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Mechanisms of Glioma Cell Invasion

Scott G. Turner, Maleeha Ahmad and
Steven A. Toms

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Abstract

Malignant gliomas are the most aggressive primary brain tumors. Although current treatment includes surgery and chemo/radiation therapy, life expectancy remains on the order of 2 years. One of the features, which make these tumors incurable, is their infiltration into normal brain tissue. This process is incompletely understood at a molecular level and appropriate targets need to be developed. This review discusses (1) the unique structure of the neural extracellular matrix (ECM), (2) the basis of the proliferation to migration transition that initiates the infiltrative process, (3) the remodeling of the ECM by degradation and synthesis of new components, and (4) trophic factors that act as chemoattractants and chemorepellents for migrating cells. Finally we briefly discuss the challenges facing the study of this complex process and future directions in attacking this important problem in neuro-oncology.

Keywords: glioma, migration, invasion, extracellular matrix, protease, signaling

1. Introduction

Malignant gliomas are the most common primary tumors of the central nervous system (CNS) accounting for over 22,000 new cases in the USA each year [1]. Glioblastoma multiforme (GBM) is the most aggressive (WHO grade IV) glioma and is characterized histologically by high mitotic activity leading to hypoxia and necrosis, nuclear atypia and cellular pleomorphism, and microvascular proliferation due to secretion of pro-angiogenic factors. These tumors are uniformly fatal with standard treatment consisting of maximal surgical resection followed by radiation and chemotherapy which targets cell proliferation (Temozolomide or other DNA modifying agents) [2] or angiogenesis (Bevacizumab) [3], as well as other newly developed methods of attacking dividing tumor cells (Optune-TTF) [4].

Gliomas differ from metastatic tumors in their ability to migrate into the surrounding brain parenchyma. While most recurrence occurs within 1–2 cm of the original tumor bed [5], seemingly multifocal disease or so-called “butterfly glioma” can develop at distant sites as a consequence of migration of cells along blood vessels and white matter tracts [6] (**Figure 1**). Although they are highly infiltrative, less than 2% of gliomas spread outside the CNS, suggesting that tumor cells are either not able to cross the basement membrane and enter the vasculature or that they require a specific neural environment containing specific molecules through which they can proliferate and migrate. Tumor cells switch from a proliferative to a migratory or mesenchymal phenotype, resorting to this more primitive state, which mimics the behavior of their migratory progenitors such as the radial glia that traversed white matter pathways and other structures during embryonic development [7]. The molecular mechanisms of this transition are currently poorly understood.

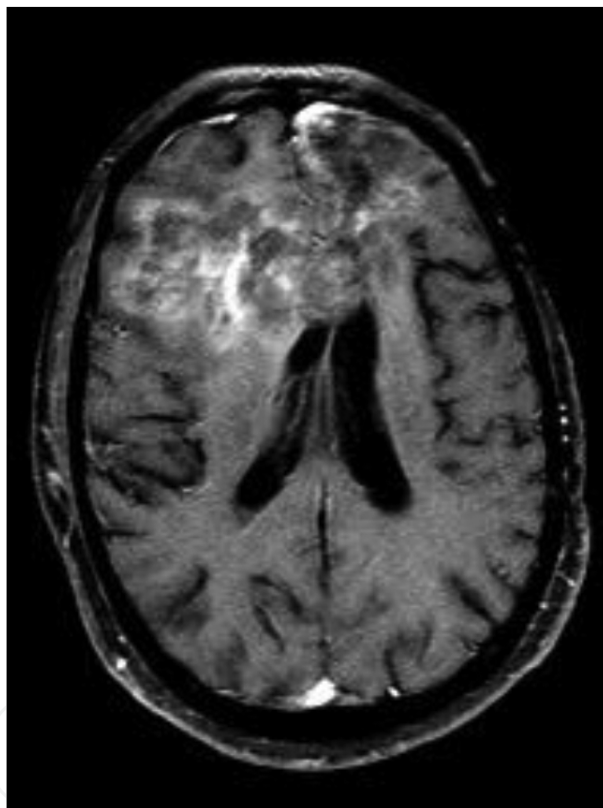


Figure 1. Butterfly glioma in a patient with bihemispheric spread due to involvement of the corpus callosum.

In this chapter, we discuss the composition of the brain’s extracellular matrix, as well as the mechanisms by which tumor cells transition to a migratory phenotype and remodel the ECM through degradation by novel proteases and their inhibitors. We discuss the search for ECM molecules expressed by the tumor cells, which then respond to chemoattractants in the environment in order to direct growth. Finally, we discuss potential targets of anti-infiltrative therapy and the obstacles that must yet be overcome to address this important neuro-oncologic problem.

2. The Extracellular Matrix

The ECM consists of three components: (1) perineural nets (PN) which surround neural cells and their processes and provide support and regulate plasticity, (2) a complex interstitial matrix between cells, and (3) basement membranes that surround blood vessels made up primarily of laminin, fibronectin, and collagens. ECM components are secreted by resident cells and serve to provide structural cell support, regulate cell-cell connectivity and communication, and sequester growth factors and chemoattractants to regulate cell motility. The composition of the ECM varies from tissue to tissue and is comprised of proteoglycans such as chondroitin sulfate, heparin sulfate, keratin sulfate, and a lattice of interconnected fibrous proteins [8].

The neural extracellular matrix comprises 10–20% by volume of the brain and spinal cord and is structurally and functionally distinct from the ECM in other tissues [9] (**Figure 2**). The most abundant component of the neural ECM is hyaluronan (HA) and its associated glycoproteins. HA is a large hygroscopic glycosaminoglycan composed of alternating D-glucuronic acid and N-acetylglucosamine which is synthesized by hyaluronan synthase anchored to cell membranes [10]. In the developing brain, HA is organized into fiber-like structures along which neural precursors migrate. HA is anchored to astrocytes through its receptor, CD44, a transmembrane glycoprotein that couples the ECM to the actin cytoskeleton [11] and to hyaluronan synthase on neurons. Overexpression of CD44 was shown to increase the length of filopodia of neuroblastoma cells *in vitro* and promote invasion into a HA-rich matrix, demonstrating how overexpression of this single gene can affect the complex sequence of events for an invading cell to detach from its substrate, adhere to and degrade the surrounding matrix, and migrate through it [12].

