RESEARCH ARTICLE

Applied physiology of IGF-I: case reports of gene therapy of malignant tumors especially glioma.

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Abstract

Introduction: Certain specific antigens, which behave as oncoproteins, are present in normal fetal/neonatal brain development, and are absent from mature brain tissues: among them, growth factors, especially insulin-like growth factor type I (IGF-I). When IGF-I reappears in the mature brain, this growth factor is over expressed in neoplastic glia, participating in the development of the most common human brain malignant tumor, glioblastoma multiforme, which is invariably fatal. Targeting the IGF-I system has emerged as a useful method to reduce glial malignant development.

Methodology: In practice, in case-control study, when human glioblastoma cells, and comparatively studied primary hepatocarcinoma and colon adenocarcinoma cells derived from cancer biopsy, are transfected *in vitro* with vectors expressing either IGF-I antisense RNA or inducing IGF RNA-DNA triple helix, the synthesis of IGF-I is stopped on translation or transcription levels, respectively (anti – gene strategy). Three cancer groups of two patients each, cancer stage I, after surgery and radiotherapy, were injected using antisense / triple helix ''vaccines'' of 1 million irradiated cells. The control groups received injected placebo.

Results: Down regulation in the expression of IGF-I coincides with the reappearance of B7 and MHC class I antigens at the surface of transfected cells (immunogenicity). When injected subcutaneously, the transfected cancer cells, "vaccines", initiate an immune reaction involving CD8+ lymphocytes, followed by tumor regression. The glioblastoma patients treated by classical surgery and radiotherapy, were "vaccinated" by three successive injections. The median survival of treated patients was 21 months (current progress in treatment of this disease involves an increase in medium survival from 8-11 months to an average of 15 months, using a chemotherapy). Using the same strategy, the patients with liver carcinoma and colon adenocarcinoma were comparatively treated. The obtained immune anti-tumor response mediated by TCD8 was similar to that of glioblastoma patients.

Conclusion: The cellular immunogene therapy using anti - gene approach constitutes one of the current efficient therapies of glioblastoma and other malignancies expressing IGF-I. The described methodology applied in Europe, and previously in the USA for glioblastoma treatment, is introduced now in university hospitals of Colombia (Bogota).

Keywords: IGF-I, glioma, antisense, immunogene therapy, TCD8

1. Introduction

The IGF system involves three ligands (insulin, IGF-I and IGF-II), their receptors IGF-I-R and IGF-II-R, and IGF binding proteins (IGFBPs, BP1 to BP6 having been characterized). Both IGF-I and IGF-II are ligands for IGF-I-R. The IGF-I-R competes with IGFBPs for free IGF-I and IGF-II (the affinity of IGF-I-R and IGFBP for IGF-I is generally of the same order of magnitude) [1-3]. The action of IGF-I on cellular metabolism depends on IGFBPs, which prolong the half-life of this growth factor and modify its interaction with its receptor. IGFBPs have growth regulatory actions that are probably independent of their capacity to bind IGF-I [4].

The IGF-I is a polypeptide of 76 amino acids, which participates in cell and tissue differentiation, stimulates both foetal and postnatal growth [2], and particularly neurogenesis and myelinisation [5,6]. As a mediator of the effects of growth hormone (GH) IGF-I is a locally acting stimulator [2]. IGF-I has characteristics of both a circulating hormone (synthesised by the liver) and a tissue growth factor with autocrine or paracrine properties. IGF-I acts through its binding to a specific IGF-I-R [1,2,7]. IGF-I-R is already present at the two-cell stage of development and is especially highly expressed from the time of neural tube formation, although glial cells express relatively little IGF-I-R mRNA [8,9].

In contrast to IGF-I-R, which is widespread in the developing brain, the expression of IGF-I is principally found in the cerebellum, sensory relay systems and the cortex. IGF-I mRNA, which is relatively scarce in the prenatal state, increases dramatically in the early postnatal-period, followed by a decline over several weeks after birth. During post-natal development IGF-I mRNA is found in the developing hippocampus, and in the anterolateral ventricle subventricular zone, suggesting a potential role in promoting proliferation of glial cells originating in these zones [5,8,10,11].

