Glioblastoma multiforme: molecular biology and new perspectives for therapy

Review Article

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Summary

Pathogenic features of glioblastoma multiforme and of other gliomas are reviewed in the present article. Emphasis is given to those genetic alterations which are involved in oncogenesis, to the process of tumor neoangiogenesis and to the role played by the immune system in controlling neoplastic growth. Aspects which are relevant to therapeutic interventions are also dissected, and gene therapy in particular. A new gene therapy approach that combines tumor suicide, via enzyme-directed prodrug activation, and cytokine-promoted immune rejection is reported, together with results from the first application of this approach in humans.

I. Introduction

The outcome of malignant gliomas remains extremely poor, in spite of aggressive use of currently available therapies. Recent advances in elucidating the molecular biology of gliomas have led to the development of innovative therapeutic strategies. The more promising approaches involve gene therapy, aiming at increasing tumor cell chemosensitivity and/or immunogenicity, by transfer of genes expressing cytokines and prodrug activating enzymes.

Glioblastoma multiforme (GBM) represents 15-20% of all intracranial tumors and 50% of gliomas (Russel and Rubinstein, 1989). It affects 5,000 Americans and 1,000 Italians every year, and typically occurs in adults, with a peak incidence in the fifth and sixth decades of life. It is a very aggressive tumor, with a uniform and profound morbidity. Because of its morbidity it contributes to the cost of cancer on a pro capite basis more than any other tumor. Despite surgery, radiotherapy and/or chemotherapy, the prognosis is extremely poor and has not substantially changed over the last two decades, death resulting in 80% of patients for tumor recurrence within 6-12 months from treatment.

Glioblastoma multiforme (grade IV astrocytoma) is usually located in the cerebral hemispheres, though it occasionally appears at other sites, such as the cerebellum, the brain stem and the spinal cord. Histology shows marked cytological diversity, ranging from tumors composed of small cells with scant cytoplasm to those composed of multinucleated giant cells. The World Health Organization (WHO) classification recognizes two distinct subvariants of the tumor: (i) giant cell glioblastoma, characterized by a predominance of enormous, multinucleated giant cells and, on occasion, an abundant stromal reticulin network; and (ii) gliosarcoma, in which hyperplastic vascular elements have undergone sarcomatous transformation.

Current therapies for malignant gliomas include surgical removal of the tumor mass, which is mandatory for precise diagnosis, and irradiation. Although surgery improves the prognosis (Levin et al, 1993), the infiltrative behavior of malignant gliomas precludes their complete
resection, and 90% of GBM recur within 2 cm of the primary site. Postoperative radiotherapy is therefore commonly administered, with a significant improvement in survival (Hochberg and Pruitt, 1980; Walker et al, 1980). Despite surgery and irradiation, however, only a few patients are alive two years after diagnosis. Results of chemotherapy trials are disappointing (Hosli et al, 1998). This is due both to the tumor intrinsic chemoresistance (Petersdorf and Berger, 1996) and to the tumor location within the central nervous system, which limits the penetration of drugs (Janzer and Raff, 1987; Mak et al, 1995). Among malignant gliomas, GBM is the least responsive to medical treatment. Available protocols include both monochemotherapy and polychemotherapy regimens. Nitrosoureas are the leading drugs in glioma chemotherapy, with response rates as single agents varying from 10% to 40% (Young et al, 1973; Fewer et al, 1972; Hoogstraten et al, 1972). Other drugs, evaluated in monochemotherapy (Forsyth and Cairncross, 1996), occasionally showed clinically and radiologically objective responses. Among these are vincristine (Smart et al, 1968), procarbazine (Rodriguez et al, 1989), paclitaxel (Chamberlain and Kormanik, 1995; Prados et al, 1996), and temozolomide (Newlands et al, 1996). However, methodological bias present in most studies raise doubts about the validity of these results. The most commonly used polychemotherapy regimens for gliomas are PVC (i.e. a combination of CCNU, procarbazine, and vincristine) and MOP (i.e. a combination of procarbazine, vincristine, and mechlorethamine). Response rates (complete or partial) of 17-37% have been reported for glioblastomas (Levin et al, 1980; Coyle et al, 1990). More recently, interesting results have been obtained in GBM patients with the ICE regimen (ifosfamide, carboplatin and etoposide), although in association with severe hematological toxicity (Sanson et al, 1996). The role of PVC as adjuvant chemotherapy is controversial (Fine et al, 1993), and, overall, there is no clear-cut evidence that survival of glioblastoma patients is improved by chemotherapy (Hosli et al, 1998).

II. Molecular biology of glioblastoma multiforme and corrective gene therapy

As for most cancers, brain tumors derive from a multi-step process of successive alterations, including loss of cell cycle control, neoangiogenesis and evasion of immune control. Figure 1 summarizes the genetic alterations associated with the malignant transformation of astrocytes. Most of these changes involve the loss of putative tumor suppressor genes or activation of proto-oncogenes.

