Chapter 2

Gliomas Biology: Angiogenesis and Invasion

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Additional information is available at the end of the chapter

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1. Introduction

Glial tumors, within neuroepitelial-derived lesions, are the most common intra-assial neoplastic histotypes. Gliomas account for about 45% of all primary central nervous system (CNS) tumors and 77% of all malignant primary CNS tumors. Gliomas can originate from neural stem cells, progenitor cells, or from de-differentiated mature neural cells transformed into cancer stem cells. Although brain tumors constitute only a small proportion of overall human malignancies, they carry high rates of morbidity and mortality. Mortality is still close to 100% and the average survival of patients with glioblastoma multiforme (GBM) is less than 1 year when classical treatment is used. Recent progresses in multimodal treatment has led to only a slight increase in average survival up to 15-18 months [1]. The effectiveness of the actual chemotherapeutic approach and multimodal targeted therapies remains modest in gliomas.

Gliomas are divided into different subtypes based on cell line from which they originate. Gliomas include astrocytomas, glioblastomas, oligodendrogliomas, ependymomas and other histological subtypes. The most common gliomas are astrocytomas, oligodendrogliomas and ependymomas. Since 1993, the 4-level grading system proposed by the World Health Organization (WHO), was the most widely accepted and widespread. It is based on histologic features: nuclear atypia, presence of mitoses, endothelial proliferation. Grade I gliomas are benign with a slow proliferation rate and include pylocitic astrocytoma most common in pediatric age. Grade II gliomas are characterized by a high degree of cellular differentiation and grow diffusely into the normal brain parenchyma and are prone to malignant progression. They include astrocytoma, oligodendroglioma and oligoastrocytoma. Grade III lesions

include anaplastic astrocytoma, anaplastic oligoastrocytoma and anaplastic oligodendroglioma. These tumors show a higher cellular density and a notable presence of atypia and mitotic cells. Grade IV tumors are the most malignant and also the most frequent gliomas and include glioblastoma and gliosarcoma. These tumors presented microvascular proliferations and pseudopalisading necrosis.

Conventional brain tumor treatments include surgery, radiation therapy and chemotherapy. Surgical treatment is invasive but represents the first approach for the vast majority of brain tumors due to difficulties arising in early stage detection. However, gross total removal is not always achievable in relation to the location of the tumor, to preserve vital nervous or vascular structures. Aggressive treatment modalities have extended the median survival from 4 months to 1 year, but the survival is often associated with significant impairment in the quality of life. Radiation therapy and chemotherapy are non-invasive options often used as adjuvant therapy, but may also be effective for curing early-stage tumors. Radiotherapy seems to be related only to a prolonged progression-free survival, with better control of seizures, but with no substantial differences in overall survival. Besides, patients treated with radiotherapy, have high risk to develop some complications such as post radiation leukoencephalopathy, that is characterized by dementia, gait disturbance, incontinence, deficit in attention and executive functions. In patients with recurrent GBM, the 6-months progression-free survival is only 21% after treatment with temozolomide [2]. The effectiveness of systemic chemotherapy is limited by toxic effects on healthy cells, generally resulting in morbidity or mortality of the patient. Moreover, the presence of the blood-brain barrier (BBB) limits the passage of a wide variety of anticancer agents. The high incidence of recurrence and poor prognosis of malignant gliomas compel the development of more powerful anti-cancer treatments. Action of alkylating agents, one of the most common class of chemotherapeutic drugs employed, is limited by activation of methylguanine-methyltransferase. The compromise of the quality of remaining life as well as the limited success of current treatment options in shrinking tumors, raise increasing concerns about the adverse effects of cancer treatment on brain function.

Glioma cell invasion into normal tissue is thought to be a multi-factorial process, consisting of cell interactions with extracellular matrix (ECM) and with adjacent cells, as well as accompanying biochemical processes supportive of active cell movement. Tumor cell invasion requires four distinct steps: (1) detachment of invading cells from the primary tumor mass, (2) adhesion to ECM, (3) degradation of ECM, and (4) cell motility and contractility. However, growth of tumors necessitates, also, the recruitment of a new blood supply. Angiogenesis, represent a key event in the progression of malignant gliomas. Tumor angiogenesis involves multiple cellular processes including endothelial cell proliferation, migration, reorganization of ECM and tube formation.

The advent of molecular studies allowed a new evaluation of the biology of gliomas with, a level of precision that promises interesting advances toward the development of specific and effective therapies. The introduction of molecularly targeted agents is one of the most significant advances in cancer therapy in recent years. Targeted therapies block activation of oncogenic pathways, either at the ligand-receptor interaction level or by inhibiting down-

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stream signal transduction pathways, thereby inhibiting growth and progression of cancer. Because of their specificity, targeted therapies should theoretically have better efficacy and safety profiles than systemic cytotoxic chemotherapy or radiotherapy.

In this chapter we evaluated the invasion and angiogenesis in the glioma progression. Moreover, new pharmacological agents on the basis of the more recent literature will be evaluated.

2. Gliomas

Approximately 50% of primary brain tumors are gliomas, arising from astrocytes, oligodendrocytes, or their precursors and ependymal cells. Gliomas are classified from I to IV according to the WHO malignancy scale. Gliomas are divided into different subtypes based on cell line from which they originate. The WHO classification of CNS tumors, now in its fourth edition, is the universal standard for classifying and grading brain neoplasms. Analysis of tumour differentiation, cellularity, cytonuclear atypia, mitotic activity, microvascular proliferation, and necrosis further enables grading of the tumour as grade 2 (diffuse infiltrating low-grade gliomas), 3 (anaplastic gliomas), or 4 (glioblastomas) with increasing aggressiveness. Unfortunately, WHO morphological classification is based on subjective criteria, lacks reproducibility, and remains imperfect in its ability to predict individual outcomes. The most recent WHO classification introduced a number of significant changes that include both additions and redefinitions or clarifications of existing entities. Three new tumor types were added to the glioma section. The first of these is angiocentric glioma. Angiocentric gliomas are slowly growing solid hemispheric tumors of children and young adults. They are strongly epileptogenic, with 95% of patients presenting with intractable seizures. These tumors are characterized histologically by elongated bipolar glial tumor cells and by their striking perivascular growth pattern. Angiocentric gliomas have a low proliferative potential (MIB-1 between 1% - 5%) and have been designated as WHO grade I neoplasms. The second, pilomyxoid astrocytoma (PMA), is now formally considered as a distinct more aggressive variant of PA. PMAs typically occur at an earlier mean age and are associated with more aggressive behavior than Pas (pilocytic astrocytomas). PMAs are composed of bipolar (piloid or hairlike) cells that lie within a distinct myxoid background matrix. PMAs are currently considered WHO grade II neoplasms. Atypical choroid plexus papilloma (CPP) is the third new tumor. Cellular atypia and increased mitotic activity are reflected in more aggressive biologic behavior, with earlier metastases and higher recurrence rates than CPP. An aCPP is designated as a WHO grade II neoplasm. The 2007 WHO version recognized, also, papillary glioneuronal tumor (PGNT), rosette-forming glioneuronal tumor (RGNT) of the fourth ventricle, and the extraventricular form of neurocytoma. PGNT is a rare relatively well-circumscribed clinically indolent tumor that is exclusively found in the cerebral hemispheres. PGNTs are biphasic tumors, with both astrocytic and neuronal elements. The histologic hallmark of PGNT is the presence of hyalinized vascular pseudopapillae. Although PGNT was not formally assigned a WHO grade in the 2007 revision, this tu-

mor generally behaves in a benign grade I fashion. RGNTs are rare slow-growing tumors of young and middle-aged adults. RGNTs contain both neurocytic and astrocytic elements. Their histologic hallmark is the formation of neurocytic perivascular pseudorosettes. RGNTs are designated as WHO grade I neoplasms. The term "central neurocytoma" describes a neuronal tumor with preferential location in the lateral ventricle body. These tumors comprise fibrillary areas mimicking neuropil plus collections of uniform round cells that have immunohistochemical and ultrastructural evidence of neuronal differentiation. A low proliferation rate is typical. Similar neoplasms have been reported outside the ventricular system, and the WHO 2007 designated the term "EVNCT" (extraventricular neurocytoma) for these uncommon tumors. Because the only distinguishing feature is location EVNCTs are included in the same histopathologic code as central neurocytoma. Both central and extraventricular neurocytomas are designated as WHO grade II neoplasms. Two new tumors of the pineal region were codified in the WHO 2007 classification, papillary tumor of the pineal region (PTPR) and pineal parenchymal tumor of intermediate differentiation (PPTID). PTPR is a rare neuroepithelial tumor that arises from the subcommissural organ and exhibits ependymal differentiation. Macroscopically, PTPRs are indistinguishable from pineocytomas. Microscopically, the tumors are easily distinguished. PTPRs show papillary architecture with pseudostratified columnar epithelium. Ultrastructural features suggesting ependymal differentiation are present. Immunohistochemistry is positive for cytokeratins. While grading of PTPRs has yet to be defined, most neuropathologists consider these as WHO grade II or III neoplasms. PPTID is a newly recognized tumor intermediate in malignancy between pineocytoma and pineoblastoma. Mild-to-moderate nuclear atypia and low-to-moderate mitotic activity result in either WHO grade II or III. PPTIDs have a much more aggressive course than pineocytomas and may warrant adjuvant therapy. PPTIDs are more common than previously recognized, accounting for up to 20% of all pineal parenchymal neoplasms. Recent reports emphasize their large size and focal invasion of adjacent structures as features that distinguish them from pineocytoma [3].

According to WHO low grade gliomas include diffuse astrocytomas (fibrillar, gemistiocytic, protoplasmatic, mixed), grade II oligodendrogliomas and oligoastrocytomas. Diagnosis of anaplastic glioma is based on the presence of mitotic activity, while vascular proliferation and necrosis are typical features of GBM. The definition of low grade glioma does not refer perhaps to I grade astrocytomas, that differ from other gliomas in relation to their biological behaviour, prognosis and genetic profile [4]. Low grade gliomas, tend perhaps to dedifferentiate: a global therapeutic approach, related to gross total (> 90%) surgical removal, radiotherapy e chemiotherapy with temozolamide, leads only to a slight prolongation of overall survival, calculated from 3 to 7 years [5]. This variability depends from various factors: age at diagnosis, tumor volume, tumor crossing the midline, neurological deficits before treatment and tumor's histology [6]. Anyway, mortality is near to 100% and only 35% of patients with glioblastoma multiforme have a median survival of one year or more.

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2.1. Genetics of malignant gliomas

Cancers originate as the result of hereditary or somatic alterations in genes that control critical biological processes. The accumulation of such genetic damage over time permits the survival and progressive transformation of abnormal cell populations that eventually lead to the formation of a tumor. Consistent with their high malignant potential, gliomas exhibit a vast array of well-documented genetic changes that likely contribute to their phenotype. Gliomagenesis is characterized by several biological events, such as activated growth factor receptor signaling pathways, downregulation of many apoptotic mechanisms and unbalance among proangiogenic and antiangiogenic factors. Several growth factor receptors, such epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDRGF), C-Kit, vascular endhotelial growth factor receptor (VEGFR) and others growth factors receptors, are overexpressed, amplified and/or mutated in gliomas. Features of the glioma cells are the loss of tumor suppressor genes, which are critical for the cell growth, differentiation and function.

The TP53 tumor suppressor gene was identified as the main driver of chromosome 17 alterations in glioblastoma, and further studies indicated that p53 plays a critical role in monitoring the genome for DNA damage and can control cell cycle arrest to permit DNA repair or can trigger apoptosis to eliminate the damaged cell [7]. It acts as a transcriptional activator and it repress the transcription of other genes, such as IL-6, c-fos, c-jun and other oncogenes. The levels of p53 increase in G1 and S phases of the cell cycle. Cells transfected with the normal gene p53 block their growth in G1, suppressing the passage S-phase. The expression of the p53 gene cause apoptosis (programmed cell death). The effects of its mutation are the loss of cell cycle regulation, through the inactivation of $p16^{ink\text{-}4a}$, the super expression of cycline dependent kinase 4 Cdk4 and Cdk6 and of ubiquitin ligase Mdm2 and Mdm4. A similar mechanism is created after mutation of retinoblastoma (Rb), a tumor suppressor gene. Probably, TP53 is a gene responsible for the progression from low-grade to high-grade gliomas, but its mutation is an early event. Many glioma cell lines expressing p53 mutations. The mutated form of p53 has a crucial role in the regulation of neovascularization. It induces increased levels of VEGF and FGFβ through an activation of the transcription of the corresponding genes. The deletion of NFKBIA (encoding nuclear factor of κ-light polypeptide gene enhancer in B-cells inhibitor-α), an inhibitor of the EGFR-signaling pathway, promotes tumorigenesis in glioblastomas that do not have alterations of EGFR. Bredel et al. analyzed 790 human GBMs for deletions, mutations, or expression of NFKBIA and EGFR. they studied the tumor-suppressor activity of NFKBIA in tumor-cell culture and compared the molecular results with the outcome of GBM in 570 affected persons. NFKBIA is often deleted but not mutated in GBM [8].

Epidermal growth factor receptor mutations include amplifications, point mutations and deletions, with the most common alteration being the variant III deletion of the extracellular domain (EGFR-vIII mutant) [9]. Platelet-derived growth factor receptor and its ligands PDGF-A and PDGF-B are also commonly over-expressed in some glioma cells, raising the possibility of autocrine or paracrine activation [10]. Other RTK genes, such as ERBB2, another member of the EGF receptor family, and MET, which encodes the hepatocyte growth fac-

tor receptor, have also been found to be mutated in GBMs [9]. RTKs mediate cell growth and proliferation via downstream effectors such as Ras and phosphatidylinositide-3-kinase (PI3K). Activity of these proteins is tightly regulated, especially by the tumor suppressors Nf1 and Pten. The PI3K pathway is another essential survival pathway for a variety of cancer cells. The tumor suppressor Pten (phosphatase and tensin homologue on chromosome 10) negatively regulates the PI3K pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) back to phosphatidylinositol-4,5-biphosphate (PIP2) [11]. Mutations in PTEN frequently involve the phosphatase domain, and mutations in PI3K typically involve the catalytic (p110α) and regulatory (p85α) domains.

Classically, GBM has been described as result endpoint from two main gliomagenesis pathways [12-13]. Primary GBM shows amplification of the EGFR, deletion or mutation of homozygous cyclin-dependent kinase (CDK) inhibitor p16INK4A/(CDKN2A), alterations in tumor-suppressor PTEN on chromosome 10, and deletion in the INK4a gene with loss of p14 and p16 [14-15]. Progression from low-grade to high-grade astrocytomas involves inactivating mutations of tumor-suppressor gene TP53 and elevated expression of PDGF ligands and receptors, accumulation of genetic alteration of retinoblastoma-associated cell cycle regulatory pathways, including deletion or mutations of cyclin-dependent kinase inhibitor p16INK4A/(CDKN2A) or the retinoblastoma susceptibility locus 1 (pRB1), as well as amplification or overexpression of cyclin-dependent kinase 4 (CDK4) and human double minute 2 (HDM2). Evolution to secondary GBM is associated with deletion of chromosome 10, which includes tumor-suppressor phosphatase and tensin homologue (PTEN). PTEN phosphatase were identified as the tumor suppressors lost on chromosomes 19 and 10, respectively. p16 can slow down cell cycle progression, whereas PTEN is a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway, [16] a major signaling pathway that stimulates cellular proliferation in response to growth factor stimulation. Interestingly, only 1 copy of the gene is mutated in the tumors, suggesting that the mutations do not result in a simple loss of function. The mutation is very specific and leads to a single amino acid change (arginine 132 usually becomes histidine) in the IDH1 active site, whereby the enzyme loses its ability to catalyze conversion of isocitrate to α-ketoglutarate. It was proposed that this might have an indirect oncogenic effect through the activation of the hypoxia-inducible factor pathway, [17] a critical step in the metabolic adaptation of tumors to anaerobic growth and for the formation of new blood vessels through the angiogenic process.

Recently, attention has been focused on the role of an enzyme, isocitrate dehydrogenase 1 (IDH1), present in cytoplasm and peroxisomes, catalyzing the oxidative decarboxylation of isocitrate to alpha-ketoglutarate, reducing NADP+ to NADPH. Studies performed with seriated biopsies from gliomas, revealed that mutation of IDH1 is always found before TP53 mutation or, in case of oligodendrogliomas, before their typical deletions of chromosomes 1p and 19q. Moreover, mutation of IDH1 is quite specific, since it is extremely rare in I grade gliomas, non glioma brain tumors and in other cancers. Yan et al. studied genetic alterations of both IDH1 and of its related gene, IDH2. Genomic analysis, showed mutation of amino acid 132 of IDH1 in over 70% of patients with both low or high grade gliomas, and many of the cases negative for IDH1 mutations, had mutation involving amino acid 172 of IDH2 [18].

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Studies suggest that mutant IDH1 loses its normal enzymatic activity in tumors while gaining a new pro-oncogenic activity, leading to the production of an onco-metabolite [18-20]. Other studies characterizing the genomic make-up of human glioblastoma have provided further insight into the genetic changes, core pathways and molecular subtypes underlying this disease [20-22]. Based on their gene expression profiles, The Cancer Genome Atlas Network (TCGA) further classified GBMs into four subtypes termed: proneural, neural, classical, and mesenchymal [9]. While the status of gene expression and mutation in EGFR, NF1, PDGFA, and IDH1 were defining components of these subtypes, it was also found that response to therapies was different for each subtype, suggesting that personalized treatment based on genomic alterations could lead to a more favorable outcome for this disease [23].

