

Antisense Molecular Therapy in Cerebral Gliomas

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Abstract: Despite innovative therapeutic strategies, the expectative of life in patients affected by cerebral gliomas remain dismal. Malignant gliomas represent a class of infiltrative and aggressive neoplasms that are generally resistant to multimodal approach. The efficacy of classical anti-cancer strategies is seriously limited by lack of specific therapies against malignant cells. Glial tumors seem to be able to create a favorable environment for the invasion of glioma cells in cerebral parenchyma when they combine with the extracellular matrix via cell surface receptors. Recent advances in molecular and tumor biology have lead to a new class of modern antitumoral agents. Antisense oligonucleotides are synthetic stretches of DNA which hybridize with specific mRNA strands. The specificity of hybridization makes antisense method an interesting strategy to selectively modulate the expression of genes involved in tumorigenesis.

In this review, molecular targets, clinical and experimental studies about the use of antisense oligonucleotides in cerebral gliomas treatment are reported.

Keywords: Angiogenesis, antisense, gliomas, oligonucleotides, targeted therapy.

INTRODUCTION

Cerebral gliomas are the most common primary brain tumor, generally characterized by highly infiltrative nature, high malignancy, and poor clinical outcome. They account for 42% of all primary CNS tumors and 77% of all malignant primary CNS tumors [1]. Conventional brain tumor treatments include surgery, radiation therapy and chemotherapy. Surgical treatment is invasive but represents the first approach for the vast majority of brain tumors due to difficulties arising in early stage detection. However, gross total removal is not always achievable in relation to the location of the tumor and to preserve vital nervous or vascular structures. Aggressive treatment modalities have extended the median survival from 4 months to 1 year but the survival is often associated with significant impairment in the quality of life. Improvements in surgical technique including intra-operative mapping of the eloquent areas of the brain may offer some benefits in prolonging survival. Radiation therapy and chemotherapy are non-invasive options often used as adjuvant therapy, but may also be effective for curing early-stage tumors. Radiotherapy, for example, seems to be related only to a prolonged progression-free survival, with better control of seizures, but with no substantial differences in overall survival. The effectiveness of systemic chemotherapy is limited by toxic effects on healthy cells, generally resulting in morbidity or mortality of the patient. Moreover, the

presence of the blood-brain barrier (BBB) limits the passage of a wide variety of anticancer agents [2].

Glioma cell invasion is a multi-factorial process, consisting of cell interactions with extracellular matrix (ECM) and with adjacent cells, as well as accompanying biochemical processes supportive of active cell movement. Angiogenesis, represent a key event in the progression of malignant gliomas and involves multiple cellular processes including endothelial cell proliferation, migration, reorganization of ECM and tube formation. It is now generally accepted that during the gliomas progression several tumor suppressor genes are inactivated, and numerous growth factors and oncogenes are progressively overexpressed.

The introduction of molecularly targeted agents is one of the most significant advances in cancer therapy in recent years. Targeted therapies control the activation of oncogenic pathways, either at the ligand-receptor interaction level or by inhibiting downstream signal transduction pathways, thereby inhibiting growth and progression of cancer. Promising approach is now represented by antisense strategy, a specific inhibition of the mutant gene expression *in vivo*, at the level of transcription or translation, by using antisense oligonucleotide directed against DNA or RNA, respectively. Oligonucleotides are increasingly recognized for their potential as therapeutic agents against a variety of human diseases. Early efforts have been mostly focused on antisense oligodeoxyribonucleotides (AONs). Other related therapeutic strategies include those that are based on microRNAs (miRNAs), small interfering RNAs

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(siRNAs), immunomodulatory CpG ODNs, ribozymes, and aptamers.

In this review we report the most relevant findings of antisense oligonucleotides strategy in cerebral gliomas treatment pointing out the attention on effectiveness and targeting modalities.

