

Review Article

Immunoexcitatory mechanisms in glioma proliferation, invasion and occasional metastasis

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Abstract

There is increasing evidence of an interaction between inflammatory cytokines and glutamate receptors among a number of neurological diseases including traumatic brain injuries, neurodegenerative diseases and central nervous system (CNS) infections. A number of recent studies have now suggested a strong relation between inflammatory mechanisms and excitatory cascades and these may play a role in glioma invasiveness and proliferation.

Chronic inflammation appears to be a major initiating mechanism in most human cancers, involving cell-signaling pathways, which are responsible for cell cycling, cancer cell migration, invasion, tumor aggressiveness, and angiogenesis. It is less well appreciated that glutamate receptors also play a significant role in both proliferation and especially glioma invasion. There is some evidence that sustained elevations in glutamate may play a role in initiating certain cancers and new studies demonstrate an interaction between inflammation and glutamate receptors that may enhance tumor invasion and metastasis by affecting a number of cell-signaling mechanisms. These mechanisms are discussed in this paper as well as novel treatment options for reducing immune-glutamate promotion of cancer growth and invasion.

Key Words: Glioblastoma, immunoexcitotoxicity, inflammatory cytokines, metastasis, tumor invasion

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INTRODUCTION

Glioblastoma (GBM) is the most commonly diagnosed primary cancer occurring in the central nervous system (CNS) and is characterized by high invasiveness, frequent central necrosis, and a short survival rate (6-18 months) in the vast majority of patients, even with aggressive treatment.^[62,80]

Inflammation plays a critical role in carcinogenesis, cancer cell proliferation, angiogenesis, invasion, and metastasis of human cancers, including gliomas.^[34] Tumor-associated inflammation is typically characterized by infiltration of

innate immune cells, such as microglia/macrophages and the production of cytokines and chemokines. In addition, tissue remodeling and angiogenesis are present in virtually all tumor types.^[26,28]

In addition to damage to the deoxyribonucleic acid (DNA) and membrane structures caused by increased levels of reactive oxygen and nitrogen species generated during chronic inflammation and excitotoxicity, one sees activation of a number of cell-signaling pathways that are increasingly recognized as being critical to the growth, progression, and invasion of cancers such as Nuclear Factor kappa B (NF- κ B), mammalian

target of rapamycin/Ak-thymoma (mTOR/Akt), and signal transducer and activator of transcription-3 (STAT3).^[1,24,29,44]

Of particular interest is the involvement of certain immune cell types in cancer biology, such as macrophage/microglia, mast cells, and T-lymphocytes, all of which have been shown to assume specific immune phenotypes that regulate tumor immunity.^[60,108] In addition, recent studies have demonstrated a primary role for cancer stem cells in tumor behavior that not only includes control of tumor proliferation but also can initiate a microenvironment associated with immunosuppression.^[32,57,108] Neural stem cells are attracted to sites of inflammation, reactive astrogliosis and angiogenesis primarily by the action of tumor necrosis factor- α (TNF- α) on NF- κ B activation.^[123,124] NF- κ B controls stem cell proliferation by up-regulation of cyclin D1.^[42] Widera *et al.* demonstrated a 10-fold increase in NF- κ B activity and a 4-fold increase in cyclinD1 positive stem cells with neural stem cell exposure to TNF- α .^[124] TNF- α is a growth factor for Hodgkin's lymphoma, cutaneous T-cell lymphoma and gliomas.^[3,91] Excitotoxicity also up-regulates NF- κ B, which may be a mechanism by which it stimulates GBM proliferation.^[77]

Various chemokines and chemokine receptors are also known to play a role in tumor biology. Of particular interest are chemokine receptors CX3CR1 and CXCR4 and their ligands. CXCR4 is the most extensively studied and is associated with aggressive forms of gliomas and poor patient survival.^[10,33] The chemokine CX3CL1 regulates microglia and controls the crosstalk between microglia and neurons. In addition, these chemokines play a significant role in tumor resistance to apoptosis, stimulation of angiogenesis and recruitment of microglia and macrophages into the tumor.^[13,130] The chemokine macrophage chemoattractant protein-1 (MCP-1) is markedly up-regulated in astrocytoma cells following exposure to TNF- α .^[103] MCP-1, which is released during inflammation, is a potent chemoattractant for neural stem cells.^[123]