IGF-I is involved in neural development, neurogenesis, glial differentiation and glucose metabolism, acting locally in a autocrine/paracrine fashion, with a predominant role compared to other growth factors [2,12,13].

Glial cells, especially astrocytes, are now considered to be involved in the regulation of many neuron functions [14-16]. In fact, normal brain function is based on neuron activity, which is regulated by glial cells. These functions involve regulation of synaptic neurotransmitters or ion concentrations, providing fuel to neurons, or control of neurogenesis and synaptogenesis [17,18]. In addition, the astrocyte calcium wave may be considered as a "parallel" electric signal, which can allow cross-talk between astrocytes themselves and between astrocytes and neurons [19]. Dysfunctions of glia are involved in many neuropathologies, in fact, when it comes to brain malignant tumours such as gliomas, cancer-dependent glial malfunctions have some deleterious consequences on brain function.

This present work reports results relevant to application of anti-gene IGF-I strategy in phase I clinical trial of glioblastoma patients.

Current treatment options for patients with advanced malignant tumors are limited in efficacy, therefore the search for new strategies such as chemotherapy [20], use of inhibitors, including antibodies, antisense oligonucleotides, short peptides and other small molecules [5, 21,22], and more specifically, cellular immune therapy [23] constitutes a current challenge. Immunogene therapy of cancer was introduced as a new domain of oncology by Trojan et al in 1992/3 [24].

Anti-gene, antisense or triple helix approach [25,26], has proven to stop the development of established animal tumors of glioma, hepatoma, melanoma and teratocarcinoma (containing three tissue derivatives). Moreover, we have signaled anti-gene therapy as a useful treatment in 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) [5,27-29].

The principal goal of this work - Phase I of cancer gene therapy - was to compare the clinical results obtained using anti gene therapy applied in three different tumors, principally neuroectodermal glioblastoma, and endodermal liver and colon cancers [5,30,31]. We expose the mechanism of this therapy based on the results of previously described studies: the immune anti-tumor phenomenon observed in the antisense anti IGF-I treatment of rat and human gliomas and signaled by the increase of CTL CD8^{+} in the tumor tissue as in peripheral blood lymphocytes [5]. In the presented work, we have used the strategy of combined antisense/ triple helix technologies to prepare the anti-gene IGF-I "vaccines" and investigate an immune response in treated patients with malignant tumors expressing IGF-I.

2. Material and methods

The IGF-I antisense strategy suppressing IGF-I expression was applied as previously

described [27,32,33], essentially with the use of episomal based vectors expressing either IGF-I RNA antisense or expressing 23 bp RNA forming the triple helix with IGF-I DNA [34].

The established cancer cells were originated from biopsies of patients with malignant glioma (glioblastoma multiforme), hepatocarcinoma and colon adenocarcinoma (University Hospital of Bromberg, Poland). Cell lines were established during 3-4 weeks in culture with conventional protocol in DMEM (Gibco-BRL, Invitrogen, UK) supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 micrograms/ml streptomycin at 37°C and 5% CO_2. Established primary cells were transfected by Ca++/Ph or comparatively with FuGENE 6 transfection reagent technique (Boehringer Mannheim, Ingelheim, Germany). 48 hours after transfection, hygromycin B was added up to 150ug/ml. Two weeks after transfection, hygromycin resistant cells were verified for expression of IGF-I by immunohistochemical [32,33] but also by RT-PCR (reverse transcriptase polymerase chain reaction) using RNA isolation kit from Roche Diagnostics (Basel, Switzerland) and application according to Reverse Transcription System from Promega Corporation (Charbonnières-les-Bains, France) with the following primers: forward, GCATCTCTTCTACCTGGCGCTG; reverse, CAGGCTTGAGGGGTGCGCAATA.

The established transfected cell lines were examined in flow cytometry with fluorescence-activated cell sorter (FACS) equipment FACSCAN and/or FACScalibur (Becton Dickinson, Le Pont-De-Claix, France), using specific monoclonal antibodies (mAbs) to major histocompatibility complex (MHC) I and II, CD80, CD86, namely W632, BU26, MEM-233 and BU63, respectively (AbD Serotec, Düsseldorf, Germany). The secondary antibody used was

Goat F(ab')2 anti-mouse Ig conjugated to FITC (fluorescein isothiocyanate) (Tebu, Le Perray-en-Yvelines, France). Thus, IGF-I antisense and triple helix transfected cells that were positive in expressing MHC-I were submitted to 5000 CGy gamma irradiation with 60Co or 137Cs (following the indications of Ethical Committee of NIH, no1602).