Gene therapy of cancer, in its most direct form, should aim at replacing a mutated gene with its correct form, or at suppressing the abnormal oncogenic function. At present, however, such a corrective gene therapy, faces the insurmountable task that gene replacement, or gene suppression, should simultaneously involve a number of different genes, and should be applied to all tumor cells to reverse the malignant phenotype. Hence, corrective gene therapy seems to be quite difficult to propose as a single therapeutic approach.

A. Genetic alterations

1. Oncogenes

Several members of the protein-tyrosine kinase receptor family are over-expressed by gene amplification in malignant gliomas, including the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor-α (PDGFRα) and the c-met genes (Furnari et al, 1995). A high percentage of glioblastomas also have EGFR gene rearrangements that may lead to the expression of a truncated, constitutively activated receptor. The transfer of a mutant human EGFR gene into glioblastoma cells caused constitutive self phosphorylation and a pronounced enhancement tumorigenicity in nude mice.

Figure 1. Simplified representation of oncogenes and tumor suppressor genes contributing to malignant progression of astrocytic tumors.
(Ekstrand et al. 1994; Nishikawa et al. 1994). Numerous strategies are currently being investigated to specifically inhibit EGFR using antibodies, immunoconjugates or antisense technology. The selective inhibition of EGFR in human GBM cells with kinase-deficient mutants inhibited cell proliferation and transforming efficiency in athymic mice (O'Rourke et al. 1997), and converted radioresistant human glioblastoma cells to a more sensitive phenotype (O'Rourke et al. 1998), providing a rationale for gene therapy applications.

Other dominant oncogenes, such as N-myc, fos, src, H-ras or N-ras, and mdm2 are amplified and highly expressed in gliomas (Collins, 1993). GBM produce high levels of insulin-like growth factor I (IGF-I). When this alteration has been targeted by a vector expressing an antisense anti-IGF-I gene, rejection of genetically altered rat C6 glioma cells was observed. Injection, even at a site distal to the tumor, caused regression of established brain GBM. Destruction of the tumor was mediated by a glioma-specific T CD8+ (CTL) response (Trojan et al. 1993).

2. Genes associated to cell immortalization

A role in cell immortalization has been proposed for telomerase, the RNA-protein complex that elongates telomeric DNA. Telomerase is expressed almost exclusively in cancer cells, but not in normal cells, suggesting the possibility that gene therapy may be applied to inhibit this function. A successful example of treatment via antisense oligonucleotides directed against human telomerase suppressed glioma cells growth and survival, both in vitro and in vivo, through the induction of apoptosis (Kondo et al. 1998).

3. Tumor suppressor genes

Molecular and cytogenetic analyses of gliomas have shown frequent losses of genetic material, suggesting the inactivation of putative tumor suppressor genes. Loss of heterozygosity (LOH) has been described in chromosome 1p, 9p, 10p, 10q, 11p, 13q, 17p, 19q, and 22q, and in some cases the tumor suppressor gene involved in LOH has been identified. This is the case of the p53 tumor suppressor gene, which maps in 17p. Wild-type p53 protein is involved in G1 cell cycle arrest and apoptosis of DNA-damaged cells and is therefore crucial in preventing mutation or deletion of functional genes. Mutations of p53 seem to be an early event in glioma tumorigenesis, being frequently detected also in low grade astrocytomas. Along with p53 mutations, amplification of the mdm2 oncogene, whose product binds to and degrades p53, accounts for p53 inactivation in gliomas. Since p53 plays a key role in the pathogenesis of most cancers, it has raised great interest as a target for cancer gene therapy. Transduction of malignant cells with wild type p53 can significantly inhibit growth and neoangiogenesis, or can induce apoptosis in p53 mutant cells in several tumor models in vitro, including gliomas (Badie et al. 1995; Van Meir et al. 1995; Gomez-Manzano et al. 1996). The presence of functional p53 has also been shown to modulate chemoresistance. Consequently, another possible advantage of the restoration of wild type p53 may be sensitization to chemotherapy and radiotherapy. Indeed, the combination of p53 gene transduction with radiation or chemotheraphy (Lowe et al. 1994) has resulted in local tumor control superior to either therapy alone (Fujirwara T et al., 1994; Gyerset et al., 1995; Nguuyen et al., 1996, Lang et al. 1998). This combined therapy is currently under investigation in clinical trials (Roth and Cristiano, 1997; Nielsen and Maneval, 1998).

