REVIEW ARTICLE

Targeted Molecular Therapy of GBM

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GBM	glioblastoma
EGFR	epidermal growth factor receptor
PDGFR	platelet-derived growth factor receptor
PTEN	phosphatase and tensin homolog
PI3K	phosphatidylinositol 3-kinase
VEGF	vascular endothelial growth factor

Major advances in molecular biology, cellular biology and genomics have substantially improved our understanding of cancer. Now, these advances are being translated into therapy. Targeted therapy directed at specific molecular alterations is already creating a shift in the treatment of cancer patients. Glioblastoma (GBM), the most common brain cancer of adults, is highly suited for this new approach. GBMs commonly overexpress the oncogenes EGFR and PDGFR, and contain mutations and deletions of tumor suppressor genes PTEN and TP53. Some of these alterations lead to activation of the PI3K/Akt and Ras/MAPK pathways, which provide targets for therapy. In this paper, we review the ways in which molecular therapies are being applied to GBM patients, and describe the tools of these approaches: pathway inhibitors, monoclonal antibodies and oncolytic viruses. We describe strategies to: *i)* target EGFR, its ligand-independent variant EGFRvIII, and PDGFR on the cell surface, *ii)* inhibit constitutively activate RAS/MAPK and PI3K/Akt signaling pathways, *iii)* target TP53 mutant tumors, and *iv)* block GBM angiogenesis and invasion. These new approaches are likely to revolutionize the treatment of GBM patients. They will also present new challenges and opportunities for neuropathology.

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What is Targeted Molecular Therapy?

The treatment of cancer is undergoing a shift. Traditionally, cancer patients are treated with radiation therapy and cytotoxic agents that aim to have a greater effect on proliferating cancer cells than they do on non-cancerous cells. For some types of cancer, this approach has translated into increased survival. Unfortunately, the effect on GBM patients has been modest, and the median survival has remained largely unchanged over the past decade (28, 45, 48). Major advances in molecular biology, cellular biology and genomics have substantially improved our understanding of the genetic and proteomic changes involved in cancer. Now, these advances are being translated into new treatments for cancer patients.

GBM is the most common malignant brain tumor of adults, and is among the most lethal of all cancers (28). GBMs commonly overexpress oncogenes such as EGFR and PDGFR and contain mutations and deletions of tumor suppressor genes such as PTEN and TP53, all of which can have an impact on the activation state of signal transduction pathways that influence their biological behavior (42). These gains and losses can lead to constitutive activation of pathways that promote malignant behavior, but which may also paradoxically be their "Achilles heel." Typically non-cancerous cells have "back-up" mechanisms that allow them to survive and even proliferate in the face of disruption of a signaling pathway. In contrast, cancer cells may become so dependent upon signaling through constitutively activated pathways, that they lose the ability to compensate for its loss (56). Thus, it may be possible to specifically block the growth/survival of cancer cells while leaving non-cancerous cells unaffected. The tools of this approach are pathway inhibitors, especially kinase inhibitors, monoclonal antibodies, and oncolytic viruses. In this paper, we will review these new approaches.

Why is GBM Suitable for Targeted Therapy?

GBMs are highly suitable for targeted molecular therapy because they have a set of defined molecular lesions and signaling pathway disruptions that present clear targets. Further, despite aggressive surgical approaches, optimized radiation therapy regimens and a wide variety of cytotoxic chemotherapies, the median survival of GBM patients is approximately one year from the time of diagnosis—the same as it was 10 years ago (28, 45, 48). New approaches are needed.

The past decade has taught us that GBMs are not a homogenous group of tumors, even if they may share similar microscopic features. Primary GBMs, those arising

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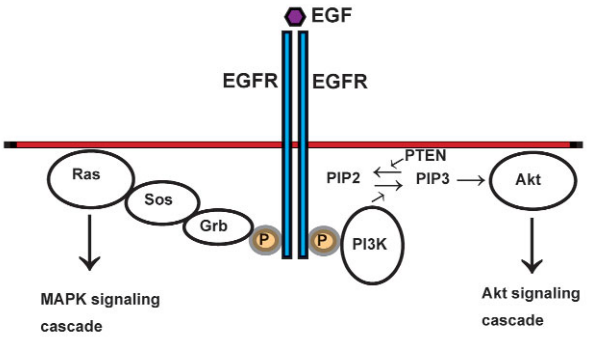

Figure 1. Schematic diagram of the signal transduction pathways activated by EGFR. Activation of the PI3K/Akt pathway and Ras/MAPK pathway are highlighted. This signaling diagram is a simplification; there are additional pathways activated, intermediate signaling molecules involved, and points of "cross-talk" between pathways.

