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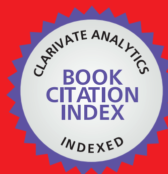

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Chapter

Neoplastic Brain, Glioblastoma, and Immunotherapy

Annabelle Trojan, Heliodor Kasprzak, Oscar Gutierrez, Pedro Penagos, Ignacio Briceno, Heber O. Siachoque, Donald D. Anthony, Alvaro Alvarez and Jerzy Trojan

Abstract

IGF-I, insulin-like growth factor 1, is present in normal fetal/neonatal brain development and reappears in the mature brain participating in the development of malignant tumor, glioblastoma multiforme. Targeting the IGF-I system has emerged as a useful method to reduce glial malignant development. Downregulation in the expression of IGF-I using antigene anti-IGF-I technology (antisense, AS, and triple helix, TH) applied in glioma cell culture established from glioblastoma biopsies induces the expression of B7 and MHC-I antigens in transfected cells (immunogenicity). The transfected cancer cells, "vaccines," after subcutaneous injection, initiated an immune response mediated by T CD8+ lymphocytes, followed by tumor regression (immunotherapy). The median survival of patients treated by surgery followed by radiotherapy and immunotherapy was 21–24 months. On the other side, the experimental work has demonstrated that IGF-I AS or TH transfected tumor cells fused with activated dendritic cells, DC, showing more striking immunogenic character. Using IGF-I TH/DC "vaccination," the efficiency in suppressing rat glioma tumors is not only relatively higher than that obtained using IGF-I TH cells but is also more rapid.

Keywords: brain neoplastic development, glioblastoma, IGF-I, antisense, triple helix, immunogene therapy, cell hybridomas, dendritic cells, CD8

1. Introduction

There is a convergence between onto-genesis and onco-genesisgenesis and the same specific oncoproteins like alpha-fetoprotein (AFP) or growth factors, such as IGF and TGF-beta, are present in embryo/fetal tissues and in neoplastic developing tissues and particularly in the central nervous system (CNS). As far as AFP and IGF-I are considered, there is an important remarque: the first antigen is present in both neural and glial developing and cancerous cells, whereas the second one is only present in glial developing and tumoral cells. This striking difference has oriented our studies toward the most malignant brain tumor expressing IGF-I gene: glioblastoma.

In this chapter, we have described our scientific approach coming from the analysis of neoplastic CNS development conducted to glioblastoma malignancy up to the establishment of immunogene therapy of this tumor: the first cancer

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immunogene therapy. The strategy of therapy consisted of blocking IGF-I synthesis in cancer cells inducing apoptotic and immunogenic phenomena. Both phenomena, related to the arrest of IGF-I expression in neoplastic glial cells, were used to prepare antitumor cell vaccines for therapy of glioblastoma. Successful clinical results were obtained in USA, EU, and China and the therapy is introduced in Colombia (Wikipedia—Gene therapy, History 1990s–2010s).

2. Neoplastic brain

To understand the morphology of CNS neoplastic development, the model of mouse teratocarcinoma derived from PCC3 and PCC4 embryonal carcinoma cell lines was investigated. Thanks to this unique model reproducing "caricatural" development of the normal CNS, after examining histologic and electron microscopy sections, the different stages of abnormal nervous tissue histogenesis [1–4] were established as follows: 1. undifferentiated carcino-embryonic structures; 2. medulloepithelial structures (composed of a mixture of ectoblastic and neuroectoblastic components); 3. neuroblastic structures; and 4. neuroepithelial structures. The final differentiation was the encephaloid tissue. These results were confirmed by studying the localization of oncoproteins as alpha-fetoprotein (AFP), serum-albumin (SA), and IGF-I directly included in normal and neoplastic histogenesis, the last using teratocarcinoma model [3, 5–8].