The clinical trial of immunogene therapy concerned two patients with glioblastoma (astrocytoma IV), two patients with primary hepatocarcinoma and two with primary colon adenocarcinoma. The inclusion criteria were as follows: age > 17 years; clinical data of glioblastoma, colon and liver cancers and radiography (TAC vs RNM); stage I of cancer; histologic confirmation of glioblastoma multiforme, colon adenocarcinoma and hepatocarcinoma; possibility of total resection in tumoral recidiv; Karnofsky > 60% or Zubrod ECOG (Eastern Cooperative Oncology Group) <2; haematologic results in agreement with surgery conditions; hope of life - superior than 3 months; no VIH. The exclusion criteria concerned: multifocal localisation of tumor or tumoral dissemination; pregnant women; Karnofsky – less than 40%; missing letter of agreement; in the case of glioblastoma: 1. subependima or leptomeningea; 2. comunication between ventrículum and cavity of resection (without possibilty of reparation using implant); 3. increase of intracranial pression due to paliative treatment or as to complication;

Every treated cancer was of stage I. Patients received 2 successive subcutaneous injections of irradiated anti - gene IGF-I (antisense / triple helix, 50:50) whole cell preparations, of 1 million transfected cells. All vaccine preparations were injected subcutaneously, generally into the left arm of cancer patients. The control clinical trial consisted also of three cancer groups, with stage I. In every cancer group were two

patients, of comparative age. The control patients received the injection of placebo (physiologic solution).

Blood samples were collected before and after vaccinations which corresponded to 2-3 weeks after the vaccine injections. PBMCs were isolated from blood red cells using ficoll-hypaque centrifugation. Flow cytometry analyses were performed as above but using mAbs conjugated to FITC and directed to cell surface markers, namely CD8. Particularly, dual staining were performed using mAbs specific to CD8-FITC and CD11b-PE (phycoerythrin), CD8-FITC and CD11b+PE. The surface markers CD3, CD4, CD19, CD56 were also explored.

Table 1. Flow cytometric 'FACS' peripheral blood lymphocyte CD marker patterns following cellular immunogene therapy in human cancers: glioblastoma multiforme, hepatocarcinoma and colon adenocarcinoma. The time of established cell line as well as the time of established transfected cell line (vaccine) are mentioned in weeks. CD molecules were labelled in peripheral blood lymphocytes (PBL) obtained from pre-vaccinated patients (C – control) and after two successive vaccinations (V1 and V2) in the same cancer patients. Two cases of each of the designated cancers were examined. Flow cytometry analysis data are expressed as percent of positive cells when compared to the isotype control. Differences in percentage of CD8+ CD11b- and CD8+ CD11b- subpopulations before and after vaccinations were strongly significant. See also figure 2.

Name of patient	Age	Type of cancer	Established line	Transfected line	PBL cells CD8+ CD11b+	PBL cells CD8+ CD11b-
B.P.	67	glioblastoma	4 weeks	4 weeks	C-23% V1-21%, V2-20%	C-14% V1-24% V2-29%
A.W.	66	glioblastoma	4 weeks	4 weeks	C-24% V1-21%, V2-19%	C-15% V1-23% V2-27%
A.B.	45	hepatocarcinoma	4 weeks	4 weeks	C-24% V1-21%, V221%	C-14% V1-23% V2-24%
H.T.	69	hepatocarcinoma	5 weeks	5 weeks	C-25% V1-22% V2-21%	C-13% V1-24% V2-25%
A.M.	56	colon adenocarcinoma	5 weeks	5 weeks	C-26% V1-25% V2-21%	C-10% V1-25% V2-30%
S.K.	48	colon adenocarcinoma	4 weeks	4 weeks	C- 27% V1-25 % V2- 22%	C-11% V1-24% V2-29%