THE ANTISENSE STRATEGY

The goal of antisense molecular approach is to selectively suppress the expression of a protein by exploiting the genetic sequence in which it is encoded acting at translational level [3-4]. AONs are short synthetic single-stranded DNA-sequences, 13-25 nucleotides long, with a complementary nucleotidic sequence to sense DNA or mRNA. In prokaryotes and eukaryotes genetic information is supported by double-stranded DNA in which only one strand (sense strand) is usually transcribed to messenger RNA. The second strand is called antisense strand because its sequence of nucleotides is the complement of message sense. These antisense molecules may be introduced into a cell to silence genic functions through inhibition of a complementary mRNA. Interactions of RNA-based AONs with target mRNA inhibit gene expression by interfering with protein translation. Differently, double-stranded DNA/RNA in mammalian cells activates the ribonuclease H- (RNase H) mediated degradation of the target mRNA. RNase H recognizes the mRNA-(DNA) oligonucleotide duplex and cleaves the mRNA strand leaving the AONs intact. AONs inhibit mRNA function by several mechanisms including modulation of splicing and inhibiting protein translation by disrupting ribosome assembly. However, the most important mechanism appears to be the utilization of endogenous RNase H enzymes [5]. The arrest of translation by oligonucleotides which hybridize within the coding region or over the initiation codon is dependent on cleavage of the targeted mRNA by RNase H.

An alternative antisense-based approach to modulate gene expression into cancer cells is RNA interference (RNAi). This method is characterized by an endogenous gene silencing mechanism physiologically used by eukaryotes to regulate gene expression. RNAi plays a fundamental role in diverse eukaryotic functions including viral defense, chromatin remodeling, genome rearrangement, developmental timing, brain morphogenesis, and stem cell maintenance. In mammalian cells RNAi can exist under two distinct forms, short interfering RNAs (siRNAs) and

short hairpin RNAs (shRNAs). These last molecules then are processed into siRNAs by the multidomains enzyme DICER in an ATP dependent process. This enzyme is an endoribonuclease that cleaves double-stranded RNA and pre-microRNA (miRNA) into short double-stranded RNA fragments called small interfering RNA. DICER then helps load its small RNA products into large multiprotein complexes denominated RNA induced silencing complexes (RISC). RISC and RISC-like complexes use the small RNAs as guides for the sequence-specific silencing of cognate genes through mRNA degradation, translational inhibition, and heterochromatin formation [6]. The target mRNA is cleaved within the complementary region of the siRNA. DsRNAs activate endogenous dsRNA-dependent protein kinase (PKR), leading to phosphorylation of the translation initiation factor EIF2 α and finally to a suppression of protein synthesis and to an up-regulation of apoptosis. The limit in therapeutic use of siRNA is represented by the transient effect of siRNA. A peculiar distinctive characteristic between RNA interference and the other antisense approaches is the extreme selectivity for siRNA. In this way siRNA may inhibit selectively the expression of oncogene containing a single point mutation, without suppresses the expression of the wild-type transcript [6].

Oligonucleotides being polyanionic macromolecules, face multiple obstacles in reaching their intracellular site of action, which presents a significant problem for development of an effective delivery system. In fact, there is no natural mechanism for these highly hydrophilic macromolecules to traverse the cellular membrane and the bioavailability of these agents on their own is minuscule. In addition, oligonucleotides are produced by chemical synthesis, which enables large-scale production of pure materials and introduction of chemical modifications that provides greater metabolic stability or improved bioavailability. Due to the high molecular weight and polyanionic characteristics, AONs are subjected to relatively rapid clearance from the blood circulation. Low cellular membrane permeability and lack of cell-type specific uptake are additional issues that need to be addressed to advance these molecules into the clinic. To overcome these limits chemical modifications such as the introduction of phosphorothioate linkages and 2'-O-methylation of the ribose moieties have been developed [7-8]. The chemical modifications can significantly improve the properties of AONs therapeutics and may be unavoidable for their clinical exploration. A variety of targeting strategies have been

studied to facilitate efficient cell or tissue-selective delivery of AONs. These strategies are generally divided into two classes: ligand-AON conjugates and ligand-targeted nanoparticles. 3' or 5' modification of AONs is a common technique for enhancing resistance to degradation and introducing targeting ligands such as peptides and aptamers. Cationic polymers and cationic lipids are two major classes of materials commonly used for incorporating AONs into nanoparticles.