Macrophages and microglia are the most abundant cell types in gliomas, but what is often ignored is that they are both sources of glutamate and other excitatory amino acids.^[49,118] Recent studies have found that glutamate and virtually all of the glutamate receptor types are involved in tumor growth, progression, invasion, and metastasis in a wide array of tumor types, including primary brain tumors.^[47,94,126] Several studies have also found that inhibiting glutamate receptors suppresses the growth and spread of a number of types of cancer.^[101,113]

INFLAMMATION, IMMUNE CELL INFILTRATION, AND GLIOMAS

Chronic inflammation (also observed in non-CNS cancers), appears to play a significant role in all

phases of brain tumor development, progression and invasion.^[23,24,98,105,109] Yet, in the case of higher grade gliomas, such as GMBs, the microenvironment is mostly immunosuppressive, yet still inflammatory.^[51,125] For example, despite the fact that GMBs are known to be immunogenic tumors neither the innate nor the adaptive immune system significantly suppresses these tumors.^[37,50]

In a recent study Tafani *et al.* demonstrated an elevation of inflammation within GBM in 10 tumors removed surgically, selectively sampling deep tumor, peritumoral tissues, and normal surrounding brain.^[116] In all samples they found a marked elevation in NF- κ B and the pro-inflammatory markers receptor for advanced glycation end-products (RAGE), cyclooxygenase 2 (COX2), nitric oxide synthase-2 (NOS2), P2X7R, and pentraxin 3 (PTX3). For most of these factors, levels were highest in the tumoral tissue with much lower levels in the surrounding brain. Similar elevations in pro-inflammatory proteins have been described in breast and prostate cancers.^[97,115] The inducible enzymes COX2 and NOS2 play a role in angiogenesis and signal a worse prognosis when elevated.^[48] Both COX2 and NOS2 are also elevated with glutamate excitotoxicity.^[2]

Interestingly, tumor reactions to TNF- α vary according to concentration. Low dose TNF- α enhances survivability of glioma cells and in higher doses increases killing of neurons.^[9] There is also a selective stimulation of glioma stem cell proliferation as well as tumor necrosis in the center of the tumor and within normal surrounding brain along its border.^[58,102] The differential effect on neurons and glia has been attributed to differences in TNF receptors, TNFR1 (p55) and TNFR2 (p75). While neurons express both types of receptors, TNFR1 is activated at lower levels of TNF- α . TNFR1 is neurodestructive, which when overstimulated can lead to necrosis of neurons as seen along the leading edge of the tumor and in the tumor core. TNFR2, primarily located on astrocytes and glioma cells, is neuroprotective.^[9] Under conditions of high glutamate levels, as seen with GBMs, stimulation of glial TNFR2 would favor glioma cell survival, thus promoting tumor growth. TNF- α also stimulates stem cell proliferation.^[123,124]

ROLE OF TUMOR TISSUE HYPOXIA IN INFLAMMATION-INDUCED INVASION

Hypoxia was also shown to enhance the inflammation and increase invasion and migration of the GBM stem cells.^[115] Because GBMs are such fast growing tumors they rapidly outgrow their blood supply, leading to hypoxia/ischemia within the tumor and frequent necrosis. It is now known that angiogenesis is an early process and is driven by hypoxia.^[25] Hypoxia inducible factor-1 (HIF-1) is present in high levels in malignant gliomas and necessary for GBM stem cell maintenance.^[96] Under

hypoxic conditions, as seen in rapidly growing tumors, HIF-1 α is stabilized and translocates to the nucleus where it binds to HIF-1 β .^[83] This dimer activates genes involved in angiogenesis, glucose transport, resistance to apoptosis, inflammation and invasion.

Hypoxia is also known to increase expression of CXCR4, which stimulates tumor cell migration and is associated with highly aggressive tumors and a poor prognosis.^[10,115] Tafani *et al.* examined the role of hypoxia on pro-inflammatory gene expression in GBMs.^[115] Using surgical samples from deep within the tumor, the peritumoral area and surrounding normal brain, they concluded that GBMs express pro-inflammatory genes and proteins that are almost exclusively localized to the tumor tissue and much less so in the peritumoral and host tissue. Under hypoxic conditions GBM stem cells overexpressed pro-inflammatory genes, such as those regulating NF- κ B, COX2, iNOS, and PTX3 and this increases migration and invasion of the tumor stem cells. Hypoxia-induced acceleration of invasion and migration of tumors stem cells also appears to play an important role in breast cancer.^[115]

In essence, hypoxia within the tumor stimulated HIF-1 α and NF- κ B activation, which in turn increased generation of pro-inflammatory cytokines and chemokines, leading to accelerated tumor cell invasion. The source of the pro-inflammatory factors was probably macrophages/microglia, since these are the predominant invading cell type. RAGE was clearly over expressed in the GBM fraction at mRNA and protein levels, which is also controlled by hypoxia. The inducible enzymes, COX-2 and NOS2 were also overexpressed in peritumoral and tumor tissue and in the case of the GBM stem cells, expression of these factors was increased by hypoxia.

