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Anti-Angiogenesis, Gene Therapy, and Immunotherapy in Malignant Gliomas

Paula Province, Alexis Bashinski Shaefer,
Benjamin McCullough and
Hassan M Fathallah-Shaykh

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1. Introduction

Malignant gliomas remain associated with poor prognosis and the cause of significant morbidity. In 2010, there was excitement that recent spectacular advancements in our basic understanding of their molecular pathogenesis, angiogenesis and new gene transfer technologies will turn the tide in our favor. The negative results of several costly phase III clinical trials are sobering; unfortunately, they take us back to the drawing board in terms of how we can improve our methods and why brain cancer has this incredible ability to resist therapy. This chapter is organized as follows. We start by an overview of the classification and significance of malignant gliomas. We proceed to reviewing the molecular pathogenesis of angiogenesis and the development of new treatment modalities against anti-angiogenesis targets, some of which were tested in Phase III clinical trials. Before considering immunotherapy strategies and targets for malignant gliomas, we review basic concepts in immunology and discuss the unique immunological features of the central nervous system (CNS). Finally, we discuss gene therapy vectors, strategies, and clinical trials in malignant gliomas. We conclude by an analysis of our current limitations, possible tumor mechanisms for resisting treatments, and what we can do to improve the outcome.

2. Overview and significance

2.1. Classification of gliomas

CNS neoplasms are diverse and demonstrate a great deal of variability in terms of clinical presentation, aggressiveness, and response to therapy, with distinctions in histology and cellular and molecular composition being primarily responsible for these variations (Brat and Mapstone 2003; Omuro and DeAngelis 2013). Gliomas are the most frequent primary brain tumors in adults and, of this group, anaplastic astrocytomas and glioblastoma (GBM) are the two highest-grade astrocytic neoplasms (Brat and Mapstone 2003; Ricard, Idbaih et al. 2012). The World Health Organization (WHO) system classifies astrocytomas into four grades. These histological grades are defined by increasing degrees of undifferentiation, anaplasia, and aggressiveness (Louis, Ohgaki et al. 2007; Omuro and DeAngelis 2013). Grade I and II tumors, the lower grade tumors, are well-differentiated with limited cell density. The characteristic features of grade III astrocytomas (anaplastic) are increased vessel and cell density, cellular atypias, and high mitotic activity. Grade IV astrocytoma (GBM) is characterized by vascular proliferation or necrosis (Westphal and Lamszus 2011; Omuro and DeAngelis 2013). Glioblastoma and other malignant gliomas are highly infiltrative tumors. Of note, there is also a WHO grading system for oligodendrogliomas and oligoastrocytomas, but they will not be discussed in this chapter (Omuro and DeAngelis 2013).

2.2. Significance of malignant gliomas

The annual incidence of malignant glioma is 5.26 per 100 thousand and this group accounts for approximately 80% of the total number of new cases of malignant primary brain tumors diagnosed in the United States each year (Omuro and DeAngelis 2013). The overall incidence of gliomas is highest among Caucasians, as compared to other ethnic groups, and is higher among males as compared to females (7.2 versus 5.0 per 100,000 persons-years) (Peak and Levin 2010). Malignant gliomas can occur in any age group; however, the incidence increases in the fifth decade of life and peaks at about 65 years of age (Brat and Mapstone 2003). GBM is the most aggressive glioma. Stupp and colleagues reported that 27.2 and 9.8 percent of GBM patients treated by concomitant and adjuvant Temozolamide and radiotherapy remained alive at 2 years and 5 years, respectively (Stupp, Mason et al. 2005; Stupp, Hegi et al. 2009). For patients diagnosed with anaplastic astrocytoma, the median survival time is higher at approximately 2 to 5 years (Wen and Kesari 2008).

3. Angiogenesis

3.1. History

The theory that tumor growth is dependent on angiogenesis and that anti-angiogenic therapy may be a potential cancer treatment was first proposed by Dr. Folkman in the 1970s (Folkman 1972). Since that time, understanding the mechanism of action of angiogenesis and developing targeted therapies have been a high priority.

3.2. Summary of angiogenesis

Angiogenesis is the process by which the vascular system is formed through growth of new capillaries from pre-existing vessels (Plate, Scholz et al. 2012). Angiogenesis plays a critical role in key physiologic and formative processes such as embryogenesis, regeneration, and wound healing. Angiogenesis is also involved in various pathologic processes including age-related macular degeneration, rheumatoid arthritis, and tumor growth and development (Wang, Fei et al. 2004).

The process of angiogenesis can be briefly summarized as follows. First, there is vasodilation, in response to nitric oxide, and increased permeability of the existing vessels. This is followed by degradation of the existing vessel's basement membrane. Next, endothelial precursor cells migrate to the area and begin to proliferate and mature into capillaries via a balance of both growth and inhibition. The final steps involve recruitment of vascular smooth muscle cells and pericytes that form a new network of mature vessels (Shinkaruk, Bayle et al. 2003).

3.3. Molecular signals of angiogenesis

Although there are numerous factors and signals that contribute to angiogenesis, the chemical signal that seems to play the most critical role in the process is Vascular Endothelial Growth Factor (VEGF). VEGF is a pro-angiogenic growth factor, which is secreted by many cells, including mesenchymal, stromal, and especially tumor cells. VEGF induces the migration of the endothelial precursor cells to sites of angiogenesis and is responsible for their proliferation and differentiation. The VEGF gene is located on chromosome 6p12 and the gene family is composed of five members, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental-derived growth factor (PlGF). Of these, VEGF-A, B, and PlGF are involved in the development of the vascular system and VEGF-C and D are involved in the development of the lymphatic system (Ahluwalia and Gladson 2010). VEGF primarily signals through its receptor VEGFR2 which is a tyrosine kinase receptor that is expressed by many cells, including endothelial cells, endothelial cell precursors, and tumor cells (Jain, di Tomaso et al. 2007). Other chemical signals that play an important role in angiogenesis are fibroblast growth factor, hepatocyte growth factor (HGF), tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), angiopoietins, and platelet derived growth factor (PDGF). Their various roles include involvement in extracellular matrix degradation, endothelial proliferation and migration, and neovessel stabilization and maturation (Martin and Jiang 2010; Ucuizian, Gassman et al. 2010).

3.4. Angiogenesis in tumors

Seven different cellular mechanisms appear to contribute to tumor angiogenesis: (1) classical sprouting angiogenesis, (2) vascular co-option, (3) myeloid cell-driven angiogenesis, (4) vessel intussusception, (5) vasculogenic mimicry, (6) bone marrow derived vasculogenesis, and (7) cancer stem-like cell derived vasculogenesis (Carmeliet and Jain 2011; Plate, Scholz et al. 2012). Of the above-listed mechanisms, the first three seem to have a clear role in glioma vascularization, as supported by pre-clinical tumor models (Plate, Scholz et al. 2012).

1. *Classical sprouting angiogenesis*. This is believed to be the primary modulator of neovascularization of the brain during development and in pathological conditions (Plate, Breier

et al. 1994; Risau 1997; Kurz, Korn et al. 2001; Plate, Scholz et al. 2012). In this model, a vascular sprout is led by tip cells toward an angiogenic stimulus that is produced by tumor cells. This sprout then elongates via dividing stalk cells. The newly formed vessel undergoes remodeling to create a vascular lumen that allows blood flow (Plate, Scholz et al. 2012). There is evidence to support that both tip and stalk cell phenotypes co-exist in the glioblastoma vasculature (Plate, Breier et al. 1994; Broholm and Laursen 2004; Dieterich, Mellberg et al. 2012; Plate, Scholz et al. 2012)

2. *Vascular co-option*. This is the process by which tumor cells infiltrate into normal tissue and adopt pre-existing vasculature (Holash, Wiegand et al. 1999; Plate, Scholz et al. 2012). This pathway seems to be enhanced through activity of pro-angiogenic molecules, like VEGF and Angiopoietin-2 (Holash, Wiegand et al. 1999).
3. *Myeloid cell-driven angiogenesis*. Tumor-associated macrophages contribute to angiogenesis by secreting pro-angiogenic factors such as fibroblast growth factor 2 (FGF2), VEGF, and matrix metalloproteinases (MMPs) (Plate, Scholz et al. 2012). Tumor-associated macrophages may also assist two vascular sprouts to form a direct connection through a process referred to as anastomosis (Plate, Scholz et al. 2012).

The role of the remaining four mechanisms in glioma angiogenesis is not yet fully understood. Briefly, *vessel intussusception* is the process by which a new vessel is formed by internal division of the pre-existing capillary plexus without sprouting through a series of steps that include vascular invagination, intra-luminal pillar formation and remodeling, and splitting (Djonov, Schmid et al. 2000; Plate, Scholz et al. 2012). *Vasculogenic mimicry* refers to the process by which cancer cells form *de novo* vasculature as a result of their high plasticity (Plate, Scholz et al. 2012; Seftor, Hess et al. 2012). *Bone marrow-derived vasculogenesis* refers to the process by which circulating endothelial precursor cells are recruited to the tumor and are incorporated into the vessel wall (Plate, Scholz et al. 2012; Huang, Peng et al. 2013). *Cancer stem-like derived vasculogenesis* is the process by which tumor-derived cells trans-differentiate into endothelial cells (Ricci-Vitiani, Pallini et al. 2010; Plate, Scholz et al. 2012). It is not the goal of this chapter to study these mechanisms in detail, but instead to provide an overview of angiogenesis in glioma and discuss key molecules involved and possible therapeutic options that target them.

4. Targets for anti-angiogenics

4.1. VEGF receptor blockers

4.1.1. Bevacizumab

Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody that targets VEGF. It was the first anti-angiogenesis agent to be approved by the United States Food and Drug Administration (FDA) in 2004. Bevacizumab was initially approved for use in metastatic colorectal cancer, but its clinical use has been extended to other cancer types (Van Meter and Kim 2010). Bevacizumab has six VEGF binding residues that neutralize the ability of VEGF to

bind to its target receptors on endothelial cells. This neutralization has been shown to have efficacy not only in *in vitro* studies, but also in *in vivo* ones.

Recently, two phase III clinical trials investigating Bevacizumab as a first-line treatment for newly diagnosed GBM tumors were completed. Unfortunately, both trials were consistent in showing no statistically-significant prolongation of overall survival time (OS) but there was a slight improvement in progression-free survival time (PFS). The two trials had a similar design, namely double-blinded prospective trials where newly diagnosed GBM patients were randomized to either standard of care with Bevacizumab or with placebo; the standard of care consisted of radiation therapy with adjuvant and concomitant Temozolomide. A total of 637 and 921 adult participants were randomized in the Radiation Therapy Oncology Group (RTOG) and AVAglio trials, respectively. The median OS was 16.1 vs. 15.7 months, in the RTOG trial ($p = 0.11$). The median PFS was longer in patients who received Bevacizumab, 7.3 vs. 10.7 months ($p = 0.004$) and 6.2 vs. 10.6 months ($p < 0.0001$) in the RTOG and Avaglio trials, respectively. In addition, the results also showed a higher incidence of adverse reactions in the Bevacizumab arm, including neutropenia, hypertension, and deep vein thromboembolism and pulmonary emboli (Gilbert, Dignam et al. 2013). The AVAglio trial noted delayed time to definitive deterioration in terms of health-related quality of life ($p < 0.0001$) and Karnofsky Performance Scale, and increased time to corticosteroid initiation (HR 0.71, 95% CI 0.57-0.88; median 12.3 vs. 3.7 months) (Henriksson, Bottomley et al. 2013). These results are discouraging and do not justify the use of Bevacizumab in a GBM patient who has had a reasonable surgical resection.

