115 Malignant Glioma Microenvironment

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THE TUMOR MICROENVIRONMENT

The microenvironment of a tumor is the set of cellular and molecular components that form the context in which the tumor originates, grows, and eventually disperses through normal tissue. These cells and molecules are in intimate contact with tumor cells and participate in a two-way communication that ultimately supports tumor progression.[1] The tumor microenvironment includes normal epithelial cells; fibroblasts that form the supporting structure—or stroma—of the tissue; blood vessels that grow in response to tumor signals; resident and infiltrating immune cells; signaling molecules provided both by tumor and normal cells; and the extracellular matrix (ECM) that is remodeled by the growing tumor.[2] Because of the rapid growth of tumor cells and their high metabolic demands, the tumor microenvironment also involves alterations and temporal fluctuations in the biochemical conditions within the tissue, such as hypoxia, low pH, and nutrient deprivation, which create additional demands on cells and impose a highly selective milieu. In malignant gliomas, the presence of neurons, astrocytes, oligodendrocyte precursors, and a distinct type of ECM results in microenvironmental components that are unique compared with other solid tumors.[3] The past decade of research has seen a momentous increase in the study of the interactions of malignant glioma cells with normal components of the neural tissue. This has generated considerable interest in strategies to identify and target key elements of the tumor microenvironment that could disrupt glioma growth and have an impact on the final outcome of the disease.[4] This chapter presents an overview of the interactions between glioma cells and the major components of the neural microenvironment, with particular emphasis on the distinctive phenomenon of glioma invasion. In addition, we review current knowledge on the interactions of tumor and immune cells, which are of great therapeutic interest and considered one of the most promising approaches to improve the outcome of this deadly disease.

MALIGNANT GLIOMA INVASION

At no time is the interaction between glioma cells and their microenvironment better illustrated than during the process of tumor cell dispersion throughout the central nervous system (CNS). Although the molecular and cellular mechanisms of tumor cell proliferation can be largely studied with isolated tumor cells in vitro, understanding tumor invasion requires a faithful replication of the interactions between tumor cells and normal cell types and ECM molecules that form the natural barriers to movement in neural tissue (as illustrated in Fig. 115-1).

The infiltration of malignant gliomas through the neural parenchyma is a hallmark of these tumors and a major factor that contributes to tumor recurrence and the eventual failure of current treatments.[5-7] Radiographic and histologic analysis of glioma invasion reveals a pattern of contiguous infiltration that results in poorly defined tumor margins followed by a scattering of malignant cells beyond the observable boundaries of the tumor.[8] Motile cells at the periphery of glioma aggregates in vitro have been compared with the cells from the core and found to be less proliferative.[9,10] A differential gene expression profile associated with the invasive phenotype has also been confirmed by analyzing glioblastoma cells recovered by laser-capture microdissection from tumor cores and their paired surrounding white matter containing infiltrated tumor cells.[11] Several differentially expressed genes in invasive glioma cells are associated with their ability to resist cytotoxic therapies and therefore become the source of tumor recurrence following therapy.[6,7] Indeed, effective control of local glioma recurrence (i.e., at the original tumor site) has been positively correlated with increased incidence of distant recurrence (at distances farther than 2 cm from the original tumor site),[12-14] suggesting that distant tumor foci result from invasive tumor cells that escaped the initial treatments.

In stark contrast to other solid tumors, glioma invasion of normal tissue is not a late feature acquired during malignant progression but instead is a defining feature of both low- and high-grade tumors, suggesting that motility could be a constitutive ability of the neural cell types that give rise to gliomas.[5,15] Indeed, neural progenitor cells have the ability to migrate through brain tissue, and a number of recent studies have suggested that specific types of progenitors (such as oligodendrocyte precursors[16,17]) are candidate cells of origin for malignant gliomas.

Malignant gliomas continuously infiltrate neural tissue as they grow (as illustrated in Video 115-1), which results in a radiographic nonenhancing penumbra around the tumor core observed by magnetic resonance imaging (MRI). Prior histologic studies of tumor infiltration have described increased frequency and distance of dispersed cells in grade II and III astrocytomas compared with the more aggressive glioblastomas.[18] The increased time for dispersion of malignant cells in lower grade gliomas, before the bulk of the tumor is detectable, could explain the reported higher frequency of distant recurrence for grade III astrocytomas compared with glioblastomas.[19] Tumor dispersion and distant recurrence have also been observed in low-grade gliomas, including ependymomas, even several years after the original treatment.[20-22] It is important to remark that these studies have used classical histologic classification of gliomas, and tumor dispersion patterns have not yet been evaluated systematically in gliomas classified by molecular phenotyping. To address this matter, a recent study analyzed the radiographic pattern or invasion in gliomas classified by their expression of the mutant gene for the enzyme isocitrate dehydrogenase 1 *(IDH1)*, which is a predominant mutation in grades II and III gliomas. This study found that tumors with *IDH1* mutation were more invasive than those with wild-type *IDH1*.[23] Because *IDH1*-mutant status is an excellent biomarker of improved prognosis, this appears a paradoxical result, but it is possible that a protracted distant recurrence of those gliomas, as opposed to faster local recurrence in *IDH1* wild-type tumors, may contribute to their extended overall survival.[24]

Despite their insidious infiltrative behavior, malignant gliomas very rarely metastasize outside the CNS,[25,26] and they grow as contained masses if implanted peripherally in animal models. On the other hand, most invasive extracranial tumors that metastasize to the brain show little to no diffuse infiltration of neural tissue.[27] The marked differences in invasive behavior between malignant gliomas and other solid tumors in the brain underscore the presence of unique interactions between the glial neoplasms and their microenvironment,[3] despite the fact that many proteases, cytokines, and ECM molecules are expressed in a similar fashion in gliomas and other solid tumors that metastasize to the CNS. In the next sections we review the interactions of glioma

