas) and Dr. Paola Ricciardi-Castagnoli (University of Milan, Italy) (Righi et al., 1989). CRL-2541, a mouse astrocyte cell line, was obtained from ATCC. Both cell lines were cultured in DMEM medium with 10% heat-inactivated FBS and 1mM sodium pyruvate.

Western blotting

Different cell lines or clones were seeded into 6-well plates at equal numbers in DMEM+10% FBS. After the cells reached 80% confluence, cells were lysed in RIPA buffer with proteases inhibitor cocktail (Sigma) and protein concentrations were measured by the Bio-Rad Bradford detergent-compatible (DC) assay. 10% SDS-PAGE was used to separate protein samples (10μg), which were transferred to polyvinylidene difluoride (PVDF) membrane, probed with mouse anti-mouse annexin A2 antibody (1:5000, BD Transduction Laboratories) or rabbit anti-mouse plasminogen antibody (1:500) and mouse anti-mouse α-tubulin antibody (1:2000, Millipore) overnight at 4°C. Goat anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:5000. HRP activity was detected with LumiGLO Chemiluminescent Substrate System. Expression levels were quantified using SCION Image software, and annexin A2 expression level was normalized to α-tubulin level.

tPA zymography

To prepare protein samples for detection of tPA activity, GL261 cells were lysed in RIPA buffer without protease inhibitors. GL261-conditioned medium was collected from a confluent GL261 plate and subjected to centrifugation to remove cells or debris. Then fresh samples were mixed with non-reducing SDS-gel loading buffer (no β-mercaptoethanol and no boiling) and loaded onto 10% SDS polyacrylamide gels containing 13 μg/ml plasminogen, as substrate for tPA, and 3 mg/ml α-casein, as

substrate for plasmin. Following electrophoresis, the gels were washed with 2.5% Triton-X 100 for 45 minutes to remove SDS and then incubated in 0.1 M Tris Buffer (pH 8.0) overnight at room temperature to allow for proteolysis of α-casein in the gels. To visualize tPA activity in the samples, the gels were stained with 0.1% Coomassie Brilliant Blue-G250 and then destained with 40% methanol and 10% acetic acid mixture. The final gels had a uniform background, except in regions where tPA cleaved its substrate (Nolin et al., 2008).

Cell proliferation assay

In vitro cell proliferation was measure with cell proliferation ELISA kit (Roche applied science) following manufacture's protocol. Briefly, 5000 cells of each clone were seeded into 96-well plates and cultured in DMEM+10% FBS for 24 hours. BrdU (1:1000) was added to culture medium for 2, 4 and 6 hours. Then the cells were fixed with FixDenat. After removing FixDenat, the anti-BrdU-POD antibody was added and the incorporated BrdU was detected by the subsequent substrate reaction. The reaction product was quantified by reading the absorbance at 370 nm. The experiment was repeated for 3 times.

Membrane-bound tPA activity

First membrane proteins from the GL261-EGFP and GL261-EGFP-annA2KD cells were extracted (Diaz et al., 2004). Briefly, confluent cells were lysed in buffer I (20mM Tris HCl, pH 6.8, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 1% Triton X-

100) without protease inhibitors. After centrifugation (13000xg) for 10 minutes, the supernatants containing cytoplasmic proteins were transferred to new tubes, and the pellets containing membrane proteins were dissolved in buffer II (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 10mM EGTA). Then the protein concentrations were measured by the Bio-Rad Bradford DC assay.

To measure tPA activity, amidolytic assay was used following the established protocol (Nolin et al., 2008). 25 µl protein samples (duplicate) were added to the reaction mixture (final volume 200 µl) containing 0.15 µM plasminogen, 0.3mM S-2251 (a chromogenic substrate of plasmin) and 1mM amiloride (uPA inhibitor). The rate of plasmin formation is measured at 405 nm after incubation in dark for 2 hours at room temperature and this photometric signal is directly proportional to plasmin activity. Different amount of recombinant tPA (Genentech) were used to generate standard curve and the tPA activity in each sample was calculated based on the standard curve and normalized to total protein amount in each sample. The experiments were repeated 3 times.

Scratch-migration assay

This assay was used to evaluate the migratory activity of glioma cells (Wesolowska et al., 2008). GL261-EGFP and GL261-EGFP-annA2KD cells were seeded in triplicate on 60-mm culture dishes at $1.5x10^6$ cells per dish. 48 hours after plating, the cells reached 80% confluence. A vertical scratch and four horizontal scratches through the plate were gently made using a pipette tip. After washing with PBS, the cells were

incubated in DMEM+10% FBS for additional 24 hours. Migrated cells were defined as cells that moved into the scratch and detached away from the cell monolayer. The horizontal scratches were used as guidance for localization of the same spot at 0 and 24 hours after the scratching. For quantification of cell migration, total number of migrated cells at 3 random fields was counted between scratch edges in each plate. For the representative images in figure 2-4, the cells were fixed in 4% paraformaldehyde (PFA) in PBS and mounted with Fluoromount-G/DAPI (Southern Biotech). The experiments were repeated 3 times.

In vivo mouse glioma model

12–16 week old C57BL/6 (wt) mice were used, which were purchased from Jackson Laboratory. Annexin A2 knockout (annA2KO) mice were kindly provided by Dr. Hajjar from Weil Cornell Medical College. All animal procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC). Mice were bred in-house under maximum isolation conditions on a 12:12 hour light: dark cycle with food *ad libitum*. Mice were anesthetized with atropine (0.6mg/kg, i.p.) and 2.5% Avertin (0.02mg/g, i.p.), and then a midline incision was made on the scalp. At stereotaxic coordinates of bregma, -1mm anteroposterior and +2mm mediolateral, a small burr hole was drilled on the skull. A 2.5 µl Hamilton syringe with a 33-gauge needle was used for tumor injection. $3x10^4$ GL261-EGFP or GL261-EGFP-annA2KD cells were delivered in 1 µl PBS at a depth of 3 mm over 2 minutes (El Andaloussi et al., 2006). After injection, the needle was left for additional 3 minutes to prevent fluid reflux and then withdrawn (Figure 2-5A).

Tumor evaluation

For each time point (day 10, 15 and 20 after tumor injection, figure 2-5B), mice were deeply anesthetized with 2.5% Avertin and transcardially perfused with 50 ml PBS followed by 50ml 4% PFA/PBS. Brains were removed and post-fixed in 4% PFA/PBS overnight at 4°C, followed by 30% sucrose at 4ºC until fully dehydrated. Brains were then frozen-embedded in optimal cutting temperature compound (Tissue-Tek) and cut using a Leica (Nussloch, Germany) cryostat. Serial coronal sections encompassing the entire tumor were generated for each animal. For samples at day 10, 10 series of 20 μm thickness section were generated, whereas 15 and 20 series of 25 μm thickness section were generated for samples at day 15 and day 20 respectively.

To measure tumor size, randomly picked serial sections from each animal were used, and the GFP-positive tumor area was measured using NIS-Elements software. The tumor sizes were calculated as tumor area x section thickness x number of series (Figure 2-5D).

To quantify tumor invasion, 5 randomly picked samples from each group were used and 5 sections of each sample were captured under fluorescent microscope. Tumor invasion was defined as the areas where isolated GFP+ glioma cells mixed with surrounding normal brain tissue or where the gliomas showed pseudopodia-like structures. Two parameters were used to evaluate tumor invasion: maximal invasion distance and percentage of invasion (invasive area / total tumor area x 100) (Guillamo et al., 2009).

To evaluate tumor angiogenesis, 5 randomly picked samples and 5 sections per sample from each group were used. The samples were immunofluorescently labeled with tomato lectin (Sigma, endothelial cells and microglia/macrophage marker) and CD45 antibody (Pharmingen, macrophage marker), and blood vessels were identified based on lectin+CD45- staining and morphology. The samples were first screened at lower magnification (10x) to find the angiogenesis "hot spot" with intense blood vessels, and then blood vessel number and diameter from at least 20 fields were quantified with NIS-Elements software under higher magnification (20x) (Campa et al., 2008; Pistolesi et al., 2004).

The brain sections were also immunostained with Ki67 antibody (Abcam), phospho-histone H3 (p-histone H3) antibody (Cell Signaling) for proliferation, and activated caspase-3 antibody (Sigma) for apoptosis. Apoptosis was also confirmed by TUNEL assay with ApopTag® red in situ apoptosis detection kit (Millipore) following manufacture's protocol. For quantification, 5 randomly selected samples per group were used and at least 15 fields in 5 different sections of the same sample were captured by confocal microscopy.

Immunofluoresent staining

For GL261 cell membrane staining, GL261 cells were seeded onto coverslips and cultured for 24 hours. After the cells attached to the coverslips, cells were fixed with 4% PFA/PBS for 15 minutes at room temperature. The cells were blocked in 5% goat serum (Sigma) in PBS for 1 hour at room temperature, and then incubated with mouse anti-

mouse annexin A2 antibody (1:500) and rabbit anti-mouse tPA antibody (Santa Cruz, 1:100) in PBS overnight at 4°C. The samples were incubated with goat anti-mouse Alexa Fluor-555 and anti-rabbit Alexa Fluor-488 secondary antibodies (1:1000, Invitrogen) in PBS for 1 hour at room temperature, and mounted with Fluoromount-G/DAPI. Single plane images were taken under confocal microscope.

For tissue staining, brain sections were blocked in 5% goat serum in PBS-T (PBS/ 0.3% Triton X-100) for 1 hour at room temperature, and then incubated with rabbit anti-mouse phosphor-histone H3 antibody (1:500), rabbit anti-mouse Ki67 antibody (1:500), or rabbit anti-mouse activated caspase-3 antibody (1:500) in PBS-T overnight at 4°C. The samples were incubated with corresponding secondary antibodies (Alexa Fluor-555, Invitrogen) for 1 hour at room temperature, and mounted with Fluoromount-G (Southern Biotech). For blood vessel staining, the samples were first labeled with biotinylated tomato lectin (1:500) and step-avidin Alexa Fluor-555 antibody (1:1000, Invitrogen), and then labeled with rat anti-CD45 (1:500) and goat anti-rat Alexa Fluor-647 antibody (1:1000, Invitrogen).

TUNEL assay

TUNEL assay was performed with ApopTag® in situ apoptosis detection kit (Millipore) following manufacture's protocol. Brain cryosections were post-fixed in ice-cold ethanol:acetic acid 2:1 for 5 minutes at -20°C. After 2 washes with PBS, the sections were incubated with equilibration buffer for 1 minute, followed by incubation with TdT enzyme at 37°C for 1 hour. Then the samples were washed 3 times with PBS and

incubated with anti-digoxigenin-rhodamine (1:200, Roche) at room temperature for 30 minutes to visualize TUNEL+ cells.

Statistical analysis

The statistical significance between two groups was determined by Student's *t*-test. For comparison of more than two groups, one-way ANOVA followed by a Bonferroni-Dunn test was used. The Kaplan-Meier survival curve was calculated with MedCalc software. Data were expressed as mean ± standard error of the mean (SEM). The statistical significance is either described in figure legends, or indicated as asterisks (*). *:P <0.05; **: P<0.01; ***:P< 0.001.

Results

Overexpression of annexin A2 in glioma cells

In this study, we used GL261, a mouse astrocytoma cell line, to investigate glioma progression. Western blot of GL261 (glioma), N9 (microglia) and CRL-2541 (control astrocytic line) protein samples (Figure 2-1A) showed that GL261 expressed elevated levels of annexin A2 when compared to the microglia cell line, whereas the astrocyte cell line lacked the expression of annexin A2, which suggested that annexin A2 could be a candidate for markers to distinguish malignant astrocytic cells from normal astrocytes. In addition, plasminogen was expressed by glioma cells but not by microglia or astrocytes. Previous studies had reported that tPA was expressed by microglia and neurons but not astrocytes in CNS under normal condition (Gravanis and Tsirka, 2005), while the zymography of GL261 cell lysate and culture medium (Figure 2-1B) showed GL261 was able to express and secret tPA. Taking these data together, GL261 has a complete tPA/plasminogen/annexin A2 proteolytic system, which was totally different from astrocytes. Furthermore, immunofluorescent staining of GL261 cell surface revealed co-localization of tPA and annexin A2 mostly in pseudopodia-like structures (Figure 2-1C).

Establishment of annexin A2 knockdown (annA2KD) clones of glioma cells

To better visualize glioma cells, we generated the stable cell line GL261-EGFP expressing GFP, and then knocked down annexin A2 expression with a RNAi construct. After selection, 3 clones were kept for subsequent experiments. As shown in figure 2-2A,

annexin A2 expression level was confirmed by western blot and normalized to α-tubulin expression. Quantification (Figure 2-2B) showed that GL261-EGFP-annA2KD clones 2, 3 and 4 had decreased levels of annexin A2 to 20.0%, 19.2% and 9.9% of wild type (wt) GL261-EGFP respectively. Since annexin A2 was reported to promote cancer cell proliferation (Bao et al., 2009; Huang et al., 2008; Zhang et al., 2009), the proliferation rate of wt and annA2KD clones was measured using BrdU incorporation assay, which showed that they had similar rates in culture (Figure 2-2C).

Knockdown of annexin A2 in glioma cells decreased membrane-bound tPA activity

Since annexin A2 is a surface receptor for tPA and plasminogen in endothelial and cancer cells (Diaz et al., 2004; Hajjar et al., 1994; Sharma et al., 2006), we next examined whether knockdown of annexin A2 would affect the tPA/plasminogen system on the glioma cell surface. Western blot of wt and annA2KD GL261 membrane proteins (Figure 2-3A) showed that annA2KD clones exhibited decreased expression of annexin A2 on the cell surface (Figure 2-3B). Using the quantitative amidolytic assay we found that in glioma cells the membrane fraction had significantly higher tPA activity than the cytoplasmic fraction (Figure 2-3C, first group). The decreased expression levels of annexin A2 also yielded decreased tPA activity (52% to 58.5% less than wt cells) on glioma cell surface, while tPA activity in the cytoplasmic fraction remained unchanged (Figure 2-3C); this result suggested that the annexin A2 knockdown clones had less tPA activity and subsequently less plasmin generation on their surface and this may result in lower potential to degrade ECM.

Knockdown of annexin A2 decreased glioma migration in culture

It has been reported that decreased annexin A2 expression leads to reduced migration of human glioma cells U87MG and U373MG *in vitro* (Tatenhorst et al., 2006). Consistent with these data, we performed scratch-migration assay and found the annexin A2 knockdown clones had significantly decreased migration activity (Figure 2-4A). The migrated cells of GL261-EGFP-annA2KD clone 2, 3 and 4 were 82.0%, 57.2% and 92.3% less than wt GL261-EGFP, respectively (Figure 2-4B). Since BrdU incorporation showed that these clones had a similar proliferation rate as wt cells (Figure 2-2C), these results suggested that the decrease of cell number between scratch edges in annA2KD group was mainly due to decreased migration ability, not proliferation. As these 3 annA2KD clones behaved similarly in culture, we used GL261-EGFP-annA2KD clone 4 (lowest annexin A2 expression) for the subsequent *in vivo* experiments.

Establishment of a mouse glioma model

Next we examined the effects of annA2KD on glioma growth *in vivo*. First, we examined whether the annA2KD was stable without blasticidin selection. GL261-EGFP-annA2KD clone 4 was cultured in regular medium without blasticidin, and cells were harvested after 10, 15 and 20 days. Western blot analysis (Figure 2-5C) showed that the annexin A2 levels slightly increased at day 10 when compared with the level under blasticidin selection, but remained unchanged at day 15 and 20. Still the annA2KD clone had overall lower levels of annexin A2 when compared with wt GL261-EGFP,

suggesting that annA2KD GL261 clone would have stably decreased levels of annexin A2 *in vivo*.

For the tumor xenograft, $3x10^4$ GL261-EGFP or GL261-EGFP-annA2KD cells were injected into the striatum of mouse brain (Figure 2-5A). 10, 15 and 20 days after tumor implantation, tumor-bearing mice were euthanized and mouse brains were subjected to histological evaluation (Figure 2-5B). As shown in figure 2-5D, a representative H&E staining of glioma sample at day 15 revealed its morphology. Since GL261 stably expressed green fluorescence, the glioma samples were observed under a fluorescent microscope. The GFP signals from the same sample (Figure 2-5E) showed that the green fluorescence corresponded very well with H&E morphology. The tumor volume was measured both after H&E histological staining and by determining GFP+ areas, which yielded 10.3 mm^3 and 11.1 mm^3 respectively. The difference between these two tumor visualization methods could be due to the excessive dehydration process in H&E staining, which led to mild tissue shrinkage. Thus, we used GFP signal to measure tumor volume in subsequent experiments.

Knockdown of annexin A2 on glioma cells slowed down glioma progression *in vivo*

Equal number of GL261-EGFP and GL261-EGFP-annA2KD clone 4 cells were injected into mouse brains, and tumor sizes were measured at day 10, 15 and 20 (Figure 2-6A). We found that the glioma sizes in annA2KD group were significantly smaller than in the wt group, especially at day 20, where the average tumor size of wt and annA2KD group was 55.62 and 25.30 mm^3 respectively. The decreased level of annexin

A2 in glioma cells led to 54.5% reduction in tumor size at day 20. We also injected wt and annA2KD clone into annA2KO mice but found the tumor sizes were similar as those in wt mice (Figure 2-12A), which suggested that absence of annexin A2 in the tumor stroma had no effect on glioma growth.

The glioma progression of tumor-bearing mice was also followed by measuring body weight loss and animal survival time. We found that at day 20 the mouse weight loss in the wt group was slightly more than that in the annA2KD group (Figure 2-6B). At day 20, the mice in wt group usually weighed around 20 grams and were very sick, which began to die around day 21 after tumor injection. A Kaplan-Meier survival curve (Figure 2-6C) of these two group revealed that annA2KD group survived significantly longer than wt group (median survival time: wt group = 24 days; annA2KD group = 27.5 days), which suggested that the expression level of annexin A2 in gliomas was related to the increasing glioma malignancy and poor prognosis, and was consistent with previous immunohistochemistry data in human glioma specimen (Reeves et al., 1992; Roseman et al., 1994).

Knockdown of annexin A2 decreased proliferation index in glioma tissue

Since the annA2KD group showed smaller tumors, we further investigated the possible mechanisms contributing to this result. Annexin A2 has been shown to stimulate cancer cell proliferation *in vitro* (Bao et al., 2009; Huang et al., 2008; Zhang et al., 2009), so we first evaluated the proliferation index in glioma samples by immunofluorescent

staining with Ki67 and p-histone H3 antibodies. We quantified the number of proliferating cells in glioma tissue as well as GFP+ glioma cells.

Ki67 is expressed by cells undergoing active cell cycle (Figure 2-7A, 2-8A) (Allegranza et al., 1991), and we found that there were more Ki67+ cells in glioma tissue at day 10 than those at day 20, indicating that tumor cell proliferation is the major mechanism for tumor expansion at an early stage, whereas other mechanisms play major roles at a later stage, such as angiogenesis, necrosis etc (Figure 2-8B). Furthermore, we found that there were more Ki67+ cells in wt tumors than in annA2KD tumors, especially at day 10 and 20 (Figure 2-8B), however, the number of Ki67+GFP+ cells was similar in both groups (Figure 2-8C), indicating the *in vivo* proliferation rate of glioma cells was similar in both groups. These data were further confirmed with p-histone H3 staining, which labeled all the mitotic cells (Figure 2-7B, 2-8D) (Colman et al., 2006). Similarly to Ki67 quantification, there were more p-histone H3+ cells in wt group at day 10 and 15 (Figure 2-8E), while p-histone H3+GFP+ cell number is similar as annA2KD group (Figure 2-8F). Taken together, these data suggested that decreased annexin A2 level in glioma cells led to decreased proliferation in stromal cells but not glioma cells themselves, a result consistent with the *in vitro* BrdU incorporation assay (Figure 2-2C). These data indicated that the overexpression of annexin A2 on glioma cells may regulate the development of tumor microenvironment.

Knockdown of annexin A2 increased glioma apoptosis

Then we evaluated the apoptosis in glioma samples by immunofluorescent staining of activated caspase-3 antibody and TUNEL assay. Caspase-3 is activated in apoptotic cells by caspase-8 and caspase-9 from extrinsic and intrinsic pathways (Figure 2-7C) (Salvesen, 2002). Staining of activated caspase-3 revealed that the apoptotic cells increased over time (Figure 2-9A, B), possibly due to the rapid tumor expansion and subsequent hypoxia at later stage. There were more apoptotic cells in total tumor tissue and GFP+ tumor cells in annA2KD group than those in wt group at all time points (Figure 2-9B, C). These findings were further confirmed by TUNEL assay, which identified sites of DNA fragmentation in apoptotic cells (Figure 2-7D) (Gavrieli et al., 1992). Similarly, the quantification of TUNEL+ cells (all apoptotic cells) and TUNEL+GFP+ cells (apoptotic glioma cells) showed that glioma samples in annA2KD group exhibited increased apoptosis both in tumor tissue and tumor cells themselves when compared to apoptosis levels in the wt group (Figure 2-9 D-F), which indicated that annexin A2 possibly regulated glioma tumor siz through positive support of the tumor microenvironment and subsequent inhibition of apoptosis.

Knockdown of annexin A2 decreased glioma invasion

The PA/plasminogen system plays an important role in tumor invasion, which is a key step for tumor growth (Diaz et al., 2004; Goh et al., 2005; Rella et al., 1993; Stack et al., 1999). Since annA2KD clones had decreased activity of membrane-bound tPA (Figure 2-3C), we further investigated whether annexin A2 could affect glioma invasion *in vivo*. Two parameters were used to evaluate tumor invasion: percentage of invasive area and maximal invasion distance. Based on the morphology of GFP+ tumor area, the

invasive areas were outlined and normalized to the total tumor area (Figure 2-10A). The quantification data showed that at days 10 and 15 the tumors in the wt group had significantly higher percentage of invasive areas than the ones in annA2KD group (Figure 2-10B). Although the difference between these two groups became smaller at day 20 (because of the huge difference in total tumor area - the tumors in wt group were twice the size of those in the annA2KD group), we found that wt glioma cells invaded much farther away from tumor mass than the annA2KD glioma cells (Figure 2-10C). These results suggested that annexin A2 promoted glioma invasion, possibly through PA/plasminogen system, which also contributed to tumor expansion.

Knockdown of annexin A2 decreased glioma angiogenesis

The PA/plasminogen system is known to promote neoangiogenesis, which is a critical step in cancer development (Stephens et al., 1998). In breast cancer, it was shown that annexin A2 is expressed on the surface of invasive cancer cells and correlates to cancer neovascularization (Sharma et al., 2010). Therefore we further investigated whether annexin A2 also regulates angiogenesis in gliomas, which was evaluated by measuring micro-vessel density (MVD) and blood vessel diameters. In normal brain striatum where the tumor cells were injected, there were numerous small blood vessels (Figure 2-11B, day 0) with an average diameter below 15 µm. In glioma tissue, MVD was lower than that of normal brain but blood vessel diameters increased over time due to blood vessel dilation and remodeling caused by glioma cells (Figure 2-11B, C) (Winkler et al., 2009). When comparing MVD of wt and annA2KD group, we found that wt tumors had significantly higher number of micro-vessels than annA2KD ones (Figure 2-

11A, B). Moreover, when MVD was sorted based on blood vessel diameter (small, < 15 µm; intermediate, 15 – 50 µm; large, > 50 µm), we found that the difference of MVD between the wt and annA2KD groups shifted from small blood vessel to intermediate blood vessel (Figure 2-11C), which indicated that annexin A2 may be involved in glioma angiogenesis and affect the pattern of tumor vasculature. The MVD was also quantified in tumors from annA2KO mice but it was similar to that of wt mice (Figure 2-12B), which suggested that the regulation of angiogenesis by annexin A2 was tumor specific.