3. Molecular biology of glioma invasion

The cellular and molecular events that initiate and promote malignant glioma development are not completely understood. Vasculogenesis and angiogenesis potentially play distinct roles in the etiology of primary and recurrent malignant gliomas, suggesting that patient therapy should perhaps be tailored specifically against the predominant vasculature pathway at a given specific stage of gliomagenesis [24]. Cerebral gliomas show a unique pattern of invasion and, with rare exceptions, do not metastasize outside of the brain. Invading glioma cells normally migrate to distinct anatomical structures. These structures include the basement membrane (BM) of blood vessels, the subependymal space, the glial limitans externa, and parallel and intersecting nerve fiber tracts in the white matter. Invasion of tumor cells into normal tissue is thought to be a multifactorial process, consisting of cell interactions with ECM and with adjacent cells, as well as accompanying biochemical processes supportive of active cell movement. Several cell types aim active movement during various stages of embryonal development, during wound healing, and in the course of immune responses. This innate activity is regulated in a very rigid manner, suggesting that the reappearance of a motile phenotype in cancer cells results from the loss or cessation of normal inhibitory controls [25]. Critical factors in tumor cell invasion, include the detachment of invading cells from the primary tumor mass, the synthesis and deposition of ECM components by tumor cells and mesenchymal cells, the release of ECM-degrading activities for remodeling interstitial space, and the expression of adhesion molecules on glioma cell surfaces that specifically recognize and adhere to ECM components.

The detachment of invading glioma cells from the primary tumor mass involves several events, including, destabilization and disorganization of the cadherin-mediated junctions that hold the primary mass together, loss of expression of neural cell adhesion molecule, which promotes adhesion to the primary tumor mass through homophilic binding, cleavage of CD44, which anchors the primary mass to the ECM by the metalloproteinase ADAM.

Cadherin superfamily are important adhesion molecules associated with glioma invasion. Cadherins (E-, P-, and N-cadherin) form adherent junctions and may function as suppressors of tumor growth and invasion. Desmosomal cadherins interact heterotypically and pro-

vide a linkage to the intermediate filament network through association with cytosolic proteins (desmoplakin, plakoglobin). Cadherins are linked to the actin-cytoskeleton via catenins (α-, β-catenin, plakoglobin), thereby establishing molecular lines of communication to other cell-cell junctions and to cell-substratum junctions [26]. During glioma progression decreased cadherin function is correlated with de-differentiation, metastasis and poor prognosis [27]. Due to its participation in processes such as morphological differentiation and contact inhibition of growth and motility, cadherins may function as suppressors of tumor growth and invasion [25]. Perego and co-worker, showed that only glioma cells with immature adherens junctions are capable of migrating and invading through poly-1-lysine coated filters. In the process of maturation of adhesions, the Lin-7 protein forms a complex with cadherin and β-catenin [28].

The second step is a decline in the expression of connexin 43, which leads to a reduction in gap junction formation. Connexin 43 is the most abundant gap junction protein in CNS and is expressed primarily in astrocytes [29]. Cell-cell communication is important in growth control and differentiation, and it is partly achieved using gap junctions and via second messengers [30]. Decreased gap junction formation may result in fewer inhibitory signals, facilitating uncontrolled cell division and de-differentiation [31]. Has been demonstrated that reduced gap junction formation is correlated with increased motility of glioma cells in vitro [32]. Increased malignancy of glioma specimens correlates with reduced in situ gap junction formation as well as reduced connexin 43 expression [33]. The third event is cleavage of CD44, which anchors the primary mass to ECM, by the metalloproteinase ADAM. CD44 is a transmembrane glycoprotein belonging to the immunoglobulin receptor superfamily, which interacts with hyaluronic acid as its ligand. Monoclonal antibodies directed against CD44 decrease intracerebral invasion of glioma cells in vivo and through matrigel matrices in vitro [34]. CD44 can be cleaved by ADAM 10 and 17, and both the extracellular and intracellular cleaved components of CD44 promote cell migration [35].

3.1. Integrins

Integrins are heterodimeric transmembrane cell surface receptors that play a key role in the crosstalk between the cell and its surrounding stroma. Integrins regulate cell adhesion, migration, differentiation, proliferation, and survival during physiological and pathological conditions, including inflammation and cancer. Upon ligation to extracellular ligands (matrix proteins such as collagens, laminins, vitronectins and fibronectins), integrins activate downstream signaling pathways in concert with growth factor receptors, including PDGFR, EGFR and VEGFR. Preclinical data indicate that integrins play a key role in cancer initiation and progression.

Integrins contain two distinct chains, calls subunit α (alpha) and β (beta). Have been identified about 18 α and 8 β subunits. The integrin subunits penetrate the plasma membrane and typically have very short cytoplasmic domains of about 40-70 amino acids. Outside the plasma membrane, α and β chains protrude to a length of about 23 nm, the last 5 of which - thanks to the termination of each chain formed dall'NH2 - a region used to form bonds to the ECM. The molecular mass of the subunits of the integrin may range from 90 kD to 160

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kD. The β subunits have 4 cysteine-rich repeat sequences. Both the α subunit β both bind many divalent cations. There are many ways to classify the integrins. For example, a subclass of the chains α has a structural domain in more inserted at the network termination NH2, the so-called alpha-domain A. The integrins that possess this characteristic usually form collagen (α1β1 integrins, and α2β1), or act as adhesion molecules between cell and cell (β2 integrin family). This domain α-1 is the active site for links to various integrins. Those who do not carry this domain, however, have a domain A in their active site, but this is in the β chain. In both cases, the domains A are connected to three active sites consisting of divalent cations. One of these is permanently occupied by physiological concentrations of divalent cations, and binds to a calcium ion or magnesium. The other two sites are occupied by cations when establishing ties - at least for the bonds involving an amino acid, aspartic acid, typically - in their interaction sites. An amino acid characterized in the active site of the integrin many proteins ECM, as part of the amino acid sequence Arginine-Glycine-Aspartic acid. The two main functions of integrins are cell adhesion to ECM and signal transduction from the ECM to the cell. They interact with two large groups of ligands: a variety of ECM proteins, such as fibronectin, vitronectin, fibrinogen, and cell surface molecules, that are members of the immunoglobulin supergene family, such as intracellular adhesion molecules (ICAM-1, ICAM-2) and vascular cell adhesion molecule (VCAM-1). In particular, the integrin αvβ3, which binds to fibronectin, vitronectin, and tenascin-C in ECM, is thought to play a central role in glioma invasion [36]. Increased expression of integrin αvβ3 leads to increased motility in human glioma cells with a concomitant decrease in apoptosis sensitivity.

Integrins combine the ECM out of the cell to the cytoskeleton (in particular microfilaments) within the cell. What site can bind the ECM is usually decided by the integrin α and β subunit which is composed of the same integrin. Bonds between the integrin are fibronectin, vitronectin, collagen, and laminin. The connection between the cell and the ECM makes the cell able to withstand the forces tie rods without being thrown out of the ECM. The connections between the integrin and the links in the ECM and microfilaments inside the cell are indirect: they are connected by proteins "armor" as talin, paxillin, and alpha-actinin. These act by regulating the kinase such as FAK (focal adhesion kinase - focal adhesion kinase) and the family of Src kinases to phosphorylated substrates such as the p130CAS or recruiting signal adapters such as Crk. The connection of the cell to the extracellular matrix is a basic requirement to form a multicellular organism. Integrins link the cytoskeleton to the extracellular matrix, and are recognized to be key regulators of tissue structure. They provide adhesive, migratory, and survival cues to tumor cells and to cells of the tumor microenvironment, including angiogenic endothelial cells. The integrins αvβ3 and αvβ5, among others, are highly expressed not only on the tumor vasculature and angiogenic endothelial cells, but also on tumor cells, including gliomas. Consequently, integrins have been considered as a promising therapeutic target in cancer. Monoclonal antibodies and peptide-based integrin inhibitors are being investigated for their potential therapeutic activity in various tumor types. This strategy is in advanced stage clinical development in glioblastoma, a highly vascular primary brain tumor [37].

The malignancy of glioma is dependent on diffusive properties of tumor cells that may invade widely as single cells anywhere in the brain. Integrins are the family of adhesive receptors that promote invasiveness of glioma. These heterodimeric transcellular membrane receptors are composed of two subunits, α and β, and are the major receptors for the ECM proteins. Immunohistopathological studies revealed an upregulation of $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha v\beta 3$ integrins on GBM when compared with normal brain [38].

Glioblastoma commonly displays enhanced expression of several integrins along with their ECM ligands: $\alpha v\beta 3$ and $\alpha v\beta 5$ (tenascin and vitronectin receptors), $\alpha 5\beta 1$ (fibronectin receptor), $\alpha 2\beta 1$ (collagens receptor), and $\alpha 3\beta 1$, $\alpha 6\beta 4$, and $\alpha 6\beta 1$ (laminins receptors). Numerous studies have focused on the αv integrin family. The integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are markers of glioblastoma malignancy and influence a variety of processes in glioblastoma progression in vivo, including proliferation, apoptosis, and angiogenesis. Furthermore, cilengitide, an $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins antagonist, extends mouse survival by delaying the tumor growth and is nowadays in clinical trial for recurrent malignant glioma. Two other integrins, $\alpha 5\beta 1$ and $\alpha 3\beta 1$, have been shown to be implicated in glioma cell adhesion and migration in vitro. The $\alpha 6$ integrin subunit associates with $\beta 1$ or $\beta 4$ subunits to form functional heterodimers that selectively bind laminins. The $\alpha 6\beta 4$ integrin is essential for the organization and maintenance of epithelial hemidesmosomes that link the intermediate filaments with the extracellular matrix. The major ligand of $\alpha 6\beta 4$ is the laminin-332, while $\alpha 6\beta 1$ is a well-characterized laminin-111 receptor. Overexpression of $\alpha 6\beta 1$ integrin has been associated with the progression of many epithelial tumors. Several studies concerning gliomas and the $\alpha 6\beta 1$ ligand laminin-111 have been reported in the literature. Using immunohistochemistry studies, Gingras et al. showed that $\alpha 6$ integrin was strongly expressed in glioblastoma tissue, whereas it was weakly expressed in normal brain [39]. Previtali et al. confirmed that the expression of $\alpha 6$ was increased in glioblastoma and in other central nervous system tumors, such as meningioma, astrocytoma, and neuroblastoma, when compared with the autologous normal tissue counterpart [40]. In glioblastoma biopsies, laminin-111 is highly expressed on tumor blood vessels, but also within the brain tumor as punctuate deposits and at the tumor invasion front. In vitro, glioma cells can both secrete laminin-111 and induce its expression in normal brain tissue. Moreover, laminin-111 is one of the most permissive substrates for adhesion and migration of glioma cells in vitro. Additionally, over laminin-111, migrating glioma cells are protected from apoptosis [41].

In malignant glioma, and in particular in GBM, overexpression of $\alpha v\beta 3$ integrin is well documented. The integrin $\alpha v\beta 3$, which binds to fibronectin, vitronectin, and tenascin-C in ECM, is thought to play a central role in glioma invasion [36]. Increased expression of integrin $\alpha v\beta 3$ leads to increased motility in human glioma cells with a concomitant decrease in apoptosis sensitivity. Importantly, $\alpha v\beta 3$ integrin is expressed both on angiogenic endothelial cells and on tumor cells. Conversely, inhibition of integrin $\alpha v\beta 3$ decreases glioma cell motility [42]. Several factors expressed in glioma cells have been found to regulate integrin expression. Particularly, uPA secreted by glioma cells has been shown to upregulate integrin $\alpha v\beta 3$ expression by autocrine mechanism [43]. Furthermore, decreasing the in vivo expression of the $\beta 1$ subunit by an antisense strategy in the intracranial C6 glioma model leads to

an inhibition of glioma associated angiogenesis at the invasive edge of the tumors [44]. Down-regulated β1 integrin protein levels in vivo probably affect interactions of glioma cells with ECM components, leading to reduced migration along vascular basement membranes. The integrin α6β1 plays an important role for the regulation of glioma-initiating cells in the perivascular niche. This integrin mediates the interaction of glioma-initiating cells to laminin, an extracellular matrix protein expressed in basement membranes, including those supporting endothelial cells. This interaction provides an anchorage for glioma-initiating cells within the perivascular niche and supports their tumorigenic potential [37].

Brown et al. evaluate the role of α9β1 integrin interaction with nerve growth factor (NGF) in glioma progression, they selected the two most malignant glioblastoma cell lines: LN229, which expresses α9β1, and LN18,in which does not. Presence of the α9 integrin subunit on LN229 and absence on LN18 cell lines were verified using several assays such as monoclonal antibody adhesion microarray, Western blot analysis of cell lysates, flow cytometry, and immunocytostaining [38]. Very high expression of the α9 integrin subunit was observed only on the LN229 cell line. Western blot analysis, using polyclonal anti-α9 antibody, showed the absence of this integrin subunit on normal astrocytes. The expression of α9β1 integrin on LN229 and lack of it on LN18 was confirmed by flow cytometry and immunocytostaining analysis. The investigation of cellular responses following binding of NGF to α9β1 integrin on LN229 and LN18 cells required evaluation of the level of two other NGF receptors, TrkA and $p75^{NTR}$. Western blot analysis of cell lysates and immunocytostaining revealed negligible expression of TrkA on both types of cells, and a stable expression of $p75^{NTR}$ in comparison with PC12 neuronal cells used as a positive control. Interestingly, the level of $p75^{NTR}$ appeared to be lower on LN18 cells, which are α9β1 integrin deficient, than on LN229 cells, which are α9β1 integrin positive. This correlation was previously observed in the mRNA level of SW480 cells transfected with α9 integrin subunit. Cells transfected with the α9 subunit showed a potent increase of $p75^{NTR}$, whereas mock-transfected cells had a significantly lower expression of this NGF receptor. The opposite situation occurred for TrkA, suggesting that this specific high affinity NGF receptor has a functional complementary association with α9β1 integrin. Several factors expressed in glioma cells have been found to regulate integrin expression. Glioma expression of focal adhesion kinase, a nonreceptor cytoplasmic tyrosine kinase, has been shown to increase phosphorylation of the enhancer of filamentation 1, which in turn stimulates PDGF-mediated stimulation of glioma integrin adhesion to ECM [45].

3.2. Proteases

Extracellular proteolytic enzymes are critical for the invasive properties of malignant neoplasms [46]. Barrier to invasion do not appear as restrictive within CNS parenchyma and matrix substrates most responsible for impeding tumor migration have not been identified [47]. Nonetheless, there is a strong evidence that the expression of specific extracellular matrix proteases promotes invasive behavior by gliomas. These include the matrix metalloproteinases (MMPs), the urokinase-dependent plasminogen activating cascade, and cathepsin

B. Wild-Bode et al. [48] found that MMP-2 and MMP-3 levels and MMP-2/MMP-9 activity correlated with glioma cell migration and invasion. NF-jB, uPA, low-density lipoprotein receptor-related protein 1, and insulin-like growth factor binding protein-2 are known to upregulate MMP expression. Induced expression of tissue inhibitor of metalloproteinases-3 (TIMP-3), a putative inhibitor of MMP activity, has been shown to suppress infiltration and also to induce apoptosis in cancer cell lines. MMPs play an important role in human brain tumor invasion, probably due to an imbalance between the production of MMPs and tissue inhibitor of metalloproteinases-1 (TIMP-1) by the tumor cells. MMP-1 is the crucial enzyme able to initiate breakdown of the interstitial collagen types; in this way, it activates the other MMPs, which allows the glioma cells to infiltrate normal brain tissue.

Matrix metalloproteinases comprise a large family of zinc-dependent endoproteinases, collectively capable of degrading all ECM components. A total of 23 families of MMPs are known and these are numbered in the sequence of their discovery [49]. Originally, MMPs were classified according to their respective substrate specificity, now they are divided into eight structural subgroups, five of which are secreted and three of which are transmembrane MMPs. A signal peptide leads them to the secretory pathway. Then, these enzymes can be secreted from the cell or anchored to the plasma membrane, thereby confining their catalytic activity to the extracellular space or to the cell surface, respectively. Their function is to degrade different protein components of the ECM and of basement membranes, therefore they are essential for the interaction of individual cells with their surrounding and for the development and function of multicellular organisms [50]. Between the various substrates of MMPs we include collagens, non collagens glicoproteins, proteoglycans and other ECM components like tenascin, fibronectin and laminin, which often show tumor specific expression are also substrates [51]. The proteolytic activities of MMPs influence essential cellular processes like cell proliferation, migration and adhesion, as well as many fundamental physiological events involving tissue remodeling, such as angiogenesis, bone development, wound healing, and uterine and mammary involution [52]. However, the increasing expression of this enzymes is associated with many pathological conditions [53-54] such as rheumatoid arthritis, cardiovascular diseases or cancer progression [55]. MMP expression and activity can be regulated at different levels including gene transcription, proenzyme activation and endogenous inhibition, which act in a coordinated manner to confine the diverse MMP proteolytic activities to those conditions and locations where they are necessary. Unfortunately, these restrictive regulatory mechanisms are frequently lost in multiple pathological conditions [56]. MMPs expression is induced by different factors like EGF, TGF-β, PDGF, and various inflammation's mediated, included TNFα and IL-1β.

MMPs or matrixins are synthesized as zymogens, inactive pro-enzymes (pro-MMPs), with the zinc ion, essential for MMP activity, hidden by a cysteine-sulphydryl residue situated near the C-terminal end of the peptide. The pro-enzyme's activations starts breaking the interaction cysteine-zinc exposing, in this way the catalytic site. The fully active enzyme is generated by proteolytic cleavage of the pro-peptide domain of the partially active intermediate enzyme [50]. Once active, MMPs are regulated by interactions with endogenous inhibitors including α2-macroglobulin, thrombospondin-2, tissue inhibitors of metallopro-

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teinases (TIMPs) and RECK (reversion-inducing cysteine-rich protein with kazal motifs) [49]. Numerous studies have investigated the expression of selected MMPs in human GBM cell lines. As it has already been reported that there are differences in the expression patterns of MMPs in different cell-lines [57]. A number of different techniques have been used to detect protease expression patterns. This may have led to dissimilar conclusions, due to disparate sensitivities and due to comparing mRNA expression with protein expression or protein activity. Variations in MMP expression may be due to in vitro selection processes or karyotype evolution, where the transcription of either the enzyme and/or its inhibitor may be affected which ultimately leads to an imbalance in the MMP-regulatory network [57]. MMP-9's production is dependent on a regulation by extracellular signal-regulated kinase (ERK), PKCα/NF-κB and jun amino-terminal kinase (JNK) signaling cascades. Glioblastomas are highly hypoxic and hypoxia upregulates MMP-2 mRNA expression in U87, U251, U373 and LN18 glioblastoma cell-lines by activation of the HIF-1α transcription factor, thereby enhancing their invasive potential. Migration and invasion of U87 and T98G GBM cells is also facilitated by NO, which can be found in high concentrations in glioblastoma tissue. NO stimulates MMP-1 expression and activity. EGF raises MMP14 expression in U251 cells, but does not influence MMP-15, -16 or MMP-24. MMP-2 expression and secretion is induced by IL-6 in U87 cells. However, IL-6 action seems to be cell-line specific, since U343 cells were not affected [57].