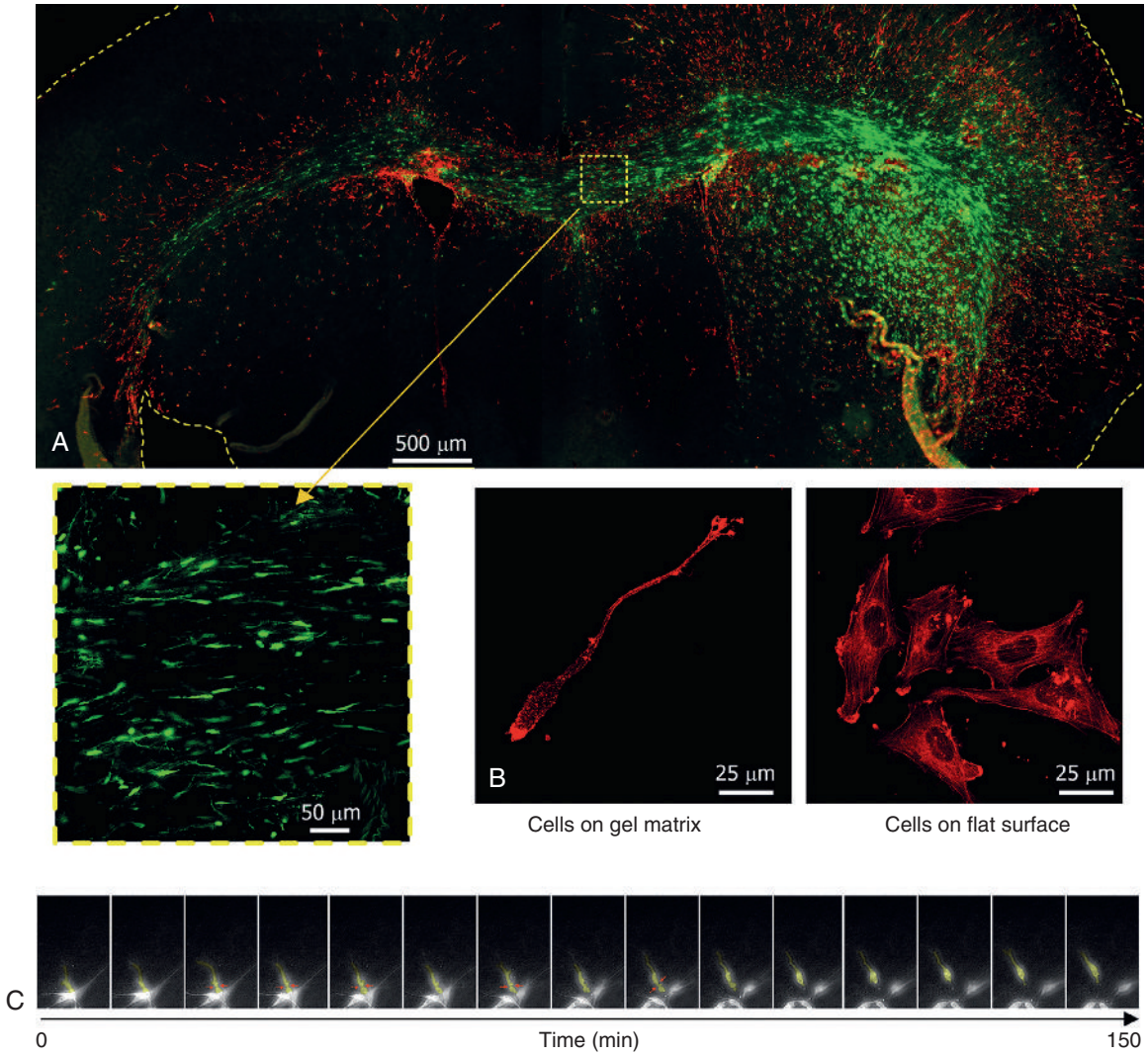

Figure 115-1. Influence of the tumor microenvironment on glioma invasion. A, A representative image of human glioblastoma–initiating cells implanted in the striatum of an immunodeficient mouse to reproduce tumor formation. Notice the extensive infiltration of glioma cells (expressing green fluorescent protein) forming a "stream" across the corpus callosum and far into the contralateral hemisphere. A magnified section of the image *(dashed rectangle)* shows the elongated bodies of individual invasive cells. Reactive astrocytes were detected with anti–mouse vimentin antibody *(orange)* and appear highly polarized toward the tumor cells. **B,** Replication of the neural microenvironment is critical to understanding the biology of glioma invasion. The same tumor cells from **A** show strikingly different morphologies when cultured on flat conventional cultureware compared with soft gels that mimic the neural extracellular matrix (ECM). **C,** Sequential frames from a time-lapse image capture of the glioblastoma cells from **A,** moving through a 250-μm thick slice of cortical tissue in culture. Notice the repeated "pinching" of one cell's body *(arrows)* as it moves away from other tumor cells. This representative behavior of glioma invasion can only be replicated in three-dimensional ECM scaffolds of appropriate stiffness. *(Courtesy of the laboratories of Mariano Viapiano [**A** and **C**] and Sean Lawler [**B**].)*

cells with the neural ECM, with vascular cells that support the tumor and can also help dispersion, with reactive astrocytes, and with cells of the immune system.

INTERACTIONS OF MALIGNANT GLIOMAS WITH THE NEURAL EXTRACELLULAR MATRIX

The neural ECM is the molecular scaffold that fills the extracellular space (ECS) in the CNS and forms the immediate physical support of all cell types. The presence of a significant neural extracellular volume was disputed until the early 1980s, owing to artifacts in the preparation of neural tissue for microscopy and deficiencies in the histologic detection of proteoglycans and mucopolysaccharides.[28,29] However, nonmorphologic methods applied to fresh tissues, based on the diffusion of charged or fluorescent molecules, demonstrated that the ECS occupies up to 20% of total CNS volume, which was later confirmed with improved fixation techniques and immunohistochemistry.[28,30] Measurements in live animals put the CNS among the tissues with the largest extracellular volume compared with other highly vascularized tissues such as kidney and skeletal muscle.[31] Measurements of the ECS have been performed in freshly resected

gliomas, revealing changes in the ECS volume and its tortuosity compared with normal brain tissue[32]: both low- and high-grade astrocytomas have larger ECS volume than normal brain, with volume increases that correlate with grade. Even in highly cell-dense glioblastomas, this acellular volume can reach up to 40% of the total tumor volume (and 58% of total volume in the necrotic regions of these tumors).

Extracellular tortuosity (not to be confounded with the tortuosity of blood vessels) is a measurement of how much the extracellular diffusion of small molecules is limited in real tissues compared with a perfect homogeneous medium. Changes in the amount and composition of the ECM can increase the extracellular tortuosity, revealing important barriers to molecule diffusion. Extracellular tortuosity is similar between low-grade astrocytomas and normal brain, but it increases significantly in anaplastic astrocytomas and glioblastomas. This increase does not correlate with tumor cell density[33] but rather with the accumulation of ECM forming fine "molecular nets" in high-grade gliomas. Interestingly, neither the ECS volume nor the extracellular tortuosity are different in grade II oligodendrogliomas compared with normal brain; this is thought to occur because these tumors are able to intermix with brain tissue without causing significant disruption of the neural architecture.[32,33]

The normal stromal ECM of the CNS, found in the white matter and gray matter neuropil, is a soft gel rich in proteoglycans, glycoproteins, and hyaluronic acid, but notoriously devoid of large fibrillar proteins (e.g., collagens, elastins, fibronectin).[3,34,35] A different type of ECM, forming a well-defined basal lamina (BL) rich in fibrillar proteins (in particular, collagen type IV, laminin type I, and fibronectin), is tightly restricted to the choroid plexus and the perivascular and subpial spaces in the CNS. The major components of the typical neural ECM and the perivascular BL in the CNS, as well as their modifications by malignant gliomas, are listed in Tables 115-1 and 115-2, respectively.

Malignant gliomas show very different interactions with both types of ECM: tumor cells usually adhere well to fibrillar proteins of the BL in vitro[36] and migrate along the BL of perivascular and

TABLE 115-1 Major Components of the Nonfibrillar Extracellular Matrix (ECM) in Neural Tissue and Gliomas

ECM Component (References)	Characteristics	Normal Neural Tissue	Malignant Glioma
Hyaluronic acid (HA)[257-259]	An extremely large (>10^6 Da) acidic polysaccharide not attached to proteins. Generates a large hydrophilic mesh with elastic spaces that facilitate cell growth and motility.	Highly expressed during early neural development but decreases in adult brain and associates noncovalently with proteoglycans, forming insoluble matrix aggregates that inhibit cell motility	Increases up to fourfold higher than in normal brain. Tumor cells proliferate and migrate through the HA mesh. HA is degraded by tumor hyaluronidases and the fragments stimulate synthesis of proteases and ECM proteins by tumor cells.
Chondroitin sulfate proteoglycans (CSPG, lectican family)[260-264]	Large, secreted glycoproteins (aggrecan, versican, neurocan, and brevican) that bind HA and cell membrane receptors, tethering cells to the ECM. CSPGs exhibit many variants generated by alternative splicing, cleavage, and posttranslational modifications.	Although each molecule has a distinctive expression profile, CSPG expression generally increases in the adult CNS. CSGPs are considered inhibitory molecules that prevent cell motility and axonal extension. They are major components of the glial scar.	The CSPGs brevican and versican are highly increased in gliomas and paradoxically promote tumor invasion (brevican) and proliferation (versican). Specific isoforms upregulated in gliomas are credited for these protumoral mechanisms.
Phosphacan[265-268]	Soluble form of the membrane tyrosine phosphatase RPTP-β (a proteoglycan carrying chondroitin- and keratan-sulfate that does not bind HA). Binds the neurite growth-promoting factor pleiotropin (*PTN* gene).	Highly expressed by neurons in the developing CNS and associated with axonal extension. Decreases in the adult CNS but is reexpressed by astrocytes following injury.	RPTP-β is increased in grades II-III gliomas, whereas the soluble form phosphacan is increased in GBM. It is postulated to increase cell migration through interaction with *PTN*.
Link proteins (*LP*)[269-273]	Small soluble glycoproteins that bind HA and CSPG forming multimolecular complexes considered the basis of the neural ECM scaffold	LPs follow an expression profile similar to CSPGs, increasing in the adult CNS and contributing to the axon-inhibitory ECM around neurons	Expression is strongly reduced in GBM tissue, but they may be found in cultures of GBM-initiating cells. They promote glioma cell migration in vitro.
Tenascin-R (TNR) and tenascin-C (TNC)[267,274-276]	Multimeric proteins that bind CSPG, fibronectin, and cell surface receptors, contributing to the structure of the neural ECM	Highly expressed in neural development but decrease and become restricted to white matter in adult CNS. They form boundaries within the ECM scaffold to regulate neural precursor migration and axonal extension	TNC is highly expressed in the perivascular space and may promote angiogenesis, tumor cell proliferation, and glioma invasion in combination with PDGF signaling.
SPARC, hevin, testicans[277-280]	A family of phosphorylated glycoproteins that bind growth factors and fibrillar proteins	Expressed in the developing CNS and involved in neural circuit formation and tissue remodeling. Expressed by astrocytes in adult CNS	Highly expressed by malignant glioma cells, regulate expression of metalloproteases, and promote adhesion and migration of glioma cells on perivascular basal lamina
Fibulins (FIB)[256,281-283]	A family of secreted proteins that associate with proteoglycans and fibrillar proteins (collagens, elastins, and fibrillins) to form large fibrillar structures in elastic matrices	Large fibulins (FIB1 and FIB2) are expressed in early neural development and can replace tenascins to form ECM scaffolds with CSPG. Their expression is much reduced in adult CNS. Small fibulins (FIB3, FIB4, and FIB5) are virtually absent in normal CNS.	FIB3 and FIB4 are highly upregulated in malignant glioma cells and promote tumor invasion and resistance to apoptosis. FIB3 is also a proangiogenic signal in the tumor vasculature.

