IGF-I triple helix gene therapy of rat and human gliomas

Trojan LA[1,2], Kopinski P[2,4,5], Mazurek A[3], Chyczewski L[6], Ly A[4], Jarocki P[2,5], Nikliński J[7], Shevelev A[4], Trzos R[5], Pan Y[1], Gitis DJ[5], Bierwagen M[2], Czapiewska JL[2], Wei MX[2], Michalkiewicz J[2], Henin D[4], Popiela T[5], Evrard F[4], Kasprzak H[2], Anthony D[1], Trojan J[2,4,5]

[1] School of Medicine, Case Western Reserve University, Cleveland, USA;
[2] Medical University and University Hospital, Bydgoszcz, Poland; [3] Department of Pathophysiology of Pregnancy, Medical University of Białystok, Poland; [4] INSERM, Robert Debre and Bichat Hospitals, France;
[5] CMUJ and University Hospital, and Polish Academy of Sciences, Cracow, Poland; [6] Department of Clinical Molecular Biology, Medical University of Białystok, Poland; [7] Department of Thoracic Surgery, Medical University of Białystok, Poland.

Abstract

Purpose: **IGF-I anti-gene technology was applied in treatment of rat and human gliomas using IGF-I triple helix approach.**

Material and methods: **CNS-1 rat glioma cell and primary human glioblastoma cell lines established from surgically removed glioblastomas multiforme were transfected in vitro with IGF-I antisense (pMT-Anti-IGF-I) or IGF-I triple helix (pMT-AG-TH) expression vectors. The transfected cells were examined for immunogenicity (immunocytochemistry and flow cytometry analysis) and apoptosis phenomena (electron microscopy). $3x10^6$ transfected cells were inoculated subcutaneously either into transgenic Lewis rats or in patients with glioblastoma. The peripheral blood lymphocytes (PBL) derived from "vaccinated" patients were immunophenotyped for the set of CD antigens (CD4, CD8 etc).**

Results: **Using immunocytochemistry and Northern blot techniques, the transfected "antisense" and "triple-helix" cells showed total inhibition of IGF. Transfected cultures were positively stained either for both MHC-I and B7 antigens – 60% of cloned lines, or for MHC-I only – 40% of cloned lines. Moreover "triple helix" cells as compared to "antisense" cells showed slightly higher expression of MHC-I or B7. Transfected cells also showed the feature of apoptosis in 60%-70% of cells. In in vivo experiments with rats bearing tumors, the injection of "triple helix" cells expressing both MHC-I and B7 interrupted tumor growth in 80% of cases. In contrast, transfected cells expressing only MHC-I stopped development in 30% of tumors. In five patients with surgically resected glioblastoma who were inoculated with "triple helix" cells, PBL showed an increased percentage of CD4+CD25+ and CD8+CD11b- cells, following two vaccinations.**

Conclusions: **The anti-tumor effectiveness of IGF-I anti-gene technology may be related to both MHC-I and B7 expression in cells used for therapy. The IGF-I anti-gene therapy of human glioblastoma multiforme increases immune response of treated patients.**

Key words: glioma, IGF-I, triple helix, immuno-gene therapy.

ADDRESS FOR CORRESPONDENCE:
Jerzy Trojan
INSERM E9935, Dept. Neurology
Robert Debre Hospital
48, bd Serurier; 75019 Paris, France
e-mail: jerzytrojan@wanadoo.fr

Received 3.07.2003 Accepted 12.07.2003

Introduction

Insulin-like growth factor-I, IGF-I, is a 70-amino acid polypeptide [1-4] involved in the growth and tissue differentiation [3,5-7]. IGF-I and the related polipeptides IGFs are expressed in many tissues, including brain, where they are involved in proliferation of neuronal and glial precursors [8]. Deregulated expression of IGF-I, is associated with growth as well as with pathology of different diseases, including tumors [5,7,9,10]. IGF-I and -II are expressed at high levels in some nervous system-derived tumors, e.g., astrocytomas and meningiomas [11] or tumor cell lines [12,13]. In contrast, the block of IGF-I synthesis, induces apoptotic and immunogenic phenomena [14].