As to the application of these observations in the pathology of human central nervous system (CNS) tumors, the model of mouse teratocarcinomas, containing neuroglial structures [3, 6, 9, 10] (described in the first studies of Stevens and then by his followers during almost 40 years of investigations [11–20]), should be useful as well in understanding human embryonic tumors of the CNS, which are able to differentiate into both neuronal and glial lineages [1, 10, 21–23], as in future gene therapies, including CNS malignant tumors [24–27].

3. IGF-I

In 1992, Trojan and his coworkers demonstrated that another oncodevelopmental antigen, an insulin like-growth factor, IGF-I [28–32], is present in glioma cells but absent in neuroblastoma cells [33]. Using the teratocarcinoma model, Trojan and his coworkers showed that neoplastic neuroblastic cells express IGF-II [34]. These observations permitted to study separately, using IGF-I and IGF-II as the oncoprotein markers, different tumors, especially glial and neural tumors [28–31, 35–40].

Comparative studies of the presence of AFP, IGF-I, and IGF-II in neoplastic cells [3, 33, 40–49] have demonstrated that IGF-I constitutes an essential target for genetic testing and therapy purpose. IGF-I, similar to AFP, is involved in tissue development and differentiation, especially in the development of the nervous system [6, 50, 51] as a mediator of growth hormone and glucose metabolism and acting locally with autocrine/paracrine, with a predominant role compared to other growth factors [29, 39, 51–55]. IGF-I is currently considered as one of the most important growth factors related to normal and neoplastic differentiation, and its overproduction is considered to be a participating factor in cancer development [32, 54, 56–58] (**Figure 3**). IGF-I reconstitutes the first step of the following signal transduction pathway: IRS/PI3K-PKC/PDK1/AKT-Bcl2/GSK3/GS [59, 60]. The elements of the said IGF-I-related transduction pathway were also considered as targets for diagnostic and therapeutic purposes [51, 59, 61–70].

Considering the IGF-1 gene, an overexpression of this gene in mature tissues is a sign of neoplastic processes, especially brain tumors [40]. IGF-I becomes useful in the molecular diagnosis of neonatal CNS malformations and tumors [9, 21, 38, 51, 71, 72]. Diagnosis and treatment should logically be related, at first using IGF-I gene testing for diagnosis [73–75] and then targeting IGF-I gene through special therapy, such as cancer gene therapy, especially therapy of gliomas [40, 76–79].

4. Gene therapy

4.1 Introduction

IGF-I and -II are expressed at high levels in nervous system–derived tumors, for example, astrocytomas and meningiomas [37, 44, 80]. In contrast, the block of IGF-I synthesis in these tumors induces apoptotic and immunogenic phenomena [81].

Our experimental approach of gene therapy has centered on the comparative use of IGF-I RNA antisense and IGF-I RNA–DNA triple helix [82, 83], to stop the translation and transcription of the IGF-I gene, respectively. Triple helix strategy [84, 85] and antisense strategy [86–88] have been applied successfully to a growing number of genes in cultured cells. However, the antisense approach has sometimes been not completely efficient due probably to insufficient antisense RNA levels [89].

We have applied the antisense strategy by employing a self-amplifying episomal vector that replicates to high copy numbers extrachromosomally [33]. The utility of episome-based expression vectors for the effective inhibition of cellular RNA expression has been subsequently confirmed by others [90]. C6 rat glioma cells expressed MHC-I [91, 92] and B7 [55, 93, 94] antigens when transfected with vectors producing IGF-I antisense RNA (IGF-I AS) or inducing IGF-I triple helix RNA-DNA (IGF-I TH) [95, 96]. IGF-I AS or IGF-I TH blockade of IGF-I syntheses changes the phenotype of transfected CNS-1 and PCC-4 cells. Moreover, it was demonstrated that transfected C6 cells become pro-apoptotic [96]. The AS and TH cells lost tumorigenicity and were able to induce a T-cell–mediated immune response in syngeneic animals against both themselves and the nontransfected tumorigenic parental cells [34, 40, 82, 97]. The experiment described here has permitted us to prepare human "vaccine" for a Phase 1 clinical trial.

