Published in final edited form as:
Neurosci Lett. 2012 October 11; 527(2): 62–70. doi:10.1016/j.neulet.2012.08.001.

Anti-angiogenic Gene Therapy in the Treatment of Malignant Gliomas

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Abstract

More than four decades ago, Dr. Judah Folkman hypothesized that angiogenesis was a critical process in tumor growth. Since that time, there have been significant advances in understanding tumor biology and groundbreaking research in cancer therapy that have validated his hypothesis. However, in spite of extensive research, glioblastoma multiforme (GBM), the most common and malignant primary brain tumor, has gained little in the way of improved median survival. There have been several angiogenesis targets that have resulted in drugs that are in clinical trials or FDA approved for clinical use in several cancers. GBM is a highly angiogenic tumor and several drugs are showing promise in clinical trials with one (bevacizumab), clinically approved for use. We will review several possible angiogenic targets in GBM as well as the vector methodologies used for delivery. In addition, GBMs present several therapeutic challenges related to structure, tumor immune microenvironment and resistance to angiogenesis. To overcome these challenges will require novel approaches to improve therapeutic gene expression and vector biodistribution in the glioma.

Keywords

VEGF; Vasculostatin; oncolytic virus; Astrocytoma; glioma; brain tumor

Glioblastoma Multiforme (GBM)

Glioblastoma multiforme, WHO grade IV astrocytoma, or malignant glioma (MG), encompassing both WHO grades III and IV, is the most common primary brain tumor and has a grim prognosis with a median overall survival of approximately 15 months[108]. Despite aggressive medical and surgical therapies as well as extensive research in the area, the major improvement in this median survival time consists of the discovery that hypermetahylation of the promoter for methyguanine methyltransferase (MGMT) is a significant beneficial prognostic factor[44]. Malignant glioma is likely to be one of the most angiogenic cancers. It has been shown to express specific angiogenic and tumorigenic markers that are useful in predicting therapeutic responses, grading of tumor and prognosis [13, 92]. Factors involved in angiogenesis are targets for multiple cancer clinical trials.

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GBM therapy presents challenges that are physiologic related to anatomy and tissue sensitivity to therapy, as well as immunologic related to immunosuppression in the neoplasm microenvironment. Many new approaches in treatment of GBM are multi-faceted including multiple chemotherapeutic agents, radiation and surgery. There is hope that gene therapy targeting different angiogenic pathways may also become a mainstay of future treatments.

Definition of Angiogenesis

Angiogenesis is a process by which new capillary blood vessels are formed from preexisting vasculature and involves endothelial cell (EC) proliferation, migration, and basement membrane degradation to form a new lumen[124]. This process can be induced in response to normal development, hypoxic and adaptive cues, injury, and neoplasm. New vessel formation is orchestrated by area ECs, monocytes, smooth muscle cells and platelets in response to growth factors, cytokines, proteins and proteolytic enzymes[26, 95–97].

Angiogenesis is a hallmark of cancer. In order for tumor cells to initiate angiogenesis, cells must first develop an angiogenic phenotype through a process termed the “angiogenic switch” [9]. This “switch” promotes cell tumorigenicity, involves activation of oncogenes, down-regulation of tumor suppressor genes and expression/signaling of angiogenic pathways[20, 103, 113]. Two other mechanisms for blood vessel formation in brain tumors have been described. The first one is vascularization, whereby circulating bone marrow cells home into the neoplasm and differentiate into endothelial cells and/or pericytes[3, 4]. The other one is vascular mimicry, where cancer cells themselves transdifferentiate and help with blood vessel formation[129]. Factors involved in these processes may be different. For this review, we will focus primarily on factors involved in angiogenesis.

Angiogenesis Target Genes

Inhibitors of angiogenesis interfere with the binding of pro-angiogenic signaling factors to their cognate receptors to initiate the step-wise process of vessel formation. Targeting angiogenesis has been approached through down-regulation of proangiogenic factors such as: vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor, insulin-like growth factor, transforming growth factor, angiopoietins, and several chemokines including the CXC family of chemokines. Alternatively, up-regulation of factors that inhibit angiogenesis, such as: thrombospondin-1 (TSP-1), angiostatin, vasculostatin and endostatin can also lead to anti-angiogenic effects [104, 116]. In the next section, we will discuss some of these factors pro-angiogenic and anti-angiogenic factors.