Classically, in order to assign a functional role of a particular gene product, one relies on naturally occurring mutants that fail to express a particular gene in normal fashion. The in vitro generation of cellular mutants has limited applicability due to the diploid nature of most genes and to the lack of adequate mutant selection. Our experimental approach in this study centers on the use of IGF-I RNA-DNA triple helix [15,16] presumed to stop the transcription of the IGF-I gene and to produce phenocopies of a null mutation for IGF-I. Triple helix strategy [17,18] and comparatively used antisense strategy [19-21] bypasses inherent limitations of functional studies dependent upon natural mutant cells or artificially mutagenized cells [22].

According to antisense concept, a nucleotide sequence is expressed in cells that complementary to a portion of the native RNA transcript (target sequence) of the gene of interest. Binding of the antisense disrupts the function of the target sequence either by blocking its function, promoting its degradation or by altering the structure of the target sequence.

Antisense strategy – i.e. the experimental inhibition of gene expression by introducing a source of nucleotide sequences complementary to a given endogenous mRNA has been applied successfully to a growing number of genes in cultured cells. Growth of transformed human astrocytes was inhibited by antisense to basic fibroblast growth factor [23]. Antisense inhibition of glial S 100 protein altered cell proliferation and cytoskeletal organization [24].

However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression [25]. Ineffective antisense RNA-mediated inhibition of gene expression, is probably a consequence of insufficient antisense RNA levels and inadequate suppression of the encoded protein product due to integrating vectors. These vectors used for most antisense experiments to date, integrate randomly into the host chromosome and are subject to local regulatory effects exerted by neighboring sequences at sites of integration. Hence, transfected cells may at times express only limited amounts of antisense RNA.

We have applied the antisense strategy by employing a self-amplifying episomal vector that replicates to high copy numbers extra-chromosomally [26]. It was showed that such a vector can be used in human T-cell clones for effective (>95%) inhibition of the T lymphocyte surface molecule CD8 [27]. The utility of episome-based expression vectors for the effective inhibition of cellular RNA expression has been subsequently confirmed by others, e.g., N-myc in primitive human neuroectodermal cell lines [28].

Johnson et al. [29] and Kiess et al. [12] previously reported that the rat glioma cell line C6 expresses high levels of IGF-I. IGF-I expression is enhanced in these tumor cells when they are grown in serum-free medium.

We have used C6 glioma cell lines as model in which to study antisense and triple helix strategies of treatement and to define a role for IGF-I in the tumorigenesis of gliomas that involves evasion of immune surveillance. IGF-I transfectants that express antisense RNA to IGF-I were shown to elicit a curative anti-tumor immune response with tumor regression at sites distant from the sites of injection of the transfectants [10,15]. We previously showed that C6 rat glioma cells expressed MHC-I [30,31] and B7 [32-34] antigens when transfected with vectors producing IGF-I antisense RNA or inducing IGF-I triple helix RNA-DNA [35,36]. Moreover, the cells lost tumorigenicity and were able to induce a T-cell mediated immune response in syngeneic animals against both themselves and the non transfected tumorigenic parental cells [6,10]. Using the IGF-I antisense and triple helix approaches we recently demonstrated that transfected C6 cells become pro-apoptotic [36]. Unfortunately, the syngeneic model of rat DBIX permitting to study C6 cells in vivo is not available. For this reason, one of the aims of the present experiment was to verify the results we obtained in the syngeneic model of Lewis rat/CNS-1 glioma cancer (see *Fig. 1*), and to determine whether or not IGF-I triple-helix blockade of IGF-I syntheses changes phenotype of transfected CNS-1 cells. Moreover, we sought to determine if the change in immunogenicity is accompanied by apoptosis in this model system and if the expression of both antigens, MHC-1 and B7 in transfected cells used for injection is necessary to stop the growth of establish rat tumors. The injection of human immunogenic glioma cells should be followed by cellular immune response shown in the patients' blood. The experiment described here using a rat glioma syngeneic model permitted us to prepare human "vaccine" and introduce a Phase 1 clinical trial (treatment of five patients with glioblastoma multiforme).