The inflammatory cytokine TNF-α and the immune-suppressive cytokine TGF-β have been implicated in migration and invasion of glioma cells in vitro. In U251 and in U373 cells, TNF-α stimulated the expression of MMP-9 and MMP-19. MMP-1 mRNA expression was significantly increased in U373 cells by TNF-α, whereas its expression in U251 cells remained unaffected. This may be due to the high basal level of MMP-1 expression displayed by U251 cells, where a further increase is not possible, or else it could also be a cell- line specific effect. Such an effect has been observed for MMP-1, -2, -3 and MMP-7 regulation by TNF-α and TGF-β1, which only caused a marked induction of expression in some GBM cell-lines. TNF-α enhances the invasiveness of T98G cells through an induction of MMP-3, but has no effect on MMP-1, -2 or MMP-9. However, in U251 cells TNF-α inhibits MMP-2 and decreases invasiveness into the extracellular matrix. In A172 cells, TNF-α induces gene expression and protein secretion of MMP-9. TGF-β1 alone had no effect on MMP-9 production. However, when it was added together with TNF-α a significant dose-dependent inhibition of MMP-9 secretion was observed. TGF-β1 displayed inconsistent effects on adhesion and invasiveness, depending on the cell-line examined. The invasive potential of U138 cells was markedly reduced, whereas U373 cell invasion remained unchanged. TGF-β1 caused a significant induction of MMP-11 and MMP-24 expression in U373 cells, whereas there was no impact on MMP expression in U251 cells. In U87 and LN229 cells, TGF-β upregulates MMP-2. Thus, the transcriptional modulation of MMP genes in response to TNF-α or TGF-β is not consistent, but extremely cell-line specific [57]. Elevated levels of several MMPs were also found in malignant glioma tissue samples. MMP-1 expression was increased in surgical specimens of GBM compared to low grade astrocytomas and normal brain. This increased expression is probably due to a single nucleotide polymorphism in the MMP-1 promoter at position-1607, creating a functional binding site for members of the ETS family of transcrip-

tion factors. MMP-1 was expressed throughout the tumor section, particularly in the highly cellular areas of the GBM [57]. Many studies have demonstrated an intimate association between MMP-9 and tumor invasiveness. Different analyses for MMP-9 were negative in NB tissue, showed weak signals in LGA and strong expression in GBM [57]. MMP-9 is strongly expressed in blood vessels at proliferating margins, as well as tumor cells, this suggests a role in the regulation of tumor neoangiogenesis [58]. Other proteases that could be related with the development of highly invasive glial tumor are MMP-11, MMP-12, MMO-14, MMP-15, MMP-19, MMP-24, MMP-25.

A second proteolytic system that interfaces with MMPs is the urokinase pathway of plasminogen activation. This system includes urokinase (also known as urokinase-type plasminogen activator, uPA), the urokinase receptor (uPAR), and plasminogen. Urokinase is secreted as a single chain, inactive proenzyme that is activated by cleavage upon binding to its cell surface receptor uPAR. Activated uPA then converts plasminogen into plasmin, a serine protease that promotes cellular migration by the degradation of extracellular matrix proteins, activation of other matrix proteases and activation of cell surface receptors that transduce intracellular signaling for migration. Plasmin activates protease activated receptor 1 (PAR1), which is normally expressed in human astrocytes, is activated in many malignancies including GBMs, and is associated with increased invasive properties [59]. Thus, uPA and plasminogen activation can occur in the immediate vicinity of the cell membrane or, following cleavage of the uPAR GPI anchor, at greater distance in the extracellular matrix. An important biologic regulator of this cascade is plasminogen activator–inhibitor 1 (PAI1), which promotes the internalization of uPA bound uPAR [60]. uPAR has roles in cellular migration distinct from its ability to activate plasminogen though its interactions with integrins, signaling through G-protein coupled receptors, and caveolin binding [61]. There is a convincing correlative relationship between uPAR and uPA expression and malignant progression in a variety of tumor types [62]. Both neoplastic cells and stromal cells contribute to the overall activity of these pathways. In co-cultures of malignant gliomas and reactive astrocytes, most uPA and uPAR activity was derived from reactive astrocytes, whereas plasminogen was expressed in largest amounts by glioma cells [63]. Levels of uPA mRNA and its enzymatic activity are higher in human anaplastic astrocytomas and GBMs than in low grade astrocytomas and normal human brain and uPA mRNA levels have an inverse correlation with survival periods [64]. uPAR levels are significantly higher in GBMs and anaplastic astrocytomas that normal brain and low grade gliomas, with uPAR mRNA localizing to both glioma cells and endothelial cells [65]. The finding of uPAR expression at the leading edge of neoplastic infiltration and within remodeling hyperplastic blood vessels suggested that it may be playing a role in invasion and tissue remodeling. mRNA levels of both uPA and uPAR genes are increased in high grade gliomas possibly suggesting increased gene transcription [66]. In the CNS, reactive astrocytes in the vicinity of invading gliomas appear to contribute for a substantial proportion of extracellular MMP activity, both by their secretion of pro-MMP2 and the components of the plasminogen activating system, uPA and uPAR [63]. Reactive astrocytes have also been shown to promote the invasive capabilities of glioma cells in vitro, presumably resulting from enhanced extracellular MMP-2 activity.

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The cathepsins are a family of lysosomal cysteine proteases. These proteases are synthesized as inactive pro-enzymes that are activated by autocatalytic activity initiated by low pH or by other proteases including cathepsin D, uPA, and pepsin. Natural regulators of cathepsins are the cystatins, which are critical, in their inhibitory properties. Cathepsins are secreted into the extracellular space where they degrade extracellular matrix components required for invasive properties in neoplastic disease. Best studied for their roles in glioma tumorigenesis and invasion are cathepsins B, D, H, L, and S. Each of these cathepsin subtypes have higher protein levels in high grade astrocytomas than low grade tumors or normal brain and enzymatic activities, in general, correlate with protein expression [67-68]. The role of cathepsins in tumor invasion has been supported by the finding that tumor cells at the invading edge of gliomas express high levels of cathepsins, especially cathepsin B [69]. In addition, protein and enzymatic levels of cathepsins B, D, L, H, and S were found to correlate with the ability of glioblastoma cells to migrate in vitro. Once in the extracellular compartment, cathepsin B physically interacts with a protein complex at the outer cell membrane containing annexin II, p11, tPA and plasminogen [70]. Its close proximity to both the plasminogen activating cascade and MMPs is critical for regulation of their combined proteolytic functions. Upon activation, cathepsin B has its own proteolytic activity on extracellular matrix components, but also activates the cascade to convert plasminogen to plasmin and is able to activate selected MMPs [71].

3.3. Extracellular matrix

The ECM affects numerous functions and processes within the brain. During brain development, the ECM modulates the migration of glial and neuronal precursor cells, guides axonal growth cones, synapse formation and cell proliferation. The roles played by the ECM in neoplastic transformation are complex and only now beginning to be uncovered. ECM is a complex network of different collagens, proteoglycans, hyaluronic acid, laminin, fibronectin, and many other glycoproteins, including proteolytic enzymes involved in degradation and remodelling of the ECM [72]. Extracellular matrix exists in two forms: interstitial matrix that fills in the intercellular space and the more specialized BM, which is a thin sheet of extracellular matrix underlying the epithelium. ECM provides the microenvironment for the cells and serves as a tissue scaffold, guiding cell migration during embryonic development and wound repair. Integrins are a class of adhesion molecules that have a major role in the adhesion and subsequent invasion of tumor cells. ECM proteins such as fibronectin, laminins or collagens form distinct protein networks that show tissue-specific variation in composition and architecture. Cell responses to contact with these networks depend on the cell's repertoire of ECM receptors. Connections from the matrix through these receptors determine the organization of cytoskeletal structures and the localization and activation of signaling molecules leading to unique tissue specific cell functions. Changes in these ECM components are felt to modulate brain tumor growth, proliferation and invasion, although specific interactions and exact mechanisms are unknown. Cell adhesion is the binding of the cells to each other and to the ECM through cell adhesion molecules such as integrins, selectins, cadherins, the Ig (immunoglobulin) superfamily and lymphocyte homing receptors. Cell adhesion mediates cell attachment, migration, and signalling to and from the extracellular matrix. Ad-

hesion complexes include focal adhesions, adherens junctions, tight junctions, desmosomes, hemi-desmosomes, and gap junctions. The best characterized adhesion molecules are the integrins and the best characterized adhesion complexes are focal adhesions. The extracellular ligands that anchor these adhesions include laminin, fibronectin, vitronectin, and various collagens. Focal adhesions can be considered both as sensors of force and as sites that originate cytoskeletal forces through anchored actin-microfilament bundles. Focal adhesions have many SH2-containing components (Src kinases, PI3K, SHP-2), as well as many tyrosine-phosphorylated molecules (focal adhesion kinase paxillin, tensin, caveolin).

Syndecans are a family of transmembrane heparin sulphate proteoglycans with four members, syndecans 1 to 4. Syndecans function mainly as co-receptors by binding to their ECM ligands in conjunction with other receptors, notably integrins. Through their heparin sulphate side chains, syndecans may further engage directly in ligand binding. Dystroglycans are heterodimeric complexes consisting of non-covalently associated α and β subunits with extracellular ligand-binding and transmembrane functions, respectively. Dystroglycans are a part of the larger dystrophin-associated protein (DAP) complex that connects basement membranes to the cytoskeleton, particularly via α2 laminins and perlecan. Ig superfamily members consist of immunoglobulin-like and fibronectin type III domains involved in homophilic and heterophilic cell–cell adhesion. The superfamily includes a variety of cell adhesion molecules (CAMs) with distinct ligand-binding specificities, including ICAM (intercellular), NCAM (neural), Ep-CAM (epithelial), L1-CAM, VCAM (vascular), ALCAM (activated leukocyte), and JAM (junctional adhesion molecule), among others. Cadherins are transmembrane proteins consisting of several tandemly repeated cadherin domains that mediate calcium-dependent homophilic cell-cell contacts. The cadherin superfamily comprises a total of more than 100 different members, with E- (epithelial) and N-cadherin (neural) most widely expressed in epithelial and neural tissues, respectively [73].

By convention, ECM components are biochemically classified fibrillar proteins (collagens), glycoproteins (laminins, fibronectin, tenascins), and several classes of proteo- glycans (heparan sulfate-, chondroitin sulfate-, dermatan sulfate-, and keratan sulfate proteoglycans). The latter mainly consist of large glycosaminoglycan (GAG) chains, covalently linked to extra- cellular or membrane bound core proteins. In contrast to other tissues, the ECM in the CNS lacks fibrillar proteins under physiological conditions. Instead the neural ECM is rich in glycoproteins and proteoglycans. It has been estimated that the neural ECM makes up about 20% of the CNS parenchyma [74]. The brain ECM is mainly deposited by astrocytes and oligodendrocytes and comprises an estimated 20% of the brain volume in adults. The main ECM components are hyaluronic acid (HA), tenascin R, and lecticans, which interconnect with each other noncovalently and form molecular networks filling the intercellular space. HA is a non-sulphated, linear, high-molecular weight glycosaminoglycan which, due to its water-binding capacity, controls the high water content of the brain interstitium. Besides tenascins and lecticans, HA binds to cell surface receptors including CD44 and ICAM-1, which together contribute to both ECM organization and cell-matrix interaction. It represents the major component of neuropil ECM and is widely distributed in the adult and embryonic brain. Malignant gliomas contain higher amounts of HA than low-grade gliomas

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in situ and in vitro, while the perivascular location in glioma biopsy suggests additional production by vascular stromal cells [75].

The tenascins (TN) are a family of large multimeric ECM proteins consisting of repeated structural modules including heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III repeats, and a globular domain shared with the fibrinogens. The TN are presumably involved in the morphogenesis of many organs and tissues [76-77]. The original tenascin discovered was TN-C, partially because of its overexpression in tumours and, inferring from cell biological studies, it has been proposed as an adhesion-modulating protein [77]. TN-C (also called neuronectin, brachinectin, myotendinous antigen, hexabrachion, glioma-mesenchymal extracellular matrix antigen, cytotactin, J1 protein, GP250 protein) has a characteristic hexabrachion structure, with as many as six arms linked to a central knob formed by disulfide bonding of cysteines in the NH2-terminal ends of polypeptides. TN-C initially appears during embryonic neural crest cell development, and it is present during brain and spinal cord organogenesis, being correlated with developmental phenomena such as cell proliferation, migration, and ECM remodelling. In addition, TN-C is transiently present in the dense mesenchyma surrounding several developing organs and tissues, including the nervous system [76-77]. Enhanced TN-C expression was detected among tumour cells, around individual cells as a fibrillary network, and around vascular channels [78]. A direct relation between the presence of TN-C and the degree of malignancy of gliomas has been reported, being TN-C reported to be expressed 5-fold higher in GBM as compared with AA and 10-fold higher as compared with juvenile pylocitic astrocytoma [79-80]. Endothelial cells in vitro attach to TN-C substrata, where they elongate, extend, and have interconnecting process. These features are lacking when endothelial cells growth on other matrix proteins, such as fibronectin, collagen, or laminin. The attachment of endothelial cells to TN-C is mediated by annexin and integrins, including $\alpha v\beta 3$ integrin, which is required for angiogenesis [79-81]. Tenascin R, another brain-specific member of the tenascin family, is a homotrimer with both lectican and integrin binding sites forming an adhesion bridge between the ECM and cells. Lecticans comprise a family of chondroitin sulphate proteoglycans with four members (brevican, versican, neurocan, and aggrecan), whereby brevican and neurocan are brain-specific. Lecticans contain HA and tenascin R binding sites and thus act as link molecules in protein–proteoglycan–glycosaminoglycan networks. Compared with peripheral interstitial tissues, a distinctive feature of the brain ECM is the absence of fibrillar collagen networks, which results in a low stiffness of the brain parenchyma. In a restricted expression pattern, fibrillar collagens I and III are, however, deposited by leptomeningeal cells, pericytes, and smooth muscle cells in blood vessels and the brain meninges, including the pia mater.

Fibronectin (FN) is a member of a family of glycoproteins that show many biological functions, including normal cell adhesion, growth, and migration. FN is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Both decreased expression and elevated degradation of FN have been shown to be responsible for some of the morphological changes observed in tumors and tumor-derived cell lines. As a peripheral protein, FN mainly acts as a bridge to link the cell surface and

ECM. Therefore, the absence or reduction of FN in tumor cells may reduce the adhesion between tumor cells and matrix components, and decrease the matrix's control of cell differentiation, proliferation, and migration. As a major protein in blood and as component of the wound provisional matrix, plasma FN contributes to tissue repair and neuronal survival following cerebral ischemia [82]. Fibronectin structure is rod-like, composed of three different types of homologous, repeating modules, types I, II, and III. These modules comprise functional domains that mediate interactions with other ECM components, with cell surface receptors and with FN itself. Twelve type I modules make up the amino-terminal and carboxy-terminal region of the molecule, and are involved mainly in fibrin and collagen binding. Only two type II modules are found in FN. They are instrumental in binding collagen. The most abundant module in FN is type III, which contains the RGD-FN receptor recognition sequence along with binding sites for other integrins and heparin. Depending on the tissue type and/or cellular conditions, the FN molecule is made up of 15-17 type III modules. In addition, there is a module that does not fall into any of these categories, called II-ICS. This module, along with EDB and EDA is regulated through alternative splicing of FN pre-mRNA. FN is an important component in the ECM of gliomas, largely in the vessel wall [83]. Expression of FN has been characterized in human GBM and in a number of astrocytoma and glioblastoma cell lines. In a study of pediatric GBM, a marked positive staining of FN in walls of small and medium size vessels was demonstrated. FN expression was seen in perivascular sheets, where it looked like a fine irregular network [83]. Knott et al. found that when normal brain tissues were invaded by glioma, ECM components such as LN, FN, and collagen type IV may are available and that tumor cells may express specific integrins, depending on the change of the interior environment, to interact with these ECM components, and enhance tumor cell invasion [84]. In vitro experiments have proven that components of ECM, LN and FN, can strongly stimulate the migration of glioma cells, which occurs after glioma cells express the relevant surface receptors [85]. Although there are many in vitro and in vivo models for FN-promoted invasion and transmigration [85], the uniqueness of these findings may be attributed to interaction between tumor cells and vascular endothelial cells (VECs), as changes of tumoral microenvironment molecules can affect tumor status and progression. Recently it has been demonstrated that FN expression is correlated with glioma migration and glioma malignancy [86]. More recently, FN has been reported to mantain extracellular matrix rigidity to promote structural rigidity, motility, and proliferation of established glioma cell lines in vitro [87]. The inhibition of FN expression in glioma cells, using short hairpin RNA-mediated silencing of gene expression, delayed cell proliferation in vitro. This delayed growth is explained, in part, by the observed reduced expression of integrin ß1 FN receptor, which was restored by the inhibition of proteosomal activity [88].

LM are a group of adhesion structural glycoproteins found in all BM as an integral part of the glia limitans externa [89]. LM the first neurite outgrowth promoting ECM identified is an adhesion glycoprotein associated with the development and regeneration of neuroectodermal tissues [89]. LM is distributed beneath vascular endothelial cells, around vascular smooth muscle cells, in the glia limitans, and beneath choroid plexus epithelial cells and plays a role in migration, neurite outgrowth, proliferation, and differentiation [90]. LM constitute the preferred substrate for growth of astrocytes and neurons, and have been found in

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all BM and in hyperplastic blood vessels in gliomas, gliosarcomas, and meningiomas [89]. Reactive astrocytes in situ, glioma cells in situ, as well as glioma cells lines, have been reported to express LM. Variants of LM may be expressed by astrocytes or neurons under different situations which adds to the complexity of the function and regulation of these large molecules in the CNS [91].