4.2 Material and methods

Cell culture. The CNS-1 cell line was offered by the Dartmouth Medical School, Hanover, NH, USA (Dr W. Hickey) and then cultivated in the Laboratory of INSERM, Salpetriere Hospital, Paris (Dr M. Sanson). The PCC-4 cell line was provided by Institut Pasteur, Paris (Dr J.F. Nicolas). The cell lines were cultivated as described earlier [97]. Primary cell cultures of human glioma derived from tumors of glioblastoma multiforme patients were established (Clinical Laboratory of Collegium Medicum, UJ University, and School of Medicine, CWRU) according to the technique described earlier [96, 98–100] (**Figure 1**).

Plasmids. The vector pMT-EP [6, 26] was described earlier [33] (**Figure 2**). IGF-I "antisense" and "triple helix" technology was used to construct episome-based plasmids expressing IGF-I RNA antisense, pMT-anti IGF-I [26], or IGF-I triple helix–inducing vector, pMT-AG TH [33, 82]. The vector pMT-EP containing cDNA expressing IGF-II antisense RNA as insert was used in control experiments [34]. In parallel, using the vector pMT-EP, the vectors expressing MHC-I and B7, as well as vectors "antisense" MHC-I and B7, were prepared [96].

Transfection. The FuGENE 6 Transfection Reagent (Boehringer Mannheim) was used. Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml was added 48 h after transfection to select for transfected cells.

Northern blot. The content of IGF-I antisense RNA was determined in 50% confluent cell cultures. Northern blot and hybridizations were done according to Maniatis [98]; the 770 bp human IGF-I cDNA and 500 bp rat IGF-I cDNA were used as probes (**Figure 3**).

Flow cytometric analysis. Cells were incubated (30 min, 40°C) with saturated amounts of monoclonal antibodies, rat or human MHC-I (HLA ABC), MHC- II, CD80, and CD86 (Becton Dickinson Pharmingen). Cells were collected (10.000 events per sample) in FACScan BD cytometer (**Figure 4**).

Ex vivo generation of dendritic cells. Two techniques for the generation of dendritic cells were used:

1. CD34+ hematopoietic progenitor cells were isolated, using the MACS CD34 Cell Isolation Kit, and functional DC cells were generated by culturing CD34+ cells in the presence of GM-CSF, TNFalpha, and SCF for 10 days [101].

2. Monocytes were isolated, using MACS CD14 MicroBeads. Monocytes were cultured in the presence of GM-CSF and IL-4 generated activated DCs [102].

Hybridomas of transfected cells with dendritic cells. The fusion of dendritic cells with tumor transfected cells was obtained as follows [103]: activated DCs (one of the two techniques mentioned above) were fusioned with tumo IGF-I antisense or triple helix transfected cells using polyethylene glycol—PEG [104]. Fusions were carried out with 40% PEG in PBS without Ca^{2+} and Mg^{2+}.

In vivo experiment. For the determination of tumorigenicity, 5 x 10^6 rat CNS-1 cells were injected subcutaneously into Lewis rats. Experimental sets were injected with: (a) parental cells; (b) IGF-I "triple helix" transfected cells expressing MHC-I; and (c) IGF-I "triple helix" transfected cells expressing both MHC-I and B7 molecules.

4.3 Results and discussion

Northern blot analysis is shown in **Figure 3**. The RNA of nontransfected cells is distributed in 7.5 and 1.0 kb bands. The RNA of anti-IGF-I transfected cells shows