3.   Results

It was observed in the group of patients with glioblastoma that the two control patients without complement immunogene therapy have only survived 9,5 and 10 months respectively while the two other patients receiving injections of cell prepara-

tions had prolonged survival up to 19 and 24 months respectively. The case relevant to the vaccinated patients with liver and colon cancers, was monitoring onward. All treated patients presented a slight increase of temperature 24-36 hours after vaccination that lasted a couple of days. A clear-cut change was observed concerning PBMCs' cell surface expression of vaccinated patients essentially relevant to the CD8+ cell subpopulation: CD8+CD11b-, which showed a significant increase in its percentage after vaccination (Table 1, and Figure 1 a, b, c). No difference was noticed about cell populations with or CD8+ CD11b+ in PBMCs harvested before and after vaccination. The interesting observation in progress concerned the PBMC subpopulation with CD8+CD28+ phenotype in vaccinated patients. The increased percentage of these cells might indicate a stimulation of immune effectors in patients vaccinated with anti - gene IGF-I modified tumor cells and might thus explain their improved survival time.

The vaccinated glioblastoma patients had a prolonged survival time (19 and 24 months, respectively) compared to the range of 12-14 months in conventional therapy. For this reason, admitting that the group of glioblastoma patients treated with antisense/triple helix cell injection has given the significant results, all liver and colon cancer patients were treated, after surgery and radiotherapy with this type of therapy. Moreover, the period of 19 months, was chosen as the end of clinical observations in all treated cancer patients. At 19 months, all liver and colon cancer patients were alive and the treatments were well tolerated (we do not include the details of clinical observations concerning treated cancers, because it is not the subject of this work).

4.   Discussion

Glioblastoma, as well as other CNS malignancies [14-16, 35], were recently successfully treated by antisense therapy targeting TGF beta, using either antisense anti TGF beta expressing vector [36] or applying oligodeoxynucleotides [37,38]. Using phosphorothioate TFG beta2 antisense oligonucleotides (AP-12009), an international phase II/III study was initiated in patients with TGF beta-overexpressing tumours 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. By 2007, overall survival was 24 months, and in the control group, survival was 20 months [37, 39]. Results from the clinical trials concerning other tumours 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 tumour diseases [37].

In anti-gene anti IGF-I approach, we have applied both antisense and triple helix technologies, stopping simultaneously the expression of IGF-I on translation and transcription levels [5]. Moreover, *in vivo* AS IGF-I approach was also developed [40]; 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 tumour recurrence – from 62 to 20%.
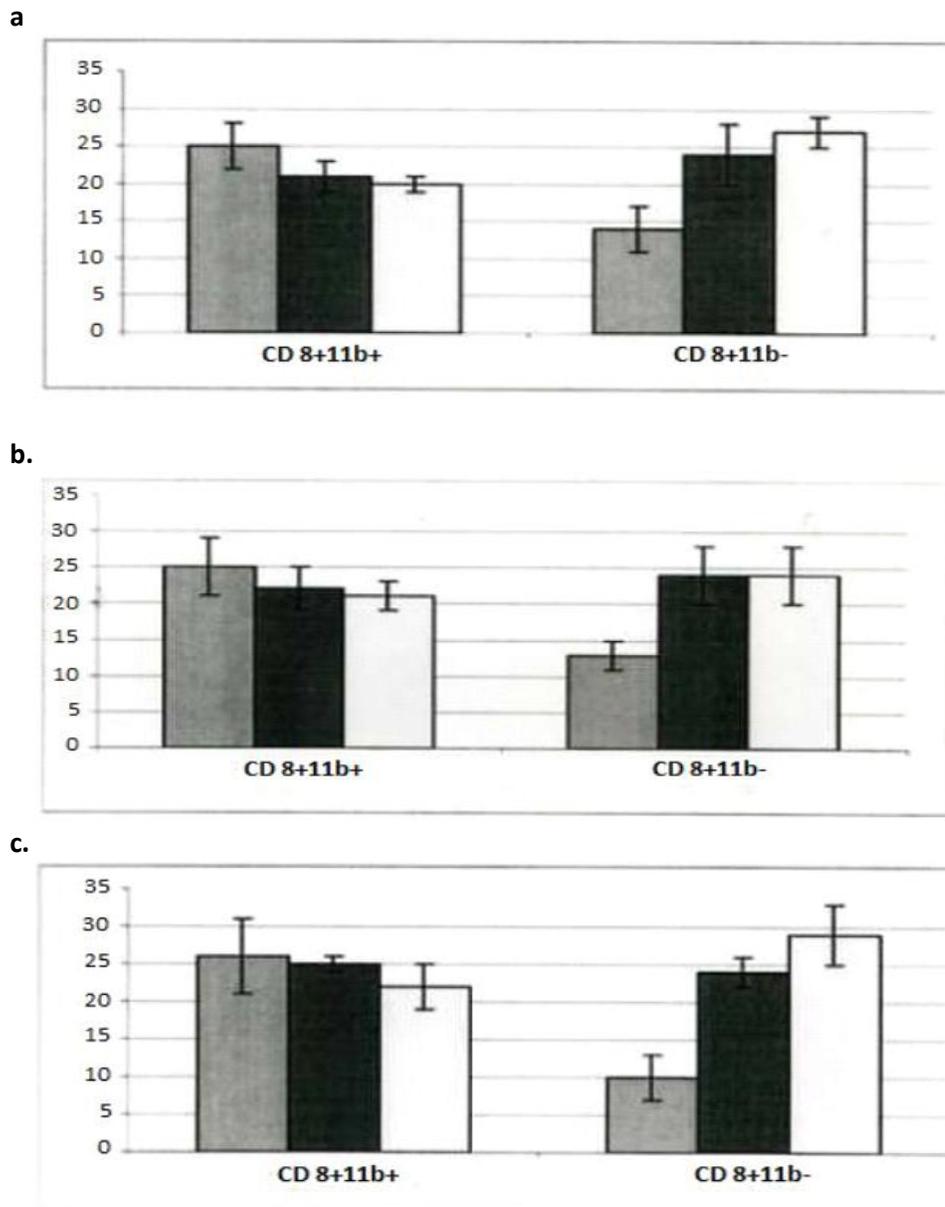
Figure 1 a, b, c