Type IV collagen (C-IV), the principal collagenous constituent of most BM, is mainly present in capillaries and large blood vessels. C-IV is associated with laminin, entactin, and the heparan sulphate proteoglycans perlecan. C-IV is found in areas of the normal brain, such as, beneath vascular endothelial cells, around vascular smooth muscle cells, in the glia limitans, and beneath choroid plexus epithelial cells. GBM cells are able of synthesising C-IV in vitro [72]. Bjerkvig et al. [92], demonstrated that C-IV was strongly expressed in tumour spheroids from rat glioma cell lines BT4C. In vitro the presence of C-IV in tumour vessels has been used to demonstrate vascular abnormalities in gliomas, such as BM duplication and disruption, as well as increased vascular density [93]. Recent studies have shown that GBM cells around areas of vascular proliferation, are also able to synthesise C-IV localised to the subendothelial BM of blood vessels [72].

4. Angiogenesis

Physiological angiogenesis, the formation of new blood vessels from pre-existing ones, is a strictly regulated fine-tuned process. The local balance between inducers and inhibitors of angiogenesis is critical in determining the generation or not of new vessels. Whenever this balance is perturbed pathological, uncontrolled, excessive angiogenesis occurs. Angiogenesis is a process that plays an essential role in cancer development. Coagulation and inflammation also play an important role in tumorigenesis. Their expression is controlled by over- or under-expression of certain genes [94]. Although a plethora of molecules can act as inducers of angiogenesis such as acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor alpha and beta (TGF-a and -h), tumor necrosis factor alpha (TNFα) and interleukin-8 (IL-8), the major growth factors specific for vascular endothelium include members of the vascular endothelium growth factor (VEGF) and angiopoietin families, and at least one member of the large ephrin family.

Angiogenesis is believed to be the primary method of vessel formation in gliomas. Malignant gliomas are characterized by extensive microvascular proliferation. Neovascularization in brain tumors correlates directly with their biological aggressiveness, degree of malignancy and clinical recurrence and inversely with the post-operative survival of patients affected by gliomas. Among all solid tumors, GBM has been reported to be the most angiogenic by displaying the highest degree of vascular proliferation and endothelial cell hyperplasia. Such intense vascularization might be responsible for the peritumoral edema, one of the pathological features of GBM [95]. The presence of endothelial glomeruloid-like proliferation and of positive immunoreaction at level of BM of tumor vascular channel are predictive of active tumor invasiveness [83]. Diffuse astrocytomas tend to progress from grade II to

grade III tumors with a time interval of several years, whereas, progression of grade III to grade IV is more rapid, typically 2 years. Primary and secondary GBMs are morphologically indistinguishable and show their histologic hallmarks, i.e., "glomeruloid" microvascular tufts and necrosis. Glioma vasculature is structurally and functionally abnormal and it correlates and leads to vasogenic edema, increased interstitial pressure, and heterogeneous delivery of oxygen and drugs [96].

Neoangiogenesis may be quantified post-operatively in brain tumors by the evaluation on surgical samples of the so-called microvessel density (MVD), which reflects the number of vessels per mm^2 within representative histological sections. Specifically, MVD is assessed in formalin-fixed and paraffin-embedded tissue sections through standard immunohistochemistry; in detail, the vessels present in the histological sections are preliminarly highlighted with peroxidase-conjugated antibodies against endothelial markers and then they are counted at light microscopy. Antibodies against several endothelial markers, such as Factor VIII, CD31, CD34 and endoglin, may be used for the quantification of MVD, with different sensitivity and specificity. For instance, following the use of antibodies against pan-endothelial markers (Factor VIII, CD31, CD34), all the vessels present in the histological section are stained, with no distinction between pre-existing and newly-formed vessels [97]. As a consequence, pan-endothelial cannot be considered as optimal markers for the quantification of neoangiogenesis. In contrast, endoglin (CD105), a 180-kDa transmembrane homodimeric glycoprotein that belongs to the TGF receptor complex [98], appears as an endothelial marker which more specifically allows the detection of vessels related to neoangiogenic process in brain tumors [97, 99-101]. Indeed, this protein is predominantly expressed by the cycling endothelial cells in the vessels of tumors [102]; even more the antibody against endoglin preferentially binds the activated endothelial cells of peri- and intra-tumor vessels that are actually involved in tumor neoangiogenesis, while a negative/weak immunoreaction for endoglin is evidenced in the vascular endothelium of normal tissues [97, 102]. Besides, in comparative studies, antibodies against endoglin have been demonstrated to be more specific in the detection of newly formed vessels in meningiomas [97], as well as in astrocytic [100] or oligodendroglial [101] neoplasias in comparison to those binding pan-endothelial markers. In addition, when MVD was assessed by using endoglin as a marker of angiogenesis, it appeared to be significantly correlated to the growth fraction and histological grade of meningiomas, and it was shown to have a prognostic impact on the overall survival and recurrence risk of these neoplasias [97]. Thus, we suggest the use of anti-endoglin antibodies instead of that of antibodies against pan-endothelial markers for the quantification of angiogenesis in brain tumors. Besides, the demonstration of endoglin expression in tumour vessels may also open therapeutic prespectives. Indeed, it has been recently shown that monoclonal antibodies against endoglin are able to induce tumour growth regression through the inhibition of the endothelial cells proliferation and angiogenesis itself [103].

The first phase in forming new blood vessels from existing vessels is the dissolution of aspects of native vessels. Glioma cells first accumulate around the existing cerebral blood vessels and lift off the astrocytic foot processes, which leads to the disruption of the normal contact between endothelial cells and the basement membrane [104]. The affected endothe-

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lial cells express angiopoietins resulting in destabilization of the vessel wall and decreased pericyte coverage. The angiopoietins are endothelial growth factors and their signal transduction pathway passes via the Tie2 receptor tyrosine kinase expressed on endothelial cells. In particular, Ang-1 and -2, have been implicated in glioma angiogenesis [105]. Ang-1 mediated activation of Tie2 is required for stabilization, remodeling and maturation of blood vessels, promotes angiogenesis and tumor growth and is associated with an increased number of highly branched vessels. Ang-1 induces phosphorylation of Tie2 and the p85 subunit of PI3K and increases PI3K activity in a dose-dependent manner, leading to endothelial cell survival via Akt signaling. In addition, Ang-1 stimulates endothelial cell migration via a PI3K-dependent activation of focal adhesion kinase (FAK), which has a key role in regulating dynamic changes in actin cytoskeletal organisation during cell migration. The biological effect of Ang-2 may depend on VEGF level. In the presence of endogenous VEGF, Ang-2 promotes vessel dilatation, remodelling of the basal lamina, proliferation and migration of endothelial cells, and stimulates sprouting of new blood vessels. In the absence of VEGF activity, Ang-2 becomes anti-angiogenic by promoting endothelial cell death and the regression of vessels [105]. Binding of Ang-2 to the Tie2 receptor on endothelial cells antagonizes this receptor's phosphorylation, thereby disrupting contacts between endothelial and periendothelial support cells and disengaging pericytes from the tumor vessels during initiation of vessel sprouting or regression. Increased expression of Ang-2 on GBM microvasculature appears early during glioma angiogenesis. Ang-2 and Tie2 expression are absent in the normal brain vasculature but are induced in tumor endothelium of coopted tumor vessels prior to their regression. Treatment of glioma cell derived mouse xenografts with a dominant negative form of Tie2 results in a significant decrease in tumor growth [106]. Maintenance of an optimal number of pericytes is necessary for successful angiogenesis in stabilizing newly formed vessels from further sprouting and to support adequate blood flow. Ang-2 may act as an antagonist to Tie2 phosphorylation, leading to destabilization of blood vessels. Therefore, Ang-2 represents a checkpoint for Ang-1/Tie2-mediated angiogenesis [107]. In brain tumor growth it's possible observe two vascular phases. In the first, the vessels are native cerebral vessels, which are co-opted by tumor cells, while in the second phase, there is true neovascularization arising from existing vessels. During the transition period between these two phases, hypoxia driven HIF-1 expression occurs which results in VEGF secretion and in the induction of neovascularization. In stage IV, angiogenesis adjacent to the necrotic area is triggered in response to increased expression of HIF-1α and VEGF. For the newly sprouting vessel is essential the deposition of proangiogenic matrix. This involves breakdown of the vascular basement membrane and extracellular matrix through the action of cathepsin B, matrix metalloproteases and other enzymes as well as the expression of matrix proteins such as fibronectin, laminin, tenascin-C and vitronectin [108]. Degradation of the vessel basement membrane and surrounding ECM, which also facilitates the invasion of endothelial cells, is an integral part of the ongoing angiogenic process. The matrix metalloproteinase family enzymes that degrade components of ECM consist of four groups according to their substrates: collagenases, gelatinases, stromelysins, and membrane-associated MMPs. Gelatinases-A (MMP-2) and gelatinases-B (MMP-9) are highly expressed in astrocytomas, and their expression levels, especially those of MMP-9, correlate with the histological grade

of tumor. MMP-2 and MMP-9 expression is strongly induced by hypoxia, and these two molecules appear to have a synergistic effect on basement membrane degradation [109]. The inhibitors of MMPs are called tissue inhibitors of metalloproteinases (TIMPs), which are comprised of TIMP-1, TIMP-2, TIMP-3, and TIMP-4. The interactions between these proteases and their inhibitors play important roles in cell morphogenesis, angiogenesis, tissue remodeling, tissue repair, tumor metastasis, cirrhosis, and arthritis. After breakdown of the basement membrane, endothelial cells proliferate and migrate toward the tumor cells expressing pro-angiogenic compounds. The activation of endothelial cells results in increased expression of cell adhesion receptors, such as integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$, and in increased cell survival, proliferation, and migration responses. In addition to migration of endothelial cells, migration of pericytes is an important part of tumor vessel formation. Platelet-derived growth factor secretion by activated endothelial cells recruits pericytes to the site of newly sprouting vessels and aids in establishing a new basement membrane [110].

The discovery of hypoxia inducible factor-1 (HIF-1) and the observation that hypoxia-induced HIF-1α expression in pseudopalisading cells, in tumoral necrotic areas, was concomitant with the expression of one of its target genes, VEGF, established a biological link between hypoxia and angiogenesis [111]. The most potent activator of angiogenic mechanisms in brain tumors is tissue hypoxia. One well-studied pathway is the HIF-1/VEGF-A pathway, which leads to endothelial cell proliferation and migration. HIF is a heterodimeric DNA-binding complex composed of two basic helix-loop-helix proteins of the PAS family (PER, AHR, ARNT and SIM family): the constitutive HIF-1b and one of either hypoxia-inducible a-subunits, HIF-1a or HIF-2a. In hypoxia, the a/b heterodimer binds to a core pentanucleotide sequence (RCGTG) in the hypoxia response elements (HREs) of target genes. The hypoxic microenvironment (1–2% O_2) caused by the increased oxygen consumption of hyperplasia and/or hypertrophy and the decreased oxygen delivery due to the increase in diffusion distance was assumed to contribute to the angiogenic switch. An important link between hypoxia and angiogenesis was the discovery that the expression of the potent vascular endothelial growth factor was induced by hypoxia [112]. VEGF, which regulates tumor edema and blood vessel formation, is an example of a gene regulated by an HIF-1 though an HRE. Angiogenesis is essential for development, wound healing, tissue or organ regeneration, but it is also part of pathological processes, such as cancer and certain retinopathies. It is an intricate multistep and temporally ordered process that involves a great number of genes, modifiers and pathways. Many of these genes are directly induced by HIF-1a, such as nitric oxide synthases, angiogenic and vascular growth factors and genes regulating matrix metabolism (urokinasetype plasminogen activator receptor; uPAR) [113]. Immunohistochemical expression of HIF-1α clearly correlated with the degree of glioma malignancies and predicted survival among patients with malignant gliomas and the degree of necrosis on MRI. In a recent study, has been demonstrated that SN38, the active metabolite of CPT11, exhibited an antiangiogenic effect. SN38 inhibited HIF-1α and VEGF mRNA and protein expression of glioma cells in a dose- and time-dependent manner [114]. Metronomic CPT11 treatment of gliomas exhibited growth inhibitory effects without systemic toxicity, that is, through comparison of body weight loss that was not observed by conventional CPT11 treatment. Tumor tissues treated with metronomic CPT11 exhibited decreased expression of

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HIF-1α protein and pimonidazole expression, which were indicative of areas of hypoxia by immunohistochemistry.

4.1. Mediators of glioma angiogenesis

Glioma angiogenesis is mediated by the release of angiogenic cytokines by the tumor cells. Numerous, different cytokines have been identified so far which are able to induce angiogenesis (Figure 1). This cytokine production is either the result of overexpressing angiogenic factors through genetic alterations or is triggered by hypoxia.

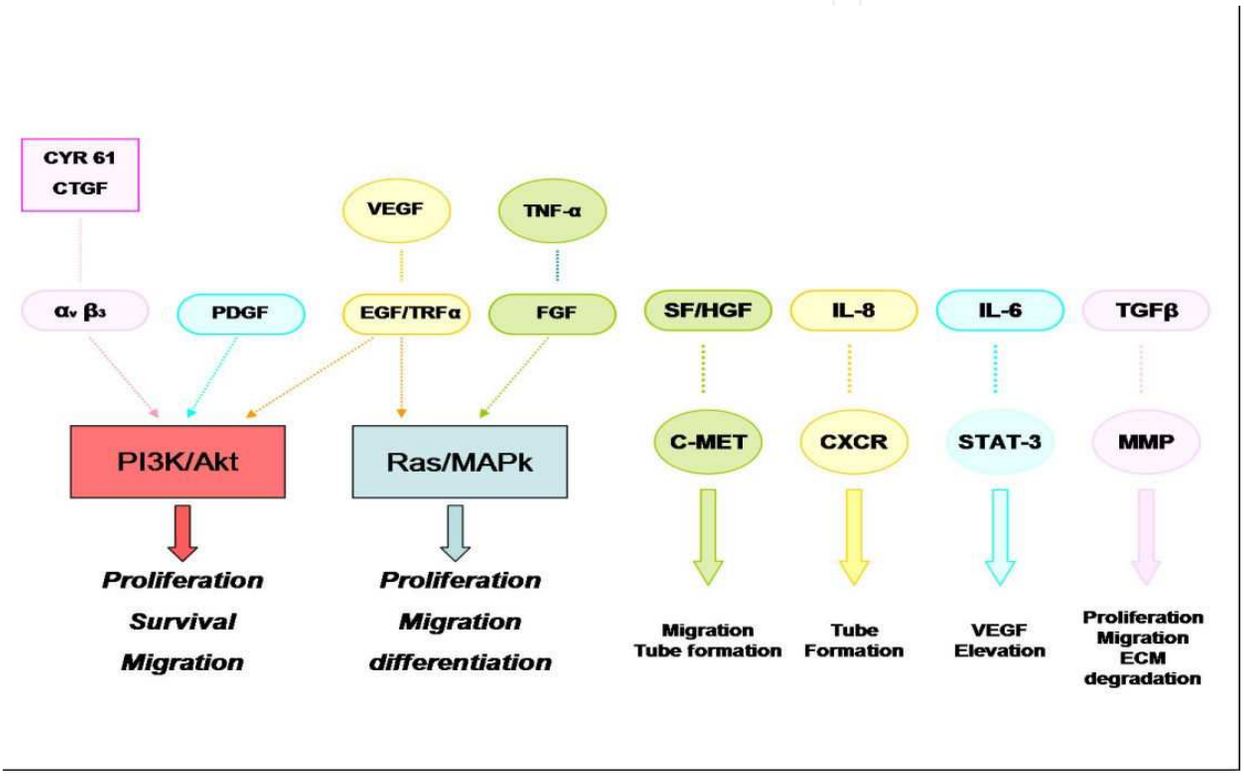

Figure 1. Representation of angiogenesis mediators in gliomas.

In the phosphatidylinositol-30 kinase (PI3K)/Akt and Ras/mitogen-activated protein kinase (MAPK) converge many angiogenic growth factors, including vascular endothelial growth factor (VEGF). These pathways modulate important cellular processes in angiogenesis, including endothelial cell proliferation, survival, migration, invasion, tube formation and extracellular matrix degradation.

CXCR = C-X-C chemokine receptor, CYR6.1 = cysteine-rich angiogenic inducer 61, CTGF = connective tissue growth factor, EGF = epidermal growth factor, FGF = fibroblast growth factor, HGF = hepatocyte growth factor, IL-6 = interleukin-6, IL-8 = interleukin-8, MMP = matrix metalloproteinases, PDGF = platelet-derived growth factor, PI3K = PI3 kinase, SF = scatter factor, TGF-a = transforming growth factor-alpha, TGF-ß = transforming growth factor-beta.