The picture that is emerging from recent research is that malignant gliomas release high levels of chemokine attractants with subsequent accumulation of large populations of macrophages/microglia immune cells within the tumor and smaller accumulations of lymphocytes and mast cells. Under normal conditions, this would result in the release of high levels of inflammatory cytokines and lead to tumor killing. While many studies have shown either *in vitro* or when using fresh tumor specimens, evidence of increased inflammation, a growing literature suggest that within the tumor there is impaired immune competence for tumor rejection, along with a reduction in the release of critical immune factors from microglia/macrophages and elevation of growth promoting cytokines, such as TGF-1 β , IL-10, IL-23, TNF- α , and IL-6.^[51] The main source of this suppressive microenvironment appears to be from factors released from GBM stem cells and the attraction of suppressor T_{regs} to the tumor, which block cytotoxic T-lymphocyte function.^[82,86,107,125]

Hypoxia also plays a significant role in malignant glioma initiation, aggressiveness, and invasion, partly by

enhancing immunosuppression and inflammation. In essence, cancer stem cells have converted the cellular immune response to one that is favorable to tumor growth, progression, and invasion.

THE ROLE OF GLUTAMATE AND GLUTAMATE RECEPTORS IN GLIOMA GROWTH AND INVASION AND THE LINK TO INFLAMMATORY CYTOKINES

A growing number of studies show that glutamate and its receptors play a major role in cancer development, progression, growth, invasion and metastasis, including gliomas.^[15,17,21,53,78,93,114] Ye and Sontheimer demonstrated using both human GBM cells lines and fresh surgical specimens of GBM a 100-fold lower uptake of glutamate from the extracellular space in comparison to normal astrocytes.^[126] They also noted a 3-fold increase in glutamate release over a 12-hour period from glioma cells by active transport. Combined, this greatly increases extraneuronal glutamate concentrations both within the tumor and at the tumor margin.

Subsequent studies have shown that glioma cells, both from GBM cell lines and fresh surgical specimens, lack the principle glutamate transport proteins, EAAT1 (GLAST) and EAAT2 (GLT-1), the latter being most important for control of extracellular glutamate levels in the adult brain.^[127] While suppression of glutamate uptake alone is sufficient to trigger excitotoxicity, of particular importance is the reverse transport of glutamate, which can occur by two mechanisms-reverse transport by EAATs and increased activity of the system X_c antiporter. The latter mechanism exchanges extracellular cystine for intracellular glutamate, leading to elevations in extracellular glutamate when the EAAT transporters are simultaneously dysfunctional. That is, normally, the expelled glutamate is rapidly taken up by GLT-1, but in the case of gliomas, GLT-1 is nonfunctional.

Recent studies have shown that glioma cells, particularly GBM cells, demonstrate a dramatic enhancement of system X_c cystine/glutamate exchange activity, which markedly elevates extracellular glutamate levels.^[22,79] Furthermore, the release of excess glutamate acts in an autocrine and paracrine manner to stimulate glutamate receptors on nearby neurons in the surrounding brain, leading to excitotoxic cell death and promotion of glioma cell migration and invasion.^[79] It has been proposed that induced excitotoxicity is at the leading edge of the expanding glioma, allowing for rapid growth of GBMs in an enclosed cranium^[117] [Figure 1]. In addition, excitotoxicity triggered by glutamate release from glioma cells is also responsible for necrosis within gliomas, a hallmark for GBM type gliomas.^[85]

Lyons *et al.*, using a variety of GBM cell lines and acute GBM surgical specimens, found that sulfasalazine,

