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Immunotherapy of Malignant Tumors Using Antisense Anti-IGF-I Approach: Case of Glioblastoma

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Abstract

The review article describes the criteria established for methodology of antisense anti IGF-I therapy of malignant tumors, particularly of glioblastoma. The cancer patients, after classical therapy of surgery, radiotherapy and chemotherapy, have undergone the injection of genetically modified autologous malignant cells—transfected by IGF-I antisense/triple helix expression vectors. For all cancer patients supervised for up to 19 months, the period corresponding to minimum survival of glioblastoma patients, the following common immune criteria for "anti IGF-I" strategy were admitted: 1) characteristics of cell "vaccines"—absence of IGF-I and expression of MHC-I in cloned transfected cells; 2) the peripheral blood lymphocytes, PBL cells, removed after every of two successive vaccinations, demonstrate an increasing level of $CD8^+$ and $CD8^+28^+$ molecules (with a switch from $CD8^+11b^+$ to $CD8^+11b^-$).

Keywords

Immunogene Therapy, Malignant Tumors, Glioblastoma, IGF-I, Antisense

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1. Introduction

1.1. Immune Aspect in Antisense Strategy

The efficiency of antisense strategy in experimental and clinical therapies [1] [2] was demonstrated for the first time, both *in vitro* and *in vivo* using rat glioma model and episomal pMT/Ep vector [3]. Such strategy of anti-gene type (antisense or triple helix, AS or TH) [4]-[6] has permitted to stop the development of established animal tumors of glioma, hepatoma, melanoma and teratocarcinoma (containing three tissue derivatives) as well as of human gliomas, mediated by immune anti-tumor $CD8^+$ T cells induced *in vivo* by injection of cellular "vaccines" presenting immunogenic character (expression of MHC-I) [7]-[10]. We have previously described the immune cellular/anti-gene anti IGF-I approach [7] targeting IGF-I, the growth factor playing a principal role in the tumor growth processes [11]-[14]. The goal of this review was to describe how the immune criteria of anti-gene IGF-I methodology for clinical trial were established (Phase I gene therapy of glioblastoma). These criteria were based on principal results of the described experimental and clinical studies—the immune anti-tumor phenomena observed in the antisense anti IGF-I treatment of rat and human gliomas, and signaled by the increase of CTL $CD8^+$ in the animal and human tumor tissues, and confirmed in human peripheral blood lymphocytes [7].

The cytotoxic T $CD8^+$ cell can exert its effect if a bridge between CD8 and the antigen of class I major histocompatibility complex (MHC) occurs [15]-[19]. Following transfection of glial cells with the antisense cDNA of IGF-I, the expression of MHC-I in transfected glioma cells *in vitro*, is greatly enhanced (5 times). This mechanism may play a role in the cytotoxic response, although not the only one involved [20]. In preclinical experiments, the bearing tumor rats receiving injections of transfected cells have revealed a very high rate of $CD8^+$ cells. This anti-tumor immune response has stopped the tumor development [3] [21]-[24]. In the presented work, we have used the strategy of combined antisense/triple helix technologies to prepare the anti-gene anti IGF-I "vaccines" and investigate an immune response in treated patients with malignant tumors expressing IGF-I. Comparatively, the tumors representing three tissue derivatives were considered: principally neuroectodermal—glioblastoma, and also entodermal—colon cancer, and mesodermal—cancers of ovary and of prostate.

1.2. Anti-Gene Strategies: Antisense and Triple Helix

Since 1978 it is known that antisense messengers are naturally produced and destroyed in the process of DNA replication [4] [25]-[28]. The authors successfully demonstrated that this phenomenon is possible in twenty different species. It is possible for the researchers to produce artificial antisense messengers in significant amount [28]-[30]. Moreover, when using antisense oligonucleotides associated with the photo luminescent amino-terminated poly amido amine dendrimer, they can be directly analyzed by fluorescence microscopy and flow cytometry [31]. The antisense sequences are capable of blocking the messenger RNA translation conducting to block of specific protein synthesis. The antisense approach has now been introduced in cancer gene therapy as well experimental as clinical trial, principally in gliomas treatment (**Table 1**).

Since the 1990's, another approach in parallel with the strategy of antisense RNA has become successful in gene therapy and clinical trials: the triple helix strategy (TH) [32]-[34]. The technology was discovered by P.B. Derwan and C. Helene [5] [6], and its action was defined as inhibition of gene expression at the level of transcription. In short, specific oligonucleotide sequences (also called triple helix-forming oligonucleotides, TFOs) are introduced into cells by transfection using chemical carriers, such as plasmid vectors which can direct synthesis TFOs. The TFOs are linked to the genomic DNA forming the triple helix structure with the target gene and inhibiting its transcription. The TFOs usually are directed against sequences located in the promoter region of genes of interest [5]. Examples of the inhibitory activity of TFOs on target genes involved in tumorigenesis are currently available [35]-[37]. Moreover, synthesis of Human Tumor Necrosis Factor (TNF), and which acts as an autocrine growth factor in various tumor cell lines including neuroblastoma and glioblastoma has been blocked by treatment with TFOs [38].

2. Methodology

2.1. Preparation of "Vaccines"

2.1.1. Plasmids

IGF-I AS and TH technologies were used to construct episome based plasmids either pMT-Anti-IGF-I expressing IGF-I RNA antisense, or pMT-AG inducing the IGF-I RNA-DNA triple helix, coming from pMT-EP "emp-

Table 1. Examples of experimental and clinical gene therapies of gliomas using antisense approach: articles since 2000's (technology antisense, AS; technology triple-helix, TH).