CNS, central nervous system; GBM, glioblastoma multiforme; PDGF, platelet-derived growth factor; RPTP-β, receptor-type protein tyrosine phosphatase beta.

TABLE 115-2 Major Components of the Basal Lamina in Perivascular Neural Tissue and Gliomas

ECM Component	Characteristics	Normal Neural Tissue	Malignant Glioma
Fibronectin[3,74,259]	A multidomain-secreted glycoprotein and major component of the basal lamina. Binds multiple ECM proteins and integrin receptors.	Expressed during fetal development by neurons and astrocytes, potentially involved in synapse formation. It is absent in the adult CNS stroma and restricted to the blood vessels (expressed by endothelial cells) and the glia limitans (expressed by meningeal epithelial cells).	Expressed by most glioma cell lines in vitro but less frequently in cultures of glioma-initiating cells. Although it is listed as one of the top upregulated genes in glioblastomas, expression in vivo is largely restricted to the tumor perivascular space. Fibronectin strongly stimulates integrin-dependent tumor cell adhesion and motility.
Laminin[101,102,284]	A secreted protein with multiple isoforms derived from the combination of three different chains. Laminin-1 is a major component of the basal lamina and the Matrigel* mixture used to study glioma invasion in vitro.	Expressed mostly around adult brain blood vessels (laminin-1) or by reactive astrocytes during development and in the adult CNS (laminin-2)	Laminins are major components of the perivascular niche where glioma-initiating cells arise and proliferate. They activate α_6 integrins and enable tumor cell growth.
Collagens[285,286]	The most abundant proteins in mammals that form a superfamily with 28 known members. Characterized by three chains forming a triple-helix structure, collagens form supramolecular, stretch-resistant fibers or participate in other ECM networks in which they provide resistance to tension forces.	Fibrillar collagens (COL-I, II, III, V, and XI) are expressed at very low levels in the CNS. Basal lamina collagens (IV and VI) are abundant during development and in the adult CNS around blood vessels. They are not expressed by astroglia and do not participate in the structure of the amorphous neural ECM.	Malignant gliomas express several types of fibrillar and sheet-forming collagens. They bind integrins and discoidin receptors and facilitate tumor cell adhesion and invasion. Collagens secreted by glioma cells (in particular, COL-IV) introduce major changes in stiffness, tortuosity, and adhesive properties of the ECM. Glioma cells further modify the collagen-rich ECM by degradation (via metalloproteases) and enzymatic cross-linking (via lysyl oxidases).
Heparan sulfate proteoglycans (HSPGs)[263,287-289]	Cell surface glycoproteins with a transmembrane domain (syndecans) or lipid anchor (glypicans). Some HSPGs are secreted into the ECM (agrin, perlecan) and form the scaffold of the basal lamina. HSPGs act as coreceptors of cell adhesion molecules and receptor tyrosine kinases.	Regulate proliferation and differentiation of neural cells by binding to and forming gradients of cytokines, growth factors, and morphogens. Interactions of HSPG with NCAM and integrins are required for migration of neural cell precursors.	Expression of HSPG and sulfation of HS chains are increased in gliomas and correlate with tumor grade. HSPGs promote activation of receptor tyrosine kinases and contribute to glioma malignancy.
Elastin[75,290-292]	Secreted fibrillar protein and major component of elastic fibers in connective tissue and arteries. Fibers are formed by covalent polymerization and cross-linking of tropoelastin monomers.	Elastin transcription is active in the developing neuroepithelium, but elastin fibers are only found in adult meninges and the brain vasculature, where they contribute to the basal lamina structure.	Glioma cells express and degrade elastin but do not form elastic fibers. Glioma cells also express proteins that can bind elastin (including elastin-binding protein and fibulins) and lysyl oxidases that can cross-link it. Elastin fragments increase glioma cell proliferation and invasion.

CNS, central nervous system; ECM, extracellular matrix; HS, heparan sulfate; NCAM, neural cell adhesion molecule.
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subpial surfaces, which form major anatomic routes for tumor dispersion.[5,37] However, glioma cells do not seem to be able to cross this BL in vivo, which would lead to intravasation or intrameningeal spread, both of which are extremely rare in these tumors.[38,39] On the other hand, adhesion of isolated glioma cells in vitro can be attenuated by stromal chondroitin sulfate, hyaluronic acid, or myelin-associated glycoprotein.[40-42] However, individual glioma cells readily traverse the ECM of the neural parenchyma, dispersing along white matter tracts and through the gray matter neuropil. It is worth noting that the interaction of glioma cells with the BL and stromal neural ECM seems to be essentially reversed in nonneural tumors that metastasize to the CNS. Metastatic cells cross the BL and readily extravasate into the brain perivascular space, but they rarely invade the inhibitory neural ECM and instead grow close to their extravasation sites as contained masses.[27]

The motility of individual glioma cells appears to involve locally restricted degradation of the ECM[43,44] and may be largely facilitated by "squeezing" of the cell body through ECM spaces (see Fig. 115-1B and C), which is driven by myosin II–dependent cell contraction[45,46] and rapid changes in total cellular volume[47,48] (Video 115-2). The resulting amoeboid movement has been largely compared with similar migratory behavior observed in neural cell precursors,[49] reinforcing the concept that glioma cells may derive from a motile neural precursor. Cell motility is, in addition, facilitated by additional mechanisms of ECM remodeling, such as incorporation of glioma-secreted matrix molecules into the neural ECM and covalent modification of the ECM scaffold. The resulting changes open intercellular spaces and increase the stiffening of the ECM, facilitating tumor cell adhesion and migration.

Most of the glioma-secreted proteases that degrade and remodel the ECM belong to the large families of matrix metalloproteases (MMPs) and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs).[34] MMPs have been classically implicated in glioma invasion owing to their ability to cleave and degrade fibrillar proteins such as collagens and fibronectin.[50,51] The collagenases MMP-2 and MMP-9 have been identified numerous times as typical MMPs upregulated in gliomas compared with normal brain tissue and shown to be required to promote tumor invasion.[52] Although these metalloproteases can also degrade chondroitin sulfate proteoglycans

in vitro,[53,54] cleavage of these proteoglycans by MMPs in vivo is less common and possibly less functionally relevant.[55,56] Instead, neural proteoglycans are primarily cleaved by ADAMTS enzymes, in particular the aggrecanases ADAMTS-4 and ADAMTS-5, with minor contribution of ADAMTS-1.[57-61] Of these, ADAMTS-5 is particularly elevated in malignant gliomas and correlates with tumor grade.[57,62] Finally, it is worth noting that, although studies of MMPs and ADAMTS have dominated the protease-oriented research for malignant glioma, other proteases such as the cysteine cathepsins also play a significant role in degrading the neuropil and BL ECM.[3,63] Even though these proteases are mostly active in the acidic lysosomal environment, their secretion into the acidified tumor stroma make them a relevant factor in ECM remodeling that may contribute to glioma invasion and angiogenesis.[3,64,65]

As indicated previously, the dispersion of single glioma cells through the neural ECM is largely dependent on their ability to open intercellular spaces and squeeze through them in an amoeboid manner.[44,66] This process may also be facilitated by the secretion of proteoglycans and hyaluronic acid by glioma cells. These high-molecular-weight, hygroscopic molecules retain water and increase the hydrated space around the tumor cell,[67] facilitating cell growth and movement. Brevican and versican are the two major chondroitin sulfate proteoglycans secreted by glioma cells and have been shown to promote tumor growth and invasion.[56,68,69] Both proteoglycans exhibit specific isoforms upregulated in glioma[70,71] and are, in addition, substrates for the ADAMTS enzymes. Work with glioblastoma cells in culture has shown that specific domains of these proteoglycans may be released from the ECM scaffold by ADAMTS cleavage, acting as signals that activate integrin and epidermal growth factor receptor–mediated signaling to promote tumor cell proliferation (versican)[72,73] and invasion (brevican).[56,74]

Finally, the ECM of the tumor microenvironment can change not only by the addition or degradation of ECM molecules but also by the chemical (covalent) modification of these molecules. For example, glioma cells secrete enzymes of the lysyl oxidase family,[75] which are copper-dependent enzymes that cross-link neighboring lysine residues in collagen and elastin.[76] In normal tissues, this cross-linking is critical to provide strength and stability to major fibrillar proteins, and absence or inhibition of lysyl oxidases results in overall weakness of bone, ligaments, and skin. In solid tumors, lysyl oxidase increases the firmness of the ECM, which facilitates cell migration and contributes to tumor invasion and metastasis.[77] The same role has been postulated for lysyl oxidases in gliomas,[78] although their specific molecular targets in the glioma ECM have not yet been identified.