The cell cycle regulator genes provide an additional target for corrective gene therapy. The p105Rb product of the retinoblastoma tumor suppressor gene (Rb) is one of the most critical regulators of cellular proliferation. The Rb protein (pRb), when unphosphorylated, is responsible for arrest of cell cycle by inhibition of the activity of the E2F family of transcription factors. Normal cell cycle progression requires inactivation of Rb through phosphorylation by cyclin-dependent kinases (CDK). This process, in turn, is regulated by CDK inhibitors. Among these, p21 protein is induced directly by p53; p16 protein, and its homologue p15, specifically bind to and inhibit CDK4, and may therefore regulate Rb phosphorylation, and cell cycle progression. Dysregulation of cell cycle control is a frequent finding in malignant gliomas, like deletion or loss of expression of p16 and p15 tumor suppressor genes (Jen et al. 1994; Nishikawa et al. 1995), amplification of CDK4 (He et al, 1994), and deletion or mutation of the Rb tumor suppressor gene (Henson et al, 1994). Interestingly, both of the latter events take place when the p16 gene is intact and correctly expressed (He et al, 1995). Restoration of wild-type p16 gene in glioma cells through an adenoviral vector arrested cells in G0-G1 phases of the cell cycle (Fueyo et al, 1996) and suppressed glioma cells invasion in vitro (Chintala et al, 1997). Overexpression of p21 increases the susceptibility of glioblastoma cells to cisplatin-induced apoptosis (Kondo et al. 1996), whereas adenovirus-mediated transfer of exogenous E2F-1 protein induced massive apoptosis and suppressed glioma growth in vivo and in vitro (Fueyo et al, 1998). The possibility that E2F-responsive promoters may be more active in tumor cells relative to normal cells, because of loss of pRb function, has been exploited to design adenoviral vectors containing transgenes driven by the E2F-1 promoter for gene therapy of gliomas (Parr et al, 1997). These vectors showed tumor-selective gene expression in vivo and reduced toxicity of the normal tissue with respect to standard adenoviral vectors.
Inhibition or inactivation of genes/factors involved in DNA repair and/or cellular SOS response could represent a gene therapy approach that potentiates radiation therapy. In fact, inhibition of the RAD51 gene by antisense oligonucleotides enhanced the radiosensitivity of mouse malignant gliomas, both in vitro and in vivo, improving survival (Ohnishi et al, 1998). This gene, a homologue of the yeast RAD51 and E. coli RecA genes, is involved in repair of DNA double-strand breaks, in recombination repair, and in various SOS responses to DNA damage caused by gamma-irradiation and alkylating agents.

Deletions of large regions or even of the entire copy of chromosome 10 are a genetic hallmark of GBM. At least two tumor suppressor genes located on chromosome 10 (one on each arm) have been demonstrated to participate to glial oncogenesis. A first candidate tumor suppressor gene, called PTEN (Phosphatase and tensin homologue deleted on chromosome TEN) was recently characterized (Li et al, 1997). The DNA region encoding PTEN is altered in glioblastoma multiforme, but not in lower grade astrocytic tumors (Tohma et al, 1998; Ichimura et al, 1998; Chiariello et al, 1998).

B. Neoangiogenesis

Tumors may remain in a state of dormancy until they establish a blood supply for receiving oxygen and nutrients. The complex process of neoangiogenesis is regulated by numerous factors, some with angiogenic properties, i.e. vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGFα), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), interleukin-8, and by endogenous inhibitors of angiogenesis, i.e. thrombospondin-1, platelet factor 4 (PF4), angiostatin, endostatin. VEGF, which binds to two specific tyrosine-kinase receptors, called Flk-1 and Flt-1, has been demonstrated to play a key role in angiogenesis of gliomas. Indeed, VEGF and its receptors are downregulated in the normal adult brain, whereas, VEGF is highly produced by GBM cells. Since both flt-1 and flk-1 genes are expressed by proliferating endothelial cells of gliomas, this leads to the establishment of a paracrine loop. Moreover, VEGF expression is higher around necrotic areas and seems to be stimulated by hypoxia.