Discussion

Cancer invasion is a complex process that includes adhesion of tumor cells to ECM proteins, proteolysis of ECM proteins by tumor-secreted proteases and ECM remodeling (Zamecnik, 2005). Through this mechanism, tumor cells create intercellular spaces for migration, an event that requires membrane synthesis and cytoskeleton rearrangement (Giese et al., 2003). During this process, overproduction of plasmin by the tumor cells and subsequent activation of other proteases such as MMPs results in localized degradation of ECM allowing tumor cell migration and invasion (McMahon and Kwaan, 2008).

Gliomas are highly infiltrative tumors that invade into and damage the surrounding normal brain tissue (Tsatas and Kaye, 2003). The overexpression of uPA and uPA signaling pathway that promote glioma invasion has been well studied (Rao, 2003), however, little has been known for the role of annexin A2, a putative receptor for tPA/plasminogen system, in glioma invasion. Annexin A2 has been shown to be upregulated in many cancers and its overexpression was found to be correlated to cancer invasion through tPA/plasminogen system (Bao et al., 2009; Diaz et al., 2004; Sharma et al., 2010; Sharma et al., 2006). In this study, we showed that glioma cells expressed tPA and annexin A2, which co-localized in the invadopodia. Moreover, knockdown of annexin A2 resulted in decreased tPA activity on glioma plasma membrane. The decrease in PA activity in turn reduced the level of plasmin generation and proteolysis on tumor cell surface. In addition, annexin A2 serves as a receptor for cathepsin B on the surface of cancer cells, including breast cancer and gliomas, which also mediates cell surface

proteolysis (Mai et al., 2000; Roshy et al., 2003). As a result, when annA2KD glioma cells were injected into mouse brain, we found that the lower expression of annexin A2 on glioma cells decreased tumor size and inhibited invasive phenotype *in vivo*.

It has been well established that neoangiogenesis is essential to tumor development and plasmin is a key component regulating angiogenesis through degradation of ECM proteins and activation of angiogenic growth factors and cytokines (Lijnen, 2001; Pepper, 2001). Annexin A2 has been shown to trigger angiogenesis (Ling et al., 2004) and its overexpression in cancer correlated to the formation of neovasculature (Sharma et al., 2010). Recent research on development of anti-angiogenesis drugs for cancer also confirmed the essential role of annexin A2. For example TM601, a synthesized chlorotoxin tested in clinical trials, was shown to inhibit tumor migration, invasion and angiogenesis, whose molecular target was found to be annexin A2 in many cancers including glioma, melanoma, pancreatic, prostate and lung cancer (Kesavan et al., 2010; Soroceanu et al., 1999). In agreement with these data, we found that there was decreased angiogenesis in annA2KD gliomas, which was regulated by tumor-associated annexin A2, indicating that tumor-released proteases were essential for development of new blood vessels. Based on these results, we suggest that a potential novel glioma therapy could arise from delivering directly annexin A2 inhibitory nucleic acids such as siRNA or antisense oligonucleotides to inhibit tumor proteolysis and angiogenesis.

In addition to cell surface proteolysis, annexin A2 is also involved in cell transformation (Hayes et al., 2004). Annexin A2 is a Ca^{2+}-dependent membrane-binding

protein and exists either as a monomer, or as a component of a heterotetrameric complex with S100A10 (Zobiack et al., 2001). The annexin A2 heterotetramer was found to localize in the cytoplasmic side of the cell membrane, and binds to actin filaments, which modulate membrane organization and trafficking events (Hayes et al., 2004; Kang et al., 1999). Annexin A2 is one of the major substrates of v-Src, which regulates cell motility and transformation including actin dynamics, cytoskeleton rearrangement and focal adhesion turnover (Boschek et al., 1981; Meijne et al., 1997). It was reported that cell transformation was absent in cells lacking annexin A2 (Hayes and Moss, 2009), which could be one possible mechanism contributing to the decreased migration activity observed in annA2KD glioma cells.

Several studies have shown that knockdown of annexin A2 in cancer cells inhibited cell proliferation *in vitro* (Bao et al., 2009; Huang et al., 2008; Zhang et al., 2009). Contrary to these data, our annA2KD glioma cells showed similar proliferation rate as wt cells both *in vitro* and *in vivo*. One possible reason for the discrepancy is that we used stable cell line in this study, while most of previous research used transient expression system. During the selection of stable cell line, the poorly-growing clones were eliminated and the remaining clones had similar growth rate as wt cells. When injected into mouse brain, the difference of tumor progression observed between wt and annA2KD group was caused mainly by proteolysis aspect but not proliferation, which made the data easier to interpret.

In annA2KD glioma samples, we found that there were less proliferating stromal cells and increased glioma apoptosis possibly due to less tumor invasion and

angiogenesis, which is essential for nutrient transport to tumor cells and tumor expansion (Pepper, 1997). Moreover, it was reported that annexin A2 was involved in cell cycle regulation (Chiang et al., 1993), and its downregulation was observed after p53 overexpression in lung cancer cells, which induced cancer apoptosis (Huang et al., 2008). This could be another possible mechanism contributing to the increased apoptosis in annA2KD tumors.

Taken together, our data demonstrated that annexin A2 enhanced glioma migration, invasion and angiogenesis as well as inhibited apoptosis possibly via tPA/plasminogen proteolytic system. The current trends for targeted cancer therapy has been aimed at anti-angiogenesis subsequent to inhibiting ECM proteolysis, which may inhibit tumor growth due to less neovascularization (Kim, 2003; Sharma and Sharma, 2007; Ulisse et al., 2009). Thus inhibition of annexin A2 in gliomas may be a potential novel interventional, adjuvant regimen to tackle gliomas.

Figure 2-1 GL261 expresses tPA, plasminogen and annexin A2. (A) Annexin A2 and plasminogen expression was detected by western blot. Annexin A2 is overexpressed in GL261 as compared to microglia and astrocytes. Plasminogen is expressed by GL261 but not microglia or astrocytes. (B) Zymography of GL261 lysate (10 µg) and conditioned medium (20 µl) showed that tPA was expressed and secreted by GL261. (C) Immunofluorescent staining of GL261 cell membrane with tPA and annexin A2 antibodies showed co-locolization of these two proteins. (scale bar = 20 µm)

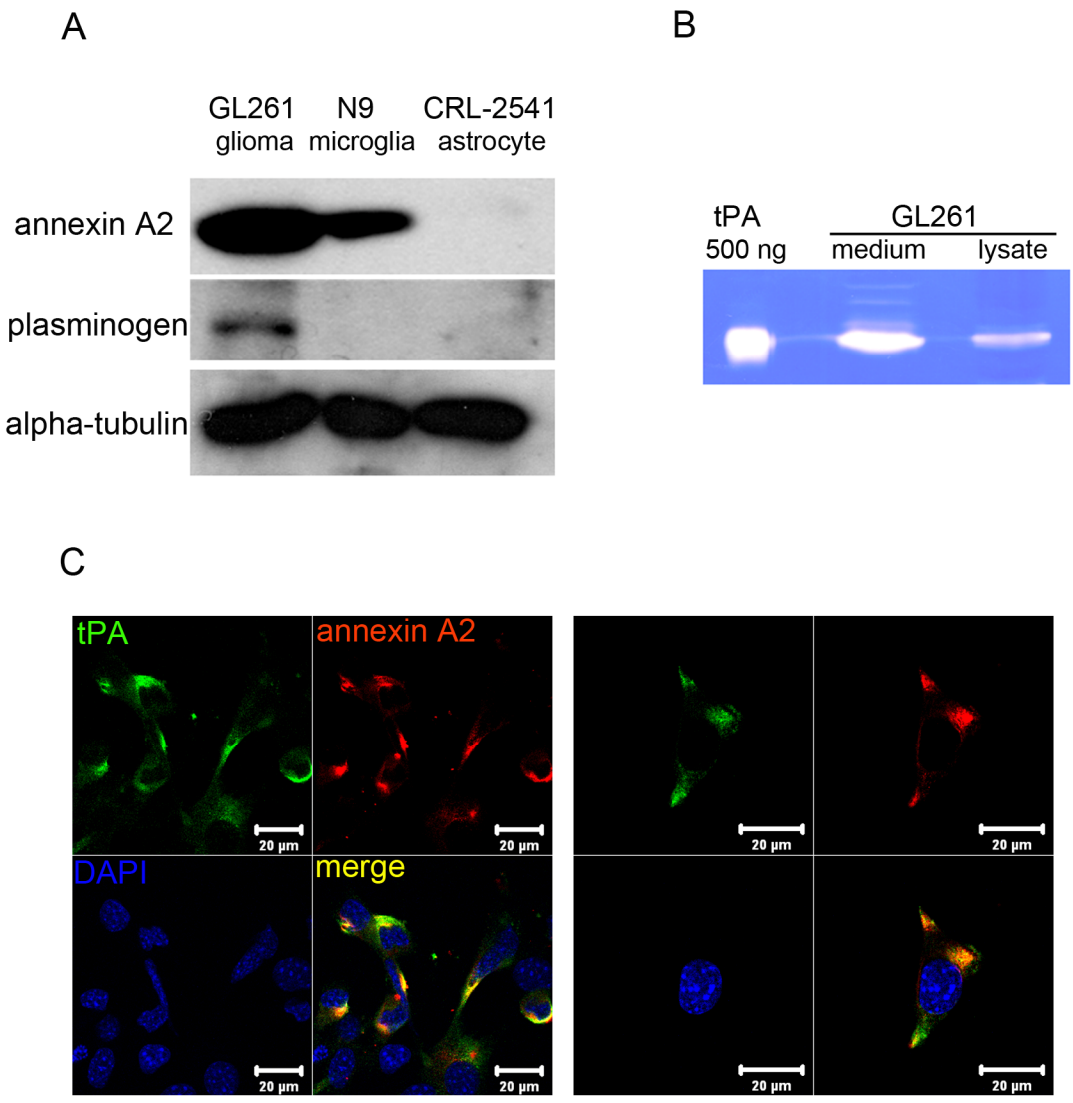


Figure 2 – 2. Establishment of stable cell lines with annexin A2 knockdown, GL261-EGFP-annA2KD. The annexin A2 expression levels of GL261-EGFP and GL261-EGFP-annA2KD clones were detected by western blot (A) and normalized to α-tubulin levels (B). (C) GL261-EGFP-annA2 KD clones had similar proliferation rate as GL261-EGFP. The cell proliferation rate was measured by BrdU incorporation rate. (n = 3)

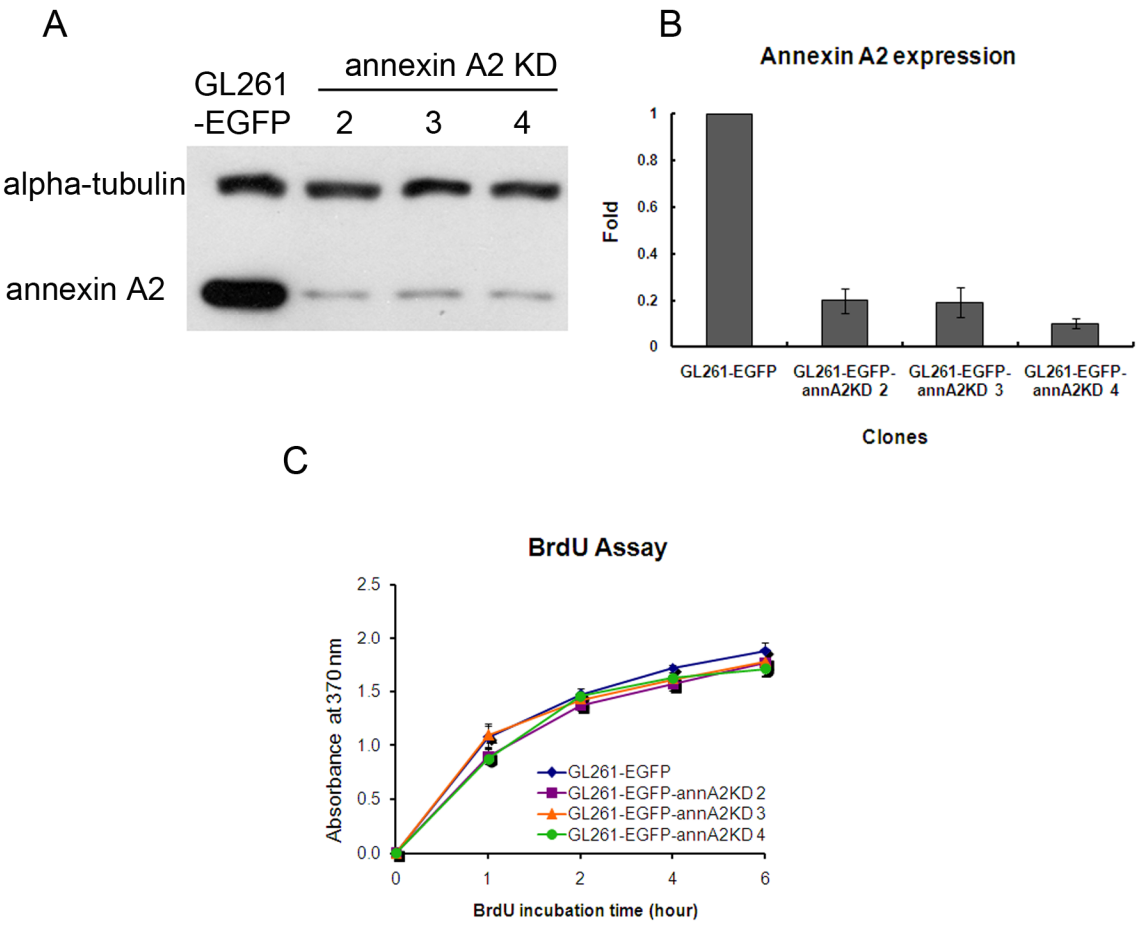


Figure 2 – 3. Knockdown of annexin A2 in glioma cells decreased membrane-bound tPA activity. The cell-surface annexin A2 expression levels of GL261-EGFP and GL261-EGFP-annA2KD clones were detected by western blot (A) and normalized to α-tubulin levels (B). (C) Amidolytic assay was used to measure tPA activity in membrane and cytoplasmic protein fraction of GL261-EGFP clones. (n = 3)

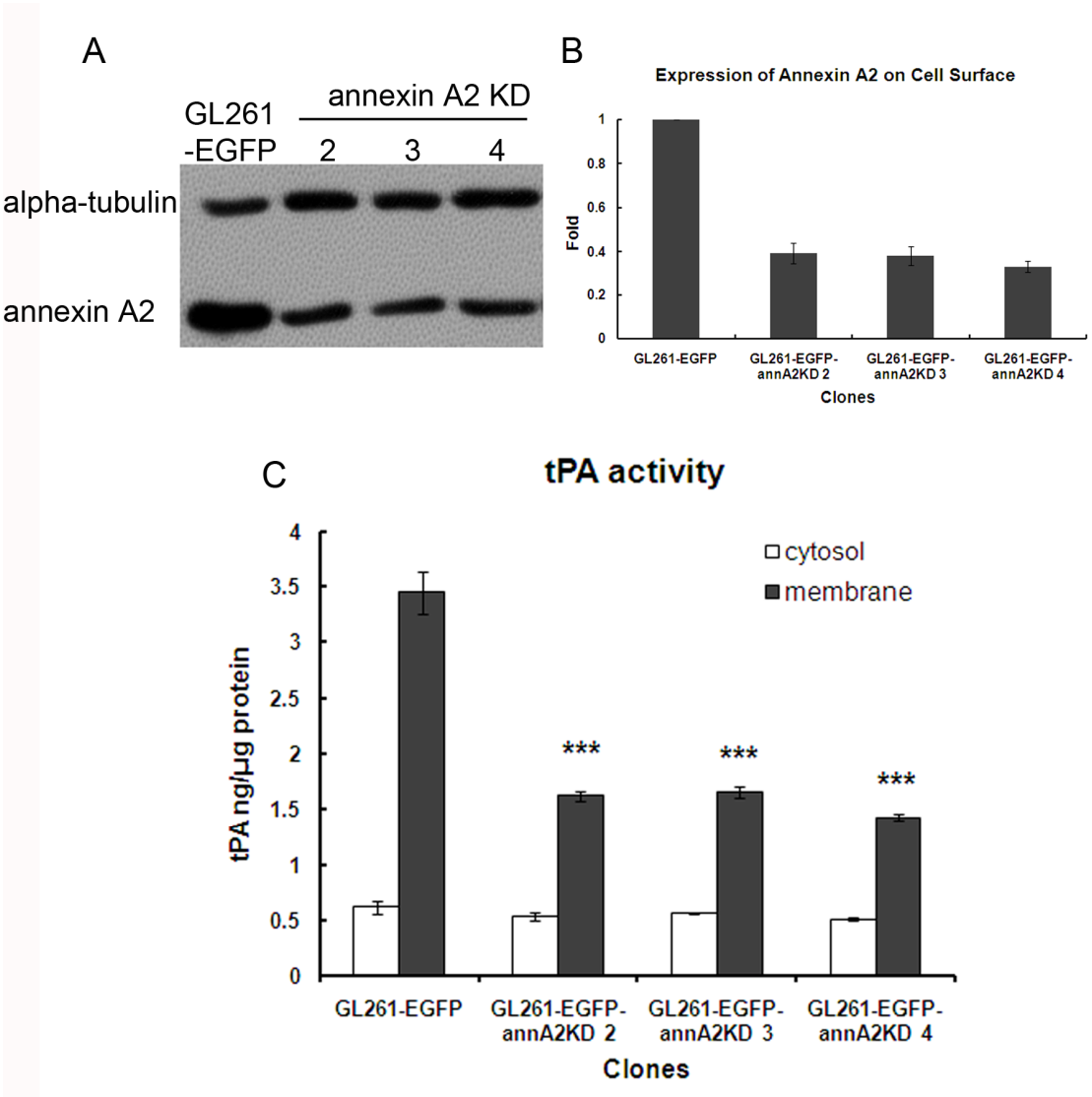


Figure 2 – 4. Knockdown of annexin A2 decreased glioma cell migration in culture. Scratch-migration assay was performed to evaluate glioma cell migration. Representative images from each clone at 0 and 24 hours are shown in (A). White dashed lines indicate the edges of scratch. (scale bar = 500 µm)The total number of migrated cells between scratch edges in 3 random fields from each plate was counted (B). (n = 3)

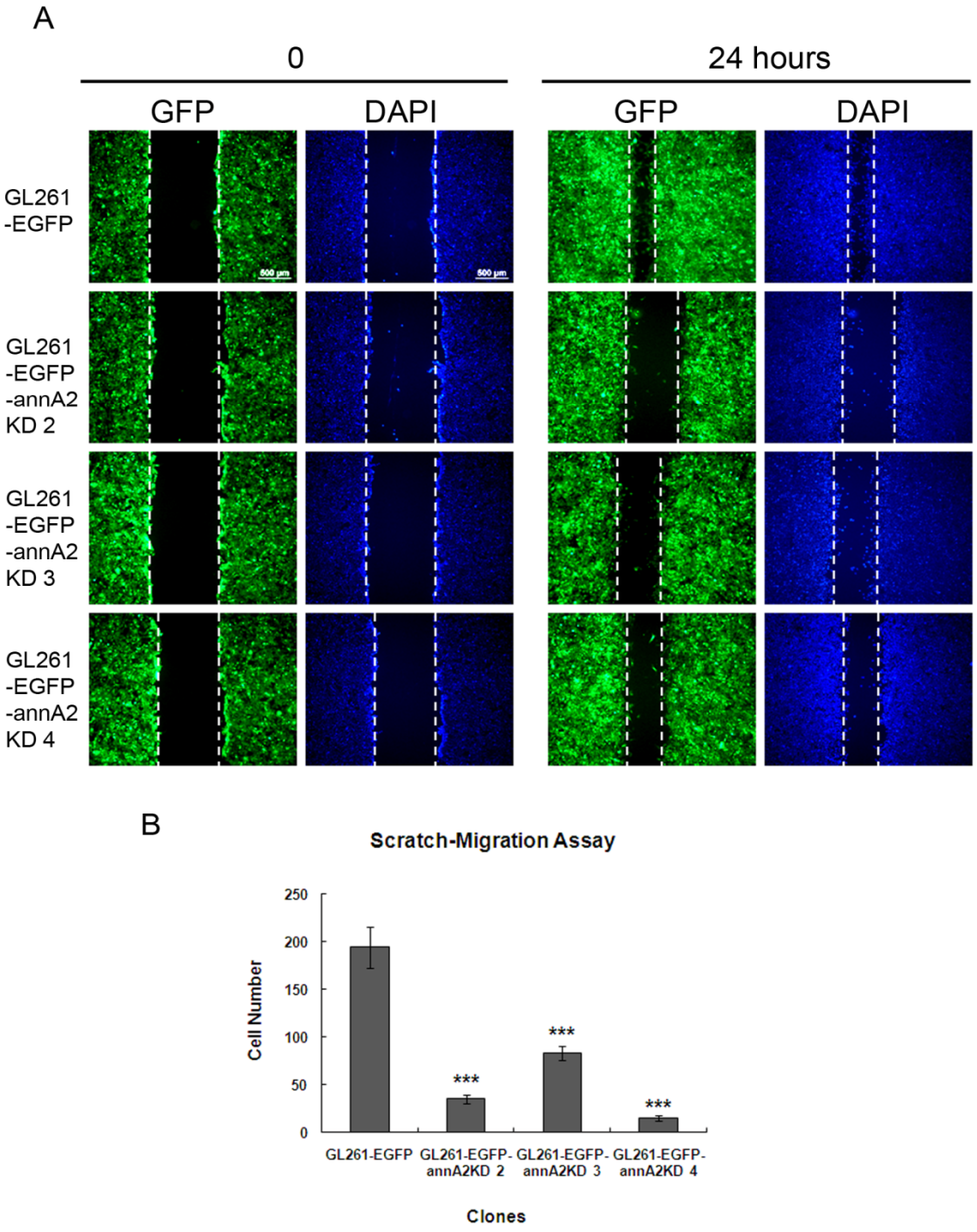


Figure 2 – 5. Establishment of a mouse glioma model. Schematic illustration of tumor injection (A) and evaluation time points (B). (C) GL261-EGFP-annA2KD 4 was cultured without blasticidin selection for 20 days, and the annexin A2 expression level was examined by western blot. A representative glioma sample from day 15 after tumor injection was shown with H&E staining (D) and GFP fluorescence (E), which indicated that the GFP signal correspond with tumor morphology very well. (scale bar = 1 mm)