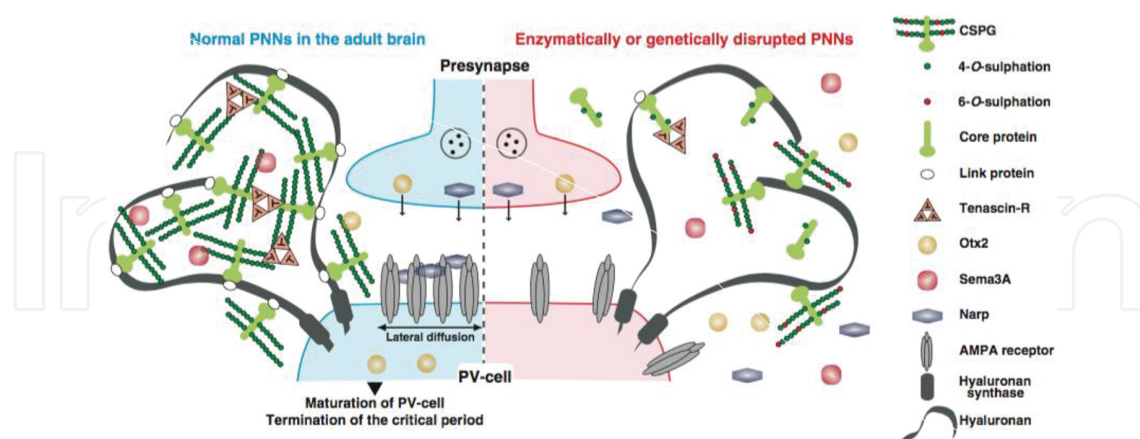
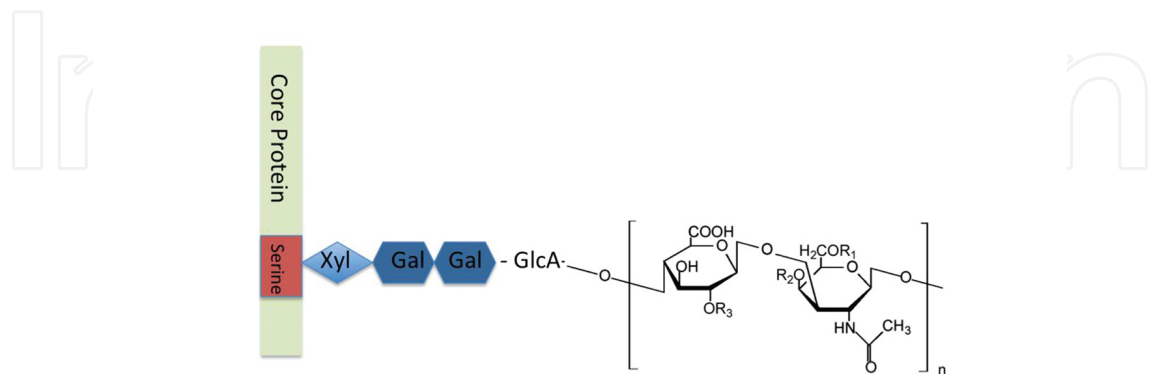


Figure 2. Structure of neural extracellular matrix (ECM). Reproduced with permission from Miyata et al. [14].

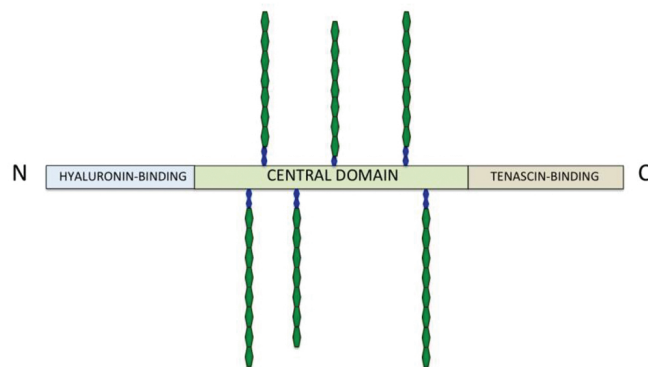
HA is associated with a number of proteins that are organized into a scaffold within the ECM. The major group is chondroitin sulfate proteoglycans (CSPGs) whose structure consists of a core protein covalently linked to chondroitin sulfate glycosaminoglycan (CS-GAG) through serine residues [13, 14]. Chondroitin sulfate is a disaccharide chain consisting of glucuronic

acid and N-acetylgalactosamine linked via a β -glycosidic bond and is polymerized into chains through the activity of chondroitin synthase and polymerizing factor [15]. Variability within CSPG derives from variation within the core protein of CSPGs as well as the number and

(A) Binding of glycosaminoglycan side chains to core protein through serine residues (Xyl= xylose, Gal = galactose). Sulfate groups added at R



(B) CSPG core protein with N-terminal hyaluronin binding domain and a C-terminal tenascin-binding domain. Central domain binds GAG side chains



(C) Structure of Lecticans (from [14]):