Target	Strategy	References
IGF-I	AS & TH vectors, experimental therapy	Ly *et al*. (2001) Mol Pathol 54:230
IGF-I-R	AS oligodeoxynucleotide, clinical trial	Andrews *et al*. (2001) J Clin Oncol 19:2189
c-myb	AS oligodeoxynucleotide, experimental therapy	Hu *et al*. (2001) Hua XI Yi 32:562
VEGF	AS vector, experimental therapy	Zhang *et al*. (2002) Zhonghua Yi 80:386
EGF-R	AS vector, experimental therapy	Zhang *et al*. (2002) J Gene Med 4:183
c-myb	AS oligodeoxynucleotide, experimental therapy	Zhao *et al*. (2002) Hua XI Yi 33:19
IGF-I	AS & TH vectors, clinical trial	Trojan *et al*. (2003) Roc Akad Med Biol 48:18
Bcl-2	AS oligodeoxynucleotide, experimental therapy	Zhu *et al*. (2003) J Neurosci Res 74:60
Laminin-8	AS oligodeoxynucleotide, experimental therapy	Khazenzon *et al*. (2003) Mol Cancer Ther 2:985
Urokinase plasmin activator	AS vector, experimental therapy	Gondi *et al*. (2003) Oncogene 22:5967
TGF-beta, FGF and VEGF	AS oligodeoxynucleotide and vector, exp. therapy	Matsumo & Nagashima (2004) Med Electron Microsc 37:158
AKT2	AS vector, experimental therapy	Pu *et al*. (2004) Tumor Biol 25:172
TGF-beta2	AS oligodeoxynucleotide (AP 12009), exp. therapy	Schligensiepen *et al*. (2005) Oligonucleotides 15:94
PKC-alpha	AS oligodeoxynucleotide (aprinocarsen), clinical trial	Grossman *et al*. (2005) Neurol Oncol 7:32
TGF-beta2	AS oligodeoxynucleotide (AP 12009), exp. ther., clin. trial	Schlingensiepen *et al*. (2006) Cytok Growth Factor Rev 17:129
Laminin-8	AS oligodeoxynucleotide, experimental therapy	Fujita *et al*. (2006) Angiogenesis 9(4):183
FAC (focal adhesive kinase)	AS oligodeoxynucleotide, experimental therapy	Wu *et al*. (2006) J Neuro Oncol 27(2):117
Galactosyl-transferase (4GalTV)	AS vector, experimental therapy	Jiang *et al*. (2006) Glycobiol 16(11):1045
PI3K regulated-ILK	AS oligodeoxynucleotide, experimental therapy	Edwards *et al*. (2006) Mol Cancer Ther 5(3):645
AKT2	AS vector, experimental therapy	Pu *et al*. (2006) J Neuro Oncol 76(1):1
VEGF	AS oligodeoxynucleotide, experimental therapy	Hong *et al*. (2006) Cancer Letters 2361:39
EGF-R	AS vector, experimental therapy	Kang *et al*. (2006) Cancer Gene Ther 13:530
EGF-R and PTEN	AS vector, experimental therapy	Tian *et al*. (2006) Neuropath 26(3):178
cMET (receptor tyrosine kinase)	AS oligodeoxynucleotide, experimental therapy	Chou *et al*. (2006) Surg Neuro 65(6):533
TGF beta 2	AS vector, clinical trial	Fakhrai *et al*. (2006) Cancer Gene Ther 13:1052
IGF-I	AS & TH vectors, clinical trial	Kasprzak *et al*. (2006) Neurol Neurochir 40(6):509
IGF-I	AS & TH vectors, clinical trial	Trojan *et al*. (2007) Int J Cancer Prev 2(4):227
IGF-I, IGF-I-R	AS &TH vectors, experimental therapy and clinical trial	Trojan *et al*. (2007) Neuroscience 145:795
TGF-beta 2	AS oligodeoxynucleotide (AP 12009) clinical trial	Hau *et al*. (2007) Oligonucleotides 17(2):201
Glycogen synthase (GS)	AS vector, experimental therapy	Ardourel *et al*. (2007) Cancer Biol Ther 6(5):719
Telomerase (hTERT)	AS adenoviruse, experimental therapy	You *et al*. (2007) Cell Mol Life Sci 64(5):621
Telomerase	AS oligonucleotide (2-5A-anti-hTR), exp. therapy	Iwado *et al*. (2007) Int J Oncol 31(5):1087
XIAP (inhibitor of apoptosis)	AS adenovirus, experimental therapy	Naumann *et al*. (2007) Gene Ther 14(2):147
c-Met (receptor tyrosin kinase)	AS oligonucleotide, experimental therapy	Chu *et al*. (2007) J Surg Res 141(2):284
Urokinase (uPAR)	AS vector, experimental therapy	Nabothula *et al*. (2007) Int J Oncol 30(3):669

Continued

Laminin & Tf and 2C5 antibod.	AS oligonucleotide, experimental therapy	Fujita *et al*. (2007) J Control Release 122(3):356
Caspases 3 & 8	AS oligonucleotide, experimental therapy	Gdynia *et al*. (2007) Mol Cancer Res 5(12):20
miR-21	AS oligonucleotide, experimental therapy	Gabriely *et al*. (2008) Mol Cell Biol 28(17):536
miR-21	AS oligonucleotide, experimental therapy	Shi *et al*. (2008) Zhonghua Yi 25(5):497
TGF-beta and immune activation	AS oligonucleotide (NPs), experimental therapy	Schneider *et al*. (2008) J Neuroimmun 195(1-2):21
TGF-beta and immune activation	AS oligonucleotide, experimental therapy	Vega *et al*. (2008) Future Oncol 4(3):433
Heat shock-protein 27 (Hsp27)	AS oligonucleotide, experimental therapy	Aloy *et al*. (2008) Int J Radiat Oncol Biol Phys 70(2):543
VEGF	AS vector, experimental therapy	Lin *et al*. (2008) Cancer Sci 99(12):2540
TGF-beta 2	AS oligodeoxynucleotide, clinical trial	Schlingensiepen *et al*. (2008) Rec Res Cancer Res 177:137
IGF-I	AS vector, clinical trial	Trojan *et al*. (2009) JAC 1:1
TGF-beta 2	AS oligodeoxynucleotide, clinical trial	Hau *et al*. (2009) Expert Rev Anticancer Ther 9(11):166
IGF BP2	AS vector, experimental therapy	Moore *et al*. (2009) Proc Natl Acad Sci USA 106(39):16675
CD133/	AS oligonucleotide, experimental therapy	Yao *et al*. (2009) Oncol Rep 22(4):781 prominin-1
EGFR	AS oligonucleotide, experimental therapy	Loew *et al*. Anticancrer Agents Med Chem 9(6):703
TGF-beta	AS oligodeoxynucleotide, clinical trial	Vallieres (2009) IDrugs 12(7):445
miR-21	AS oligonucleotide, experimental therapy	Li *et al*. (2009) Brain Res 25(1286):13
miR221/222	AS oligonucleotide, experimental therapy	Zhang *et al*. (2009) Int J Oncol 34(6):1653
IGF-I	AS vector, clinical trial	Trojan *et al*. (2010) Biomed & Pharmaother 64(8):57
miR-21	AS oligonucleotide, experimental therapy	Zhou *et al*. (2010) Oncol Rep 24(1):195
c-Met	AS oligonucleotide, experimental therapy	Chu *et al*. (2010) Oncol Rep 24(1):189
AKT2	AS oligonucleotide, experimental therapy	Zhang *et al*. (2010) Oncol Rep 24(1):65
EGFR	AS oligonucleotide, experimental therapy	Li *et al*. (2010) Oncol Rep 23(6):1585
PED/PEA-15 (ERK1/2-inter. protein)	AS oligonucleotide, experimental therapy	Botta *et al*. (2010) Hum Gene Ther 21(9):1067
miR-21 & 5FU	AS oligonucleotide, experimental therapy	Ren *et al*. (2010) J Biomater Sci Polym Ed 21(3):303
miR-21	AS oligonucleotide, experimental therapy	Zhou *et al*. (2010) Lab Invest 90(2):144
EGFR	AS oligonucleotide, experimental therapy	Kang *et al*. (2010) J Biomed Mater Res A 93(2):585
Laminin-411	AS oligodeoxynucleotide, clinical trial	Ding *et al*. (2010) Proc Natl Acad Sci USA 107(42):18143
TGF-beta & T cell therapy	AS oligodeoxynucleotide, clinical trial	Dietrich *et al*. (2010) Curr Opin Oncol 2010; 22(6):604
Telomerase & taxifen	AS oligonucleotide, experimental therapy	Wang *et al*. (2010) Mol Med Report 3(6):935
VEGF	AS vector, experimental therapy	Yang *et al*. (2011) J Neurooncol 103(1):33
IGF-I	AS vector, clinical trial	Trojan & Anthony (2011) Curr Signal Transd Ther 6(3):411
TGF-beta	AS oligodeoxynucleotide, clinical trial	Jashinsky *et al*. (2011) Curr Pharm Biotechnol 12(12):220
TGF-beta	AS oligodeoxynucleotide, clinical trial	Hau *et al*. (2011) Curr Pharm Biotechnol 12(12):2250
PrPc	AS oligonucleotide, experimental therapy	Barbieri *et al*. (2011) Autophagy 2011 Aug 1; 7(8). [Epub]
miR-10b	AS oligonucleotide, experimental therapy	Sun *et al*. (2011) Brain Res 1389:9
uPAR	AS vector, experimental therapy	Raghu *et al*. (2011) Mol Cancer 10:130
microRNA-7	AS oligonucleotide, experimental therapy	Lee *et al*. (2011) Radiother Oncol 101(1):171
miR-221/222	AS oligonucleotide, experimental therapy	Hao *et al*. (2012) Oncol Rep 27(5):1504