Therapeutic Relevance of the Glioma-Associated Extracellular Matrix

Glioma-induced changes in the composition and structure of the ECM not only promote the growth and invasion of these tumors but also limit the efficacy of current therapeutic strategies. For example, the increased extracellular tortuosity in gliomas, coupled with increased interstitial pressure, results in reduced solute diffusion. This limits the ability of therapeutic agents to spread in the tumor and reduces their efficacy, even when they are delivered intraoperatively.[33]

The critical role of the ECM as a scaffold that supports tumor cell division and dispersion makes it also a particularly relevant molecular target. Strategies targeting the glioma ECM have largely focused on approaches to inhibit ECM-degrading metalloproteases and ECM-binding integrins, with the goal of limiting tumor invasion. The MMP inhibitor marimastat has been tested in clinical trials for high-grade glioma following radiotherapy, both as single agent[79] and in combination with temozolomide.[80] Results have been disappointing, with no significant advantages of marimastat as single agent and minimal increase in progression-free survival for the marimastat-temozolomide combination, but with considerable toxicity. The cyclopeptide cilengitide, which inhibits the binding of α_V integrins to ECM substrates, has also been tested as an anti-invasive and antiangiogenic agent and is described later (see "Therapeutic Relevance of the Glioma-Associated Vasculature" section). An alternative approach has consisted of direct targeting of ECM molecules to deliver a toxic payload. The most relevant example of this approach has been the iodine 131–conjugated anti–tenascin-C monoclonal antibody 81C6 (Neuradiab). A phase 2 clinical trial for recurrent glioblastoma, in which 81C6 was added to standard chemoradiation, reported increased progression-free survival[81] and has cleared the way for two future studies of this reagent: a randomized, phase 3 trial for recurrent high-grade glioma and a pilot study for newly diagnosed glioblastoma.

INTERACTIONS OF MALIGNANT GLIOMAS WITH ASTROCYTES

Glial cells of the astrocytic lineage are the most abundant cell type in the adult neural parenchyma and the major source of molecules that form the stromal ECM of the CNS. Astrocytes are, in addition, a key cell type in the perivascular niche where most gliomas arise by transformation of neural precursors of the glial lineage into glioma-initiating cells.[1] Astrocytes are therefore in intimate contact with neoplastic cells and contribute to the metabolic and functional support of nascent gliomas through the delivery of nutrients and cytokines and removal of metabolic byproducts. It has been hypothesized that newly formed glioma cells can functionally "co-opt" surrounding astrocytes, which would help in the initial stages of tumor dispersion.[82,83] A recent experimental model has confirmed this co-option by showing that glioma cells secrete molecules of the tumor necrosis factor (TNF) superfamily that do not promote their own invasion but activate astrocytes instead, which, in turn, respond with their own proinvasive factors in vivo.[84]

Histologic analysis of the tissue surrounding human gliomas reveals a broad band of astrocytes with the characteristic reactive phenotype that is observed in other neural injuries: hypertrophic and highly ramified bodies, high expression of intermediate filament proteins such as glial fibrillary acidic protein (GFAP) and vimentin (see Fig. 115-1A), and formation of a glial scar rich in chondroitin sulfate proteoglycans, which extends several hundreds of microns around the diffuse borders of the tumor.[42,85] The glial scar isolates damaged neural tissue and restricts cell motility and axonal extension into or through the injury epicenter. However, current evidence suggests that reactive astrogliosis around malignant gliomas does not limit the dispersion of the tumor cells and may even contribute to tumor invasion through the accumulation of astrocyte-secreted trophic factors in the periphery of the tumor.[4,85,86]

The communication between glioma cells and the reactive astrocytes found within and around the tumor is complex and bidirectional. Soluble factors secreted by glioma cells promote astrocyte proliferation and likely contribute to exacerbating the peritumoral gliosis.[87] These soluble factors have not been completely elucidated but are known to include the transforming growth factors α and β (TGF-α and TGF-β), platelet-derived growth factor (PDGF), TNF molecules (mentioned previously),[84] and cytokines such as CXCL12.[4,88] Astrocytes and astrocyte precursors not only proliferate and increase their GFAP immunoreactivity in the presence of glioma cells but also exhibit changes in their expression of molecules that regulate ECM structure. For example, astrocytes co-cultured with glioblastoma cells show increased expression of MMP-2[82,89] and decreased expression of the MMP inhibitor tissue inhibitor of metalloproteinase 2 (TIMP2),[89] both of which may contribute to increased

peritumoral ECM degradation and therefore to tumor expansion and invasion. Similarly, GFAP-reactive astrocytes recovered from PDGF-induced glioblastomas have been shown to upregulate MMP-10 (a protease from the stromelysin family), tenascin-C (an ECM protein in the perivascular niche), and BL-associated collagen VI,[86] all of which can also contribute to increased tumor cell adhesion and migration. Indeed, glioma-initiating cells co-cultured with astrocytes or their conditioned medium exhibit increased expression of promitotic and proinvasive genes as well as increased migration toward glial cells.[83]

Interestingly, glioma cells and astrocytes interact not only through a two-way paracrine communication mediated by soluble factors but also by direct physical association through the formation of gap junctions between normal and malignant cells.[90] These gap junctions, identified by expression of connexin 43, have been demonstrated as functional connections that permit coordinated propagation of intercellular calcium signaling[91] and may increase the invasive potential of glioblastoma cells through the neural parenchyma.[92,93] More recently, co-culture experiments have shown that astrocytes increase the resistance of glioma cells to the standard chemotherapeutics temozolomide, doxorubicin, and vincristine.[94] The increased apoptotic resistance is, at least in part, mediated by intercellular communication through gap junctions between glioma cells and astrocytes.[95]

Therapeutic Relevance of Glioma-Associated Astrocytes

Although astroglia seem to play an important role in supporting glioma proliferation and invasion, there are currently no therapeutic strategies formulated to disrupt this support or to revert the reactive status of tumor-associated astrocytes. However, experimental work has demonstrated that astrocytes can be used as an associated target to induce apoptosis of glioma cells. For example, the adenosine reuptake inhibitor propentofylline does not affect glioma cells directly but can increase glutamate uptake in co-cultured astrocytes,[96] reducing the availability of glutamate and glutamine for tumor cells and increasing their apoptosis. Similarly, it has been proposed that gap junctions between astrocytes and glioma cells could be inhibited with gap-channel blockers to reduce astrocyte support and potentially increase glioma chemosensitivity.[95] Alternatively, these gap junctions could be exploited to improve the efficacy of suicide gene therapy whereby cytotoxic products generated in one cell can induce apoptosis in neighboring cells ("bystander effect"). Cytotoxic products that do not diffuse between cells and must cross intercellular junctions (such as nucleoside analogues that are generated by the suicide gene thymidine kinase[97]) could show increased efficacy if the suicide gene were locally delivered not only to glioma cells but also to neighboring reactive astrocytes.[98]

INTERACTION OF MALIGNANT GLIOMAS WITH VASCULAR CELLS

The relationship of glioma cells with the neural vasculature is perhaps the best and most extensively studied interaction between malignant brain tumors and their microenvironment. The origin of gliomas as transformed neural precursors that arise in a perivascular niche[99] and the extensive interaction of glioma cells with preexisting blood vessels during the initial stages of tumor growth have been extensively reviewed elsewhere and are not described in detail here. This section briefly focuses on the cell-to-cell communication mechanisms that have been observed between neoplastic cells and the major cell types that form the tumor-associated vasculature and on the strategies that could disrupt these interactions with the vascular microenvironment.