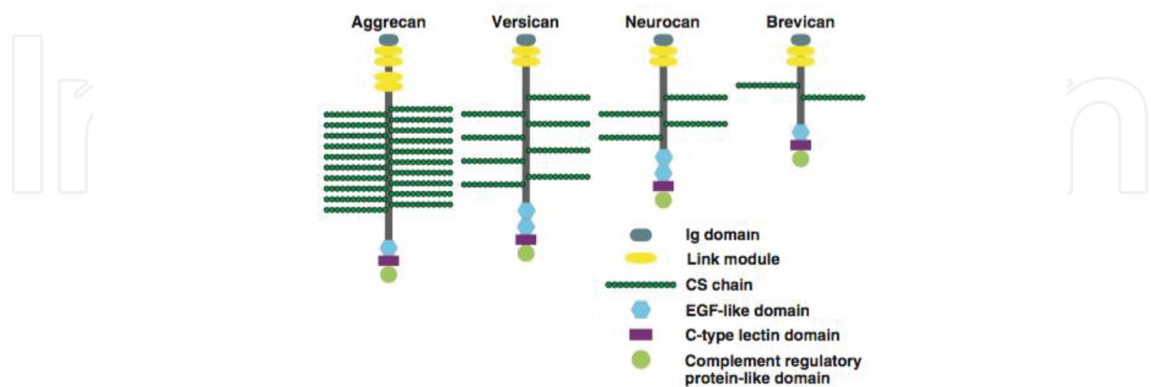


Figure 3. Chondroitin sulfate proteoglycan structure. (A) Binding of glycosaminoglycan side chains to core protein through serine residues (Xyl, xylose; Gal, galactose). Sulfate groups added at R. (B) CSPG core protein with N-terminal hyaluronan binding domain and a C-terminal tenascin-binding domain. Central domain binds GAG side chains. (C) Structure of Lecticans (Reproduced with permission from Miyata et al. [14]).

positions of sulfate groups, which are added via chondroitin sulfotransferases [16, 17]. This variability determines the CSPG binding properties and their function. Link proteins such as Bral-1 and 2, [18], Crtl1 [19], and HAPLN1 [20] stabilize the interaction between HA and CSPG within the PN. Mice lacking Crtl1 have attenuated PN and persistent plasticity in the visual cortex [21].

The classes of CSPG include (1) lecticans such as aggrecan, brevican, neurocan, and versican [22], (2) phosphacan (a tyrosine phosphatase) [23], and (3) small leucine-rich proteoglycans [24]. CSPG can be associated with the plasma membrane through a membrane-spanning domain [25] or a GPI-anchor [26], or can be secreted into the ECM (e.g., lecticans and phosphacan). In the CNS, the chondroitin sulfate side chains act as chemorepellents, and CSPGs are known to inhibit axon projection and cell motility and limit neural plasticity [27]. The lecticans are the principal CSPGs in the CNS, whose core proteins consist of an N-terminal HA-binding domain and a C-terminal domain that binds tenascin-R [28] and tenascin-C [29] (**Figure 3**).

The tenascin family of glycoproteins has four members, tenascin-R, -C, -X, and -W, which are encoded by four genes with a number of splice variants [30] and are believed to modulate cell adhesion and migration. Tenascin-R (formerly called restrictin) is found exclusively in the adult CNS and forms trimers which crosslink CSPGs. Tenascin-R inhibits adhesion of neural cells to fibronectin [31]. Tenascin-C is expressed during embryonic development by migrating neural crest cells, is re-expressed during wound healing and in gliomas, and is thought to be involved with increasing glioma cell proliferation and migration [32]. In tenascin-C knockout mice, CSPGs aggregate and fewer PN form [33]. Tenascin-X is not found in the nervous system. Tenascin-W is expressed in blood vessels within gliomas and may be involved in angiogenesis [34].

The interstitial ECM forms a highly compressible network of HA and CSPG filaments that is resistant to cell migration by virtue of the inhibitory actions of CSPG, especially their CS components, paucity of anchorage points in the water-rich environment, and the presence of sequestered inhibitory molecules such as slits [35], semaphorins [36], and netrins [37].

In contrast to the hydrated PN that surrounds neurons and the loose interstitial matrix of the brain parenchyma, the ECM around the brain's vasculature and subpial surfaces forms a more rigid basal lamina that contains laminin, fibronectin, and type IV and VI collagens and is similar to the ECM in other tissues [35]. This substrate is more likely to allow adhesion of migrating cells and for this reason, gliomas tend to follow blood vessels and subpial surfaces as they invade into the surrounding tissue [36]. They do not, however, degrade the basal lamina and thus do not generally intravasate and spread hematogenously to distant sites.

3. Migration vs. proliferation

One of the characteristics of malignant gliomas that makes them universally fatal is their ability to infiltrate normal brain parenchyma. This diffuse spread makes surgical cure impossible and

makes treatment with radiation and chemotherapy difficult and inefficient. The rapid proliferation of cells in malignant gliomas changes the tumor microenvironment, which becomes hypoxic, acidic, and devoid of glucose and other nutrients. Tumor cells must adapt to these changes to survive and thus change from a proliferative to a migratory phenotype in order to reach a more favorable environment. The mechanism by which tumor cells transition to this migratory state and the factors which trigger this process are therefore important to understand as it serves as an excellent target for therapy. This complex metabolic change, sometimes called the “epithelial-mesenchymal transition,” is poorly understood, however, and involves multiple signal transduction pathways with many molecules needed to effect the changes in gene expression needed to bring about this transition.

One of the key molecules involved with sensing stress in a cell's microenvironment is the AMP-activated serine/threonine protein kinase (AMPK) [37]. AMPK is activated by a high AMP/ATP ratio and other conditions of metabolic stress and causes cells to conserve energy, thus regulating their cellular homeostasis in response to environmental cues. AMPK is activated in response to environmental stress through phosphorylation by three known protein kinases: liver kinase-B1 (LKB1) and the two calmodulin-dependent protein kinases, CaMKK α and CaMKK β , that phosphorylate AMPK in response to high intracellular calcium levels [38]. Once activated, AMPK exerts its effects on cellular metabolism through many downstream molecules, one of which is cyclooxygenase-2 (COX2), whose inhibition by AMPK leads to more aggressive tumor growth and invasion [39]. Overexpression of COX2 has been seen in many types of cancer including colon, breast, and lung [40].

LKB1, a tumor suppressor gene, is constitutively active and is the primary AMPK kinase. Its phosphorylation of AMPK sets off a cascade that results in liberation of intracellular ATP and conservation of energy through regulation of biosynthetic pathways [41]. Mutations in LKB1 are found in Peutz-Jeghers syndrome [42] as well as melanoma [43], lung [44], and pancreatic cancers [45]. LKB1 signaling pathways also are involved in cell migration by virtue of their control of cytoskeletal proteins involved in cell polarization and migration. LKB1 deficiency leads to alterations in cell polarity and impaired migration of neural progenitor cells *in vivo* [46], while LKB1 activation is known to inhibit cell proliferation and can affect cellular polarity, which is essential for cell migration [47]. The latter effect is thought to be mediated, in part, through phosphorylation of MAP/microtubule affinity regulating kinase-3 (MARK-3), which regulates phosphorylation of microtubule-associated proteins [48] and phosphorylation of myosin light chain-2 directly by AMPK [49].