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miR-143 and miR-145	AS oligonucleotide, experimental therapy	Koo *et al*. (2012) BMC Cancer 12:143
miR-1275	AS oligonucleotide, experimental therapy	Katsushima *et al*. (2012) J Biol Chem 2012 Jun 26 [Epub]
PTBP1	AS oligonucleotide, experimental therapy	Izaquirre *et al*. (2012) Mol Carcinog 51(11):895
miR-21	AS oligonucleotide, experimental therapy	Zhou *et al*. (2012) Zhonqhua Znonq Liu Za Zhi 33(10):74
ATM	AS vector, experimental therapy	Chuah *et al*. (2012) Int J Oncol 40(6):1963
miR-143 &-145	AS vector, experimental therapy	Koo *et al*. (2012) BMC Cancer 12:143
18-kDa-TSPO	AS vector, experimental therapy	Veenman *et al*. (2012) Pharmacogenet Genomics 22(8):606
miR-1275	AS oligonucleotide, experimental therapy	Katsushima *et al*. (2012) J Biol Chem 287(33):27396
miR-21	AS oligonucleotide, experimental therapy	Wong *et al*. (2012) Anticancer Res 32(7):2835
Metallo-thionein 1E	AS vector, experimental therapy	Ryu *et al*. (2012) Int J Oncol 41(4):1305
miR-92a	AS oligonucleotide, experimental therapy	Niu *et al*. (2012) Oncol Rep 28(5):1771
14-3-3-beta	AS vector, experimental therapy	Park *et al*. (2012) Neurol Res 34(9):893
MMP-9	AS vector, experimental therapy	Sun *et al*. (2013) 29(1):83
miR106b	AS oligonucleotide, experimental therapy	Zhang *et al*. (2013) J Neuro-oncol 112(2):179
miR-30a-5p	AS oligonucleotide, experimental therapy	Jia *et al*. (2013) PLoS One 8(1):e55008
IGF-I	AS vector, experimental therapy	Pan *et al*. (2013) PLoS One 8(3):e58428
EGF-R	AS vector, experimental therapy	Kalman *et al*. (2013) Neuromolecular Med 15(2):420
miR-21	AS oligonucleotide, experimental therapy	Costa *et al*. (2013) Mol Ther Nucleic Acids 2:e100.

ty" vector [3] [37]. The cassette contains the Epstein-BarrVirus origin of replication and the gene encoding nuclear antigen I, which together drive extrachromosomal replication. (This type of vector based on EBV elementsconfer higher transgene expression in primary human tumors [39]). In the pMT-AG triple helix, the cassette consists of a 23 bp DNA fragment cloned into the pMT-EP vector, which transcribes a third RNA strand forming a triple helix structure within the target region of the human IGF-I gene (**Figure 1**). The triple helix structure forming IGF-I RNA-DNA structure, giving rise to used IGF-I triple helix gene therapy approach, was largely described in previously published papers; moreover, the experimental data *in vitro* accompanied by control experiments constituted by use of either antisense technique or by use of control "empty" vectors were also performed [24] [37]. The vector and the cells transfected with these vectors (see below) were tested for the presence of DNA sequence of EBV virus—in the vector, the 4.4 Kb sequence of EBV is inserted. The tests of PCR EBV have given the negative results (Texcell-Institut Pasteur, ref. 114/01/1054D-02/07 and -01/03; report 27.03.1996). Although the tests date back from 1996, the results are still valid as the total sequence of used vectors were never changed.

2.1.2. Primary Parental and Transfected Cell Cultures

Surgically removed biopsies of primary malignant tumors as follows: glioblastoma (astrocytoma grade IV, glioblastoma multiforme), colon carcinoma (differenciated adenocarcinoma), ovary carcinoma (cystadeno-carcinoma) and prostate carcinoma (adenocarcinoma, cytologic malignancy, grade I) were used to establish primary cell cultures. Two cases of each malignant tumor were investigated. Surgical resections were done in the University Hospital of Bromberg (Bydgoszcz), Poland, and in National Institute of Cancerology in collaboration with INS, Bogota. Primary cell lines originated from every biopsy were established during 3 - 4 weeks [24] simultaneously in three countries (Bromberg and Cracow, Poland, Paris, France, and Bogota, Colombia).

Cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37˚C and 5% CO_2 (**Figure 2**). In the case of glioblastoma and colon cancer, primary human cell lines established previously (CWRU, Cleveland, and Paul Brousse Hospital, Villejuif) have played a role of "cell line controls" for verifications of IGF-I presence (immunocytochemical reaction for IGF-I, using antibodies anti IGF-I, and confirmed by RT-PCR), and MHC-I and B7 antigens absence (immunocytochemical or flow cytometry analysis using antibodies anti MHC-I and anti B7) [23] [24] [40] [41].

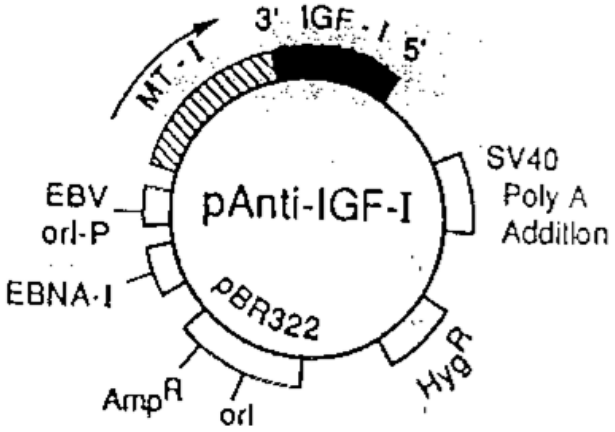

Figure 1. Vector encoding IGF-I cDNA in antisense orientation. Episome based plasmid (pMT-Anti-IGF-I) expressing IGF-I RNA antisense coming from pMT-EP "empty"). vector [3] (see Methodology The cassette contains the Epstein-Barr Virus origin of replication and the gene encoding nuclear antigen I, which together drive extrachromosomal replication.

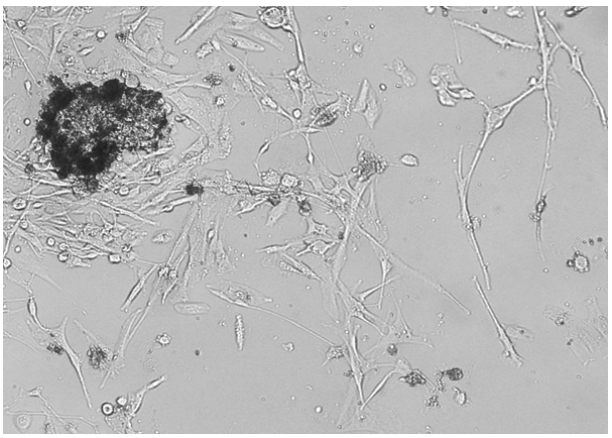

Figure 2. *In vitro* culture of primary human malignant cells derived from surgical biopsy of glioblastoma. Parental tumour cells, proliferating from the tissue of biopsy (note dark black cluster—left up), are attached efficiently after two weeks of primary culture [×200].