Material and methods

Cell culture

The CNS-1 rat cell line was used for experiments. The cell line was offered by Dr William Hickey of the Dartmouth Medical School, Hanover, NH, USA. The cell line was cultivated in the laboratory of INSERM by Dr M. Sanson (Salpetrier Hospital, Paris) and then subcloned. Cells were cultured in DMEM (GIBCO-BRL) supplemented with 10 % FCS, 2 mM glutamine, 100 U/ml penicillin and 100 microg/ml streptomycin, at 37°C and 5% CO_2. Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml was added 48 hours after transfection to select for transfected cells. After one week, concentration of hygromycin B was changed to 0.15 mg/ml and maintained with each change of fresh medium over the next 3-4 month.

Primary cell cultures of human glioma were derived from tumors of 5 glioblastoma multiforme patients during surgical resection in the University Hospital of Bydgoszcz, Poland. Cell lines were established according to the technique described earlier [36-39].

Histology

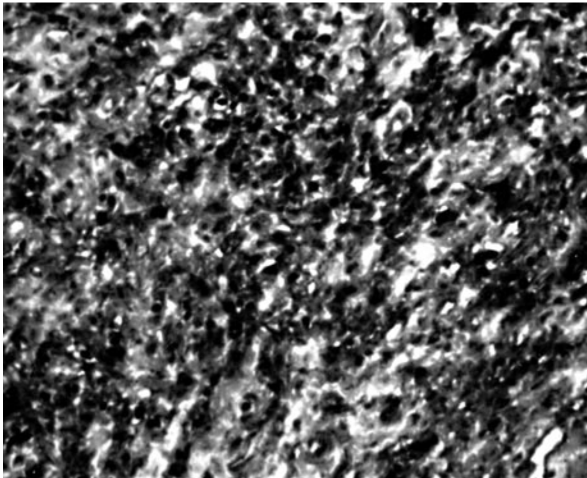
Male Lewis rats, 7 week-old male, were provided from CERJ, Lyon, France. Rats were inoculated subcutaneusly with 5 mln cloned CNS-1 cells expressing IGF-I. The removed tumors were fixed in 4% para-formaldehyde, and paraffin embedded sections were stained for IGF-I by immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) using polyclonal antibodies to IGF-I (Valbiotech, Paris, France) (*Fig. 1*).

Plasmids

The vector pMT-EP [6,26] was prepared in The School of Medicine, Case Western Reserve University (Joseph Ilan Laboratory) (*Fig. 2*). IGF-I "antisense" and "triple helix" technology was used (*Fig. 3*) to construct episome based plasmids expressing either IGF-I RNA antisense, pMT-Anti IGF-I [26], or IGF-I triple helix inducing vector, pMT-AG TH [15]. The pMT-EP is under control of the metallothionein, MT-I, inducible promotor. The casette contains the Epstein-Barr Virus origin of replication and the gene encoding nuclear antigen I which together drive extrachromosomal replication. Down stream of the insertion site is a poly A termination signal followed by the hygromycin B and ampilicin resistance genes (*Fig. 2*).

In pMT-AG triple helix the cassette consists of a 23 bp DNA fragment cloned into the vector pMT-EP which tran-

Figure 1. Histology. IGF-I immunoperoxidase staining of CNS1 rat glioma [55] resulting from subcutaneous injection of cloned cells into Lewis rat (counterstaining with hematoxyline). (X 200)



scribes a third RNA strand forming a triple helix structure within the target region of the human IGF-I gene, between its transcription and translation initiation sites. The vector pMT-EP containing cDNA expressing IGF-II antisense RNA as insert was used in control experiments [6].