A) Reducing pro-angiogenic gene expression

1) Vascular endothelial growth factor (VEGF)—Among the listed targets, one the most well studied has been VEGF. This has become even more relevant to glioma clinical therapy since the approval of the humanized monoclonal antibody bevacizumab (Avastin®) which binds VEGF-A to block receptor activation. Bevacizumab was the first clinically available angiogenesis inhibitor, shown to slow tumor growth and extend the median survival of patients[73, 98]. The U.S. Food and Drug Administration (FDA) approved bevacizumab to be used alone for glioblastoma with poor or refractory response to other therapies. Aside from CNS tumors, bevacizumab is FDA approved for used in combination with other drugs or therapies in treatment of metastatic renal cell and colorectal cancers and some non-small cell lung cancers[73]. The drug was determined to be not both safe and effective for use in breast cancer patients[18]. Other medical therapies with antiangiogenic activity include sorafenib (Nexavar®), a multi-kinase inhibitor[130, 142]; sunitinib

(Sutent®), a multi-kinase inhibitor[80], pazopanib (Votrient®), another multi-kinase inhibitor[21, 48, 106], and everolimus (Afinitor®), a mammalian target of rapamycin inhibitor[65]. Although several of these are also in clinical trials for GBM, thaey have not been approved for use, yet. There is also interest in gene therapy targeting VEGF. For instance, using both viral and non viral vectors encoding for soluble VEGF receptor conjugated to Fc, VEGF mediated endothelial growth and migration was shown to be inhibited [87, 120]. Blockade of VEGF expression by gene therapy approaches with siRNA or targeted repressors have also been tested and found efficacious in animal models [54, 139]. Therefore, VEGF remains a highly interesting target for medical, but also gene-based, therapies.

2) Fibroblast growth factor (FGF)—FGF is a growth factor that binds to its receptor on cancer and endothelial cells, thereby initiating receptor dimerization and autophosphorylation, This leads to a signaling cascade that promotes proliferation, migration and angiogenesis. Given its pleiotropic effects on both cancer cells and tumor endothelium, blockade of its signaling by dominant negative FGFR expressed by an oncolytic HSV-1 (bG47Delta-dnFGFR) was tested [70]. Mice bearing intracranial glioma treated with bG47Delta-dnFGFR showed significant improvement in their survival [70]. Therefore, this appears to also be an interesting target for further exploitation.

3) Interleukin-8 (IL8)—Interleukin-8 is a pro-inflammatory and angiogenic cytokine that increases VEGF expression and signaling [79]. Administration of IL-8 to cancer cells has been shown to increase their proliferation. Retroviral mediated transfer of antisense IL-8 showed reduced growth suggesting that the translation of such an approach to glioma patients could be beneficial [132]. Based on these observations a short hairpin ribozyme against IL-8 incorporated in a conditionally replicating adenovirus vector (Ad-DeltaE1-U6shIL80) was engineered and tested for therapeutic efficacy in mice bearing glioma [138]. Ad-DeltaE1-U6shIL8 efficiently suppressed endothelial cell migration, tube formation and sprouting, and also showed significant improvement in antitumor efficacy [138]. This thus shows that targeting IL8 could provide an important anti-angiogenic effect in gliomas.

B) Increasing anti-angiogenic gene expression

1) Brain Angiogenesis Inhibitor (BAI1)—BAI1 was identified as a p53 responsive gene in a genomic screen for genes containing a p53 response element in 1997 [85]. The presence of TSP type 1 repeats in its extracellular domain suggested an anti-angiogenic function. Consistent with this, its expression was found to be reduced in a majority of glioblastoma specimens and glioma cell lines. Glioblastoma multiforme (GBM) with low BAI1 and high VEGF expression correlated with a poor progression [55]. Reduced expression of BAI1 has also been noted in several different malignancies including colorectal, renal cell carcinoma, pulmonary adenocarcinoma, and gastric cancer [31, 43, 50, 67]. Collectively, these studies suggested an anti-angiogenic/anti-tumorigenic function for BAI1 and reconstitution of BAI1 was found to suppress tumor angiogenesis and growth [24, 62]. Based on these studies a recombinant adenovirus expressing the coding sequence of full length BAI1 (AdBAI1) was tested for antitumor efficacy. *In vivo* inoculation of established subcutaneous and intra-cerebral xenografts with AdBAI1 reduced tumor growth and reduced tumor vascularity [53, 131].