The data support the idea that Bevacizumab may be reserved until the time of recurrence as several prospective phase II clinical trials have shown prolongation of the 6-month PFS rates ranging from 25 to 42.6 percent and median OS times from 6.5 to 9.2 months. However, a significant limitation of these trials is that the comparison was made to historical controls (Friedman, Prados et al. 2009; Kreisl, Kim et al. 2009; Raizer, Grimm et al. 2010).

4.1.2. VEGF-trap

VEGF-Trap (drug name Aflibercept) is a recombinant fusion protein that acts as a decoy receptor for VEGF, thereby blocking its interaction with its normal receptors and interrupting the VEGF signaling pathway (Holash, Davis et al. 2002). VEGF-Trap was developed by incorporating domains of both VEGF receptor 1 and VEGF receptor 2 fused to the constant region of human immunoglobulin G1. VEGF Trap has a high affinity for all isoforms of VEGF-A, as well as for PlGF, another pro-angiogenic agent that primarily acts on VEGF receptor 1 (Holash, Davis et al. 2002; Gomez-Manzano, Holash et al. 2008; de Groot, Lamborn et al. 2011). Preclinical studies demonstrated efficacy of VEGF-trap in glioma animal models (Haapa-Paananen, Chen et al. 2013). de Groot et al. conducted a Phase II study of Aflibercept in recurrent malignant glioma; unfortunately, their results revealed that Aflibercept had minimal activity as a single-agent against recurrent GBM (de Groot, Lamborn et al. 2011).

4.1.3. Sunitinib

Sunitinib is a small-molecule inhibitor of VEGF receptors 1 and 2, PDGFR alpha and beta, stem-cell factor receptor (SCFR), fms-like tyrosine kinase 3 (FLT-3), colony-stimulating factor-1 receptor (CSF-1R), and the *RET* oncogene tyrosine kinase (*RET*) (Chow and Eckhardt 2007; Kreisl, Smith et al. 2013). It has FDA approval for use in metastatic renal-cell carcinoma, gastorointestinal stromal tumors refractory to imatinib mesylate, and advanced pancreatic neuroendocrine neoplasms (Kreisl, Smith et al. 2013). Recently, a phase II clinical trial was completed investigating the role of continuous daily Sunitinib in recurrent GBM in both Bevacizumab exposed and Bevacizumab naïve patients (Kreisl, Smith et al. 2013). Unfortunately, the results did not demonstrate an improvement in PFS in either population. Recent evidence by Costa et al suggests that silencing of micro-RNA 21 (miR-21), a small, non-coding RNA that regulates gene expression, may enhance the anti-tumoral effect of Sunitinib (Costa, Cardoso et al. 2013).

4.1.4. Nintedanib

Nintedanib (BIBF 1120) is a small, orally available triple angio-kinase inhibitor that targets VEGF receptors 1-3, FGFRs 1-3, and PDGFR alpha and beta. It is still in phase III development, but preclinical models demonstrated effective growth inhibition of both endothelial and perivascular cells when the above listed pathways were simultaneously interrupted (Hilberg, Roth et al. 2008; Muhic, Poulsen et al. 2013). Phase I/II clinical trial results have demonstrated tumor stabilization rates of 46-76%, when tested in various tumor types (Mross, Stefanic et al. 2010; Okamoto, Kaneda et al. 2010; Richeldi, Costabel et al. 2011; Muhic, Poulsen et al. 2013) Muhic et al. conducted an uncontrolled phase II trial assessing the efficacy of single-agent Nintedanib in patients with recurrent GBM who had previously failed 1-2 lines of therapy; unfortunately, this study was stopped prematurely secondary to futility (Muhic, Poulsen et al. 2013).

4.1.5. Vandetanib

Vandetanib is a multi-targeted tyrosine kinase inhibitor of VEGF receptor 2, epidermal growth factor receptor (EGFR) 2, and the rearranged-during-transfection oncogene that results in the simultaneous blocking of several pathways, including angiogenesis (Kreisl, McNeill et al. 2012). Preclinical rat and mice glioma xenografts have shown anti-tumor effects of Vandetanib (Sandstrom, Johansson et al. 2004; Rich, Sathornsumetee et al. 2005; Kreisl, McNeill et al. 2012). Kreisl et al. conducted a phase I/II trial of Vandetanib in patients with recurrent malignant glioma and found that it did not have activity as a single agent in this population (Kreisl, McNeill et al. 2012).

4.2. Integrins

Integrins are cell surface receptors that play key roles in mediating the migration of endothelial cells. They are receptors for many different extracellular matrix (ECM) ligands and they play an important role in angiogenesis via the processes of integrin-mediated adhesion, migration,

proliferation, survival, and differentiation of cells that form the vasculature (Hynes, Bader et al. 1999; Tchaicha, Mobley et al. 2010). The αv integrin subfamily has five members- $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, and $\alpha v\beta 8$ - and the $\alpha v\beta 8$ member, in particular, has been shown in mouse models to be a central regulator of angiogenesis in the developing brain (McCarty, Monahan-Earley et al. 2002; Zhu, Motejlek et al. 2002; Tchaicha, Reyes et al. 2011).

Cilengitide, a selective inhibitor of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, demonstrated preclinical activity against angiogenesis in GBMs and it is also being investigated clinically (MacDonald, Taga et al. 2001; Onishi, Kurozumi et al. 2013). A phase II study of Cilengitide conducted by Reardon et al. was associated with a median survival of 9.9 months and a PFS rate of 15% in recurrent glioma patients. Unfortunately, the CENTRIC phase III trial revealed that Cilengitide failed to prolong PFS or OS in patients with newly diagnosed GBM and a methylated MGMT promoter (Onishi, Kurozumi et al. 2013).

4.3. Notch signaling

4.3.1. Ligands

Notch signaling in host endothelial cells is important for angiogenesis. Recent evidence has shown that delta-like ligand 4 (DLL4), a member of the Notch ligand family, is expressed in tumor cells and can activate Notch signaling in host endothelial cells and can therefore affect the vascular function of tumors. In fact, DLL4 expression appears to be regulated by VEGF and the tumor's hypoxic microenvironment (Patel, Li et al. 2005; Li, Gong et al. 2012). Increased levels of VEGF lead to an up-regulation of DLL4 expression which results in endothelial cells expressing Notch receptors to down-regulate VEGF-induced vessel sprouting and branching and ultimately resulting in productive and efficient angiogenesis (Li, Gong et al. 2012). Furthermore, it has also been demonstrated that blockade of DLL4 can result in non-productive angiogenesis by causing tumor growth inhibition and a decrease in tissue perfusion (Scehnet, Jiang et al. 2007; Li, Gong et al. 2012). Li et al. recently conducted a study to investigate the role of DLL4 in malignant gliomas, specifically in terms of vascular quantity and quality and showed that DLL4 expression was significantly up-regulated in malignant human gliomas as compared to normal brain tissue. Additionally, they also demonstrated that DLL4-positive malignant glioma tissues have increased proliferation of vascular endothelial cells and pericyte recruitment, as compared to DLL4-negative malignant glioma tissue, and that DLL4-positive tissues had a higher vessel maturation index (VMI). These results provide evidence that DLL4 inhibition may alter glioma vessel maturity and, in turn, may improve the effects of anti-angiogenic agents (Li, Gong et al. 2012).

4.3.2. Gamma secretase

Gamma secretase is a pre-senilin dependent protease that acts as a regulator of angiogenesis through a series of complex steps that are beyond the scope of this chapter. However, part of its role in angiogenesis is related to Notch signaling (Jain, di Tomaso et al. 2007; Boulton, Cai et al. 2008). RO4929097 is a potent and selective gamma secretase inhibitor of Notch signaling that is being investigated as an anti-tumor agent. Phase I studies have demonstrated safety

and phase II studies are underway to assess its role in recurrent GBM when given alone and in combination with Bevacizumab (Tolcher, Messersmith et al. 2012).

4.4. Transforming growth factor beta (TGF- β)

TGF- β is a multifunctional protein that is involved in the regulation of proliferation, differentiation, and survival of many cells, including glioma cells and endothelial cells (Bertolino, Deckers et al. 2005). TGF- β 1 and TGF- β 2, members of the TGF- β family, stimulate expression of VEGF, the plasminogen activator inhibitor, and some metalloproteinases that are involved in vascular remodeling, angiogenesis, and degradation of the extracellular matrix. Animal models demonstrate that inhibitors of TGF- β signaling reduce viability and invasion of gliomas (Kaminska, Kocyk et al. 2013). Fresolimumab, a human monoclonal antibody that inactivates all forms of TGF- β , is being investigated as a potential therapeutic for glioma (Trachtman, Ferverza et al. 2011).

4.5. Topoisomerase I inhibitors

Topoisomerase I is critical for efficient DNA replication and cell division. Topoisomerase I activity is increased in malignant gliomas and inhibitors of topoisomerase I activity, such as Camptothecin, Irinotecan, and the indolocarbazoles, have been tested as potential glioma therapies (Pommier 2006; Feun and Savaraj 2008; Vredenburg, Desjardins et al. 2009; Lampropoulou, Manioudaki et al. 2011). Recently, Lampropoulou et al. have shown that inhibition of topoisomerase I activity by the pyrrolo[2,3- α]carbazole derivatives may be linked to a decrease in the number of viable glioma and endothelial cells *in vitro* and may also be related to inhibition of angiogenesis *in vivo* (Lampropoulou, Manioudaki et al. 2011).

4.6. Oncoproteins

B-cell specific Moloney murine leukemia virus integration site 1 (Bmi-1) is an oncoprotein that plays a role in the development and progression of cancers including breast, lung, prostate, and interestingly, brain (Jagani, Wiederschain et al. 2010). Bmi-1 is a member of the Polycomb gene family of proteins that function as epigenetic silencers of genes that control self-renewal, differentiation, and proliferation; dysregulation of Bmi-1 has been associated with cancer cell proliferation, invasion, and repression of apoptosis or senescence (Jiang, Song et al. 2013). In particular, Bmi-1 promotes growth and survival of glioma tumor cells (Li, Gong et al. 2010; Jiang, Song et al. 2013); furthermore, Bmi-1 promotes angiogenesis of gliomas by activating the NF- κ B signaling pathway *in vitro* as well as *in vivo* (Jiang, Song et al. 2013). Thus, targeting Bmi-1 is a promising aim in gliomas.