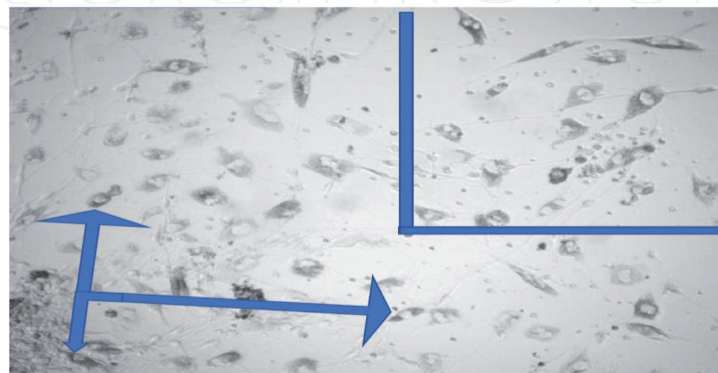

Figure 1.
In vitro *staining of IGF-1 biomarker human in human glioma cell culture. The tissue and cells are stained for IGF-1 using anti-IGF-1 antibodies applied in immunoperoxidase technique. Nine days of culture established from human glioblastoma biopsy. (left down) Note the cells (head arrows) proliferating from compact tissue of biopsy (left down corner) (200×); (right up) Note the cluster of cells showing dark cytoplasm of staining (400×).*

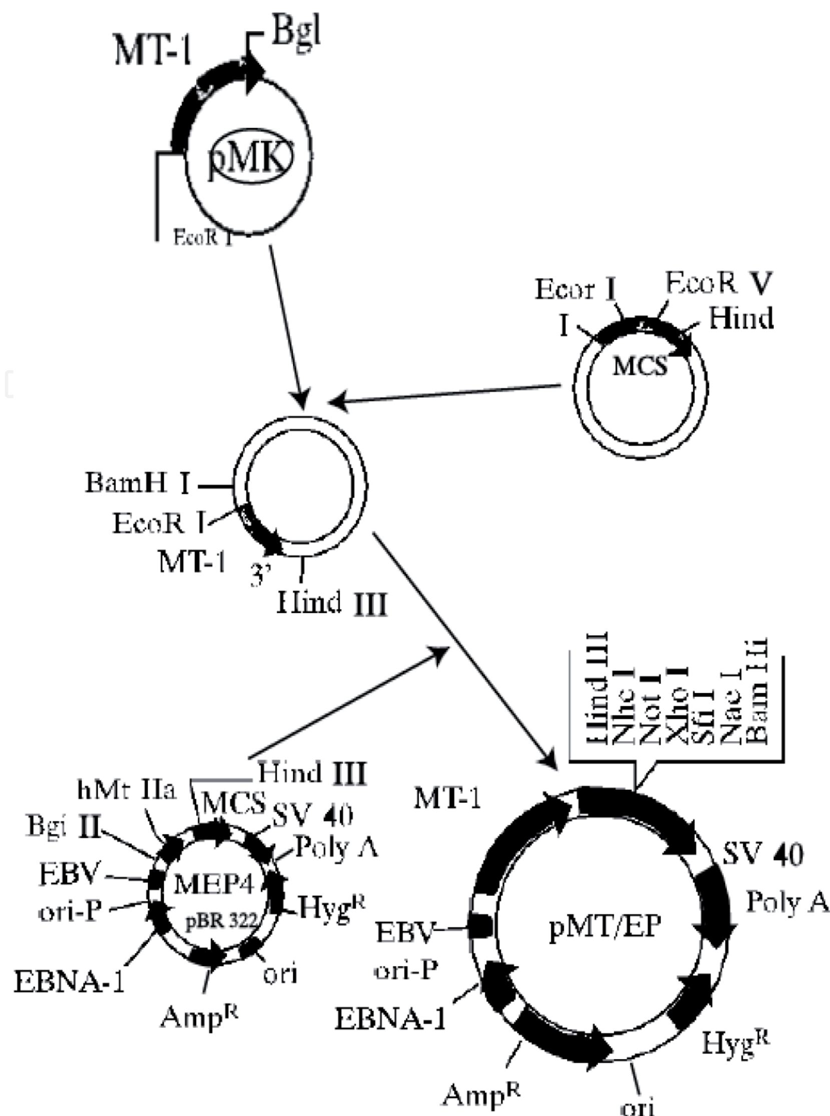

Figure 2.
Diagrammatic representation of steps employed to construct the episomal vector pMT/EP used for preparation of IGF-I antisense and triple helix expression vectors.