Nanoparticles delivery systems in cancer therapy provide better penetration of therapeutic agents with a reduced risk in comparison to classical treatment. Nanoparticles distribution within the body is related to their relatively small size (resulting in longer circulation times) and their ability to take advantage of tumor characteristics [9]. Furthermore, NP size allows for interactions with biomolecules on cell surfaces and within the cells without altering the behavior and biochemical properties of those molecules. Drugs delivery into the brain is a major challenge due to the presence of the BBB. Through nanoplateform-based intracerebral drug delivery systems, drugs and therapeutic probes in general could be delivered into cerebral areas also with intact BBB. Nanoscale drug delivery vehicles have shown the ability to encapsulate a variety of therapeutic agents such as small molecules, peptides, protein-based drugs, AONs, and nucleic acids. Encapsulated molecules can be released from nanocarriers in a controlled manner over time to maintain a drug concentration within a therapeutic window, or the release can be triggered by some stimulus unique in the delivery site [10]. The surface of the nanocarrier can be engineered to increase the blood circulation half-life and influence the bio-distribution, while attachment of targeting ligands to the surface can result in enhanced uptake by target tissues [11]. The result is a lower systemic toxicity of the therapeutic agent, while increasing of the agent concentration in the area of interest will result in a higher therapeutic index [12].

One of the most investigated approaches uses liposomes as sub-micron delivery vehicles. Liposomes consist of a lipid shell surrounding a core containing a therapeutic molecule or gene. They are particularly useful as gene therapy devices due to their ability to pass through lipid bi-layers and cell membranes. Lipid encapsulation is an attractive delivery approach because of the biocompatibility of constituents and facile assembly of the complexes, which requires only mixing and incubation of components. In addition,

these complexes can be engineered for specific delivery through conjugation of targeting moieties directly to the lipid molecules prior to liposome production. Glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine (DOPE) are among the most widely used neutral lipids. Simply mixing siRNA with DOPC results in more than 65 percent encapsulation, and these complexes have been shown to bring about siRNA-mediated silencing in cancer cells *in vivo* [13]. Cationic lipids, such as 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane (DOTAP), can complex electrostatically with siRNAs and be used to create a more effective liposome as the positively charged lipids provide enhanced cell entry and increased protection against serum enzymes [14]. Recently, a new wave of work in lipid-based delivery systems has demonstrated that some synthetic lipid-like materials (termed "lipidoids") form complexes with siRNA or miRNA that facilitate intracellular delivery of the oligonucleotides [15]. Polymeric nanospheres are capable of translocation into the cytoplasm of a cell, but transport to the nucleus has not been established [16]. Polyamidoamine (PAMAM) and poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) are low toxic polymers which have shown great potential as carriers. Polycaprolactone (PCL) is another promising delivery system. PCL-g-PDMAEMA nanoparticle/DNA complexes could escape from the endosome and release their payloads effectively in cytoplasm, which may be induced by the enhanced interaction between the complexes and cell membrane due to hydrophobic modification [17].

Generally speaking systemic AONs treatment is well tolerated and side-effects are dose-dependent. Dose-limiting toxicities include thrombocytopenia, hypotension, fever and asthenia. Furthermore, elevation of the liver enzymes aspartate aminotransferase and alanine aminotransferase, as well as complement activation and a prolonged partial thromboplastin time have been reported [18].

MOLECULAR TARGETS

The choice of target gene is crucial to the potential success of the therapeutic approach. Growth factors comprise a complex family of polypeptide hormones, steroid hormones, or biological factors that are capable of stimulating cell growth, proliferation and differentiation. Growth factors are important for regulating a variety of cellular processes, including regulating tissue morphogenesis, angiogenesis, cell

differentiation, and neurite outgrowth. Activities of growth factors are mediated *via* binding to transmembrane receptors that often contain cytoplasmic tyrosine kinase domains. Growth factors such as epidermal growth factor, vascular-endothelial growth factor, transforming growth factor, and insulin-like growth factor type I, are present during the development of the CNS. When they reappear in the mature brain they are overexpressed in neoplastic glia, participating in the development of malignant gliomas. However, the most investigated targets are involved in apoptosis, cell proliferation, neoangiogenesis and invasion (Tab.1).