4.7. Other potential therapeutics in development

4.7.1. Carboxyamidotriazole orotate (CTO)

CTO is a triazole orotate formulation of carboxyamidotriazole (CAI), which is an inhibitor of receptor-operated calcium channel-mediated calcium influx. CTO has anti-proliferative, anti-

invasive, as well as anti-angiogenic properties in several human cancer cell lines including glioblastoma (Ge, Rempel et al. 2000; Fiorio Pla, Grange et al. 2008; Karmali, Maxuitenko et al. 2011). In initial clinical development, CAI was shown to have poor bioavailability, limited efficacy, and high toxicity. CTO, however, appears to have much better bioavailability and less toxicity (Grover, Kelly et al. 2007; Karmali, Maxuitenko et al. 2011).

4.7.2. TRC105

TRC105 is a novel, first-in-class antibody against endoglin (CD 105), an endothelial cell receptor that is essential to angiogenesis and acts primarily through its effects on TGF- β and BMP-9 signaling. A phase I trial conducted by Rosen et al. demonstrated that this drug is well-tolerated at clinically relevant doses and multiple phase II trials are ongoing to evaluate its potential role in other malignancies, including malignant glioma (Rosen, Hurwitz et al. 2012).

4.7.3. Thalidomide and lenalidomide

Thalidomide and its analogue Lenalidomide have both been shown to have anti-angiogenic and anti-tumor effects in preclinical models (D'Amato, Loughnan et al. 1994; Short, Traish et al. 2001). The anti-angiogenic effects are thought to be related to a hepatic metabolite that inhibits endothelial growth, although the exact mechanism is unclear (Short, Traish et al. 2001). Additionally, an early clinical trial conducted by Baumann et al. showed that the combination of Thalidomide with Temozolomide appeared to be more effective than Thalidomide alone in the treatment of GBM (Baumann, Bjeljac et al. 2004). Additional studies are underway to examine the role of Thalidomide in combination with other anti-glioma agents.

4.7.4. Tandutinib

Tandutinib (MLN0518) is an active inhibitor of type III receptor kinases with activity against PDGF receptors alpha and beta, FLT3, and c-KIT. Its anti-angiogenic effects appear to be mediated by interruption of PDGF/PDGFR. It is currently being investigated in combination therapy with other agents against malignant glioma (Boult, Terkelsen et al. 2013).

5. Immunotherapy for malignant gliomas

Our immune system can be viewed as an intricate balance of opposing functions that lead to either immunity or tolerance. Perturbations that disrupt this stable equilibrium could lead to autoimmune disease or tolerance to malignant cells. In general, the immune system has the ability to recognize and to react to foreign antigens, which leads to their removal as well as to the destruction of cells that express them. Before attempting immunotherapy for cancer, one needs to understand the crucial balancing acts of the immune response that eventually lead to a desired outcome; in addition, the central nervous system has unique features that require special considerations.

In this section, our goals are to introduce the readers to the basics of peripheral immunology focusing on how foreign antigens activate the immune response leading to immunity vs. tolerance. Nevertheless, a detailed discussion of immunity is not within our scope; in some disciplines, we will just be scratching the surface. We will detail antigen processing and presentation, T cell priming, with attention to the synapse between T cells and the antigen-presenting cell (APC) and clonal expansion. Because of our interest in brain tumors, we will compare the systemic immune response to that of the CNS, discussing the historical thoughts of the immune privileges of the CNS and more recent evidence of processing of CNS antigens via the glymphatic pathway. The stage will be set for a discussion of immunotherapy for brain tumors, including priming in the periphery, priming in the CNS, and passive transfer of immunity. The last section lists the clinical trials that employ immunotherapy for brain tumors and their proposed modes of action.

5.1. Peripheral immunology

As part of the adaptive immune system, antigens enter the body through epithelium and are immediately met in the infected tissue by APCs, most commonly dendritic cells (DCs), which then process the antigens into protein fragments (Hugues 2010; Joffre, Segura et al. 2012; Abbas, Lichtman et al. 2014). DCs are a type of APC that can induce priming of naïve CD4⁺ and CD8⁺ T cells into helper and cytotoxic T cells through a series of steps that include antigen processing, antigen presentation, and interactions with co-stimulatory molecules, in addition to the secretion of various cytokines (Hivroz, Chemin et al. 2012). If not processed locally by an APC, the antigens drain into lymph nodes via lymphatic vessels where an APC will be waiting (Abbas, Lichtman et al. 2014). These antigens are processed internally through degradation in the cytosol, processing in the endoplasmic reticulum, and transportation to the cell surface by the Golgi apparatus (Joffre, Segura et al. 2012). APCs then travel to the lymph nodes, where naïve T cells can recognize displayed protein fragments of antigens (Abbas, Lichtman et al. 2014). DCs serve as the most specialized of the APCs and assist in differentiating naïve T cells into both effector and memory cells. Once activated, effector cells then travel via the blood stream to the site of infection where they can recognize antigens being presented by other types of cells and initiate cytotoxic responses (Abbas, Lichtman et al. 2014).

All nucleated cells in the body display a major histocompatibility complex (MHC) I molecule for presenting processed pathogens or infected cells to T lymphocytes once detected (Joffre, Segura et al. 2012). Only CD8⁺ T cells bear receptors for MHC I; CD4⁺ T cells bear receptors for MHC II, typically expressed by dendritic cells, macrophages, and B cells (Abbas, Lichtman et al. 2014). Nucleated cells produce peptide antigens from viruses living in the cell, phagocytosed and endocytosed organisms, and proteins derived from mutated self-genes (Joffre, Segura et al. 2012; Abbas, Lichtman et al. 2014). Traditionally, exogenous antigens are presented via MHC II-bearing cells and endogenous antigens by MHC I cells, but cross-presentation permits MHC I cells to present exogenous antigens (Jarry, Jeannin et al. 2013). Additionally, DCs can ingest virally-infected host cells and present the processed antigens via MHC I to CD8⁺ naïve T cells through cross-priming (Abbas, Lichtman et al. 2014). Similarly, infected DCs can prime CD8⁺ T cells via MHC I by directly presenting the processed antigen

(Joffre, Segura et al. 2012). This cross-priming process has been implicated in immune responses not only to infection, but also to cancer and autoimmune disease (Jarry, Jeannin et al. 2013)

5.1.2. T cell priming and activation

T cell priming by DCs induces activation, cytokine secretion, and clonal proliferation (Mempel, Henrickson et al. 2004). For T-cell activation, both the MHC-bound antigen and the MHC itself must be recognized by the T-cell receptor (TCR) and the co-receptor, respectively (Abbas, Lichtman et al. 2014). This process of priming naïve T cells into effector and helper cells occurs in lymphoid organs (Joffre, Segura et al. 2012). DCs prime the T cells during three stages: 1. Contact for exchange of information between the T cell and the dendrite in the lymphocyte pool, 2. The formation of a stable bond followed by secretion of interleukin-2 and interferon- γ , and 3. Rapid movement and clonal expansion (Mempel, Henrickson et al. 2004).

The synapse between the T cell and the APC requires the interaction of not only the TCR and MHC, but also adhesion molecules and co-receptors to receive signals from the APC (see section 5.1.3 below). Early on during this process various cytokines are released. Certain cytokines lead to clonal expansion of antigen-specific lymphocytes, some of which become differentiated into effector T cells that can remove infected cells. Others differentiate into memory T cells that serve to remain inactive until re-exposed to the same antigen (Abbas, Lichtman et al. 2014). During future encounters, DC-bearing antigens will migrate to the paracortical region in the lymph node to search for a T cell that recognizes its antigen, ultimately activating clonal expansion (Bousso, 2003).

5.1.2.1. T lymphocyte – Antigen presenting cell contact

To begin the process of priming T cells, the DC must physically contact the naïve T cell. This process tends to occur in lymphoid tissue, specifically in the draining lymph nodes, spleen, and Peyer's patches, after infiltration of antigen-APC complexes from peripheral tissue through lymph vessels (Mempel, Henrickson et al. 2004; Hugues 2010). Mempel et al. showed that naïve T cells re-circulate continually between the blood and lymph nodes searching for antigen. In the absence of antigen, the T cells move randomly in the three dimensions in a stop-and-go manner leading to approximately 500-5000 T cells contacting one DC per hour (Mempel, Henrickson et al. 2004; Miller, Hejazi et al. 2004; Hugues 2010). In the absence of antigen, DCs enter the lymph node via the sub-capsular cortex and travel to the paracortex where T cells are localized. Then, the dendrites on DCs scan the T cells which results in transient interactions of up to a few minutes (Hugues 2010). In the presence of antigen, the data supports a three-phase model. First, within a few hours of lymph node entry, contact between naïve T cell-DC with peptide increases in duration, now lasting up to five minutes. Within ten hours of antigen entry into the lymph node, mobility slows dramatically as T cells and DCs form more stable bonds, which will persist from two to twenty four hours. This step also promotes the up-regulation of activation markers. After thirty hours, the bonds separate and this is followed by increased mobility of T cells, corresponding with T cell proliferation (Miller, Wei et al. 2002; Mempel, Henrickson et al. 2004; Hugues 2010).

5.1.2.2. Naïve T lymphocyte – Antigen presenting cell synapse

Naïve T cells are constantly searching for presented antigen on the MHC-antigen complex of mature DCs, from which the T cell and its receptor will require co-stimulation (Mempel, Henrickson et al. 2004). T cell activation relies on the successful synapse of the T cell receptor (TCR) with the peptide-MHC complex on the APC. Additionally, several signaling complexes must connect between the TCR and the adaptor protein linker for activation of T cells and subsequent filamentous actin (F-actin)-dependent TCR cluster formation (Dustin and Depoil 2011). The role of co-stimulatory and co-inhibitory proteins is to modulate the TCR signal to increase or decrease activation of the T cell or to direct the response of that cell down a particular differentiation pathway (Dustin and Depoil 2011).

Antigen recognition and adhesion involves simultaneous recognition of many molecules. In the receptor layer, antigen recognition occurs by the TCR to the peptide with its co-receptor CD4 or CD8 binding MHC II or MHC I, respectively. This is the first step in the signal cascade. For signal transduction and co-stimulation, several transmembrane signaling molecules, including CD3 and ζ chain, form part of the TCR complex and bind the MHC/antigen complex. Additionally, CD28 (and CTLA-4) on the T cell binds B7-1 (CD80)/B7-2 (CD86) on the APC (Dustin and Depoil 2011). The B7 proteins are created by APC in response to an antigen to ensure that T cells are not activated by self-antigens. This key bond is essential for signaling and thus activation of naïve T cells. Concurrently, the CD40 Ligand on the T cell and CD40 on the APC unite and promote increased production of B7 and secretion of cytokines in the APC in order to further encourage T cell activation (Abbas, Lichtman et al. 2014). For adhesion, the T-cell integrin LFA-1 (Leukocyte function-associated antigen 1) binds ICAM-1 (Intercellular adhesion molecule) on the APC (Dustin and Depoil 2011; Abbas, Lichtman et al. 2014).