RT-PCR technique was applied as described earlier [42]. RNA from cells was isolated using High Pure RNA Isolation Kit (Roche Diagnostics GmbH n˚ 1828665. The applied components of RT PCR were used according Reverse Transcription System Promega Corporation (n˚ A3500. The following primers were used for RT PCR study of human IGF-I:

Forward primer IGF-I: GCATCTCTTCTACCTGGCGCTG, and

Reverse primer IGF-I: caggcttgaggggtgcgcaata (sequence according "rgd" Human Genome Database).

In addition, the quality control of the tumor cultured cells has concerned the test for mycoplasma, endotoxin and aerobic and anaerobic bacteria.

Total transfection of cell cultures was obtained after 2 - 3 weeks, using AS and TH vectors, by either Ca^{++}/Ph technique or FuGENE 6 Transfection Reagent (Boehringer Mannheim). 48 hours after applied FuGENE techbniquet, the selection of transfected cells was done in the presence of Hygromycin B (Boehringer Mannheim) at a concentration of 0.005 mg/ml. After one week, concentration of hygromycin B was changed to 0.015 mg/ml, and progressively increased up to 0.15 mg/ml and maintained with each change of fresh medium over the next 2 - 3 months. Two weeks after application of FuGENE, cell lines derived of the same tumor, were verified for ab-

sence of IGF-I using immunocytochemistry technique, confirmed by RT PCR technique [42] [43] (**Figure 3**), and for presence of MHC-I and B7 molecules using flow cytometry analysis (**Figure 4**): monoclonal antibodies, labeling human MHC-I (HLA), MHC-II, CD80 and CD86 (B7) antigens were used for direct immunostaining (Becton Dickinson Pharmingen) [40]. The expression of IGF-I, MHC-I and B7 in non transfected and transfected separately "antisense" and "triple helix" cancer cell lines, was verified in the laboratories of Bromberg and Krakow (Poland), Cleveland (USA), Villejuif (France) and Bogota (Colombia). The cell lines transfected with "empty" vector have constituted a negative control for both "antisense" and "triple helix" lines.

AS and TH cell clones expressing MHC-I and B7 molecules were used for preparation of cell vaccines (in general, 40% - 50% of clones were positively stained for MHC-I, and 50% - 60% of clones were negative for MHC-I). The cultures of these clones, four weeks after transfection have presented about 50% - 60% of apoptotic cells, and 40% - 50% of no apoptotic cells which were IGF-I (−), MHC (+) and B7 (+). The MHC-I and B7 expression, as well as apoptosis were verified as described earlier [8] [43] (**Figure 4**).

The established cell cultures were divided in three parts. The first part, 200,000 cells, was used for the preparation of cell membranes, prepared according to the technique of Matlib [44]. These cell membranes have constituted the material for first, non-cell, "membrane vaccination". When the cells growing in culture were numerous enough, 3 - 5 million "antisense cells" and "triple helix cells", respectively, one part of them was used for "cellular vaccination"—injection of 3 million of cells: 1.5 million of "antisense cells" mixed with 1.5 million "triple helix cells", and another part was frozen as "back-up" in liquid nitrogen.

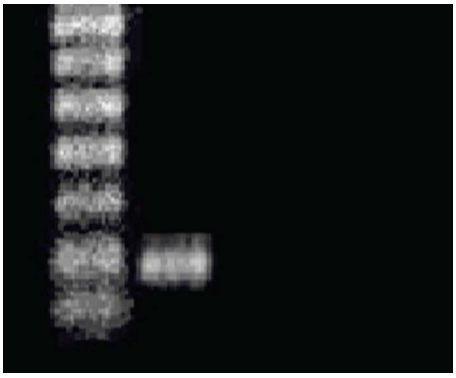

Figure 3. Expression of IGF-I in primary human glioma cell lines, parental and transfected cells. RT PCR technique. M: marker (down/up: from 100 bp to 700 bp band). a: presence of IGF-I in parental cells (200 bp band of amplified DNA using IGF-I primer). b: absence of IGF-I expression in cells transfected with antisense anti IGF-I vector. c: absence of IGF-I expression in cells transfected with triple helix anti IGF-I vector.

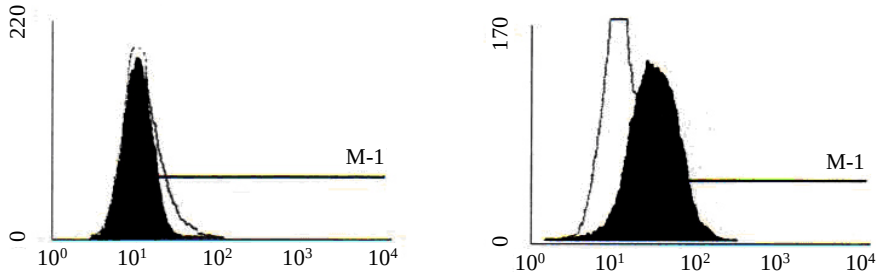

Figure 4. Expression of MHC-I in an established primary human glioma cell line. Flow cytometry analysis (FACScan Becton Dickinson). Panel left: parental non transfected cells. Panel right: cells transfected with both antisense and triple helix anti IGF-I vectors.

2.2. Vaccination of Cancer Patients

Six patients in University Hospital of Bydgoszcz and six patients in CWRU Hospital of Cleveland, were treated for glioblastoma, respectively. The patients in Bydgoszcz were divided in three groups of two cases each (considering that this clinical trial was the beginning of Phase 1 presenting a limited number of patients, the statistical analysis was not included in the study). In every group the age of patients were about twenty years and sixty years, respectively (the individual characteristics are not a subject of the presented work). In the first group, the patients treated by surgery, radiotherapy and chemotherapy (low dose chemotherapy—temozolomide 75 mg/m^2/day, applied during the period of radiotherapy) have followed three successive subcutaneous injections of cellular membranes isolated from IGF-I antisense/triple helix transfected cells ("membrane vaccinations"). In the second group after surgery, radiotherapy and chemotherapy (low dose), the first membrane injection was followed by two successive subcutaneous "cellular vaccinations" composed of IGF-I AS/TH cells containing both apoptotic and no apoptotic cells. The third group, a control group, was treated by surgery, radiotherapy and chemotherapy.

On the other side, six glioblastoma patients in Cleveland were treated by unique therapy: surgery, radiotherapy and chemotherapy was followed by successive subcutaneous "cellular vaccinations" composed of IGF-I AS cells containing both apoptotic and no apoptotic cells. In parallel, the patients with cancers of colon, ovary and prostate (Bydgoszcz) have followed the treatment composed of surgery, radiotherapy and successive "cellular vaccinations" (see Section 3.3).

The injections were done with interval of four - five weeks. 48 hours before every vaccination, the membrane or cell pellets ("membrane" or "cellular" therapies) were irradiated with 5000 Gy gamma (Co60 or Cs137) [40]. The membranes or cells were injected subcutaneously, into the left arm of operated cancer patients, in 1 ml of PBS solution. The blood was collected three times: before vaccinations and after every of two cell vaccinations (2 - 3 weeks after the second and the third injection). Flow cytometric analysis of PBL cells was done as follows; Peripheral blood lymphocyte (PBL) typing was performed after hemolysis by incubation of peripheral blood with monoclonal antibodies against the cell antigens: CD3, CD4, CD5, CD8, $CD8^+11b^+$, $CD8^+11b^-$, $CD8^+28^+$, CD19, CD3-$(16^+56)^+$(NK), CD25, CD44, CD45 (Becton Dickinson Pharmingen, direct immunstaining). Paraformaldehyde-fixed cells were examined in FACSscan BD cytometer. Double direct immunotyping with pairs of monoclonal antibodies conjugated with FITC and PE were used. Lymphocyte gate was defined according to the CD45 back gating. Data were presented as percentage of positive cells [42]. The PBL labelling of cells was done simultaneously in two laboratories (Bydgoszcz and Villejuif).