While the reconstitution of full length BAI1 coding sequence revealed an anti-angiogenic and anti-tumorigenic function of BAI1, it was not clear how a membrane bound receptor could function as a paracrine anti-angiogenic factor. The first clue about its function came from the observation that its extracellular fragment contained a conserved GPS proteolytic cleavage site that was processed to release its 120 kDa extracellular portion (Vasculostatin:

Vstat120) [56]. Vstat120 expressed in glioma cells was efficiently secreted and recognized CD36 on endothelial cells to inhibit blood vessel growth *in vitro* and *in vivo* [57]. This extracellular portion was thus designated Vasculostatin (Vstat120) and reconstitution of its expression in glioma cells revealed significantly reduced tumor growth and angiogenesis in vivo [56, 57]. These observations led to the creation of RAMBO (Rapid Antiangiogenesis Mediated By Oncolytic virus) an oncolytic HSV-1 based virus that encoded for Vstat120 cds [41]. Treatment of mice bearing subcutaneous and intracranial glioma with RAMBO led to a significant improvement in survival compared to control oncolytic virus treated mice [41]. The encouraging results observed with RAMBO treatment led the investigators to develop a second oncolytic HSV-1 vector which expressed Vstat120 within the backbone of a transcriptionally driven oncolytic virus [137]. Treatment of mice bearing very high nestin positive glioma cells with 34.5ENVE led to a significant improvement in survival of mice bearing intracranial glioma with long term survivors [57]. Interestingly a second secreted 40kDa fragment of BAI1 (Vstat40)was recently reported and also found to have antiangiogenic activity [17]. Gene therapy approaches with this fragment have not been described to date.

2) Angiostatin—Angiostatin is produced by the proteolytic cleavage of the first four kringle domains of plasminogen [86]. Treatment of tumor bearing mice with angiostatin has been shown to inhibit both angiogenesis and glioma growth *in vivo* [60]. Although several cell surface receptors are known to bind to angiostatin, it is also thought to be also internalized by endothelial cells where it can induce apoptosis via down-regulation of mitochondrial BCL-2 [68]. Adeno associated virus (AAV) vectors have been utilized for sustained delivery of angiostatin in vivo. Treatment of animals bearing intracranial glioma showed long term survival of 40% of rats treated with AAV-angiostatin given by direct inratumoral injection or intramuscularly [75, 76]. Angiostatin expressed by a replication defective adenovirus given intratumorally to rats bearing intracranial tumors also showed increased efficacy in combination with radiation [37]. In spite of these promising results, there has not been an application to humans with GBM in a clinical trial setting, yet

3) Endostatin—Endostatin, is a 20-kDa antyiangiogenic protein produced by cleavage of collagen XVIII. It has been shown to inhibit endothelial cell proliferation and migration and induce their apoptosis [22]. It has also been shown to inhibit MMP-2 activity leading to reduced migration of both endothelial cells and tumor cells [59]. Treatment of rats bearing intracranial glioma with endostatin has been shown to prolong survival [38]. Gene therapy approaches using endostatin have been explored and delivery of endostatin by human mesenchymal and neural stem cells, adenovirus vectors, plasmid, and alginate encapsulated cells have shown antitumor efficacy [7, 72, 93, 94, 111, 136]. Gene therapy with recombinant endostatin and angiostatin fusion proteins using both viral and non viral gene transfer using sleeping beauty transposon of mice bearing glioma xenografts with a fusion protein of soluble vascular endothelial growth factor receptor (sFlt-1) and an angiostatin-endostatin fusion protein also showed antitumor activity [87, 147]. Therefore, it seems that there has been high interest in this anti-angiogenic factor in gene therapy approaches.

4) Thrombospondin—Thrombospondin (TSP) was the first naturally occurring inhibitor of angiogenesis identified in 1978[66]. It functions as a matricellular glycoprotein that binds EC receptors[116, 124]. The thrombospondin family, which consists of five extracellular calcium-binding multifunctional proteins: TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5. TSP-1 is the best studied of this group and is expressed by a variety of normal cells, including endothelial cells, fibroblasts, adipocytes, smooth muscle cells, monocytes, macrophages, and transformed cells such as malignant glioma cells[35, 124].