The co-stimulatory signals play a key role in determining immunity or tolerance. Due to the required co-receptors and signal transduction, many mechanisms are set in place to prevent T cells from activating against self-protein. Through early central tolerance mechanisms designed to prevent autoimmune disease, immature T cells that react to self-proteins undergo apoptosis early in development (Luptrawan, Liu et al. 2008). This activation-induced cell death is assisted through the interaction of Fas, which is expressed everywhere and in high concentration in the thymus, with Fas Ligand on T lymphocytes and NK cells (Maher, Toomey et al. 2002); a similar process results in clonally expanded T cells after they are no longer needed. Similarly, if a T cell encounters an antigen on an APC without the appropriate co-stimulation, it is susceptible to developing tolerance to that antigen such that on future encounters it will ignore it, even if given the appropriate co-stimulation (Luptrawan, Liu et al. 2008; Abbas, Lichtman et al. 2014). On cross-presentation by dendritic cells with MHC I and CD8+ T cells, clonal deletion, functional inactivation (anergy) or programming into a suppressive (regulatory) T cell phenotype can result (Joffre, Segura et al. 2012).

5.1.2.3. Clonal expansion

To amplify activation, T cells and APCs secrete various cytokines. Initially, T cells secrete interleukin-2 (IL-2), which facilitates the binding of IL-2 by augmenting the presence of IL-2

receptors. IL-2, by acting on the T cell that secreted it, supports the production of T cells specific to the antigen. IL-2 is also needed to maintain regulatory T cells (Abbas, Lichtman et al. 2014).

Clonal expansion transpires in 1-2 days, leading to the creation of antigen-specific CD4+ and CD8+ cells. CD8+ cells develop into effector cells that ultimately migrate to the site of infection to interact with the specific antigen to which they were primed. CD4+ cells further develop into T helper I (Th1) and T helper 2 (Th2) lymphocytes. Antigen-exposed macrophages and DCs release IL-12 and natural killer cells secrete interferon- γ , thereby promoting the differentiation of Th 1 cells (Abbas, Lichtman et al. 2014). Th1 cells secrete IL-2, interferon- γ , and lymphotoxin-a which leads to type 1 immunity with enhanced macrophage activation and phagocytosis (Spellberg and Edwards 2001). Interferon- γ also increases the expression of MHC I and II molecules to amplify antigen presentation (Spellberg and Edwards 2001). Th2 cells, stimulated by IL-4, also release IL-4, IL-5, IL-9, IL-10, and IL-13 promoting production of antibodies and type 2 immunity, which minimizes phagocytosis and decreases inflammation (Spellberg and Edwards 2001). In times of overwhelming systemic response or immunosuppression, a type 2 response can supersede the appropriate type 1 response (Spellberg and Edwards 2001).

All the aforementioned steps have to be executed flawlessly to achieve immunity against a tumor or a tumor antigen. The body is set up to have low affinity to self-antigens, as would be expressed on tumor cells (Luptrawan, Liu et al. 2008). Along those lines, dendritic cells, which have been discovered in tumors, play a large role in presentation of tumor antigens; however, that does not necessarily predict the nature of the immune response. In particular, any flaw in dendritic cells, from cross presentation to IL-12 production, will lead to tolerance and impaired CD8+ T cell response to tumors (Joffre, Segura et al. 2012).

5.2. Central nervous system immunology

The CNS was once thought to be immunologically-privileged because of its unique immunological features, including 1) lack of immunological surveillance due to low expression of MHC molecules, 2) lack of distinct lymphatic drainage, and 3) protection by the blood brain barrier (BBB), which limits the movement of naïve T cells into the CNS (Chavarria and Cardenas 2013). Nonetheless, the CNS has more recently been discovered to have a finely tuned immune surveillance managed by APC, believed to be microglia, DC, perivascular macrophages and meningeal dendritic cells (Fathallah-Shaykh, Gao et al. 1998; Yang, Han et al. 2010; D'Agostino, Gottfried-Blackmore et al. 2012; Ousman and Kubes 2012; Romo-Gonzalez, Chavarria et al. 2012; Chavarria and Cardenas 2013). Furthermore, more recent evidence, as can be found in gliomas and multiple sclerosis, suggests that the CNS microglia coordinate with peripheral T cells and APC (Yang, Han et al. 2010). Such evidence describes more active inspection of the BBB in specific regions of the brain most notably the meninges, circumventricular organs, and choroid plexus (D'Agostino, Gottfried-Blackmore et al. 2012).

5.2.1. Centrally-acting peripheral immune cells

In addition to resident microglia, the primary immune cell in the CNS, peripheral immune cells including peripherally activated T lymphocytes, macrophages, and DC circulate in small numbers within the CNS. They are predominantly in specialized CNS compartments located outside the parenchyma with ability to gain access to the parenchyma through various mechanisms that include the choroid plexus, perivascular or Virchow-Robin spaces, meningeal vessel branch points into the subarachnoid space, and through post-capillary venules (Ousman and Kubes 2012). As in the periphery, these cells are capable of mounting an activated immune response if they encounter an antigen (Ousman and Kubes 2012; Jarry, Jeannin et al. 2013). Additionally, perivascular macrophages sample CSF and can phagocytose suspected antigens (Ousman and Kubes 2012). There is also separate evidence of drainage of CNS antigens to deep cervical lymph nodes, based on intracranial injection of labeled antigen (D'Agostino, Gottfried-Blackmore et al. 2012; Ousman and Kubes 2012). Despite controversy over poor immune surveillance due to low expression of MHC II, it is thought that pre-activated T cells can release IFN- γ and TNF- α to simulate MHC II molecule expression (Romo-Gonzalez, Chavarria et al. 2012). Also of debate is the function of central antigen presentation by central DCs. It is known that the integrity of the BBB is compromised during times of infection, trauma, aging, and autoimmunity due to weakening of the vascular endothelium as a result of cytokine release by astrocytes and microglia (D'Agostino, Gottfried-Blackmore et al. 2012; Romo-Gonzalez, Chavarria et al. 2012).

5.2.2. Microglia

Microglia play a large role in both innate and adaptive immune responses, in addition to regulatory roles in the CNS (Yang, Han et al. 2010). They comprise 5-12% of all CNS cells and are uniformly distributed throughout the CNS parenchyma (D'Agostino, Gottfried-Blackmore et al. 2012). Similar to the peripheral immune APCs, microglia express MHC I and II molecules and CD 80/86 and CD40 co-stimulatory molecules that once activated, proliferate and phagocytose in response to both CD4+ and CD8+ T cells (Fathallah-Shaykh, Gao et al. 1998; Yang, Han et al. 2010; Ousman and Kubes 2012). In the latent state, microglia survey the microenvironment via pinocytosis. Once they sense infection, neuronal injury, or neurodegenerative disease, they up-regulate the expression of MHC and co-stimulatory molecules and release cytokines including IL-1, IL-6, and TNF-alpha as well as neurotrophic and cytotoxic factors, and chemokines for lymphocyte recruitment (Yang, Han et al. 2010; Jarry, Jeannin et al. 2013). These pro-inflammatory cytokines then make the BBB more soluble for entry of peripheral immune cells and potentially naïve T lymphocytes (Yang, Han et al. 2010). Microglia's phagocytic and cytotoxic features are also up-regulated with the triggering of an immune response (Yang, Han et al. 2010). As in the peripheral immune response, microglia CD80/CD86 and CD40 bind the T cell's CD28 and CD40L, respectively (Yang, Han et al. 2010). IFN- γ release sustains this response and promotes phagocytosis and direct tumor-cell cytotoxicity (Fathallah-Shaykh, Gao et al. 1998; Yang, Han et al. 2010; D'Agostino, Gottfried-Blackmore et al. 2012).

Similar to peripheral tolerance, if there is insufficient co-stimulatory response, the interaction of Fas ligand (FASL) on microglia and Fas receptor on the T cell leads to activation-induced T

cell apoptosis. Microglia also express FAS molecules themselves, which induce apoptosis upon binding FASL (Yang, Han et al. 2010). Nitric oxide released by microglia in response to activation can also potentiate effector cell death (Yang, Han et al. 2010). Furthermore, microglia display B7-H1 molecules which also support immunosuppression by stimulating T cell apoptosis (Yang, Han et al. 2010). Additionally, glycoprotein CD200 down regulates activated microglia (via CD200 ligand on microglia) and perivascular macrophages in the CNS, acting as an anti-inflammatory and serving to keep microglia in a quiescent state (Ousman and Kubes 2012; Chavarria and Cardenas 2013).

5.2.3. *The glymphatic pathway*

The CNS lacks lymphoid tissue. For appropriate immune surveillance, both antigens and APC must be able to travel to lymphoid tissue, ideally via lymphatic channels, for T cell priming (Romo-Gonzalez, Chavarria et al. 2012). For small and hydrophobic molecules as well as transporter substrates, exit through the BBB is easy. Other substances are cleared from CSF through arachnoid granulations or peripheral lymphatics on cranial nerves. Clearance of large particles and matter deep within the parenchyma is more difficult and is ascribed to a high rate of flow of interstitial fluid (Iliff and Nedergaard 2013). This flow of fluid transports antigens from the brain parenchyma for presentation in cervical lymph nodes (Romo-Gonzalez, Chavarria et al. 2012). Through what has been termed the glio-vascular or glymphatic pathway, interstitial solutes are cleared from the brain to the peripheral lymphatic system via perivascular water channels from the para-arterial CSF influx pathway through the interstitium and along the para-venous clearance route (Iliff and Nedergaard 2013). Once filtered from the CNS, antigens are captured by APCs in the cervical lymph nodes and activate lymphocytes that then migrate to the CNS in search for remaining antigens (Romo-Gonzalez, Chavarria et al. 2012). As noted above, several hypotheses for lymph-like drainage of antigens exist including efferent flow via CSF and interstitial fluid past the optic, trigeminal, and acoustic nerves to the cervical lymph nodes, reabsorption through arachnoid villi into the venous sinuses, and through perivascular APC including macrophages and DC (Romo-Gonzalez, Chavarria et al. 2012).

A more recent study by Jarry et al. showed that adult microglia can cross-present antigen to naïve CD8+ T cells for priming if there is appropriate microglial activation (Jarry, Jeannin et al. 2013). Their study involved injecting naïve T cells into the brain, as the natural presence of naïve T cells in the brain is limited, with restriction of entry to activated T cells instead. Their study suggests that if naïve T lymphocytes are given the ability for entry into the brain, typically during stressful inflammatory illnesses, coupled with the appropriate microglial response, cross-priming of naïve T cells is possible (Jarry, Jeannin et al. 2013).