Figure 1 a, b, c.   Flow cytometric ("FACS") peripheral blood lymphocyte CD marker patterns following cellular gene therapy in human cancers. a – glioblastoma multiforme; b – hepatocarci-noma; c - colon adenocarcinoma. 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 first and second 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. Differences in percentage of CD8+CD11b- and CD8+CD11+ subpopulations before and after vaccination were strongly significant (with a range of p below 0.01) and are illustrated in the bar graph for statistical significance. (The original FACS data concerning PBL cells are in the archives of Collegium Medicum of Nicolas Copernic University, Bromberg, Poland).

After here described 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 (Table 1, and Figure 2a).   The work in progress has also shown in different treated tumours 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 [5].

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-tumour response involving APC activating CD8+ T cells [5,28, 32,41,42] (Figure 2a).

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 compensatory 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 [33]. Indeed, the increase of IGF-I receptor level could explain the expression of B7. There is a relationship 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 [43]. 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 [14,44].

The immunogene therapy represents a novel approach for cancer therapy. The understanding of molecular biology of cancer cells has progressively helped to identify the different molecular pathways altered in various cancers. 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, TGF beta, CSF and especially IGF-I, whose receptor, IGF-I-R, plays a principal role in the tumor growth process [3,4,5] (Figure 2b).

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 transfected AS GS cells were also immunogenic (MHC-I expression) [48,49]. 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 tumour 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 GF-I-R can be inappropriate or exaggerated [4]. 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) [3,5]. The final result 3,5of 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 [50].