Glioblastoma multiforme is one of the most highly vascularized solid neoplasms; therefore, treatments that target neoangiogenesis would be of great interest in clinical practice. Co-injection of rat C6 glioma cells, either subcutaneously or intracerebrally in nude mice, together with cells producing retroviral vectors encoding a dominant-negative mutant of the Flk-1 receptor showed inhibition of neoangiogenesis, reduction of tumor growth, and survival improvement (Millauer et al, 1994; 1996). Antisense VEGF oligonucleotides (Saleh et al, 1996) and ribozymes against VEGF mRNA have been successfully employed to reduce VEGF expression in glioma cells (Ke et al 1998), once more suggesting a potential role for antiangiogenic gene therapy. Similarly, bFGF antisense cDNA decreased C6 glioma cells proliferation (Redekop and Naus, 1995). Besides inhibiting the production of angiogenic factors, a therapeutic intervention could also consist of providing tumors with antiangiogenic factors. Indeed, retroviral and adeno-associated viral vectors expressing a modified PF4 were reported to inhibit endothelial cells proliferation in vitro and the growth of intracerebrally implanted gliomas (Tanaka et al, 1997). Retroviral and adeno viral vectors transducing angiostatin gene increased apoptotic death of glioma tumor cells (Tanaka et al, 1998). Additionally, the intratumoral delivery of angiostatin gene by an adeno viral vector produced inhibition of tumor growth in vivo, suppression of neovascularization, and a marked increase of tumor cells apoptosis (Griscelli et al, 1998). Damage of tumor microvasculature was reported also in human malignant glioma xenografts, after gene therapy followed by radiotherapy. The treatment consisted of intratumoral injection of adeno viral vectors expressing tumor necrosis factor-α (TNF-α), under control of the Egr-1 promoter (Staba et al, 1998). The use of viral vectors containing radiation-inducible promoters, such as Egr-1, has the advantage of selectively, spatially, and temporally limiting the effects of the therapeutic gene in the radiation field. Recently, this strategy has yielded interesting results in rat 9L glioma cells (Manome et al, 1998).

III. Suicide gene therapy

Suicide gene therapy operates by tumor transduction of genes converting a prodrug into a toxic substance; independently, the gene product and the prodrug are non-toxic. The prototype of this approach exploits the selective intracellular phosphorylation of ganciclovir (GCV), driven by the herpes simplex virus thymidine kinase gene product (HSV-TK). This activation generates a toxic drug metabolite that inhibits DNA synthesis, inducing cell death. For in vivo gene transfer of the HSV-TK gene to malignant cells, packaging cells that produces retroviral vectors expressing HSV-TK, have been injected directly into the tumor to transduce replicating cells. An interesting feature of the HSV-TK/GCV system is the bystander killing of nontransduced cells.

The mechanisms that are responsible for this effect have not been fully defined, but are likely to include: (i) transfer of non-diffusible, phosphorylated GCV to neighboring cells through gap junctions; (ii) endocytosis by nontransduced cells of cellular debris containing toxic GCV; and (iii) stimulation of host antitumor immune response. The therapeutic efficacy of the HSV-TK/GCV
system may be further increased by the use of adenoviral vectors, since these vectors can also transduce resting tumor cells. However, adenoviral vectors will infect also normal cells; hence, the inclusion of sequences able to restrict gene expression only in tumor cells can circumvent this problem. Selective tumor toxicity was obtained positioning the suicide gene under control of the E2F-responsive promoter elements which are de-repressed in glioma cells (Parr et al, 1997).

The effectiveness of suicide gene therapy has been explored for a variety of neoplasms, especially for refractory and localized diseases such as GBM. The HSV-TK/GCV scheme has been demonstrated to be effective in animal models, by ex vivo and in vivo transduction with retroviral, adenoviral, or adenoviral-associated vectors expressing HSV-TK (Ezzeddine et al, 1991; Culver et al, 1992; Takamiya et al, 1992, 1993; Barba et al, 1993, 1994; Ram et al, 1993; Kim et al, 1994; Vincent et al, 1996; Maron et al, 1996; Mizuno et al, 1998). Novel gene delivery systems were also applied in a mouse model for gene therapy of meningeal gliomatosis. Liposomes coated with Sendai virus envelope protein were highly efficient in delivering the therapeutic gene in disseminated glioma cells (Mabuchi et al, 1997).

A factor that may limit the effectiveness of HSV-TK/GCV therapy is the GCV crossing through the blood-brain barrier. This can be circumvented by the use of the bradykinin analogue and potent blood-brain barrier permeabilizer RMP-7, which, administered intravenously, increase the delivery of GCV into rat brain tumors, enhancing the cytotoxic and bystander effects of HSV-TK/GCV (LeMay et al, 1998).

Several clinical trials exploiting the HSV-TK/GCV system have been initiated. It is too early to estimate the effectiveness of these therapeutic procedures. Notwithstanding the evidence for growth-suppressive activities of HSV-TK plus GCV, cure rates are low. Explanation for lack of complete response in humans may reside in the different biological behavior of GBM cells when injected into animals (Sturtz et al, 1997).

The antitumor effects elicited by HSV-TK/GCV prompted to explore other prodrug activating enzymes. A promising system exploits the ability of E. coli cytosine deaminase (CD) to convert the relatively non toxic 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Significant antitumor effects of CD/5-FC were observed in nude mice bearing tumors derived from C6 glioma cells and transduced with CD. A "bystander" effect could also be demonstrated (Ge et al, 1997), suggesting a potential role for gene therapy of glioblastoma.