5.2.4. *Glioma-associated microglia*

Glioma-associated microglia/macrophages cannot mount a successful anti-tumor T cell response (Yang, Han et al. 2010). Microglia, along with some T lymphocytes, infiltrate gliomas in a pattern that was initially thought to be an immune response against tumor cells but has been more recently realized to actually encourage tumor growth by promoting immunosup-

pression (Yang, Han et al. 2010). Pathological examination typically reveals a large numbers of microglia dispersed within the tumor and not just in necrotic tissue (Yang, Han et al. 2010). The data of Okada et al. suggest that the glioma-infiltrating cells may compose up to 30% of the glioma tumor, correlating in volume with degree of malignancy (Okada, Kohanbash et al. 2009). The lack of phagocytosis by the microglia is thought be related to decreased expression of MHC II and co-stimulatory molecules CD80/86 and CD40, thus prohibiting appropriate T cell activation (Okada, Kohanbash et al. 2009; Yang, Han et al. 2010). Glioma cells appear to attract microglia by secreting chemoattractants and growth factors including Macrophage Chemoattractive Protein-1 (MCP-1), which binds to the microglial MCP-1 receptor, as well as colony stimulating factor-1, Granulocyte-CSF, and hepatocyte growth factor/scatter factor (Yang, Han et al. 2010). Microglial secretion of epidermal growth factor (EGF), VEGF and MCP-1 promote tumor propagation and angiogenesis (Okada, Kohanbash et al. 2009; Yang, Han et al. 2010). Additionally, the release by microglia of MMPs assists in tumor dispersal (Yang, Han et al. 2010). Interestingly, tumors depleted of microglia actually become less invasive (Okada, Kohanbash et al. 2009).

In addition to altering the response of microglial cells, gliomas take an active role in down-regulating the immune response. Recent data has shown that reduced phagocytic activity by glioma-associated microglia stems from defective antigen presentation for T cell activation due to decreased MHC II expression as well as suppression of pro-inflammatory cytokine (TNF- α) release, especially in high-grade gliomas (Yang, Han et al. 2010). Instead, glioma cells favor TGF- β , IL-10, and PGE2 secretion, which inhibits both cytotoxic function of T cells and IFN- γ -induced MHC II expression in microglial cells (Luptrawan, Liu et al. 2008; Okada, Kohanbash et al. 2009; Yang, Han et al. 2010). PGE2 specifically inhibits T cell activation, suppresses natural killers cell activity, and favors a Th2 response by increasing cytokines Il-4, Il-10, and Il-6 while suppressing the Th1 cytokines Il-2, IFN-gamma, and TNF- α (Luptrawan, Liu et al. 2008). Additionally, glioma cells do not express adequate co-stimulatory molecules required for appropriate T cell activation, potentiating anergy through tolerance (Luptrawan, Liu et al. 2008). A homologue to the B7 family (B71/2 (CD80/86)), B7-H1 expression on the surface of glioma cells inhibits CD4+ and CD8+ T cell activation. IFN- γ not only enhances antigen processing but also promotes increased B7-H1 expression, ultimately reducing T lymphocyte effectiveness in the presence of gliomas (Okada, Kohanbash et al. 2009). Additionally, some gliomas display Fas-L leading to apoptosis of Fas-labeled T cells contacting the tumor cells (Okada, Kohanbash et al. 2009).

6. Immunotherapy strategies and targets

Immunotherapy for malignant gliomas is based on various strategies aimed at the induction of anti-tumor immunity. Nevertheless, though curing a mouse from a brain tumor using immunotherapy is rather easy, this goal has proven to be more challenging in humans especially when coupled with the globally impaired immune response and increased tumor tolerance in patients with GBM (Luptrawan, Liu et al. 2008). Because of the aforementioned data, the goal of immunotherapy for gliomas should be not only to activate the cytotoxic T cell

response, but also to counteract the active immunological depressive effects by the tumor itself. We will not list an exhaustive search of all immunotherapeutic strategies but will instead discuss an outline of the approaches that can be used. A thorough discussion can be found in Okada et al. (Okada, Kohanbash et al. 2009). Here, we will emphasize the different categories and discuss limitations of immunotherapy.

6.1. Priming in the periphery

Initiating an immune response against tumors is typically difficult due to poor antigen presentation and the active immunosuppressive effects by tumor cells (Luptrawan, Liu et al. 2008). Peripheral vaccination has been performed using purified antigen and irradiated genetically modified tumor cells. Through vaccination with a tumor antigen, one hopes to induce an immune response peripherally, which translates to CNS immunity as activated T cells cross the BBB. This goal may be achieved by processing the antigen via APCs at the subcutaneous injection site, migration to lymph nodes, and priming naïve T cells. Nevertheless, choosing an appropriate antigen is crucial so as to avoid an autoimmune response causing encephalitis (Okada, Kohanbash et al. 2009).

Peptide-based vaccines (see Table 1) for glioma epitopes are synthetically derived for specific antigens and run less risk of autoimmune encephalitis. This process has the potential to be individually tailored based on assessment of the patient's peripheral blood for positive response to the various antigens (Okada, Kohanbash et al. 2009). Many antigen epitopes exist and will be briefly covered. IL-13R α 2 appears as a membrane protein in more than 80% of gliomas but not in normal brain tissue, making it a target for immunotherapy (Debinski, Gibo et al. 1999). The tyrosine kinase receptor EphA2, which is involved in cell-cell contact in normal cells, contributes to malignant nature of tumor cells (Kinch, Moore et al. 2003). T-cell epitopes of Survivin, an apoptosis inhibitor protein present in several human cancers, have shown promise via vaccination for patients with pancreatic cancer and melanoma (Otto, Andersen et al. 2005; Wobser, Keikavoussi et al. 2006). These proteins are found in 100% of astrocytomas but not in normal brain tissue (Uematsu, Ohsawa et al. 2005; Okada, Kohanbash et al. 2009). Wilm's Tumor 1 gene, a transcription factor oncogene, is also present in many tumor types, including the majority of GBM but not in normal glial cells (Sugiyama 2002). The transcriptional cofactor family SOX, Sry-Related High-Mobility Group Box, is present in normal tissue development and is upregulated in various tumors, including gliomas. Vaccinations with SOX have been shown to be therapeutic in mice with gliomas (Ueda, Kinoshita et al. 2008; Okada, Kohanbash et al. 2009). HER-2/neu, in the EGFR family, promotes tumor growth by inhibiting apoptosis and stimulating migration, adhesion, and angiogenesis in many tumor-types, most notably breast, ovarian, colorectal, pancreatic, renal-cell, and GBM (Meric-Bernstam and Hung 2006; Okada, Kohanbash et al. 2009). Additional epitopes have been identified involving EGFR variant III, found in 30-50% of GBMs, Squamous Cell Carcinoma Antigen Recognized by T Cells 1 (SART-1), a gene-coding tumor antigen in many cancer types, including glioma but not in normal tissue, and Cytomegalovirus, which infects a large number of gliomas and may contribute to glioma pathogenesis (Cobbs, Harkins et al. 2002; Saikali, Avril et al. 2007; Okada, Kohanbash et al. 2009).

In addition to using purified antigen as above, whole glioma cells may be used for vaccination (See Table 2). In this process, tumor cells, either autologous or allogeneic, are grown *in vitro*, irradiated, and injected back into the patient (Wikstrand and Bigner 1980; Zhang, Eguchi et al. 2007). The benefit of whole cell vaccinations is the availability of multiple associated antigens and, specifically, the ones expressed by the individual patient's glioma (Okada, Kohanbash et al. 2009).

As a means of bypassing local antigen presentation at the site of the tumor, DC vaccination has also been a source for many clinical trials with various techniques of uniting the DC with the antigen (See Table 3). Some have used DCs pulsed with autologous glioma cell peptides and have shown promise when the DC vaccines were given both into the tumor and subcutaneously (Yamanaka, Homma et al. 2005; Okada, Kohanbash et al. 2009; D'Agostino, Gottfried-Blackmore et al. 2012). Through loading autologous DCs, one can use either tumor lysates, apoptotic tumor cells or tumor-based cDNA (D'Agostino, Gottfried-Blackmore et al. 2012). DC-glioma cell fusion, to create a multinucleated cell such that the DC can present tumor antigen, has also shown potential (D'Agostino, Gottfried-Blackmore et al. 2012). The results of DC vaccinations are encouraging; in one study, repeat surgical resection showed infiltration into the tumor of appropriate CD8⁺ T cells (Luptrawan, Liu et al. 2008). Furthermore, DC vaccination was well tolerated by 12 GBM patients; the median OS was 23.4 months as compared to 18.3 months in controls. In addition to best method of preparing the vaccine, several questions remain unanswered including the best DC subtypes to use, ideal conditions and co-stimulation, prime route of administration, and the correct vaccination dosing and frequency (Okada, Kohanbash et al. 2009). Additional obstacles include the initial immune state of the host prior to vaccination; for example, patients with increased tumor burden have elevated levels of TGF- β and IL-10, which inhibit entry into a cytotoxic response (Luptrawan, Liu et al. 2008).

6.2. Priming in the brain

Fathallah-Shaykh et al. showed that priming in the brain elicits an anti-tumor response leading to destruction of the brain tumor as well as to anti-tumor systemic immunity in animals (Fathallah-Shaykh, Gao et al. 1998). The basic mechanisms for eliciting such an immune response in the CNS are detailed above. One possible method consists of injecting DCs directly into the tumor; the goal is to enhance local antigen processing followed by glymphatic drainage and priming in cervical lymph nodes (Luptrawan, Liu et al. 2008). Early preliminary results in humans are encouraging. In a study of 10 patients with glioma, half received subcutaneous vaccination of pulsed DC with autologous tumor lysate and the other half received both subcutaneous vaccine and intra-tumoral injection of immature autologous DC. On follow-up imaging, the patients who received both therapies showed diminution of contrast-enhancing tumor (Yamanaka, Yajima et al. 2003; Luptrawan, Liu et al. 2008). A phase I/II trial including 24 patients with Grade III or IV glioma at first recurrence evaluated the safety and benefits of DC immunotherapy given either via subcutaneous injection near a cervical lymph node or both subcutaneously and intra-tumorally via an Ommaya reservoir. The study revealed that patients with both intratumoral and intradermal administrations had a longer survival times

than patients with intradermal administration only (Yamanaka, Homma et al. 2005; Luptrawan, Liu et al. 2008). Another method, which has shown promise in animal models was used by Choi et al and involves the injection of chimeric antigen receptors-transduced T cells targeting EGFR variant III into mice gliomas. The results show a dose-dependent increase in survival, while at the same time sparing cytotoxicity to normal brain tissue (Choi, Suryadevara et al. 2013).

6.3. Passive transfer of immunity

In passive immunotherapy, the patient is given *effector* cells or molecules. Such therapies include monoclonal antibodies, radio-nucleotides that are conjugated to monoclonal antibodies, coupled toxins, and T cells.

6.3.1. Transfer of monoclonal antibodies

The use of monoclonal antibodies (see Table 4) for CNS targets necessitates overcoming important barriers (Okada, Kohanbash et al. 2009); for instance, the size of monoclonal antibodies, around 150kDa, impairs their diffusion into the CNS. However, evidence suggests that the BBB both in normal patients and those with malignancy tolerates the entry of monoclonal antibodies (Chen and Mitchell 2012). Additionally, antibodies bound to the tumor boundary layer create a concentration gradient that makes it difficult for additional antibodies to permeate against a concentration gradient, essentially not being able to reach the core of the tumor. This option may be more valid for use in conjunction with surgical resection and convection enhanced delivery (CED) where the agent of choice is given at high pressure and in bulk through an intracranial catheter into the brain tumor and parenchyma (Okada, Kohanbash et al. 2009). As opposed to using diffusion, this method uses bulk flow and has been implemented in several clinical trials. While bypassing the BBB and limiting systemic toxicities, a limitation of this method is that it can be slow and thus difficult to deliver high volumes of molecules (Bobo, Laske et al. 1994; Ferguson and Lesniak 2007; Okada, Kohanbash et al. 2009).