While the activation of AMPK leads to energy conservation in nutrient-poor environments, the mammalian target of rapamycin complex-1 (mTORC1) is a serine/threonine kinase, which promotes cell growth and proliferation. Inhibition of apoptosis [50] by mTOR overactivity has been observed in several types of cancers [51]. The balance between AMPK and mTOR is maintained in part by the tuberous sclerosis complex-2 gene (TSC-2), which is activated by AMPK and which, in turn, inactivates mTOR [50] (**Figure 4**). In addition, AMPK directly phosphorylates Raptor, a scaffold protein in the mTOR1 complex, resulting in direct inactivation of mTORC1 [52]. Mammalian target of rapamycin complex-2 (mTORC2) is considered resistant to rapamycin and is not sensitive to nutrients in the cellular

microenvironment. It activates PKC- α and AKT to regulate the structure of the actin cytoskeleton [53].

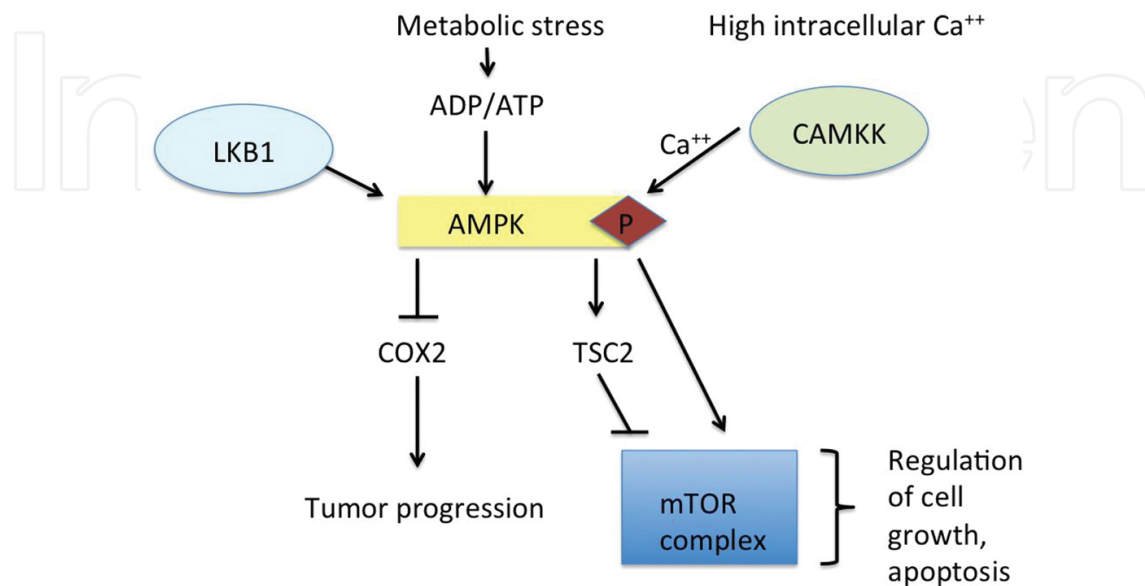


Figure 4. The AMPK/mTOR system. AMPK, AMP-activated serine/threonine protein kinase; CAMKK, Ca(2+)/calmodulin-dependent protein kinase kinase; COX2, cyclooxygenase-2; LKB1, liver kinase B1, mTOR, mammalian target of rapamycin; TSC2, tuberous sclerosis-2.

In addition to intracellular energy levels, hypoxia and acidity are triggers for cells to regulate their gene expression to adapt to a hostile environment. In tumors, hypoxia occurs due to rapid cell proliferation and inadequate blood supply from aberrant blood vessels. It leads to resistance to radiation and chemotherapy and is associated with a more aggressive disease and a poorer outcome. Oxygen homeostasis is mediated by the hypoxia-inducible factor (HIF) family of basic helix-loop-helix transcription factors, which consist of heterodimer of a constitutively expressed beta-subunit and an alpha-subunit which, when translated, is only stabilized under hypoxic conditions and is degraded once hypoxia has been corrected [54–56]. HIF-1 induces expression of dozens of target genes involved in the regulation of angiogenesis, cellular metabolism, and cell migration by binding to hypoxia-responsive elements (HREs) in their promoters. HIF-1 α directly activates transcription of vascular endothelial growth factor (VEGF) [57, 58], which is the major regulator of angiogenesis and directs new blood vessel growth into hypoxic areas. HIF-2 α knockdown leads to reduced levels of VEGF and poorly vascularized, highly necrotic tumors in neuroblastoma [59]. In order to adapt to hypoxia, cells switch from aerobic to anaerobic metabolism, and this shift is regulated, in part, by HIF-1. Glycolytic enzymes such as pyruvate kinase M2, phosphoglycerate kinase, and aldolase are induced by HIF-1 [60, 61] as are the glucose transporters, GLUT-1 and GLUT-3 [62]. Additionally, pyruvate dehydrogenase kinase-1 is activated, reducing mitochondrial oxygen consumption by preventing pyruvate from entering the citric acid cycle [63]. Finally,

HIF-1 is essential in the epithelial-mesenchymal transition by directly regulating the expression of Twist, which is essential for cancer metastasis [64]. Twist is a basic helix-loop-helix transcription factor whose expression is regulated through a number of signal transduction pathways including Akt, Ras, and Wnt and whose expression correlates with higher tumor grade [65]. It inhibits the E-cadherin-mediated adhesion between cells, which enables tumor cells to adopt a more motile phenotype [66]. Twist also serves as a survival factor by inhibiting p53-induced apoptosis by counteracting the effects of c-MYC in neuroblastoma [67]. HIF-1 regulates expression of a number of adhesion molecules, such as alpha- and beta-integrins and E-cadherin [68–70], matrix metalloproteinase-2 and -9 [71, 72] as well as a number of chemokines and their receptors including c-Met and CXCR4 [73–75], suggesting how hypoxia may play a role in triggering cell migration and digestion of the ECM.