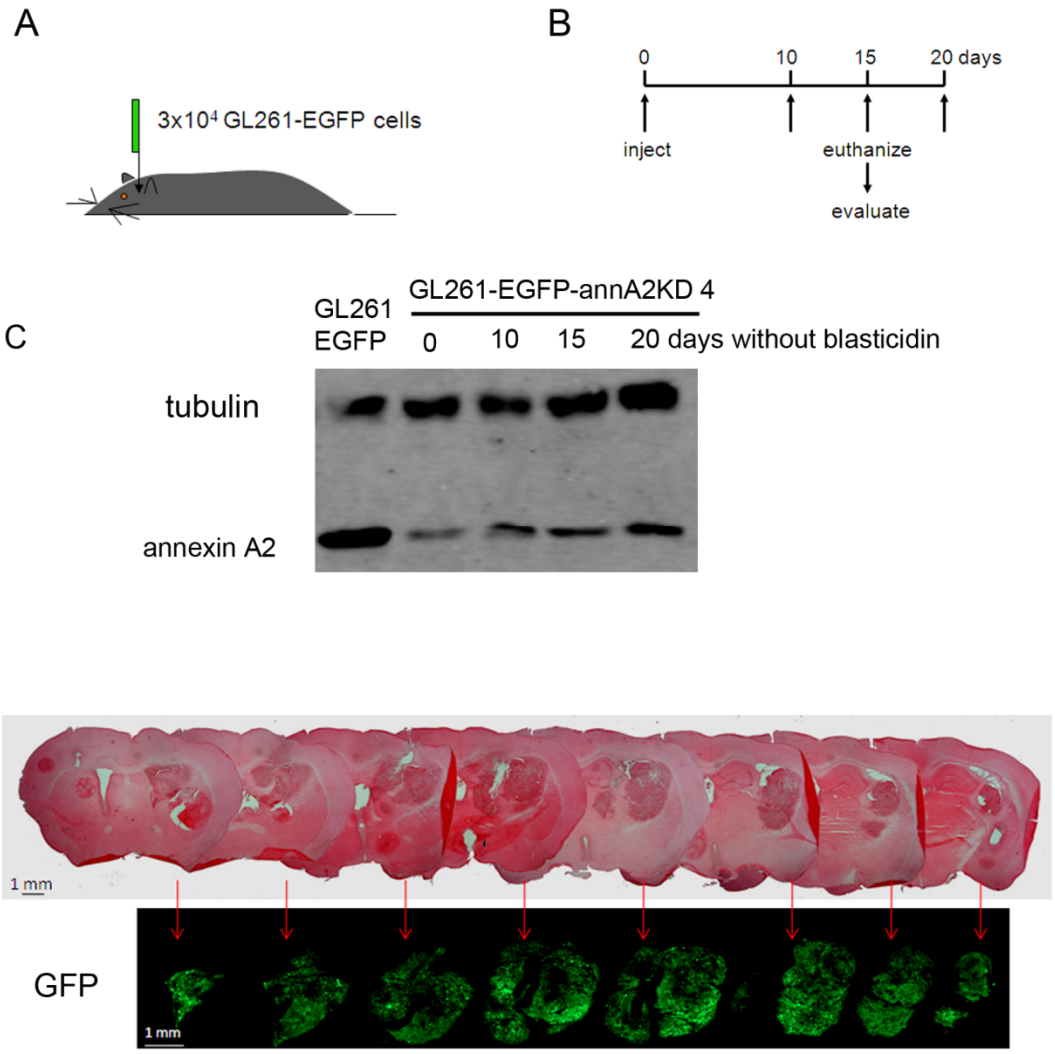
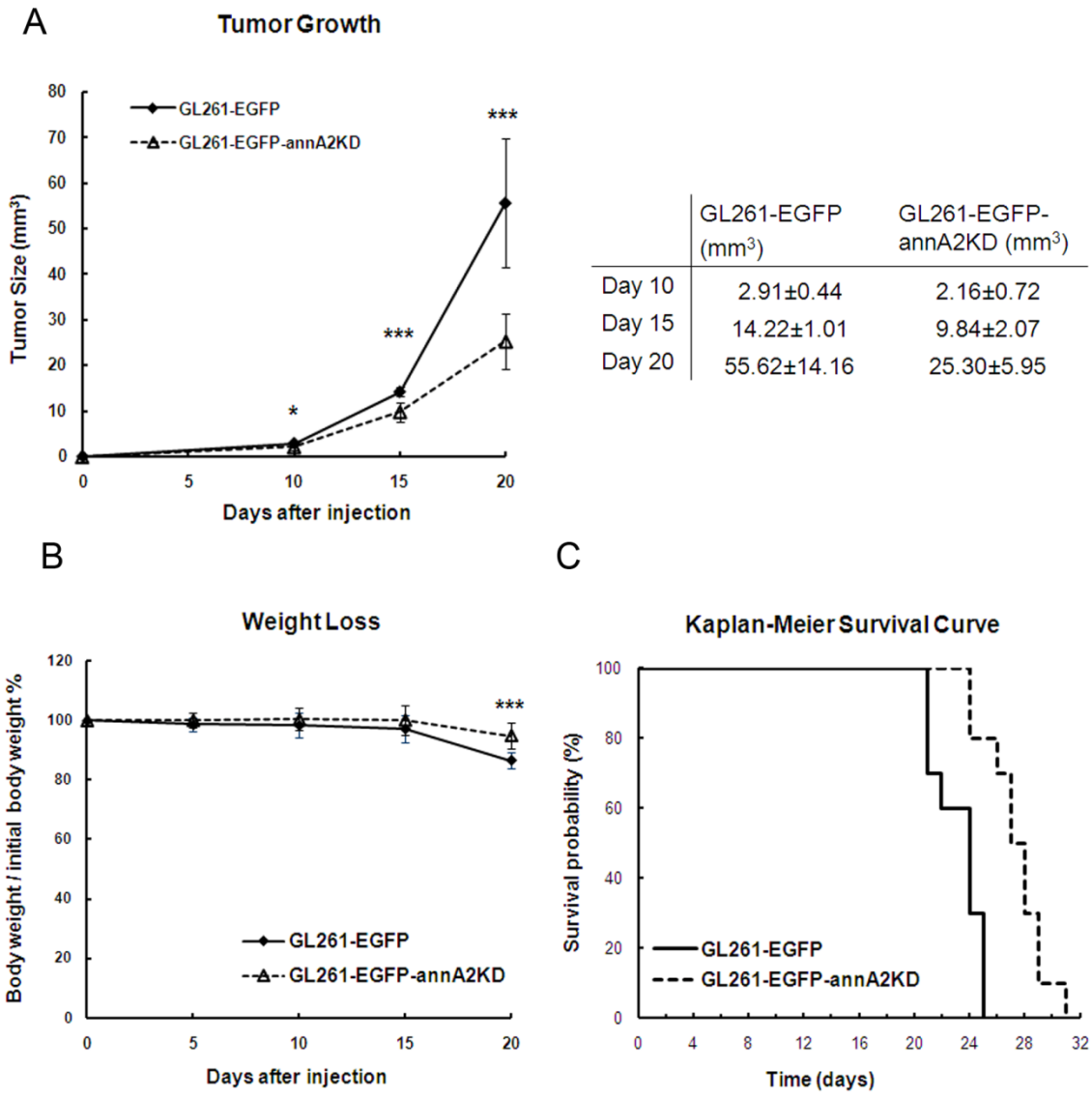


Figure 2 – 6. Knockdown of annexin A2 in glioma cells slowed down tumor progression *in vivo*. (A) Tumor sizes were measured at 10, 15 and 20 days after tumor cell injection (n = 8, from 4 independent injections). (B) Weight loss of tumor-bearing mice was monitored at 10, 15 and 20 days after tumor injection. (n = 15) (C) Kaplan-Meier survival curves of tumor-bearing mice (**p = 0.0003**, n = 10).



	GL261-EGFP (mm^3)	GL261-EGFP-annA2KD (mm^3)
Day 10	2.91±0.44	2.16±0.72
Day 15	14.22±1.01	9.84±2.07
Day 20	55.62±14.16	25.30±5.95

Figure 2 – 7. High magnification pictures of immunofluorescent staining with Ki67 (A), phospho-histone H3 (B) and activated caspase-3 (C) antibodies as well as TUNEL assay (D) to demonstrate the cell specificity. (scale bar = 20 µm)

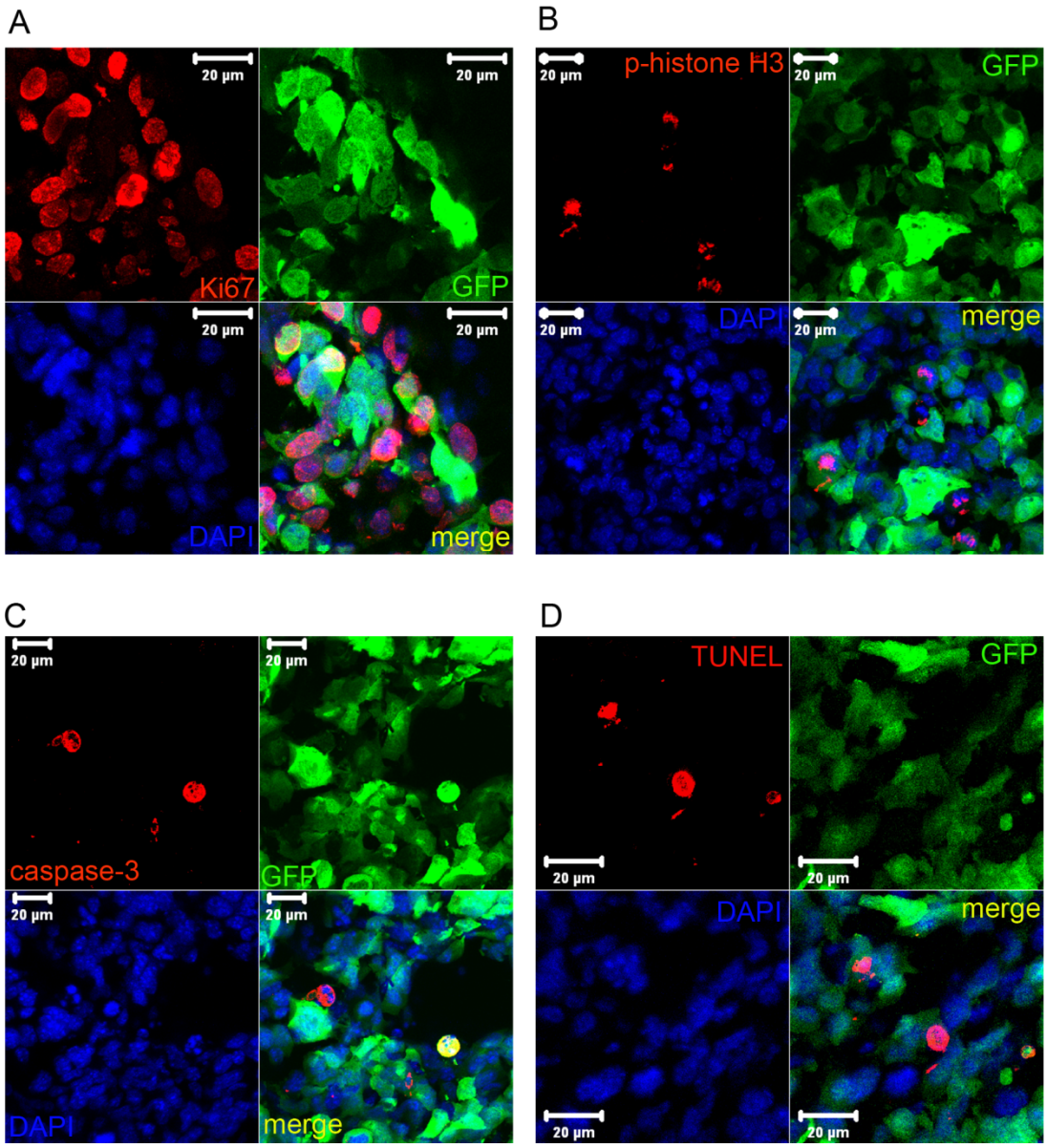


Figure 2 – 8A. Animals receiving AnnA2KD glioma displayed less proliferation within the tumor tissue. The proliferation of tumor tissue (B) and tumor cells (C) were quantified after Ki67 (A) immunofluorescent staining. (n = 5) (scale bar = 50 µm)

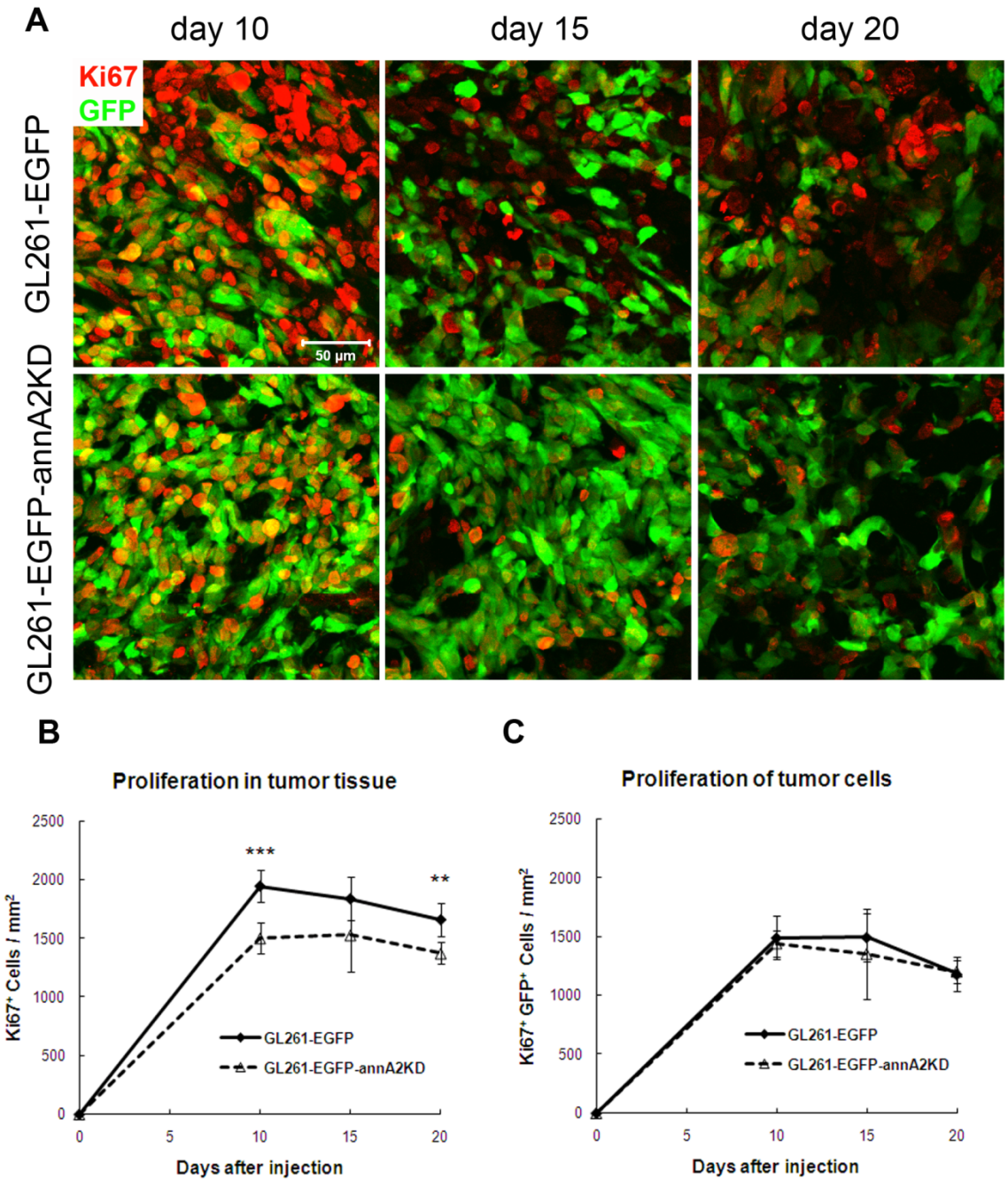


Figure 2 – 8B. Animals receiving AnnA2KD glioma displayed less proliferation in tumor tissue. The proliferation of tumor tissue (E) and tumor cells (F) were quantified after p-histone H3 (D) immunofluorescent staining. (n = 5) (scale bar = 50 µm)

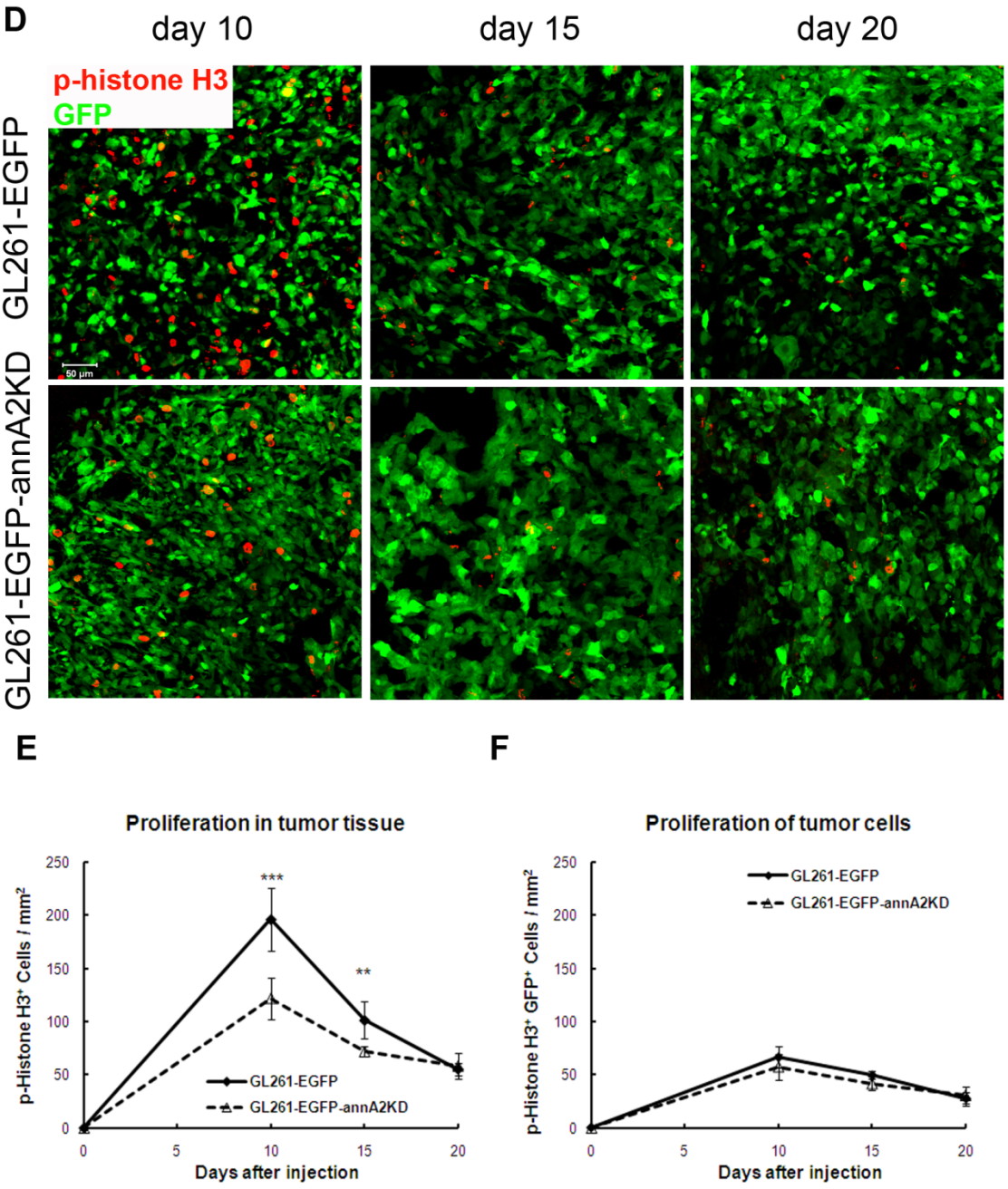


Figure 2 – 9A. Knockdown of annexin A2 in glioma cells increased glioma apoptosis. The apoptosis of tumor tissue (B) and tumor cells (C) were quantified after activated caspase-3 immunofluorescent staining (A). (n = 5) (scale bar = 100 µm)

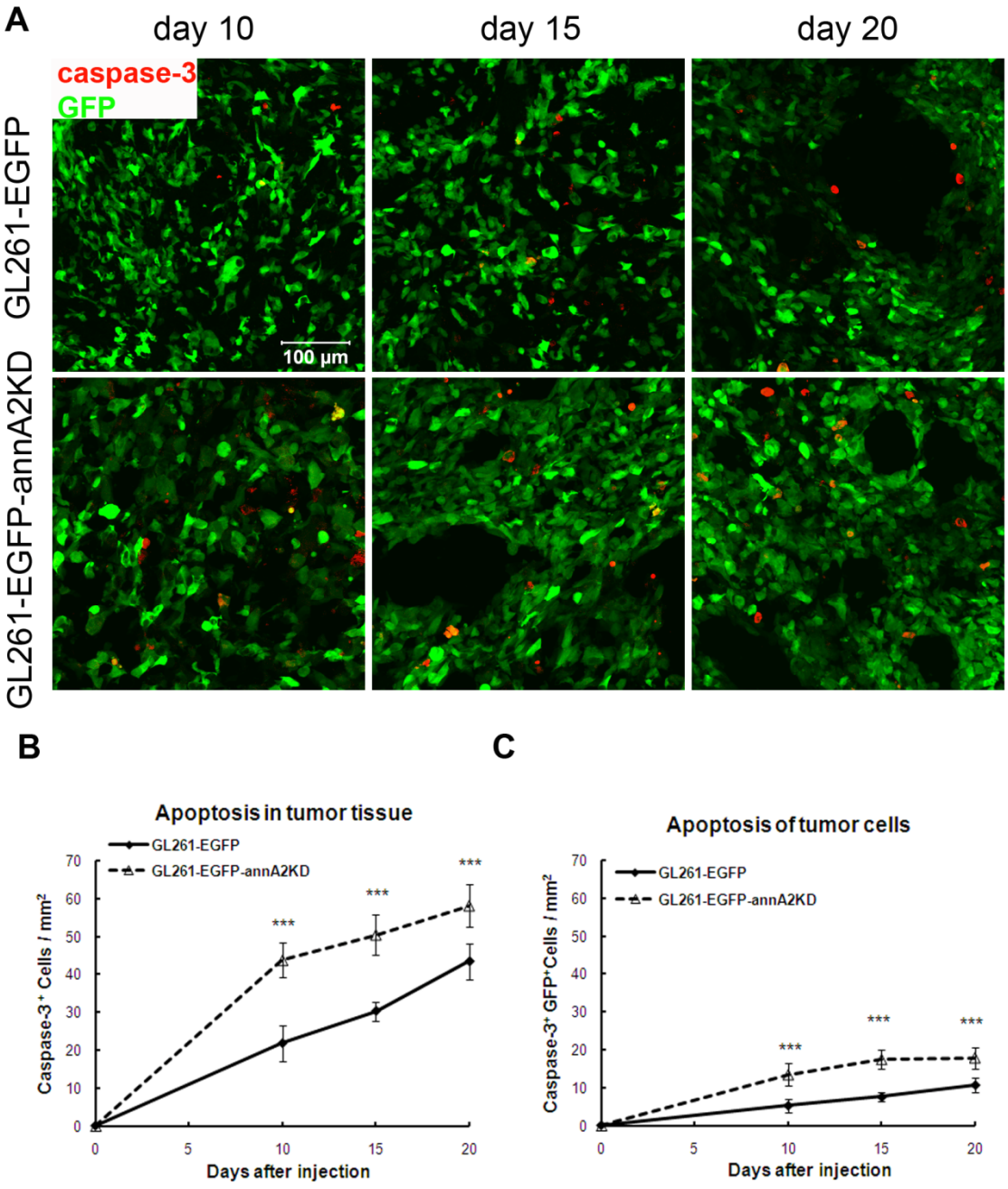


Figure 2 – 9B. Knockdown of annexin A2 in glioma cells increased glioma apoptosis. The apoptosis of tumor tissue (E) and tumor cells (F) were quantified after TUNEL assay (D). (n = 5) (scale bar = 100 µm)