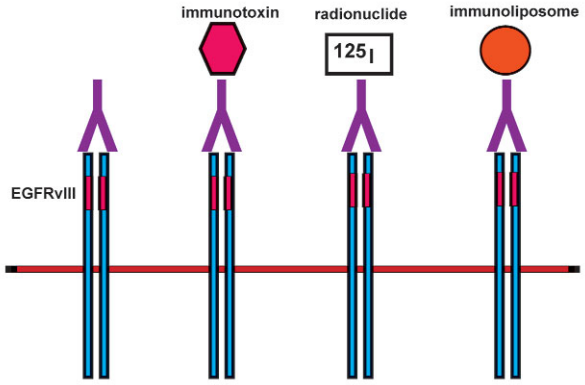

Figure 2. Schematic diagram demonstrating the use of monoclonal antibodies as targeted therapy. These antibodies can be used as "naked" unarmed antibodies, or fused to immunotoxins, radionuclides or liposomes.

as de novo lesions, commonly overexpress the epidermal growth factor receptor EGFR and its ligand-independent mutant EGFRvIII (16, 23, 30, 42, 43, 53, 57, 69, 79, 84). This results in signaling through RAS-MAPK and PI3K/Akt pathways, among others (Figure 1) (17). Downstream of these receptors, the PTEN tumor suppressor gene is also commonly mutated (42, 47, 64, 72), which further promotes activation of the Akt pathway (Figure 1) (56). Secondary GBMs, which arise from lower grade gliomas, commonly contain TP53 mutations (42). The PDGFR is also frequently overexpressed, resulting in activation of many of the same pathways as EGFR and EGFRvIII (48). Therefore, EGFR, EGFRvIII, PDGFR, and the RAS/MAPK and PI3K pathways, as well as TP53, present attractive targets for GBM therapy.

In this paper, we will discuss new treatment strategies for GBM patients that focus on these pathways. We will describe strategies to: *i)* target EGFR, EGFRvIII and PDGFR on the cell surface, *ii)* target constitutively activated RAS/MAPK and PI3K/Akt signaling pathways, *iii)* target TP53 mutant tumors, and *iv)* target GBM angiogenesis and invasion. These are not the only important mutations in GBMs; CDK4, pRB, MDM2, and Ink4a/Arf also play critical roles in GBM pathogenesis and may potentially provide targets for therapy.

Monoclonal Antibodies as Targeted Therapy

Cancer cells commonly express different proteins on their cell surface relative to their non-neoplastic counterparts. Overexpressed cell-surface growth factor receptors are attractive targets because they are often amplified and highly expressed, and because they activate signaling pathways that are critical for tumor growth and survival. In GBMs, EGFR overexpression is detected in nearly 50% of cases, including over two-thirds of those cases presenting as de novo "primary" GBMs. Amplification is detected in most of these cases (16, 23, 30, 42, 43, 53, 57, 69, 79). In addition a mutant EGFR receptor, EGFRvIII, is co-expressed in nearly 50% of GBMs with EGFR amplification (16, 23, 30, 42, 43, 53, 57, 69, 77, 79, 84). EGFRvIII, which arises from genomic deletion of exons 2 to 7, cannot bind ligand, but is constitutively active (16, 43, 53, 57, 84). A number of other mutant EGFRs have been described that may also play a role in GBM pathogenesis (23).

"Naked" (unarmed) antibodies. Growth factor receptor overexpression is not unique to GBM. In fact, the critical role of ErbB2/Her2 overexpression in breast cancer has been clearly demonstrated (65, 67, 76). Herceptin, a monoclonal antibody against Her2 improves the survival of a subset of breast cancer patients (12, 68), thus demonstrating the principle that monoclonal antibodies can be effective cancer treatments. A series of monoclonal anti-EGFRvIII antibodies have been generated for GBM therapy (Figure 2) (52, 81, 82). Mechanistically, these antibodies promote receptor internalization resulting in attenuated receptor phosphorylation and downstream signaling (43, 52). In addition, these antibodies can potentially support antibody-dependent cellular cytotoxicity, which may be important for their efficacy against GBMs (63). In vivo xenograft studies demonstrate considerable efficacy for these unarmed antibodies (52, 63).