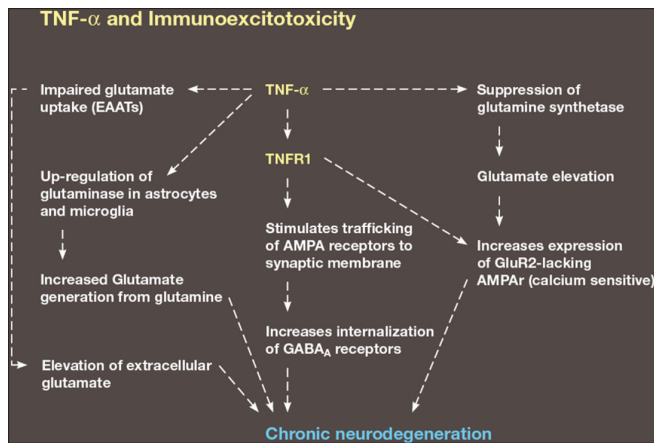


Figure 1: Diagram demonstrating the interactions of TNF- α with membrane receptors leading to an up-regulation of GluR2-lacking AMPA receptor trafficking to synaptic membranes and internalization of GABA receptors. TNF- α also triggers an up-regulation of glutaminase, which stimulates glioma invasion and provides energy molecules for cellular growth and proliferation. In addition, it also suppresses GLT-1 regulated glutamate uptake, leading to excitotoxic levels of extracellular glutamate. The glioma stem cells are mostly resistant to immunoexcitotoxicity

an inhibitor of the system Xc cysteine/glutamate antiporter, reduced glioma cell migration by 40-50% for cells tested *in vitro*.^[79] Using a glioma mouse model they injected D54-MG GBM (GBM cell line) cells into the cerebrum of severe combined immunodeficiency (SCID) mice and compared tumor growth and invasion when system Xc was inhibited versus controls with an intact system Xc. They used sulfasalazine and S-(4)-CPG to suppress system Xc.^[41,92]

Control animals demonstrated large, highly invasive tumors with distant satellite tumors, whereas both the sulfasalazine and S-(4)-CPG treated animals had much smaller tumors, no evidence of tumor invasion at the boundary and few if any satellite tumors. Others have also shown that inhibition of system Xc slows tumor growth and significantly extends the life of the animal.^[22] Lyons *et al.* demonstrated a dramatic up-regulation of system Xc and GluR2-lacking, Ca²⁺-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in glioma cells lines and surgical GBM specimens. Elevations in extracellular glutamate and associated calcium oscillations were blocked when system Xc was inhibited.^[79]

Sulfasalazine, a drug used to treat ulcerative colitis, penetrates the brain easily and has a good safety profile.^[110] The doses used in the above study were comparable to that used in human treatment of bowel diseases. Cystine is rapidly generated by oxidation of cysteine.^[68] Lyons *et al.* demonstrated that cystine loading increased glioma cell Ca²⁺ oscillations by stimulating glutamate release. Cystine is also used by glial cells to generate glutathione, which protects cancer cells from apoptosis.

In vivo studies using spectroscopic magnetic resonance imaging (MRI) have confirmed that the peritumoral fluid surrounding high grade oligodendrogliomas contains increased levels of glutamate.^[99] This localized elevation in glutamate has also been shown using microdialysis studies from necrotic tissues of GBMs.^[100] It should be appreciated that glutamate accumulation also causes rapid activation of adjacent microglia leading to immunoexcitotoxicity^[11,12] [Figure 1]. Glutamate, as well as chemokines, stimulate migration of microglia to the tumor, explaining the dense population of microglia seen in high-grade astrocytomas.^[76] Microglia are major sources of both glutamate and pro-inflammatory cytokines.

Glutamate and its receptors play a major role in cell migration during brain development.^[63] This has been extensively studied in the case of cerebellar granule cell migration during neurodevelopment and has been shown to be dependent on GluR2-lacking, Ca²⁺-permeable AMPA glutamate receptors, which also play a major role in human malignant glioma and GBM migration and invasion.^[31,53,79]

LINK BETWEEN INFLAMMATORY TNF-A AND EXCITATORY GLUTAMATE RECEPTOR ENHANCED SENSITIVITY

In the normal brain, TNF- α is release in low concentrations, whereas most other cytokines are undetectable. A number of studies have shown that TNF- α plays a major role in constitutive plasticity, neural circuit function and LTP by regulating AMPA receptor (AMPA) trafficking to the neural cell membrane by a process of exocytosis from nearby endoplasmic reticulum.^[8,112] AMPA receptor insertion points include the cell body and dendrite spines, with the latter having the highest concentration. Most of the inserted AMPARs are extrasynaptic but approximately 25% will move laterally to be inserted in the postsynaptic density, significantly increasing glutamate excitation.