Much remains to be elucidated regarding the molecular cascades through which cells transition to a migratory phenotype. Rapid proliferation creates a toxic microenvironment that, when sensed by the cell, sends a signal through the AMPK-mTORC axis or by HIF-1 and others to effect the changes in transcription needed to bring about the transformation to a migratory phenotype so the cell may escape to a more favorable environment. As they leave the main tumor mass and move into the brain parenchyma which limits and inhibits their migration, glioma cells remodel their environment by secreting degradative enzymes and novel ECM components which attempt to recapitulate the more permissive, primitive structure of the developing brain.

4. ECM Remodeling

4.1. Degradation

In 1946, Fisher [76] proposed that metastatic spread of tumors may be mediated by proteolytic degradation of the ECM. Since then, several classes of intracellular and extracellular proteases, both secreted and membrane-bound, have been identified which play roles in tumorigenesis including cell proliferation, adhesion, migration, angiogenesis, and apoptosis. The coordination between ECM degradation and subsequent cell adhesion to ECM components through integrin and other receptors followed by migration is the basis of glioma infiltration into brain parenchyma.

Lysosomal cathepsins are proteases, which are critical in removing other proteases which are in turn critical in removing unwanted cellular and extracellular components such as collagens, fibronectin, and laminin [77], and secreted cathepsins also mediate the activity of matrix metalloproteases (MMPs) by degrading their inhibitors TIMP-1 and TIMP-2 [78]. Cathepsin B promoter activity and protein levels are higher in high grade gliomas than in low-grade gliomas, and the protein is maximally expressed at the leading infiltrating edge of enlarging tumors [79]. Additionally, upregulation of cathepsins B and D has been shown to correlate with glioma tumor grade and invasiveness [80, 81], while inhibition inhibits glioma cell invasion *in vitro* [82]. Another class of intracellular proteases is the caspases, a family of intracellular cysteine proteases essential for apoptosis that are synthesized as inactive pro-

caspases and activated by pro-apoptotic signals [83]. Malignant cells have the ability to escape apoptosis and, in neuroblastoma cells, the caspase 8 gene (CASP8) has been shown to be either deleted or silenced [84]. Loss of CASP8 correlates with increased risk of metastasis in patients with neuroblastoma. Other caspases, including CASP10, 3, 5, 6, and 7, have also found to be mutated in various tumor types [85–89].

Extracellular proteases are secreted by migrating tumor cells in order to degrade ECM components and to release chemoattractant and chemorepellent molecules to direct further tumor cell migration [90]. MMPs are a class of 28 zinc-dependent endopeptidases whose expression by tumor cells has been linked to increased invasion, proliferation, angiogenesis, and morbidity [91]. There are both membrane-bound MMPs and secreted forms which are released as inactive zymogens in response to growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor beta (TGF- β) [92], as well as in response to cell–cell and cell–ECM interactions and signaling [93]. These inactive proenzymes contain a pro-peptide residue at the N-terminus, which masks the zinc ion within the catalytic region. Proteolytic cleavage of this region exposes the catalytic domain and activates the enzyme [94]. MMP2 and MMP9 are both expressed in human glioma cells *in vitro* [95, 96] and overexpression in gliomas correlates with higher tumor grade and poorer prognosis [97]. The MMPs localize to the leading edges of migrating cells, and many components of the neural ECM have been identified as substrates for MMP2 and MMP9 including the lecticans-aggrecan [98], versican [99], brevican [100], and neurocan, as well as link protein and tenascin [101] and components of the vascular basement membrane such as laminin, fibronectin, and collagen [102]. Although these substrates have been demonstrated *in vitro*, the exact role of MMP *in vivo* is unclear. Versican, for example, appears not be a major MMP substrate *in vivo*, and glioma cells do not degrade the basal laminae of blood vessels to enter the bloodstream. MMP activity is tightly regulated at the level of transcription, activation of the zymogen, and by activity of tissue inhibitors of metalloproteases (TIMS) [103]; the degree of ECM digestion during migration is clearly a complex and carefully regulated process. MMPs are also involved in the “pro-angiogenic switch” that stimulates the production of new blood vessels into the growing tumor mass [104], a process mediated, in large part, by the VEGF signaling pathway [105]. The transmembrane metalloprotease, MT1-MMP, can directly degrade the ECM and also activate pro-MMP2 and upregulate VEGF expression [106], suggesting that this MMP may play a major role in tumor invasiveness and angiogenesis and may serve as a potential target for therapy.

Another class of zinc-dependent metalloproteases is the ADAMS (a disintegrin and metalloproteinase) and the related ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs). ADAMS are membrane-bound, zinc-dependent metalloproteases that contain a pro-domain which is cleaved to activate the metalloprotease, a disintegrin domain which can bind to integrin receptors, a cysteine-rich domain, an EGF-like domain, and a transmembrane domain at the C terminus [107] (**Figure 5**). ADAMTS are extracellular proteases similar in structure but lack the EGF and transmembrane domains and contain an additional thrombospondin type I motif at the C terminus which may bind them to the ECM [108]. ADAM-8 and ADAM-19 are expressed at high levels in gliomas, and its expression

correlates with invasiveness [109]. ADAM-17 activates the EGF/phosphoinositide-3 kinase/serine/threonine kinase signal transduction pathway under hypoxic conditions and leads to increased tumor cell invasion [110]. ADAM-22 normally inhibits astrocyte proliferation in normal brain via interactions between its disintegrin domain and cell surface integrins. It is downregulated in high-grade gliomas leading to elimination of this growth inhibition [111]. ADAMTS-4 and ADAMTS-5 degrade the lectican and small leucine-rich repeat families of proteoglycans, and their expression also correlates with glioma invasiveness [112].