Transfection

Cells, 60-80% confluent, were transfected in 6-well plates in a ratio of 1 ug of plasmid per 3-4 x 10^5 cells. The FuGENE 6 Transfection Reagent (Boehringer Mannheim) was used according to the supplier's instructions (3 μl of Reagent per 1 ug DNA). To determine the efficiency of transfection, the process was carried out using pMT-EP construct containing lac-Z as a reporter gene. Cell cultures were washed in PBS and incubated at 37°C in the presence of the staining solution which contained 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.8 mg/ml X-gal made in PBS.

Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml was added 48 hours after transfection to select for transfected cells. After one week, concentration of hygromycin B was changed to 0.10 mg/ml and maintained with each change of fresh medium over the next 3-4 month.

Parental, non transfected cells and transfected cells were verified for IGF-I presence using antibodies to IGF-I by immunoperoxidase technique (*Fig. 4*).

Northern blot

Content of IGF-I antisense RNA was determined in 50% confluent cell cultures. Cells were deprived of serum and cultured overnight in DMEM containing 0.1% BSA; 60μM $ZnSO_4$ (Sigma) was then added for 5 hours to induce the MTI promoter. The cells were then prepared for Northern blot as described earlier [37]. Labeling of rat and human IGF-I cDNA and chicken beta actin cDNA and hybridizations were done according to Maniatis and procedures previously described [37]; the 770 bp human IGF-I cDNA and 500 bp rat IGF-I cDNA used as probes were a gift from J. Ilan (CWRU,

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.

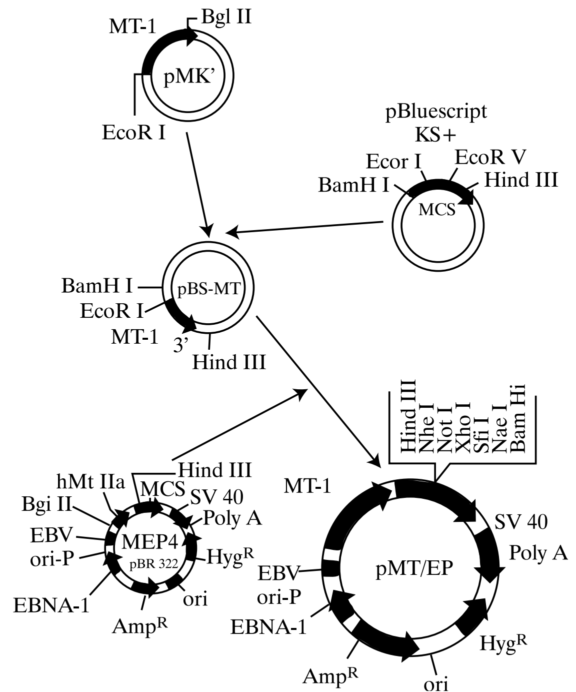
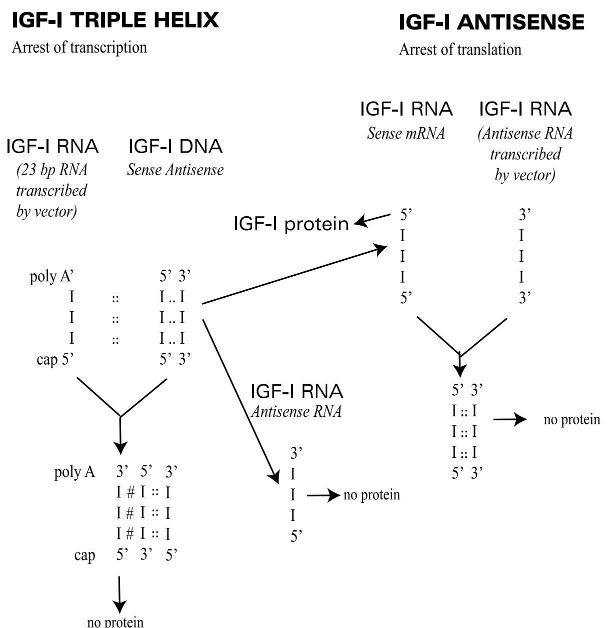
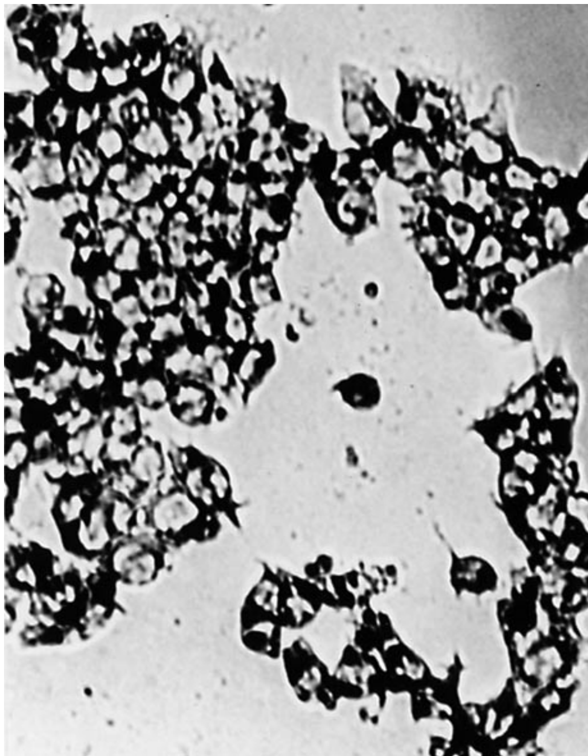