As already reported for p53, both the CD and HSV-TK systems sensitize cancer cells to radiation. Animal models have shown encouraging results both in vitro and in vivo (Khil et al, 1995; Kim et al, 1997). 9L glioma cells transduced with a retrovirus encoding a CDHSV-TK fusion gene exhibited enhanced sensitivity to both GCV and 5-FC, as well as increased radiosensitivity (Rogulski et al, 1997). This experiment suggests the feasibility of a combined approach with two suicide genes associated with radiotherapy.

Another prodrug activation system is represented by cytochrome P450 2B1 (the liver enzyme catalyzing cyclophosphamide and ifosfamide activation) gene transfer followed by cyclophosphamide or ifosfamide administration. Metabolites of these drugs produce interstrand DNA cross-linking in a cell cycle-independent fashion. C6 and 9L rat glioma cells, when stably transfected with the P450 2B1 gene, become highly sensitive to cyclophosphamide in in vitro and in vivo models (Wei et al, 1994; Manome et al, 1996). Rabbit cytochrome P450 isozyme CYP4B1, which converts the inert prodrugs 2-amionanthracene (2-AA) and 4-ipomeanol (4-IM) into highly toxic alkylating metabolites, also showed high antitumor effects, both in vitro and in vivo. The treatment had relatively low toxicity and was associated with a bystander effect, not requiring cell-to-cell contact (Rainov et al, 1998).

Another suicide gene system is based on E. coli purine nucleoside phosphorylase (PNP), which generates toxic purine nucleoside analogues intracellularly, either from 6-methylpurine-2'-deoxyriboside or arabinofuranosyl-2-fluoroadenine monophosphate. Significant antitumor activity and low systemic toxicity were reported in nude mice bearing human malignant D54MG glioma tumors expressing PNP (Parker et al, 1997).

Phosphorylation of the prodrg cytosome arabinoside (ara-C) by deoxycytidine kinase (dCK) is a limiting step for activation. Thus, ara-C, a potent antitumor agent for hematological malignancies, has only minimal activity against most solid tumors. Transduction of the dCK cDNA by retroviral and adenoviral vectors also resulted in marked sensitization of glioma cell lines to ara-C in vitro, and in significant antitumor activity in vivo (Manome et al, 1996).

Unlike other prodrug activating enzymes, E. coli gpt sensitizes cells to the prodrugs 6-thioxanthine (6TX) and 6-thiouguanine (6-TG), and confers resistance to different regimens (mycophenolic acid, xantine, and hypoxanthine), providing a means to select for gpt-positive cells. Rat C6 glioma cells transduced with a retroviral vector expressing the gpt gene exhibited significant 6TX and 6GT susceptibility and a "bystander" effect in vitro. An antiproliferative effect was demonstrated in vitro and in vivo (Tamiya et al, 1996; Ono et al, 1997).
Tumor suicide can also be achieved by direct infection of tumor cells with a conditionally replicative virus, i.e., an infectious agent able to replicate and to kill only dividing cells. In the case of tumors highly proliferating in the context of a completely post-mitotic tissue, such as the brain, gene transfer can ideally be obtained by using neurotropic herpes viral vectors, which are rendered conditionally replicative after deletion of non-essential genes (Lachmann and Efstathiou, 1997).

Tumor specific cell death has already been provided in animal glioma models by HSV vectors, deleted in neurovirulence genes, such as γ 34.5, thymidine kinase and ribonucleotide reductase (Chambers et al, 1995; Mineta et al, 1995; Boviatisis et al, 1994; Miyatake et al, 1997; McKie et al, 1996; Andreansky et al, 1996). Their direct injection into gliomas produced tumor regression with minimal bystander effects on surrounding normal tissue. Enhancement of replication of defective HSV vectors lacking γ 34.5 gene and a significant reduction of tumor mass was observed combining ionizing radiation (Advani et al, 1998).

Another way to treat malignant gliomas emerged from the discovery that these tumors often express functional Fas (CD95) (Weller et al, 1994). Fas is a transmembrane glycoprotein belonging to the nerve growth factor/TNF receptor superfamily: when activated, Fas can transduce an apoptotic signal through its cytoplasmic domain. Apoptosis is triggered by the binding of Fas to its natural ligand (FasL) or by cross-linking with anti-Fas antibodies. A high proportion of human glioma cell lines are sensitive to apoptosis mediated by anti-Fas antibodies in vitro. Some other cell lines are resistant, but may be rendered sensitive after stable transfection with human Fas cDNA (Weller et al, 1995). These results offer new possibilities for treating gliomas with anti-Fas antibodies or soluble FasL. One possible drawback of such an approach is that other Fas-positive cells may be affected, like infiltrating leukocytes. Thus their activity may be reduced, restricting strategies relying upon simultaneous immune response enhancement.