3. Results

Each primary cell line was subcloned to obtain IGF-I positive clones (the percent of IGF-I positive cells in the human cell lines ranged from 50% to 70%). All the primary cells lines were also transfected, showing a change of phenotype. The examples of primary cell lines as well as of transfected cell lines of ovary and prostate cancers are shown in **Figure 5**.

Using RT PCR, analysis of RNA of cancer cells four weeks after transfection was compared to RNA of parental cells. Then the transfected cells (not expressing IGF-I), serving as "vaccines" for different types of cancers were cloned to obtain MHC-I postive cell lines. The transfected MHC-I postive cancers cells have also expressed B7 antigen with exception of hepatoma transfected cells as described earlier [8]. Human cancer cells transfected with "empty" vector, pMT/EP, serving as negative control, were stained positively for IGF-I and negatively for MHC-I and B.

In treated patients, with higher mentioned "vaccines", the typing of PBL cells removed from blood samples was performed. Clear-cut phenotypic changes in peripheral blood lymphocytes (PBL) was observed in all cancer diseases treated with "cellular therapy": after the first cell vaccination, the increase of CTL $CD8^+$, particularly $CD8^+11b^-$, was observed. There was a characteristic switching from $CD8^+11b^+$ to $CD8^+11b^-$ which was practically not significant in the group of "membrane therapy" applied for two glioblastoma patients. This increasing switching was also observed after the second cell vaccination in all treated cancers. Moreover, the PBL cells have demonstrated, in all types of tumor diseases, an increasing expression of cell surface markers $CD8^+CD28^+$ confirming the effectiveness of "cellular therapy" (**Figures 6(A)-(D)**).

The specificity of immune response was every time confirmed as follows. As demanded by approved clinical trials (see Section 4—Ethical Consideration), the removed PBL of patients were tested *in vitro* for immune

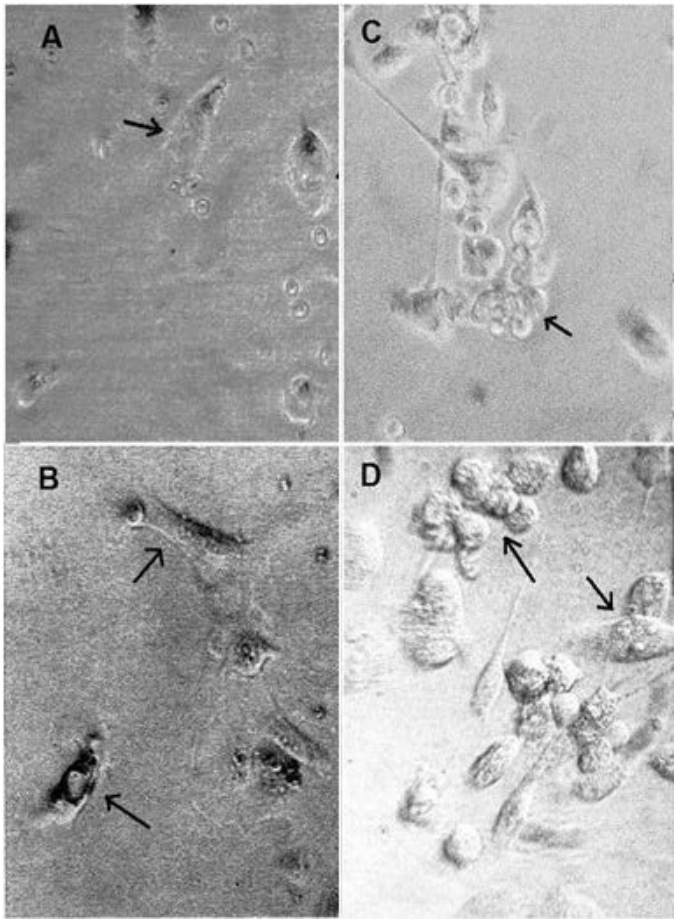

Figure 5. Examples of *in vitro* culture of primary human cancer cells derived from surgical biopsies. Panel (A) and (C)—primary ovary cyst-adenocarcinoma derived cells. Panel (B) and (D)—primary prostate adenocarcinoma derived cells. Parental ovary cancer cells (A) and prostate cancer cells (B) are attached efficiently in the fourth day of culture (arrows). "Antisense/triple helix" anti-IGF-I ovary cancer cells (C), and prostate cancer cells (D), both twenty days after transfection, form the established lines characterized often by the clusters of round apoptotic cells, becoming progressively small ((C), arrow; (D), arrow up). They are accompanied by no apoptotic and more voluminous cells ((D), arrow down) presenting generally elongated shape ((C) and (D)). The transfected cells are always different from non transfected parental cells ((A), (B)), as it was demonstrated previously in cases of human glioma and hepatoma cell lines established from primary tumours of glioblastoma and hepatocarcinoma [20] [23] [24], ×400.

response after contact with autologous tumor cells to demonstrate antitumor activation through high percentage of specific T cells observed after vaccination compared to controls—before vaccination (the tumor cells were labeled with Cr51 before the test of lyses in the presence of CTL cells [45] [46]; data not shown). Other examined CD molecules as CD3 or CD19, CD45 (data not shown) were not shown the significant values in all treated cancers. In the case of CD4, slightly decreased values were registered (as only two cases of each tumor were studied, these observations not be statistically valuable). That is important to add that no significant change in CTL CD8 level was observed before and after the surgery, and before and after radiotherapy treatments as compared to "cellular therapy". The control "empty" vector, without antisense anti IGF-I cDNA, used to prepare "vaccines" in animal models previously described, has never shown influence for immune anti-tumor response [3] [43].