only an abundant 1.0 kb band. The transfected cultures were positively stained either for both MHC-I and B7 antigens (in 60% of cloned lines) or for MHC-I (only in 40% of cloned lines). The data show that transfection with "antisense" and "triple helix" vectors induced a significant increase in the expression of MHC-I and B7 (**Table 1**). The "triple helix" rat and human cells as compared to "antisense" cells showed slightly higher expression of MHC-I or B7. As to apoptosis, it was detected in approximately 70% of the IGF-I antisense and triple helix transfected cells. As expected, the hybridomas of IGF-I triple helix or IGF-I antisense cells fused to activated dendritic cells, IGF-I TH//DC or IGF-I AS//DC, were negative for IGF-I. The most important observations concerned the increased level of MHC-I and MHC-II, and especially the presence of B7 in IGF-I TH//DC and IGF-I AS//DC hybridomas (**Table 1**). No tumors were observed in animals injected subcutaneously with CNS-1 cells transfected with IGF-I "triple helix" vector, expressing both MHC-I and B7.

The simultaneous increase in the presence and role of B7 and MHC-I antigens in the induction of T-cell immunity against tumors has been extensively investigated [33, 93, 94]. The injection of IGF-I antisense and triple helix transfected cells presenting both MHC-I and B7 molecules stopped effectively the established rat glioma tumors. This was not the case for cells expressing MHC-I only (**Table 1**). Injection of cell hybridomas composed of IGF-I antisense cells and activated dendritic cells (IGF-I AS//DC) into tumor-bearing animals suppressed the established glioma

Figure 3.
Antisense transcripts in cultured C6 glioma cells. Molecular sizes of IGF-I transcripts are shown in kilobases. Lane 1, parental nontransfected C6 glial cells exposed to serum-free medium. Lanes 2 and 3, transfected C6 glioma cells incubated in serum-free medium in the absence (lane 2) or presence (lane 3) of ZnSO4. For lanes 2 and 3, nick-translated rat IGF-I cDNA was used.

tumors in 4/6 cases of Lewis rats. The experience was repeated using cell hybridomas composed of IGF-I triple helix cells and activated dendritic cells (IGF-I TH//DC). In this case, the subcutaneous injection of the hybridomas into glioma-bearing animals completely suppressed tumors in a ratio 6/6.

MHC-I molecules were in general not sufficient to stimulate T-cell response. In the absence of B7 molecule, MHC-peptide complexes could selectively inactivate T cells [105]. B7 molecules bound to the counter-receptor CD28 and/or CTLA4 expressed on the T cells [9, 106, 107]; enhancement in B7 costimulation through a cAMP mechanism linked to tyrosine kinase of the CD28 receptor has been previously reported [108]. The mechanism of signaling (tyrosine kinase activates IRS-1, and then IRS-1 activates PI3K [109, 110]) could be considered in the cytokine induced B7-1 expression demonstrated in fetal human microglia in culture [111].

Using CNS-1 glioma, we have confirmed the relation between the immunogenicity and apoptosis found in IGF-I transfected cells [96]. The phenotypic modifications due to apoptosis may explain the recognition of the transfected cells by the immune system like tumor-specific immunity mediated by CD8+ T described earlier by us [40, 98]. Apoptotic cells, in the context of MHC-I, are recognized by dendritic cells activating lymphocytes T-CD8 [112, 113]. B7 molecules can be included in this mechanism, because both MHC-I and B7 molecules are necessary for T-cell activation [4, 55, 79, 93, 114–116]. Considering the role of dendritic cells, the presented results may be useful in introducing IGF-I TH//DC "vaccines" into cellular therapy of human gliomas. Moreover, the obtained results of tumor suppression are in agreement with the immunogenic character of used "vaccines"—the efficiency of "vaccines" being related to the expression level of MHC-I, -II, and B7 (**Table 1**) [97, 117–119].