VEGF Family

The VEGF family of growth factors and their receptors are the most important mediators of glioma angiogenesis. The VEGF acts as a major vascular permeability factor and as a mitogen/survival promoter for endothelial cells [19]. VEGF-A and its receptors are the best characterized signaling pathway in angiogenesis and binds to two receptor tyrosine kinases (RTK) – VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). It is generally agreed that VEGFR-2 is the major receptor mediating the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. VEGFR-1 is involved in the activation of MMPs associated with matrix degradation and in the production of growth factors from endothelial cells. VEGF-A gene expression is up-regulated by hypoxia, mediated by the transcription factor HIF and the product of the von Hippel–Lindau (VHL) tumor suppressor gene. The ligands for VEGF3 (VEGF-C&D) are expressed by multiple cell types that surround the angiogenic vessels, suggesting the existence of a novel pro-angiogenic paracrine signaling pathways in these neoplasms. VEGF enhances vascular permeability through the MAPK signaling cascade by rearranging cadherin/catenin complexes and loosening adhering junctions between endothelial cells. The activation of MAPK/ERK is associated with inhibition of the Jun-N terminal kinase (JNK) pathway in mediating the anti-apoptotic effect of VEGF. Activated VEGFR-2 mediates the phosphorylation of Akt, which potently inhibits endothelial cell apoptosis by interfering with various apoptosis signaling pathways [20]. VEGF stimulates endothelial production of urokinase-type plasminogen activator (uPA), which induces conversion of plasminogen to plasmin, causing the breakdown of ECM components and leading to ECM remodeling [21]. The end result of VEGF signaling in tumors is the production of immature, highly permeable blood

vessels with subsequent poor maintenance of BBB and parenchymal edema [22]. Edema in glioma tumors is considered one of the most pathological characteristics, but the mechanism of regulating vascular permeability is still unclear. In tumorigenic mice generated by subcutaneous injection of glioma cell lines, overexpression of antisense VEGF (C6-VEGF(-/-) mice) significantly suppressed tumor growth, decreased angiogenesis and reduced tumoral edema. Further studies by electron microscope revealed that tumor-induced hyperpermeability was mediated by formation of vesiculo-vacuolar organelles (VVO), specifically reducing the number of vesicle and caveolae in VVO, and this effect was partially blocked by antisense VEGF [23]. In a successive study, in C6 cells with expression vectors containing sense (C6/VEGF+) or antisense (C6/VEGF-) VEGF complementary DNA (cDNA) or an empty vector (C6/vec), VEGF expression, water content, and morphological characteristics were investigated. The observations demonstrated that VEGF can aggravate edema in tumor tissues by increasing VVO and plays critical roles in the stickiness of tumor cells to vessel wall and in the integrity and continuity of the basal lamina of vessels [24].

EGFR Family

Epidermal growth factor receptor (EGFR, ErbB1, HER1) is a tyrosine kinase receptor that is abnormally activated in 70% of solid cancers. EGFR overexpression and immunoreactivity are more common in primary tumors than in secondary glioblastomas. The EGFR transmembrane protein comprises three domains: the extracellular domain, the transmembrane domain, and the cytoplasmic domain, which harbors the tyrosine kinase activity. Ligand binding (amphiregulin, EGF, transforming growth factor TGF- β , decorin, betacellulin) to the extracellular domain of a monomer results in its homo- or heterodimerization, inducing phosphorylation of the tyrosine kinase domain, activating several signaling pathways, in particular: phosphatidylinositol 3'-kinase/Akt/mammalian target of rapamycin (mTOR), Ras/mitogen-activated protein kinase (MAPK), phospholipase C (PLC)/protein kinase C (PKC), and (d) c-Src [25]. These activated pathways are involved in several cell biological processes, including cell proliferation, angiogenesis, migration/adhesion, survival, and differentiation. Recently was evaluated the suitability of folate-PAMAM dendrimer conjugates for efficient EGFR ASODN delivery into glioma cells, wherein they release the ASODN from the FA-PAMAM