Figure 3. Schema of IGF-I antisense and IGF-I triple helix IGF-I technology. In antisense technology the end result is the inhibition of IGF-I mRNA (sense RNA) activity by binding to the antisense RNA [15]. In IGF-I triple helix technology the oligopurine third strand (23 bp) prepared by PCR forms RNA-DNA triple helix with IGF-I gene [16,36].



Homopurine triple helix is formed in 'in vitro' experiment (oligopurine strand poly A 3' GAG…GAG 5' cap) and DNA of IGF- I). We admit that similar mechanism occurs in 'in vivo' studies. # Hoogsteen hydrogen bonds; :: Watson-Crick bonds.

Figure 4. IGF-I immunoperoxidase staining of CNS-1 rat glioma cells. These cells after transfection with IGF-I antisense or IGF-I triple helix expression vectors lose their capacity to synthetize IGF-I (see also *Fig. 5*) (X 200).



Cleveland). The Northern blot was used also to verify expression of IGF-I in solid glioblastomas.

Immunocytochemical stainning

Localization of IGF-I and other antigens was done by immunofluorescence technique [6,10]. Cells were fixed in 4% methanol. Antibodies to rat MHC-I (OX-18) (from Valbiotech, Paris, France) and antibodies to mouse MHC-I (5 F1.3, anti Kb; from ATCC, Bethesda, MD, USA) gave the same staining. To stain B-7, a fusion protein CTLA4-Ig was used (Bristol Myers Squib, Seattle, WA, USA) [35].

Flow cytometric analysis

Cells were washed in PBS and incubated (30 min., 4^0C) with saturated amounts of monoclonal antibodies, labeling rat or human MHC-I (HLA ABC), MHC-II, CD80 and CD86 antigens (Becton Dickinson Pharmingen, direct immunostaining). Paraformaldehyde-fixed cells were collected (10.000 events per sample) in FACScan BD cytometer. Data were presented as percentage of positive cells. The human or rat glioma cells examined by flow cytometry were tested in two parallel groups of samples: one group of non irradiated cells; another group after irradiation with ^{60}Co, 5000 rads.

Electron microscopy

Cells were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.l M phosphate buffer, pH 7.2. The fixed cells were then rinsed in 0.1 M phosphate buffer (3x) followed by a post-fixation in phosphate – buffered 1% OsO_4. After rinsing in distilled water for 20 min, the cells were stained with 2% uranyl acetate for 40 min at 60°C. They were then rinsed in distilled water (3x), dehydrated in progressive concentration of ethanol and 100% propylene oxide and embedded in Epon resin. 80-nm sections were cut, placed on 200 mesh copper grids, and contrasted with lead citrate and uranyl acetate. Photomicrographs were taken with a Philips EM-2000 electron microscope operating at 80 kV.