The perivascular environment of the CNS is considered the anatomic and functional niche where glioma initiating cells first appear and proliferate,[1,100] in a manner regulated by components of the perivascular ECM.[101,102] Glioma cells can use preexisting vessels for nutrient supply (vascular co-option)[103,104] and as anatomic avenues for dispersion, giving rise to stereotypical patterns of perivascular growth.[5] Because of the high density of blood vessels in the CNS, infiltrative gliomas can even grow to macroscopic levels by taking advantage of the preexisting vascular network, which challenges the paradigm of neovascularization as an absolute requirement for macroscopic tumor growth.[105] Nevertheless, accumulation of glioma cells triggers robust vascular proliferation that is essential for the progression of high-grade gliomas toward their more aggressive phenotype.[5,106] Accordingly, antiangiogenic treatments to dissociate tumor cells from the vasculature, reduce aberrant vascular growth, and normalize tissue blood flow have become the most common adjuvant strategy complementing cytotoxic antitumor therapies.[107,108]

Glioma cells promote the formation of new blood vessels by secreting proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF),[109] and angiopoietins (released by endothelial cells in response to glioma signals).[110] These soluble factors, together with ECM-remodeling proteases such as MMP-2, induce proliferation and migration of local endothelial cells and elongation of existing vessels.[111] At the same time, these angiogenic factors enhance the homing of hematopoietic precursors into the tumor, resulting in aberrant, de novo vasculogenesis.[112,113] Activation of VEGF receptor (VEGFR) signaling results in upregulation of Notch receptors and their ligands in endothelial cells,[114] and subsequent activation of Notch signaling.[115] Endothelial cell-to-cell communication mediated by Notch receptors and the Notch ligand DLL4 is necessary to regulate the effect of proangiogenic factors and stabilize a functional vascular network.[116] Recent work has shown that glioma cells can regulate this process by secreting ECM proteins (e.g., fibulin-3) that activate Notch signaling independently of VEGFR in tumor-associated endothelial cells.[117]

Glioma cells that grow in the perivascular space eventually attach to the BL of preexisting capillaries and migrate alongside the vessel surface.[118] Tumor cells displace the network of glial end feet that are tightly wrapped around blood vessels, causing a local breach in the blood-brain barrier (BBB).[119] The displacement of astrocytic end feet effectively disrupts the ability of astroglia to control vascular tone and blood flow, resulting in local alterations of blood flow even in areas distant to the tumor core but invaded by tumor cells.[119,120] Glioma cells in close contact with endothelial cells can establish long-lasting communication mechanisms to control vascular function. For example, in vitro studies have shown that glioma cells can form gap junctions with co-cultured endothelial cells[121] and can also release extracellular vesicles loaded with RNA and microRNA molecules that reprogram endothelial cell function.[122] These mechanisms illustrate that glioma cells do not just stimulate vascular formation but also actively control blood flow, vessel permeability, and vascular architecture for tumor support.

Glioma cells also interact directly with mural cells (pericytes and smooth muscle cells) of the tumor-associated vasculature. Both pericytes (usually identified by expression of the cell-surface proteoglycan NG2/CSPG4[123]) and vascular smooth muscle cells (identified by expression of smooth muscle actin[124]) proliferate and accumulate early in tumor-associated vessels, likely responding to similar paracrine and juxtacrine signals produced by glioma cells as observed in endothelial cells. Although glioma cells that migrate along blood vessels disrupt the coupling between astroglia and mural cells that is necessary to regulate vascular tone, the tumor cells appear to take control of this function: glioma cells replace astrocytes in releasing potassium (through calcium-dependent potassium channels), which regulates smooth muscle cell contraction and therefore local blood pressure.[119]

Finally, it is worth noting that glioma cells can transdifferentiate into vascular phenotypes, and therefore they not only stimulate vascular growth but also become, in fact, an active part of the aberrant tumor-associated vasculature. Activation of VEGFR2 has been shown to induce transdifferentiation of glioma-initiating cells into endothelial-like cells, which can integrate into functional blood vessels or form new blood-perfused vascular channels, thus contributing to tumor neovascularization.[125,126] Although the direct contribution of tumor cells to forming vascular endothelium is considered limited compared with the proliferation of preexisting endothelial cells, it illustrates the extreme extent of control that glioma cells can exert on the neural microenvironment.[127,128]

Glioma-initiating cells can also transdifferentiate into pericytes in response to VEGF and TGF-β signaling.[129,130] Selective elimination of these glioma-derived pericytes in animal models disrupts tumor vascularization and growth, suggesting that, in contrast to tumor-derived endothelial cells, glioma cells may contribute to a large proportion of pericyte-like cells found in the tumor vasculature.[130]

Therapeutic Relevance of the Glioma-Associated Vasculature

Glioblastomas, the most aggressive type of gliomas, are some of the most highly vascularized types of solid tumors.[106] Exuberant and aberrant tumor vascularization is a defining feature of glioblastomas and, therefore, is an appealing target in the tumor microenvironment. Accordingly, antiangiogenic therapies have become a predominant chemotherapeutic strategy used in combination with standard or other experimental treatments for high-grade gliomas.

A large number of antiangiogenic agents have been evaluated in clinical trials for glioblastoma, including small-molecule inhibitors of VEGFR (e.g., cediranib); the integrin inhibitor cilengitide; and the anti-VEGF antibody bevacizumab (Avastin),[131,132] the latter being approved by the U.S. Food and Drug Administration for recurrent glioblastoma. Both cilengitide and bevacizumab have shown positive results in the treatment of recurrent glioblastoma, such as extension of progression-free survival and improvement in quality-of-life indicators.[133,134] However, recent results from large, randomized phase 3 trials have failed to show any significant benefits for bevacizumab or cilengitide in newly diagnosed glioblastoma, or for the VEGFR inhibitor cediranib in recurrent glioblastoma.[132,134,135] These results dramatically underscore the need for improved approaches to overcome the mechanisms of resistance to antiangiogenic therapy.

Alternative molecular targets proposed to disrupt glioblastoma vascularization include the angiopoietins that promote early vascularization[104,136]; the endothelial Notch/DLL4 signaling axis that regulates vascular sprouting[137,138]; and the signaling mechanisms that support the conversion of glioma cells into functional vascular cells.[128,130] New inhibitors could then be combined with anti-VEGF to overcome antiangiogenic resistance through blockade of multiple mechanisms.[2,136] As expected, a major challenge of these approaches will be the increased toxicity caused by overlapping and cumulative toxic effects of the inhibitors.[136]

In addition to the multiple mechanisms that may need to be inhibited to disrupt glioma vascularization, experimental and clinical results have revealed a growing concern with escape mechanisms triggered in tumor cells facing starvation due to antiangiogenic treatments. Evidence from experimental models has suggested that antiangiogenic therapies may trigger an invasive program in tumor cells, promoting diffuse tumor dispersion that is difficult to detect by MRI.[139,140] Accordingly, patients who eventually relapse after antiangiogenic therapy usually show a radiographic response typical of infiltrative tumor progression,[141,142] which correlates with increased tumor infiltration detected histologically[143] and a more aggressive phenotype at recurrence.[144] Interestingly, it is possible that the invasion program may depend on the antiangiogenic treatment used: increased glioma infiltration has been reported in clinical studies and experimental models after bevacizumab treatment[143,145]; in contrast, patients with newly diagnosed glioblastoma treated with cilengitide did not show a more infiltrative pattern of tumor progression.[146] Indeed integrin inhibition in experimental models may suppress bevacizumab-induced glioma invasion[147] and has been proposed as a potentially useful therapeutic combination. A phase 2 clinical trial for cilengitide plus bevacizumab in recurrent glioblastoma (NCT01782976) was initiated in 2013 but interrupted at the time of this writing.

INTERACTION OF GLIOMA CELLS WITH MICROGLIA AND MACROPHAGES

Malignant gliomas are heavily infiltrated by microglia and macrophages, which represent the predominant immune cell population in the tumor.[148] Experiments in rats implanted with allogeneic gliomas have shown that monocytic cells can account for up to 30% of the total cell mass in experimental tumor models.[149] Quantitative immunohistochemistry has revealed a similar upper boundary for the microglia and macrophage burden in low-grade human astrocytomas (range, 4% to 21% of all cells) and glioblastoma (range, 3% to 31% of all cells).[150] The presence of these myeloid-derived cells in the tumor parenchyma is so conspicuous that it was originally described by Wilder Penfield and Pio del Rio Hortega in the mid-1920s, before the advent of modern immunohistochemical techniques.[151] Despite their prominence, the role of microglia and macrophages in high-grade gliomas has been a subject of lively debate, which has only recently reached consensus regarding the potential tumor-promoting role of these immune cells.[152]