to knock down EGFR expression in C6 glioma cells, both *in vitro* and *in vivo*. Folic acid was coupled to the surface amino groups of G5-PAMAM dendrimer (G5D) through a 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide bond, and ASODNs corresponding to rat EGFR were then complexed with FA-PAMAM. The ASODN transfection rates mediated by FA-PAMAM and PAMAM resulting in greater suppression of EGFR expression and glioma cell growth. Stereotactic injection of EGFR-ASODN:FA-PAMAM complexes into established rat C6 intracranial gliomas resulted in greater suppression of tumor growth and longer survival time of tumor-bearing rats compared with PAMAM and oligofectamine-mediated EGFR-ASODN therapy [26]. In a previous study, has been demonstrated that the cotransfection of U87MG cells with wild-type PTEN and antisense EGFR constructs could inhibit the cellular growth by 91.7% [27]. Studies in human GBM cells suggest that EGFRvIII, a mutated EGFR isoform, promotes tumor growth and progression *via* constitutive activation of the PI3-K/Akt pathway; it also induces up-regulation of cell proliferation *via* the MAPK/ERK1/2 signal transduction pathway. The use of antisense RNA into murine models of human glioma facilitates tumor targeting and induces reduction of HER1/EGFR expression increasing survival [28].

PI3K Pathway

Over-activation of the PI3K/AKT/mTOR pathway seems to play a key role in the downstream signaling pathways promoting growth and survival in several types of tumor cells, including glioma. Pathway activation is triggered by stimulation of growth factor receptors (including EGFR, PDGFR, fibroblast growth factor receptor, and insulin-like growth factor-I receptor) and the Ras pathway, leading to activation of PI3K. pAKT promotes phosphorylation of several downstream effectors, including MDM2, p21/p27, Bad, FKHR, nuclear factor- κ B, caspase-9, glycogen synthase kinase-3 α , and mTOR. mTOR plays a key role in downstream signaling of the PI3K/AKT pathway through the regulation of cellular catabolism, anabolism, proliferation, cell cycle control, autophagy, angiogenesis, and apoptosis. In a murine model, Pu *et al.* showed the down-regulation of proliferation rate of C6 glioma cells transfected with antisense AKT2 cDNA construct. Parental C6 cells and C6 cells, transfected with antisense construct, were implanted into the right caudate nucleus of rats through lipofectamine complexes. Antisense inhibition of AKT2 resulted in the reduction of growth rate and proliferation of C6 cells, as

well as in the up-regulation of GFAP expression and in the induction of apoptosis [29].

The protein kinase C- α (PKC- α), translated from the mRNA of PKC- α gene, located on chromosome 16p11.2-q12.1, is involved in growth-factor-mediated signal transduction pathways. Numerous pre-clinical studies in animal models have showed the efficacy of AONs against PKC- α in the cancer growth inhibition. Inhibition of PKC- α expression, at mRNA level, by a syntetic AON has shown to inhibit proliferation of C6 glioma cells and *in vitro* and *in vivo* growth inhibition of transformed U-87 cells transfected with a PRSV vector loading antisense anti-PKC- α oligonucleotide, and, respectively, *in vivo* subcutaneous tumorigenicity lost [30-31]. Grossman *et al.* has studied the therapeutic efficacy and toxicity of a phosphorothioate AON (Aprinocarsen, Eli Lilly LY9000003) directed against PKC- α in patients with recurrent gliomas. In this clinical phase II study no clinical benefit was seen, probably because of tumor growth or the effect of Aprinocarsen on BBB, whose mechanisms are unknown [32]. Other potential therapeutic targets are human C-raf kinase and c-Ki-RAS proteins and signal transduction kinase proteins, involved in the control of proliferation, cellular migration and differentiation, and cytoskeletal rearrangements. Monia *et al.* demonstrated the antitumor activity of phosphorothioate antisense inhibitor C-raf Kinase (ISIS 5132 or CGP69846A) in tumor-bearing mice, demonstrating an important reduction of tumor growth [33].

IGF Family

Neuroglial cell growth and CNS development are normally strongly regulated by IGF-1 and IGF-2 *via* IGF-1R. By introducing a plasmid expressing an antisense RNA to IGF-1R RNA, Resnicoff *et al.*, demonstrated the reduction of IGF-1R levels in cell growth *in vitro*, anchorage-independent growth and prevention of the development of tumors in rats [34]. Recently, in an *in vivo* murine model, IGF-1R AONs-treated autologous glioma cells induced toward apoptosis, gave rise to a host response [35]. In an *in vitro* and *in vivo* study (U87 and LN229 glioma cell lines and mice bearing intracranial glioma xenografts) has been showed that TAE226, an ATP-competitive inhibitor of several tyrosine kinases, down-regulated proliferation and invasion of glioma cells and inhibited cell cycle progression at the G2-M checkpoint. TAE226 also gave rise to a concomitant reduction of the expression of p-cdc2 (Tyr15) and cyclin B1, increase in apoptosis and reduction in glioma invasion in an *in vitro*