4.1.1. VEGF, VEGF-receptors and VEGF pathway

The VEGF family of growth factors and their receptors are the most important mediators of glioma angiogenesis. The VEGF family includes six glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor. The VEGF acts as a major vascular permeability factor and as a mitogen/survival promoter for endothelial cells [115, 116]. VEGF-A and its receptors are the best characterized signaling pathway in angiogenesis and binds to two receptor tyrosine kinases (RTK) – VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1) [117]. It is generally agreed that VEGFR-2 is the major receptor mediating the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. Recent evidence has suggested that VEGFR-1 participates in haematopoiesis and in the recruitment of monocytes and other bone marrow derived cells to promote tumor angiogenesis [118]. In addition, VEGFR-1 is involved in the activation of MMPs associated with matrix degradation and in the production of growth factors from endothelial cells. VEGF-A gene expression is up-regulated by hypoxia, mediated by the transcription factor HIF and the product of the von Hippel–Lindau (VHL) tumor suppressor gene. Other transcription factors capable of up-regulating VEGF transcription include the ETS-1 proto-oncogene and STAT-3. ETS proteins activate many genes involved in angiogenesis, including those that regulate VEGFR-1 and VEGFR-2, integrin b3, some MMPs and urokinase-type plasminogen activator (uPA). ETS-1 is expressed more frequently in GBM being most prominently observed in the glomeruloid tufts of GBM [119]. VEGF promotes endothelial proliferation via the activation of the MAPK pathway. VEGF also enhances vascular permeability through the MAPK signaling cascade by rearranging cadherin/catenin complexes and loosening adhering junctions between endothelial cells [120]. The VEGF-A is secreted by tumor cells as well as by stromal and inflammatory cells. VEGF-A can be linked in the extracellular matrix through the interaction with proteoglycans or glycosaminoglycans. The expression of the receptors VEGFR1 and VEGFR2 is regulated on the endothelial cells in gliomas. VEGF-A activation causes endothelial cell differentiation into a “tip” cell and a VEGF-A gradient induces “stalk” cell proliferation along an opening in the BM in forming of a new vessel sprout [121]. The ligands for VEGF3 (VEGF-C&D) are expressed by multiple cell types that surround the angiogenic vessels, suggesting the existence of a novel pro-angiogenic paracrine signaling pathways in these neoplasms. In addition to transcription factors, VEGF expression is also probably correlates with many other growth factors and their specific receptors, including transforming growth factor (TGF)-b, platelet-derived growth factor (PDGF)-B, epidermal growth factor (EGF), basic fibroblast growth factors (FGF). VEGF promotes endothelial proliferation via activation of the MAPK pathway. The activation of MAPK/ERK is associated with inhibition of the Jun-N terminal kinase (JNK) pathway in mediating the anti-apoptotic effect of VEGF. The PI3K/Akt pathway is of central importance in VEGF signaling. Activated VEGFR-2 mediates the phosphorylation of Akt, which potently inhibits endothelial cell apoptosis by interfering with various apoptosis signaling pathways. Akt also promotes endothelial cell migration, and increases the expression of HIF, leading to enhanced VEGF expression [122]. VEGF stimulates endothelial production of urokinase-type plasminogen activator (uPA), which induces conversion of plasminogen to plasmin, causing the breakdown of ECM components and leading to ECM remodeling [123]. The end result of VEGF signaling in tumors

is the production of immature, highly permeable blood vessels with subsequent poor maintenance of BBB and parenchymal edema [96, 124]. Alternatively, the angiogenic effect of VEGF can be mediated through integrins, a1b1, a2b1 and avb3, which promote cell migration, proliferation and matrix remodeling.

4.1.2. FGF and FGF receptors

The FGF family of proteins and their receptors are overexpressed in various types of cancer. Binding of FGF to its receptor causes transphosphorylation and activation of intrinsic tyrosine kinase, which results in signal transduction. Both acidic FGF (aFGF) and basic FGF (bFGF) are up-regulated in GBM [125] and are responsible for resistance of endothelial cells to apoptosis. Basic fibroblast growth factor (bFGF) is expressed by vascular cells and, focally, by the tumor cells. The receptors for bFGF include FGFR1, expressed by the tumor cells and the tumor endothelial cells and the FGFR2 expressed only by the tumor cells. The antiapoptotic effect of bFGF is mediated by increased expression of Bcl-XL and Bcl-2 via the MEK-dependent signaling pathway. In addition, bFGF stimulates the expression of VEGFR in endothelial cells.145 Similar to VEGF, aFGF and bFGF induce endothelial cell proliferation and migration. Furthermore, FGF activation leads to remodeling of ECM and degradation of the basement membrane by inducing production of plasminogen activator, collagenase and MMP in endothelial cells [127].

4.1.3. PDGF Family

PDGF-B and platelet-derived growth factor b receptor (PDGFRb) have important roles in the development and differentiation of the vessel wall [128]. PDGF is a mitogen for multiple cells of mesenchymal and neuroectodermal origin that acts through the PDGF receptors α and β. PDGF-B is required for recruitment of pericytes and maturation of the microvasculature. PDGFR-β is expressed on endothelial cells of astroglial tumors and that PDGF expression correlates with astroglial malignancy and angiogenic activity

PDGF contributes indirectly to tumor angiogenesis acting as a potent mitogenic and chemotactic stimulus for angiogenesis associated stromal cells, such as smooth muscle cells or pericytes. However, PDGF's effects on angiogenesis are mediated partly by VEGF. The angiogenic effects of PDGF are mediated through PI3K/Akt, MAPK/ERK and STAT3 signalling [129].

4.1.4. Molecules of the inflammatory cascade

Molecules of the inflammatory cascade act indirectly on angiogenesis via modulating expression of direct angiogenic factors. Interleukin-8 (IL-8) is a potent chemoattractant. But recent data suggest that it has a critical role in glial tumor angiogenesis and progression. High expression levels of PGES-1 (Prostaglandine E 1 Sinthase) and IL-8 in malignant gliomas cells and microglial cells, strongly correlated with grading tumor, are been demonstrated [130]. IL-8 expression is first observed in low grade astrocytoma in perivascular tumor areas expressing inflammatory cytokines. In malignant gliomas, IL-8 further localizes in oxygen-

deprived cells surrounding necrosis. IL-8, however, stimulates angiogenesis via the interaction with the CXC chemokine receptor 1 (CXCR1), CXCR2 and Duffy antigen receptor for cytokines (DARC). DARC expression has been detected on tumour-associated endothelial cells, whereas CXCR1 and CXCR2 are found on infiltrating leukocytes near blood vessels. Macrophages are known to produce high levels of IL-8, which has a tumorigenic activity, by inducing tumor growth and angiogenesis; IL-8 is an inflammatory chemoattractant responding to the tumor microenvironment. Tumor pseudopalisading cells secrete HIF which induces IL-8 secretion. On the basis of preliminary results, the role of IL-8 as crucial angiogenesis mediator within HIF-1α pathway and crosstalk between hypoxia-induced high levels of HIF-1α and VEGF expression has been demonstrated.

The cyclooxygenase isoforms (COX-1 and COX-2) catalyze the synthesis of prostaglandins from arachidonic acid. While COX-1 is ubiquitously expressed in a wide range of tissues, COX-2 is cytokine inducible. COX-2 is expressed in human glioma cells where its expression correlates with malignancy, being highest in glioblastoma. The anti-angiogenic efficacy of COX-2 inhibitors is currently explained first by downregulation of VEGF, which results in blocking of endothelial cell proliferation and induction of endothelial cell apoptosis and second by inhibition of integrin function and signaling.

Tumor necrosis factor-α (TNF-α) is a potent proinflammatory cytokine with a quite complex role in endothelial cell survival and migration. It has impact on endothelial cell survival and migration via its TNF-receptor 1 and 2 which are both expressed on endothelial cells.

Membrane type 1-matrix metalloproteinase (MT1-MMP) expressed by the tip cell of an endothelial sprout opens up the surrounding matrix and is only later down-regulated when stalk cells come into contact with pericytes. Re-established contact between endothelial cells and pericytes induces expression of tissue inhibitor of metalloproteinases-2 (TIMP-2) in endothelial cells and TIMP-3 in pericytes in switching off the proteolytic phenotype in endothelial cells [131]. MT1-MMPs on the endothelial cell surface are also required for the subsequent step in the angiogenesis cascade of tube formation by playing a role in endothelial intracellular vacuole and lumen formation. The vessel lumen is tightly sealed by adjoining endothelial cells held together by tight and adherens junctions. A basement membrane is then produced by endothelial cells in cooperation with surrounding cells in providing structural support and maintaining endothelial cell quiescence. The BM is built up of scaffolding laminins and essential components such as collagen IV, perlecan, nidogens, and collagen XVIII [132-133]. During the maturation of new capillaries, pruning of excess or unneeded vessels for optimal perfusion is proposed to represent an adjustment to oxygen surplus while the surrounding ECM exerts mechanical strain providing traction and orientation for angiogenic microvessels [134].

Many other proangiogenic factors are upregulated in gliomas and this aspect might explain the failure of many actual antiangiogenic therapeutic strategies in gliomas management. TGF-b and its receptors are highly expressed in malignant gliomas, especially in areas of vascular hyperplasia and around necrotic regions. In glioma cells, the angiogenic effect of TGF-b is probably mediated through the enhanced expression of VEGF. TGF-b also promotes angiogenesis via the integrin signaling pathway. TGF-b upregulates expression of

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$\alpha v\beta 3$ integrin that, in turn, binds to MMP-2, which leads to degradation of the ECM and enhanced endothelial cell invasion [135]. An important role has absolved by IL-1beta, IL-6, TNF-alpha and stromal-cell-derived factor (SDF)-1 alpha [46].

5. Microglia

Glioma tissues as obtained by surgical resection contain also a considerable amount of non-transformed cells. The majority of these cells are tumor-associated macrophages (TAMs) [136-137]. Resident macrophages of the brain are termed microglia. These cells invade the brain early in development and differentiate into so-called resident, ramified microglia. These tumor-associated microglia displayed an ameboid morphology similar to that described in other pathologies. Since microglia share many properties with macrophages found in non-neuronal tissues including the blood, it was possible that glioma-associated microglia could be recruited de novo from the microglial population resident in the brain, or alternatively, migrate into brain tumors from the periphery. To address this question, Badie and Schartner [138] used CD45 labeling of cells in rodent tumor models to distinguish brain microglia (CD45 low) from peripheral macrophages (CD45 high). They used CD11b/c as a general marker for macrophages and sorted the CD11b/c2 positive cells into two populations showing high and low CD45 expression. From this, they concluded that the macrophages are primarily found within tumors, while microglia was detected in all brain tissue. Thus, the precise origin of tumor microglia remains to be determined. Regardless of origin, we can define any glioma-associated monocytic cell with macrophage characteristics as a tumor-associated microglia. Candidate chemoattractants of tumor-associated microglia include monocyte chemotactic protein-3(MCP-3), colony-stimulating factor 1 (CSF-1), granulocyte-colony stimulatory factor (G-CSF), and hepathocyte growth factor/scatter factor: each of these has been shown to be released by gliomas and microglia are known to express receptors for these chemoattractant growth factors [139-140].

TAMs promote cancer progression through several mechanisms, including promotion of angiogenesis, induction of tumour growth, and enhancement of tumour cell migration and invasion. The hypoxic stress in the tumour mass leads to the expression of inflammatory molecules, which promote the recruitment of macrophages followed by conversion to the M2 phenotype. TAMs are capable to modulate and induce neovascularization and functions related to stroma formation. When TAMs are activated, in response to specific stimuli, these cells can express a repertoire of substances that promote angiogenesis. Growth factors such as acidic fibroblasts growth factor (aFGF/FGF1), basic fibroblasts growth factor (bFGF/FGF2), vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (GM-CSF), transforming growth factor-α, insulin-like growth factor-1, platelet derived growth factor (PDGF), tumour growth factor-β (TGF-β) and other monokines (e.g. tumour necrosis factor-α (TNF-α), interleukin-1, interleukin-6, interleukin-8, substance P, prostaglandins, interferons and thrombospondin which are released by tumour cells leads to the activation of macrophages and have the capability to influence the angiogenic process [141]. Therefore, macrophages recruited in situ represent an indirect pathway of amplification of

angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. In experimental in vitro studies, glioma cell lines seem produce high levels of a monocyte-macrophages-derived cytokine, IL-8, that induces formation of tube-like structures by human microvascular endothelial cells [142]. We previously demonstrated significant increase in IL-8 protein level in astrocytic cultures treated with PGE2. The ability of PGE2 to increase IL-8 expression in glioma cells has a significant biological impact on tumorigenesis, as shown by increased growth and reduced apoptosis in PGE2-treated cells [143]. Macrophages can exert a dual influence on blood vessel formation. On the one hand macrophages produce proangiogenic molecules on the other hand they can express anti-angiogenic molecules and damage the integrity of blood vessels. In general the pro-angiogenic functions of TAMs prevail [144].

Angiogenesis is also facilitated by TAM-derived proteases released in tumours, as extracellular proteolysis is an absolute requirement for new blood vessel formation. Macrophages can express proteases to release a number of pro-angiogenic molecules bound to heparan sulfate in proteoglycans, and fragment of fibrin and collagen, which facilitate angiogenesis. Among these, matrix metalloproteases (MMPs 1, 2, 3, 9 and 12), plasmin, urokinase plasminogen activator and receptor are the prominent ones which promotes tumour directed angiogenesis. MMPs are a family of matrix degrading enzymes including collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), matrilysin (MMP-7), gelatinase B (MMP-9), and other MMPs. TAMs have been reported to correlate with the metastatic potential of a variety of human cancers, and they have also been shown to be a major source of MMP-9. In addition, urokinase-type plasminogen activator is a serine protease synthesized by TAMs in various human tumour types [145]. TAMs were shown to express CXCL8, which like VEGF, binds heparin in the ECM and stimulate angiogenesis [146]. Thus TAMs have the capacity to affect each phase of the angiogenic process, including degradation of the extracellular matrix, endothelial cell proliferation and endothelial cell migration. TAMs can also secrete cysteine-type lysosomal proteases and a wide variety of growth factors that can stimulate cancer growth.

New blood vessels in tumours are usually disorganized and prone to collapse, resulting in areas of inadequate perfusion and hypoxia (low oxygen tension). Additionally, rapid tumour cell proliferation in some areas may outpace the rate of new blood vessel growth, causing hypoxic areas to form [147]. The level of TAMs in tumours appears to be affected by hypoxia, a trait commonly found in these tissues. TAM numbers are generally higher in tumours containing high overall levels of hypoxia, as seen in primary human breast carcinomas and various animal tumours. Hypoxic tumours secrete higher amounts of chemoattractants and/or other factors that enhance monocyte attachment to and migration through the tumour vasculature. Once targeted to hypoxic sites, TAM functions are greatly affected by hypoxia-related factors. Because macrophages are phagocytes, they may also be attracted to hypoxic, perinecrotic areas along a trail of necrotic debris emanating from dead cells. Hypoxia also entraps TAMs by decreasing their mobility in a number of ways. One such approach involves the hypoxic up-regulation of the enzyme mitogen-activated protein kinase phosphatase (MKP-1) by macrophages. This is important because various chemoat-

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tractant receptors, including those for CCL2, VEGF, and endothelin 2, stimulate cell migration by phosphorylating the signaling enzymes MEK, ERK1/2, and p38 MAPK. Up-regulated MKP-1 rapidly dephosphorylates these molecules in TAMs, thus terminating the chemotactic response of TAMs to these chemokines [148]. Hypoxia also inhibits macrophage expression of the chemokine receptors CCR2 and CCR5 [149], further helping to immobilize TAMs. Hypoxia also induces a profound change in the phenotype of macrophages, promoting increased expression of a wide range of genes.

This is brought about by the hypoxic up-regulation of such transcription factors as hypoxia-inducible factors, HIF-1α and HIF-2α. TAMs respond to hypoxia by up-regulating a broad array of genes encoding proteins that promote the proliferation, invasion, and metastasis of tumour cells as well as tumour angiogenesis. Genes coding for M-CSF, a growth factor that promotes the survival and differentiation of macrophages, is over expressed in some human tumours, and elevated M-CSF levels correlate with high TAM numbers and poor prognosis [150]. Hypoxic macrophages are also likely to promote the invasive and/or metastatic behavior of tumour cells by releasing such pro-invasive factors as macrophage inhibitory factor [151]. Macrophage inhibitory factor is known to modulate the activities of a number of cell types in tumours, including stimulation of tumour cell motility [152]. This may involve indirect effects such as macrophage inhibitory factor-stimulated release of matrix metalloproteinase 9, which in turn degrades components of the basement membrane and extracellular matrix, thereby increasing the motility of tumour cells.

In a cultured mouse brain slice model, the invasion and growth of glioma cells was compared between normal and microglia-depleted slices. Glioma cell invasion was significantly reduced in microglia depleted slices relative to control slices [153]. The impact of microglia on glioma migration might relate to the production of membrane type 1 metalloprotease (MT1-MMP or MMP-14) that are produced by microglia in response to soluble factors released from glioma cells. Glioma cells also release metalloproteases 2 that is fully activated by MT1-MMP released from microglia. The consequent degradation of the extracellular matrix has been postulated to enhance the invasion of glioma cells into the brain parenchyma. The importance of microglia for glioma growth was further substantiated by studying animals in which microglia was depleted. The microglia-depletion in vivo was achieved by using the CD11b-HSVTK mouse model. Seven days after intracerebral glioma inoculation, ganciclovir (a specific substrate for the viral thymidine kinase HSVTK) was infused via mini-pumps into the tumor area for a further 7 days. To restrict the effect of ganciclovir on the intrinsic microglial population, the mice were irradiated before bone marrow transplanted with wild-type monocytes. The ganciclovir treatment led to a considerable depletion of microglia and to an 80% reduction in glioma volume [153].

CD68 is a monoclonal mouse antibody that labels human monocytes and macrophages. The antigen recognized by CD68 is absent from resting microglia but readily detectable in phagocytic microglia, perivascular cells and brain macrophages. In a recent study, the presence of CD68 positive cells neighboring of neoplastic cells, contiguous to necrotic and hypoxic areas within neoplastic tissue has been evidenced. The presence of TAMs that passed through vessel wall also in low-grade astrocytomas was, also, observed [154]. In anaplastic cases, neo-

plastic cells seem to be guided towards microglia. These data, can reinforced the hypothesis that macrophage infiltration could be closely associated with neovascularization and malignancy in human gliomas. Matrix metalloprotease activity is also regulated by the CX3CL1/CX3CR1 signaling pathway. For example, matrix metalloproteases 2, 9, and 14 are upregulated in microglia following activation of CX3CL1/CX3CR1signaling. Notably, CX3CR1 is upregulated in glioma associated microglia [155] and polymorphisms in the chemokine receptor CX3CR1 have been associated with prognosis among patients with glioma. The common CX3CR1 allele (termed V249I) was a favorable prognostic factor. Patients who had only this CX3CR1 allele had a more than 1.5 longer mean survival time. This common allele was associated with reduced microglial cell infiltration in primary tumor biopsies [155].