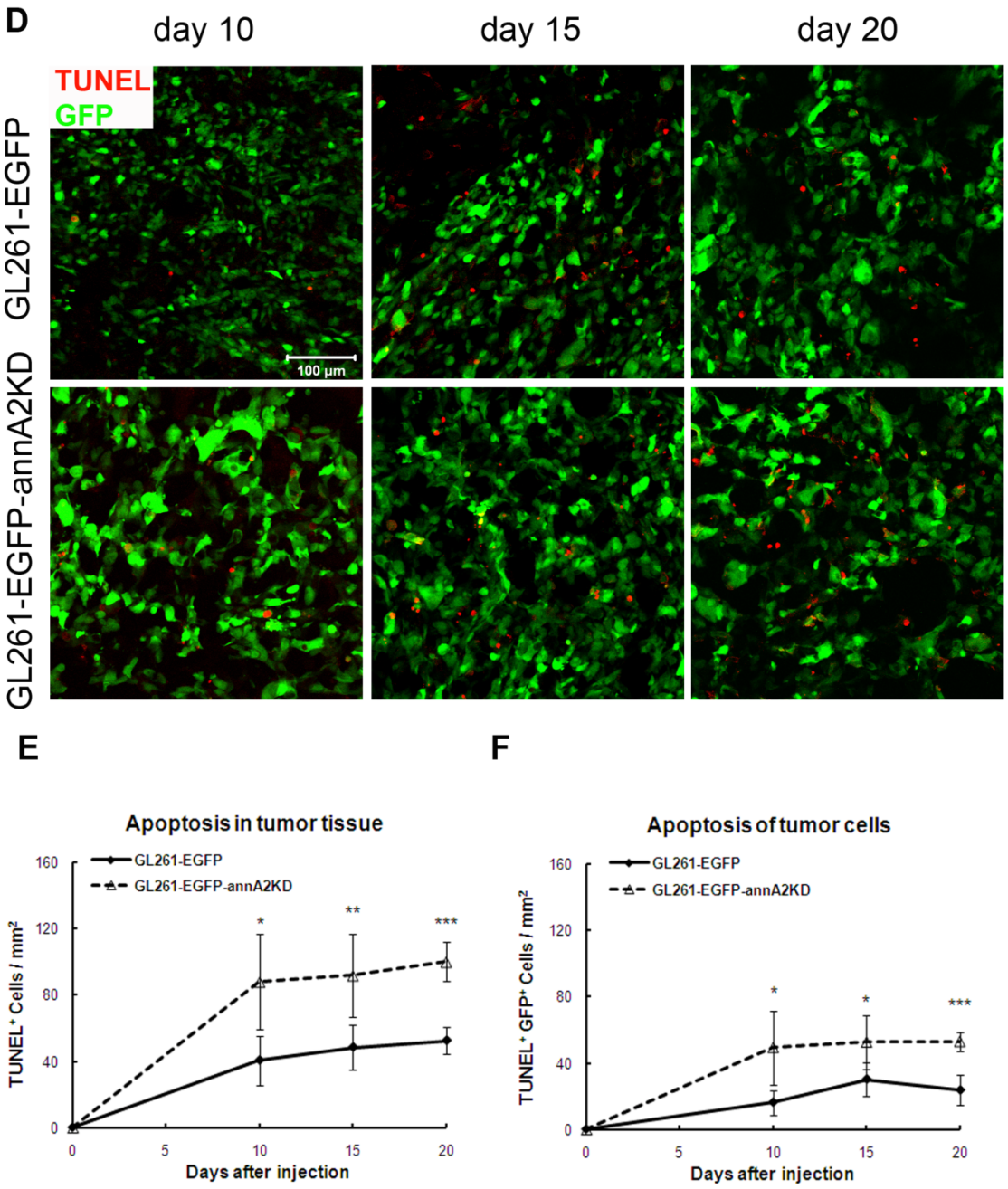


Figure 2 – 10. Knockdown of annexin A2 decreased glioma invasion *in vivo*. The tumor invasion was quantified by measuring percentage of invasion (A, B) and maximal invasion distance (C). (n = 5) White dashed lines indicate the border between tumor mass and invasive area. (scale bar: in each group, right column - 200 µm, left column – 500 µm for day 10 and 15, 1000 µm for day 20)

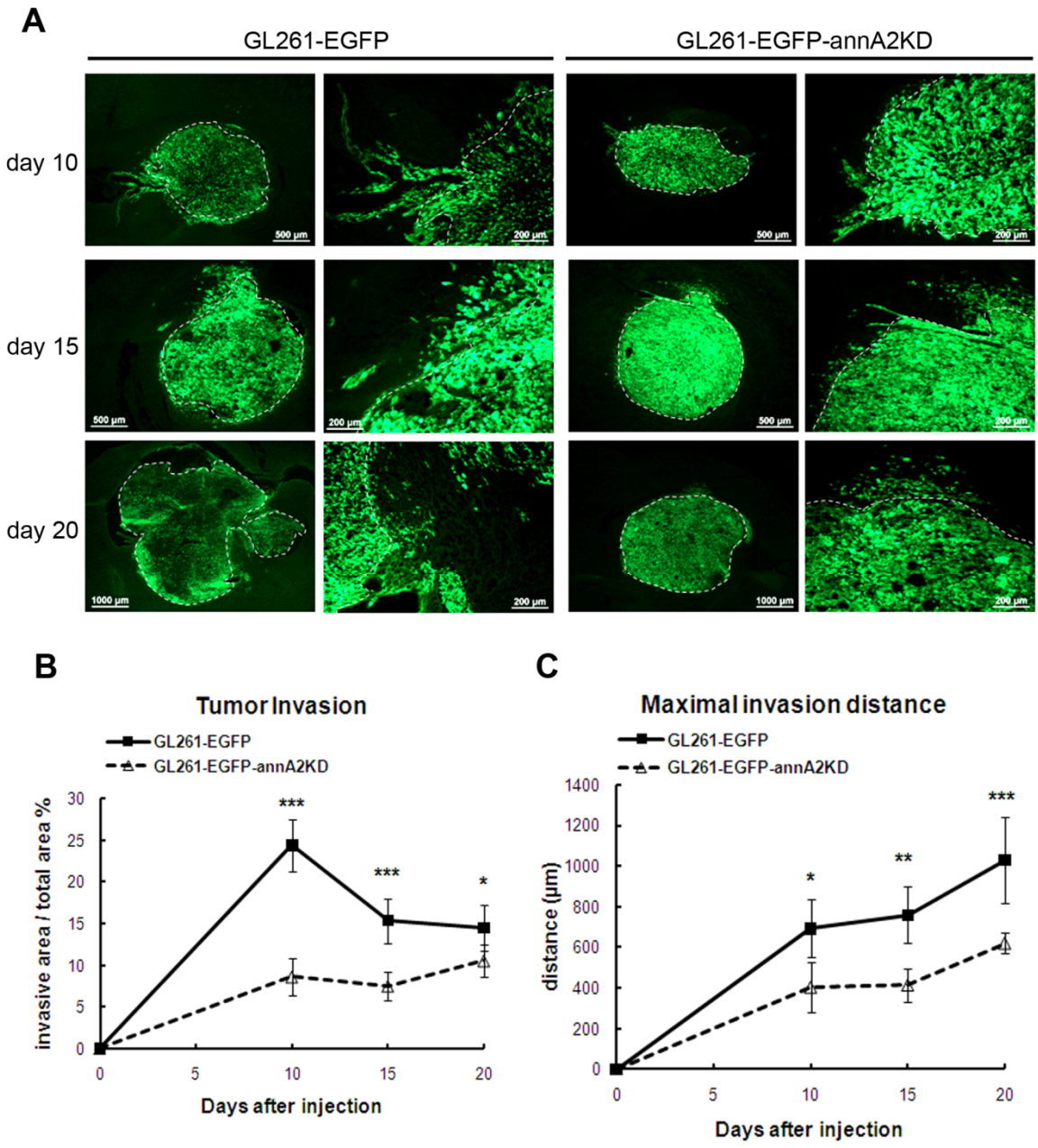


Figure 2 – 11A. Knockdown of annexin A2 decreased glioma angiogenesis. The blood vessels were indentified based on immunofluorescent staining of endothelial cells (A). MVD (B) in glioma tissue was quantified by counting blood vessel number in twenty 20x fields. (n = 5) (scale bar = 100 µm)

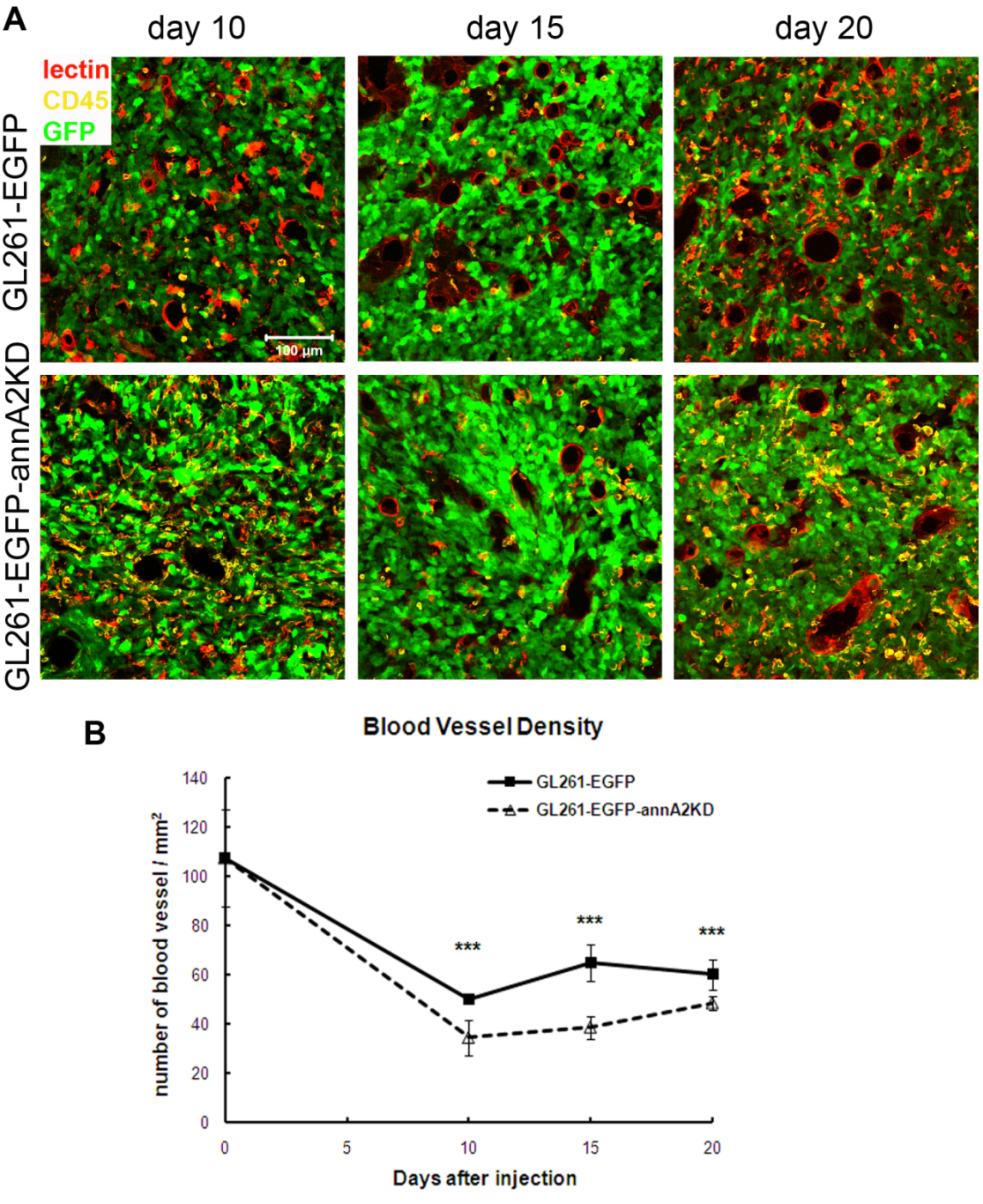


Figure 2 – 11B. Knockdown of annexin A2 in glioma cells decreased glioma angiogenesis. (C) The blood vessel diameters were measured and used to sort MVD based on the sizes. (n = 5)

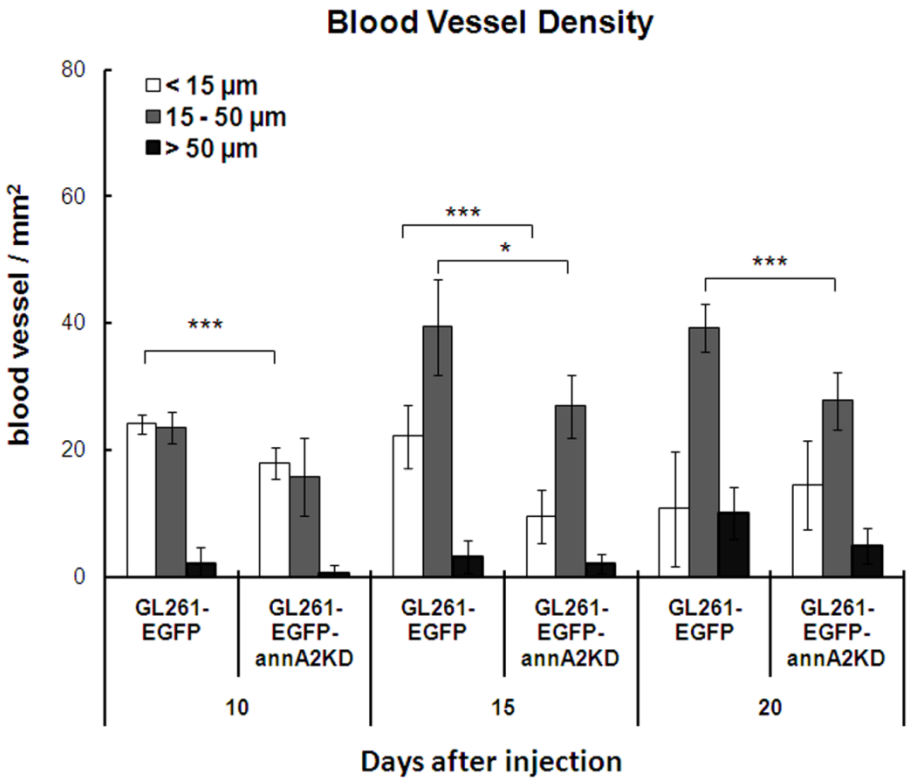
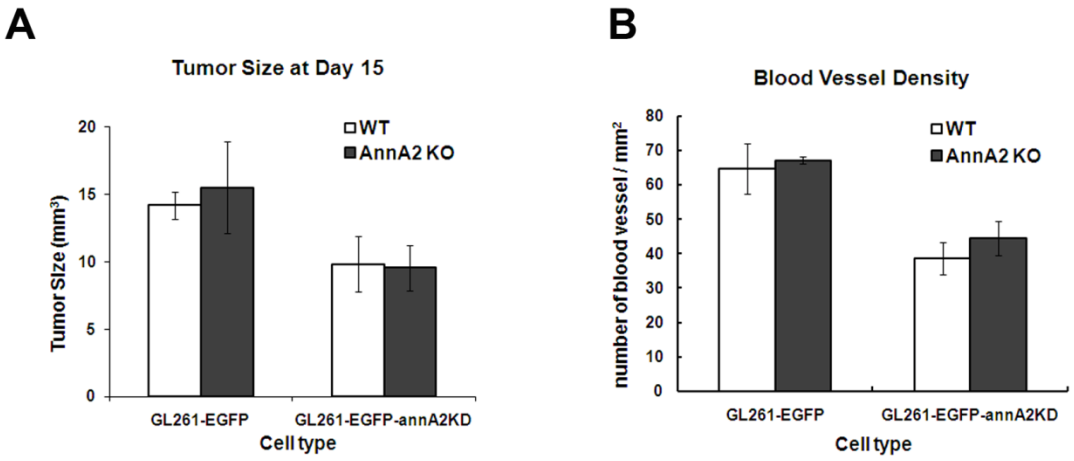


Figure 2 – 12. Absence of annexin A2 in tumor stroma had no effect on glioma growth. Tumor size (A) and MVD (B) were measured 15 days after tumor injection into WT and annexin knockout (annA2KO) mice. (WT mice: tumor size, n = 8; MVD n = 5. AnnA2KO mice: n = 2)



Chapter 3

Microglia/Macrophages Promotes Glioma Progression

Introduction

A long-standing characteristic of gliomas has been the observation that the tumors are infiltrated by large numbers of MG/MP, and in fact, the extent of MG/MP infiltration correlates positively with the degree of malignancy (Morimura et al., 1990; Morris and Esiri, 1991; Roggendorf et al., 1996). Microglia are brain resident macrophages capable of antigen presentation to T cells patrolling the CNS (Kreutzberg, 1996). Upon injury, microglia undergo a process of activation characterized by morphological change, changes in gene expression, increased proliferation and phagocytic capacity, and migration towards the site of injury. The microglia up-regulate cell adhesion molecules and major histocompatibility complex (MHC) proteins needed to present antigen, and secrete complement components, cytokines, growth factors, reactive oxygen species, and chemokines to regulate immune responses (Kreutzberg, 1996; Streit et al., 1999).

The role of MG/MP in glioma progression remains controversial. Some studies have found that the immune defensive functions of glioma-infiltrating MG/MP (GIMs) are compromised. Moreover GIMs have been proposed to promote glioma growth by secreting growth factors, such as TGF-β, or chemokines, immune-suppressive cytokines

and angiogenic factors (Alterman and Stanley, 1994; Demuth et al., 2007; Galasso et al., 2000; Lafuente et al., 1999; Wagner et al., 1999; Wesolowska et al., 2008). These studies have stimulated interest in developing therapeutic methods that involve modulation of MG/MP activity/function. However, such approaches have yielded conflicting results. For example, injection of CpG-containing oligonucleotides, which stimulate microglia, induced apoptosis of glioma cells and prolonged survival times of tumor-bearing animals in one report, whereas the same approach caused increased animal tumor size in others (El Andaloussi et al., 2006; Ginzkey et al., 2009).

In this study, we investigated the consequences of interaction of microglia and glioma cells in culture, using microglial activation and glioma cell proliferation as functional endpoints. We also examined tumor progression in a mouse glioma model using pharmacogenetics to locally ablate MG/MP, or a pharmacological approach to exaggerate MG/MP activation. We show that manipulation of the activation state of MG/MP appears to be a potentially promising interventional approach for the treatment of gliomas.

Materials and methods

Animals

C57BL/6 mice (wild type, WT) were purchased from Jackson Laboratory. CD11b-HSVTK transgenic mice were described previously (Heppner et al., 2005). Female CD11b-HSVTK (+/-) mice were bred with male C57BL/6 mice and the offspring genotyped by PCR using primers 5'-GACTTCCGTGGCTTCTTGCTGC-3' and 5'-GTGCTGGCATTACAGGCGTGAG-3'. All animal procedures were approved by the Stony Brook University IACUC. Mice were bred in-house under maximum isolation conditions on a 12:12 hour light: dark cycle with food *ad libitum*.

Primary microglia cultures

Mixed cortical cultures from newborn C57BL/6 mice (day 0 to 3) were prepared using a standard protocol (Rogove and Tsirka, 1998). Briefly, the pups were decapitated and the cerebral cortices isolated by removing midbrain, olfactory bulbs, hippocampi and meninges. The cortices were incubated with 0.05% trypsin/EDTA (Fisher Mediatech) at 37°C for 15 minutes and then dissociated in DMEM with 10% FBS, 1 mM sodium pyruvate and 40 µg/ml gentamicin. The homogenized mixture was filtered through a 40 µm cell strainer (Fisher). The resulting mixed cortical cells were plated on poly-D-lysine (Sigma) coated plates. Ten days after plating, primary microglia were isolated by treatment with 15 mM lidocaine (Sigma) for 15 minutes at room temperature and gentle rocking, which released the microglia into the culture medium. The supernatant was collected and centrifuged at 1500 rpm for 5 minutes. The microglial pellet was

resuspended in an appropriate volume of ice-cold DMEM with 1% FBS. After 24 – 48 hours of culture, microglia were used for experiments as described.

Microglia and glioma cell co-culture

Microglia-glioma co-cultures were performed by co-plating GL261-EGFP glioma cells with rhodamine-labeled primary microglia. In brief, after isolation of primary microglia as above, the cells were resuspended at a density of $5x10^4$ cells / ml in DMEM with 1% FBS and 20 μg/ml mini-ruby (a rhodamine-conjugated dextran, Invitrogen), and then cultured in 12-well plates (Ullrich et al., 2001). After 48 hours, the medium was removed and $2x10^4$ GL261-EGFP cells seeded on top of the microglia in DMEM with 10% FBS. As controls, primary microglia were either switched to the same medium without the addition of glioma cells, or the microglia were seeded with $2x10^4$ of the CRL-2541-EGFP astrocytes. To evaluate the microglia-glioma interactions over time, the cells were followed by confocal imaging over a period of 5 days. To evaluate the growth rate of the two populations, cells were detached from the plates and cell numbers were counted by a hemacytometer for 5 days (as glioma or astrocytic cells were labeled with EGFP and the microglia were loaded with Mini-Ruby). The experiments were repeated 3 times with duplicate samples for each group.

Segregated microglia-glioma co-cultures were performed by plating GL261 cells in 24-well plates below primary microglia adherent to 0.4 μm cell culture inserts (Millicell). Briefly, $5x10^4$ primary microglia were seeded on the inserts in DMEM with 1% FBS. After 48 hours, the inserts were moved to 24-well plates containing 5,000

GL261 cells/well in DMEM with 10% FBS. 150 µg/ml tuftsin or MIF (Bachem) was added to the medium above the inserts. Empty inserts with the same medium were used as control. GL261 proliferation was measured every other day. The experiments were repeated 3 times with duplicate samples for each group.

Cell proliferation assay

The CCK-8 kit (Dojindo Molecular Technologies) was used to quantify cell proliferation following the manufacture's protocol. In brief, equal numbers of GL261 cells were seeded in 96-well plates or 24-well plates (co-culture) with respective treatments (duplicate samples for each treatment). At each time point, the culture medium was changed to include a 1:10 dilution of CCK-8. After 2 hours incubation at 37°C, absorbance values were read at 450 nm by a plate reader. Cell proliferation rates were plotted by normalizing the absorbance values at each time point over the ones at day 0.

Western blotting of microglial markers

Primary microglia were seeded into 6-well plates at equal numbers in DMEM + 1% FBS. After two days, the medium was replaced with DMEM + 10% FBS or conditioned medium. Glioma-conditioned medium (GCM) was collected from an 80%-confluent GL261 plate and centrifuged at 10,000 rpm for 10 minutes to remove cell debris. Negative controls included GCM boiled for 10 minutes and astrocyte-conditioned medium (ACM) collected from a CRL-2541 plate. At different time points, cells were lysed and protein concentrations were. 15% SDS-PAGE was used to separate protein

samples (10 µg), which then were transferred to a PVDF membrane. Membranes were blocked by 3% bovine serum albumin (BSA) in PBS / 0.05% Tween 20 for 1 hour at room temperature, and then incubated with rabbit anti-mouse Iba1 antibody (1:1000, Wako) and mouse anti-mouse α-tubulin antibody (1:2000, Upstate, Millipore) overnight at 4°C. Goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were used at 1:5000, and HRP activity detected with LumiGLO Chemiluminescent Substrate System. Expression levels were quantified using SCION Image software, and Iba1 expression level normalized to the α-tubulin expression level. The experiment was repeated 3 times.

In vivo mouse glioma model and local drug delivery

12 – 16 week old male CD11b-HSVTK (+/-) mice weighing 25 – 30 grams were used for intracranial injections. WT littermates (CD11b-HSVTK (-/-) mice) were used as controls. For the tuftsin group, 12 – 16 week old C57BL/6 mice were used. The tumor injection process was same as the one used in Chapter 2.

Local drug administration was performed using 14-day or 28-day mini-osmotic pumps (Alzet®, DURECT Corporation) containing different concentrations of ganciclovir (GCV) (Calbiochem), 25 µg/ml tuftsin, or 25 µg/ml MIF. Each pump was connected to a plastic tubing and a 4 mm guide cannula (PlasticsOne). The pumps were incubated in normal saline overnight at 37 °C and then placed subcutaneously on the mouse back with the cannulas inserted to the injection site. The drugs were infused at a rate of 0.25 µl per hour over 14 or 28 days. Pumps with normal saline inserted right after

tumor cell injection were used as negative control. GCV pumps were inserted either immediately after (D0 GCV pump), or five days after (D5 GCV pump) tumor injection. Animals accepting GCV pumps were administered 50 μg/g GCV i.p. one day before tumor injection to ensure the maximum ablation of MG/MP.

Tumor evaluation

At each time point of tumor evaluation, brain specimens were collected as described in Chapter 2. For general morphology, sections were stained with hematoxylin and eosin (H&E). The tumor sizes were measured based GFP+ areas. The sections were immunostained using an Iba1 antibody or biotinylated tomato lectin (Sigma) to visualize MG/MP infiltration, a Ki67 antibody (Abcam) to quantify proliferation, an activated caspase-3 antibody (Sigma) to measure apoptosis and a CCL21 (PeproTech) antibody to measure cytokine level. For quantification, 3 randomly selected samples from each group were used and at least 15 fields in different sections of the same sample captured by confocal microscopy.