ADAM



ADAMTS



Figure 5. ADAM/ADAMT structure. ADAM and ADAMT members contain a propeptide domain (Pro) followed by the enzymatic metalloprotease domain containing the Zn-binding site followed by the disintegrin- and cysteine-rich domains. ADAM members contain an EGF-like domain and ADAMTS contain additional thrombospondin type 1 motifs (TSP1) transmembrane (TM).

Finally, plasmin degrades ECM components and activates several MMPs, and the plasminogen activators, urokinase-type PA (uPA), expressed by glioma cells, and tissue-type PA (tPA) transmembrane (TM) expressed in the endothelium of blood vessels, both play an important role in tumor cell invasion and angiogenesis [113, 114]. There is a higher level of expression of uPA in higher-grade gliomas than in lower-grade gliomas [115] and its binding to its receptor (uPAR) causes it to form a heteromeric complex with integrin receptors, which are also highly expressed on glioma cells. These then initiate signal transduction cascades that result in the upregulation of uPA, MMP, and other molecules that promote cell migration through the ECM [116].

4.2. Synthesis

It is evident that intracellular and extracellular proteases play important roles in the complex process of glioma cell-cell adhesion and attachment and detachment to ECM components during migration. This process is mediated by a well-regulated cascade of signal transduction pathways that also lead to the production of novel ECM components to create a new scaffold on which tumor cells can migrate. As migrating cells degrade the ECM, they

further change their microenvironment by secreting novel proteins and liberating peptides on which to migrate and generating chemoattractants and chemorepellents for guidance. In many ways, this new environment is reminiscent of that of the primitive CNS in which cell migration was abundant in setting up the organization of the brain.

One of the earliest events in glioma formation is the loss of the p53 tumor suppressor gene and upregulation of secreted protein acidic and rich in cysteine (SPARC), an ECM-associated glycoprotein that has an anti-adhesive role and leads to cell rounding and detachment from the ECM [117]. This is thought to be accomplished through the activation of P38 mitogen-activated protein kinase (MAPK)-heat shock protein (HSP)-27, Akt, and Shc-Raf-extracellular signal-regulated kinase (ERK) signaling pathways [118]. SPARC is secreted at the leading edge of the invading cells [119] and has been shown to increase invasion *in vitro* [120] and *in vivo* [121]. Additionally, the combination of SPARC overexpression and loss of p53 may play a role in promoting cell survival by escaping immune surveillance [122]. SPARC is highly expressed in gliomas, and increased levels are associated with poorer prognosis [123]. Interestingly, SPARC levels are higher in developing brain, where cell migration is necessary for setting up the architecture of the developing brain, so it is not surprising that invading glioma cells try to recapitulate this more permissive environment.

The Receptor-type protein tyrosine phosphatase mu (PTP μ) is a cell adhesion molecule normally found in neurons and glia but is absent in higher-grade, infiltrative gliomas. It is hypothesized to be involved with cell-cell adhesion and contact inhibition and that its loss allows for cell migration [124]. PTP μ is cleaved in human GBM tumor tissue and cell lines by a number of proteases including ADAMS, calpain, and serine proteases to generate protein fragments with unique biologic functions affecting cell adhesion and migration [125]. In addition to the degradation of the protein component of the ECM, glioma cells secrete hyaluronidases, which break down HA in the ECM, generating soluble HA, which activates MMP and promotes invasion [126]. Increased levels of HA and hyaluronectin are found in peripherally invasive regions of certain tumors [127] creating a more disorganized matrix through which cells can migrate. Receptor-type protein tyrosine phosphatase zeta (RPTP- ζ) is a membrane-bound proteoglycan expressed in developing and adult brains as well as in migrating glioma cells [128]. The soluble factor pleiotrophin is overexpressed in gliomas and, through binding to RPTP- ζ , promotes cytoskeletal changes through modification of beta-catenin, beta-adducin, and Fyn [129]. RPTP- ζ undergoes differential splicing and one splice variant, phosphacan, is a soluble factor lacking the cytoplasmic phosphatase domain. Phosphacan is also highly expressed during embryogenesis and in migrating glioma cells and may regulate glioma migration through interactions with tenascin in the ECM [130] and axonin [131].

Two members of the lectican family, which are normally inhibitory to cell migration, versican and brevican, have unique isoforms that are present at different times of development, and these tumor-specific isoforms have been shown to promote invasion. Versican undergoes differential splicing to generate four different isoforms (V0, V1, V2, and V3), which vary in their GAG-binding domains. The V2 isoform is predominant in the adult CNS and is a potent inhibitor of axonal growth into the ECM [132]. The V0/V1 isoform, however, is found in the

primitive developing brain and is upregulated by TGF- β in malignant glioma [133], where it acts as a pro-migratory factor by upregulating membrane type 1 matrix metalloprotease (MT1-MMP) through the activation of microglial Toll-like receptor 2 (TLR2) [134]. Brevican, on the other hand, undergoes differential glycosylation and there are novel glycoforms in gliomas in developing and mature brain [135]. It is overexpressed in malignant gliomas, and its brevicin knockdown inhibits proliferation, invasion, and spread of brevicin-expressing glioma cells *in vitro* [136]. Not only is brevicin overexpressed in gliomas, it is also proteolytically cleaved by metalloproteases of the ADAMTS family including ADAMTS-4 and -5, which are also overexpressed in gliomas [137]. If this posttranslational cleavage is blocked, brevicin does not enhance glioma cell invasion *in vitro* or tumor progression *in vivo* [138].

Because the basal lamina of blood vessels presents a more favorable substrate for migration, it is not surprising that migrating glioma cells secrete basal lamina components to travel on. For example, certain laminin isoforms are secreted by glioma cells, and these cells interact with these isoforms and others on the tumor vasculature through the $\alpha 3 \beta 1$ integrin during migration [139]. As tumor cells proliferate, the tumor mass becomes denser, and this mechanical stress induces secretion of collagens, their crosslinker LOX, and the angiogenic factor VEGF [140]. Collagens bind to integrins via integrin-binding domains at the cell surface and can thus activate signal transduction pathways that control proliferation, angiogenesis, and migration [141]. Integrins are associated with the actin cytoskeleton through the interaction with talin and with the microtubule network via paxillin and binding to components of the cytoskeleton modulates the affinity of integrins for the ECM [142]. Glioma cells can migrate along fibronectin in the vascular ECM, and both versican and brevicin can increase synthesis of fibronectin through an EGFR-dependent mechanism by binding to β -1 integrin and β -3 integrin, respectively [143–145]. These newly synthesized fibronectin fibrils accumulate at the migrating cell surface and serve to reorganize the ECM and promote cell attachment [146, 147].