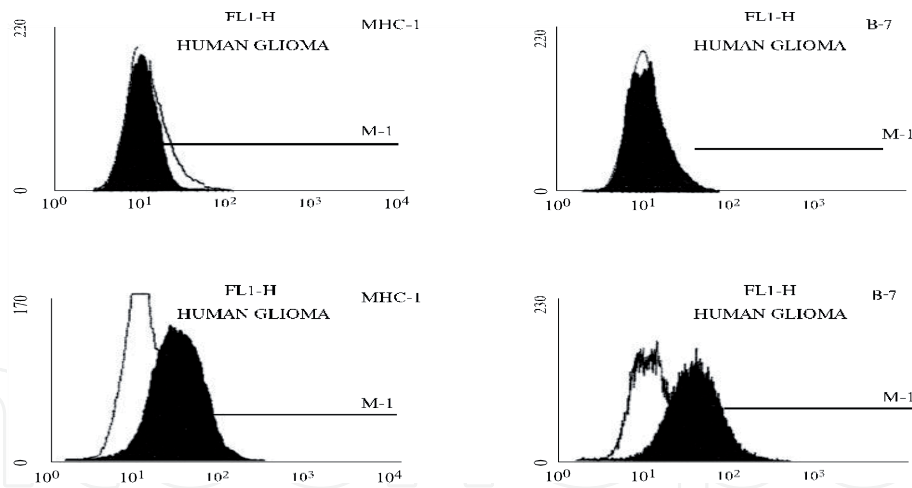

Figure 4.
Flow cytometry analysis (FACScan Becton Dickinson). Expression of MHC-I (left) and B7 (right) in primary human glioblastoma cell line. Upper panels: non transfected cells; lower panels: transfected cells (upregulation of MHC-I and B7).

Cells	Rat glioma CNS-1 cells		
	MHC-I	MHC-II	B7
NT	<0.5	<0.5	<0.5
IGF-I AS	12.3	<0.5	18.1
IGF-I TH	14.6	<0.5	19.6
IGF-I AS/IGF-I TH	12.8	<0.5	18.4
IGF-I AS/MHC-I AS/B7 AS	1.0	<0.5	1.0
IGF-I TH/MHC-I AS/B7 AS	1.0	<0.5	1.0
IGF-I AS//DC	14.7	3.8	19.3
IGF-ITH//DC	16.9	4.2	21.9

NT: parental nontransfected cells; pMT-EP: cells transfected with "empty vector"; IGF-I AS or IGF-II AS: cells transfected with IGF-I or IGF-II antisense expression vector; IGF-I TH: cells transfected with IGF-I triple helix expression vector; IGF-I AS/IGF-I TH: cotransfection with antisense and triple helix vectors; IGF-I AS/MHC-I AS/B7 AS, and IGF-I TH/MHC-I AS/B7 AS: triple cotransfection with IGF-I antisense or triple helix, MHC-I antisense and B7 antisense expression vectors; IGF-I TH//DC or IGF-I AS//DC: cells transfected with IGF-I antisense or triple helix expression vectors, and fused to dendritic cells.
**The data of flow cytometry (the average of three experiments) are presented as percent change in value of fluorescence relative to fluorescence in control nontransfected cells (CC). The increase in MHC-I, -II, and B7 is significant at the $P < 0.01$ level (Wilcoxon's signed rank test).*

Table 1.
*Expression of MHC-I, MHC-II, and B7 in the cells of rat glioma.**

5. Clinical gene therapy

5.1 Methodology

Using radiotherapy and chemotherapy, the mortality of glioblastoma remains close to 100% and the median survival, using conventional therapy, is 9–14 months. Current pharmacology increases the survival to 15 and rarely to 18 months [120]. The etiology of glioma is still being investigated using molecular biology techniques [64]. New or proposed therapies are based either on immune treatment or on immuno-gene strategies [121]. The AS and TH technologies [84–87] have permitted us to establish new and successful immuno-gene therapy strategies targeting glioma's growth factors [40, 122]. Other technologies include those of potentially useful siRNA [123, 124] and

miRNA (microRNA) [125]. The role of 21–23mer double-stranded RNA (siRNA) in the silencing of genes is strongly similar to that of the TH DNA mechanism, which also involves 23mer RNA [85]. Whether or not siRNA technology or miRNA knock-down will supplant the AS oligodeoxynucleotide approaches remains in question at this time [124, 126, 127]. The AS oligodeoxynucleotides reinforced by association with polycations (polyethyleneimine), polylysine, or cationic lipids (DOTMA, DOTAP) were also used for transfection of cells with plasmids encoding antisense RNA [128].