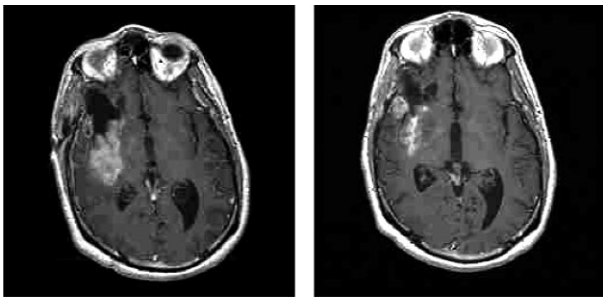

Figure 3. MRI's demonstrating a response to ZD-1839 (IRESSA) in one of the patients on the NABTC sponsored clinical trial. Note the decrease in tumor size between the initial MRI on the left, done at baseline, and the follow up MRI performed after treatment with ZD-1839.

"Armed" antibodies—immunotoxins, radionuclides and immunoliposomes. Clinical experience with other monoclonal antibodies such as Herceptin (for erbB2 positive breast cancer patients) and Rituximab (anti-CD20 for non-Hodgkin lymphoma patients) suggest that monoclonal antibodies alone may not be sufficient to arrest disease (7, 12, 51). Current strategies are now focused on combining monoclonal antibodies with conventional cytotoxic therapies (68). Alternatively, monoclonal antibodies can be "armed" with toxins or radionuclides (7). An anti-EGFRvIII antibody fused to pseudomonas exotoxin A generates a potent immunotoxin (Figure 2) (49). To a similar aim, an immunotoxin consisting of the EGFR ligand TGF-α fused to the pseudomonas exotoxin was generated (78). Because a single toxin molecule can kill an individual cell, this approach efficiently kills tumor cells. Monoclonal antibodies against EGFR and EGFRvIII have also been used to deliver ^{125}I to GBM cells in animal xenografts and in patients (Figure 2) (5, 22). This has the added benefit of potentially enhanced efficacy due to penetratration of the radiation to surrounding tumor cells. However, it also poses the risk of increased toxicity to non-neoplastic tissue.

Immunoliposomes present another promising alternative. This technology, which is in its relatively early stages of development, uses antibody fragments to deliver lipsomes to tumor cells (Figure 2). These liposomes, which are attached to antibody fragments, can be generated to contain a variety of cytotoxic agents, toxins, or even genes for therapy (7, 61, 66, 88).

In addition to serving as a "portal of entry" for toxins or radiation, these receptors, particularly EGFRvIII can be used to induce humoral reactivity (31, 43, 81), either directly or via presentation by dendritic cells to elicit an anti-tumor response (19, 43, 81). These immune-based approaches are the focus of considerable research (19, 43, 46).

Targeting Signaling Pathways

Kinase inhibition at the receptor level—EGFR, EGFRvIII and PDGFR. One of the most obvious points at which to inhibit a signaling cascade is at its origin—the growth factor receptor. A number of synthetic EGFR kinase inhibitors have been developed and are in clinical trials. Reversible inhibitors ZD1839 (IRESSA-AstraZeneca), OSI-774 (Roche/OSI), PKI166 (Novartis), and irreversible inhibitors CI1033 (Pfizer/Warner-Lambert) and EKB-569 (Wyeth-Ayerst) compete with ATP for binding to the receptor, and thus inhibit the kinase activity and downstream signaling through EGFR (65, 73). CI1033 also blocks activation of EGFRvIII (73), as some of these other inhibitors probably also do. These compounds are currently in clinical trials for a variety of cancers (65).

PDGFR is also an attractive target for therapy because PDGFR signaling promotes GBM proliferation and survival (48). STI-571 (Gleevec; Novartis) is an ATP competitive inhibitor of Abl that also has a high level of activity against PDGFR (and Kit) (48, 65, 73). STI-571 has already dramatically impacted the survival of CML patients (15). STI-571 also inhibits the growth of GBM xenografts in vivo (38).