IV. Immunotherapy of cancer

Glioblastoma multiforme has the propensity to microscopically infiltrate normal structures since its early stage of development. This characteristic makes therapeutic success rather difficult by any approach. Moreover, it becomes virtually impossible to obtain specific targeting of all tumor cells and sparing of normal ones. Therefore, inducing an efficient immune response against malignant cells becomes an attractive and essential treatment strategy. In this perspective, the natural circulatory properties of cells of the immune system offer also an important support for the recognition of secondary lesions.

Despite the location in the central nervous system (CNS), a long-believed “immunologically privileged site”, glioblastoma cells may interact with immune cells. These interactions are mediated by receptor-ligand recognition during cell to cell contact and by a plethora of cytokines. An imbalance in the tumor-host relationship, resulting in deficit in some components of the response, may explain the aggressive growth of malignant gliomas. Before discussing the designed strategies to increase the immune response against glioblastoma cells, we review the more recent acquisitions in the “dialogue” between these neoplastic cells and the immune system (Dietrich et al, 1997).

A. Antigenicity of glioblastoma cells

The presence of specific antigens at the surface of tumor cells to be recognized by cells of the immune system is essential for the generation of a specific antitumor immune response. At present, no tumor antigen able to elicit an immune response has been identified in glioblastoma in vivo. MAGE-1 (melanoma antigen) was the first tumor-specific antigen to be identified (Van der Bruggen et al, 1991), and MAGE family members are expressed by some glioblastoma cell lines (Rimoldi et al, 1993), but not in uncultured tumors (De Smet et al, 1994). This observation could be explained with different levels of DNA methylation induced by culture, where MAGE expression was regulated by methylation (De Smet et al, 1995).

Proteins that are structurally altered during malignant transformation, or that contribute to this process, are possible tumor antigen candidates. The frequent alterations of p53 in the early stages of carcinogenesis, for example, may provide new antigenic peptides that could trigger an immune response. Consistent with this possibility, specific cytotoxic T-cell clones were generated in vitro against mutated p53 protein (Houbiers et al, 1993). Additionally, in vivo immunization with mutated p53 peptide was shown to induce specific CTL clones able to lyse MHC-matched tumor cells expressing the mutated p53 gene (Noguchi et al, 1994).

The future identification of glioblastoma-specific antigens would aid new treatment strategies.

B. Tumor-induced immunosuppression

A high proportion of glioblastomas have shown to be infiltrated by lymphocytes, mostly T lymphocytes, but also B and NK cells. Differently from what reported for other tumors, the level of lymphocyte infiltration does not relate with a better prognosis. In fact, tumor-infiltrating lymphocytes (TIL) of gliomas appear to be functionally defective. Abnormalities range from abnormal
hypersensitivity responses, depressed response to mitogens, decreased humoral responses (CD4+ T helper cells deficit?), and impaired T cell-mediated cytotoxicity. These functional alterations may be explained, at least in part, by a defective high-affinity IL-2 receptor (IL-2 R) (Roszman et al, 1991).

It has been demonstrated that glioblastoma cells produce and release soluble factors that are responsible for a depressed immune response. T lymphocytes from normal individuals exhibit immunologic abnormalities when grown in presence of supernatant obtained from glioblastoma cell line cultures (Roszman et al, 1991). The most important soluble suppressing factor seems to be TGF-β2. This cytokine acts as a growth-inhibitory factor (Sporn et al, 1986; Sporn et al, 1987), and has a defined variety of immunoregulatory properties, including: inhibition of: (i) T cell proliferation; (ii) IL-2R induction (Kehrl et al, 1986); (iii) cytokine production (Espevik et al, 1987; Espevik et al, 1988); (iv) natural killer cell activity (Rook et al, 1986); (v) cytotoxic T cell development (Jin et al, 1987; Ranges et al, 1987); (vi) LAK cell generation (Espevik et al, 1987; Jin et al, 1987); and (vii) production of tumor-infiltrating lymphocytes (Kuppner et al, 1989). Most cells secrete TGF-β in a latent form (Sporn et al, 1986), but glioblastoma cells have also the capacity to convert it to an active form, through proteolytic cleavage. This was demonstrated by an experimental work in which T-cell suppression mediated by TGF-β2 was inhibited when proteolytic enzymes were blocked by protease inhibitors (Huber et al, 1992).

Other soluble factors, namely prostaglandin E2 (PGE2), IL-1 receptor (IL-1 R) antagonist, and interleukin-10 (IL-10) may be implicated in immunosuppression, although in vivo intervention has not been fully defined. A potential down regulation of some immune functions was shown for IL-10 cytokine, which was produced both by GBM cells and normal brain tissue (Nitta et al, 1994; Merlo et al, 1993). Furthermore, in different animal models, human and murine IL-10 was demonstrated to stimulate the acquisition of a specific and efficient antitumor immune response (Berman et al, 1996).