This is an interesting target as it is endogenous and elicits minimal immune responses as a component of combination therapy. A recent study used human neural stem cells that over-expressed TSP-1 and showed that they acted on vascular components of gliomas, limiting tumor vessel-density and tumor growth[121]. In a phase 1 trial a TSP-1 mimetic drug was used in combination with temozolomide and radiotherapy and it reported increased dose tolerance and a median survival time of about 16 months, slightly improved over the currently reported median survival for GBM[81]. This study used the drug delivered as a water soluble acetate salt in daily subcutaneous injections. The drawback to this agent is its bioavailability and rapid enzyme degradation. These drawbacks could be potentially overcome with the use of vector delivery system to deliver the TSP1 gene. Furthermore, as a naturally occurring antiangiogenic factor, TSP-1 would an excellent candidate because of probable lack of immunogenicity.

5) Tissue inhibitor of Matrix metalloproteases—Matrix metalloproteases (MMPs) are a family of secreted proteases that degrade extracellular matrix and hence increase both cancer cell and endothelial cell migration [58]. Apart from direct remodeling of the tumor microenvironment these proteases also facilitate the release of growth factors sequestered by the extracellular matrix [28]. Increased expression and activity of MMP with reduced TIMP expression has been associated with invasive GBM cells in patient tissue [82]. Consistent with this, proteomic analysis of angiogenic factors in the serum of 36 GBM patients showed that low serum level of TIMP-1 were associated with longer survival [19]. Thus gene therapeutic approaches to increase TIMP expression and reduce MMP activity have been tested out. Transduction of glioma cells with a nonreplicating adenovirus and HSV expressing TIMP-2 reduced their invasiveness *in vitro* [47, 74]. Interestingly the expression of TIMP-3 in a conditionally replicating adenovirus was found to inhibit MMP activity but did not affect antitumor efficacy in mice bearing intracranial glioma [64]. Therefore, the therapeutic effect of TIMPs may depend on the context of tumor type and/or vector type.

6) Platelet factor 4 Chemokine, CXC Motif, Ligand 4—Platelet factor 4 is a small chemokine that is sequestered in platelets and rapidly secreted upon activation. Its main function is to neutralize the effects of heparin on endothelial cells thereby promoting coagulation [25]. Apart from its effect on coagulation, PF-4 is also known to inhibit endothelial cell migration and proliferation *in vitro* and also inhibit angiogenesis *in vivo* [33, 77]. Its anti-angiogenic effect has been attributed to its ability to bind to FGF, and block its dimerization, as well as its ability to block VEGF signaling [89, 109]. Recombinant PF-4 was tested in patients with colorectal carcinoma. While the treatment was found safe, no clinical response was identified in this clinical trial. The lack of a clinical response was attributed to low local concentration of PF-4 in tumors. Thus to counter this, gene therapy approaches to deliver sustained levels of PF-4 are currently being investigated in preclinical animal models. Treatment of mice bearing intracranial glioma with replication competent and non replicating viruses expressing PF-4 has been found to increase survival of tumor bearing mice [71, 115]. Therefore, this also appears to be a promising anti-angiogenic gene to consider in gene therapy for GBM.

7) Interleukin-12 (IL-12)—Il-12 is a secreted cytokine produced by macrophages, that exhibits potent antiangiogenic effects in vivo [112]. Because it is a heterodimer, composed of a heavy and light chains, simultaneous transduction of cells with both enes is essential to express functional IL-12. An oncolytic HSV-1 encoding for murine IL-12 (M002) has been created and found to increase survival of mice bearing intracranial tumors *in vivo*. Based on the safety and preclinical efficacy of this vector, future testing of an oncolytic HSV-1 expressing human IL-12 has been proposed [78]. Apart from replicating virus vectors, Semiliki forest virus virus like particle, non replicating adenovirus, AAV mediated

expression of IL-12 by microglia, mesenchymal and neural stem cells has shown efficacy in preclinical models of glioma [16, 99, 101, 135, 141]. Therefore, this cytokine has been widely studied and is now poised to be tested in human trials.

Vectors for Anti-Angiogenic GBM Therapy

Two classes of delivery methods or vectors have been utilized for delivery of genes into brain tumors, nonviral- and viral-based. In general, research and clinical experience has been greatest with the latter mode of delivery, in spite of concerns for toxicity. We will thus review research and clinical experience with these two methods of delivery.