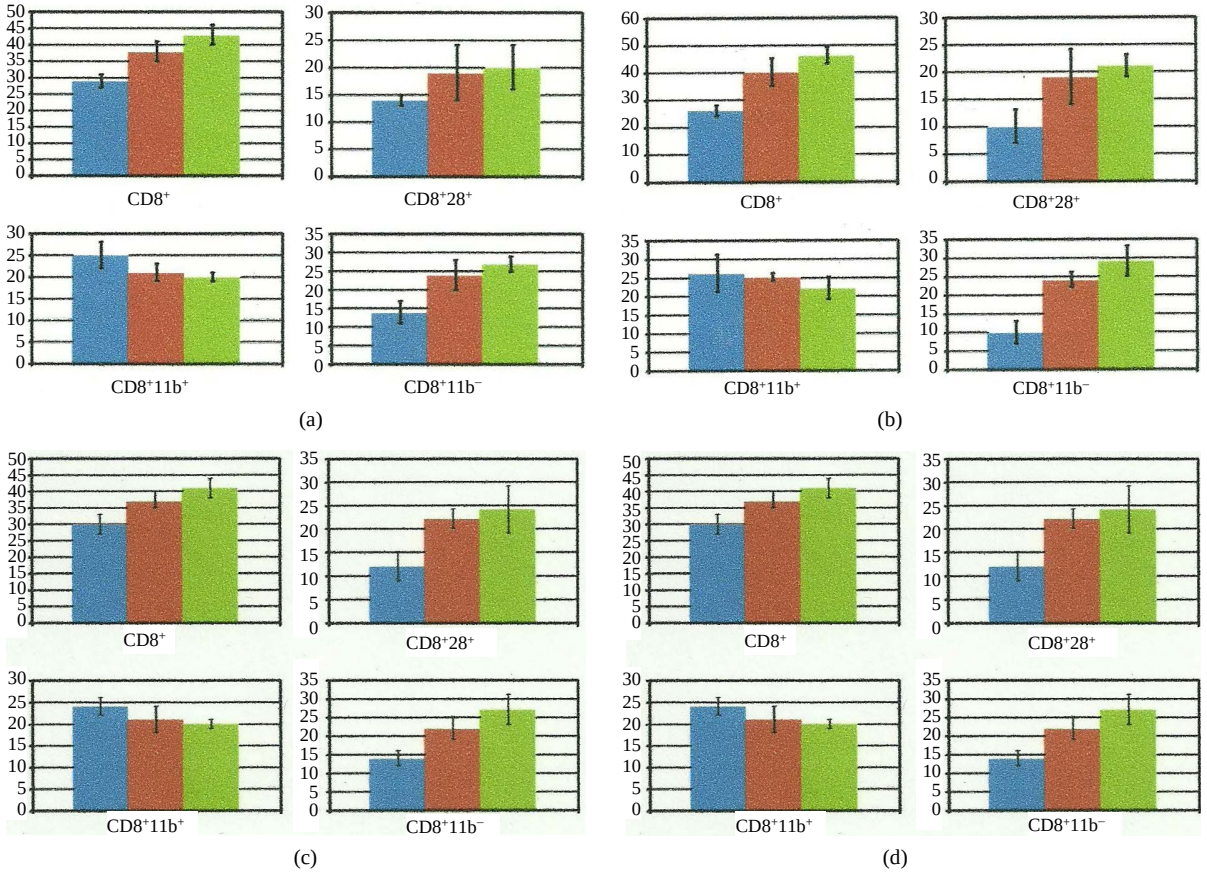

Figure 6. Flow cytometric peripheral blood lymphocyte CD marker patterns following cellular gene therapy in human cancers. Examples of cancers of three tissue derivatives are shown as follows: neuroectodermal—glioblastoma multiforme (a); entodermal—colon adenocarcinoma (b); mesodermal—ovarian carcinoma (c) and prostate adenocarcinoma (d). CD molecules were labelled in peripheral blood lymphocytes (PBL) obtained from pre-vaccinated and "vaccinated" cancer patients. Each of the first column corresponds to data obtained before vaccinations; each second and third column corresponds to data obtained after one and two successive cellular vaccinations (IGF-I antisense/triple helix cells). Two cases of each of the designated cancers were examined (bar graphs represent the median value of the two cases). Data are expressed as percent of positive cells when compared to the isotype control. Difference in percentage of $CD8^+$ $CD11b^-$ and $CD8^+$ $CD28^+$ subpopulations before and after vaccination was strongly significant with a range of p from 0.001 to 0.02 according to the Student's t test, and weakly significant concerning the decreasing $CD8^+$ $CD11b^+$ subpopulation from the relevant patients. The p value for $CD8^+$, $CD8^+28^+$ and $CD8^+11b^-$ (below 0.01) are illustrated in the bar graph for statistical significance.

The clinical results obtained in the University Hospital of Bromberg were as follows: two glioblastoma patients included in the group of "cellular therapy" have survived 19 and 24 months, respectively (beginning from day 0—diagnosis of malignant glioma, followed by surgery, radiotherapy and antisense/triple helix cell injections). Two glioblastoma patients included in the group of "membrane therapy" have survived 9.5 and 10 months respectively—starting from surgery followed by radiotherapy and injection of cell membranes. The results observed in the group of glioblastoma patients treated with "membrane therapy", were not so different from those obtained in the third group treated by classical therapy; in the last group median survival was as 10 and 11 months. For this reason, admitting that the group of glioblastoma patients treated with antisense/triple helix cell injection has given the significant results, all other cancer patients (age 20 - 65 years: two cases of liver, colon, ovary, uterus and prostate cancer diseases) were treated, after surgery and radiotherapy with this type of "cellular therapy". Moreover the period of 19 months, was chosen as the end of clinical observations in all treated cancer patients. At 19 months, all these cancer patients were alive and the treatments were well tolerated (we do not include the details of clinical observations concerning different types of treated cancers, because it is not the subject of this work). The only secondary observed effect including glioblastoma patients, was that of increased temperature up to 38˚C - 39˚C persisting during two-three days after every of cell vaccination.

As to the patients treated in University Hospitals of Cleveland using antisense IGF-I "cellular therapy" (two cell injections), two of the treated patients forming a group of maximum median OS have both survived 19 months. Other group of three patients, have not responded so positively to the therapy, showing the median survival compared with that of "membrane" therapy. The therapy done in the USA has shown that the number of cell vaccinations (between one and four) was not related to the median OS. Concerning serial MRI/CT performed in USA patients: 1 - 2 month intervals before vaccine showed continuous growth of the intra cerebral tumor. MRI one month post vaccination showed first evidence of an unequivocal decrease in size of tumors viewed by radiology in University Hospitals of Cleveland. Moreover, all patients treated in the USA had advanced disease with cerebral edema at the time of first treatment with vaccine, and also were receiving treatment with high dose of decadron or related steroids to reduce the effect of CNS edema. This of course has caused further jeopardy to the immune system, and can explain the relatively negative results in three last treated cases.

To summarize the obtained results, the following schema of immuno-gene therapy mechanism was proposed (**Figure 7**).

4. Ethical Consideration

Human experiments were conducted in accordance with the Declaration of Helsinki (1964). The experiment was conducted with the understanding and the consent of the human subject. The responsible Ethical Committees have approved the experiments.

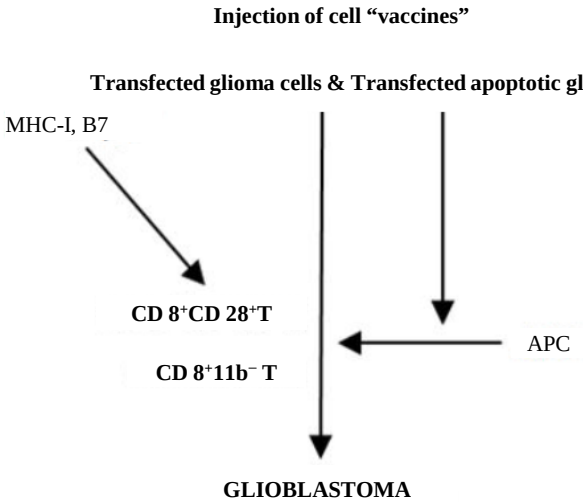

Figure 7. Mechanism of anti-gene anti IGF-I (antisense/triple helix) therapy of malignant tumours. The case of glioblastoma therapy; hypothetically, the same mechanism should exist in the treatment of other tumours expressing IGF-I. Tumour cells are transfected *in vitro* with a vector encoding IGF-I cDNA in antisense orientation (**Figure 1**), or with a vector inducing a formation of triple helix IGF-I structure. The transfected tumor glial cells, in absence of IGF-I, become immunogenic—expressing MHC-I and B7 molecules, and apoptotic as follows. The expression of MHC-I is due to the presence of TAP1; the expression of B7 is related directly with signal transduction pathway: TK/IRS/PI3K/PKC; the phenomenon of apoptosis is also related with signal transduction pathway: TK/ IRS/PI3K/AKT/Bcl2 [7] [10] [18] [47]-[51]. After *in vivo* injection, together with the antigen presenting cells, APC, they activate the T CD8 (CD8CD28) lymphocytes inducing immune anti tumor response against the malignant glioma (expressing MHC-I) [7] [19] [52] [53]. In conclusion, the mechanism of antisense therapy is a combination of an augmentation of the immune anti-tumour response and of an inhibition of signal transduction pathway that is involved in the transformed phenotype of the tumour.