A real time study of individual living neurons demonstrated rapid (milliseconds) insertion of AMPARs following stimulation by TNF- α .^[128] Neuronal sensitivity to glutamate receptor activation was regulated both by constitutional TNF- α release and higher levels of release under pathological conditions, such as with inflammation.^[18,43] TNFR1 has been shown to be necessary for AMPAR trafficking, demonstrating a strong link between inflammation and excitatory glutamate receptor activation.^[112] Activation of AMPAR trafficking is mostly limited to TNF- α and less so IL-1 β . IL-6 and IL-10 had no effect on AMPAR insertion into cell membranes. Phosphatidylinositol-3 kinase (PI3K) is an essential cell-signaling molecule for TNFR1 activation of AMPAR trafficking. GBMs have been shown to have high concentrations of PI3K.^[74]

IL-6 is frequently produced by glioma cells and may play a role in tumor growth, peritumoral edema and angiogenesis. Chang *et al.* found that patients with IL-6 negative GBM tumors had a median survival of 16 months, while those with IL-6 positive GBMs had a median survival of only 7 months.^[16] Three patients had survivals longer than 1 year and these showed no IL-6 expression. IL-6, IL-10, and IL-27 can all activate STAT3 (IL-6-STAT3 axis) and thereby can suppress macrophage immunocompetence.^[66] In addition, elevations in glutamate were found to stimulate the release of IL-6 in rat hippocampus.^[19] As a result, one would expect higher levels of IL-6 in patients with the highest tumoral glutamate levels.

CA²⁺-PERMEABLE AMPA RECEPTORS, INFLAMMATION AND GLIOMA INVASION

Normally, in the adult brain, AMPA receptors are calcium impermeable, primarily because they contain the GluR2 subunit. Of the ionotropic receptors, N-methyl-D-aspartate (NMDA), AMPA and kainate receptors, it is the NMDA receptors that normally move extracellular calcium inside the cell, but gliomas do not contain functional NMDA receptors, leaving Ca²⁺-permeable (GluR2 subunit-lacking) AMPA receptors as the principle ionotropic receptor in gliomas.^[53,79] Ca²⁺-permeable AMPA receptors are increasingly found in a number of neuropathological conditions including amyotrophic lateral sclerosis (ALS), strokes, chronic pain syndromes, spinal cord injury, and brain trauma chronic traumatic encephalopathy (CTE).^[14,38,75,119]

Under physiological conditions, transient insertion of Ca²⁺-permeable AMPA receptors occurs in various brain

areas associated with memory and learning processes.^[95] The persistent presence and even transient presence of these Ca²⁺-permeable receptors on neuronal membranes increases the risk of excitotoxicity.^[67] One of the most consistent driving forces for pathological insertion of Ca²⁺-permeable AMPA receptors is the presence of high levels of pro-inflammatory cytokines, in particular TNF- α ^[67,75,112] [Figure 2]. These observations further emphasize the importance of the interaction between immune factors and glutamate receptors (immunoexcitotoxicity).

Studies have consistently shown rapid, robust insertion of GluR1-containing AMPARs on dendritic membranes following exposure to TNF- α .^[8] More recently, it was shown that initially these inserted AMPARs are composed of the GluR2-lacking subunit, which makes them highly calcium permeable. Even a transient presence of Ca²⁺-permeable AMPA receptors to synaptic membranes increases excitotoxic sensitivity^[112,128] [Figure 3].

Compelling evidence suggest Ca²⁺-permeable AMPA receptors also play a critical role in glioma invasion.^[53,79] In the Lyons *et al.* study, all glioma cell lines and surgical specimens of human gliomas demonstrated that GluR2-lacking AMPARs were universally present, with higher concentrations in GBM cells.^[79] Blocking these receptors, either with GYKI, a broad-spectrum AMPAR blocker or Joro-spider toxin, a specific GluR2-lacking-AMPA blocker, reduced glioma cell migration 60%.^[31] NASPM, a synthetic joro spider toxin analog, which also blocks Ca²⁺-permeable GluR2-lacking AMPARs, has been shown to reduce TNF- α enhancement of excitotoxicity, thus demonstrating a possible link between enhanced AMPAR sensitivity and increase glioma cell migration.^[67]

Another important process in glioma invasion is glutamate-induced migration along blood vessels and