In vivo experiment

For determination of tumorigenicity 5 mln CNS-1 rat cells were injected subcutaneously into Lewis rats. Animals were separated into groups of eight. Experimental sets were injected with: a) parental cells; b) IGF-I «triple-helix» transfected cells expressing MHC-I; c) IGF-I «triple-helix» transfected cells expressing both MHC-I and B-7 molecules.

Vaccination of glioblastoma patients

Human glioma cell lines were transfected with the pMT–AG TH plasmid vector. Clones of transfected cells (down-regulated for IGF-I and expressing MHC-I and B-7 molecules) were selected two months after day of transfection. Before injection, cells were irradiated as described previously and 5 mln cells were injected subcutaneously into the left arm of operated glioblastoma patients. Blood was collected before the first vaccination, and again 3 weeks after the first and the second injection. Peripheral blood lymphocyte (PBL) typing was performed using mouse monoclonal antibodies directed against the superficial cell antigens (*Tab. 1*).

Results

Parental rat glioma CNS-1 line was subcloned using cloning cylinders. The subcloned cell line expressing IGF-I (*Fig. 1*) was used for in vitro transfection and for in vivo injection experiments. Endogenous IGF-I was down-regulated, when CNS-1 cells were stably transfected with either IGF-I “antisense” or “triple helix” vector and grown in presence of Zn^{2+}, in order to activate the MT-I promoter. Transfected cells examined by northern blot analysis show the IGF-I RNA in “antisense” orientation (*Fig. 5*). The RNA of non-transfected cells is distributed in 7.5 kb and l.0 kb bands corresponding to the different processing stages of endogenous IGF-I RNA (lane a). The RNA of pMT-Anti IGF-I transfected cells shows only an abundant 1. 0 kb band (lane b). As expected, RNA of pMT-AG triple helix transfected cells shows no band when rat IGF-I cDNA is used as probe (lane c). The internal control to this experiment was the beta actine bands (*Fig. 5*).

IGF-I «triple helix» or «antisense» cells were up-regulated in expression of MHC-I and B-7 molecules as measured by flow cytometry and immunocytochemical staining (*Fig. 6A, B*). Transfection with either pMT-EP «empty vector» or with vector expressing IGF-II antisense RNA [6] in place of IGF-I antisense RNA, produced no increase in MHC-I or B-7 molecules. The transfected cultures were positively stained either for both MHC-I and B-7 antigens (in 60% of cloned lines), or for MHC-I (only in 40% of cloned lines). The data show that trans-

Table 1. Peripheral blood lymphocyte (PBL) typing. Monoclonal antibodies (MoAb) used for direct immunofluorescence. FL1 and FL2 indicate standard fluorescence channels of flow cytometer, FITC, fluoresceine isothiocyanate, PE, phycoerythrin.

Sample no	FL 1 MoAb FITC conjugated	FL 2 MoAb PE conjugated	Notes
1	CD45	CD14	PBL gate defining, contamination of monocytes
2	CD4	CD8	CD4/CD8 ratio
3	CD3	CD16+CD56	T & NK cells
4, 5	CD25	CD4 (CD8)	Activated Th (Tc) cells – Th (Tc) cells positive with interleukine-2 receptor
6, 7	CD45RO	CD4(CD8)	Th (Tc) memory cells
8	CD19	CD5	B cell subsets (T dependent and T independent B cells)
9	CD8	CD11 b	Tc & Ts cells
10	control antibody (IgG-1)	control antibody (IgG-2a)	Negative isotype control

Table 2. Expression of IGF-I, MHC-I and B-7 in “antisense” and “triple helix” transfected cells*.