As to growth factors, historically, first IGF-I and its receptor and then TGF-beta were targeted in experimental preclinical studies [40, 57, 122, 129, 130] and then glycogen synthase, GS [51]. The absence of IGF-I, TGF-beta, and GS synthesis in "AS" transfected cells leads to a compensated increase in IGF-I-receptor [51] (relation between the signal transduction pathway of tyrosine kinase (IGF-I-R) and the induction of B7 [131]). Other growth factors such as EGF and VEGF, and their receptors, have also been investigated by AS technology in preclinical studies. The *in vitro* and *in vivo* results were similar to the results obtained with AS IGF-I technology [51, 130]. Thus IGF-I via IGF-I-R not only increases cell proliferation but also "supervises" mitogenic action of other growth factors (EGF, PDGF, etc.) by its autocrine-paracrine stimulation, becoming some kind of growth factor director. In clinical IGF-I antisense/triple helix immunotherapy, the cells used for "vaccination" were downregulated in IGF-I and presented both MHC-I and B7.1 molecules (**Figure 4**).

5.2 Results and discussion

The first clinical assay for human GBM using AS IGF-I approach was performed by Anthony et al. and by Trojan et al. [96, 100, 114]. After each of three AS IGF-I vaccinations, there was an increase in the percentage of CD8+ T cells in peripheral blood lymphocytes with a characteristic phenotype—switch