Immunofluoresent staining

Primary microglia treated with GCM were fixed with 4% PFA/PBS for 15 minutes at room temperature. Cells or brain sections were blocked in 5% goat serum in PBS-T (0.3% Triton X-100 in PBS) for 1 hour at room temperature, and then incubated with Iba1 antibody (1:500), Ki67 antibody (1:500), activated caspase-3 (1:500), and biotinylated tomato lectin (1:500) in PBS-T overnight at 4°C. The samples were

incubated with corresponding different secondary antibodies (Alexa Fluor 555 or 647) for 1 hour at room temperature, and then mounted with Fluoromount-G.

For CCL-21 immunostaining, brain sections were first subjected to antigen retrieval with Target Retrieval Solution, Citrate pH 6 (Dako). First the stock solution was diluted 1:10 with distilled water and then the slides were immersed with the solution in a plastic coplin jar. The covered coplin jar was heated to 95°C by microwave oven at 30% power for 1 to 1.5 minutes, followed by incubation at 55°C for 10 minutes and room temperature for 20 minutes. After 3 washes with PBS, the slides were blocked and incubated with CCL21 antibody (1:500) overnight at 4°C. In order to amplify the signals, the sections were first incubated with goat anti-rabbit biotinylated secondary antibody (1:200) for 1 hour at room temperature and then strep-avidin Alexa Fluor 555 antibody (1:1000) for 30 minutes at room temperature.

Statistical analysis

The statistical significance between two groups was determined by Student's *t*-test. For comparison of more than two groups, one-way ANOVA followed by a Bonferroni-Dunn test was used. The Kaplan-Meier survival curve was calculated with MedCalc software. Data were expressed as mean ± SEM. The statistical significance is either described in figure legends, or indicated as asterisks (*). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Results

Glioma cells produce soluble factors that activate microglia

We began by exposing primary microglia to glioma-conditioned medium (GCM) to determine whether soluble factors secreted by glioma cells alter the state of microglial activation. Microglial activation can be assessed by changes in cellular morphology. Resting microglia appear rod-like shaped in culture, and upon activation, they transform into an ameboid shape with pseudopodia (Siao and Tsirka, 2002). At time 0, the resting microglia had elongated cell bodies and thin processes, and continuing the culture for another 24 h in the same (microglia, DMEM + 1% FBS) or GL261-control medium (DMEM + 10% FBS) elicited no change in the morphology (Fig. 3-1A, B). When exposed to GCM for 16-24 hours, however, the microglia retracted their processes and became ameboid in shape. Iba1 (ionized calcium binding adaptor molecule 1) is a marker for MG/MP that is upregulated upon activation (Ito et al., 1998). The Iba1 expression level increased 3-4 fold with GCM treatment (Fig. 3-1B, C), concomitant with the observed morphological changes. However, no increase in Iba1 was observed when the microglia were cultured with boiled GCM or with astrocyte-conditioned medium (ACM), indicating that the secreted factor is glioma-specific as well as heat-labile, suggesting that it is most likely protein in nature.

Microglia in culture promote a modest increase in glioma cell growth

Microglia are derived from monocytic progenitors and play a major role in both innate and adaptive immune responses in the CNS. As part of the innate immune

response, they phagocytose pathogens and release proteases, cytokines and other molecules that function to protect the host (Kreutzberg, 1996); this function may be compromised in a glioma-harboring environment (Hussain et al., 2006). To directly assess the effect of glioma cells on microglial function, we co-cultured rhodamine-labeled microglia and glioma GL261-EGFP cells. As a control the microglia were also plated alone, or with the CRL-2541 astrocytic line. Microglia plated alone remained rod-like throughout the 5 day experiment (Fig. 3-2A). Microglia co-cultured with CRL-2541-EGFP cells exhibited modest signs of morphological activation and phagocytic activity throughout the 5-day period (Fig. 3-2B, arrows and insets where the red –microglia-channel has been removed to display the green (astrocytic) fluorescence within the specific microglia cells, indicative of phagocytic events). In contrast, within one day of being co-plated with GL261 cells, the microglia exhibited activated morphology and their pseudopodia were in contact with the GL261 cells (Fig. 3-2C). At day 2 and day 3, a few phagocytic microglia containing GFP fluorescence in their cell bodies (arrows in figure 3-2C-D) could be observed. However, after day 4, phagocytosis of GL261 cells was only rarely observed.

We next examined the growth rate of GL261 and microglia in the co-culture system by counting the cell numbers of each population every 24 hours (Fig. 3-3). When primary microglia were switched to DMEM + 10% FBS (the glioma medium), the primary cells didn't proliferate in culture. In the presence of GL261, microglia remained in similar numbers as in the control group. At plating, the initial numbers of GL261 ($2x10^4$) cells were lower than those of microglia ($5x10^4$), in an attempt to mimic the beginning stages of glioma growth in the CNS. Three days after plating, there were more

GL261 cells than microglia. Moreover, when GL261 cells were cultured together with microglia, the GL261 growth rate was higher than the one of GL261 alone (15.3% more cells on day 5, slope of growth curve: no microglia – 6.0248, with microglia – 6.9347). Overall the growth (cell numbers) of GL261 cells in culture was modestly greater (and the difference was statistically significant) in the presence of microglia than in their absence. These results suggest that both the innate phagocytic response of microglia in culture is inhibited by GL261 cells, and that microglia may support glioma growth.

Establishment of an *in vivo* mouse glioma model with local MG/MP ablation

To have a better understanding of the *in vivo* response of microglia cells to a developing glioma, we decided to use a model of local microglial ablation. The CD11b-HSVTK transgenic mice express the herpes simplex thymidine kinase (HSVTK) protein in monocytic cells, including MG/MP (Heppner et al., 2005). When these animals are exposed to GCV, the cells that express HSVTK are eliminated. Systemic administration (100 µg/g GCV i.p. every 2 days) resulted in the death of all proliferating monocytic cells, which led to severe anemia and death around 10 days after the GCV injection (Heppner et al., 2005). We modified this protocol by using local infusion of GCV (Mirrione et al., 2010) at the tumor injection site, which ablated MG/MP in and around the tumor.

We first investigated whether GCV could affect GL261-EGFP growth in culture and *in vivo*. When GL261-EGFP cells were treated with 25 and 50 µg/ml GCV (every other day for 8 days, at day 4, 6 and 8), the cell growth was significantly inhibited as

compared to control group ($P < 0.001$), while 2.5 µg/ml and 10 µg/ml GCV treatments had no effect on GL261-EGFP growth (Figure 3-4A). We then tested different GCV concentrations *in vivo* in WT mice (Fig. 3-4B) and found that concentrations up to 1 mg/ml and 1.25 mg/ml GCV had no significant effect on tumor size at day 14 after tumor cell injection. When we tested higher concentrations, the tumor size decreased significantly. These results suggested that high concentrations of GCV were per se toxic to GL261-EGFP growth, as all cells have intrinsic thymidine kinases; however the HSV thymidine kinases are ~ 1000-fold more sensitive to GCV. For all subsequent experiments we used 1 mg/ml GCV.

To investigate the role of MG/MP in the temporal progression of glioma, we used CD11b-HSVTK mice and treated them with GCV: one group received GCV immediately after tumor injection (D0 GCV group), and the other one was treated with GCV five days after tumor implantation (D5 GCV group) to mimic an early stage glioma (Figure 3-4C). Control animals were either WT treated with saline or with GCV and CD11b-HSVTK mice treated with saline.

MG/MP ablation inhibits glioma progression

It has been reported that glioma invasion was attenuated in organotypic brain slices after administration of clodronate, which depletes microglia; provision of exogenous microglia restored the tumor invasiveness (Markovic et al., 2005). Similar effects were recently observed *in vivo*: Markovic and colleagues used the CD11b-HSVTK mice to study the effect of depletion of microglia-derived MT1-MMP on glioma

growth. The elimination of MG/MP resulted in 80% reduction of tumor volume (Markovic et al., 2009). Using our protocol for local MG/MP ablation (Mirrione et al., 2010) we performed a detailed evaluation of tumor growth in the presence and absence of microglia. Using the experimental and control groups described above, we found that the tumor size in control mice (either WT or CD11b-HSVTK that only received saline) was similar at 14 days after GL261 injection, whereas in MG/MP-depleted mice was significantly smaller ($P<0.001$; Figure 3-4D). At day 14 after tumor implantation, the average tumor size in the D0 GCV group and D5 GCV group of CD11b-HSVTK mice was 1.55 mm^3 and 2.94 mm^3 respectively (87.5% and 76.3% less than that of CD11b-HSVTK mice receiving only saline, which was 12.38 mm^3). To investigate the long-term effects of MG/MP depletion on glioma progression, we followed the tumor growth in WT mice treated with saline (saline WT group) and CD11b-HSVTK mice receiving GCV at the day of glioma infusion (D0 GCV group) at later time points. Twenty days after tumor inoculation, WT mice receiving saline were very sick with an average tumor size of ~ 55.6 mm^3 (Figures 3-4E, G). These mice suffered from extensive brain hemorrhage, brain swelling and weight loss. In contrast, the tumor size of mice within the HSVTK D0 GCV group decreased to almost undetectable (~ 0.15 mm^3) 28 days after inoculation, and was not detectable any more at day 42 (Figures 3-4E, G).

We then assessed the survival of tumor-bearing mice in the presence or absence of MG/MP (Figure 3-4F), and found no difference between WT and CD11b-HSVTK mice treated with saline (median survival time 24 days and 22 days respectively). In contrast, CD11b-HSVTK mice receiving GCV from the time point of tumor inoculation

(HSVTK D0 GCV) survived. These results indicate that glioma growth is dependent on the presence of MG/MP in this model system.

To evaluate tumor morphology, H&E-stained sections (Figure 3-4G) were correlated with the GFP signal derived from GL261-GFP cells by fluorescent microscopy (Figures 3-4H, I). Both saline- and GCV-treated WT mice as well as saline-treated CD11b-HSVTK (labeled as HSVTK) mice showed intact glioma tissue with numerous MG/MP in and around the tumor, which were labeled with Iba1 antibody (Figure 3-4J, K). GCV treatment of CD11b-HSVTK mice significantly decreased MG/MP, especially in the animals that received the D0 pump (although there was a significant decrease in those with the D5 pump as well). The decrease of MG/MP was accompanied by a reduction of tumor size as visible by few residual GFP signals. Again the animals with the D0 pump were carrying the smaller size tumors. At later time points, day 20 and day 42, the tumor was almost undetectable.

MG/MP labeling by Iba1 revealed that the number of MG/MP, after the initial decrease at the earlier timepoints, increased later (day 42), and the microglial morphology indicated that MG/MP were activated, as they presumably were recruited to the injury site to clean up tissue debris (Figure 3-4J, K).

Exaggerated activation of MG/MP increases glioma growth

Tuftsin (threonine-lysine-proline-arginine, TKPR) derives from the proteolytic degradation of immunoglobulin (Ig) G (Nishioka et al., 1973) and is a potent stimulator of MG/MP; it can enhance the phagocytic activity, migration and antigen presentation of

monocytic cells, including neutrophils and MG/MP (Siemion and Kluczyk, 1999). Tuftsin has been widely used as an anti-tumor agent in animal cancer models, including leukemia and melanoma (Banks et al., 1985; Noyes et al., 1981; Wleklik et al., 1986). In this study, we investigated the effects of tuftsin on glioma growth. When culturing GL261-EGFP cells in the presence of different concentrations of tuftsin, we found no effect on cell proliferation as assessed by a CCK-8 kit or by counting cell numbers (Figure 3-5A). Next, the effect of tuftsin on the growth of GL261-EGFP cells was measured in the presence of microglia. To this effect we first used a tissue-culture insert system to study the effects of soluble factors released by microglia on GL261 growth. Tissue culture inserts containing microglia were placed in wells containing GL261-EGFP cells. Microglia were treated with or without tuftsin for different periods of time. Then the inserts containing microglia were removed to exclude physical interaction between microglia and GL261-EGFP and the proliferation of GL261-EGFP was measured (Figure 3-5C). When compared to GL261-EGFP alone, GL261-EGFP growth was slower in the presence of microglia; but after day 6 of co-culture, glioma cell proliferation was increased ($P<0.01$). Similar results were obtained when using a glioma co-culture system with physical interaction between microglia and glioma cells (Figure 3-5B, E).

To assess the effects of tuftsin on glioma growth *in vivo*, tuftsin was delivered either at day 0 or 5 days after tumor injection to the tumor implantation site by mini-osmotic pumps and the size of tumors analyzed at day 14 after tumor inoculation. Tuftsin treatment at day 0 (immediately after tumor implantation) significantly increased the tumor size (mean = 21.0 mm^3) when compared to the average tumor size of the control group (mean = 11.7 mm^3) ($P<0.05$), while infusion of tuftsin starting 5 days after tumor

injection had no significant effect (Figure 3-7A, B). Immunofluorescent staining with Iba1 revealed that tuftsin activated MG/MP (with morphology as an output) in the area surrounding the infusion site in the absence of glioma cell implantation (Figure 3-6). Upon glioma inoculation, MG/MP numbers and the intensity of Iba1 signal increased in and around the tumor by tuftsin administration immediately after tumor cell injection (Figure 3-7A, C, 3-8B). After we quantified the proliferation (Ki67) and apoptosis (activated caspase-3) in the saline and D0 tuftsin group, we found that the infusion of tuftsin increased the proliferation both in tumor tissue volume and tumor cell number as compared to those of the saline control group (Figure 3-8). Meanwhile, tuftsin also decreased the apoptosis of both tumor cells and GIMs (Figure 3-9).

MIF inhibits the growth of glioma cells in the presence of microglia

Based on our results and previous findings (Ginzkey et al., 2009), the size of gliomas *in vivo* appears to increase upon administration of stimulators of MG/MP. We used therefore an inhibitor of MG/MP, MIF, to evaluate whether it would reduce glioma growth. MIF (tuftsin fragment 1-3, TKP) is a tripeptide and blocks the activity of monocytic cells *in vitro* and *in vivo* (Thanos et al., 1993). The infusion of MIF to the site of excitotoxic injury inhibits microglia activation, and MIF treatment of primary microglia inhibits lipopolysaccharide (LPS)-mediated tumor necrosis factor (TNF) -α release (Rogove and Tsirka, 1998). As shown above, an indirect and direct co-culture system was utilized to assess the effects of MIF on glioma cell growth. In the indirect system, in the presence of MIF and microglia the growth of GL261-EGFP cells slightly slowed down 6 days after co-culture compared to that of GL261-EGFP cells with

microglia only ($P<0.05$) (Figure 3-5C). In the direct co-culture in the presence of microglia, MIF treatment had a very modest effect on GL261-EGFP growth 2 days after co-culture compared to that of GL261-EGFP cells with microglia only ($P<0.01$) (Figure 3-5E). MIF-treated microglia resulted in lower total GL261-EGFP cell numbers than GL261 alone at day 4 ($P<0.01$). Since MIF had no effect on GL261-EGFP growth in the absence of microglia (Figure 3-5B, D), the MIF-mediated inhibitory effect on glioma cell proliferation in the presence of microglia was probably due to the attenuated microglial activation. These results were then explored *in vivo*: delivery of MIF 5 days after tumor injection inhibited tumor growth and MG/MP infiltration (Figure 3-7). The quantification of Ki67 (cell proliferation) and activated caspase-3 (cell death) in saline control mice and D5 MIF-treated mice revealed that MIF treatment inhibited glioma cell proliferation, but had little effect on glioma cell apoptosis (Figures 3-8, 3-9). However MIF induced apoptosis of GIMs, which may contribute to the lower level of MG/MP infiltration (Figure 3-9).

Tuftsin and MIF treatment had no significant effects on glioma angiogenesis

Since we labeled MG/MP with tomato lectin, a marker for both MG/MP and endothelial cells, we next measured blood vessel density to quantify the angiogenic effects of tuftsin and MIF on gliomas (Figure 3-12). However, there was no significant difference in saline, D0 tuftsin and D5 MIF group based on blood vessel density and diameters. These results indicated that the different MG/MP infiltration induced by tuftsin and MIF treatment, but not endothelial cells, contribute majorly to the difference observed in tomato lectin staining (Figure 3-7C).

Tuftsin and MIF treatment had different effects on CCL21 expression in glioma tissue

The chemokine CCL21 is expressed by many cancers, including breast cancer and melanoma, and was recently shown to function as an immune suppressive chemokine, which facilitates the immune escape of tumors (Shields et al., 2007; Shields et al., 2010). Here we examined whether the treatment of tuftsin and MIF could modulate CCL21 expression in glioma tissue. The immunofluorescent staining of CCL21 revealed that primarily stromal cells and some glioma cells expressed CCL21 (Figure 3-10), while the adjacent normal brain tissue did not show any positive staining (Figure 3-11A). The D0 tuftsin group had more CCL21-positive cells in glioma tissue compared to saline control group, however, the D5 MIF group revealed a significant decrease in the number of CCL21-expressing cells (Figures 3-11A, B). These results suggested that modulating microglial activation, potentially using MIF as a treatment, may be beneficial for the treatment of glioma by attenuating the immune microenvironment surrounding the gliomas.

Discussion

The components of tumor mass are tumor and stromal cells, which interact with each other and ultimately also affect tumor growth. Among the stromal cells, macrophages have been shown to have dual roles, which are both tumor-suppressing and tumor-promoting (Allavena et al., 2008; Solinas et al., 2009). Macrophages can secrete anti-tumor cytokines and interact with T cells to destroy tumor cells (Galani et al., 2009). On the other hand they can be recruited by tumor-released chemokines and switch their immune response to tumor-supporting function by the tumor microenvironment (Demuth and Berens, 2004; Pollard, 2004).

Glioma cells release chemokines and growth factors such as CSF-1, GM-CSF, MCP-1, and HGF/SF, which recruit and promote the growth of tumor-infiltrating MG/MP (Alterman and Stanley, 1994; Badie et al., 1999; Kielian et al., 2002; Leung et al., 1997; Nitta et al., 1992). Moreover, tumor-infiltrating MG/MP are commonly activated and proliferate in gliomas (Klein and Roggendorf, 2001). In our study, we show that glioma conditioned medium, but not astrocyte-derived medium, activated microglia, which is consistent with previous *in vivo* data (Klein and Roggendorf, 2001). In the GL261 mouse glioma model, in agreement with data from human glioma specimens, we found strong infiltration of activated MG/MP within and around tumor tissue, and the density of MG/MP in the tumor area was higher than the density of MG/MP that surrounded relatively normal brain tissue.

MG/MP are crucial to the innate and adaptive immune responses in CNS. Patients with gliomas often have deficiency in immunologic responses against the tumor,

including cutaneous anergy, lymphopenia, decreased Ig production, and impaired T cell responses (Morford et al., 1997). Although up to one third of all cells in glioma specimens were MG/MP, T cells were rarely seen in gliomas (Morimura et al., 1990; Roggendorf et al., 1996). Independent studies showed that the antigen-presenting function of microglia was compromised in gliomas, since the MHC class II molecules and co-stimulatory molecules exhibited decreased their expression levels (Badie et al., 2002; Flugel et al., 1999). The studies on the innate immune response of GIMs isolated from glioma tissue demonstrated that they expressed TLRs and mediated phagocytosis, but lacked production of IL-1β and TNF-α, which are important cytokines for tumor rejection (Frei et al., 1994; Hussain et al., 2006). Moreover in a CD11b-HSVTK glioma model similar to ours, Markovic et al. (2009) reported that activation of MMPs in glioma is promoted via activation of TLRs and MT1-MMP in microglia. Our data also shed light on the innate immune response of MG/MP against gliomas. When microglia encountered glioma cells in culture, they first exhibited morphological changes, phagocytosis and up-regulation of surface activation markers. However, after three days in co-culture, phagocytosis was rarely observed and the growth of glioma cells were unaffected by the microglia, which suggested the presence of an immunosuppressive environment within gliomas. The components contributing to this suppression could be Th2/Th3 cytokines released by glioma cells, such as IL-6, IL-11, LIF, OSM, and TGF-β, which are able to inhibit cytotoxic T-cell activation and MG/MP activation (Constam et al., 1992; Goswami et al., 1998; Halfter et al., 1998; Hao et al., 2002; Murphy et al., 1995).

On the other hand, GIMs themselves release many factors, such as cytokines, growth factors and proteases, which directly or indirectly influence tumor progression

(Graeber et al., 2002). It has been shown that GL261 cell migration occurred earlier and faster in the presence of primary microglia or microglia-conditioned medium, which indicated that microglia-released soluble factors can promote glioma cell migration (Bettinger et al., 2002). GIMs are the major source of IL-10, which is a strong immunosuppressive cytokine, able to promote the proliferation and migration of glioma cells (Huettner et al., 1997; Wagner et al., 1999). Moreover, the glioma-derived and GIM-derived TGF-β has multiple effects on glioma progression: it can inhibit the proliferation of microglia and release of cytokines *in vitro* (Suzumura et al., 1993). It can also induce MMP expression and promote the invasion of glioma cells (Markovic et al., 2009; Wesolowska et al., 2008; Wick et al., 2001). Both GIMs and glioma cells secrete VEGF (Lafuente et al., 1999; Tsai et al., 1995), which is a well-documented factor in mediating angiogenesis. These findings suggested that gliomas recruited MG/MP, inhibit their immune response and induce the MG/MP production of tumor survival factors, which in turn facilitate glioma growth and malignancy.

Our experiments further supported this hypothesis and provide new evidence of the tumor-promoting function of GIMs. In culture, we showed that glioma cells grew faster in the presence of primary microglia. *In vivo,* we demonstrated that the depletion of GIMs led to significant decrease in glioma size and prolonged survival of tumor-bearing mice. The results presented here are in agreement with recent data by Markovic et al. (Markovic et al., 2009), but provide more detailed characterization of the model. Since GIMs are a significant source of tumor survival factors, the loss of GIMs may result in decrease of immunosuppressive cytokines, growth factors and proteases, which are essential for glioma growth. In addition, the GCV / HSVTK system has been shown to

have “bystander effect”, which is mediated by the transfer of toxic GCV metabolites through gap junctions of adjacent cells (Culver et al., 1992; Mesnil et al., 1996). Since primary microglia have close interactions with glioma cells in culture, it is possible that the decrease of glioma cells in the GIM-ablated model was partially induced by the death of adjacent HSVTK-positive GIMs. Thus our data demonstrate that the presence of GIMs is crucial for glioma growth, and GIMs could potentially be manipulated to interfere with glioma progression.

In a previous report by Galarneau et al., it was shown that the depletion of macrophages in CD11b-TK^{mt-30} mice via systemic injection of GCV resulted in increase of glioma growth (Galarneau et al., 2007). These results may agree with the early-time points of our data, but the final outcome is different. The difference may lie in the experimental system (administration route of GCV, reconstitution of microglia with bone-marrow derived wild-type cells).

The recent strategies for glioma therapy are – among many others - interventions aiming to manipulate the activity of tumor-associated macrophages, including inhibition of their recruitment to the tumor, their survival in tumor tissue as well as restoration of their anti-tumor immunity (Allavena et al., 2005; Sessa et al., 2005; Wu et al., 2009). However, the development of therapeutic anti-glioma options by modulating the activity of GIM has not been thoroughly investigated. Glioma cell lines used in animal studies are also of different immunogenicity and thus probably one of the reasons for controversial results found in the literature. For example, the injection of CpG oligonucleotides to GL261 mouse glioma cells resulted in contradictory data in one 9L rat glioma model (El

Andaloussi et al., 2006; Ginzkey et al., 2009), which suggests that MG/MP stimulators may have different effects on MG/MP activities depending on the tumor microenvironment. Our results also suggest that the use of the MG/MP activator tuftsin will not be an effective, i.e. useful treatment option for glioma patients. Although tuftsin has been widely used in different anticancer studies (Banks et al., 1985; Noyes et al., 1981; Wleklik et al., 1986), its infusion into the mouse glioma model presented here exaggerated tumor growth. It has been shown that tuftsin can activate monocytes and macrophages leading to the release of IL-1, TNF-α and nitric oxide (Cillari et al., 1994; Robey et al., 1987; Wleklik et al., 1987), which possibly contributed to the initial slowing down of glioma cell growth. However, due to the immunosuppressive environment within gliomas, the activation effects of tuftsin on GIMs may switch them towards a rather tumor-promoting phenotype at later stages. Interestingly, similar results were obtained in a mouse model of multiple sclerosis, where the infusion of tuftsin altered the host immune response towards favoring the expression of immunosuppressive Th2 genes (Bhasin et al., 2007).