Several targets for monoclonal antibodies have been investigated in clinical trials. Epidermal growth factor receptor (EGFR) antibodies target the EGFR on glioma cells, over-expressed on 40-50% of tumors (Rivera, Vega-Villegas et al. 2008; Okada, Kohanbash et al. 2009). EGFR is a transmembrane receptor responsible for initiating gene transcription and thus increased tumor growth and spread (Baselga 2001). A variant of EGFR, EGFR variant III, is often found in GBM (Batra, Castelino-Prabhu et al. 1995). The monoclonal antibody Cetuximab inhibits this EGFR pathway, including glioma cells expression variant EGFR (Fukai, Nishio et al. 2008). Nimotuzumab works similarly (Ramos, Figueredo et al. 2006).

Radio-immunotherapy (RIT) via radionucleotides conjugated to monoclonal antibodies is another technique for targeting specific tumor antigens (see Table 5). This technique delivers localized, cytotoxic radiation to tumor cells resulting in cell death. This method is used concurrently with surgical resection into the surgical cavity. Specifically, antitenascin has been most studied for RIT due to high prevalence of the glycoprotein tenascin on the surface of high-

grade gliomas, including 90% of GBMs (Zalutsky 2004). Duke University has developed the specific antibody 81C6, which has shown promise when given into the tumor cavity concurrently with resection (Zalutsky, Moseley et al. 1989; Okada, Kohanbash et al. 2009). Several other clinical trials have used similar approaches with RIT and glycoprotein tenascin. Other targets include the DNA/Histone H1 complex, the extra domain B of fibronectin, and the alpha chain of the IL-2 receptor (Okada, Kohanbash et al. 2009).

6.3.2. *Transfer of ligands (cytokines)*

Via coupled targeted toxins, cytokines fused with toxins can be delivered to tumor cells (see Table 6). Specifically, IL-4R and IL-13R α 2 expression is increased in high-grade gliomas making them ideal targets for chimeric fused proteins. For these chimeras, pseudomonas exotoxin is fused to IL-4 and IL-13, creating IL4-PE and IL13-PE, respectively (Debinski, Obiri et al. 1995; Joshi, Leland et al. 2001). These proteins are then delivered via CED (Okada, Kohanbash et al. 2009). By combining toxins with cytokines, one can target tumor receptors and induce cytotoxicity. Additional chimeras have been made using diphtheria toxin, which bonds to transferrin, and TGF α , which binds to Pseudomonas exotoxin.

6.3.3. *Transfer of cells*

For the adoptive transfer of tumor-reactive autologous cytotoxic T lymphocytes (see Table 7), various techniques are used to create an antigen-specific receptor on a CD8⁺ T cell that can prompt T cell activation (Okada, Kohanbash et al. 2009). This process has previously been used in conjunction with IL-2 infusion for the treatment of melanoma. Antigen-specific cytotoxic T cells from peripheral blood or from tumor nodules are isolated from the patient. The T cells will then undergo clonal expansion *in vitro* with specificity for tumor antigen, possibly with the aid of IL-2. These cells are then returned to the patient where they would in theory perform cytotoxic responses upon recognition of tumor-associated antigen in the brain parenchyma (Okada, Kohanbash et al. 2009). In terms of usage in gliomas, the first steps would be creating a library of highly avid cytotoxic T cell clones from which to build highly selective TCR gene pairs to create transgenic cytotoxic T cells. Adoptive therapy is not limited to CD8⁺ T cells and has been also tried using NK cells and CD4⁺ T cells, both of which can be similarly removed, expanded, and injected back into the patient. Blancher et al treated 13 GBM by recombinant IL-2, with and without lymphokine activated killer cells, given directly via a catheter into the tumor resection bed. Unfortunately, the treatment had no effects on tumor progression. The adverse reactions included cerebral edema, confusion, and fever (Blancher, Roubinet et al. 1993).

Some obstacles with the adoptive process include creating T cells with TCR of appropriate avidity (Okada, Kohanbash et al. 2009). Further difficulties arise with T cell reproduction; many of these specialized T cell populations are thought to be terminally differentiated and thus unable to propagate long-term existence (Wherry, Teichgraber et al. 2003). Additionally, these transgenic T cells must also overcome the immunosuppressive features of GBM and, in fact, do so better than natural T cells due to the ability to manipulate them and strengthen them with specific chemokines and integrin receptors (Okada, Kohanbash et al. 2009).

6.4. Limitations of immunotherapy

The limitations of immunotherapy for malignant gliomas include: 1) physical obstacles of drug administration due to the BBB, 2) direct and indirect down-regulation of the immune response by gliomas, 3) the high mutation rate of the tumor, which will select for tumor cells that do not express the target of the immune response. In fact, cancer genomes are unstable as evidenced by microsatellite instability of the tumor cells, which aids in tumor evolution and progression (van de Kelft and Verlooy 1994; Yip, Miao et al. 2009; Milinkovic, Bankovic et al. 2012). Additional limitations include difficulty in monitoring the tumor response to treatment because inducing an inflammatory response may create MRI changes that mimic tumor growth. Immunotherapy is also complicated by the common use of steroids, which suppress the immune system.

In our opinion, the most significant limitation of immunotherapy is the limited understanding of the dynamics of the interactions of cytotoxic T lymphocytes with the tumor microenvironment. Clinical trials using immunotherapy have failed to show a clinically significant therapeutic response despite demonstrating the presence of circulating tumor-specific CTL (Lasalvia-Prisco, Garcia-Giralt et al. 2008; Leffers, Lambeck et al. 2009). The key obstacle that we need to overcome is not the induction of a systemic anti-tumor immune response, but making that immune response effective within the tumor microenvironment

7. Immunotherapy clinical trials for brain tumors

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- A Pilot Study of Glioma Associated Antigen Vaccines in Conjunction With Poly-ICLC in Pediatric Gliomas
 - A Study of Rindopepimut/GM-CSF in Patients With Relapsed EGFRvIII-Positive Glioblastoma
 - Biological Therapy Following Surgery and Radiation Therapy in Treating Patients With Primary or Recurrent Astrocytoma or Oligodendroglioma
 - Effects of Vaccinations With HLA-A2-Restricted Glioma Antigen-Peptides in Combination With Poly-ICLC for Adults With High-Risk WHO Grade II Astrocytomas and Oligo-Astrocytomas
 - GP96 Heat Shock Protein-Peptide Complex Vaccine in Treating Patients With Recurrent or Progressive Glioma
 - HLA-A2-Restricted Glioma Antigen-Peptides Vaccinations With Poly-ICLC for Recurrent WHO Grade II Gliomas
 - HSPPC-96 Vaccine With Temozolomide in Patients With Newly Diagnosed GBM
 - Immunotherapy for Recurrent Ependymomas in Children Treatment for Recurrent Ependymomas Using HLA-A2 Restricted Tumor Antigen Peptides in Combination With Imiquimod
 - Peptide Vaccine for Glioblastoma Against Cytomegalovirus Antigens
 - Peptide-based Glioma Vaccine IMA950 in Patients With Glioblastoma
 - Phase I Study of Safety and Immunogenicity of ADU-623
 - Phase I/II Trial of IMA950 Multi-peptide Vaccine Plus Poly-ICLC in Glioblastoma
 - Phase II Study of Rindopepimut (CDX-110) in Patients With Glioblastoma Multiforme

- Phase III Study of Rindopepimut/GM-CSF in Patients With Newly Diagnosed Glioblastoma (uses EGFR)
- Poliovirus Vaccine for Recurrent Glioblastoma Multiforme (GBM)
- Vaccine Therapy and Sargramostim in Treating Patients With Malignant Glioma
- Vaccine Therapy and Sargramostim in Treating Patients With Sarcoma or Brain Tumor
- Vaccine Therapy in Treating Patients With Newly Diagnosed Glioblastoma Multiforme
- Vaccine Therapy, Temozolomide, and Radiation Therapy in Treating Patients With Newly Diagnosed Glioblastoma Multiforme

Table 1. Tumor Antigen Vaccine

www.clinicaltrials.gov

The above table lists several current clinical trials using tumor-derived antigens for developing peripheral vaccination against CNS tumors. See section 4.1 above.

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- Chemotherapy and Vaccine Therapy Followed by Bone Marrow or Peripheral Stem Cell Transplantation and Interleukin-2 in Treating Patients With Recurrent or Refractory Brain Cancer
 - Derivation of Tumor Specific Hybridomas
 - Imiquimod/Brain Tumor Initiating Cell (BTIC) Vaccine in Brain Stem Glioma
 - Phase I/II Study To Test The Safety and Efficacy of TVI-Brain-1 As A Treatment For Recurrent Grade IV Glioma
 - Pilot Immunotherapy Trial for Recurrent Malignant Gliomas
 - Study to Evaluate the Effects of Imiquimod and Tumor Lysate Vaccine Immunotherapy in Adults With High Risk or Recurrent/Post-Chemotherapy WHO Grade II Gliomas
 - Study To Test the Safety and Efficacy of TVI-Brain-1 As A Treatment for Recurrent Grade IV Glioma
 - Vaccination With Lethally Irradiated Glioma Cells Mixed With GM-K562 Cells in Patients Undergoing Craniotomy For Recurrent Tumor

Table 2. Tumor Lysate or Cell Vaccine

The above table lists several current clinical trials using whole tumor cells to develop peripheral vaccines against multiple antigens found on CNS tumors. See section 4.1 above.

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- A Study of ICT-107 Immunotherapy in Glioblastoma Multiforme (GBM)
 - Biological Therapy in Treating Patients With Glioblastoma Multiforme
 - Daclizumab in Treating Patients With Newly Diagnosed Glioblastoma Multiforme Undergoing Targeted Immunotherapy and Temozolomide-Caused Lymphopenia
 - Dendritic Cell Cancer Vaccine for High-grade Glioma
 - Dendritic Cell Vaccine For Malignant Glioma and Glioblastoma Multiforme in Adult and Pediatric Subjects

- Dendritic Cell Vaccine for Patients With Brain Tumors
- Dendritic Cell Vaccine Therapy With In Situ Maturation in Pediatric Brain Tumors
- Dendritic Cell Vaccine With Imiquimod for Patients With Malignant Glioma
- Efficacy & Safety of Autologous Dendritic Cell Vaccination in Glioblastoma Multiforme After Complete Surgical Resection
- Immunotherapy for Patients With Brain Stem Glioma and Glioblastoma
- Phase II Feasibility Study of Dendritic Cell Vaccination for Newly Diagnosed Glioblastoma Multiforme
- Proteome-based Personalized Immunotherapy of Glioblastoma
- Safe Study of Dendritic Cell (DC) Based Therapy Targeting Tumor Stem Cells in Glioblastoma
- Study of a Drug [DCVax®-L] to Treat Newly Diagnosed GBM Brain Cancer
- Study of DC Vaccination Against Glioblastoma
- Surgical Resection With Gliadel Wafer Followed by Dendritic Cells Vaccination for Malignant Glioma Patients
- Tumor Lysate Pulsed Dendritic Cell Immunotherapy for Patients With Brain Tumors
- Vaccination With Dendritic Cells Loaded With Brain Tumor Stem Cells for Progressive Malignant Brain Tumor
- Vaccination-Dendritic Cells With Peptides for Recurrent Malignant Gliomas
- Vaccine for Patients With Newly Diagnosed or Recurrent Low-Grade Glioma
- Vaccine Immunotherapy for Recurrent Medulloblastoma and Primitive Neuroectodermal Tumor
- Vaccine Therapy and Temozolomide in Treating Patients With Newly Diagnosed Glioblastoma
- Vaccine Therapy in Treating Patients Undergoing Surgery for Recurrent Glioblastoma Multiforme
- Vaccine Therapy in Treating Patients With Malignant Glioma
- Vaccine Therapy in Treating Patients With Newly Diagnosed Glioblastoma Multiforme
- Vaccine Therapy in Treating Young Patients Who Are Undergoing Surgery for Malignant Glioma
- Vaccine Therapy With or Without Sunitinib in Treating Patients With NY-ESO-1 Expressing Solid Tumors

Table 3. Dendritic Cell Vaccine

The above table lists several current clinical trials using dendritic cells combined with tumor antigen as a method of delivering peripheral vaccination against CNS tumors. See section 4.1 above.