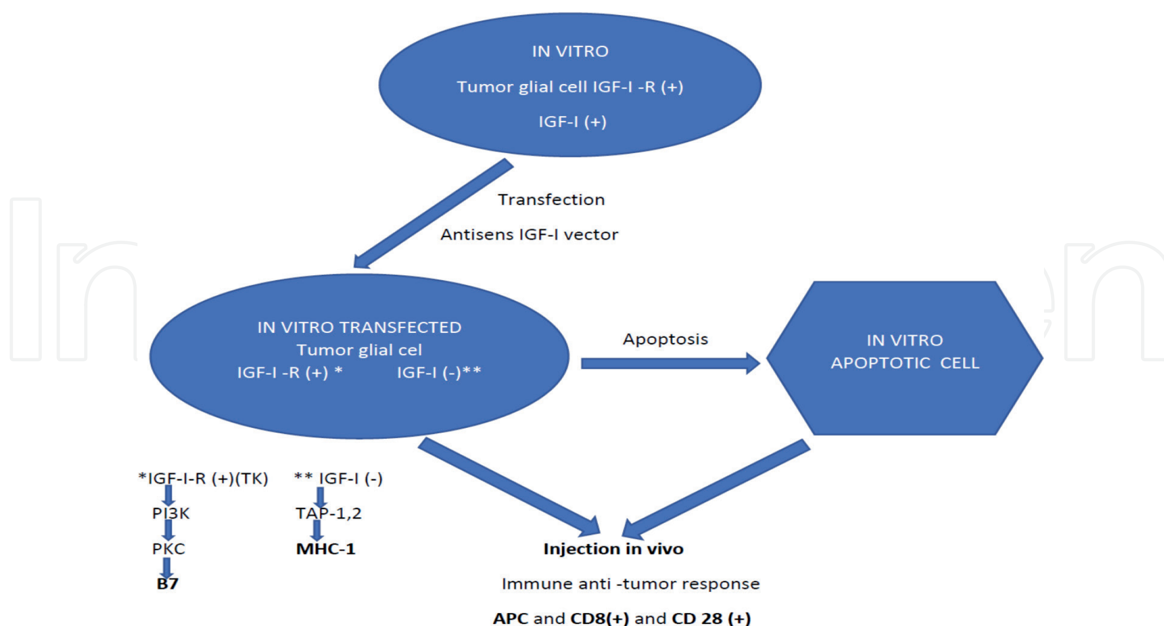

Figure 5.
Antisense immunotherapy. Example of antisense anti-IGF-I treatment of glial malignant tumor—glioblastoma. The schema of therapy shows transfected in vitro *brain tumor glial cells using a vector containing cDNA of IGF-I in antisense orientation. After transfection, the cells express IGF-I RNA antisense stopping the IGF-I synthesis characteristic for tumor cells. They become MHC-I [+] and B7 [+], and partially apoptotic. The transfected cells, together with apoptotic cells and APC cells induced* in vivo, *activate T lymphocytes (CTL CD8+CD28+). Abbreviations used in signal transduction pathway: TK (tyrosine kinase of growth factors receptor); PI3K (phosphatidylinositol 3 kinase); PKC (protein kinase C); TAP 1,2 (transporter associated with antigen processing antigen); APC (antigen presenting cell).*

CD8+CD11b+/CD8+CD11b− (**Figure 5**). In patients with GBM treated in Bromberg (NATO Science Programme—U.S.A./France/Poland), life from time of diagnosis to time of demise was 19 and 24 months.

Histopathologic examination of resected tumors showed peritumor necrosis and infiltration by lymphocytes CD8+ T and CD4+ T cells [100]. Moreover, we can underline, as described in our previous studies [51], that using anti-IGF-I approach without chemotherapy, median survival in GBM-treated patients has reached 19 months and has increased to more than 21 months (NATO Programme) when applied in combination with chemotherapy (temozolomide). The individualized therapy using IGF-I antigene treatment and pharmacology (temozolomide) has been applied in phase I/II trials [132].

In 2001, simultaneously with the first assay with AS IGF-I, Andrews et al. [133] treated 12 patients with recurrent glioblastoma and anaplastic astrocytoma using an antisense to IGF-I receptor, AS IGF-I-R, strategy. Histological analysis of tumors resected from patients with disease progression revealed lymphocytic infiltration and necrosis [133]. As new experimental therapies and efficient viral vectors expressing AS IGF-I-R are being developed, clinical trials using this approach will increase [66, 133, 134].

The approach of AS TGF-beta using an AS oligodeoxynucleotide, compound AP 12009, has given satisfactory results [135–137]. In another clinical AS TGF-beta study, a phase I clinical trial in grade IV astrocytoma (GBM) was performed using autologous tumor cells modified by an AS TGF-beta2 vector. There were indications of humoral and cellular immunity induced by the vaccine [138].

6. Conclusions

The clinical strategies of glioma treatment, using either inhibitors (i.e., imatinib and gefitinib) or antibodies (i.e., Avastin) targeting growth factors and their receptors [139–143], are currently focusing on antisense technology combined with pharmacological treatment.

The neuro-oncology research on glial cells focuses on the PI3K/AKT pathway becoming a potential target in antisense/triple helix strategy for the treatment of glioblastoma patients [59, 69]. The arrest of at least two links either IGF-I or TGF-beta or VEGF and GS of the pathway TK/PI3K/AKT/GSK3/GS [64] seems to be in line for a future clinical gene therapy trial strategy for treatment of GBM. The final result of this signal transduction pathway element inhibition is an immune response mediated *in vivo* by lymphocytes T CD8 and APC cells (**Figure 5**). But the near future in treating this group of disorders belongs to a combination of treatment [4, 42, 70, 79, 115, 130, 144–150]: classical surgery; radiotherapy with immunotherapy, including the use of dendritic cells pharmacologic therapy; growth factor inhibitors; and the use of the antisense/triple helix gene blockade approach targeting signal transduction pathway elements of cancer processes.

Conflict of interest

No conflict of interest.

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