4.3. Transcriptional control

The regulation of the expression of novel proteins in gliomas is poorly understood but is surely a complex process involving many signal transduction pathways and transcription factors, and some candidates have emerged that may regulate cell migration. The Oct-3/4 transcription factor is involved in regulating self-renewal in stem cells and was recently found to be overexpressed in malignant gliomas. Oct-3/4 expressing-glioblastoma cells exhibited increased migration and invasion *in vitro* and resulted in upregulated FAK and c-Src expression, which mediate integrin signals as well as increased MMP-13 expression [148]. ATF2, another transcription factor expressed in malignant glioma, is thought to be involved in the regulation of cell invasion as its level of expression is correlated with cell invasion *in vitro* [149]. Finally, suppressor of fused (Sufu) is a tumor suppressor which downregulates hedgehog, WNT, and other signaling pathways to prevent tumorigenesis [150, 151]. Overexpression suppresses glioma cell proliferation and invasiveness, angiogenesis, and *in vivo* tumor growth, while knockdown of Sufu promoted these effects, possibly by directly affecting Gli1, a transcription factor in the hedgehog signaling pathway [152].

4.4. Chemotaxis

Once glial cells switch to a migratory phenotype, they degrade the ECM surrounding them, detach from the matrix and extend “invadopodia”, actin-rich protrusions with ECM proteolytic activity that bind to and digest ECM components as a result of complex signal transduction pathways linking the extracellular microenvironment to the actin cytoskeleton [153]. Cells respond to soluble molecules in this environment and use these cues to direct migration through various signal transduction pathways. These include growth factors, soluble peptides generated by proteolysis of cell surface adhesion molecules [118], and small chemotactic cytokines.

The EGF family of growth factors is known to stimulate cell proliferation and migration [154], and overexpression of EGF receptor (EGFR) is an important feature distinguishing high-grade from low-grade gliomas [155], and the highest level of expression is found at the invasive border of the expanding tumor [156]. EGFR is amplified in 40% of GBM and of these, half have a mutant form of the receptor (EGFRvIII) lacking the ligand-binding domain leading to constitutive activation [157]. Ligand binding induces dimerization and activation of EGFR, a receptor tyrosine kinase (RTK), whose signaling results in cell proliferation, angiogenesis as well as metastatic spread through the activation of PI3K-AKT-GSK3b-Rac1 and Ras-Raf-MEK-ERK signal transduction pathways [158]. However, the activation of wild-type EGFR promotes invasion independent of angiogenesis, whereas loss of its activity results in angiogenic tumor growth. EGFRvIII might only be involved in stimulating angiogenic tumor growth when wild-type EGFR expression is lost [159,160]. Formylpeptide receptor (FPR) is a G-protein-coupled receptor that has been shown to be expressed in highly malignant gliomas [161]. Necrotic GBM cells release a number of potential ligands for FPR, and the activation of this pathway promotes chemotaxis as well as the production of VEGF [162, 163]. In addition, FPR has been shown to transactivate EGFR leading to increased chemotaxis and proliferation [164].

Scatter factor/hepatocyte growth factor (SF/HGF) as well as its receptor which is encoded by the c-MET proto-oncogene are both upregulated in malignant gliomas. MET is a transmembrane RTK whose signal transduction cascade leads to increased gliomas motility *in vitro* as well as survival and angiogenesis [165]. Fibroblast growth factor (FGF) may act synergistically with upregulated VEGF and SF/HGF in GBM cells to enhance malignancy [166].

Serine-threonine kinases also play a role in tumorigenesis. TGF- β is an important growth factor whose signaling is involved in invasion as well as proliferation and survival of glioma cells [167]. Its receptor is a serine-threonine kinase that, on ligand binding, oligomerizes and activates a signal transduction cascade that results in the translocation of activated Smads to the nucleus where they interact with other transcription factors to regulate expression of genes involved in cell motility and proliferation [168]. TGF- β signaling upregulates MMP expression and suppresses tissue inhibitors of metalloproteinase (TIMP), thus promoting invasion [169] and inhibition of TGF- β 1 or knockdown of its receptor reduces invasiveness *in vitro* [170].

Chemotactic cytokines are a group of small molecules that have been found to regulate the migration of leukocytes in the immune system and have been found to be involved in

metastatic behavior of certain cancers [171]. Chemokine receptors are G-protein-coupled transmembrane receptors whose signaling pathways regulate many cellular activities including motility. Chemokines and their receptors are expressed throughout the CNS by neurons and glia and are overexpressed in high-grade gliomas [172]. Stromal-derived factor 1 (SDF-1) also called CXCL2 is a chemotactic cytokine, which, along with its receptor CXCR4, is overexpressed in gliomas as well as within the vascular endothelium along the hypoxic rim of the tumor [173, 174]. SDF-1 has been shown to promote the migration of glioma cells *in vitro* [175] by upregulating the expression of membrane type-2 matrix metalloproteinase (MT2-MMP) [176]. CXCL1 is another small chemokine known to be involved in the metastatic spread in melanoma [177] and has been shown to be highly expressed in glioma samples and promotes migration *in vitro* by upregulating MMP-2 and β 1-integrin [178]. TGF- β signaling promotes invasion by reducing expression of neurotactin, a chemokine also known as CX3CL1 whose pro-adhesive properties must be overcome to allow cells to detach and migrate. The treatment of glioma cells with recombinant TGF- β 1 reduced CX3CL1, expression and facilitated glioma cell detachment and dispersion [179].