In a previous study has been demonstrated, in a series of GBM, the presence of laminin, fibronectin and type IV collagen in hyper-plastic vessels, in and around vascular channel, in vascular walls and at level of BM associated with endothelial glomerulus-like proliferations [83]. Laminin within the BM can bind to both endothelial cells and tumor cells and is involved in angiogenesis and tumor growth. We can hypothesized that in pilocytic astrocytoma, the ECM integrity cause a reduced macrophagic/microglial migration. In this view, an incompetent control of interactions occurs between microglial adhesive molecules and ECM substrates. In low-grade astrocytomas is achievable that low macrophage/microglial recruitment is also correlated with a lower vascular neo-angiogenesis. This relationship may influence microglial morphology, blocking microglial migration and phagocytosis [154]. To support these findings, a recent study showed an increase of CD68 positive cells which correlated with trends toward worse event-free survival [156].

6. Therapeutic strategies for malignant gliomas

The treatment of brain cancer is one of the most difficult challenge in neurosurgery and oncology. Malignant gliomas involve, in their progression, multiple aberrant signaling pathways and the blood-brain barrier (BBB) restricts the delivery of many chemotherapeutic agents. Current conventional treatments protocols include maximally safe surgical resection followed by fractionated radiation therapy of the tumor and surrounding brain parenchyma and systemic chemotherapy. However, radiation therapy is limited to a largely palliative role, and chemotherapy has provided only a modest benefit in clinical outcome. There are several factors underlying the disappointing results in brain cancer therapeutics including limited tumor cell drug uptake, intracellular drug metabolism, inherent tumor sensitivity to chemotherapy, and cellular mechanisms of resistance. The BBB protects the brain from toxins and fluctuations in systemic chemical concentrations, but it also excludes many therapeutic agents. Multimodal therapeutic approaches and molecular-based targeted therapies are modern and complex strategies potentially very efficacious and applicable to up-regulate the selectivity of therapeutic effects and down-regulate systemic toxicity and side effect toward peritumoral and safe brain tissue. Developments in molecular biology have led to a clearer understanding of the mechanisms of tumor development and resistance to therapy. As a results, new treatment strategies are emerging that target steps in the molecular patho-

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genesis of brain gliomas. An optimal realization of a system that overcomes the problems associated with developing effective brain tumor treatments requires the identification of neoplastic markers, and understanding their evolution over time, and the development of technology for the biomarker-targeted delivery of multiple therapeutic agents, and for the simultaneous capability of avoiding biological and biophysical barriers. In this complex phenomenon it is fundamental to improve specific selective drugs delivery systems: in this way, drugs, antisense oligonucleotides (AONs), small interference RNAs, engineered monoclonal antibodies and other therapeutic molecules may diffuse into CNS overcoming the BBB.

Glioma gene expression and its development during gliomagenesis may help to better understand the role of important molecules involved in tumor-safe brain parenchyma relationships. These molecules, such as ECM proteases, cell adhesion molecules, and their related signaling pathways, show an important role in glioma cell migration and invasion and could selectively attack to inhibit the glioma invasive rim. Various small molecule inhibitors and antibodies has been developed, and is continuing to be developed, to target key components of the signal transduction machinery, with a particular focus on growth factors and their corresponding receptors and canonical downstream signal transduction intermediates. Many of these inhibitors have potential for the treatment of glioblastoma. Testing some of these inhibitors, as well as using genetic approaches to untangling these signaling networks in well-designed model systems (including both highly representative xenograft models and mouse genetic models), is beginning to provide an emerging picture of the targetable molecular phenotype of glioblastoma.

Angiogenesis is tightly correlated with the histological grading and prognosis of gliomas. Glioma vasculature is structurally and functionally abnormal, leading to vasogenic edema, increased interstitial pressure, and heterogeneous delivery of oxygen and drugs. VEGF is the key factor implicated in the angiogenesis of gliomas. It acts as a major vascular permeability factor and as a mitogen/survival promoter for endothelial cells. VEGF expression is stimulated by hypoxia, acidosis, and many growth factors (EGFR, PDGFR, HGFR, CKit, insulin-like growth factor receptor), and their downstream signaling pathways (PI3K–Akt, Ras–MAPK) are commonly activated in gliomas. However, many other proangiogenic factors are upregulated in gliomas, and this might explain how gliomas may escape a specific antiangiogenic therapy. Antiangiogenic therapy is able to normalize the structure and function of abnormal neovasculature [96]. The normalization hypothesis states that antiangiogenic therapies may augment the effects of chemotherapy and radiotherapy by normalizing tumor vessels. Furthermore, normalizing tumor vessels might also reduce hypoxia, and thus make the tumor cells more sensitive to chemotherapy and radiation therapy [96].

6.1. Molecular targeted therapy

Elevated expression or mutation of receptors and intracellular downstream effectors has been demonstrated in gliomas. These pathways are controlled by several growth factors linked to tyrosine kinase. Specific targeting of these signaling pathways that lead to altered cellular proliferation and cell migration and invasion could provide new molecularly targeted options for glioma treatment. The introduction of molecularly targeted agents is one of

the most significant advances in cancer therapy in recent years. Targeted therapies block activation of oncogenic pathways, either at the ligand–receptor interaction level or by inhibiting downstream signal transduction pathways, thereby inhibiting growth and progression of cancer. Because of their specificity, targeted therapies should theoretically have better efficacy and safety profiles than systemic cytotoxic chemotherapy or radiotherapy. The main rationale for using antiangiogenic therapies in glioblastoma is to normalize the vasculature, restoring the selective permeability of the blood-brain barrier.

6.1.1. VEGF, VEGF-receptors and VEGF pathway

VEGF-A is a member of the VEGF family that acts as a key proangiogenic factor because of its specificity to endothelial cells and the multitude of responses that it can elicit. These include ECM degradation, endothelial cell proliferation, migration and tube formation, and expression of other proangiogenic factors, such as urokinase-type plasminogen activator, plasminogen activator inhibitor-1, urokinase-type plasminogen activator receptor, and matrix metalloproteinase-1. Overexpression of VEGF-A occurs in response to hypoxia, PDGF, EGF, transforming growth factor-h, interleukin-1h, and tumor necrosis factor-a. The best-studied receptor is VEGFR-2, a potent tyrosine kinase that mediates endothelial cell signaling through the activation of Ras/Raf/MEK/MAPK, PI3K/AKT/PKB, and protein kinase C pathways. Has been suggested that blocking VEGF pathways may normalize tumor vasculature and improve chemotherapy delivery, allowing higher drug concentrations [157].

Several strategies for targeting VEGF have been proposed, such as VEGFR TKI, anti-VEGF-A and VEGFR-2 monoclonal antibodies, antisense oligonucleotides, and ribozymes. The best-studied drugs in gliomas have been vatalanib (PTK787/ZK222584), ZD6474, sorafenib (BAY 43-9006), sunitinib (SU11248), and cediranib (AZD2171). Difficulties include defining the optimal biological dose of drugs that are rarely toxic, determining the relevance of combinations with cytotoxic chemotherapy and radiotherapy, and elucidating adequate surrogate markers.

Vatalanib is a VEGFR-1 and VEGFR-2 TKI that showed activity in glioma cell lines and xenograft models [158]. A phase I study of vatalanib, and c-kit in patients with newly diagnosed GBM receiving radiation, temozolomide, and an enzyme-inducing anti-epileptic drug in order to determine the MTD of vatalanib in this patient population was evaluated. Vatalanib was well tolerated with only 2 DLTs (thrombocytopenia and elevated transaminases). Other grade 3/4 toxicities included leukopenia, lymphopenia, neutropenia, and hand-foot syndrome. Of the 13 patients evaluable for a radiographic response, 2 had a partial response and 9 had stable disease. Vatalanib significantly increased PlGF and sVEGFR1 in plasma circulation and decreased sVEGFR2 and sTie2. Vatalanib was well tolerated and this study demonstrates the safety of oral small molecule inhibitors in newly diagnosed GBM patients. Blood biomarkers may be useful as pharmacodynamic markers of response to anti-angiogenic therapies [159].

ZD6474 is a VEGFR-2 TKI with additional VEGR-3 and EGFR inhibition properties. In vivo models showed a broader spectrum of action, suggesting an antiangiogenic and anti-VEGFR preponderant effect [160]. In a glioma xenograft study, ZD6474 decreased tumor volume,

lowered the tumor cell proliferation index (Ki-67), and increased tumor cell apoptosis. However, microvascular density, a typical marker of angiogenic activity, surprisingly increased, raising doubts on the antiangiogenic effects of the drug [161].

The effect of ZD6474, was also evaluated in combination with either radiotherapy or temozolomide. ZD6474 in combination with radiotherapy significantly decreased tumour area by 66% compared with controls whereas the combination with temozolomide decreased tumour area by 74% [162].

Recently, the AEE788, a reversible TK inhibitor that inhibits EGFR and VEGFR, in recurrent glioblastoma patients, was evaluated [163]. Continuous, once-daily AEE788 was associated with unacceptable toxicity and minimal activity for the treatment of recurrent glioblastoma. The study was, therefore, discontinued prematurely.

Cediranib (AZD2171) is a potent, orally available, small-molecule inhibitor of VEGF-receptor (VEGFR) tyrosine kinase activity that rapidly normalizes tumor blood vessels in patients with glioblastoma, leading to a clinical improvement in cerebral edema [164]. In a recent study, drug effects were evaluated over time through the use of magnetic resonance imaging techniques (including analysis of perfusion, permeability, and relative vessel size) and assessment of circulating progenitor cells, circulating endothelial cells, and plasma levels of several proangiogenic proteins. Results suggest that cediranib leads to a normalization of vasculature and decreased edema; however, such effects disappear overtime, which is accompanied by an increase in circulating basic fibroblast growth factor, stromal cell-derived factor-1a, and circulating endothelial cells. In mouse models, improvement in edema was associated with increased survival, despite continued tumor growth [165]. The first clinical data of the REGAL trial of cediranib plus lomustine (CCNU) to investigate whether preclinical findings will translate into improvements for patients with recurrent glioma have been negative [166].

Single-agent sunitinib, an oral small molecule inhibitor of multiple tyrosine kinase receptors, was evaluated for treatment of patients with recurrent glioblastoma and anaplastic astrocytoma (AA). For AA patients, the most common side effects were fatigue, diarrhea, hand-foot syndrome, neutropenia, thrombocytopenia, and nausea. In the GBM cohort, the most common side effects were fatigue, diarrhea, neutropenia, and thrombocytopenia. Median overall survival was 12.1 months (AA) and 12.6 months (GBM). Nonetheless, sunitinib did not demonstrate significant anti-glioma activity in patients with recurrent malignant astrocytic gliomas [167]. Recent preclinical studies suggest that treating GBM with a combination of targeted chemotherapy and radiotherapy may enhance the anti-tumor effects of both therapies. In a recent study, have been evaluated the effects of combination therapy in a mouse gliomamodel that utilizes a PDGF-IRES-Cre-expressing retrovirus to infect adult glial progenitors in mice carrying conditional deletions of Pten and p53. The addition of sunitinib to low-dose radiation caused a modest, but significant delay in tumor growth. However, no significant survival benefit was seen as tumors progressed in 100% of animals. Histological analysis revealed a reduction in vascular proliferation and a marked increase in brain invasion. The results showed that the addition of Sunitinib to radiotherapy fails to significantly alter survival in GBM despite enhancement of the effects of radiation [168].

In gliomas, despite evidence of activity in preclinical models, clinical development of bevacizumab was delayed initially because of the fear of central nervous system hemorrhage [169]. Bevacizumab is a monoclonal antibody against VEGF inhibiting angiogenesis by preventing receptor activation. Phase II clinical trials using bevacizumab in both newly diagnosed and recurrent high-grade glioma showed promising results. Bevacizumab has been shown to be safe and tolerable in malignant gliomas. In the recurrent disease setting, bevacizumab alone might be sufficient for a clinical benefit and is currently approved as a single agent for this indication. In a phase II study was evaluated the efficacy and safety of bevacizumab in Japanese patients with recurrent malignant glioma. The 6-month progression-free survival rate in the 29 patients with recurrent glioblastoma was 33.9% and the median progression-free survival was 3.3 months. The 1-year survival rate was 34.5% with a median overall survival of 10.5 months. There were eight responders (all partial responses) giving an objective response rate of 27.6%. The disease control rate was 79.3% [170].

Norden et al. [171] analyzed the pattern of recurrence in patients treated with bevacizumab and irinotecan, and their observations suggest that bevacizumab might more efficiently suppress enhancing tumor recurrence than infiltrative tumor growth. A single-institution phase II trial using the humanized monoclonal VEGF antibody bevacizumab combined with irinotecan in malignant gliomas has been reported [172]. No central nervous system hemorrhages were observed. The 6-month PFS rates were 30% and 56% in grade 4 and grade 3 gliomas, respectively, comparing favorably with historical controls. In a similar study, bevacizumab plus irinotecan in recurrent malignant gliomas improves responses, progression-free survival, and overall survival compared with historical data. Karnofsky performance status of at least 80% was a predictive factor for response and overall survival [173].

In a recent research was evaluated concurrent bevacizumab with hypofractionated stereotactic radiation therapy (HSRT) for the treatment of recurrent malignant gliomas. Despite the promising initial response seen with the addition of HSRT to bevacizumab as salvage treatment for recurrent gliomas, approximately half of patients ultimately still experience failure within the radiation field. The rate of local failure with the addition of HSRT seems to be lower than that seen with bevacizumab alone in the salvage setting [174].

Several studies of bevacizumab in combination with metronomic dosing of temozolomide or etoposide or with daily erlotinib in recurrent glioma patients are ongoing.

6.1.2. Epidermal growth factor receptor

Epidermal growth factor receptor (EGFR, ErbB1, HER1) is a tyrosine kinase receptor that is abnormally activated in 70% of solid cancers. EGFR overexpression and immunoreactivity are more common in primary tumors than in secondary glioblastomas. Activation of EGFR pathways in cancer cells has been linked to increased motility, adhesion, invasion, and proliferation of tumor cells as well as inhibition of apoptosis and induction of angiogenesis.

The EGFR transmembrane protein comprises three domains: the extracellular domain, the transmembrane domain, and the cytoplasmic domain, which harbors the tyrosine kinase activity. Ligand binding (amphiregulin, EGF, transforming growth factor TGF-β, decorin, be-

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tacellulin, epiregulin, neuroregulin) to the extracellular domain of a monomer results in its homo- or heterodimerization, inducing phosphorylation of the tyrosine kinase domain, activating several signaling pathways, in particular: phosphatidylinositol 3'-kinase/Akt/mammalian target of rapamycin (mTOR), Ras/mitogen-activated protein kinase (MAPK), phospholipase C (PLC)/protein kinase C (PKC), and (d) c-Src [175]. These activated pathways are involved in several cell biological processes, including cell proliferation, angiogenesis, migration/adhesion, survival, and differentiation. Approximately 50% of glioblastomas overexpress EGFR and 25% express a constitutively active mutated form of EGFR. This activation occurs through several molecular mechanisms: protein overexpression, reported in ~60% of cases; gene amplification, reported in ~40% of cases; truncated transcripts encoding for a constitutionally active receptor, reported in ~20% (mainly EGFRvIII) of cases; and mutation of the extracellular domain (15%) [175]. These alterations, which are quite frequently combined in the same tumor, activate the EGFR downstream signaling pathways, promoting the oncogenic process. Therefore, several strategies have been developed in order to block the EGFR signaling pathway, including small molecule tyrosine kinase inhibitors (TKIs), monoclonal antibodies, toxin-linked conjugates, and vaccine therapies. However, caution is needed with EGFR inhibitors, because hypoxia and low glucose levels might convert the cytotoxic effects of EGFR inhibition into a cytoprotective effect.

Two main EGFR small molecule TKIs have been evaluated in gliomas: gefitinib and erlotinib. These small molecules, which are orally delivered, block the ATP pocket of the EGFR intracellular tyrosine kinase domain and thus inhibit activation of downstream signaling pathways.

Growth factor pathway expression using epidermal growth factor receptor (EGFR), mutant EGFR (EGFRvIII), platelet derived growth factor receptor (PDGFR), C-Kit and C-Abl together with phosphatase and tensin homolog (PTEN) expression and downstream activation of AKT and phosphorylated ribosomal protein S6 (P70S6K) was analysed in 26 primary glioma cultures treated with the tyrosine kinase inhibitors (TKIs) erlotinib, gefitinib and imatinib. Response for each culture was compared with the EGFR/PDGFR immunocytochemical pathway profile using hierarchical cluster analysis (HCA) and principal component analysis (PCA). Erlotinib response was not strongly associated with high expression of the growth factor pathway components. Increased EGFR expression was associated with gefitinib response; increased PDGFR-α expression was associated with imatinib response. The results of this in vitro study suggest gefitinib and imatinib may have therapeutic potential in gliomas with a corresponding growth factor receptor expression profile [176].

Gefitinib is a selective epidermal growth factor receptor tyrosine kinase inhibitor that inhibits cell growth and induces apoptosis in human glioma cells. Gefitinib also induces death of H4 cells with characteristics of the intrinsic apoptotic pathway, including Bax mitochondrial translocation, mitochondrial outer membrane permeabilization, cytochrome c cytosolic release, and caspase-9/caspase-3 activation. Gefitinib caused Bad dephosphorylation, and increased its binding preference to Bcl-2 and Bcl-xL. The dephosphorylation of Bad in gefitinib-treated cells was accompanied by reduced intracellular cyclic AMP content and protein kinase A (PKA) activity. Adenylyl cyclase activator forskolin attenuated, but PKA

inhibitor H89 augmented, gefitinib-induced Bad dephosphorylation, Bax mitochondrial translocation, caspase-9/caspase-3 activation, and viability loss. Inactivation of PKA sensitized H4, T98G, and U87 cells to gefitinib cytotoxicity, Bad dephosphorylation in serine-112, and caspase-9/caspase-3 activation [177].