Our presented work has permitted to start Phase I and II in South America (Colombia). This way we have established criteria for selection of vaccines (expression of IGF-I, MHC-I, B7) and of PBL cells markers (CD 8+ related molecules) in patients presenting the arrest of growing tumors.
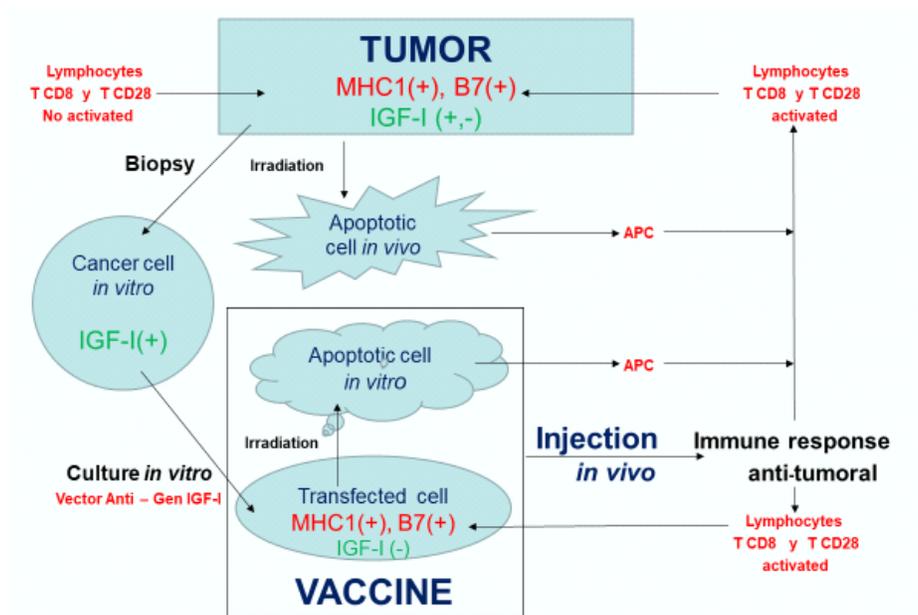
Figure 2a. Schema of cancer immunogene therapy. The cells isolated from tumor biopsy are growing in tissue culture. The established cell line is transfected by vector of anti -gene type (antisense and triple helix IGF-I). The transfected cells, and originated apoptotic cells, are injected in proportion 50-50 ('vaccine') in the cancer patients. The presence of MHC-I and B7 molecules present in tissues and cells, as well as induced immune response mediated by lymphocyte and APC cells participate in shown immunogene mechanism.
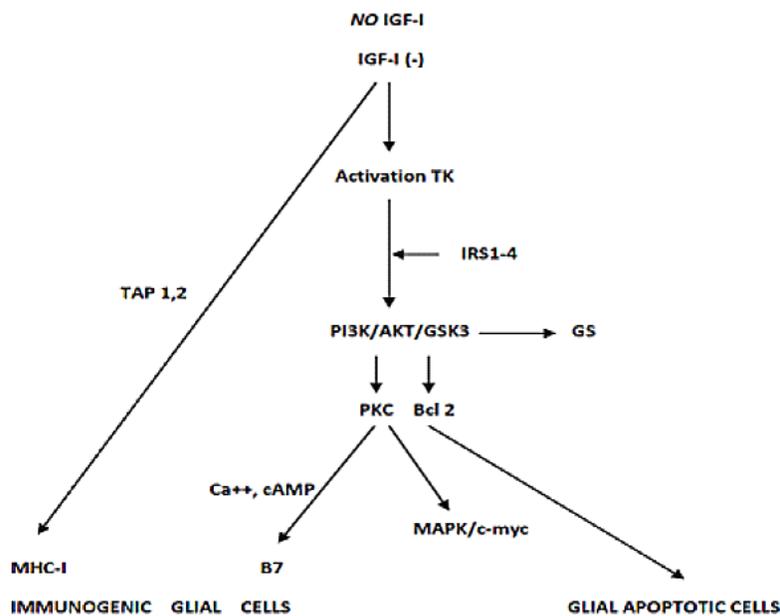

Figure 2b. Schema: arrest of IGF-I in anti – gene IGF-I transfected glial cells. The cells stop to express IGF-I but they become immunogenic expressing MHC-I and B7. One part of transfected cells enters in apoptosis [5,43,45-47]. Abbreviations: TAP 1,2 (transporter associated with antigen processing antigen); TK (tyrosine kinase); PI3K (phosphatidyinositol 3 kinase); PDK1 (phosphoinositide-dependent kinase 1); AKT (PKB, protein kinase B); Bcl 2 (key molecule of apoptose); GSK3 (glycogene synthetase kinase 3); GS (glycogene synthetase); PKC (protein kinase C).

5.   Agreements of Ethical Committees

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.

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´s University, 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).

6.   Acknowledgements

7.   Conflict of interest

No conflict of interest.

8.   References

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