C. Costimulatory molecules

A complete T-cell effector function needs not only antigen presentation, but also the delivering of activation signals to the T cell, which is mediated by the so-called costimulatory molecules. Unresponsiveness of T cells (anergy) may be due to absence of the second signal, essentially given by the B7-CD28 interaction (June et al, 1994). Two other members of the B7 family have been cloned, B7.1 and B7.2; their counter-receptors on T cells are CD28 and CTLA-4, respectively. CD28 mediates stimulatory effects, while CTLA-4 appears to be a negative regulator of T cell responses. Glioblastoma cells and monocytes that infiltrate the tumor are not expressing B7 costimulatory molecules, while monocytes in the normal tissue that surrounds the tumor are B7-positive (Tada et al, 1996). This suggests the possible intervention of local mechanisms able to down-regulate B7 expression in glioblastomas, impeding efficient T-cell priming and favoring T-cell anergy. Moreover, B7-CD28 interactions in the CNS have been shown to be essential to generate a valid CTL response towards viral antigens (Kuendig et al, 1996). Hence, restoring B7 expression by gene transfer might become an interesting task to elicit a proper immune recognition of glioblastoma cells, and an appropriate immune response.

V. Restoring a proper immune response

Many approaches have been tented to restore a proper immune response towards malignant gliomas. As previously stated, T lymphocytes play a major role in the antitumor response, and priming of T lymphocytes requires antigen recognition, with or without help from APC. Since no specific antigens have yet been identified for glioblastomas, a vaccination approach has been proposed by administration of genetically modified tumor cells. Moreover, tumor cells transfected to produce various cytokines have been used to enhance lymphocyte responsiveness in animal models.

The most interesting results were obtained with cells of murine glioma transfected with an expression vector containing the murine interleukin 7 cDNA (Aoki et al, 1992). IL-7 transfected glioma cells were vigorously rejected by a CD8+ T-cell-mediated immune response, that was proportional to the level of IL-7 production. Moreover, the response was tumor-specific, since no effect was observed against other syngeneic tumor cells (melanoma and fibrosarcoma cells). IL-7 is a very interesting cytokine being able to increase IL-2R α chain expression on CD4+ T lymphocytes and to inhibit TGF-β mRNA expression and production by murine macrophages (Dubinett et al, 1993). IL-12 can also be considered a promising agent to enhance the antitumor response, since it augments T-cell and natural killer-cell activities, induces IFN-γ production, and promotes the differentiation of uncommitted T cells to Th1 cells (Hendrzak and Brunda, 1995). Vector-mediated delivery of IL-12 into established tumors suppresses tumor growth (Caruso et al, 1996) and can induce immune responses against challenge tumors (Branson et al, 1996). Moreover, IL-12 has other non-immune properties such as anti-angiogenic effects (Voest et al, 1995).

IL-4, another cytokine with pleiotropic functions, increases T cell proliferation and cytotoxicity, and enhances eosinophil and B cell proliferation and differentiation. It
also exerts direct anti-proliferative effects in vitro against many tumor cell lines (Tepper, 1993). Antitumor effects induced by IL-4-transfected cells were reported in nude mice, suggesting T cell-independent mechanisms (Tepper, 1993; Yu et al, 1993). A strong recruitment of eosinophils and subsequent inhibition of the tumor growth was noted. Eosinophil depletion was not performed as a control; hence a direct anti-proliferative effect mediated by IL-4 cannot be excluded. In any respect, a possible non-T-cell-dependent mechanism could be an advantage in the glioblastoma setting, considering the various abnormalities of T cell function.

In an attempt to overcome the local immunosuppression mediated by TGF-β2 Fakhrai et al conducted an experimental work on rats by antisense gene therapy. 9L gliosarcoma transfected cells inoculated subcutaneously became highly immunogenic and were able to induce eradication of an established wild-type tumor (Fakhrai et al, 1996).

The enhancement of antigen presentation and T cell co-stimulation has also been considered and may be achieved with genes coding for cytokines, like GM-CSF, co-stimulatory molecules, like B7, or CIITA, a transcription factor playing a critical role in the regulation of MHC class II molecules. Encouraging results have been reported in a murine melanoma model located in the CNS, whereby an efficient antitumor response was induced by subcutaneous vaccination with irradiated, GM-CSF-producing tumor cells (Sampson et al, 1996). Vaccination with cells co-transfected with B7 and IL-2 was able to mediate rejection of established tumors (Gaken et al, 1997), suggesting a possible application of such an intervention for the treatment of glioblastomas.

VI. Combined gene therapy approach in humans

The strict localization of glioblastomas in the CNS, with only exceptional metastases, makes these tumors candidates for approaches of direct intra-tumoral gene delivery. Retrovirus-mediated gene therapy of GBM is particularly attractive, since these viral vectors transduce only mitotically active cells, sparing the normal neuronal tissue composed of non-replicating cells.