Table 1: Principal Molecular Pathways Involved in Gliomagenesis and Glioma Angiogenesis

Molecular target	Normal function	Kind of mutation	Effect of mutation
VEGF	Mitogen/survival promoter for endothelial cells. Angiogenesis.	Overexpression; gene amplification	Cell invasiveness and angiogenesis by stimulating secretion of MMPs
EGFR	Increased motility, invasion, and proliferation of tumor cells.	Overexpression	Upregulation of cancer cell, proliferation and tumor growth.
PKC- α	Involved in growthfactor-mediated signal transduction pathways	Overexpression	Proliferation and tumor growth.
Insulin-like growth factor I	Transmembrane receptor activated by IGF-1 and by IGF-2. It belongs to the large class of tyrosine kinase receptors.	Overexpression Gene amplification	Cancer cell proliferation, downregulation of apoptosis.
TGF- β 2	Suppression of the effects of interleukin dependent T-cell tumors.	Overexpression	Downregulation of apoptosis and tumor growth.
PDGF, FGF (involved in PI3K/AKT/PKB pathway)	Pathway involved in cell survival.	Overexpression Gene amplification	Upregulation of cancer cell proliferation

Matrigel [36]. By using anti-FAK phosphothioate AONs packaging into liposomes in U251 MG cells, Wu *et al.* showed the down-regulation of expression levels of FAK and activation of apoptosis, through increase in caspase-3 activity, the key-mediator of apoptosis in mammalian cells. In fact, FAK activates the PI3K survival pathway with the concomitant activation of nuclear factor Kappa B (NF- κ B) and induction of inhibitor of apoptosis [37].

TGF Family

Jachimczak *et al.* demonstrated the efficacy of specific antisense therapy in down-regulation of TGF- β 2 through stimulation of antitumoral immunosurveillance [38]. In HTZ-153, HTZ-209 and HTZ-243 glioblastoma and malignant astrocytomas cell lines, TGF- β 2 specific phosphorothioate AONs enhanced lymphocyte proliferation up to 2.5 fold and autologous tumor cytotoxicity up to 60% [39]. TGF- β 2 binds to TGF- β receptors (TBR) and gives the start to a signaling cascade *via* cytoplasmic signaling mediators (SMAD) into the nucleus, inducing regulation of target gene expression [40]. During tumorigenesis, TGF- β increases extracellular matrix invasion and induces escape of tumor from immunosurveillance. The antisense compound AP 12009 (AON anti TGF- β 2 mRNA) enhanced the immune cell mediated cytotoxic antitumor response [41-43]. Schneider *et al.* have examined a “double-punched” approach to overcome the escape of glioblastoma cells to the immune

surveillance, through an active specific immunization (ASI) with Newcastle-Disease-Virus infected tumor cells and blocked the TGF- β production by delivery of TGF- β AONs using polybutyl cyanoacrylate NPs. This approach induced a significant decrease in plasma TGF- β 2 level and, at the same time, an increase in rate of high affinity IL-2 receptor (CD25) on lymphocytes and consequently of antitumoral cytotoxicity [44].

PDGF and FGF

The FGF family of proteins and their receptors are overexpressed in various types of cancer. Binding of FGF to its receptor causes transphosphorylation and activation of intrinsic tyrosine kinase, which results in signal transduction. Both acidic FGF (aFGF) and basic FGF (bFGF) are up-regulated in GBM [45] and are responsible for resistance of endothelial cells to apoptosis. Basic fibroblast growth factor (bFGF) is expressed by vascular cells and, focally, by the tumor cells. The anti-apoptotic effect of bFGF is mediated by increased expression of Bcl-XL and Bcl-2 *via* the MEK-dependent signaling pathway. aFGF and bFGF induce endothelial cell proliferation and migration. PDGF-B and platelet-derived growth factor b receptor (PDGFRb) have important roles in the development and differentiation of the vessel wall [46]. PDGF is a mitogen for multiple cells of mesenchymal and neuroectodermal origin that acts through the PDGF receptors α and β . PDGF expression correlates with astroglial malignancy and angiogenic activity and

contributes indirectly to tumor angiogenesis acting as a potent mitogenic and chemotactic stimulus for angiogenesis associated stromal cells, such as smooth muscle cells or pericytes. The angiogenic effects of PDGF are mediated through PI3K/Akt, MAPK/ERK and STAT3 signaling [47].