In contrast, the use of MIF in a glioma-microglia co-culture led to optimistic results, since the presence of MIF and microglia slowed down glioma cell growth. Notably, also *in vivo* delivery of MIF to the tumor site inhibited tumor proliferation and induced apoptosis of GIM, which is in agreement with the current trend of tumor therapy to inhibit macrophage recruitment and survival at the tumor site (Allavena et al., 2005; Meng et al., 2010; Miselis et al., 2008). Along this line, it has been shown that delivery of MIF to the injury site after intracerebral hemorrhage (ICH) in the mouse brain inhibited

MG/MP infiltration and activation, which had protective effects (Wang and Tsirka, 2005).

One possible mechanism contributing to the above results could be the change of CCL21 expression level in glioma tissue. A recent report (Shields et al., 2010) showed that melanoma cells could shift their microenvironment to an immune tolerant one by expressing CCL21. This led to the formation of a lymphoid-like stromal structure, increased the levels of TGF-β1 and the number of myeloid-derived suppressor cells. Melanoma cells upregulated CCL21 in stromal cells, and this induced expression was a major contributor to an immunotolerant microenvironment, which resulted in promotion of melanoma progression. Consistent with these findings, we find that glioma, but mostly surrounding stromal cells expressed CCL21 two weeks after glioma inoculation, which suggested that the glioma microenvironment is immune suppressive. Moreover, tuftsin treatment increased CCL21 levels in the glioma tissue, whereas MIF treatment had the opposite effect. In GCV-treated CD11b-HSVTK mice, the expression of CCL21 was not detectable (data not shown), indicating that the modulation of GIM activities can possibly change the tumor microenvironment and modulate tumor progression.

Taken together, our data demonstrate that GIMs promote glioma growth and invasion, and, that depletion or pharmacological inhibition of GIMs is in the longer-term beneficial to glioma-bearing animals. Thus, inhibiting the activity of GIM by local delivery of factors such as MIF may be a potential novel interventional, adjuvant regimen to tackle gliomas.

Figure 3 - 1. GCM activated primary microglia. Primary microglia were treated with GCM or with DMEM + 1% FBS (microglial medium) or 10% FBS (glioma medium) as control. At each time point, cells were observed under bright field microscopy (A), were immunofluorescently stained with Iba1 antibody and labeled with DAPI to visualize nuclei (B), and lysed to obtain protein extracts for western blotting (C). Iba1 expression levels were normalized to α-tubulin. The experiment was repeated 3 times. Arrows: microglia with amoeboid shapes. Scale bar: A – 50 µm; B – 20 µm.

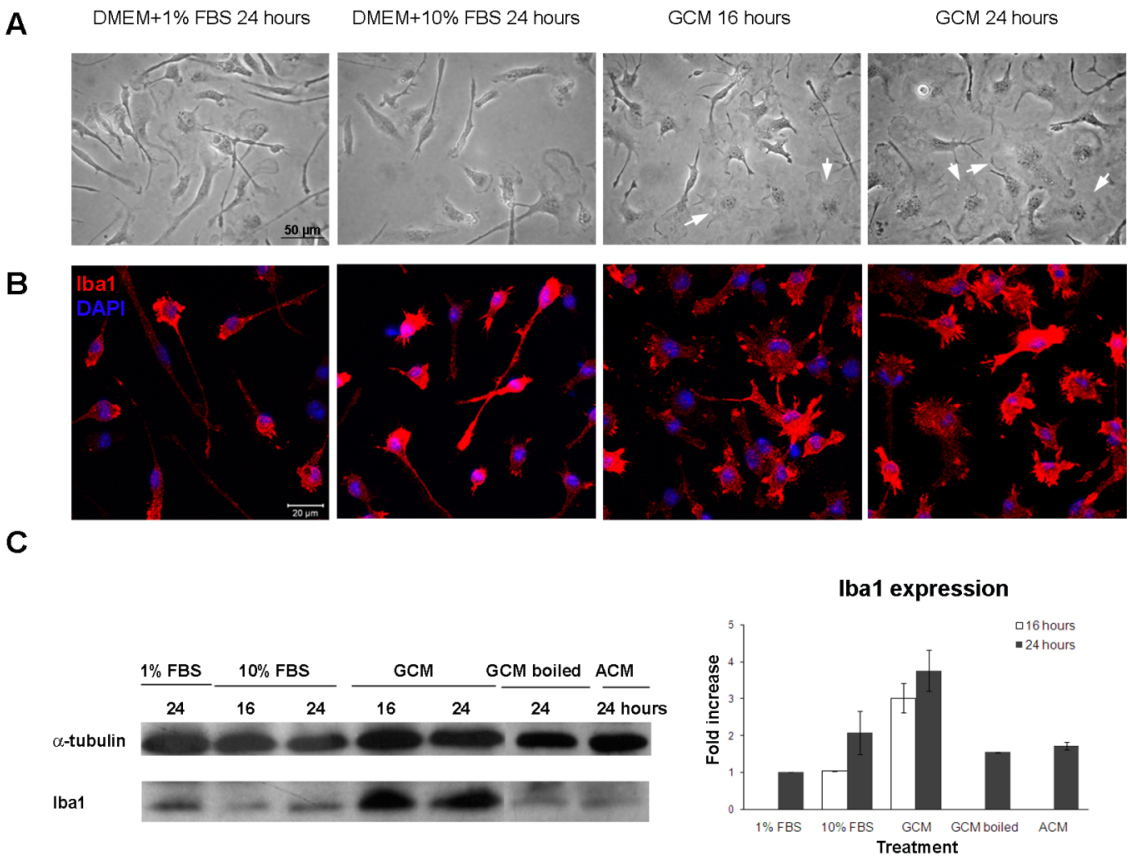


Figure 3 - 2. Microglia-glioma co-culture. $5x10^4$ rhodamine-labeled primary microglia (A) were plated and left at resting conditions in DMEM + 1% FBS (microglial medium) for 48 hours before $2x10^4$ CRL-2541-EGFP (B) or GL261-EGFP (C, D) were plated in the same well. DMEM + 10% FBS was used as a negative control. Confocal images (A - D) were taken every day for 5 days. (A – D) red fluorescent cells: primary microglia; green fluorescent cells: CRL-2541-EGFP or GL261-EGFP as indicated. Arrows: phagocytic microglia with GFP protein in cell body. Insets: the red –microglia- channel has been removed to display the green fluorescence within microglial cells, indicative of phagocytic events. The scale bar in panels A-C is 50µm, whereas in panel D it is 20µm.

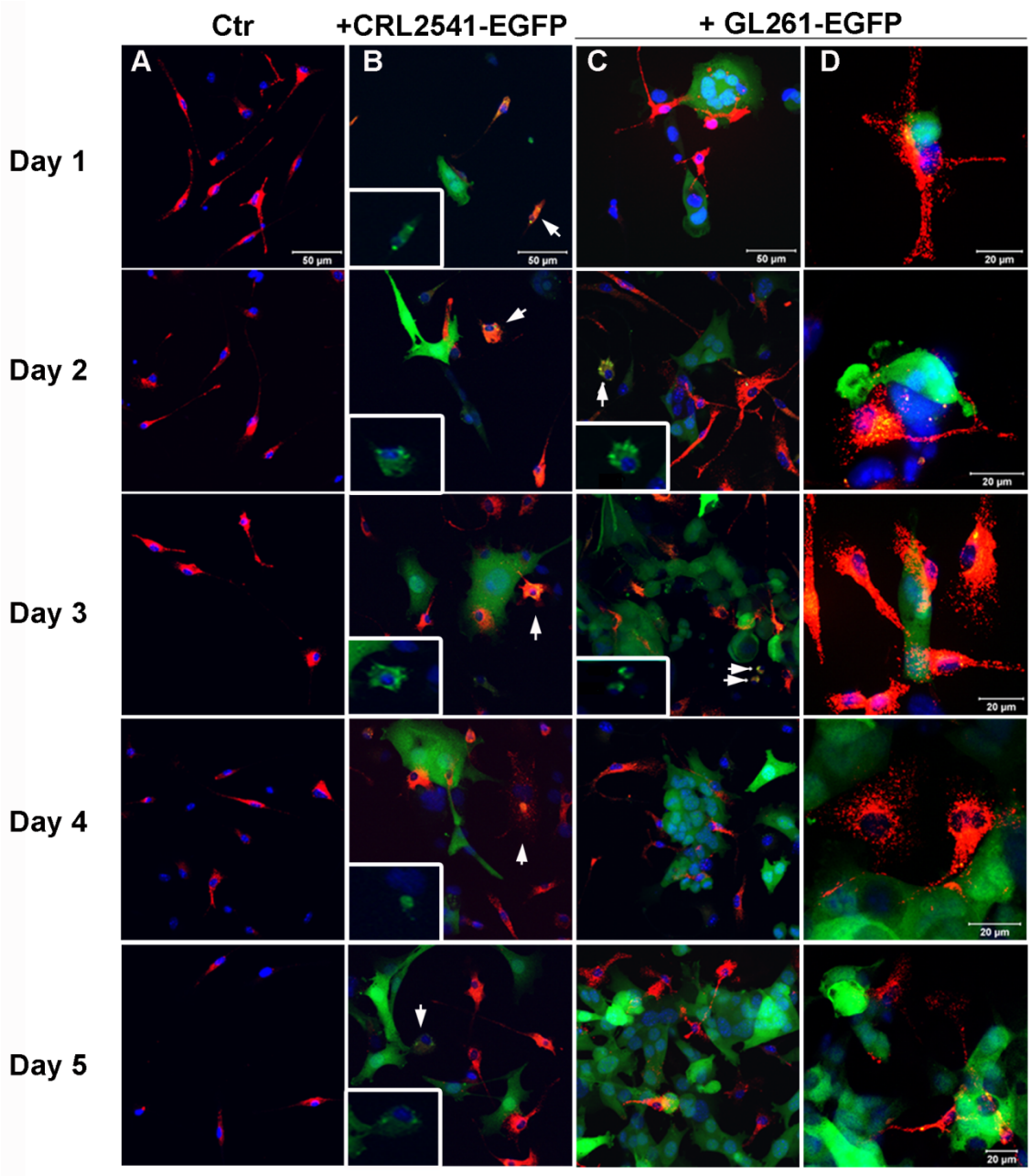


Figure 3 - 3. Microglia promote glioma growth in culture. $5x10^4$ rhodamine-labeled primary microglia were plated and left at resting conditions in DMEM + 1% FBS (microglial medium) for 48 hours before $2x10^4$ CRL-2541-EGFP or GL261-EGFP were plated in the same well. Cell number of each population was counted every day for 7 days. *** indicates the statistical significance between the cell numbers of GL261 alone and GL261 in co-culture. The experiment was repeated 3 times. (B) Slope of growth curves were calculated using linear data from day 2 to 5: GL261 alone – 6.0248; GL261 in co-culture with microglia – 6.9347.

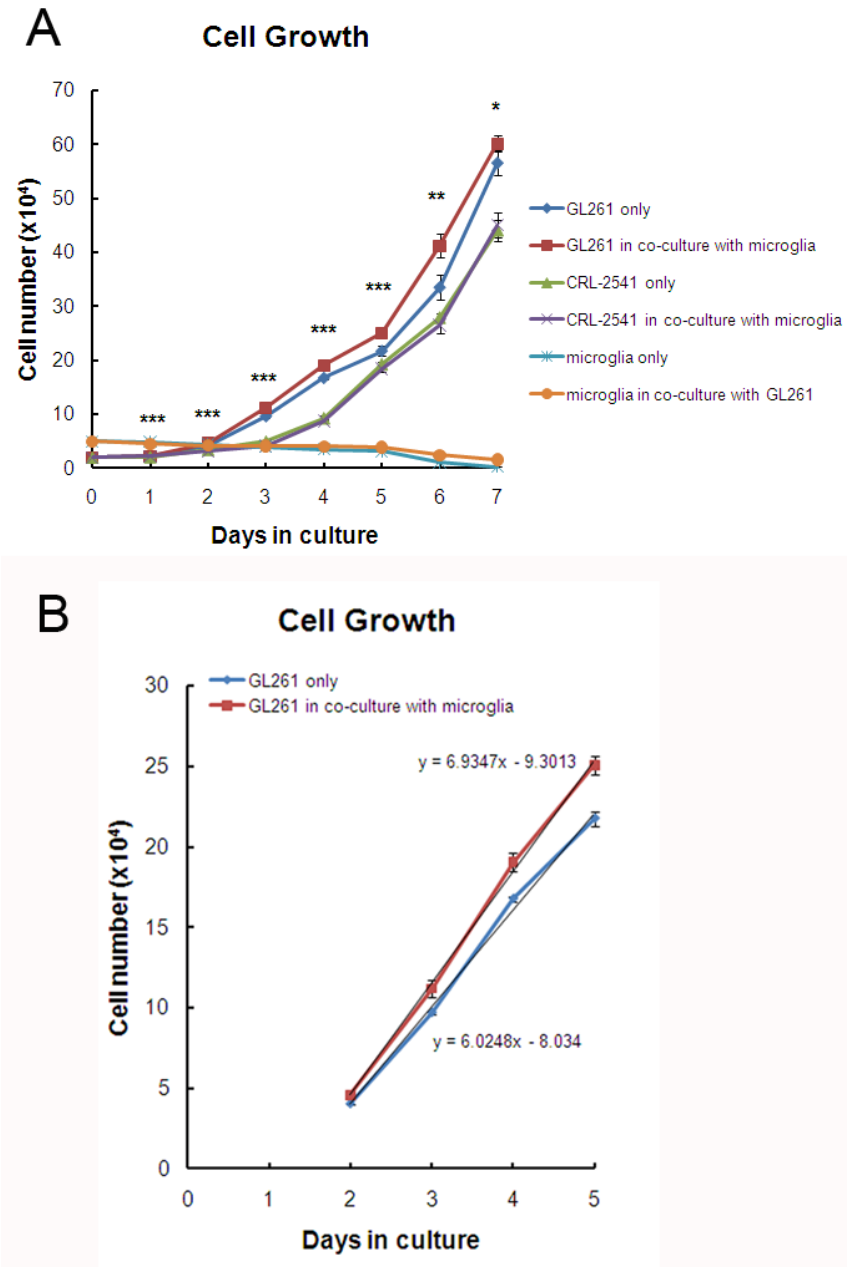


Figure 3 – 4A. MG/MP ablation slowed down glioma progression. (A) GL261-EGFP cells were treated with different concentrations of GCV, and cell proliferation was quantified with the CCK-8 kit every 2 days for 8 days (25, 50 μg/ml group at day 4, 6 and 8: $P < 0.01$). (B) WT mice were injected with GL261-EGFP and infused with different concentrations of GCV. Tumor sizes were measured 14 days after injection. (0, 1 mg/ml group: n = 5, 1.25, 1.5 mg/ml group: n = 2). (C) Schematic illustration of tumor injection and GCV infusion. (D) Tumor sizes in WT and HSVTK mice were measured 14 days after GL261-EGFP injection and infusion of 1 mg/ml GCV or saline (WT saline and GCV group: n = 5; HSVTK saline and D0 GCV group: n = 6; HSVTK D5 GCV group: n = 4).

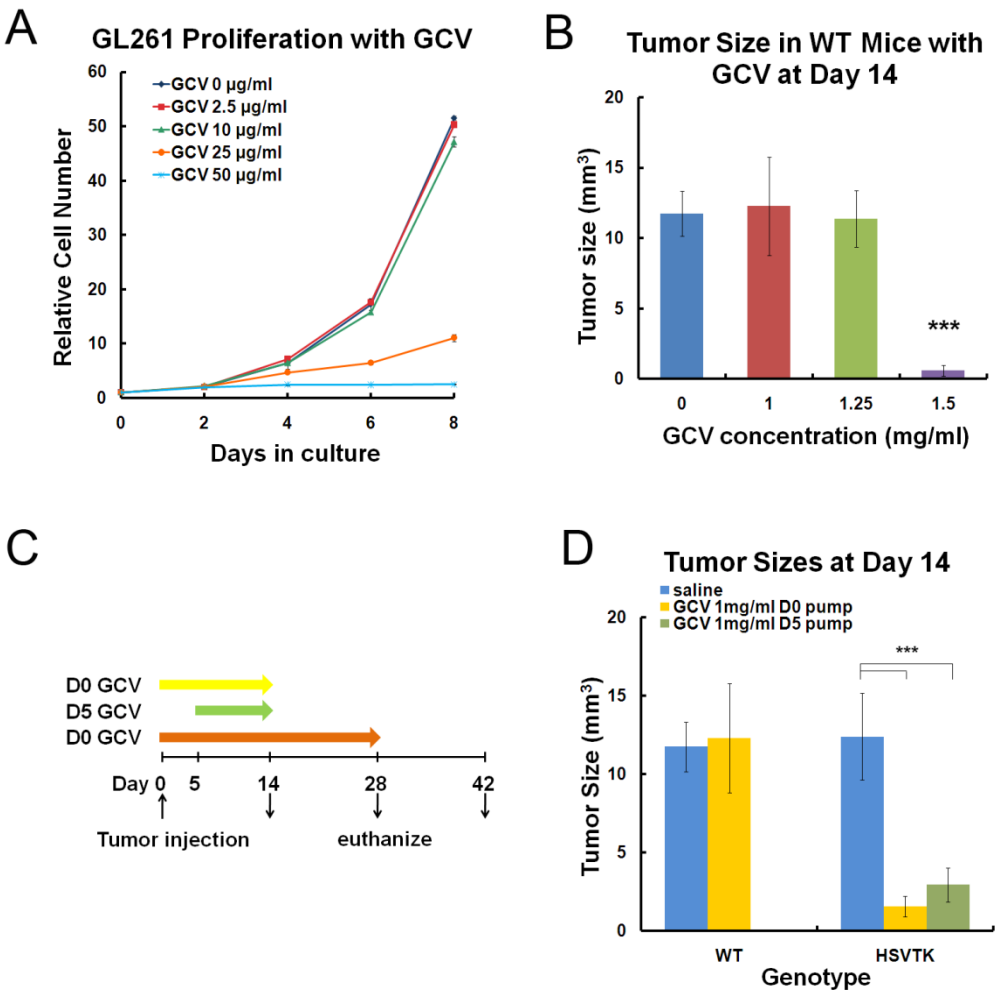


Figure 3 – 4B. MG/MP ablation slowed down glioma progression. (E) Tumor sizes were measured at different time points after GL261-EGFP injection and infusion of 1 mg/ml GCV or saline (WT saline group: day 10 n = 6, day 14 n = 5, day 20 n = 8; HSVTK D0 GCV group: day 14 n = 6, day 28 n = 3, day 42 n = 2). (F) Kaplan-Meier survival curves of tumor-bearing mice. WT saline group: n = 10, HSVTK saline and D0 GCV groups: n = 5. Saline group of WT and HSVTK mice: no significant difference; HSVTK GCV group vs. saline groups: $P < 0.01$. (G-K) Tumor morphology and MG/MP infiltration was evaluated at each time point. Representative images of (G) H&E staining (scale bar = 1000 µm; the tumor is demarcated by the dashed line); (H-I) GFP fluorescent signals (H: scale bar = 1000 µm; I: scale bar = 100 µm); (J-K) Iba1 immunofluorescence (J: scale bar = 100 µm; K: scale bar = 50 µm) is also shown.

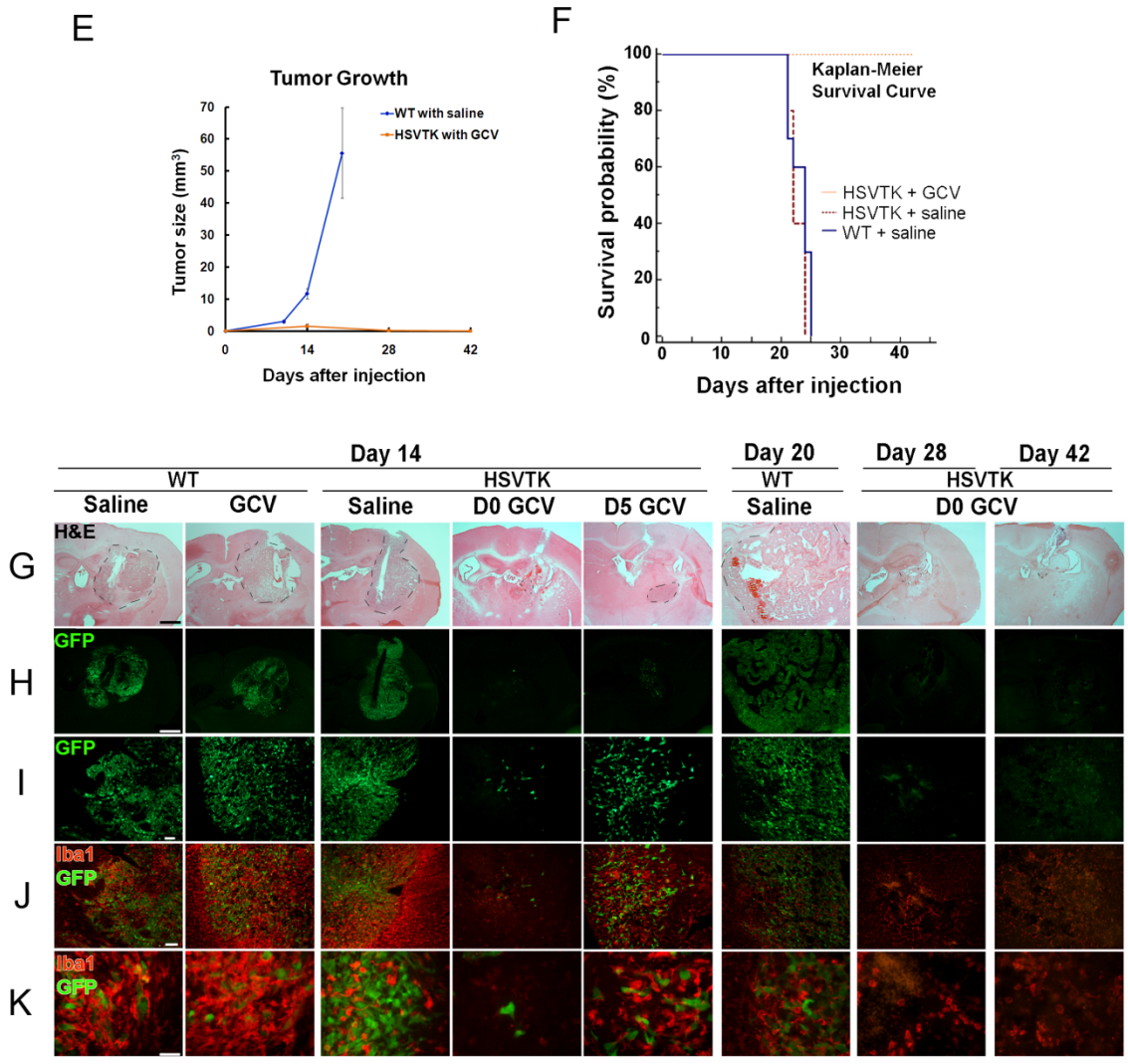


Figure 3 - 5. Different effects of tuftsin and MIF on GL261 growth in the presence of microglia. (A and B) GL261 were treated with different concentrations of tuftsin (A) or MIF (B), and cell proliferation was measured by CCK-8 kit every 2 days for 6 days. (C) GL261 were incubated with inserts containing primary microglia and treated with 150 µg/ml tuftsin or MIF. GL261 proliferation was measured every 2 days. Statistical analysis: GL261 only – M1, GL261 with microglia – M2, GL261 with microglia and tuftsin – M3, GL261 with microglia and MIF – M4. Day 2, 4 and 6, M1 vs. M2 or M3 or M4: $P < 0.01$; day 4, M2 vs. M3: $P < 0.05$; day 6, M2 vs. M3: $P < 0.01$, M2 vs. M4: $P < 0.05$, M3 vs. M4: $P < 0.01$. (D) GL261-EGFP cells were incubated with 150 µg/ml tuftsin or MIF and cell number was counted every day for 5 days. (E) GL261-EGFP cells were cultured with primary microglia in the presence of 150 µg/ml tuftsin or MIF, and GL261-EGFP cell number was counted every day for 5 days. Statistical analysis: M1 vs. M2, M2 vs. M4: day 2 –5 $P< 0.01$; M1 vs. M3, M3 vs. M4 day 2, 4 and 5 $P<0.01$; M1 vs. M4 day 4 and 5 $P<0.01$; M2 vs. M3 day3 $P<0.05$. All the experiments were repeated 3 times.