Traditionally, pharmaceutical companies have not focused on the need to test small molecule inhibitors in GBM patients. To rectify this problem, the National Cancer Institute's (NCI) Clinical Trial Evaluation Program (CTEP) has developed a partnership with pharmaceutical companies to develop Cooperative Research and Development Agreements (CRADA) (2). Through CTEP, the North American brain tumor consortium has Phase I/II trials on going for the EGFR inhibitors ZD-1839 (IRESSA) and OSI-774, as well as for STI-571 (Gleevec). Although the results are preliminary, a subset of patients have shown stabilization of growth or reduction in tumor size in response to IRESSA in these clinical trials (Figure 3) (80). In the final analysis of these trials, it will be critical to determine the efficacy of these drugs, and to determine the whether patient sensitivity correlates with receptor expression level and activation of the PI3K/Akt and Ras/MAPK pathways.

Inhibition of PTEN/Akt pathway. Phosphatidylinositol 3-kinase (PI3K) is activated by growth factor

receptors, including EGFR, EGFRvIII and PDGFR (Figure 4). PI3K catalyzes the addition of a phosphate to phosphatidylinositol-4,5,-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5,-triphosphate (PIP3), which initiates many of its tumorigenic activities via Akt (75). Akt is recruited to the plasma membrane by PI3K-mediated formation of PIP3, leading to Akt phosphorylation at thr308 and Ser473 (via phosphoinositide-dependent kinase-1 and phosphoinositide-dependent kinase-2, respectively). Akt phopshoryation at these 2 sites activates its kinase function, leading to downstream signaling that promotes proliferation and inhibits apoptosis (75). The PTEN tumor suppressor gene encodes a phosphatase that catalyzes the dephosphorylation of PIP3, thus inhibiting activation of the Akt pathway (75). When PTEN is altered, the Akt pathway can become constitutively active (Figure 1) (56, 75).

Bi-allelic PTEN loss occurs in up to 40% of GBMs, suggesting that deregulation of the Akt pathway is common in GBMs (47, 64, 69). Since this pathway is so critical for tumor cell proliferation and survival (as well as for downstream angiogenesis), the PTEN/Akt pathway is an attractive target for therapy. PI3K inhibitors such as Wortmannin and LY294002 are effective, but also toxic. Specific Akt inhibitors have been difficult to develop. An alternative approach focuses on mTOR/FRAP kinase, which is activated by Akt, and which can be inhibited with relatively little systemic toxicity (Figure 1) (56). The immunosuppressant drug rapamycin, which is well-tolerated by patients, forms a complex with the immunophilin FKB12 that binds specifically to mTOR and inhibits its kinase activity (56, 75). Rapamycin and its ester analogue CCI-779 (Wyeth-Ayerst) inhibit GBM cell proliferation in culture and in intracerebral xenografts (56). Most promisingly, PTEN-deficiency is associated with enhanced sensitivity to CCI-779 (56). This suggests that rapamycin/CCI-779 may be an effective targeted molecular therapy for GBM. Because EGFR, EGFRvIII and PDGFR all initiate activation of the PI3K pathway, CCI-779/rapamycin may benefit a large number of GBM patients. Some patients have shown clear clinical and radiographic response in early clinical trials (Figure 4), suggesting a proof of this principle. It will be critical to determine the efficacy of CCI-779/rapamycin in these clinical trials, and to determine whether patient sensitivity correlates with PTEN loss and activation of the PI3K/Akt. It will also be important to determine whether EGFR and EGFRvIII expression are associated with response.

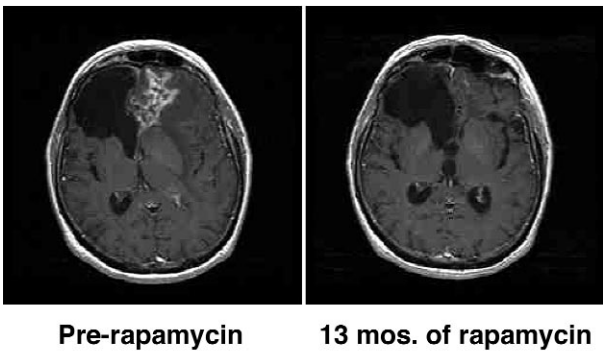

Figure 4. MRI's demonstrating a response to rapamycin. The MRI on the left was taken at baseline; the MRI on the right was performed after 13 months of treatment.