Experimental evidence has shown that microglia in the normal adult brain are independent of bone marrow–derived adult macrophages and derive instead from primitive yolk sac macrophages that colonize the CNS during early embryogenesis.[153] Nevertheless, there is evidence that gliomas are infiltrated both by local microglia and by peripheral macrophages recruited from the circulation. Immunohistochemical studies in pediatric low-grade astrocytomas[154] and analysis of mononuclear cells isolated from intracranial gliomas in mice[155] have revealed that at least two populations of cells can be identified in the tumor, corresponding to local microglia ($CD45^{low}/CD11b^{+}/CD11c^{+}$) and peripheral macrophages ($CD45^{high}/CD11b^{+}/CD11c^{+}$). However, differentiation between these populations has been difficult because there is no robust consensus to identify a "microglia-specific" marker.[152] Current approaches are based on the differential expression of cell surface markers (in particular CD45),[156] although transcriptomic studies have suggested new and potentially more robust markers (such as the genes *HexB* and *P2RY12*).[157] More important, both originally resident microglia and recruited macrophages appear to have the same functions regarding their contribution to tumor progression.[152] Therefore we can collectively refer to these innate immune cells in gliomas as *glioma-associated microglial cells*. Recent experimental results suggest that, in absence of BBB breach, most glioma-associated microglial cells derive from the original resident microglia in the CNS. These cells can even upregulate the expression of the CD45 marker commonly used to identify peripheral macrophages.[158]

Macrophages activated by immunologic triggers are usually classified into two major states known as M1 and M2[159]: M1 macrophages are "typically" activated and exhibit proinflammatory responses such as antigen presentation, phagocytosis, and release of cytokines that trigger immune stimulation. M2 macrophages are "alternatively" activated cells with immunosuppressive and tumor-promoting functions. These categories are not rigidly

separated but rather are the phenotypic extremes of a continuum that defines the net contribution of these cells to tissue inflammation and immune activation. M1/M2 states can be identified by the expression of characteristic genes and antigens that have been extensively reviewed (e.g., inducible nitric oxide synthase [iNOS], signal transducer and activator of transcription 1 [STAT1], interleukin-12 [IL-12], and TNF-α for M1; and STAT3, arginase, and TGF-β for M2).[152] The degree of polarization of macrophages toward the M1 or M2 phenotypes is regulated by microenvironmental cues provided by neighboring cells and the surrounding ECM (as shown in Fig. 115-2). In the case of glioma-associated microglia, glioma cells provide these environmental cues by extensive two-way paracrine communication that ultimately subverts the reactive phenotype of microglia into a tumor-promoting phenotype. A recent transcriptomic study has suggested that the genes expressed by glioma-associated microglia do not clearly match the M1 and M2 gene sets observed in peripheral macrophages from other solid tumors and may be therefore a unique phenotype that responds to unique signals from glioma cells.[160]

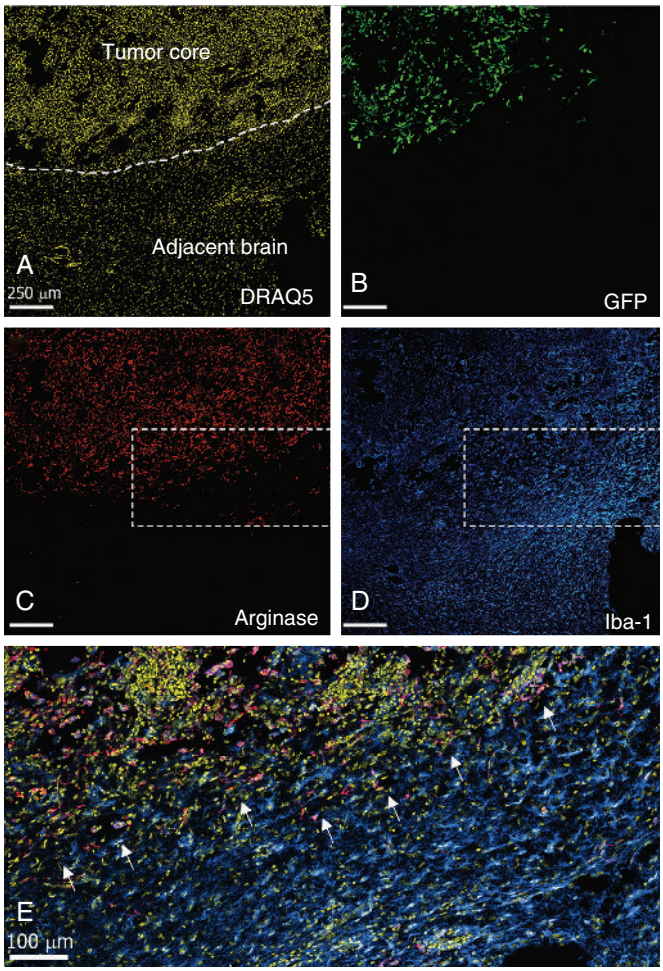

Figure 115-2. Gliomas induce heterogeneity in their microenvironment. Representative, false-color images of an experimental syngeneic glioblastoma model developed in immunocompetent mice. Tissue sections were processed for immunohistochemistry to detect: cell nuclei (using the nuclear dye DRAQ5) **(A);** tumor cells (expressing green fluorescent protein [GFP]) **(B);** M2-activated microglia (expressing arginase) **(C);** and overall macrophage population (expressing the monocyte-specific marker Iba-1) **(D).** Notice the high density of microglia and macrophages in and around the tumor border. The tumor introduces heterogeneity in this population by inducing the conversion of microglia to the M2 phenotype, which is restricted to the tumor core and margin. **E,** Magnified image from the area indicated in **C** and **D** *(dashed rectangle),* with *arrows* showing the boundary between the periphery of the tumor, infiltrated by highly ramified microglia, and the tumor core with less ramified M2 microglia. *(Courtesy of the laboratory of Mariano Viapiano and Dr. Aneta Kwiatkowska.)*

Microglia that infiltrate gliomas exhibit the typical ramified morphology observed in activated macrophages that accumulate in sites of neural injury or inflammation. These cells retain certain innate immune functions, such as phagocytosis and direct cytotoxicity when co-cultured with tumor cells, although to a lesser extent than normal microglia.[161,162] However, glioma-associated microglia do not perform antigen presentation, respond to Toll-like receptor activation, or release pro-inflammatory cytokines, indicating that their immune responses have been largely stunted by the immunosuppressive environment of the tumor.[161,162]

Glioma cells contribute to microglia recruitment into the tumor through a variety of chemoattractant factors, including macrophage colony-stimulating factor (M-CSF/CSF-1),[163] monocyte chemotactic protein-1 (MCP-1/CCL2),[164] MCP-3,[165] and hepatocyte growth factor/scatter factor (HGF/SF, which may be released by glioma-associated astrocytes).[166,167] In addition to these chemokines, glioma cells secrete TGF-β and prostaglandins (described in detail by Li and Graeber[151]), which, together with CSF-1, induce downregulation of major histocompatibility complex II in microglia and strong polarization toward the immunosuppressive M2 phenotype.

As a result of the paracrine influence of glioma cells, glioma-associated microglia are deeply "re-educated" not only to lack antitumor responses but also to contribute to tumor progression. Li[168] and Rolle[169] have summarized a large number of studies showing that glioma-associated microglia release multiple cytokines that promote glioma cell proliferation and invasion. Of these, the cytokine IL-10 secreted by microglia in response to gliomas has a potent role as immunosuppressant in other immune cells and promotes glioma cell proliferation.[169,170] At the same time, IL-10 may activate STAT3 signaling in neighboring microglia and glioma cells,[171,172] which is one of the key signaling mechanisms that drive glioma progression toward a more aggressive phenotype.[173] Other cytokines released by microglia that also regulate STAT3 signaling include IL-1β, IL-6, IL-8, and TNF-α,[151,174] all of which reduce activation of adaptive immune responses and facilitate tumor cell proliferation and invasion.

A major contribution of microglia to glioma progression seems to be the enhancement of tumor invasion[175]: under the influence of glioma cells, microglial cells produce metalloproteases (such as secreted MMP-2 and MMP-9, as well as membrane-bound MT1-MMP) that degrade the peritumoral ECM.[176] Using experimental ex vivo models, microglia have been detected in association with migratory glioma cells detaching from the tumor core.[175] Evidence following inhibition of microglia-derived proteases and cytokines in vitro and in animal models strongly suggests that glioma-associated microglia are necessary to facilitate tumor dispersion in the CNS.[163,177,178] Similar results have been observed with microglia isolated from glioma patients and co-cultured with glioma cells.[179]

Finally, it is worth noting that a recent hypothesis has proposed that, in addition to local resident microglia and recruited peripheral macrophages, a subpopulation of glioma-associated microglia could be in fact derived from tumor cells differentiated into a microglial phenotype.[180] In contrast to the transdifferentiation of glioma cells into endothelial and mural cells, these "neoplastic microglia" could arise from fusions of glioma cells with resident microglia that had abortive phagocytosis. Histologic evidence suggests that some cells in the tumor stroma present cell surface markers and morphologic features of both glioma and

mononuclear cells,[181] and they have been postulated to contribute to the overall tumor spread.