Both positive and negative signals exist within the microenvironment of glioma cells, and hypoxia is an important chemorepellent as described previously which induces cell migration away from the tumor mass. HIF-1 α is stabilized at the leading tumor edge and mediates cell invasion and angiogenesis through integrin and RTK signaling pathways [180]. Slit glycoproteins are secreted into the ECM and normally serve as chemorepulsive factors but whose expression is diminished in invasive gliomas through promoter methylation. They normally bind to members of the Roundabout (Robo) family of transmembrane receptors and lead to depolymerization of the actin cytoskeleton within the invadopodia to promote cell adhesion [181, 182]. This may be accomplished through the inactivation of Cdc42, a Rho GTPase known to be involved in cell motility [183].

Semaphorins and their receptors (plexins and neuropilins) have been found to be involved in cell migration and metastasis as well as proliferation and angiogenesis in several types of cancers [184–188], and different members of the family have different functions depending on the type of tumor involved. For example, Sema 3A inhibits migration in GBM and has anti-angiogenic properties in meningioma [189, 190]. Secreted semaphorins contain an N-terminal sema domain followed by variable numbers of PSI (plexins, semaphorins, and integrins) and immunoglobulin-like domains in their extracellular regions [191]. Sema 3A binds to the Neuropilin-1 receptor that recruits the PlexinA1 receptor to transduce a chemorepulsive signal. Sema3A also binds Neuropilin-2 but at a lower affinity than Neuropilin-1, and the binding of Neuropilin-2 acts to modulate cell signaling and converts the repulsive signal into an attractive one. Blocking Neuropilin-1 or Plexin A1 switches the Sema3A response from repulsion to attraction, while blockade of Neuropilin-2 suppresses Sema3A's typical chemorepulsive effect [192]. Similarly, Sema 4D which binds to PlexinB1 and acts through Rho [193], and Sema5A which binds to PlexinB3 and acts through Rac1, both act as chemorepellents by ultimately affecting the actin cytoskeleton and altering cell morphology [194].

Finally, Netrins are secreted laminin-associated chemotactic molecules that regulate embryonic axon migration [195] which have also been shown to be involved in glioma cell migra-

tion, mediated by binding to their receptors, deleted in colorectal cancer (DCC), neogenin, and uncoordinated-5 (UNC5) [196–198]. Netrins have been shown to localize to cell surfaces and interact with laminins in the basement membrane of blood vessels. Netrin-1 binding to DCC receptors on migrating glia promotes the formation of focal adhesions, limiting migration. GBMs have been shown to downregulate Netrins, thus releasing the inhibition and promoting loss of cell-cell interaction, promoting migration along basement membranes [199]. Though Netrin-1 binding to the DCC receptor tends to promote adhesion, limiting migration, UNC5 binding transforms this to repulsion [200]. This switch from attachment to motility is reminiscent of that described earlier with the semaphorins.

5. Future Directions

Current therapy for malignant gliomas is aimed at reducing tumor burden and targeting dividing cells with cytotoxic chemotherapy, anti-angiogenic agents, or tumor-treating fields. New agents targeting new pathways are desperately needed as survival still remains extremely poor and glioma cells become resistant over time to current therapies. Delivering therapy to normal brain parenchyma containing infiltrating tumor cells is also difficult as the blood-brain barrier remains largely intact, though several strategies have been attempted to overcome this [201]. Limiting the invasiveness of these aggressive tumors is desirable; however, the molecular pathways involved in this complex process remain incompletely understood and new targets and therapies are lacking. Additionally, because tumor cells seem to adopt either a proliferative or migratory phenotype, preventing migration may impose a selective advantage for cells to proliferate rather than migrate leading to more rapid local recurrences. There is also no way to visualize invading cells until they stop migrating and proliferate to create a radiographically evident tumor mass. Two-dimensional *in vitro* assays have been used to study this process using glass or plastic substrates and more recently, assays using hydrogels or nanofiber scaffolds have been developed to better simulate the three-dimensional microenvironment encountered by migrating tumor cells [202–204].

Identifying molecular targets has been a priority in developing new therapies for GBM, and the complex process of cell migration offers many potential targets including ECM components, proteases, and members of signal transduction pathways. The neural ECM has many unique components that are potential targets for therapy. Because tenascin is upregulated in malignant gliomas and may be a stem cell marker [205], it is an attractive target and phase I trials are underway exploring the use of radiolabeled monoclonal antibodies to tenascin-C or tenascin-R to deliver a radiation boost to the resection cavity. Since many signal transduction pathways involved in glioma invasion often involve RTKs, and since various RTKs are mutated or overexpressed in GBM, RTK inhibitors are an obvious choice of targeted therapy. The results, however, have been disappointing, and no clear RTK inhibitor or combination has demonstrated a significant survival benefit [206]. Similarly, several inhibitors of MMPs have been investigated and, though some have shown efficacy *in vitro*, no clear clinical benefit has yet been demonstrated [207–209]. Cilengitide is an arginine-glycine-aspartic acid containing peptide that targets integrins and, though it was promising in preclinical studies, it failed to

show improvement in progression-free survival or overall survival in both the CENTRIC [210] and CORE [211] trials investigating the addition of cilengitide to standard therapy in patients with newly diagnosed GBM with or without MGMT promoter methylation.

Many molecules involved in regulating cell migration in malignant gliomas are also involved in angiogenesis, cell proliferation, and avoidance of apoptosis; so agents targeting these molecules would be expected to have multiple antitumor effects. However, results have been mixed and, though *in vitro* data are encouraging for many agents, none has proven successful in showing clinical improvement in survival. There is apparently much redundancy in these signaling pathways, requiring a more complete understanding of these molecular events as well as more accurate modeling with which to study the complex processes involved in tumor spread. The earliest events leading to a migratory phenotype would be ideal candidates for therapy, though inhibiting migration could select for a proliferating phenotype leading to faster local recurrence. Hopefully, as we achieve a better understanding of the genetics and molecular alterations leading to glioma invasion, new therapies will arise to limit this aggressive, deadly disease.

Author details

Scott G. Turner*, Maleeha Ahmad and Steven A. Toms

*Address all correspondence to: sgturner@geisinger.edu

Departments of Neurology and Neurosurgery, Geisinger Medical Center, Danville, PA, USA

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