In a recent study, the molecular effects of the tyrosine kinase inhibitor gefitinib on the EGFR signaling pathway in human glioblastoma were investigated. Resected glioblastoma tissues exhibited high concentrations of gefitinib, 20 times higher than respective plasma. However, no significant effect on 12 pathway constituents was detected. In contrast, in vitro treatment of a glioblastoma cell line, BS-153, with endogenous EGFRwt amplification and EGFRvIII expression resulted not only in dephosphorylation of the EGFR, but also of key regulators in the pathway such as AKT. Treating established xenografts of the same cell line as an in vivo model showed dephosphorylation of the EGFR without affecting downstream signal transductors, similar to the human glioblastoma. Taken together, gefitinib reaches high concentrations in the tumor tissue and efficiently dephosphorylates its target [178]. A phase II study was designed to assess the safety and efficacy of gefitinib given with and following radiation therapy in children newly diagnosed with a poor prognosis brainstem glioma. The observation that a subset of children with this generally fatal tumor experienced long-term progression-free survival, coupled with recent observations regarding the molecular features of brainstem gliomas, raises the possibility that prospective molecular characterization may allow enrichment of treatment responders and improvement in outcome results in future studies of biologically targeted agents [179].

Comparative analysis of tissue obtained from patients before and after the start of treatment suggested that EGFR phosphorylation and downstream signaling were not markedly inhibited after treatment was started. Two main mechanisms of resistance to small molecule TKIs have been proposed. First, Stommel et al. [180] showed that several RTKs could be activated simultaneously in gliomas maintaining activation of RTKs downstream signaling pathways. Thus, inhibition of one single activated RTK is insufficient and could be easily bypassed by other activated RTKs [181]. Second, in addition to RTK activation, growth factor receptor downstream signaling pathways can be activated through a mutation of ras or PTEN or an amplification of PI3K, inducing redundant activation of the signaling pathways [182].

Haas-Kogan et al. reported that glioblastoma patients whose tumor overexpressed and amplified EGFR gene had a better tumor response to small TKIs than patients whose glioblastoma did not have these molecular abnormalities. In addition, these investigators showed that low phospho-PKB/Akt level is associated with a good tumor response to erlotinib [183].

The combination of EGFR inhibitors with inhibitors of mTOR, a distal target of the growth factor receptor signaling cascade (temsirolimus, sirolimus, everolimus), is a promising strategy. It became clear that the mechanisms of sensitivity and resistance in gliomas differ from other types of tumors, such as lung cancer, in that the sensitivity in gliomas does not seem to be linked to tyrosine kinase domain mutations. In order to achieve higher dosages than previously used in clinical trials, we conducted a phase I trial to determine the maximum tolerated dose (MTD) for the combination of erlotinib and sirolimus for the treatments of recurrent malignant gliomas. The MTD was determined to be 150 mg daily for erlotinib and

5 mg daily (after a 15 mg loading dose) for sirolimus. The dose-limiting toxicity s included rash and mucositis, hypophosphatemia, altered mental status, and neutropenia. The combination oferlotinib and sirolimus is difficult to tolerate at dosages higher than previously reported in phase II trials [184].

Ongoing trials of gefitinib, erlotinib, and other EGFR TKIs, such as lapatinib, as well as further tissue analysis of finished trials, may clarify whether selection of patients based on EGFR overexpression, EGFRvIII, pAKT, and/or PTEN expression could improve results. Other attempts to overcome resistance include trials combining EGFR TKI with cytotoxic chemotherapy and radiotherapy as well as the use of new agents that are capable of inhibiting EGFRvIII-overexpressing cell lines in vitro, such as HKI-272 (an irreversible EGFR TKI) and AEE788 [185].

A phase 2 study assessed the efficacy and safety of concurrent radiation therapy (RT) and temozolomide with pharmacodynamic dose escalation of erlotinib in patients with newly diagnosed GBM. Twenty-seven patients were treated in this study. Twenty-two (81%) patients came off study for progressive disease (18 [67%]) or adverse events (4 [15%]). Eighteen patients (67%) have died. Median progression-free survival was 2.8 months, and the median overall survival was 8.6 months. Erlotinib co-administered with RT and temozolomide was not efficacious and had an unacceptable toxicity [186].

Recently, an EGFR murine humanized monoclonal antibody, cetuximab was developed. Little is known about the efficacy of cetuximab in glioma patients. Recently, a phase I/II study combining cetuximab, radiation, and temozolomide was initiated in order to assess the safety and efficacy of this combination as first-line treatment for primary glioblastoma patients [187].

6.1.3. Platelet-derived growth factor receptor PDGFR

PDGFs are a growth factor family composed of four different polypeptide chains (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) that exert their cellular effects through two types of protein tyrosine kinase receptors: PDGFR-a and PDGFR-h. Ligand binding induces receptor dimerization, activation, and autophosphorylation of the tyrosine kinase domain, which results in activation of several signal transduction pathways, including Ras-MAPK, PI3K, Src family kinase, signal transducers and activators of transcription factors (Stat), and phospholipase Cg. Overexpression of PDGF and PDGFR has been shown to play a role in the development of cancer through autocrine stimulation of cancer cells, development of angiogenesis, and control of tumor interstitial pressure. Animal gliomagenesis models have suggested that PDGFR pathways not only play a role in proliferation but also have effects on cell differentiation through dedifferentiation of mature cells, prevention of glial cell differentiation, and even promotion of cancer stem cells [188]. Inhibition of PDGFR was correlated with decreased phosphorylated extracellular signal-regulated kinase and pAKT levels, suggesting inhibition of MAPK and PI3K pathways. PDGFR and PDGF are frequently expressed in gliomas, particularly in secondary glioblastomas and seems to be associated with a poorer prognosis.

Clinical studies using the tyrosine kinase inhibitor, imatinib mesylate, in malignant gliomas, have shown no major inhibition of tumor growth or extension of survival for patients, unlike those in chronic myeloid leukemia and gastrointestinal stromal tumors. Phase I data in malignant gliomas have suggested that use of enzyme-inducing drugs significantly affects drug metabolization [189]. However, preliminary phase II results of single-agent imatinib have found only limited efficacy in unselected patients [189-190].

The effects of imatinib on the PDGFR downstream signaling pathways as well as on other cellular functions in human glioblastoma cells were studied. Western blot analysis demonstrated that imatinib was more effective in inhibiting the activated rather than the quiescent forms of the target proteins. Furthermore, the imatinib treatment induced the sustained activation of extracellular signal-regulated kinase (ERK 1/2) signaling as well as components of other downstream signaling pathways, such as PI3K/Akt, STAT3 and p38MAPK. Further analysis indicated that the activation of ERK induced by the imatinib treatment was related to the S-phase re-entry of the cell cycle in one of the three glioma cells. Imatinib significantly inhibited cell migration but not cell growth. The combination treatment of imatinib with a MEK or PI3K inhibitor resulted in significant growth inhibition but did not inhibit cell migration beyond the inhibition achieved with the imatinib treatment alone [191. In a recent study has been demonstrated that long-term culture with imatinib mesylate against PDGFR and c-Kit (stem cell factor receptor) resulted in reduced cancer stem cell ability in glioblastoma cells through cell differentiation. Derived from RG glioblastoma cells co-cultured with imatinib for 3 months, RG-IM cells showed distinct properties of cell cycle distribution and morphology in addition to significantly decreased ability to form aggregates and colonies in vitro and tumorigenicity in vivo. Furthermore, decreased expression of stem cell markers, i.e., CD133, Oct-3/4, nestin, and Bmi1, and increased terminal neural cell markers, GFAP, Tuj1, etc., were identified in RG-IM at the mRNA level [192]. Conversely, using a combination of imatinib and hydroxyurea achieved better results compared with imatinib alone or historical controls [193]. It has been hypothesized that imatinib potentializes hydroxyurea cytotoxic effects through a decrease in tumor interstitial pressure (which would increase hydroxyurea delivery to tumor cells) and a decrease in DNA repair secondary to imatinib-induced reduction of Rad 51 expression. The combination of imatinib, hydroxyurea, and vatalanib, a VEGFR inhibitor, was well tolerated in a phase I trial and has been suggested as a possible multitargeted regimen for GBM [194].

Sorafenib is an orally available antiangiogenic agent that inhibits tumor cell growth and proliferation by blocking the action of intracellular and receptor kinases, including PDGFR, RAF kinase, VEGFR2, and c-KIT. In human glioblastoma cell lines, sorafenib inhibited proliferation synergistically in combination with bortezomib, a proteosome inhibitor, and rottlerin, an experimental inhibitor of protein kinase C[195-196]. A phase II trial found that first-line TMZ and radiotherapy followed by TMZ plus sorafenib was tolerated by patients with glioblastoma, although preliminary efficacy data for this regimen were similar to data for standard therapy [197]. It has been postulated that sorafenib and protracted, daily temozolomide may provide complementary therapeutic benefit among recurrent GBM patients.

The conclusions of this study showed that sorafenib can be safely administered with daily temozolomide with a regimen with limited activity for recurrent GBM [198].

Tandutinib is an orally active inhibitor of PDGFR, FLT3, and c-KIT tyrosine kinase activity. Although no preclinical data have been reported for tandutinib in glioblastoma, 2 early-phase trials are assessing tandutinib in recurrent or progressive glioblastoma as monotherapy or combined with bevacizumab. In a novel study, targeted PDGFR-A mediated tumor growth in vitro and in vivo using the tyrosine kinase inhibitor,tandutinib (MLN-518), which strongly inhibits PDGFR-A has been evaluated. Although PDGFR-A inhibition by this agent resulted in reduced mouse tumor cell growth and increased apoptosis in vitro, and reduced tumor cell proliferation in vivo, tandutinib did reduce tumor volume at the doses tested (360 mg/kg) in vivo [199].

6.1.4. P13K and related pathways

Overactivation of the PI3K/AKT/mTOR pathway seems to play a key role in the downstream signaling pathways promoting growth and survival in several types of tumor cells, including glioma. Activation of such a pathway is triggered by stimulation of growth factor receptors (including EGFR, PDGFR, fibroblast growth factor receptor, and insulin-like growth factor-I receptor) and the Ras pathway, leading to activation of PI3K. Activated PIP3 promotes phosphorylation of AKT through translocation near the cell membrane and activation of PDK1 and PDK2. pAKT promotes phosphorylation of several downstream effectors, including MDM2, p21/p27, Bad, FKHR, nuclear factor-nB, caspase-9, glycogen synthase kinase-3h, and mTOR. mTOR plays a key role in downstream signaling of the PI3K/AKT pathway through the regulation of cellular catabolism, anabolism, proliferation, cell cycle control, autophagy, angiogenesis, and apoptosis.

In vitro studies have suggested that mTOR activity is particularly highly activated in cells with deficient PTEN function, including glioma cell lines. Alterations of PTEN expression are frequent in high-grade gliomas, with PTEN mutations being present in 15% to 40% of primary glioblastomas [200]. Preclinical studies in gliomas have suggested that PTEN-deficient tumors show enhanced sensitivity to mTOR inhibition; this provided the rationale for clinical trials of mTOR inhibitors in glioblastomas [201].

Temsirolimus (CCI-779) is a lipid soluble analog of rapamycin that inhibits mTOR by binding to FKBP-12, resulting in cell cycle arrest and decreased growth of several human cancer cell lines. In a mouse model of PDGF-B driven low-grade gliomas the use of CCI-779 demonstrated dramatic anti-proliferative effect in these tumors [202]. Again, data from other studies, demonstrated that the blockade of mTOR with CCI-779 resulted in regional apoptosis and conversion in the character of surviving tumor cells from astrocytoma to oligodendroglioma in a mouse model of Akt+KRas-induced GBMs [203]. In a recent research, a combination of perifosine and CCI-779 to inhibit Akt and mTOR respectively was adopted. In vivo, perifosine and CCI-779 showed synergy in inhibiting the PI3K/mTOR axis corresponding with decreased tumor proliferation and induction of apoptosis [204].

Recently, was reported that the combination of focal RT and mammalian target of rapamycin (mTOR) inhibition using clinically relevant concentrations of temsirolimus (CCI-779) prolongs survival in a syngeneic mouse glioma model through additive cytostatic effects. In vitro, the mTOR inhibitor CCI-779 exerted marked anti-invasive effects, irrespective of the phosphatase and tensin homolog deleted on chromosome 10 status and counteracted the proinvasive effect of sublethal irradiation. In this study, the G-protein signaling 4 (RGS4) was identified as a novel target of mTOR inhibition and a key driver of glioblastoma invasiveness, sensitive to the anti-invasive properties of CCI-779 [205]. A phase II study of temsirolimus was conducted in children and adolescents with high-grade glioma, neuroblastoma or rhabdomyosarcoma. Temsirolimus administered weekly at the dose of 75 mg/m(2) did not meet the primary objective efficacy threshold in children with high-grade glioma, neuroblastoma or rhabdomyosarcoma; however, meaningful prolonged stable disease merits further evaluation in combination therapy [206].

Prompted by in vitro evidence of synergism between mTOR inhibitors and EGFR TKI, ongoing clinical research is concentrating efforts on such combinations, including trials of temsirolimus, everolimus (RAD001), or sirolimus (rapamycin) combined with gefitinib, erlotinib, or AEE788. A phase I study combining gefitinib and sirolimus in malignant gliomas found no significant pharmacokinetic interaction between the two drugs. Preliminary results of a study using gefitinib and everolimus in unselected recurrent glioblastoma patients were recently presented. Using modified radiographic criteria, responses were found in 31% of patients, including partial and minor responses. However, median OS and PFS were not different from historical controls [207].

Enzastaurin suppress proliferation and induced apoptosis via a caspase-dependent mechanism in glioblastoma cells in vitro and inhibited growth of human glioblastoma xenografts, which was accompanied by decreased phosphorylation of downstream signaling molecules, including GSK-3b [208]. Enzastaurin is a potent a PKC/PI3K/AKT inhibitor, that reduces angiogenesis and has direct cytotoxic activity against glioma cells in preclinical studies. In a novel study, therapy was well tolerated with thrombosis, thrombocytopenia, hemorrhage, and elevated alanine aminotransferase as the most commonly observed drug-associated grade 3 or higher toxicities. The 6-month PFS was 7% for patients with glioblastoma and 16% for patients with anaplastic glioma. Enzastaurin showed, an anti-glioma activity in patients with recurrent high-grade glioma, but does not appear to have enough single-agent activity to be useful as monotherapy [209].

A recent study was conducted in patients with recurrent glioblastoma or newly diagnosed disease that was not treatable with standard (chemo)radiotherapy. In this phase 1 research, the safety and recommended dose of the oral protein kinase C-beta inhibitor (anti-angiogenic) enzastaurin in combination with single-agent temozolomide, was evaluated. The recommended dose for enzastaurin in combination with standard 4-weekly temozolomide is therefore 500 mg OD. Temozolomide did not appear to effect enzastaurin exposures at the 250 mg or 500 mg OD dose levels [210].

In vivo models showed that enzastaurin combined with radiotherapy synergistically reduced tumor volume, radiation-induced satellite tumor formation, upregulation of VEGF

expression, neovascularization, and GSK-3b phosphorylation [211]. An open-label, single-arm, phase II study combined enzastaurin with temozolomide plus radiation therapy (RT) to treat glioblastoma multiforme (GBM) and gliosarcoma was reported. Progression-free survival (PFS), toxicity, and correlations between efficacy and molecular markers analyzed from tumor tissue samples were also evaluated. The treatment regimen was well tolerated. Overall survival (median, 74 weeks) and progression-free survival (median, 36 weeks) results from the current trial were comparable to those from a prior phase II study using erlotinib and were significantly better than those from 2 other previous studies that used thalidomide or cis-retinoic acid, all in combination with temozolomide plus RT [212].

In a phase II study of enzastaurin in patients with recurrent heavily pretreated glioblastoma, an interim analysis showed that objective radiographic responses occurred in ~20% of patients. The subsequent phase III trial comparing lomustine and enzastaurin at first or second recurrence was the firstphase III trial to evaluate a targeted therapy for recurrent glioblastoma. However, a planned interim analysis found that enzastaurin treatment did not significantly increase PFS, leading to enrolment being halted [213].

6.1.5. SRC and SRC-family kinases

SRC and SFKs are frequently activated in glioblastoma cell lines and patient samples. SRC and SFKs are promiscuous regulators of multiple signaling pathways regulating cell growth, proliferation, adhesion, migration, and invasion, which are important processes in tumor invasion and metastasis. In particular, SFKs mediate signaling from growth factor receptors that are commonly overexpressed in GBM. Recently, SRC and FYN (an SFK) were shown to mediate oncogenic EGFR and EGFRvIII signaling in a rodent GBM model [214].

Dasatinib is a potent inhibitor of SRC and SFK tyrosine kinase activity. Preclinical studies in a wide variety of solid tumor cell lines, including prostate, breast and glioma, have shown that dasatinib acts as a cytostatic agent, inhibiting the processes of cell proliferation, invasion and metastasis. Dasatinib also inhibits the activity of osteoclasts, which have a major role in the development of metastatic bone lesions. Dasatinib also has inhibitory activity against c-KIT and PDGFR.

In GBM cells, dasatinib inhibited migration and induced autophagic cell death, and autophagy was increased by combining dasatinib with TMZ [214-215]. In vivo, dasatinib inhibited invasion, promoted tumor regression, induced apoptosis in EGFRvIII-expressing glioblastomas, and enhanced the activity of anti-EGFR antibodies.19 Recently, it has been showed that dasatinib interacts synergistically with JSI-124, an STAT3/JAK pathway inhibitor. Depletion of Src and STAT3 by siRNA inhibits cell proliferation and migration, suggesting that Src and STA3 play a key role in these cellular responses [216].

Recently the efficacy of dasatinib, in patients with recurrent GBM after bevacizumab failure, was explored. Adult patients were treated with dasatinib 70-100 mg twice daily in combination with bevacizumab (n = 14). Of the thirteen evaluable patients, none had a complete or partial response and only one patient had stable disease after an 8 week interval. Median progression-free survival (PFS) was 28 days. Median overall survival was 78 days. The

study demonstrated that Dasatinib in conjunction with bevacizumab does not appear to have activity in patients with recurrent, heavily pretreated GBM [217].