Gene therapy of brain tumors by intra-tumoral injection of retroviral vector producing cells (RVPC) in human patients was initiated by Oldfield and colleagues in 1993 (Oldfield et al, 1993). The gene being transferred was that expressing the herpes simplex thymidine kinase (HSV-TK), which conferred sensitivity to the anti-herpes drug ganciclovir. This treatment has proved free of toxicity and safe for there was no evidence of systemic spread of the retroviral vector (Long et al, 1998). However, a clinical benefit was limited to very small tumors (1.5 ml), probably because only malignant cells adjacent to the RVPC were transfected (Ram et al, 1997).

A new treatment strategy combining two different modalities, enzyme-directed prodrug activation (tumor suicide) along with cytokine-promoted tumor rejection, has been recently devised to amplify the antitumor response, and proved to be efficacious in animal models (Castleden et al, 1997) (Figure 2). A bicistronic retroviral vector co-expressing HSV-TK and human interleukin-2 genes has been designed to pursue this new approach of cancer gene therapy in humans (Pizzato et al, 1998).

![Structure of a bicistronic retroviral vector for transduction of genes coding for a cytokine and a prodrug-activating enzyme, expressed via a cap-dependent and an internal ribosome entry site (IRES)-dependent mechanism, respectively. A selectable marker (neomycin phosphotransferase gene, neo) is expressed under the control of a SV40 promoter.](image-url)
**Figure 3.** Gene therapy approach for treatment of glioblastoma multiforme, via intratumoral stereotactic injection of cells producing a triple gene retroviral vector.

**Figure 4.** Contrast-enhanced MRI sagittal images of left parietal GBM lesion before (left), and one month after completion of GCV treatment (to the right).
Figure 5. Histology of stereotactic biopsies from patients treated by HSV/TK-IL-2 combined gene therapy. A) Toluidine blue staining - Evidence of a large number of infiltrating inflammatory cells; Immunostaining with B) monoclonal antibodies marking CD3⁺ cells; C) Mac 387 antibodies recognizing young/activated macrophages; D) monoclonal antibodies marking CD1⁺ infiltrating cells.

After in vitro characterization of efficacy and safety (Pizzato et al, 1998), the vector was employed in a pilot study to treat four patients with recurrent glioblastoma multiforme (Colombo et al, 1997; Palù et al, 1998) (Figure 3). A significant and sustained reduction (>50% of the initial volume) of the tumor mass (80 ml) was demonstrated by magnetic resonance imaging (MRI) and computerized tomography (CT) in one patient (Figure 4). In this case, the objective response was associated with a dramatic clinical improvement. The other three patients showed areas of tumor necrosis (2 ml) around the site of stereotactic RVPC injection and stabilized disease for a long period of time (11-12 months).

In stereotactic biopsies taken before ganciclovir administration, large tumor infiltrates of immune-inflammatory cells (T lymphocytes, mostly CD4⁺ but also CD8⁺ granzyme B-positive cells, activated macrophages, NK cells, neutrophils) were present, notwithstanding the standard steroid therapy (Figure 5). The observed inflammatory response has never been reported in previous trials with thymidine kinase (Ram et al, 1997; Ostertag and Chiocca, personal communications).

Interestingly, endothelial cells stained positive for TK by in situ hybridization, indicating that the vector had targeted the neo-vascular component, a highly replicative population in glioblastomas. This is consistent with an anti-angiogenic effect of this therapeutic approach, that, in addition to direct tumor suicide and immune activation, may be relevant to the bystander phenomenon and to the clinical response. It is noteworthy that IL-2 was measurable in the cerebral-spinal fluid, even after GCV treatment. This cytokine might have derived from an autocrine-paracrine secretion of recruited infiltrating immune-inflammatory cells, after primary expression in transduced cells.

Efforts to achieve more efficient gene transfer systems are being sought for. These include the development of new generation retroviral vectors, produced at higher titres and characterized by higher transduction efficiency. Strategies involving envelope pseudotyping, use of new
packaging cell lines of human origin, and substitution of promoter elements will contribute to the improvement of current available vectors.

New therapeutic gene combinations should also be accomplished in order to promote a more generalized immune response. Genes for cytokines other than IL-2 (i.e., IL-4, IL-7, IL-12, GM-CSF) as well as genes targeting neoangiogenesis deserve further consideration for combined treatment approaches.

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transduction

nucleus

integrated viral vector

mRNA

IL-2

TK

GCV

GCV-P

GCV-3P

TIL recruitment

bystander effect

producer cells inoculation
IRES

LTRLTR

cytokine
cytokine

TK

SV

NEO

Ψ

regulatory elements

therapeutic genes

gene for positive selection