Therapeutic Relevance of Glioma-Associated Microglia

The paracrine influence of glioma, which re-educates M1 microglia that was attracted to the tumor into the M2 tumor-promoting phenotype, is a clear example of tumor cells introducing heterogeneity to control their microenvironment. Normal functions of microglia, such as immune surveillance, scavenging of cell debris, and termination of immune responses, are aberrantly used to favor glioma escape from its local microenvironment as the tumor grows. Numerous examples from experimental models have shown that depletion of glioma-associated microglia is sufficient to reduce tumor growth and invasion.[182,183] Similar responses can be achieved by preventing M2 polarization or by blocking the immunosuppressive signaling mechanisms initiated by M2-polarized microglia.[155,184] Evidence from these studies suggests that therapies that could target immunosuppressive microglia or polarize the cells back into a proinflammatory phenotype would contribute to improved therapeutic efficacy for glioma.[185] For example, a recent experimental gene therapy strategy for glioblastoma used adeno-associated virus to deliver IL-12 in the tumor; this interleukin elicited strong, local proinflammatory and cytotoxic activity in microglia, resulting in enhanced antitumor activity.[186] On the other hand, the immune functions of microglia that remain in the immunosuppressive environment of gliomas have been shown to be sufficient to prevent other therapeutic strategies from working well, in particular those involving delivery of therapeutic genes or oncolytic viruses against the tumors.[97,187] Therefore "reactivation" of microglia should be pursued, keeping in mind that the expected results may introduce inflammation, toxicity, or reduced efficacy for complementary therapies.

As an alternative strategy, the fact that a proportion of glioma-associated microglia arises from recruited peripheral macrophages has spurred significant interest in the use of these cells as potential Trojan horses to disrupt glioma growth. Li and collaborators have proposed that autologous monocytes could be harvested from the patient, genetically manipulated, and re-introduced to deliver a toxic payload once they are recruited into the tumor.[168]

INTERACTION OF GLIOMA CELLS WITH ADAPTIVE IMMUNE CELLS

The growth of gliomas is accompanied by immune suppression, which occurs both systemically and in the local tumor microenvironment. As with other solid tumors, gliomas induce a tolerant state that reduces inflammatory and immune responses against neoplastic cells.[188,189] Interestingly, gliomas are widely infiltrated by circulating lymphocytes, a phenomenon that was first described systematically in 1971 and proposed to be a thwarted "antitumor response."[190,191] However, current studies on the types of infiltrating lymphocytes and their functions in the tumor stroma suggest that the lymphoid infiltration in glioma plays multiple roles, including active immunosuppression following regulatory cues provided by the tumor cells.[188,192]

The CNS is typically regarded as an immune-privileged environment with highly regulated and restricted immune events, which have been extensively described elsewhere.[193,194] Access of circulating T cells to the neural stroma is, in part, limited by the BBB,[195] although activated T cells can readily extravasate in response to recruitment signals.[196] Access of T cells to the tumor stroma is facilitated by the local breach of the BBB but at the same time is limited by proangiogenic factors released by tumor cells, which reduce T-cell anchorage to blood vessels.[197,198] Nevertheless, infiltration of T-cell lymphocytes is observed in 30% to 60% of all gliomas and increases with tumor grade.[199] These cells include effector T cells (both cytotoxic $CD8^+$ and helper $CD4^+$ cells) as well as induced regulatory T cells ($CD4^+/CD25^+$ Tregs) identified by expression of the transcription factor *FOXP3*.[200]

Tregs are a key subpopulation of T cells needed to prevent immune response against self-antigens and to suppress the pathologic activation and proliferation of effector T cells in the CNS.[200] Intratumoral accumulation of $CD4^+/CD25^+/FOXP3^+$ Tregs has been shown to correlate with tumor grade and worsened outcome,[199,201,202] which has put these cells in the limelight as relevant therapeutic targets in the glioma microenvironment.

Tregs are recruited to the tumor by immunomodulatory signals released by glioma cells and by glioma-associated microglia, such as the cytokines CCL2 and CCL22,[203] IL-10 (described earlier in the "Interaction of Glioma Cells with Microglia and Macrophages" section), and TGF-β.[204] TGF-β in particular has been shown to induce the development of Tregs (inducible Tregs or iTregs, $CD4^+$ $FOXP3^+$) from naïve T cells ($CD4^+$ $FOXP3^-$) recruited to gliomas.[205-207] Tregs are also potently recruited to the tumor by tryptophan metabolites of the kynurenine pathway, produced by the enzyme indoleamine 2,3-dioxygenase (IDO) that is highly upregulated in gliomas.[208,209]

Both inhibition of Treg recruitment and depletion of these immunosuppressive cells have been shown to enhance antitumor responses. For example, Treg depletion using anti-CD25 antibodies in a mouse glioblastoma model was sufficient to increase overall survival.[210] Similarly, stimulation of Toll-like receptor 9 using synthetic oligonucleotides in another mouse glioma model was sufficient to increase the proinflammatory polarization of microglia and reduce Treg recruitment, reducing tumor growth and prolonging survival.[211] Multiple strategies to inhibit IDO in gliomas have also resulted in reduced Treg recruitment and enhanced T-cell–mediated tumor rejection.[209,212] Importantly, studies in animal models have shown that the amount of time elapsed between initial tumor growth and Treg depletion is critical to ensure success of anti-Treg strategies because no improvement in survival was observed when Tregs were depleted after tumors reached a high tissue burden.[213]

Therapeutic Relevance of Glioma-Associated T Cells

Immunotherapeutic strategies for glioma have focused on reversing the immunosuppressed state that prevents a strong immune attack on tumor cells. As described previously, some of those strategies attempt to deplete or inhibit Tregs in the local glioma microenvironment to reduce local tolerance. As a complement, other strategies have focused on activating cytotoxic T-cell responses by inhibiting immune checkpoint effectors.

Immune checkpoints are a host of signaling mechanisms that inhibit T-cell cytotoxicity and regulate the extent of immune responses.[214] Inhibition of immune checkpoint receptors on the surface of effector T cells, such as programmed cell death protein-1 (PD-1/CD279), cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4/CD-152), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), triggers a strong immune response against tumor-specific antigens.[215-218] Since 2011 (when the first antibody against CTLA-4 was approved by the U.S. Food and Drug Administration), antibodies blocking CTLA-4 (ipilimumab) and PD-1 (nivolumab and pembrolizumab) have shown impressive results as monotherapy agents for metastatic melanoma, a tumor with dismal prognosis.[219,220] These agents are being tested as monotherapy and in combination therapies for recurrent glioblastoma[221]: recent clinical trials initiated in 2014 will test combinations of ipilimumab and nivolumab in addition to temozolomide (National Institutes of Health trials NCT02311920 and NCT02017717). A few caveats for these

therapies have been raised, such as the uncertainty about the extent to which cytotoxic T cells will effectively infiltrate the CNS after checkpoint inhibition and whether they will remain activated after infiltration. Results are expected to depend largely on the extent of local T-cell responses in the CNS and the innate immunogenicity of the tumors.[222] Future strategies may combine conventional chemotherapy with checkpoint inhibitors and Treg suppression; a strategy combining IDO inhibition and checkpoint inhibitors has already shown sustained therapeutic effects in animal models.[223]

Because the efficacy of immunotherapies against glioma is expected to depend on the degree of immunogenicity of the tumor, it is worth noting that epidemiologic studies have shown that a heightened immune status may be sufficient to reduce the risk for glioma. Studies in large patient cohorts have established a negative correlation between patients with a history of asthma and glioma incidence.[204] This has led to the hypothesis that stimulation of cellular and humoral immune responses may be sufficient to prevent glioma development.[224,225]

CYTOMEGALOVIRUS IN THE MICROENVIRONMENT OF GLIOMAS

Evidence from multiple laboratories has shown that DNA, RNA, and viral proteins from human cytomegalovirus (CMV) can be detected in most clinical glioblastoma multiforme (GBM) specimens (this evidence has been extensively reviewed by Cobbs[226] and Lawler[227]). Moreover, functional studies have shown that some CMV gene products are pro-oncogenic[226] and promote tumor growth and progression. This has led to the controversial hypothesis that CMV may play a significant role in glioma formation or progression.[228]

CMV is a member of the Herpesviridae family, characterized by a double-stranded DNA genome and an enveloped viral capsid. The genome of CMV is approximately 230 kb and contains some 200 genes as well as regulatory micro-RNAs and other noncoding transcripts.[229,230] CMV has wide tropism for human cells and can infect neural cells and monocytes,[231,232] although the natural latent reservoir appears to be the bone marrow–derived progenitors of dendritic and myeloid cells.[233] CMV is endemic in the human population, with seroprevalence in adults ranging from 50% to almost 100%.[234-236] Spontaneous CMV infection occurs mostly during the first year of life, resulting in lifelong latent persistence of the virus in circulating reservoirs. Clinical effects caused by CMV in healthy adults are rare and usually triggered by systemic immunosuppression, as observed in AIDS patients or recipients of transplants.[237] CMV may also activate during pregnancy, leading to neurological disabilities in children.[238,239]