-
- A Phase 2 Evaluation of TRC105 in Combination with Bevacizumab for the Treatment of Recurrent or Progressive Glioblastoma That Has Progressed on Bevacizumab
 - Chemotherapy, Radiation Therapy, and Vaccine Therapy With Basiliximab in Treating Patients With Glioblastoma Multiforme That Has Been Removed by Surgery
 - Use of Racotumomab in Patients With Pediatric Tumors Expressing N-glycosylated Gangliosides (anti-idiotypic antibody)

- Vaccine Therapy With Bevacizumab Versus Bevacizumab Alone in Treating Patients With Recurrent Glioblastoma Multiforme That Can Be Removed by Surgery

Table 4. Vaccine with monoclonal antibody

The above table lists several current clinical trials using passive immunotherapy by means of delivering monoclonal antibodies directed at tumor cells. See section 4.3.1 above.

-
- Convection-Enhanced Delivery of 124I-8H9 for Patients With Non-Progressive Diffuse Pontine Gliomas Previously Treated With External Beam Radiation Therapy
 - Intrathecal Radioimmunotherapy, Radiation Therapy, and Chemotherapy After Surgery in Treating Patients with Medulloblastoma
 - Radiolabeled Monoclonal Antibody Therapy in Treating Patients with Primary or Metastatic Brain Tumors
 - Radiosurgery Plus Bevacizumab in Glioblastoma
-

Table 5. Radioimmunotherapy with monoclonal antibody

The above table lists current clinical trials using cytotoxic radiation coupled to monoclonal antibodies to kill tumor cells. See section 4.3.1 above.

-
- IL-4 (38-37)-PE38KDEL Immunotoxin in Treating Patients With Recurrent Malignant Astrocytoma
 - Imaging Study of the Distribution of IL13-PE38QQR Infused Before and After Surgery in Adult Patients With Recurrent Malignant Glioma
 - NBI-3001 Followed by Surgery in Treating Patients with Recurrent Glioblastoma Multiforme
 - TP-38 Toxin in Treating Young Patients with Recurrent or Progressive Supratentorial High-Grade Glioma
-

Table 6. Transfer of Ligands

The above table lists current clinical trials using molecules fused with toxins as a means of killing tumor cells.

-
- A Phase I Study to Investigate Tolerability and Efficacy of ALECSAT Administered to Glioblastoma Multiforme Patients
 - Autologous Natural Killer T Cells Infusion for the Treatment of Cancer
 - Cellular Adoptive Immunotherapy in Treating Patients With Glioblastoma Multiforme
 - Cellular Adoptive Immunotherapy Using Genetically Modified T-Lymphocytes in Treating Patients With Recurrent or Refractory High-Grade Malignant Glioma
 - Cellular Immunotherapy Study for Brain Cancer
 - CMV-specific Cytotoxic T Lymphocytes Expressing CAR Targeting HER2 in Patients with GBM (HERT-GBM)

- Evaluation of Recovery From Drug-Induced Lymphopenia Using Cytomegalovirus-specific T-cell Adoptive Transfer
- Phase I Study of Cellular Immunotherapy for Recurrent/Refractory Malignant Glioma Using Intratumoral Infusions of GRm13Z40-2, An Allogeneic CD8+ Cytotoxic T-Cell Line Genetically Modified to Express the IL 13-Zetakine and HyTK and to be Resistant to Glucocorticoids, in Combination With Interleukin-2
- Safety and Effectiveness Study of Autologous Natural Killer and Natural Killer T Cells on Cancer
- White Blood Cells With Anti-EGFR-III for Malignant Gliomas

Table 7. Adoptive Immunotherapy

The above table lists several current clinical trials using adoptive immunotherapy as a way of applying passive immunotherapy to return autologous tumor-antigen-specific T cells back to the patient as a means of targeting CNS tumors. See section 4.3.3 above.

8. Gene therapy

Gene therapy as it relates to malignant gliomas is based on tumor-specific introduction of genetic material for the purpose of treatment. It involves direct injection of a gene transfer vector or vector producing cells (VPC) into the tumor itself or into the cavity left after resection. Although preclinical studies have been quite promising, unfortunately therapeutic response to gene therapy clinical trials remains low (Tobias, Ahmed et al. 2013). Three classes of genetic therapy treatment have taken center stage over the last several decades: prodrug/suicide genes, oncolytic viruses, and gene immunotherapy. Although each is its own distinct entity, they all facilitate delivery of genetic material through the use of one or more vectors as described below.

8.1. Vectors

8.1.1. Retroviral vectors

Retroviruses and retroviral vector producing cells (RVPCs) may be used to deliver specific genes to glioma cells; they are perhaps the most widely-studied class of vectors for treatment of GBM. This class of virus is advantageous in that its transduction is limited to rapidly dividing cells, meaning that normal brain cells remain unaltered. However, the transduction rate is low secondary to rapid inactivation of free retroviral vectors by complement as well as a lack of movement of virus to sites distant to the injection. It should be noted that transduction of circulating cells by vectors may occur, thus putting the patient at risk of cancer initiation via insertional mutagenesis (Barzon, Zanusso et al. 2006).

8.1.2. Adenoviral vectors

Adenoviruses belong to a family of 90-100 nm non-enveloped viruses made up of a nucleocapsid and double-stranded linear DNA. They account for roughly one tenth of all upper

respiratory tract infections in children, infecting the host via introduction of their genome into the nucleus of the host organism's cells where the viral DNA remains free. This is in opposition to the retroviral mechanism involving incorporation of genetic material into the host cell's genomic structure.

Adenovirus enters the host cell by way of 2 distinct sets of interactions. Firstly, the knob domain of the virus's fiber protein binds to the cell receptor (either CD46 or coxsackievirus adenovirus receptor). This is followed by the interaction of a specialized motif in the penton base protein with an integrin molecule, which prompts internalization of the virus via an endosome. Thereafter the capsid components dissociate and the virion is released into the cytoplasm. Viral DNA enters the nucleus via the nuclear pore, later associating with histones. Following nuclear invasion, the viral genome is reproduced along with the host cell's DNA. However, the progeny of the original host cell will not carry the newly-introduced viral DNA. This necessitates numerous rounds of viral introduction in the treatment of cancer (Doloff and Waxman 2013).

8.1.3. *Reoviral vectors*

The genome of Reoviridae is segmented, double-stranded RNA, and the virus has the ability to make use of a non-functional protein kinase R (PKR) pathway in glioma cells to allow for viral replication. This is advantageous as the virus does not require genetic engineering. Other advantages include small size (70-80nm) and an absence of known consequent encephalitis in humans (Clarke, Debiasi et al. 2005).

8.1.4. *Nonviral vectors*

There are several nonviral vectors either currently in use or being considered for use in gene therapy such as synthetic vectors, nanoparticles, and stem cells/progenitor cells. From this group, perhaps the most studied is the liposome (included in the category of nanoparticles). Cationic Liposomes are easy to produce, have relatively low immunogenicity and toxicity, and typically exhibit long-term stability (Tobias, Ahmed et al. 2013).

8.2. Gene therapy strategies

8.2.1. *Prodrug activating genes/suicide genes*

Prodrug/suicide genes represent an ingenious wing of gene therapy. The basis of this anti-tumor modality is introduction of genes, either into the host genome or the intranuclear milieu, which imparts susceptibility to a subsequent therapeutic agent. The vectors themselves are genetically modified to produce an enzyme which converts a prodrug, given systemically, into toxic metabolites which act specifically on the malignancy.

Perhaps the earliest/most-studied example of prodrug/suicide gene utility when addressing gliomas is that of Herpes Simplex Type 1 Thymidine Kinase (HSV-tk). After incorporation of this gene into tumor cells (often residual cells status-post resection) and the endothelium of their vasculature, the host is treated with an antiviral such as gancyclovir (GCV). HSV-TK

phosphorylates the prodrug of GCV into its active compound, whose mechanism of action involves DNA cross-linking, which leads to cell death. Following treatment with GCV, there may also be an observed “bystander effect” which involves the killing of non-transduced adjacent cells or even distant cells via immune response (T Cells, NK Cells) and toxic metabolites received via gap junctions (Ram, Culver et al. 1997; Floeth, Shand et al. 2001; Matuskova, Hlubinova et al. 2010). In a xenograft glioma model, a significant therapeutic effect was found when only approximately 10% of tumor cells were transduced with HSV-tk (Chen, Chang et al. 1995; Sandmair, Loimas et al. 2000). Introduction of HSV-tk/GCV may also increase response to standard measures such as radio- and chemotherapy (Rainov, Fels et al. 2001; Chiocca, Broaddus et al. 2004). This method has also been hypothesized to stimulate an immune response and provide an anti-angiogenic effect (Culver, Ram et al. 1992; Ayala, Satoh et al. 2006; Chiocca, Aguilar et al. 2011). Although there have been numerous enzyme-prodrug clinical trials ranging from Phase I to Phase III, endpoints such as median survival have not been overly impressive (Iwami, Natsume et al. 2010; Kroeger, Muhammad et al. 2010).

8.2.2. Retrovirally-mediated therapy

Intratumor injection of RVPCs has shown a high percentage of tumor regression in some studies (Ram, Culver et al. 1997; Pulkkanen and Yla-Herttuala 2005). Rainov et al. conducted a Phase III, multicenter, open-label, randomized trial of newly diagnosed GBM comparing standard therapy vs. standard therapy with adjuvant gene therapy of the tumor bed by HSV-tk. Although this mode of treatment was shown to be safe, there was no significant difference in 12-month survival rates or progression-free median survival (Rainov 2000). A recent Phase I head-to-head trial of intra-operative HSV-tk introduction via retrovirus vs. adenovirus showed promising results for adenoviral vectors in a small number of patients (Sandmair, Loimas et al. 2000).