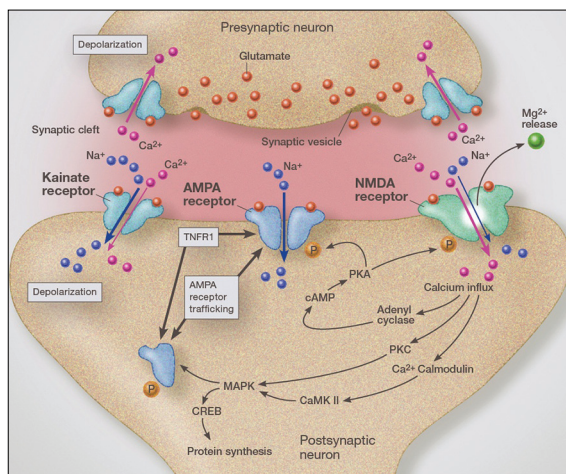


Figure 2: Illustration demonstrating the trafficking of AMPA receptors stimulated by TNF- α activation of the neurodestructive TNFR1 receptor. This can lead to immunoexcitotoxic destruction of the brain surrounding the tumor, thus allowing for expansion. It can also result in central tumor necrosis commonly seen with in glioblastomas

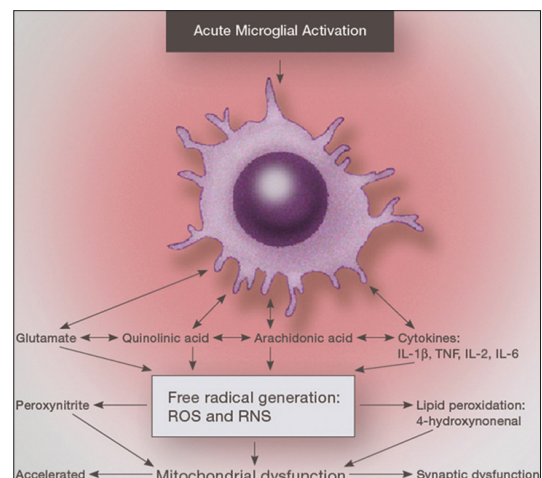


Figure 3: Illustration of immune and excitotoxic factors released by an activated microglia and the effects on mitochondrial function caused by the generation of free radicals, lipid peroxidation products, fatty acid molecules (arachidonic acid), pro-inflammatory cytokines and their interaction resulting in immunoexcitotoxicity

neural pathways. Piao *et al.* found that AMPA receptors containing high levels of the GluR1, such as in the case of GBMs, promoted perivascular glioma invasion by promoting β -integrin-dependent adhesions to the extracellular matrix.^[93] The GluR1 subunit may be linked to the integrin receptor by way of an erythrocyte membrane protein (EMP). Piao *et al.* found that expression of AMPA receptors directly correlated with the level of glioma cell invasion and distant metastasis via perivascular and subpial invasion both *in vitro* and *in vivo*. With TNF- α rapidly increasing AMPA receptors containing GluR1 and GluR2-lacking (Ca²⁺ permeable) AMPA receptors, one would expect increased invasion potential. The TNF- α amplification of AMPA receptor trafficking is dose dependent, meaning that the greater the tumor-associated inflammation, the greater the excitotoxic stimulation of tumor invasion and proliferation.

Using a Matrigel Transwell migration/invasion assay they confirmed a direct correlation between GluR1 and GluR4 expression and the degree of glial migration and invasion.^[93] This finding shows that subunit composition of AMPA receptors determines the behavior of glioma cells in terms of invasiveness and that gliomas (especially the higher grade gliomas) have a special, pro-invasive subunit composition (High GluR 1 and low or absent GluR2).

Piao *et al.* also found that cells overexpressing the GluR1 subunit have significantly more focal adhesions (Fas) than control cells.^[93] A dynamic process of adhesion and release determines migration along perivascular pathways, and both processes can be controlled by GluR1 expression.^[30,93] Other studies have confirmed that glutamate can increase integrin surface expression in neurons.^[72]

Invasion of normal brain requires penetration of the extracellular matrix, mainly by proteolytic enzymes. Matrix metalloproteinase (MMPs), a group of proteolytic enzymes, can erode through the extracellular matrix, and are released from the invadopodia. Studies have shown that their expression is increased by elevated glutamate levels.^[55]

GLUTAMATE-INDUCED CELL SIGNALING AND GLIOMA INVASION

A number of studies have examined cell signaling linkage to glutamate receptor activation and how these pathways enhance glioma invasion and metastasis.^[53-55,93] Several key processes seem to be involved and include PTEN, NF- κ B, Akt, and calpain2, all of which are linked to glutamate receptors, either directly or indirectly.^[4,53,55,84,85,90]

PTEN, a phosphatase and tensin homolog protein, is produced by the PTEN gene as a tumor suppressor and is frequently mutated in astrocytomas; more often in GBM than low-grade astrocytomas.^[3,69] Interestingly, PTEN is

more frequently mutated in primary than in secondary GBM and is known to accelerate progression of low-grade astrocytomas to GBM.^[61,65]

PTEN suppresses tumor growth and acceleration by inhibiting Akt, another tumor growth promoter.^[46] Akt expression is linked to NF- κ B activation, which is in turn activated by glutamate.^[56,84] The latter observation provides a link between inflammation, glutamate receptor activation (immunoexcitotoxicity), and tumor invasion and aggressiveness.