Inhibition of the RAS/MAPK pathway. Ras proteins are a family of membrane-associated small GTPases that transmit signals from cell surface receptors such as EGFR, EGFRvIII, and PDGFR, promoting diverse cellular effects such as proliferation, survival and angiogenesis (Figure 1) (17, 48). To transmit signals, Ras must attach to the plasma membrane, and this is dependent upon the post-translational addition of a farnesyl group to the C-terminal amino acid group known as CAAX box by the enzyme farnesyl-transferase (1, 37). Farnesyl transferase inhibitors (FTIs) block Ras-mediated signaling; they may also inhibit signaling through other pathways requiring farnesylation, including Rho B and the PI3K/Akt pathway (34). In some types of cancer, Ras mutations lead to its constitutive activation (37). This does not appear to be the case in GBMs. Rather, constitutive Ras activation in GBMs arises primarily from EGFR and EGFRvIII, and PDGFR signaling, suggesting that patients whose tumors overexpress these receptors may derive benefit. The synthetic FTIs SCH66336 (Schering-Plough) and R115777 (Janssen Research Foundation) demonstrate promising results in pre-clinical models, including GBM cell lines (18, 27). Further, preliminary evidence suggests that EGFR overexpression in GBM cells can confer enhanced sensitivity to SCH66336 (27). In early clinical trials, these drugs have been effective in some patients with a variety of cancers, particularly in combination with conventional cytotoxic agents (37). NABTC has completed enrollment on one arm of their phase II trial using R115777 and confirmed responses have been obtained in 2 patients with partial response and 2 with stable disease, with response lasting greater than 16 months (Figure 5) (11). In the future, it will be critical to determine the efficacy of this drug in a larger series of patients, and to determine the parameters of sensitivity.

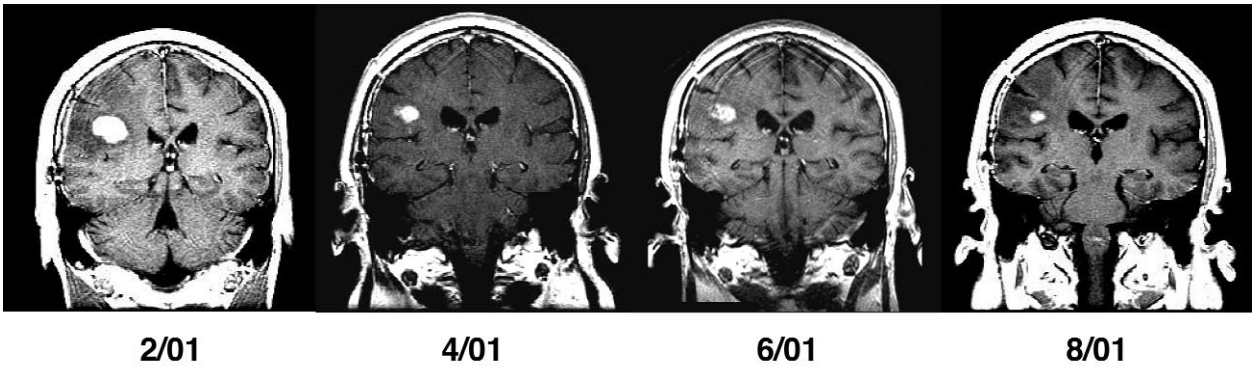

Figure 5. MRI's demonstrating a response to R115777 in one of the patients on the NABTC sponsored clinical trial. These are serial MRI's taken at baseline (left), followed by repeat examination at two month intervals. Note the continued decrease in tumor size up to six months later.