The approval for the gene therapy clinical trial (based on NIH clinical protocol n˚ 1602, Bethesda, Maryland, 24/11/1993), containing scientific basis of methodology, cell therapy product standardization of preparation, detailed clinical protocol including inclusion criteria and exclusion criteria (*i.e.* HIV and EBV active infection) and the letter of agreement, was administrated by the Bioethical Commissions of the L. Rydygier Medical University, Bromberg (Bydgoszcz), Jagiellonian University, Cracow, Poland (n˚ KB/176/2001, 28/06/2002, and n˚ KBET/184/L/2000, 21/09/2000), La Sabana University, Chia, Colombia, no P 004-10, 15/12/2010, Cartagena University Hospital of the Caribbean (preclinical study), Colombia, no 3—19/10/2011, and registered by international Wiley Gene Therapy Clinical Trial database, Stockholm, n˚ 635 and 636 (J Gene Med, updated 2002). The protocol was verified by Ministry of Health, AFSSAPS Committee, Paris, France, 03/06/2005, and by NATO Science program 2003-2007 (n˚ LST 980517).

5. Discussion

The immunogene and immune therapies may represent a novel approach for cancer therapy [54]. The glioblastoma, as well as other malignant tumors, were recently successfully treated by antisense therapy targeting TGF beta, using either antisense anti TGF beta expressing vector [55] or particularly applying the oligodeoxynucleotides [56] [57]. Using phosphorothioate TFG beta2 antisense oligonucleotides (AP-12009), an international phase II/III study was initiated in patients with TGF beta-overexpressing tumors such as high-grade gliomas, and by 2005-2006 the trial was ongoing in over 140 patients with anaplastic astrocytoma (AA) or glioblastoma; the treatment was very well tolerated. In 2007, overall survival was 24 months, and in the control group, survival was 20 months [56] [58] [59]. Results from the clinical trials concerning other tumors over expressing TGF beta were also recently published (pancreatic carcinoma, metastatic melanoma or advanced colorectal carcinoma); the treatment was well tolerated in all types of tumor diseases [56]. Other antisense approaches of malignant tumor treatment have been developed recently, since 2001, especially those of antisense anti IGF-I-Receptor [11]. AS IGF-I-R strategy of treatment of glioblastoma [Andrews *et al.*, 2001] was not continued. It seems that this therapy could be more efficient if the cell "vaccines" were prepared after cloning of IGF-I-R antisense cells for MHC-I expression. In anti-gene anti IGF-I approach, we have applied both antisense and triple helix technologies, permitting to stop simultaneously the expression of IGF-I on translation and transcription levels [7]. Moreover, *in vivo* AS IGF-I approach was also developed [60]; 45 patients with PHC were co-transfected *in vivo* with antisense IGF-I expression vector and sense B7.1 expression vector. At two years following treatment of PHC stage II, there was marked reduction in tumor recurrence—from 62% to 20%. Using described here IGF-I antisense/triple helix strategy, all treated patients have well tolerated the three injections of transfected cancer cells. The PBL cells have shown an increase in $CD8^+CD28^+$ molecules with a characteristic switching from $CD8^+11b^+$ to $CD8^+11b^-$ phenotype, observed after two cell vaccinations, reflecting the enhanced activation of cytotoxic T-cells in blood. These results concerning the switching $CD8^+11b^+$ to $CD8^+11b^-$ in different treated tumors have confirmed previously obtained data in glioblastoma and hepatoma treatment using antisense anti IGF-I approach [40] [43]. The work in progress has also shown in different treated tumors described here, an increased percentage of T CD25 (interleukin-2 receptor), in the context of CD4, which has confirmed the results obtained in glioblastoma treatment. The only secondary observed effect was increased temperature, 38˚C - 39˚C, confirming the immune response induced by antisense/triple helix "vaccines". Regarding injection of cell membranes, the switching mentioned earlier was not significant. The challenge of injection of membranes, isolated from IGF-I antisense/triple helix transfected cells expressing MHC-I, has proved that the whole transfected cell population is necessary to produce an *in vivo* anti-tumor effect. At first, the cytoplasm of the transfected cells contains the IGF-I antisense RNA and IGF-I triple helix RNA-DNA structures constituting the principle of anti-gene cellular therapy [37]. Next, the cellular therapy described here has shown that both cell populations, as well MHC-I and B7 expressing transfected cells as apoptotic cells, are necessary to induce *in vivo* an immune anti-tumor response involving APC activating $CD8^+$ T cells [23] [61]; it was previously demonstrated that doubly transfected cells, using antisense anti MHC-I and anti B7 vectors, lose their apoptotic and immune anti-tumor characters [20] [24]. This way, it was shown that both processes—immunogenicity (MHC-I and B7 expression) and apoptosis, "work" together. On the other side we have previously compared the efficiency of gene therapy—using the injection of IGF-I AS nucleotides and that of described here "cellular therapy" much more promising. In gene therapy approach, after the injection of IGF-I AS nucleotides directly to the tumor, the cancer cells internalizing AS nucleotides could not become immediately immunogenic to induce the

rapid immune response, and for the same reason to develop the efficient apoptosis. Moreover, in the gene therapy, the co-transfection with B7 expression vector was necessary to reinforce the immune response [43] [60].

The immune criteria of used vaccines were strongly related to the preparation of cancer cells to be used as vaccine. Cancer cells cultivated under stem cell-permissive conditions more closely reflect the tumor of origin, including the genetic profile, than the parental tumor adherently growing cells under conventional cultivation conditions [62] [63]. In our experimental clinical trial, to avoid this effect of "contamination" increased by numerous passages, the primary cancers cells and transfected cancer cells "vaccines" were systematically cloned after every passage to obtain *in vitro*, in the first case 100% IGF-I (+), MHC-I (−), and in the second case 100% IGF-I (−), MHC-I (+) expression. Moreover, in parallel to these criteria, another criteria was obligatory to produce the immune anti-tumor effect of "vaccines": the cell vaccines were composed of higher characterized cancer transfected cells and of apoptotic derived cancer transfected cells (mixture 50:50).

The mechanism of antisense therapy targeting growth factors and their receptors is a combination of an augmentation of the immune anti-tumor response and of an inhibition of the signal transduction pathway—PI3K/AKT/GWK3/GS—that is involved in the transformed phenotype of the tumor [7] [49]. Activation of the PI3K/AKT/GWK3/GS pathway is mediated by some tyrosine kinase receptors, under the control of several growth factors and cytokines as EGF, PDGF, VEGF, TGFbeta, CSF and especially IGF-I, whose receptor, IGF-I-R, plays a principal role in the tumor growth process [7] [13] [14]. As far as PI3K/AKT/GWK3/GS pathway (in relation with glioma) is considered, it was recently demonstrated that in experimental antisense anti-glycogene synthetase, GS, tumor therapy, the transfeted AS GS cells were also immunogenic (MHC-I expression) [49] [50]. Anyway in AS GS strategy an immune anti-tumor response was not as striking as when using AS IGF-I approach. This shows that AS IGF-I appears as a dominant tool for the arrest of tumor progression. Moreover, targeting IGF-I instead of IGF-I receptor seems more efficient: because of downstream elements involved in the IGF-I-R transduction pathway, signals from IGF-I-R can be inappropriate or exaggerated [13]. Nevertheless, if crosstalk of IGF-I's related different pathways is considered, IGF-I, through its binding to IGF-I-R, which activates PI3K/AKT transduction cascade, has been reported to block the apoptosis pathway (IRS/PI3K/AKT/Bcl or AKT/GSK3 or Ca^{2+} or caspases). As to PI3K/AKT/GWK3/GS pathways of IGF-I AS or TGFbeta AS therapies, we cannot avoid the relation with PI3K/PKC/RAF/MAPK chain, and we cannot exclude that the inhibition of TK/PI3K/AKT pathway using AS IGF-I approach, can be reinforced by "side" effect of MAPK inhibition. The inhibitors of RAF targeting the ATP binding site, as well as the inhibitors of MAPK at a non-ATP site, were also introduced in cancer clinical trials [64] [65]. For most of the pathways that have been disclosed it has been a problem to develop selective molecules having a relevant clinical impact in malignant diseases, including uncured glioblastoma [66]. To target specific genetic defects, the antisense oligonucleotides have become one of the important anti-cancer approaches used in clinical trials [28].