Akt expression has been shown to confer a more aggressive phenotype to malignant tumor cells by suppression of tumor cell apoptosis.^[6] The combination of PTEN mutations along with elevated extracellular glutamate levels leads to Akt pathway activation resulting in tumor growth stimulation.^[85]

One of the principle cell signaling pathways linking tumor aggressiveness to glutamate receptor activation is calpain2, which is activated by Ca²⁺ entry into glial cells.^[55] One of the early links between calpain and cancer invasion was demonstrated by Shiba *et al.*, who found increased levels of calpain in breast cancer tissue compared with normal breast tissue cells.^[106] Subsequently it was found that knocking down calpain2 reduced breast cancer invasion by ~50%, mainly by regulating invadopodia projections necessary for movement through the extracellular matrix.^[27]

Ca²⁺-permeable AMPA receptors allow entry of Ca²⁺ into the glioma cells, which not only increases intracellular Ca²⁺ levels but also initiates Ca²⁺ oscillations that extend from the Ca²⁺- channel inward. It is the Ca²⁺ oscillations that control migration of glioma cells and invadopodia activity.^[79] These oscillations have also been shown to play a critical role in focal adhesion assembly and disassembly necessary for invasion of GBM cells into surrounding tissues.^[40]

Jang *et al.*, using a Matrigel Transwell migration/invasion assay of U87MG human GBM cells with knockdown of calpain2 expression, found that invasion was ~90% lower in the calpain2 knockdown cells compared with control glioma.^[55] They also observed a 39% lower level of MMP-2 in the knockdown cells, a major mechanism of glioma invasion. Reactive oxygen species also play important roles in signaling activation pathways of invadopodia and are abundantly generated during immunoexcitotoxicity.^[122]

More than 95% of human primary brain tumors express astrocyte-elevated gene-1 (AEG-1) a factor that may provide GBMs with significant growth stimulation.^[35,57] AEG-1 protects glioma cells from apoptosis and acts via the Akt pathway.^[68] AEG-1 also activates the promoter of NF- κ B and enhances formation of the NF- κ B transcription activator p50/p65 complex in the nucleus, which activates numerous pro-inflammatory cytokines, several of which act as tumor growth promoters.^[35]

Interestingly, AEG-1, like NF- κ B and TNF- α , have a negative effect on the EAAT2 (GLT-1) glutamate transporter, which, when impaired, raises extracellular glutamate levels.^[57] Thus, there appears to be a cyclic buildup of glutamate involved in glioma progression and invasion, involving numerous interacting mechanism and this significantly enhances the proliferation, aggressiveness and invasion potential of glial tumors.

FUTURE DIRECTION IN CONTROLLING GLIOMA GROWTH AND INVASIVENESS

Conventional treatment of anaplastic astrocytomas and GBMs has been overall a failure. Death rates and length of survival of GBM patients is essentially the same as it was 40 years ago.^[87] This failure is based on the fact that most such treatments are directed against single cancer cell mechanisms that can be quickly overcome by the cancer cells. Recent evidence suggest that of more importance to resistance is the finding that malignant gliomas arise from GBM stem cells that are resistant to virtually all chemotherapy agents and radiation.^[7,32,36,39] These tumor stem cells create an immunosuppressive microenvironment within the tumor that prevents successful immune system elimination of the tumor but yet the tumor itself is associated with significant inflammation at the same time. In addition, the pro-inflammatory cytokine TNF- α has been shown to stimulate neural stem cell proliferation via NF- κ B signaling, which is also activated by glutamate stimulation. This suggests that glutamate may stimulate GBM stem cell proliferation via NF- κ B activation as well.