Cells	Rat CNS-1 glioma			Human primary glioma		
	IGF-I	MHC-I	B-7	IGF-I	MHC-I	B-7
Non transfected	++	<0.5	<0.5	++	<0.5	<0.5
IGF-I antisense	--	12.5	18.0	--	9.0	11.0
IGF-I triple helix	--	14.5	19.5	--	10.0	12.5
IGF-II antisense	++	<0.5	<0.5	++	<0.5	<0.5

* Cells were analyzed by immunocytochemistry for IGF-I (ABC method with immunoperoxidase staining), and by flow cytometry (FACScan Becton Dickinson) for MHC-I and B7. The data of flow cytometry are presented as percent change in value of fluorescence relative to fluorescence in control non transfected cells. The average values of three experiments are shown; the increase in MHC-I and B7 expression in antisense and triple helix transfected cells are significant at the $P<0.01$ level (Wilcoxon’s signed rank test).

fection with pMT-Anti IGF-I and pMT-AG triple helix vectors induced a significant increase in expression of MHC-I and B-7 (*Tab. 2*). The “triple helix” rat and human cells as compared to “antisense” cells showed slightly higher expression of MHC-I or B7: i.e. transfection of rat glioma with either the pMT-Anti IGF-I or pMT-AG triple helix vector induced mean increases of 12,5% and 14,5% in the expression of MHC-I, respectively.

Apoptosis induced morphological changes in pMT-Anti-IGF-I and pMT-AG triple-helix transfected cells were detected using electron microscopy (*Fig. 7A,B*). The apoptotic changes occurred within 5-6 hours after incubation of IGF-I «triple-helix» or «antisense» transfected cells in presence of 55-60 μM $ZnSO_4$. Apoptosis was also analyzed in culture cell population using the May-Grünwald-Giemsa and Hematoxylin/Eosine stainings. Apoptosis was detected in approximately 70% of the IGF-I antisense and triple helix transfected cells. In contrast, evidence of apoptosis was observed only in 3-5% of the non-transfected cells. These results, confirmed in three separate experiments, are in agreement with electron micrographies images of transfected IGF-I «antisense» and «triple-helix» cells.

When 5 mln of CNS cells were injected subcutaneously into Lewis rats, tumor was observed in 6-10 days. In contrast, no tumors were observed in animals injected subcutaneously with

Figure 5. Northern blot analysis of rat CNS-1 glioma cell line: lane a – parental cells showing two bands; lane b – IGF-I antisense transfected cells showing one band; lane c – IGF-I triple helix transfected cells presenting negative blocks. Beta-actin was used as the control (lowest band).

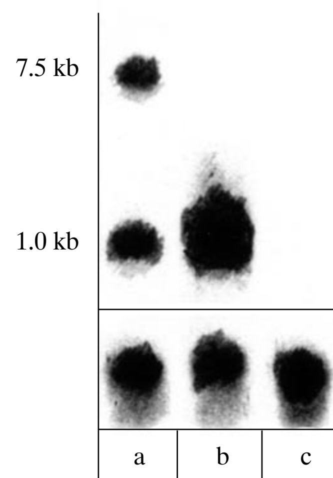


Figure 6. Immunofluorescence localization of MHC-I *A)* and B-7 *B)* in IGF-I "triple helix" transfected rat glioma cells using polyclonal antibodies (X400).