Nonviral Vectors

In general, use of nonviral vectors has been limited by decreased specificity for target tissue, poor stability and low potency or transfection rates[119]. Nonviral vectors that have shown promise in research and could be applicable to glioma therapy include:

1. Injection of naked nucleic acids. Examples of this would include antisense oligonucleotide's double stranded RNAs, or small interfering RNAs (siRNAs or RNA interference, RNAi), or plasmid DNAs. RNA-based methods typically include short sequences of 15–30 nucleotides in length that function by hybridizing to complementary sequences of a target mRNA, that activates its degradation by RNAseH (Antisense) or by a RNA silencing complex (siRNA). They thus act by suppressing expression of oncogenes or pro-oncogenic factors. For instance, antisense VEGF sequences were shown to reduce levels of endogenous VEGF with resultant decrease in glioma growth in a subcutaneous model, albeit after delivery with an adenoviral vector[49]. Recently, a clinical trial in Poland was reported where humans with malignant gliomas underwent surgical resection of their tumor, followed by intracavitary injection with a RNAi against tenascin[100]. This is an extracellular matrix protein highly expressed in high grade gliomas and involved in tumor invasion and angiogenesis[12, 34, 46, 144, 145]. They reported a significant improvement in patient survival. They attributed this directly to effects against tenascin expression rather than stimulation of intracellular innate responses based on interferon from *in vitro* studies. However, this would not exclude an immune effect in humans *in vivo*. Plasmid DNA therapy was utilized in a rat model of glioma where direct intra-arterial injection of an expression plasmid encoding endostatin demonstrated improved survival time of 47%[7]. In general, these techniques are limited by rapid degradation and clearance of the nucleic acid by nucleases in the circulation and by the fact that their effect is short-lived and may require repeated injections. In addition, it remains unclear whether *in vivo* effects could still be mediated by interferon activation sensing atypical nucleic acid structures.
2. Utilization of physical or chemical methods to shield delivered genes from degradation and increase delivery into target glioma cells. Several chemical and/or physical methods have been employed to deliver anti-angiogenic or other genes into glioma cells and glioma tumors. A particularly sophisticated strategy using a polyamidine dendrimer (PAMAM) linked to a Tat peptide and to a bacterial magnetic nanoparticle has been employed to deliver siRNAs against EGFR in glioma cells and in glioma tumors *in vivo*[40]. The rationale behind the various components was that PAMAM provides the vector structure to ferry the gene, Tat provides the ability to pass across cell membranes and the bacterial magnetic nanoparticle provides the ability to disperse well because of their coverage with a stable lipid bilayer. Delivery of the siRNA EGFR in glioma cells in vitro and in

vivo resulted in improved anticancer effects and the authors concluded that this could be a novel synthetic gene delivery method. Another interesting method consists of cell encapsulation whereby cells engineered to secrete a protein with therapeutic potential are enclosed in a polysaccharide alginate. The cells can be implanted in the brain parenchyma next to the tumor or in the ventricular system to provide continuous release of the anticancer compound[123]. A variety of other methods, such as electroporation, various liposome complexes, aptamers, high-frequency ultrasound and others have been utilized and extensively reviewed[8, 11, 88, 117, 146], albeit not in glioma therapy as of yet.

Viral Vectors

As opposed to nonviral, viral vectors are generally recognized as being more efficient in delivery of genes to tumor cells and providing more prolonged periods of gene expression. However, when compared to noviral, viral vectors may be more toxic, immunogenic and possibly lead to more undesirable side-effects. A number of viruses have been altered to retain the ability to deliver the anticancer gene of interest with little or no expression of endogenous viral genes. In some applications, though, viruses are engineered to retain the ability to replicate as well as delivering cytotoxic anticancer genes. These are designated as oncolytic viruses (OV). Gene therapy vectors, based on retroviruses, lentiviruses, and adeno-associated viruses (AAV) integrate into the host genome, allowing for stable transgene production. In the case of retroviruses, integration and successful gene expression is limited to actively dividing cells[119]. Commonly used viral vectors in the treatment of glioma include:

1. Herpes simplex -1 viruses (HSV1): HSV has been well studied for gene delivery in the CNS and is attractive for such properties as infection of a wide host range, persistence in neuronal cells, and ability to harbor large genes for transfer[15, 126] [36, 125]. There are two types of HSV1 vectors for gene transfer: recombinant vectors and amplicons. The former is an HSV1 where viral genes essential for replication have been deleted[27]. The latter instead is a plasmid that contains the origin of replication of HSV1, its packaging signal, and occasionally a HSV1 gene, such as ICP0, to improve expression[102, 110]. Another type consists of oncolytic HSV1 (oHSV1), where some of the viral genes have been deleted and/or tumor specific promoters are used to drive expression of HSV genes needed for replication[5, 51]. The main concern with HSV1 vectors in the brain is the potential for encephalitis and/or meningitis. We recently reported use of 34.5ENVE (viral ICP34.5 Expressed by Nestin Expressing) and RAMBO (Rapid Antiangiogenesis Mediated By Oncolytic Virus), both novel oHSV1 for the treatment of subcutaneous and intracranial gliomas in mouse models[42, 137], as discussed above. Others have also employed similar approaches[134]. In summary, it is evident that HSV vectors and oHSV can be useful to deliver antiangiogenic genes into glioma models.
2. Adenoviruses (Ad) are also DNA viruses of different serotypes, whose surface capsid proteins and fibers can bind specifically to a different cellular receptor, dependent on the serotype. After entry into the cells and escape from the endosomal/lysosomal compartment, Ad capsids enter the nucleus. The Ad genome and its expressed genes will remain extrachromosomal. Clinical and research experience with Ad is amongst, if nit the highest of all vectors. Some of the reported disadvantages are transient gene expression and elicitation of an inflammatory response[116]. Use of adenoviral vectors for the delivery of siRNA for bFGF was reported to block glioma growth, progression and vascularity[69]. In another approach, adenoviruses (Ads) expressing the VEGF promoter-targeted

transcriptional repressor ZFP, F435-KOX (designated Ad-DeltaE1-KOX) was shown to significantly reduce VEGF expression and inhibited angiogenesis. These authors were also able to show that, in vivo, an oncolytic Ad expressing F435-KOX, namely, Ad-DeltaB7-KOX, elicited a pronounced antitumor effect against a human glioblastoma U87 xenograft[54]. Another approach related to the multifunctional growth factor scatter factor/hepatocyte growth factor (SF/HGF) and its receptor c-met[2]. To down-regulate the expression of these pro-angiogenic factors, the authors utilized novel chimeric U1snRNA/ribozymes..Treatment of animals bearing intracranial glioma xenografts with anti-SF/HGF and anti-c-met U1snRNA/ribozymes by either intratumoral injections of adenoviruses expressing the transgenes or intravenous injections of U1snRNA/ribozyme-liposome complexes substantially inhibited tumor growth and promoted animal survival. We have shown that adenoviral mediated delivery of PTEN inhibits angiogenesis in a glioma model[1]. These are just a few examples of applications of adenoviral vector mediated anti-angiogenic gene delivery.

3. Adeno-Associated viruses (AAV) have been used less often in gliomas, primarily because they tend to infect neuronal more than glial cells. Conversely, the reduced immunogenicity, high titer production, and lack of side-effects in clinical trials may make these vectors advantageous, if targeting can be improved. One example of AAV vector use for gliomas related to the delivery of angiostatin by AAV in intracranial glioma models leading to improved animal survival[75]. There are a few other examples of AAV mediated delivery of different anti-angiogenic factors[107, 133], but overall experience with this vector has been less than the previous aforementioned ones.

4. Lentiviral vectors are considered more recent vector technology with the benefit of fairly long term transgene expression through host genome integration which is not limited to dividing cells and works well in terminally differentiated cells of the CNS. Development of lentiviral vectors expressing matrix metalloproteinase-2 (MMP-2), angiostatin and endostatin have been reported[91, 105]. Recombinant lentiviral expression of a gene that makes the secreted protein alphastatin, an endogenous angiogenesis inhibitor, decreased glioma vascularization, inhibited the growth of tumors and suppressed the early angiogenic “switch” steps by reducing Jnk and ERK phosphorylation in vitro[39]. Therefore, experience with lentiviral vectors to alter glioma angiogenesis remains in development.\

5. Recent applications of retroviral vector mediated gene delivery have entailed using the vector to generate stably transduced glioma lines expressing the anti-angiogenic gene, rather than direct injections of the vector into established tumors[45, 84, 90]. As such, these vectors have been primarily employed for biologic studies rather than for gene therapy.