An alternative approach to blocking the growth of Ras-active GBMs comes for the use of the reovirus. Reovirus is replication competent double-stranded RNA virus capable of initiating a cytolytic infection in cells. Reovirus infects and lyses tumor cells, but not non-neoplastic cells (13). This selectivity is associated with Ras activation; transformation of cells with activated Ras, or with the v-erbB oncogene leads to enhanced infection. Mechanistically, a double-stranded RNA-activated protein kinase (PKR) becomes activated by early viral transcripts and inhibits viral replication. Activated Ras, or something downstream of activated Ras, inhibits PKR activation and permits viral replication and oncolysis. In vivo xenograft studies of glioma cell lines have demonstrated efficacious tumor cell lysis (85). Early clinical trials of this promising approach are currently underway.

Lastly, a number of small molecular kinase inhibitors that block signaling, both upstream and downstream of Ras, have been developed, although few have been identified to be safe for patients. In the future, it is likely that a number of such clinically useful compounds will be identified.

Targeting TP53 mutant cells—the role of oncolytic viruses. Loss of the TP53 tumor suppressor gene is strongly associated with secondary GBMs; it is also implicated in their malignant progression (42, 58). Because this represents a loss of function, with loss of pathway activation, it is a harder pathway to target. Oncolytic viruses are a novel approach to this problem. Taking advantage of the natural cytolytic effects of viruses, adenoviruses and some retroviruses have been used as to promote tumor cell lysis (39, 50). Advances in genetic engineering have promoted the development of viruses that can, at least in theory, specifically target TP53-deficient tumor cells. The p53 protein can inhibit replication. Therefore, adenoviruses encode a protein, EIB, that binds to host cell p53 and inhibits its activity. This enables adenoviruses to replicate in TP53 competent cells. To exploit this attribute, a mutant virus has been generated by deletion of an 800 bp fragment of the E1B gene (ONYX-015; Onyx Pharmaceuticals). This renders the adenovirus incapable of replicating in TP53 wild type cells (25), but allows for replication and tumor lysis in TP53 deficient tumor cells.

ONYX-015 is currently in clinical trials for a variety of cancers with some promising initial results (40). However, recent studies call into question both the specificity of this approach, and the role of p53 in blocking the cytolytic effect. ONYX-015 inhibits the growth of GBM xenografts in nude mice independent of TP53 status (25), and some data even suggest that functional p53 may play a role in promoting adenovirus-mediated cytolysis (29). A variety of other oncolytic viruses are currently being developed that may lead to greater efficacy and specificity in the future.

Targeting Angiogenesis and Invasion

So far, we have focused on approaches that target proliferation and survival. However, the dismal prognosis of GBM reflects 2 additional aspects of its biology: angiogenesis and invasion. Angiogenesis is essential for tumor growth (20), and a form of angiogenesis, glomeruloid vascular proliferation (vascular endothelial hyperplasia) is a prominent feature of most GBMs (41). Signaling through VEGF/VEGFR pathway (6, 70) is critical for angiogenesis, although other signaling pathways also contribute to this process. Integrin-mediated signaling, and cell surface interactions between

integrins and matrix metalloproteinases also regulates GBM angiogenesis (3, 87). Further, tumors endogenously secrete anti-angiogenic factors such as angiostatin (8, 59), endostatin (8, 59) and thrombospondin-1 (TSP-1) (33, 44), which inhibit angiogenesis. All of these processes present potential targets for anti-angiogenic therapy.

VEGF/VEGFR signaling can be inhibited at the level of the receptor, or via downstream signaling pathways. Since VEGFR uses many of the same signaling pathways as EGFR, EGFRvIII and PDGFR, including the PI3K/Akt and Ras-MAPK pathways (26), many of the inhibitors described above may also target VEGFR-mediated signaling. At the receptor level, two VEGFR inhibitors PTK787 (Novartis) and SU5416 (Semaxanib; Sugen/Pharmacia) are currently being evaluated, including in NABTC-sponsored clinical trials (65, 73). PTK787 inhibits all three VEGF receptors (VEGFR2-KDR/Flk-1; VEGFR1-FLT-1 and VEGFR3-FLT-4) and reduces the number of tumor microvessels in an animal model (14). PTK878 is currently being evaluated in GBM patients in a phase I clinical trial (86). SU5416 targets VEGFR2. It too has demonstrated impressive results in in vivo animal models of a variety of cancers (21) including GBM (24, 74). As an alternative approach to inhibit the pathway at the level of the receptor, anti-VEGF (Bevacizumab, Avastin; Genentech) and anti-VEGFR2 (IMC-IC11; ImClone) monoclonal antibodies have been developed and are being evaluated in clinical trials (32, 62).