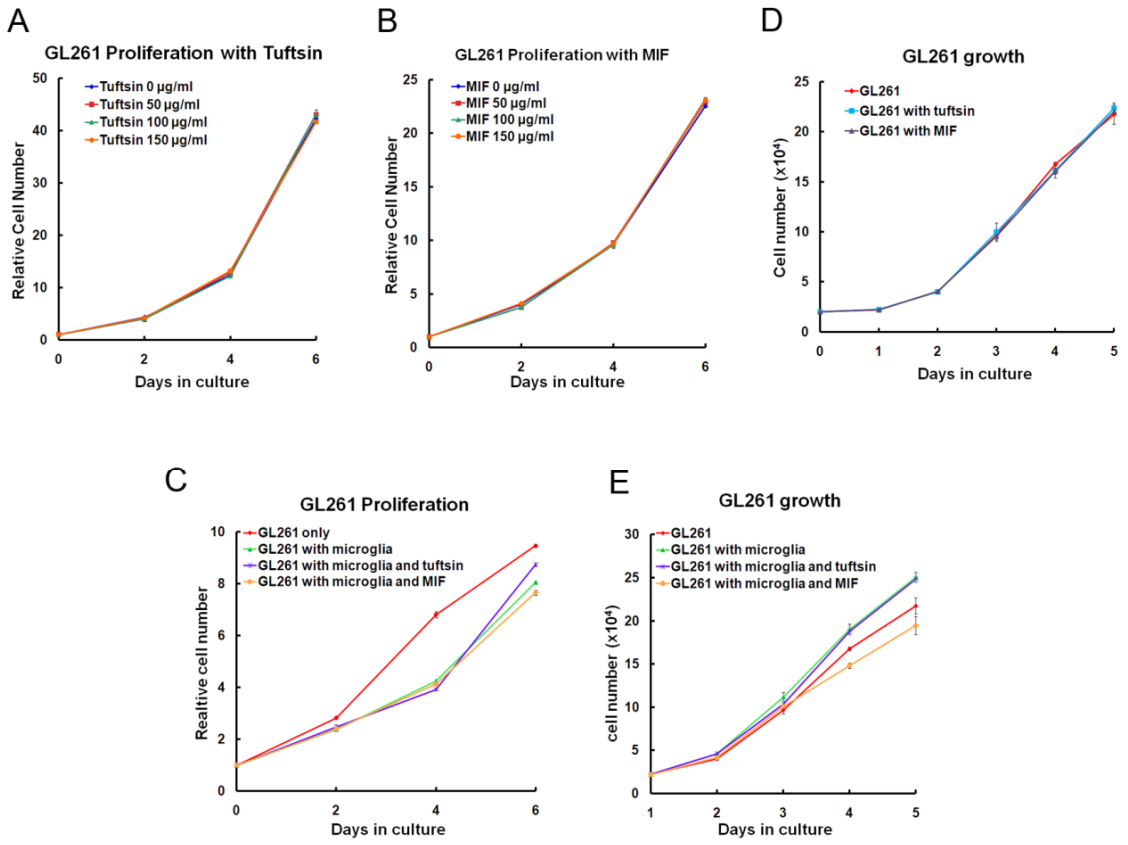


Figure 3 - 6. Tuftsin can activate MG/MP. Infusion of tuftsin into the mouse brain without injection of glioma cells activated MG/MP locally. Arrow indicates infusion canula track.

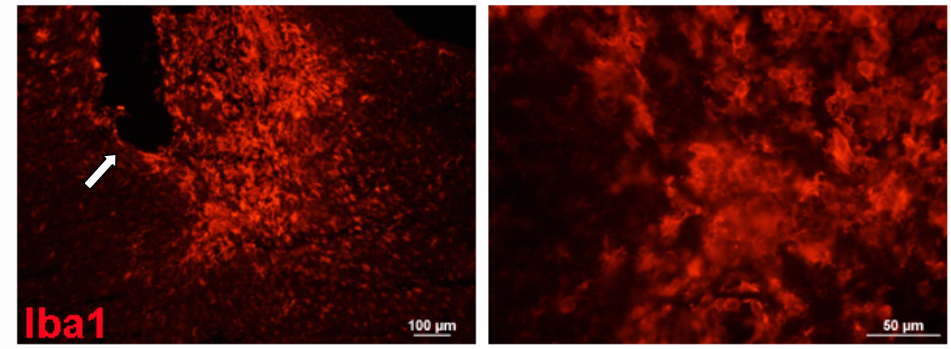


Figure 3 - 7. Tuftsin and MIF treatment had different effects on glioma progression *in vivo*. Tumor sizes (B) and MG/MP infiltration (A) (Iba1^{+} cells) were evaluated 14 days after GL261-EGFP injection and infusion of 25µg/ml tuftsin (TF), MIF or saline (scale bar 500 µm). The GIM density (C) in saline group, D0 tuftsin group and D5 MIF group were quantified (n = 3).

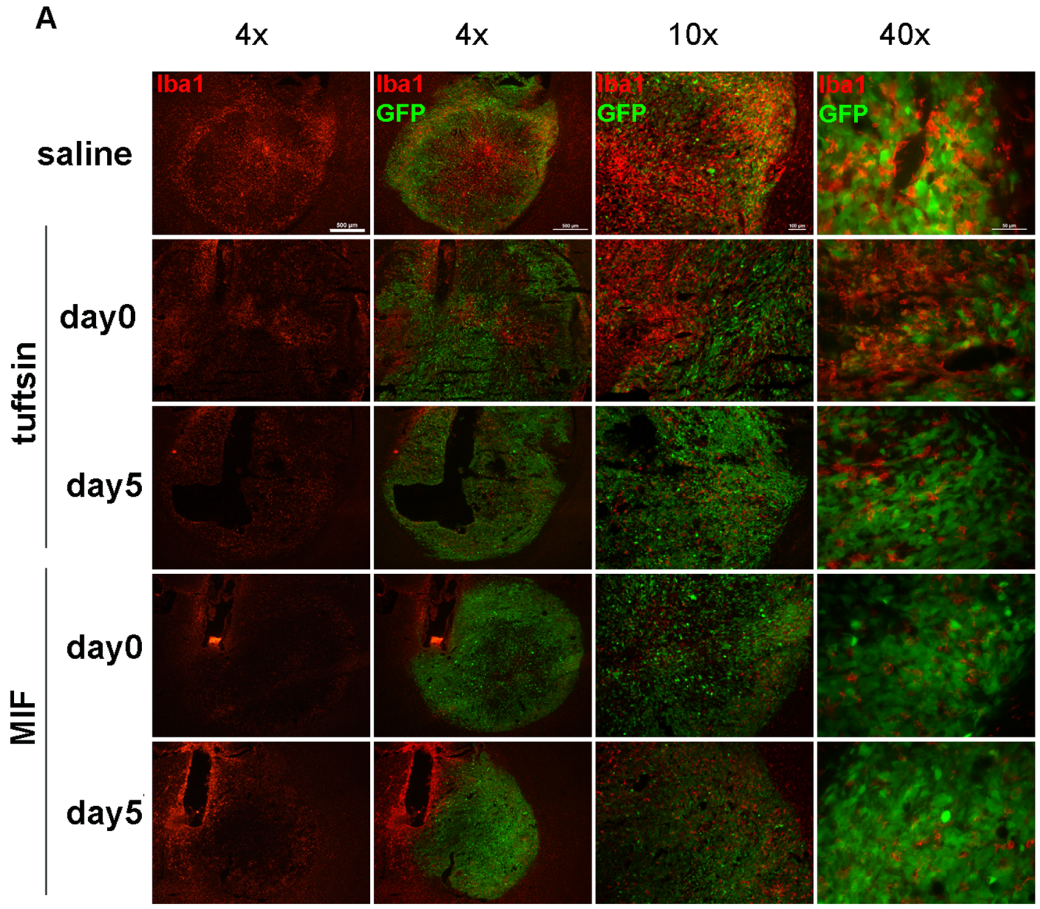


Figure 3 - 8. Tuftsin and MIF treatment had different effects on glioma proliferation *in vivo*. (A) Higher magnification (scale bar 20 µm) pictures of immuofluorescent staining with tomato lectin and Ki67 to demonstrate the cell specificity. The proliferation of tumor tissue and tumor cells in saline group, D0 tuftsin group and D5 MIF group were quantified (C) after (B) Ki67 (scale bar = 50 µm) immunofluorescent staining (n = 3).

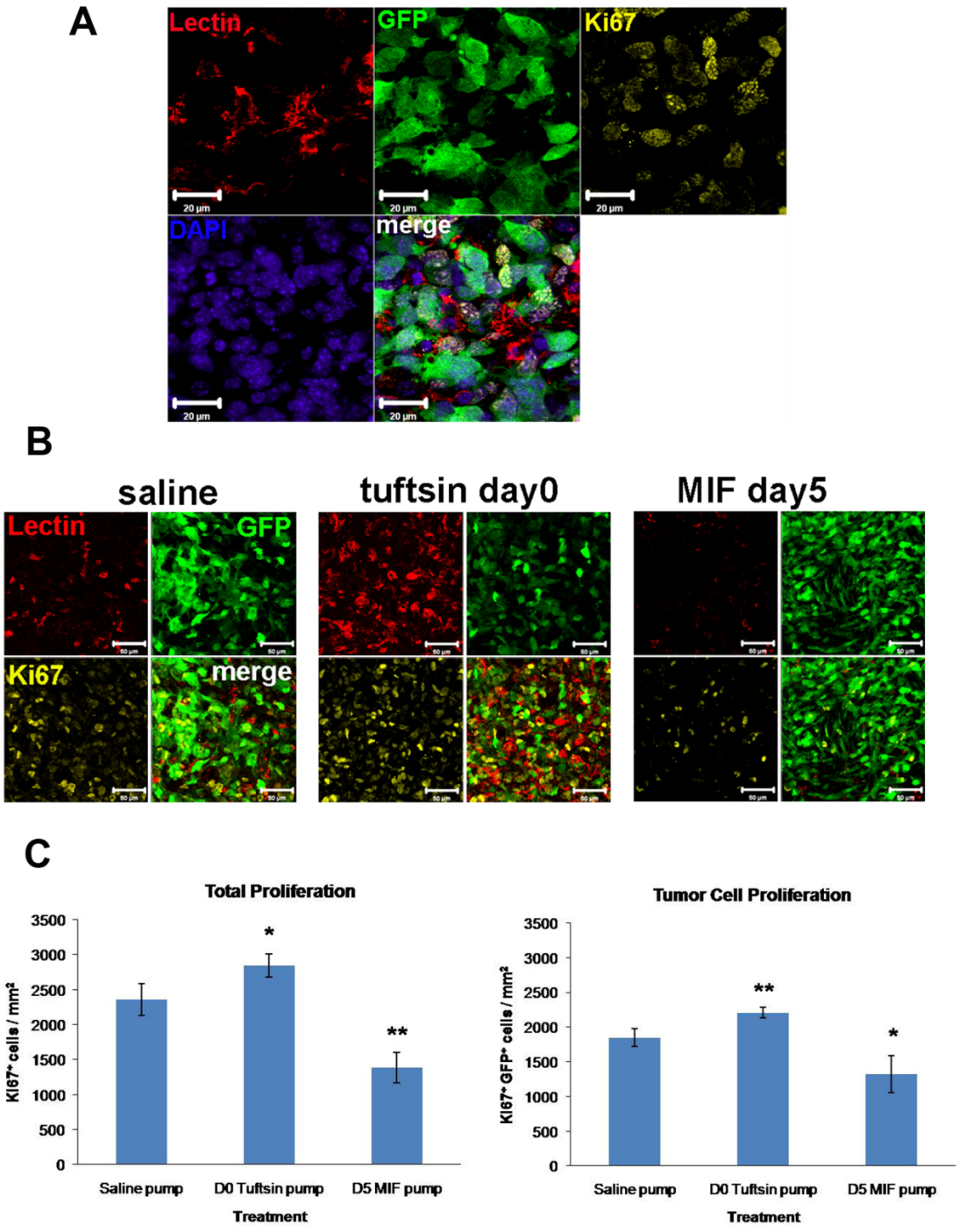


Figure 3 - 9. Tuftsin and MIF treatment had different effects on glioma apoptosis *in vivo*. (A) Higher magnification (scale bar 20 µm) pictures of immuofluorescent staining with tomato lectin and activated caspase-3 to demonstrate the cell specificity. The proliferation of tumor tissue and tumor cells in saline group, D0 tuftsin group and D5 MIF group were quantified (C) after (B) activated caspase-3 (scale bar = 100 µm) immunofluorescent staining (n = 3).

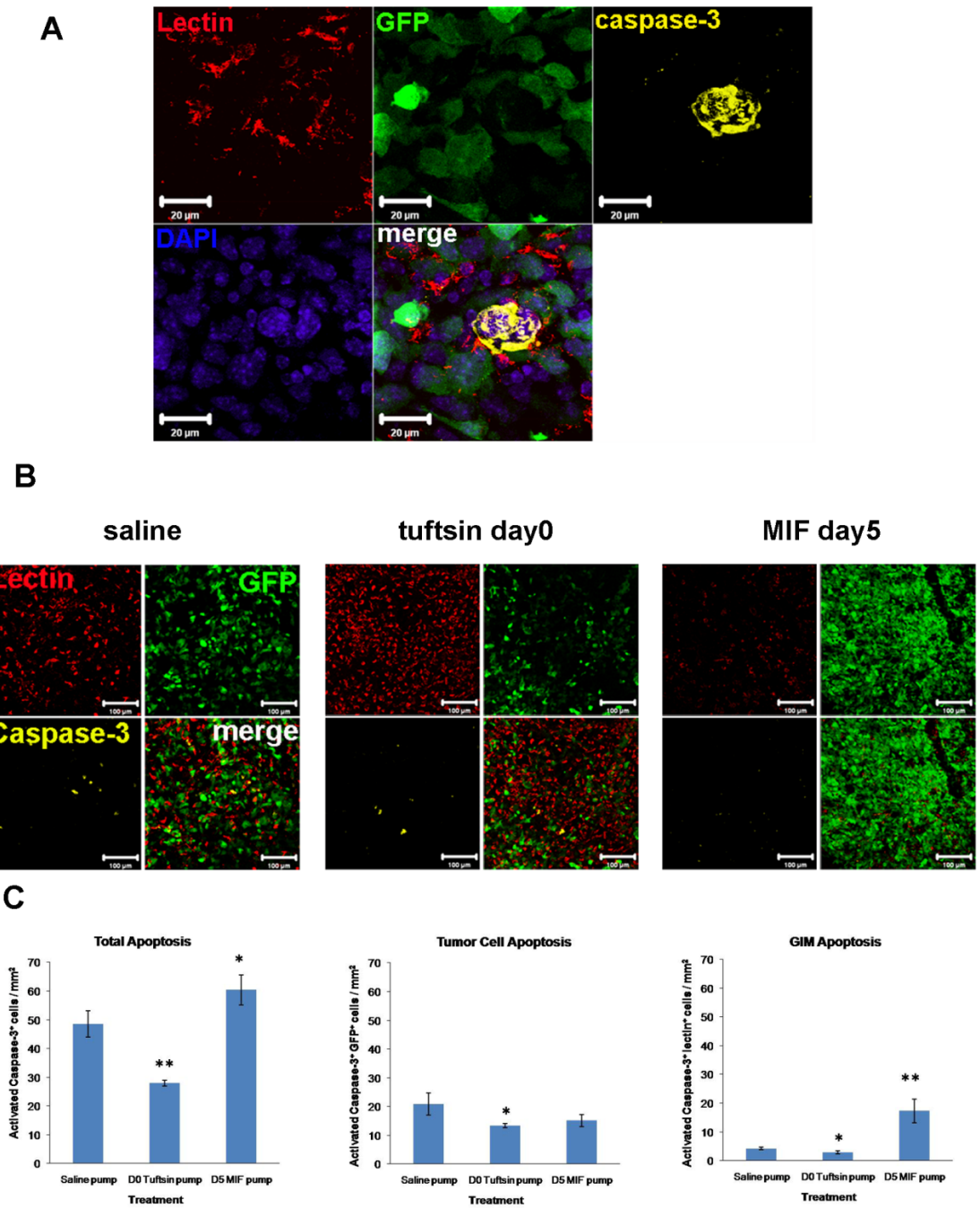


Figure 3 - 10. CCL21 is expressed by both tumor cells and stromal cells. Higher magnification pictures of immunofluorescent staining with CCL21 show the cell localization of CCL21 expression. Arrows indicate the expression of CCL21 in stromal cells (not expressing GFP). (scale bar = 20 µm)

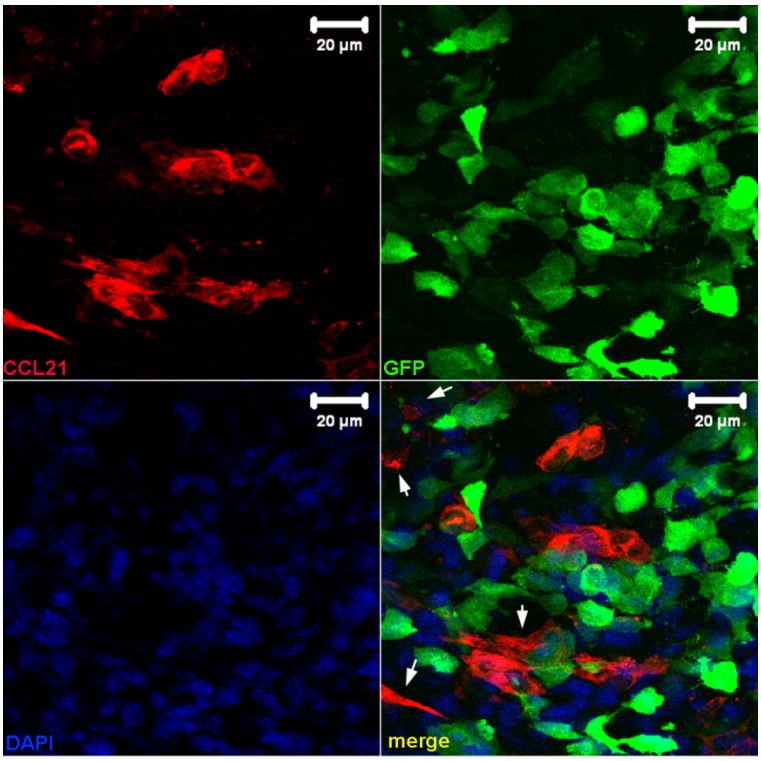


Figure 3 - 11. Tuftsin and MIF treatment had different effects on CCL21 expression in glioma tissue. 14 days after GL261-EGFP injection and infusion of 25µg/ml tuftsin (TF), MIF or saline, CCL21 expression levels were quantified by counting $CCL21^+$ cells within the tumor tissue (B) (n = 3). Representative images from tumor border and center are shown (A). White dashed lines indicate the tumor borders. (scale bar = 100 µm)

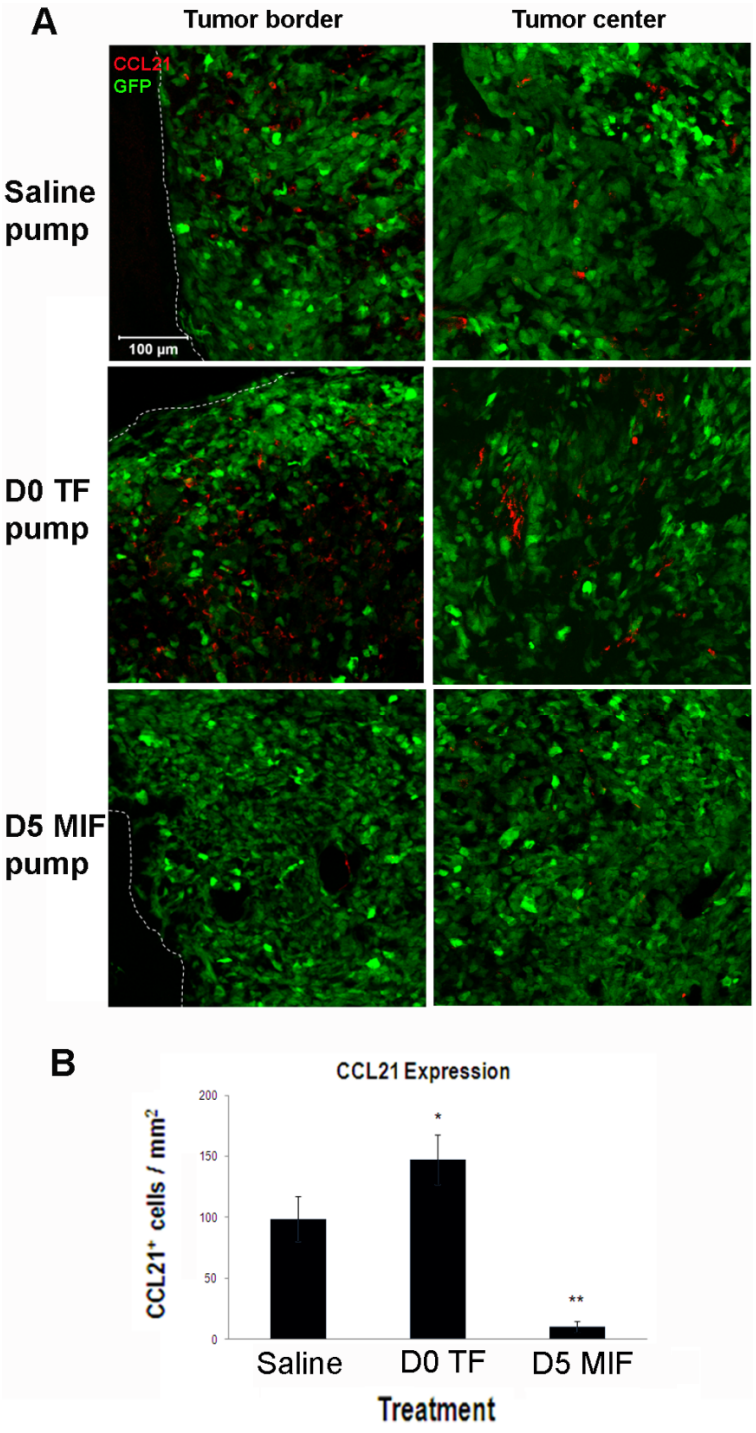
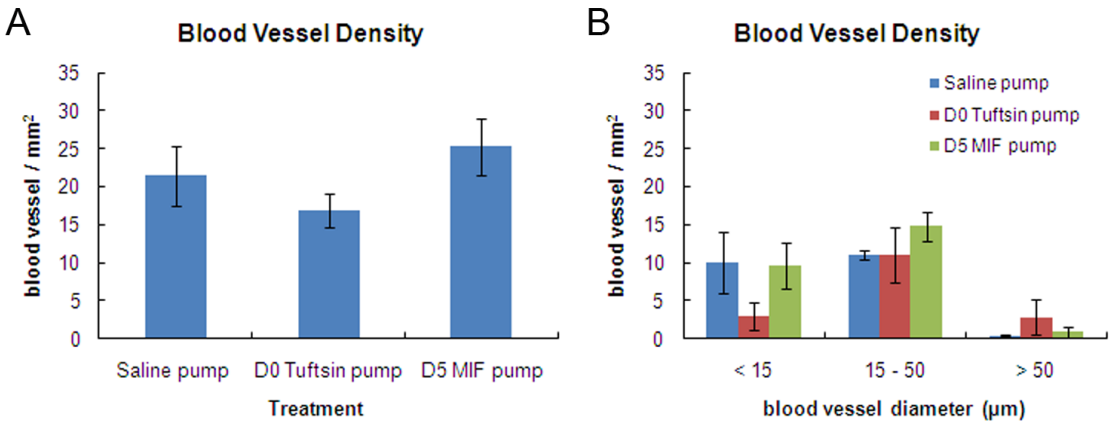


Figure 3 - 12. Tuftsin and MIF treatment had no significant effects on glioma angiogenesis. The blood vessels were identified based on immunofluorescent staining of endothelial cells (tomato lectin). Blood vessel density (A) and diameter (B) were measured in glioma tissue from twenty 20x fields. (n = 3)



Chapter 4

Conclusions and future directions

Summary of conclusions

The components of tumor mass are excessively proliferating tumor cells and stromal cells constituting the tumor microenvironment, which interact with each other and ultimately affect tumor growth. In this study, we investigated the contribution of both components of gliomas and their interactions.