Challenges to Anti-angiogenic Glioma Therapy

Tumor Physical Properties

Solid tumors have been reported to have increased interstitial pressure related to increased vascular permeability as a known feature in tumor development. This along with the tissue barrier that results from the tumor tight extracellular matrix composition has been associated with overall poor delivery of drug therapy to the site[30, 52, 83]. Interestingly, our own findings also show that increased vascular hyperpermeability drives the anti-viral immune response, increases inflammatory reactions and limits therapies that involve viral vectors[63]. Therefore, we were able to show that modification of this tumor micro-

environment with either anti-angiogenic drugs[63] or with enzymes that disrupt the glioma extracellular matrix[23] enhances intratumoral distribution of injected viral vectors.

Glioma Tumor Immune Microenvironment

The highly regulated CNS immune microenvironment gives the organ the designation as "immune privileged" and serves as a mechanism for protecting the terminally differentiated tissues of the brain. The brain has few native antigen presenting cells, selective permeability and an altered T cell costimulatory profile. The discovery of toll-like receptors on brain tissues is evidence that, while the CNS is immunoprivileged, it still responds to immune challenges, including utilizing innate immunity[10, 61, 118, 122]. Innate immune responses can be a signal to resident microglia and circulating macrophages to destroy vectors and vector infected glioma cells, thereby lowering viral titers and reducing the anticancer effect in injected gliomas[6, 14, 29, 32, 127, 128]. Conversely, immunocompetence and stimulation of immunity is also necessary to enhance antiglioma immunity. Therefore, understanding the need for a balance between immunosuppression to allow the vector/virus to replicate in the glioma and its microenvironment and immunocompetence to allow for robust antiglioma immunity is paramount to the future success of any gene therapy.

Resistance to VEGF anti-angiogenic therapy

The use of bevacizumab as the primary antiangioegenic treatment against gliomas Vascular endothelial growth factor (VEGF) is localized within tumor cytoplasm and endothelium situated predominantly in the pseudopallisading hypoxic areas of the tumor[114]. VEGF levels are lower in low-grade compared to high-grade gliomas and even lower to nil in normal tissues, rendering it an ideal target for therapy[113]. However, it is likely that, after tumorigenic cell transition and turning on of the angiogenic switch, targeting only VEGF expression could become linked to resistance. Malignant gliomas have been reported to have developed resistance to antiangiogenic therapies due to the presence of hypoxia[113]. Hypoxia induces upregulation of hypoxia-inducible factor 1α (HIF1α), downstream of VEGF. Therefore, anti-VEGF therapies may not take into account expression of this factor in hypoxic areas. In fact, presence of HIF-1α on glioma cells bodes negatively for prognostication[104]. Downregulation of HIF-1α in glioma cells using siRNA inhibited other pro-oncogenic signals[140]. Therefore, it is clear that therapies that only focus on VEGF will ultimately lead to anti-angiogenic therapy resistance.

Tracking Viral Vector Biodistribution

Use of viral vectors has clear advantages and is effective within the tumor area of delivery. However, it has been impossible to know with certainty the therapeutic field, or area of vector activity, after delivery. Recent *in vivo* studies using superparamagnetic iron oxide nanoparticle labeled adenoviral vectors helps to monitor delivery and potentially guide therapy[143]. This technology could also be important for imaging and visualizing vector biodistribution in injected gliomas.

Conclusions

GBM has the most grim prognosis of all primary brain tumors with median survival time less than 15 months. Ongoing research in antiangiogenesis using oncolytic viral vectors has shown promise in delivering targeted and durable gene expression to slow tumor progression and extend survival. There are multiple angiogenic targets and genes suitable to alter the angioegenic milieu of the glioma. One approach to express anti-angiogenesis genes includes the use of viral or nonviral vectors for delivery in the treatment of gliomas. Success of this technique rests on the vector's ability to target, infect and express the anti-angiogenic gene long term. The vector must also survive both the host immune response as well as

biodistribute within the tumor microenvironment. Attenuation of the vector immunogenic proteins, down regulation of host anti-viral responses, and harnessing the natural processes viruses employ to evade the host immune system will aid anti-angiogenic gene therapy techniques.

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Highlights

1. Angiogenesis is one of the major determinants of glioblastoma progression and maintenance.
2. The process is maintained by a balance between pro- and anti-angiogenic factors
3. The action and function of several of these factors has been elucidated
4. While several of these factors are “druggable”, the ability to use gene therapy methodologies to alter the angiogenic milieu appears to be a logical avenue for translational research and possible clinical applications
5. In this review, we will highlight examples of pro- and anti-angiogenic targets, vectors utilized for delivery of target genes and challenges facing applications of gene therapy against glioma angiogenesis.