8.2.3. Adenovirally-mediated therapy

It should also be noted that unlike retroviral vectors, adenovirus can transduce both dividing and non-dividing cells. The majority of adenoviruses used for this purpose are E1-deleted adenoviral vectors, which may be injected at a higher titer than RVPCs; however high doses may indeed lead to serious side effects, including confusion, seizures, fever, leukocytosis, and hyponatremia that appear to be secondary to immune response to the vector (Trask, Trask et al. 2000). This same immune response lowers the yield of viral delivery but also aids in tumor reduction (Trask, Trask et al. 2000; Lang, Bruner et al. 2003). Notably, the adenoviral vector may be found transiently in blood but has not been found as a replication-competent entity.

Preliminary clinical data suggest that adenoviral mediated gene transfer of suicide genes (AdvHSV-tk) may have clinical utility (Germano, Fable et al. 2003; Immonen, Vapalahti et al. 2004). A Phase IIB randomized controlled trial of patients with malignant gliomas reported a significant increase in OS from 37.7 weeks in the control arm (n=19) to 62.4 weeks in the adenoviral treated arm (AdvHSV-tk, n=17) (Immonen, Vapalahti et al. 2004). A recent Phase 1B trial showed treatment with adenovirus-HSV-tk followed by Valacyclovir, when paired

with resection, chemotherapy and radiotherapy, was safe and without dose-limiting toxicity (Chiocca, Aguilar et al. 2011).

Despite the aforementioned promising results from a small number of patients, the Phase III international open-label, randomized ASPECT clinical trial, which studied the intra-operative administration of adenoviral-HSV-tk followed by GCV (n=124) as compared to resection and standard of care alone (n=126), was not positive. Unfortunately, the data revealed no difference between the groups in terms of OS; furthermore, more patients in the experimental group had one or more treatment-related adverse events than those in the control group (88 [71%] vs 51 [43%]) (Westphal, Yla-Herttuala et al. 2013).

8.2.4. Nanoparticle/Neural stem cell-mediated therapy

Synthetic vectors, including nanoparticles have been applied to deliver DNA plasmids, RNA and siRNA (Jin and Ye 2007; Germano and Binello 2009; Jin, Bae et al. 2011). Liposomes are perhaps the most-researched of all nanoparticles (Tobias, Ahmed et al. 2013). Given through convection-enhanced delivery via stereotactically-placed catheters a liposome-DNA complex has been used to deliver HSV-tk in a small number of patients. The treatment was well-tolerated without major side effects (Jacobs, Voges et al. 2001; Voges, Reszka et al. 2003).

Pleuripotent neural stem cells procured from the subgranular zone of the hippocampus and the areas surrounding the lateral ventricles have the ability to migrate to areas of parenchymal damage (Luskin 1993). Neural stem cell clones may migrate to areas of tumor infiltration and thus were examined as vehicles for delivery of suicide genes, cytokines, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); there is evidence of potential efficacy in animal models but no clinical utility data yet (Aboody, Brown et al. 2000; Marsh, Goldfarb et al. 2013).

8.2.5. Tumor suppressor gene replacement

A well-documented characteristic of GBM is its inherent inactivation of the p53 tumor suppressor gene. Animal trials have shown that re-introduction of the wild-type p53 gene is pro-apoptotic leading to increased sensitivity to current modalities of treatment such as chemo- and radiotherapy. A Phase 1 trial of adenoviral gene transfer of intra-tumoral wild-type p53 in recurrent malignant glioma proved to be safe, but the transfected cells were not found in a radius large enough to be therapeutically effective (Lang, Bruner et al. 2003).

8.3. Oncolytic gene therapy

The realm of oncolytic virus therapy involves the use of replication-competent viruses with the ability to selectively replicate and kill cancer cells, with or without gene transfer. This is in opposition to prodrug/suicide gene therapy which makes use of replication-incompetent modalities. In order to combat the inefficiency of suicide gene therapy, oncolytic treatment employs tumor-specific, conditionally replicating viral vectors (Tobias, Ahmed et al. 2013). The mechanism of action involves viral replication which eventually leads to lysis of the host tumor cell and subsequent release of additional copies of competent virus which may lead to

further tumor reduction. This method is tumor-specific as it makes use of either attenuated viruses containing inactivated genes which replicate in tumor cells only, or viruses with replication-essential genes in tumor-specific promoters (Chiocca 2002). This method employs herpes simplex virus (HSV), Adenovirus, Reovirus, Poliovirus, Newcastle Disease Virus (NDV), and Measles virus.

8.3.1. *Oncolytic HSV-1*

Herpes Simplex is an enveloped, double-stranded DNA virus which exhibits inherent action upon the human nervous system; it can replicate in both active and quiescent cells. Consequently, safety was an original concern with this viral vector. Approximately 8 different HSV-1 genes have been altered or deleted to promote tumor specificity and lower collateral CNS damage (Tobias, Ahmed et al. 2013). There are two strains of replication-competent HSV-1 which have been significantly studied: G207 and HSV1716. G207 is the more widely-examined of the two and possesses a mechanism of action involving alteration of the gene which produces ribonucleotide reductase. In a recent phase 1B clinical trial, patients received injections of this virus both before and after tumor resection. Although viral replication was observed, treatment efficacy was sparse (Markert, Liechty et al. 2009). Additional studies have likewise shown adequate safety but minimal efficacy (Todo, Martuza et al. 2001).

G207 overcomes host defenses mediated by protein kinase R (PKR), which normally shuts down translation in infected cells through phosphorylation of eIF-2 alpha (Barzon, Zanusso et al. 2006). In a Phase I study by Markert et al., conditionally replicating G207 virus (given by stereotactic intratumor injection) was not found to lead to the development of herpes encephalitis (Markert, Medlock et al. 2000). Additionally, replication-competent HSV1716 administration in a Phase 1 dose-escalation study by Rampling et al. did not lead to encephalitis. Furthermore, no viral shedding was noted and no viral genome was found in tumor biopsies performed months after treatment (Rampling, Cruickshank et al. 2000).

8.3.2. *Oncolytic adenoviruses*

Adenoviruses carrying mutations in E1A or E1B can also act on GBM via oncolysis. Their mechanism of action involves tumor-specific binding and inactivation of apoptotic proteins like pRB family and p53. Of note, adenovirus is inherently non-neurotropic, which may lend itself to superior safety versus HSV. One adenovirus, *ONYX-015*, has been found to preferentially replicate in p53 deficient cells secondary to its deletion for p53-inactivating protein E1B-55K. In one clinical trial it was injected into the surgical cavity after resection and found to have no serious adverse effects; however; almost all patients involved in the trial had progression of their GBM (Chiocca, Abbed et al. 2004). It should also be noted that Georger et al found human xenografts to be responsive to ONYX-015 without correlation to their p53 status (Georger, Grill et al. 2003).

8.3.3. Oncolytic NDV

NDV is an avian paramyxovirus, which does not harm humans except for rare pulmonary infection in poultry farmers; certain strains harm neoplastic cells via a currently unknown mechanism (Reichard, Lorence et al. 1992). Interestingly, NDV also has pleiotropic immunomodulatory properties (Schirmacher, Haas et al. 1999). It should be noted that treatment with NDV necessitates starting at a low dose as there have been examples of treatment-related death with *NDV PV701 and solid cancers* (Pecora, Rizvi et al. 2002). *MTH-68/H*, a live attenuated oncolytic viral strain of NDV, has shown promising results in a small number of GBM patients (Csatary, Gosztanyi et al. 2004).

8.3.2. Oncolytic reoviruses

Reovirus is a double-stranded RNA-containing virus that replicates in GBM cells because of a hyperactive ras signaling; it distinctively does not replicate in normal brain cells. A phase I clinical trial of intratumoral administration of genetically unmodified virus was well tolerated by patients with recurrent malignant gliomas (Forsyth, Roldan et al. 2008). Further studies involving reovirus are currently underway.

8.4. Gene immunotherapy

Treatment of gliomas with immune therapy is based on harnessing of the patient's T-Cell mediated response to tumor cells. Typically, gene-immune therapy falls into the category of priming in the brain by the transfer of cytokine genes, like IL-2, IL-4, IL-12, and interferons gamma and beta (Freeman, Abboud et al. 1993; Borden, Lindner et al. 2000; Candolfi, Xiong et al. 2010; Denbo, Williams et al. 2011; Ryu, Park et al. 2011; Markert, Cody et al. 2012). A phase I clinical trial of the injection of cationic liposomes carrying the human IFN-Beta gene into the postsurgical cavity showed low toxicity (Wakabayashi, Natsume et al. 2008). A phase 1 trial of adenovirus-mediated gene transfer of INF-Beta was also well tolerated (Chiocca, Smith et al. 2008). Furthermore, a small pilot study of liposomal-mediated IFN-Beta gene transfer into the postsurgical cavity showed promising results (Yoshida, Mizuno et al. 2004).

Another important strategy combines cytokine gene transfer (human IL-2) paired with HSV-TK/GCV treatment (Palu, Cavaggioni et al. 1999; Colombo, Barzon et al. 2005). The results are promising in a small number of patients (Colombo, Barzon et al. 2005); in particular, biopsy following treatment showed tumor necrosis at site of administration as well as significant immune response in the form of activated cytotoxic T cells, macrophages and T-Helper/inducer lymphocytes (Barzon, Zanusso et al. 2006).

9. Conclusion

The aforementioned negative results of several key phase III clinical trials in GBM demonstrate that current proof of efficacy in preclinical models is a necessary but not sufficient condition

for clinical utility. The consistency in obtaining negative results in GBM is remarkable. How can we improve and what do we do to turn the tide in our favor?

It is becoming evident that the phenotypes of GBM are not created by few solitary molecules but rather by dynamic networks with positive and negative loops that react and respond to a therapeutic intervention. The good news is that these networks are finite dimensional. Furthermore, because of the instability of cancer genomes, random mutations are introduced in the population of rapidly dividing glioma cells; hence, a particular therapy could merely delay growth by selecting a resistant subpopulation. We suggest that we should elevate the threshold by mandating stringent criteria before proceeding to very costly phase III clinical trials, as follows.

1. Phase II clinical trials must include a control arm with appropriate stratification instead of historical controls.
2. Preclinical models must include proof of feasibility in at least 8-10 different cell lines/ animal models.
3. We ought to invest in developing a better understanding of the structure of the oncogenic molecular networks in GBM and demand laboratory data depicting the reactions of these networks to a new therapeutic strategy.
4. We need to develop mathematical models, results, and simulations of these molecular networks and acquire the ability to test therapeutic strategies in silico.

We believe that investing in the aforementioned endeavors will increase the likelihood that a chosen therapy will have proven clinical utility against GBM. Maintaining the status quo, by forging ahead with large phase III clinical trials costing about \$50-100 million each, is not attractive.

Author details

Paula Province, Alexis Bashinski Shaefer, Benjamin McCullough and
Hassan M Fathallah-Shaykh

The University of Alabama at Birmingham, Birmingham, Alabama, USA

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