The influence of endogenous basic FGF on glioblastoma cell growth *in vitro* was evaluated using basic FGF-specific antisense oligonucleotides. In two glioblastomas cell lines (HTZ-146, HTZ-17) the expression of growth factors by northern blotting and immunochemical methods were evaluated. To investigate the possible pathophysiological role of PDGF and bFGF, antisense technology was employed with chemically modified nuclease-stable 14-mer phosphorothioate oligodeoxynucleotides. Antisense phosphorothioate oligodeoxynucleotides *in vitro*, targeted against PDGF-A-chain-, -B-chain-, and -bFGF-messenger ribonucleic acid, reduced the proliferation index. These data indicate autocrine stimulatory loops for PDGF and bFGF, which may be blocked, and may have interesting therapeutic implications [48]. In another study, the influence of bFGF on additional parameters of glioma cell malignancy was evaluated utilizing also antisense oligonucleotide primers to suppress bFGF expression or activity. The addition of 30 microM bFGF-specific antisense oligonucleotide primer to the human glioma cell line SNB-19 resulted in a 55% inhibition in colony formation in soft agar. This effect was dose-dependent and specific, as sense strand primer was ineffective in suppressing growth. In addition to exhibiting fewer colonies, antisense treatment significantly altered colony morphology [49].

EXTRACELLULAR MATRIX PROTEINS

Khazenon *et al.* have demonstrated the effects of antisense inhibition of laminin-8 expression in glioma therapy through an *in vitro* model using human GBM cell lines M059K and U-87MG co-cultured with normal human brain microvascular endothelial cells (HBMVEC). Endothelial cells contribute laminins containing $\alpha 4$ and $\alpha 5$ chains, whereas glial cells synthesize laminins containing $\alpha 1$ and $\alpha 2$ chains. During progression of human gliomas, the expression of capillary BM laminins containing $\alpha 4$ chain switches from the predominant laminin-9 into laminin-8. Laminin-8 plays an important role in glioma cell invasiveness, in combination with other proteins associated with glioma progression, such as tenascin-C, MMP-2 and MMP-9. In fact, up-regulation of laminin-8 expression in both glioma cells and glioma-adjacent capillary endothelial

cells may reduce glial cell adhesion and enhance migration. The result of this study was a significant reduction of invasion of co-cultures through Matrigel and through the use of morpholino oligos against $\alpha 4$ and $\beta 1$ chains of laminin-8 [50]. Polycefin, a bioconjugate drug delivery system based on slime-mold *Physarum polycephalum*-derived poly(malic acid), is used to inhibit the expression of laminin-8 constituents in GBM *in vitro* and *in vivo*. It carries an attached transferrin receptor antibody to target tumor cells and to deliver two conjugated morpholino antisense oligonucleotides against laminin-8 $\alpha 4$ and $\beta 1$ chains. Polycefin efficiently inhibited the expression of the $\alpha 4$ and $\beta 1$ laminin-8 chains by cultured GBM cells. Intracranial Polycefin treatment of human U87MG glioblastoma-bearing nude rats reduced incorporation of both tumor-derived laminin-8 chains into vascular basement membranes. The treatment also significantly reduced tumor microvessel density and increased animal survival [51]. $\alpha 4$ chain is a component of laminin-8 and laminin-9 both of which are overexpressed in approximately 75% of GBM cases and in low grade gliomas. Laminin $\alpha 4$ chain appears to be an important factor in glioma migration and invasion, both *in vitro* and *in vivo*. Nagato *et al.* have demonstrated that the downregulation of laminin $\alpha 4$ chain using AONs inhibits the motility of human glioma cells [52]. Increased expression of matrix metalloproteinases (MMPs) has been associated with human glioblastoma tumor progression. MMP-9 expression was significantly reduced by the use of a plasmid vector capable of expressing an antisense transcript complementary to a 528-bp segment at the 5' end of human MMP-9 cDNA. Co-cultures of tumor spheroids and fetal rat brain aggregates showed that the antisense-transfected stable clones showed no invasion of the rat brain aggregates. Intracerebral injection of antisense stable transfectants in nude mice produced no tumors or very small tumors [53].