In the first part (Figure 4 – 2B), we studied how glioma-released proteases regulate the glioma microenvironment and tumor progression. We found that GL261, a mouse glioma cell line, overexpresses annexin A2, a surface receptor for tPA and plasminogen. We knocked down annexin A2 expression in glioma cells, and found that the annA2KD decreased membrane-bound tPA activity, which subsequently led to reduced level of plasmin generation on the glioma cell surface. Annexin A2 is a substrate of v-Src kinase and is involved in cell transformation (Hayes and Moss, 2009), which could explain the decreased glioma cell migration *in vitro* observed in annA2KD group. Plasmin is capable of ECM degradation and remodeling, which correlates with cancer invasion and angiogenesis (Dano et al., 1985; DeClerck et al., 1997; Levicar et al., 2003b; Lijnen, 2001). Thus knockdown of annexin A2 in glioma cells would potentially lead to reduction of invasion and angiogenesis *in vivo*. Confirming this theory, we found

that knockdown of annexin A2 in glioma cells decreased tumor size and slowed down tumor progression, characterized by decreased invasion and angiogenesis, which are two key steps for tumor development and expansion. As a result, we found that there was less proliferation and more apoptosis in tumor tissue of the annA2KD group. Taken together, our findings demonstrated that inhibition of annexin A2 expression in glioma cells may become a novel therapy for glioma patients.

In the second part (Figure 4 – 2A), we studied the contribution of inflammatory cells in tumor microenvironment to glioma progression. MG/MP have an important regulatory role in CNS immunity. They become an attractive candidate in immunotherapy for gliomas because of their function of communicating between immune-privileged CNS and the peripheral immune system, as well as their significant infiltration in gliomas. A detailed study of MG/MP–glioma interactions and manipulation of the glioma microenvironment may allow the generation of a specific and durable anti-glioma immunity. Here, we used a glioma-microglia co-culture system to observe the effects the tumor and microglia have on each other, and found that glioma cells activated microglia but inhibited their phagocytic activities. We then assessed glioma progression *in vivo* after MG/MP ablation or exaggerated activation. Local genetic depletion or pharmacological inhibition of MG/MP *in vivo* significantly decreased tumor size and improved survival curves, suggesting that MG/MP are required for glioma development. Conversely, pharmacological activation of MG/MP increased glioma size through stimulating tumor proliferation and inhibiting apoptosis. The chemokine CCL21 is expressed by many cancers, including breast cancer and melanoma, and was recently shown to function as an immune suppressive chemokine, which facilitated the immune

escape of tumors (Shields et al., 2007; Shields et al., 2010). Consistently we found that expression of CCL21 was enhanced after MG/MP activation and correlated with tumor growth. Taken together, our findings demonstrate that inhibition of MG/MP activation may constitute a new and effective glioma therapy by suppressing tumor proliferation.

Future directions

Changes in signaling pathways in annA2KD glioma cells

In this study, we focused on the role of annexin A2 in enhancing proteolysis on glioma cell surface. However annexin A2 has been shown to participate several signaling pathways (Bao et al., 2009; Hayes and Moss, 2009; Inokuchi et al., 2009; Shiozawa et al., 2008), thus investigation of alterations in signaling pathways and gene expression in annA2KD glioma cells can provide new insights into the function of annexin A2 in glioma progression.

As discussed before, annexin A2 heteotetramer can bind and bundle actin filaments, which regulates cell motility (Hayes et al., 2004). The phosphorylation of annexin A2 by v-Src inhibits annexin A2-actin interactions (Hubaishy et al., 1995). v-Src phosphorylates focal adhesion kinase (FAK) and form a complex, which regulates focal adhesion turnover (Webb et al., 2004). Fibroblasts lacking annexin A2 failed to undergo transformation and anchorage-dependent growth, characterized by defective FAK phosphorylation and failure in targeting activated v-Src to cell membrane and its subsequent internalization (Hayes and Moss, 2009). These data suggest a dual role of

annexin A2, a v-Src effector in actin reorganization and a regulator for v-Src trafficking. In figure 2-4, we showed that annA2KD decreased glioma cell migratory activity in culture; however the mechanism contributing to this phenomenon is still not clear. In the future, we can investigate the regulation of the v-Src/FAK signaling pathway in these annA2KD glioma cells, which may also show defects in phosphorylation and molecule trafficking.

Annexin A2 has been shown to promote cancer cell proliferation and invasion through multiple signaling pathways. In prostate cancer, annexin A2 facilitates cancer cell growth partially via MAPK pathway (Shiozawa et al., 2008), as well as induction of IL-6 secretion, which is overexpressed in prostate cancer and enhance cell proliferation and survival (Inokuchi et al., 2009). Moreover, in myeloma cells, silencing annexin A2 gene by siRNA decreases mRNA level of VEGF, MMP-2, MMP-9 and MT1-MMP (Bao et al., 2009), which are important angiogenic factors for tumors. In addition, annexin A2 is involved in cell cycle regulation (Chiang et al., 1993), and p53 overexpression induces its downregulation in lung cancer cells (Huang et al., 2008). In our data (Figure 2-8, 2-9), we showed that annA2KD decreased proliferation and increased apoptosis of glioma cells *in vivo*. Further experiments investigating the above signaling pathways can be carried out to elucidate the mechanism, such as downstream effectors of annexin A2.

Anti-angiogenesis therapy for gliomas

Recently anti-angiogenesis therapy becomes an attractive strategy for cancer therapy, which has been demonstrated to an effective method for limiting tumor growth (Cao et al., 1998; Kesavan et al., 2010; Wu et al., 1997). In our data (Figure 2-3C, 2-10,

2-11), we showed that annA2KD in glioma cells led to decreased surface tPA activity and subsequent decreased invasion and angiogenesis *in vivo*, which is consistent with previous research in other cancers (Diaz et al., 2004; Sharma et al., 2010; Sharma et al., 2006). Angiostatin has been reported to inhibit tumor growth, metastasis and angiogenesis in mouse cancer models (O'Reilly et al., 1994; Sim et al., 1997; Wu et al., 1997), and its binding partner is found be annexin A2, which blocks plasmin generation (Sharma et al., 2006). In addition, anti-annexin A2 monoclonal antibody is shown to significantly inhibit lung cancer cell growth (Sharma et al., 2006). Moreover TM601, a synthesized chlorotoxin tested in clinical trials, was shown to inhibit tumor migration, invasion and angiogenesis, whose molecular target was found to be annexin A2 in many cancers including glioma, melanoma, pancreatic, prostate and lung cancer (Kesavan et al., 2010; Soroceanu et al., 1999). These observations are consistent with results obtained in plasminogen knockout mice, which failed to induce invasion, angiogenesis and metastasis of tumors suggesting an absolute requirement of plasmin in angiogenesis dependent tumor progression and metastasis (Bajou et al., 2001). Thus Annexin A2 could be an important therapeutic target for anti-angiogenesis glioma therapy.

However the delivery methods need to be investigated in the future. In chapter 3, we used mini-osmotic pumps to infuse drugs locally to the tumor area, which could be a promising method for glioma treatment. The current standard method for glioma chemotherapy is systemic administration, however, many forms of systemic chemotherapy are excluded from the CNS by BBB (Groothuis, 2000). A few compounds, such as antiproliferative drugs called nitrosoureas (carmustine and lomustine) or alkylating agents (temozolomide), have some ability to cross the BBB (Mason and

Cairncross, 2005). Thus systemic delivery of these agents only offer modest benefit as a supplement to radiotherapy (Sawyer et al., 2006). Direct intracranial drug delivery would eliminate the need for a chemotherapeutic agent to cross the BBB as well as decrease the systemic toxicity, which provide new delivery method for not only chemotherapy but also immune therapy and anti-cancer vectors (Ferguson and Lesniak, 2007). Meanwhile using mini-osmotic pumps to deliver drugs into CNS can keep constant drug infusion speed (Mirrione et al., 2010), which is another advantage over systemic administration. Thus inhibitory molecules of annexin A2, such as siRNA, antisense oligonucleotides, monoclonal antibody, competitors of annexin A2 binding, can be delivered locally to glioma site, which may potentially inhibit tumor proteolysis and angiogenesis. Further *in vivo* experiments need to be carried out to optimize the outcomes.

tPA and annexin A2 in MG/MP and glioma cells interaction

The interaction between tPA and annexin A2 plays an important role in microglia activation. tPA is upregulated upon microglial activation and affects microglial activation independently of its proteolytic activity, an event that reveals a cytokine-like function of tPA (Rogove et al., 1999). This activation is mediated by tPA via its amino-terminal finger domain through interaction with annexin A2 (Siao and Tsirka, 2002). In human peripheral monocytes, annexin A2 heterotetramer was shown to be the surface receptor for the plasmin-induced signaling, including the release of TNF-α (Laumonnier et al., 2006). Thus tPA and annexin A2 interaction emerges as a new candidate involved in the signaling events of glioma-MG/MP interaction.

As shown in figure 4 – 1, I treated the N9 mouse microglial cell line with conditioned medium from GL261 (GCM) or DMEM + 10% FBS as a negative control. The supernatant from N9 cultures and N9 cell lysates were collected at 0, 2, 4, 6 and 24 hours after the treatment. 15 μl medium and 20 μg protein from each sample were used to detect tPA activity via zymography. With the treatment of DMEM + 10% FBS, N9 cells only expressed very low levels of tPA (Figure 4 – 1B). However upon treatment with GCM, N9 cells took up tPA from the medium and the tPA levels in the cell lysates increased over time (Figure 4 – 1A). tPA activity is detectable up to 24 hours, which suggests that tPA may function as a link between the GL261 glioma cells and N9 microglia.

In the future, we can further investigate the downstream effectors of tPA and annexin A2 interaction in microglia. For example, we can use GCM to treat wt, tPA knockout (KO) and annA2KO microglia, and compare the changes in signaling pathways after the treatment, such as MAPK cascade, cytokine release. Since plasminogen KO microglia showed defect in MCP-1 induced migration (Sheehan et al., 2007), the microglial migratory activity of tPA KO, annA2 KO and plasminogen KO in response to GCM can also be examined by Boyden chemotaxis chamber, which could possibly discover the receptor responsible for MCP-1 induced microglia migration.

CCL21 in glioma progression

In Chapter 3 (Figure 3-10, 3-11), we found that gliomas expressed CCL21 two weeks after glioma inoculation, which suggested that the glioma microenvironment is immune suppressive based on a recent report about CCL21 in melanoma (Shields et al.,

2010). During the early phase of tumor development, CCL21-secreting tumors attracted more $CCR7^+$ cells (naïve T cells, T_{Reg} cells and APCs), and increased the levels of TGF-β1 and the number of myeloid-derived suppressor cells, which induces the formation of lymph node-like structures to support tumor development. Furthermore, tumor cells induce $gp38^+$ reticular stromal cells to secrete CCL21, and this induced expression was a major contributor to an immunotolerant microenvironment, which resulted in promotion of melanoma progression. Consistent with this theory, we also found that tuftsin treatment increased CCL21 levels in the glioma tissue, whereas MIF treatment had the opposite effect. In GCV-treated CD11b-HSVTK mice, the expression of CCL21 was not detectable.

However the investigation of CCL21 in glioma is not complete. Previously it has been shown that CCL21 is up-regulated in neurons *in vivo* and *in vitro* after injury, which activates microglia through the chemokine receptor CXCR3 and induces microglia migration (Biber et al., 2001; de Jong et al., 2005; Rappert et al., 2002). There is no literature showing the expression of CCL21 in cells other than neurons in CNS. Thus the source of CCL21 in glioma specimens needs to be identified, which will provide new insights into glioma microenvironment. In addition, we need to investigate the mechanism contributing to the decreased level of CCL21 by MG/MP inhibition. One possible explanation could be that MG/MP function as a link between glioma cells and other stromal cells. We can treat primary microglia with CCL21 under normal or glioma-conditioned environment, and compare the changes in signaling pathways and cytokine release. We would expect that glioma-harboring microenvironment could shift the response of microglia to CCL21 from anti-inflammatory to pro-inflammatory.

Moreover CCL21^{low} melanomas can evoke an antitumor immune response, where APCs, IFN-γ and IL-2 promote effector T cell proliferation (Shields et al., 2010), which makes CCL21 an attractive candidate for modulation of tumor microenvironment. In the future, we can also inject glioma cells with low CCL21 expression into mouse brain to follow the tumor progression. Injection of CCL21 antibody or inhibitory molecules such as siRNA can also be applied to glioma-bearing animals, which can provide a novel regimen for glioma immunotherapy.

Potential application of MIF in glioma immunotherapy

One recent development of tumor therapy treatments is to manipulate the activity of tumor-associated macrophages, including inhibition of their recruitment to and survival in tumor tissue and restoration of their anti-tumor immunity (Allavena et al., 2005; Sessa et al., 2005; Wu et al., 2009). In chapter 3, we showed that MIF can inhibit glioma growth *in vivo* characterized by decreased MG/MP infiltration, tumor proliferation and CCL21 expression, as well as increased apoptosis, which suggested that MIF may be a potential novel regimen for glioma treatment.

More research on the outcome of MIF treatment needs to be done for a comprehensive evaluation of MIF as an anti-glioma drug. The changes in gene expression in both MG/MP and glioma cells needs to be investigated, such as cytokine release and expression of angiogenic factors, which may provide insights into the mechanism contributing to the inhibitory effects of MIF on glioma. The dosage and delivery method also need to be optimized. Here we used 25μg/ml MIF *in vivo*, which was reported to efficiently suppress MG/MP activation under neurological diseases

(Wang and Tsirka, 2005); however this dosage may need to be adjusted in the context of glioma microenvironment. The long-term effect of MIF on glioma progression should also be investigated, such as the survival time of tumor-bearing animals, tumor morphology at later stage, behavior tests to examine potential neurological side effects. In addition, our lab showed that incubation of MIF at 37°C for 14 days reduced nearly half of its ability to inhibit of microglial TNF-α release (results from Jaime Emmetsberger), thus the long-term delivery method of MIF also need to be optimized to gain maximal glioma inhibition with minimal adverse effects.

Proposed model

Based on our study and previous research, we proposed a model for the interactions between glioma cells and MG/MP. Glioma cells release cytokines, chemokines and growth factors to attract MG/MP infiltration from surrounding tissue and remote locations, and support their proliferation. The activated MG/MP under glioma-harboring microenvironment in turn secrete cytokines, growth factors and proteases to promote the development of tumor stroma, which facilitates the immune escape of glioma cells (Figure 4 – 2A). Glioma cells also release proteases, among which tPA and plasminogen are secreted and bound to annexin A2 tetramer on the surface of glioma cells and MG/MP. This binding speeds up the generation of plasmin, which degrades ECM and activates latent growth factors to promote tumor migration, invasion, proliferation and angiogenesis (Figure 4 – 2B). Taken together, our study showed that

glioma progression can be modulated by manipulation of tumor-released proteases and tumor-associated MG/MP.

Figure 4 – 1. tPA functions as a link between glioma cells and microglia. Equal numbers of N9 microglial cells were treated with GCM (A) or DMEM + 10% FBS (B) as a negative control. The supernatants from N9 culture and N9 cell lysates were collected at different time points, and then subjected to zymographic gels to detect tPA activity.

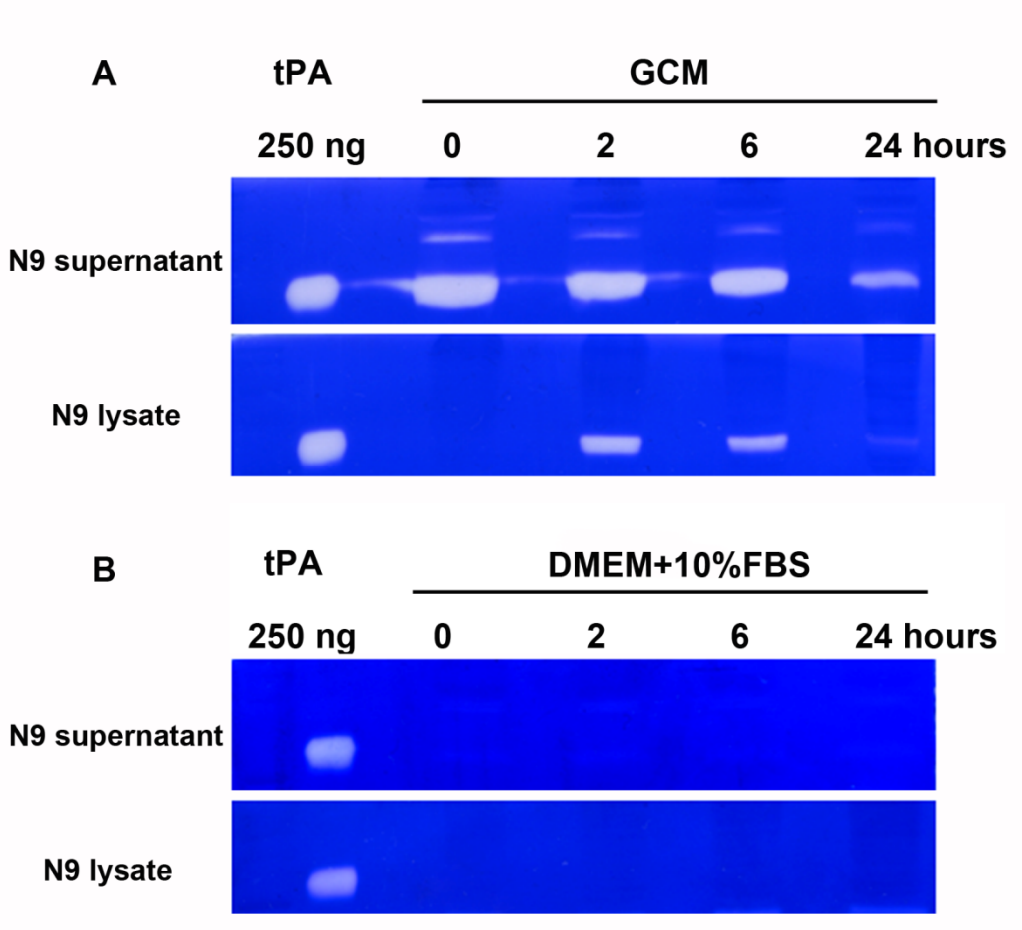
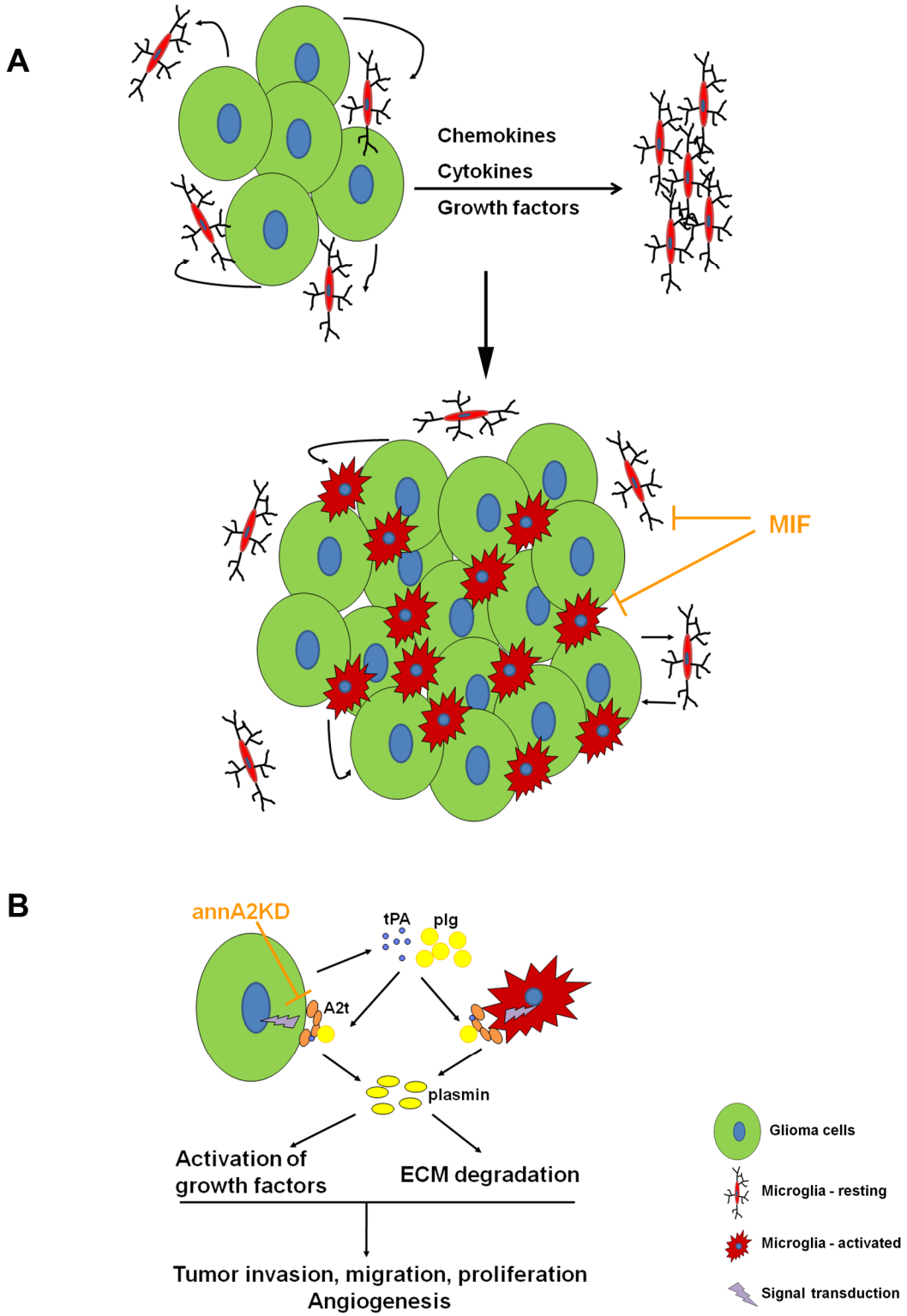


Figure 4 – 2. Proposed model. (A) Interactions between glioma cells and MG/MP. (B) The role of annexin A2 in glioma progression. (A2t – annexin A2 tetramer; plg – plasminogen)



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