At present, there is no direct evidence showing that CMV causes glioma, but it is plausible that in the immune-suppressed glioma microenvironment, CMV could experience partial or total reactivation in tumor cells, neural stem cells, or glioma-associated myeloid cells. This reactivation would result in expression of oncomodulatory CMV proteins that would increase tumor aggressiveness. For example, the CMV genome encodes a homologue of human IL-10 that has a similar immunosuppressant role to native IL-10. CMV IL-10 has been shown to promote the M2 tumor-promoting phenotype in glioma-associated microglia, which could hasten tumor progression, as described earlier in this chapter.[240]

A key piece of evidence supporting a role for CMV in glioma progression was recently obtained in the transgenic mouse model *Mut3* that forms spontaneous gliomas in early adulthood (genotype: GFAP-Cre; $NF1^{flox/+}$; $p53^{+/-}$).[241] Mut3 mice infected with mouse CMV at birth did not show any complications from the infection, which became undetectable in the blood after a few days. However, infected mice developed gliomas earlier than controls and had shorter overall survival and higher incidence of progression from grade III astrocytoma to grade IV glioblastoma.[242] In contrast, mice infected with herpes simplex virus were not different from mock-infected mice, confirming that the accelerated glioma pathology was specific to CMV infection. CMV was specifically detected in $CD45^+$ monocytes in the tumor, suggesting that its protumoral role was mediated by modulation of the tumor microenvironment.[242] Although these results in mouse models are highly supportive of a role for CMV in brain tumors, viral particles have not been identified in glioma patients and active transcription of CMV messenger RNA has not been detected in sequencing studies of clinical tumors. Therefore the gliomagenic or glioma-promoting role of human CMV remains to be fully validated.

Therapeutic Relevance of Glioma-Associated Cytomegalovirus

The potential role of CMV in glioma pathobiology has spurred potential antitumor treatments in which targeting the virus is a main focus of the combinatorial therapy. In a randomized, double-blind study in Sweden, the antiviral agent valganciclovir (Valcyte), normally used to treat CMV infections, was added to standard-of-care chemotherapy for glioblastoma. Preliminary results were encouraging but have not yet been replicated or extended.[243,244]

More commonly, the potential role of CMV reactivation as a tumor-promoting factor has become the target of immunotherapeutic strategies. The presence of CMV antigens in glioma tissue but not in normal brain has prompted attempts to boost antitumor responses by targeting CMV proteins as "non-self" tumor markers. Specific approaches (described in detail by Schuessler and colleagues[245] and Nair and associates[246]) tested in clinical studies have included using autologous dendritic cells pulsed with CMV antigens to activate cellular immune response; selecting CMV-specific autologous T cells for reintroduction in the patient; and using CMV peptides for autologous vaccination. An early case report of these strategies described the detection of anti-CMV immunoreactivity as a serendipitous finding: a glioblastoma patient with robust anti-CMV response was identified during a phase 1 trial in which patients had been vaccinated with autologous dendritic cells treated with their own tumor lysates. This patient had exceptional response to therapy and, on inspection, it was observed that the tumor was infected with CMV. The patient developed a CMV-specific $CD8^+$ T-cell response against a dominant epitope of the viral protein pp65, indicating that CMV antigens could become targets for glioma immunotherapy.[247]

The fact that CMV epitopes may act as tumor-specific antigens is being exploited as an opportunity to use antiviral immunity as a driver of immunotherapies that do not require the identification of other tumor-specific antigens produced by mutations. The ongoing phases 1 and 2 clinical trial NCT00639639 for recurrent glioblastoma uses autologous dendritic cells pulsed with CMV-pp65 RNA to vaccinate patients in an attempt to induce antitumor immunity. Preliminary results (unpublished at the time of this writing but described during the first international Symposium on Human CMV and Glioma in 2011[248]) have reported a median survival of 21 months for patients with recurrent glioblastoma, which is an unprecedented increase in overall survival for patients with these tumors.

In addition to using the patients' own dendritic cells to induce antitumor immunity, other strategies have focused directly on activating and expanding CMV-specific cytotoxic T cells in vitro before reintroducing them in the patients. This approach has the advantage that antigenic challenge, T-cell activation, T-cell clone selection, and expansion occur in vitro under optimized conditions for immune stimulation that are not counteracted by the

immunosuppressive tumor environment. In a recent study, CMV-pp65 RNA was chosen to induce vaccination responses in autologous dendritic and T cells obtained from glioblastoma patients. The resulting CMV-specific T cells had high efficacy to kill cultured tumor cells derived from the same patients,[249] although no antitumor studies were performed in vivo.

Cytotoxic T cells with specificity for the CMV antigens IE1 and pp65 have also been identified in the blood of glioblastoma patients. These cells have been expanded and shown to be toxic against autologous tumor cells.[250,251] A recent phase 1 trial used synthetic CMV epitopes to isolate and expand these CMV-specific T cells from glioblastoma patients. The cells were then reinfused in the patients in combination with chemotherapy. The primary end point of this study was to demonstrate the safety of the procedure, which was well tolerated and reported only minor adverse events.[252] Most important, the patients showed significant responses and much improved survival compared with the historical median overall survival for recurrent glioblastoma (with one patient surviving for as long as 4 years after treatment). Overall, these studies strongly suggest that immunotherapeutic approaches against CMV (as an associated tumor-specific target) are a promising approach to malignant gliomas. Even if the role of CMV as a possible "driver" versus "passenger" factor in glioma progression is still under debate, results from clinical trials are likely to continue sparking anti-CMV therapies that will help the brain tumor community to assess the relevance of this pathogen in glioma biology.

CONCLUSION AND FUTURE PERSPECTIVES

Comprehensive genomic and proteomic profiling studies have demonstrated with exquisite detail that gliomas of all grades are highly heterogeneous tumors, both genetically and phenotypically. Gliomas that appear histologically similar and used to be grouped as tumors of the same type or grade can instead be classified in a variety of molecular subgroups defined by specific mutations, epigenetic alterations, and metabolic features.[111] More strikingly, high-resolution studies have shown extensive regional heterogeneity even within individual tumors,[253] underscoring that each tumor is indeed a collection of malignant clones with unique molecular properties and survival mechanisms. Glioma heterogeneity is therefore one of the most challenging aspects for the successful treatment of these tumors because therapies must face a diverse, ever-evolving population of malignant cells.

Molecular and genetic profiling of gliomas has also revealed extensive heterogeneity of the tumor microenvironment, although systematic classification of the microenvironmental heterogeneity is far from complete. A clear example is illustrated by the regional changes in tumor cell metabolism due to local hypoxia and nutrient depletion: although tumor-associated cells lack the extensive metabolic reprogramming of glioma cells, they show changes in glucose and acetate metabolism that parallel those observed in tumor cells.[85] Another example is the different changes in the ECM in response to tumor cell proliferation (at the tumor core) or invasion (at the tumor border)[44]; changes in the local structural properties of the stroma that must accommodate more cells or facilitate cell dispersion are just starting to be quantified in gliomas.[254] The M1-to-M2 reprogramming of glioma-associated microglia is yet another example of stable molecular and epigenetic heterogeneity in tumor associated cells.[155,160] A comprehensive characterization of reprogramming for other glioma-associated cells such as astrocytes, pericytes, or endothelial cells is yet to be described, but it is expected that it will show stable molecular changes and regional heterogeneity of the reprogrammed cells. Moreover, the nature and extent of those changes are likely to depend on the genetic alterations of the tumor cells directing the reprogramming.[255]

In summary, because the tumor microenvironment is as much part of the tumor as the neoplastic cells themselves, the molecular and genetic heterogeneity of glioma cells is reflected by similar heterogeneity in glioma-associated cells and ECM. Understanding this heterogeneity is critical to identifying mechanisms of tumor growth and therapy resistance that may not be located in the tumor cells themselves but instead in the tumor-associated cells. Because there are no known genomic aberrations in tumor-associated cells, targeting these cells is likely to meet less resistance than targeting the neoplastic clones.[2,97] On the other hand, a major challenge lies in identifying molecular targets restricted to the tumor microenvironment and absent from the normal CNS. Better understanding of the genetic and molecular landscape of the tumor microenvironment will help define novel glioma biomarkers and identify key mechanisms of tumor support that can be disrupted to advance glioma therapy.

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