DISCUSSION

Malignant gliomas remain a poorly understood form of cancer associated with high rates of morbidity and mortality. Current conventional treatments protocols include maximally safe surgical resection followed by fractionated radiation therapy of the tumor and surrounding brain parenchyma and systemic chemotherapy. However, radiation therapy is limited to a largely palliative role, and chemotherapy has provided only a modest benefit in clinical outcome. Targeted therapies are designed to disrupt specific functions or deliver toxins to tumor cells expressing the targets. Developments in molecular biology have led to

a clearer understanding of the mechanisms of tumor development and resistance to therapy. The knowledge of the gliomas genetic bases and of their invasive behavior may suggest new molecular targets to overcome the mechanisms of multi-drug resistance of the actual therapeutic approaches. Genetic heterogeneity of tumor, redundant and overlapping signal transduction pathways, and limited drug delivery to the tumor are among several mechanisms underlying therapeutic failure.

The antisense oligonucleotide strategy is emerging as a valid approach to selectively modulate gene expression. Many studies have demonstrated convincing *in vitro* reduction in target gene expression and promising activity against a variety of human tumors. Besides, clinical trials have evidenced that antisense oligonucleotide treatment is well tolerated and do not increase the toxicity of conventional treatments. This new strategy has showed interesting results in glioma cell lines and *in vivo* murine models. On the basis of these preliminary results and the limits of actual standard therapeutic protocol, antisense therapy may be an interesting approach to modify the biological development of gliomas, probably trying to modulate crucial pathways of gliomagenesis during precocious steps of tumor progression and possibly two or more molecular targets of the same pathway or of two different pathways. In consideration of the complex pathological intratumoral gene expression and of multiple phenotypes inside the same glioma, we consider very interesting the idea to detect more molecular targets within the same therapeutic scheme [2, 54].

Angiogenesis seem to represent the key rate-limiting step to evolution from a low-grade glioma, anatomically limited, to a high grade glioma, diffuse and anatomically and biologically uncontrolled. On the base of this pathophysiological consideration, anti-angiogenic approach appears to be an important therapeutic target for malignant gliomas [55]. Future possible molecular target may be clusterin, eIF-4E (eukaryotic initiation factor-4E), integrins, metalloproteinases and other key molecules involved in invasion and angiogenesis. Most researchers agree that anti-angiogenic therapy should be combined with a cytotoxic agent for maximal benefit. In the light of these considerations, we are investigating the role of HIF (Hypoxic inducing factor) isoforms and IL-8 (Interleukine-8) in gliomas progression and the possibility to block their pathways through an antisense molecule. HIF-1 α actively regulates downstream

processes and it is also influenced by the tumor microenvironment in many different ways. HIF is a transcriptional factor produced in response to hypoxic conditions within HIF-1 α /VEGF-regulation expression-dependent angiogenic pathway. Our investigations have recently demonstrated high expression levels of PGES-1 (Prostaglandine E 1 Synthase) and IL-8 in glioma cells and microglial cells strongly correlated with grading tumor [4, 57]. During malignancy progression, leukocyte infiltration and necrosis are associated with the development of neovascularization. In malignant gliomas IL-8 further localizes in oxygen-deprived cells surrounding necrosis. Macrophages are known to produce high levels of IL-8 by inducing tumor growth and angiogenesis; IL-8 is an inflammatory chemoattractant responding to the tumor microenvironment. These preliminary results demonstrate an important role of IL-8 as crucial angiogenesis mediator within HIF-1 α pathway and crosstalk between hypoxia-induced high levels of HIF-1 α and VEGF expression.

Future treatment strategies for malignant gliomas will likely involve synergistic combinations of agents aimed at different pathways in the molecular pathogenesis of this type of cancer. Major steps to improve AON delivery into the central nervous system and the efficacy of AON therapy for malignant brain tumors include the design of more effective vector systems and the development of new techniques to enhance delivery of genetic vectors into brain tumors and for monitoring gene delivery into tumors. The future potential of antisense therapy depends on the design of multiple drugs based on the major knowledge of genes and their functions.

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