NVP-AEW541is an inhibitor of insulin-like growth factor-I receptor (IGF-IR) kinase activity on growth and signaling in a panel of glioma cell lines. NVP-AEW541 blocked phosphorylation of IGF-IR in a dose- and time-dependent manner and inhibited proliferation and clonogenicity. NVP-AEW541 also induced loss of mitochondrial membrane potential and release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria. Combined treatment with dasatinib and NVP-AEW541 induced significantly more apoptosis than either agent alone in glioma cells, but not non-neoplastic astrocytes, and synergistically inhibited clonogenic survival. Mechanistic studies indicated that combination of NVP-AEW541 and dasatinib significantly reduced pERK and pAkt and markedly increased AIF release, Bax oligomerization and loss of mitochondrial potential compared to each agent alone. These data indicate that activation of Bax plays a critical role in mediating NVP-AEW541 and dasatinib-induced apoptosis, and suggest the potential value of combining IGFR inhibition with other classes of tyrosine kinase inhibitors to potentiate therapeutic efficacy [218].

6.1.6. Integrin inhibitors

Integrins are transmembrane receptors for the ECM that play a role in cell adhesion and cell migration, namely, in endothelial cell migration, adhesion, and proliferation during angiogenesis. Cilengitide is a cyclic arginine– glycine–aspartic acid containing peptide that binds to and inhibits $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, which are specifically involved in angiogenesis.

Cilengitide induces apoptosis in U87 glioma cells by preventing adherence to vitronectin and tenascin, matrix protein mediators of brain tumor invasion, and growth [219]. In a phase I study (n = 51) of recurrent high grade glioma patients, cilengitide was well tolerated and was demonstrated to have some activity. An objective response was observed in five patients, with two patients demonstrating prolonged complete responses [220]. The most notable trial to date was a randomized phase II study of cilengitide, which was associated with a median survival of 10 months in recurrent glioma patients [221].

A novel study was designed to evaluate the efficacy and tumor delivery of cilengitide in patients with recurrent glioblastoma. The study accrued 30 patients with recurrent glioblastoma and, after recovery from surgery, patients were treated with cilengitide (2000 mg i.v. twice weekly, maximum of 2 years of treatment). The results confirm drug delivery and possibly retention in tumor. This study provides evidence that with established dosing, cilengitide is adequately delivered to the tumor, although as a single agent, efficacy in recurrent glioblastoma is modest [222].

In Phase I and II GBM trials, cilengitide and the combination of cilengitide with standard temozolomide and radiation demonstrate consistent antitumor activity and a favorable safety profile. Preliminary results of a phase II trial of cilengitide added to radiotherapy and temozolomide conducted in 52 patients with newly diagnosed glioblastomas suggested efficacy in a subgroup of patients, with little or no additional toxicity [223]. A multicenter, phase I/IIa study, investigated the efficacy and safety of cilengitide in combination

with standard chemoradiotherapy in newly diagnosed glioblastoma. Patients were treated with cilengitide (500 mg) administered twice weekly intravenously in addition to standard radiotherapy with concomitant and adjuvant temozolomide. Treatment was continued until disease progression or for up to 35 weeks. The conclusions demonstrated that the combination of cilengitide with temozolomide and radiotherapy was well tolerated, with no additional toxicity [224]. In a recent study, the mechanisms of cilengitide-induced cytotoxicity in glioma cells was evaluated. The final data showed that cilengitide treatment induced cell detachment in glioma cells plated on vitronectin in a dose-dependent manner and decreased cell viability with increasing doses. Moreover, the authors evidenced that the treatment of glioma cells with cilengitide induced autophagy in these cells, as demonstrated by puncta LC3-GFP appearance, the increased expression of LC3II, and increased acidic vacuole formation. Some level of cell apoptosis was observed in the cilengitide-treated cells only after 48 hours of treatment, following the induction of autophagy. The pretreatment of the cells with cilengitide could increase the level of autophagy induced by γ-irradiation and significantly decreased the viability of the cells [225]. Nabors et al. examined, in a randomized phase 2 trial, the safety and efficacy of cilengitide when combined with radiation and temozolomide for patients with newly diagnosed glioblastoma multiforme. The authors showed that cilengitide was well tolerated when combined with standard chemoradiation and may improve survival for patients newly diagnosed with glioblastoma multiforme regardless of MGMT methylation status. The authors concluded that, from an efficacy and safety standpoint, future trials of this agent in this population should use the 2000 mg dose [226].

6.2. Antisense strategy

Several approaches are available to manipulate gene expression at the DNA or RNA stage of protein synthesis. In eukaryotic organism, pre-mRNA is transcribed in the nucleus, introns are spliced out and then the mature mRNA is exported from the nucleus to cytoplasm. The small subunit of the ribosome usually starts by binding to one end of the mRNA and is joined there by other eukaryotic initiation factors, forming the initiation complex. This multi-enzymatic complex scans along the mRNA strand until it reaches a start codon, and then the large subunit of ribosome attaches to the small subunit so that the translation of a protein begins. This process, by which the information of a gene is converted into protein, is referred to as “gene expression”.

Several approaches are available to specifically manipulate gene expression at the DNA or RNA stage of protein synthesis. An interesting molecular targeting strategy is the introduction of single-stranded antisense oligonucleotides, specific molecules involved in cell proliferation and cell death, to modify gene expression at the translational level. Antisense oligonucleotides (AONs) are short synthetic single-stranded DNA-sequences, 13-25 nucleotides long, that bind to and induce the cleavage of homologous stretches of mRNA sequences. These result in targeted destruction of mRNA and correction of genetic aberrations. AONs thus can act as drug molecules and potentially rectify many disease conditions. RNA and DNA based oligonucleotides are the most prevalent and most practical antisense drugs.

Interactions of RNA based AONs with target mRNA inhibit gene expression by interfering with protein translation without necessarily altering mRNA stability. AONs inhibit mRNA function by several mechanisms including modulation of splicing and inhibiting protein translation by disrupting ribosome assembly.

Two different and adjunctive antisense-based approaches to modulate gene expression into cancer cells are RNA interference (RNAi) and ribozymes. RNAi is mainly responsible for the post-transcriptional regulation of gene expression but is involved in transcriptional regulation. RNAi is a process within living cells that moderates the activity of their genes. RNA interference plays a fundamental role in diverse eukaryotic functions including viral defence, chromatin remodeling, genome rearrangement, developmental timing, brain morphogenesis, and stem cell maintenance. The power of RNAi lies in the key discovery that endogenous RNAi gene silencing machinery can be hijacked to artificially regulate genes of interest. The selective effect of RNAi on gene expression makes it a valuable research tool, both in cell culture and in living organisms because synthetic double-stranded RNA (dsRNA) introduced into cells can induce suppression of specific genes of interest.

Ribozymes are small oligo-ribo-nucleotides which have a specific base sequence with natural self-splicing activity. This activity can be directed against virtually any RNA target by the inclusion of an antisense region into the ribozyme. Some ribozymes have a self-cleavage catalytic action while other ones are true catalysts and can carry out RNA slicing by transesterification (splicesome) and peptidyl transfer (in ribosomes). A major disadvantage of ribozymes at present is that, being ribonucleic acids, they are particularly sensitive to nuclease degradation. Ribozymes are not generally being considered as agents which may be exogenously administered. Whilst it should be possible to develop nuclease-resistant ribonucleotide analogues, present strategies employing ribozymes achieve their delivery by genetic means, in the form of mini-gene constructs. Ribozymes are now utilized to study gene function in HIV disease and in cancer research.

6.2.1. Antisense oligonucleotides

The choice of the target is crucial to the potential success of the therapeutic approach. The most interesting targets are involved in apoptosis, cell proliferation, neoangiogenesis and invasion.

In tumorigenic mice generated by subcutaneous injection of glioma cell lines, overexpression of antisense VEGF (C6-VEGF(-/-) mice) significantly suppressed tumor growth, decreased angiogenesis and reduced tumoral edema. Further studies by electron microscope revealed that tumor-induced hyperpermeability was mediated by formation of vesiculo-vacuolar organelles (VVO), specifically reducing the number of vesicle and caveolae in VVO, and this effect was partially blocked by antisense VEGF [227].

Recently was evaluated the suitability of folate-PAMAM dendrimer conjugates for efficient EGFR AON delivery into glioma cells, wherein they release the AON from the FA-PAMAM to knock down EGFR expression in C6 glioma cells, both in vitro and in vivo. Folic acid was

coupled to the surface amino groups of G5-PAMAM dendrimer (G5D) through a 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide bond, and AONs corresponding to rat EGFR were then complexed with FA-PAMAM. The AON transfection rates mediated by FA-PAMAM and PAMAM resulting in greater suppression of EGFR expression and glioma cell growth [228]. In a previous study, was demonstrated that antisense-EGFR transfection inhibited the growth and transforming phenotype of U87MG cells. In this research, the co-transfection of U87MG cells with wild-type PTEN and antisense EGFR constructs could inhibit the cellular growth by 91.7% [229].

Inhibition of PKC-α expression by a synthetic AON inhibits proliferation of C6 glioma cells. In addition, inhibition of PKC-α expression, has also showed growth inhibition of transformed U-87 cells transfected with antisense anti-PKC-α oligonucleotide [230]. PKC- α antisense oligonucleotide treatment in GBM and in A172 GBM cells was accompanied by reduction in PKC-α levels and the induction of wild-type p53 and insulin-like growth factor-binding protein-3 (IGFBP3) 24-72 h after treatment. Increased IGFBP3 levels were accompanied by increased mRNA levels. Recombinant human IGFBP3 induced an apoptotic effect that was similar to the PKC- α antisense oligonucleotide, and its effect was blocked by IGF-I [231]. Other potential therapeutic targets are human C-raf kinase and c-Ki-RAS proteins and signal transduction kinase proteins, involved in the control of proliferation, cellular migration and differentiation, and cytoskeletal rearrangements.

In a murine model, was showed the down-regulation of proliferation rate of C6 glioma cells transfected with antisense AKT2 cDNA construct. Parental C6 cells and C6 cells, transfected with antisense construct, were implanted through lipofectamine complexes. Dominant-negative (DN-AKT2) and antisense AKT2 constructs (AS-AKT2) were transfected into rat C6 glioma cells with elevated endogenous AKT2 expression. AKT2 expression was inhibited in C6 cells transfected with AS-AKT2 but did not significantly change in cells transfected with DN-AKT2. The cell migration distance was reduced in cells transfected with DN-AKT2 or AS-AKT2 compared to the control cells and gelatin zymography showed that the production of MMP2 and MMP9 was inhibited in transfected cells [232]. Edwards et al. targeted the phosphatidylinositol 3-kinase/protein kinase B (PKB)/Akt and the Ras/MAPK pathway for their involvement in cell survival and cell proliferation. The glioblastoma cell lines U87MG, SF-188, and U251MG were transiently transfected with an antisense oligonucleotide targeting ILK (ILKAS) alone or in combination with the Raf-1 inhibitor GW5074 or with the MEK inhibitor U0126. Glioblastoma cells transfected with ILKAS exhibited reduced levels of ILK and phosphorylated PKB/Akt on Ser473. These results confirmed that combinations targeting ILK and components of the Ras/MAPK pathway result in synergy and could potentially be more effective against glioblastoma cancer than monotherapy [233].

During progression of human gliomas, the expression of capillary BM laminins containing α4 chain switches from the predominant laminin-9 into lanimin-8. Effects of antisense inhibition of laminin-8 expression in glioma therapy through an in vitro model using human GBM cell lines M059K and U-87MG co-cultured with normal human brain microvascular endothelial cells (HBMVEC) have been observed. Laminin-8 and its receptor, integrins α3β1 and α6β1, are important for the functioning of endothelial cell BMs, which play a role in the

maintenance of the BBB. Laminin-8 plays an important role in glioma cell invasiveness, in combination with other proteins associated with glioma progression, such as tenascin-C, MMP-2 and MMP-9. In this study was demonstrated a significant reduction of invasion of co-cultures through Matrigel and through the use of morpholino oligos against α4 and β1 chains of laminin-8 [234]. The downregulation of laminin α4 chain using AONs inhibits the motility of human glioma cells was shown. Laminin α4 chain appears to be an important factor in glioma migration and invasion, both in vitro and in vivo [235].

The antisense modulation of Bcl-2 expression could increase the effectiveness of conventional chemotherapeutic agent. Antisense human bcl-2 cDNA was transfected into human malignant glioma cells. The effects of bcl-2 protein down-regulation on glioma cell morphology, in vitro tumor growth, and tumorigenicity in nude mice, as well as chemosensitivity to cisplatin, were studied. Expression of antisense bcl-2 cDNA decreased bcl-2 protein by more than sixfold. Antisense bcl-2 stable transfectants (AS-bcl-2) showed profound morphological change, marked retarded cell growth in vitro and significantly increased cytotoxicity of cisplatin [236]. Phase I and II studies are also being done to test G3139 in combination with docetaxel in patients with advanced breast cancer, hormone-refractory prostate cancer, and other solid tumors. By using AONs against the first six codons of the human Bcl-2 gene transfected into malignant glioma cells (Jon52 and Roc GBM cell lines), has been demonstrated a decrease in cell growth and an increase in apoptotic death [237]. There is a link between resistance to chemotherapy in glioblastoma and expression of antiapoptotic bcl-2 family members, including bcl-XL. Anti-bcl-XL AONs (ISIS 16009, ISIS 16967) in M059K GBM cell lines have been used and demonstrated a valid correlation between reduction of bcl-XL protein expression, induction of intrinsic apoptotic pathway and enhancement of cytotoxic responses to paclitaxel treatment, resulting in a chemosensitizing effect of anti-bcl-XL therapy [238].

7. Conclusion

Cerebral gliomas remain essentially resistant to traditional cancer therapy. Radiation therapy is limited to a largely palliative role, and chemotherapy has provided only a modest benefit in the clinical outcome. Ideally we would analyze tumor tissue in order to confirm adequate concentrations of our agents; however, the difficulties of accessing brain tumor samples compared with other tumor samples in systemic cancers make this a difficult task. This also impedes our validation of biological effects in vivo, a crucial step in the assessment of new interventions and therapies.

Understanding the genetic bases of gliomas and of the invasive behavior may suggest new molecular targets to overcome the mechanisms of multi-drug-resistance of the actual therapeutic approaches and to attack at the same time different crucial biological events of gliomagenesis. In recent years, basic and preclinical studies have revealed multiple new mechanisms of gliomagenesis and corresponding targets for treatment. Glioma gene expression and its development during gliomagenesis will may help to better understand the role

of important molecules involved in tumor-safe brain parenchyma relationships. These molecules, such as ECM proteases, cell adhesion molecules and their related signaling pathways show an important role in glioma cell migration and invasion and could be selectively attack to inhibit the glioma invasive rim. Targeted therapies are adopted to disrupt the function of their targets or deliver toxins to tumor cells expressing the target. However, drugs targeting single molecules show a limited efficacy, because multiple abnormalities are simultaneously present in glioma patients. It is clear that the complexity and cross-talk between signal transduction pathways limits the potential efficacy of targeting a single receptor or molecule. As a result of a tested preclinical study, clusterin, eukaryotic initiation factor-4E (eIF-4E), integrins, metalloproteinases and other key molecules (involved in invasion and angiogenesis) might be possible future interesting molecular targets in glioma therapy. Targeting of multiple signaling pathways by multitargeted kinase inhibitors or combinations of single-targeted kinase inhibitors may increase treatment efficacies. Parallel to this is the task of determining the unique molecular and genetic profiles of individual patient tumors so that the most effective therapies can be selected for those individuals. Targeted agents are likely to have the greatest potential when used in combination to increase the activity of standard chemotherapies, broadening the range of pathways inhibited by treatment and/or counteracting mechanisms of resistance. In addition, as for classic cytotoxic agents, an intact BBB may represent an important impediment limiting the efficacy of targeted therapies.

Nanotechnology provides a unique opportunity to combat cancer on the molecular scale through careful engineering of nanomedicines to specifically interact with cancer cells and inhibit cancer cell function. It is also possible to take into neoplastic tissue, novel selective contrast enhancement molecules to visualize brain tumors and to study in vivo all of their characteristics, such as cellular proliferation, angiogenesis, necrosis, tumor-safe tissue interface, and edema. There are significant opportunities to investigate the use of nuclear imaging techniques such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) for development of radiolabeled nanoparticles targeting cancer. The versatility, sensitivity and tomographic imaging capabilities of these imaging modalities will provide excellent opportunities for future development of targeted nanoparticle formulations. The surface of nanoparticles can be modified to achieve targeted delivery and improved biocompatibility. Compounds may also be encapsulated within the interior core of nanoparticles for multiple functions. Nanoparticle-based delivery systems could increase the overcoming of the BBB by the use of drugs with a targeted-cell specificity modality. This approach permits the use of a lower dose of drug, a selective drug delivery to target tumor cells, both into the central core of tumor and into the distal foci of tumor cells within areas often characterized from integrity of the BBB [239-240]. Considerable effort has been made toward the research and development of multifunctional nano-particle systems for cancer targeted imaging and therapy. Theranostic nanomedicine represent an integrated nanotherapeutic system, which can diagnose, deliver targeted therapy, and monitor the response to therapy. This integration of diagnostic imaging capability with therapeutic interventions is critical to addressing the challenges of brain tumor hetereogeneity and adaptation. As a platform technology, nanomedicine has the advantage of being able to target multiple tumor markers and deliver multiple agents simultaneously for synergy in ad-

dressing the challenges of cancer hetereogeneity and adaptative resistance. Nanoparticle drug delivery vehicles have shown the ability to encapsulate a variety of therapeutic agents such as small molecules (hydrophilic and/or hydrophobic), peptides, protein-based drugs, and nucleic acids. By encapsulating these molecules inside a nanocarrier, the solubility and stability of the drugs can be improved, providing an opportunity to reevaluate potential drugs previously ignored because of poor pharmacokinetics. Encapsulated molecules can be released from nanocarriers in a controlled manner over time to maintain a drug concentration within a therapeutic window or the release can be triggered by some stimulus unique to the delivery site [241]. The surface of the nanocarrier can be engineered to increase the blood circulation half-life and influence the bio-distribution, while attachment of targeting ligands to the surface can result in enhanced uptake by target tissues.

On the basis of the data collected and of the limits of actual standard therapeutic protocol, we think that targeted therapy represents an interesting approach to modify the biological development of gliomas, probably trying to modulate crucial pathways of gliomagenesis during precocious steps of tumor progression and more molecular targets of the same pathway or of two different pathways.

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