The immune anti-tumor response was signalled as a principal mechanism of antisense technology, as well using anti TGF beta technique as anti IGF-I and IGF-I-R inhibiting growth factors and their signalling pathway [24] [55]. The final result of AS IGF-I approach including the TK/PI3K/AKT pathway elements inhibition is an immune response mediated *in vivo* by lymphocytes T CD8 and APC cells [7] [14]. As far as the relationship between anti-gene anti IGF-I technology and immunogenicity is considered, the absence of IGF-I synthesis in "antisense" and "triple-helix" transfected cells, could lead to a compensative increase in IGF-I receptor (tyrosine kinase); IGF-I and IGF-II present in foetal calf serum of culture medium, as well as intracellular IGF-II can interact with the type I receptor [22]. Indeed, the increase of IGF-I receptor level could explain the expression of B7. There is a known relation between the signal transduction pathway of tyrosine kinase and the induction of B7 molecules: enhancement in B-7 co-stimulation through a cAMP mechanism linked to tyrosine kinase of the CD 28 receptor has been previously reported [18]. The co-stimulatory B7 molecule in antigen presenting cells (APCs) is bound to the counter-receptor CD28 and/or CTLA4 expressed on the T-cells [52] [67]. B7 was present in different antisense and triple helix anti IGF-I transfected cancer cells but absent in transfected human hepatoma and in previously described murine hepatomas.

As to the MHC-I (HLA) expression in AS and TH cells, this molecule is strictly related to TAP-1 presence; TAP-1, TAP-2 tightly linked to LMP-2 and LMP-7 [17] [68]-[71] are multiple components of the endogenous, antigen presentation pathway machinery. Deficiencies in expression of TAP-1, TAP-2 and LMP-7 were observed human glioma cell lines. Following down-regulation of IGF-1 by transfection with AS IGF-I vector, the deficiencies in components of the MHC-1 antigen presentation pathway were up-regulated (with restoration of

TAP and LMP) [10]. Our previous results have also demonstrated that the expression of MHC-I in human transfected hepatoma cells was much higher than that in transfected human glioma cells [23]. This strong expression of MHC-I in human transfectedhepatoma lines (5 times, compared to human transfected glioma lines) could explain that the presence ofMHC-I was sufficient to induce T CD8 lymphocytes response in the absence of B7 antigen. To summarize the immune anti-tumor mechanism of anti-gene anti IGF-I strategy, this aspect was published previously [22] [43]. As far as largely studied glioma treatment is considered, and similarly other concerned tumors, the mechanism concerns the reaction between activated lymphocytes expressing CD8CD28, and immune molecules MHC-I and B7. The following chain reaction could occur: cultured cloned glioma cells [IGF-I (+), MHC-I (−), B7 (−)] => cultured transfected anti-gene IGF-I cells [IGF-I (−), MHC-I (+), B7 (+)] => injection (glioblastoma patients) => induction of CTL CD8 (+) CD28 (+) => destruction of injected transfected anti-gene IGF-I cells [IGF-I (−), MHC-I (+), B7 (+)] and arrest of a solid glioma tumor [IGF-I (+/−), MHC-I (+), B7 (+)] (see also legend of **Figure 7**).

6. Conclusions

The treatment options for patients with advanced malignant tumors, including brain tumor glioblastoma (current mortality 100%), such as surgery, radiation or hormone therapy are limited in efficacy, therefore the search for new strategies like chemotherapy [72], use of inhibitors, including antibodies, antisense oligonucleotides, short peptides and other small molecules [1] [2] [7] [73]-[75], or cellular immune therapy [19] [76] represents a permanent challenge. The current clinical strategies for the treatment of gliomas are usually a combination of chemotherapy and use of different types of inhibitors (imatinib, gefitinib) including antibodies (*i.e.* avastin), targeting growth factors and their receptors [72] [73] [77] [78]. A pharmacological strategy—the use of temozolomide introduced by R. Stupp, has offered a new hope for treatment of glioblastoma. However, although median survival has reached almost a year and a half, we are still far from victory [7] [72] [73] [79] [80].

Our presented work focuses on the criteria established for methodology of anti IGF-I gene therapy analyzing our different previous basic and clinical results obtained in Europe, USA and Asia, following our previous NATO science program (see Acknowledgement), and published recently [43] [51], permitting to start Phase I and II in South America (Colombia). This way we have established the common criteria for selection of vaccines (expression of IGF-I, MHC-I, B7) and of PBL cells markers ($CD8^+$ related molecules) in patients presenting the arrest of growing tumors.

The various therapies in the treatment of cancer are still experimental [81]. A number of strategies for inhibiting gene expression have been developed including the triple helix approach, antisense cDNA and oligodeoxynucleotides. Among the new strategies in the efforts of treating malignant tumors expressing different growth factors, and more specifically IGF-I, TGF beta, VEGF or EGF [14] [65] [74] [82], the anti-gene therapy approach, either antisense or triple helix, appears as a promising solution [57] (**Table 1**). Although in the presented work only limited numbers of glioblastoma patients were treated, the clinical results obtained are positive (minimum survival has reached 19 months). The anti-gene anti IGF-I therapy, giving a strong immune anti-tumor response in different comparatively studied tumor diseases presents all characteristics of cell immunotherapy ($CD8^+$ and $CD28^+$ expression in T lymphocytes, and MHC-I in relation with TAP-1 and -2 molecules in "vaccine" cells) [10] [83] including apoptotic phenomenon [7] [18] [44] [84]. We suggest that anti-gene cell therapy, giving comparable results to those of currently applied chemotherapy, inhibitors or antibodies [72] [73] [85], could be used either alone [57] [86] or as combined therapies *i.e.* antisense targeting simultaneously different elements of growth factors signalling pathway [49] [50] [63] [64], or as antisense/chemotherapy. The combined anti-cancer strategies considering the role of immune anti-tumor response [65] [77] [78] [87]-[91], including study of control CD8 (+) T-cell effect or functions [92], new tools of cell transfection [93] and especially the search for new onco-proteins [94] and growth factor targets [14] [50] [65] [95] [96] appear as the near future challenge. Among growth factors, targeting IGF-I system in relation with cancer therapy constitutes ongoing basic and clinical research [97]-[99]; the IGF-I being considered as one of the principal precancerous markers [3] [100] [101], has conducted to experiments on suppression of IGF-I expression in tumors, following directly by immuno-gene therapy of malignant tumors. Gene therapy, particularly cellular imunogene therapy, and cellular immunotherapy are currently among the most promising approaches for treatment of cancer diseases [78] [91] [102] [103]. As far as gene therapy is concerned, the technologies permitting higher transgene expression using either viral vectors or synthetic vectors, are in permanent study [39] [104].

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