The role of glutamate excess in tumor growth, progression, invasion, and on rare occasions, metastasis, has been firmly established for a great number of cancers, including primary brain cancers. A number of recent studies have shown that reducing glutamate levels or blocking specific glutamate receptors can dramatically reduce tumor induction, proliferation and in particular, invasion.^[79,101,113]

Now that glutamate receptor activation has been linked to both tumor expansion by increased proliferation as well as other mechanisms, such as a stimulation of invasion and progressive necrosis of the normal brain along its leading edge, what should the neurosurgeon do to reduce this immunoexcitotoxic effect? That is, should we change our targets for a more successful treatment protocol?

Olney and other studies have shown that many commonly eaten foods contain free glutamate levels that can produce brain lesions in animals when orally ingested.^[88,89,111] Because the blood-brain barrier is designed only to prevent brain injury from acute elevations in glutamate, chronic ingestion of high glutamate-containing foods presents a special problem. A great number of processed foods contain one of more sources of free glutamate and/or aspartate.

During chronic ingestion the elevated levels of plasma glutamate can enter the brain via the circumventricular organs (CVO) and in the case of gliomas, especially GBM, the blood-brain barrier is often compromised, allowing glutamate to freely enter the area of the tumor.

With impaired glutamate transport, orally ingested and intrinsically generated glutamate can add to the already elevated extracellular glutamate levels seen in gliomas and could potentially increase tumor invasion. A number of commercial enteral feeding formulas have high concentrations of free glutamate, cysteine, and aspartate manufactured from processed hydrolyzed casein, hydrolyzed whey protein, crystalline L-amino acids, and soy protein isolate, all known to provide very high concentrations of excitotoxic amino acids.^[81]

Neurosurgeons and neurologists should counsel their patients to avoid foods high in glutamate and processed foods containing excitotoxin additives, such as monosodium glutamate, hydrolyzed protein, soy protein concentrates and isolates, whey protein isolates, autolyzed yeast, sodium or calcium caseinate, and other food label names for disguised forms of glutamate.

In terms of clinical treatment, a wide array of natural substances have been shown to regulate cancer cell signaling pathways and reduce cancer growth, aggressiveness and invasion by a great number of well-defined mechanisms.^[45,59,129] By inhibiting a large number of essential cellular signaling mechanisms used by cancer cells for survival, growth, and invasion, these natural products may hold more promise than conventional chemotherapy and can also enhance the effectiveness and safety of traditional treatments.

Of particular interest is curcumin, an extract from the spice turmeric, which inhibits a number of cancer signaling pathways including NF- κ B, the oncogenes c-myc, c-fos, c-jun, MAPKs, ERK, PI3K, Akt, CDKs, iNOS, mTOR, PKC, and EGFR tyrosine kinase.^[73] Studies have demonstrated antiangiogenesis effects and reduced metastasis and invasion in cancer models.^[64] In addition, curcumin has been shown to be a chemosensitizer and radiosensitizer for cancer cells and protects surrounding normal cells from damage by these same treatments.^[70,71]

Of considerable interest is the ability of curcumin to down-regulate multidrug-resistance (MDR) and inhibit STAT3.^[20,52] Curcumin has a very high safety profile even when given in large doses. Curcumin also shows significant activity against GBM cells *in vitro* and *in vivo*.^[5] Quercetin suppresses NF- κ B, LOX, and PI3K, which are linked to inflammation, tumor proliferation, and AMPAR trafficking.^[120]

Clinically, the main difficulty with curcumin, and some of the other flavonoids, is with poor absorption orally when used in a powdered form. Newer techniques in

pharmacology and drug delivery, such as nanosizing, lipid encapsulation and mixing it with certain oils, such as extravirgin olive oil, medium-chain triglycerides (MCT) oil, and coconut oil, have greatly increased the bioavailability of these extracts. Once in the blood, curcumin readily enters the brain.

Other cancer inhibiting extracts, such as quercetin, ellagic acid, hesperidin, silymarin, resveratrol, tea catechins, epigallocatechin gallate, and luteolin hold great promise in providing safer and more effective treatments for gliomas. A discussion of these extracts will be covered in an upcoming article.

Another approach is the suppression of glutaminase, the enzyme that converts glutamine to glutamate within glial cells. Studies targeting this rate-limiting enzyme have shown slowed glioma cell growth *in vitro*.^[104,121]

In conclusion, given the multifactorial nature of the GBMs, the rationale behind conventional chemotherapeutic treatment methods aimed at targeting a single pathway should be reconsidered. A much better option for treating these highly aggressive tumors may instead, lie in a multi-prong attack on diverse cooperating cell signaling and metabolic factors, which are essential for cancer cell survival and invasion. In this regard, several natural compounds hold a great and yet much underutilized therapeutic potential.

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