The anti-angiogenic factors angiostatin, which is generated from the proteolytic cleavage of plasminogen, and endostatin, which is generated from the proteolytic cleavage of collagen type 18 (60), promote tumor regression in animal models (4, 20, 60), including subcutaneous U87 xenografts (35). However, the past year few years have shown that optimization of anti-angiogenic compounds may be more complex than initially thought (55). It is nonetheless likely that they these agents will eventually have an impact on GBM patient care. Thrombospondin-1 (TSP-1), another endogenously occurring protein that inhibits angiogenesis (44, 71) may also hold hope as a future therapy for GBM patients (71).

In the process of angiogenesis, new blood vessels invade the surrounding tissue, using many of the same proteins that GBM cells use to invade surrounding tissue (3, 54). Matrix metalloproteinases (MMPs) are critical for this process, and thus represent a potential target for both anti-angiogenic and anti-invasive therapy (54). A number of clinical trials using MMP inhibitors are underway (54). Our group has previously demonstrated that MMP-9 activation is strongly associated with primary GBM subtype, suggesting that MMP-9 inhibition ought to be targeted to a particular subset of GBMs. We also showed that MMP-9 activation is highly correlated with EGFRvIII expression and MAPK activation (10), suggesting potential points at which to block its activation. MMP activation is regulated by a set of interactions between MMP pro-enzymes, membrane bound MMPs, integrins and endogenous inhibitors (TIMPs) that regulate their activation (54). Further elucidation of these complex pathways will likely yield development of more specific and effective MMP inhibitors.

Future Challenges and Opportunities

The development of targeted molecular therapy presents new opportunities, as well as new challenges. It will require a different kind of diagnosis from the neuropathologist. In addition to classifying tumors using our well-developed criteria (41), we will need to provide information about cell surface receptors and the activation state of critical signal transduction pathways. To accomplish this, we will need to develop the tools to identify activation states of critical signal transduction pathways in paraffin-embedded patient biopsy tissue. Our laboratory is currently actively involved in this process. But this will take the concerted effort of multiple groups to achieve this goal.

Continuing research and collaboration between pharmaceutical companies and glioma researchers will be need to develop additional targeted inhibitors, monoclonal antibodies and oncolyltic viruses, and we will need to further identify the molecular determinants of their sensitivity. While pathway inhibition in vitro and in animal xenografts is an important proof of principle, the real determination will require confirmation in clinical trials. Again, this will further require accurate detection of pathway activation states in patient biopsy material. In addition, we will need to refine these tools to serve as molecular inclusion criteria for clinical trials, and then we will need to determine whether the parameters of sensitivity identified in our pre-clinical studies correlate with patient response.

Finally, we will need to develop an expanded molecular sub-classification of GBMs. The primary versus secondary GBM distinction, and the finding of associated molecular lesions has dramatically advanced our understanding of GBM biology. The new challenge will be to tease out additional molecular subclasses, particular those that might require distinct sets of targeted therapies.

This will require genomic approaches such as gene expression profiling coupled with careful biological validation and development of new clinical markers. These challenges are formidable, but they also present an unprecedented opportunity for neuropathology to contribute to cancer therapy.

The inherent molecular heterogeneity of GBMs posses a challenge for any targeted molecular therapy. Micro-dissection studies of GBMs clearly indicate intra-tumor heterogeneity (9, 36). The patchy immunohistochemical expression of EGFRvIII is consistent with this finding (83). Thus, any single treatment may target a subset of tumor cells—but not all of them. Kinase inhibitors such as CCI-779/rapamycin that target downstream effectors common to multiple upstream mutations (such as Akt pathway activation by either PTEN alteration or EGFR/EGFRvIII signaling) may prove to be more efficacious in heterogeneous GBMs. Alternatively, it is probable that synergistic combinations of targeted therapies will be required. This will force us to begin to rigorously confront and determine the extent of molecular heterogeneity within GBMs, particularly as it modulates activation of critical signaling pathways. The challenges imposed by targeted molecular therapy will stretch us, but they may also bring new hope to cancer patients.

Acknowledgments

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