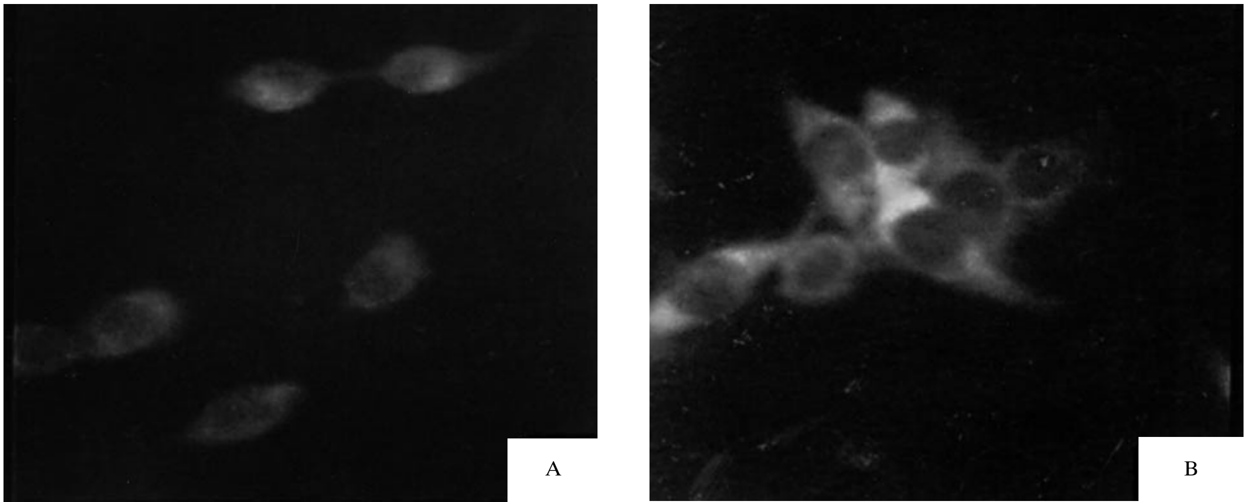
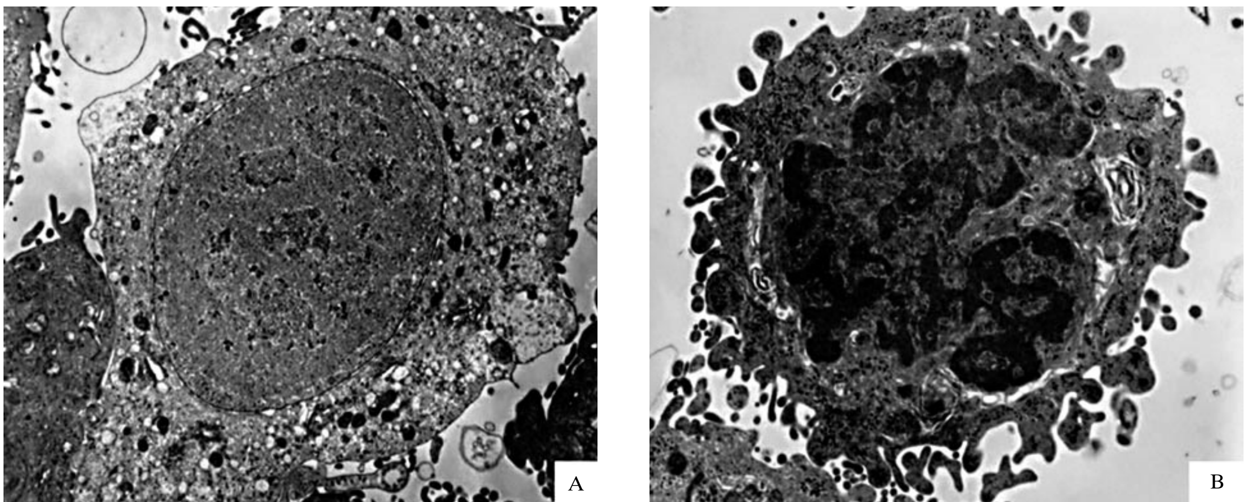


Figure 7. Ultrastructure characteristics of CNS-1 rat glioma cells: (a) parental non-transfected cells; (b) IGF-I triple-helix transfected cells. a) Parental cell with a nucleus which is uniform and well separated from cytoplasm. b) Transfected cell has characteristics of apoptotic cell with distorted nucleus and condensed electron dense chromatin (original magnification X7000).



5×10^6 CNS-1 cells stable transfected with IGF-I «triple-helix» vector, and expressing either MHC-I or both MHC-I and B-7. All eight of these mice remained tumor free for 8-10 months. The efficiency of triple helix approach to suppress CNS-1 rat glioma established tumors was 80% using the transfected cells expressing both MHC-I and B-7, and 30% using the transfected cells expressing only MHC-I (*Fig. 8*).

Human glioma cell cultures established from five glioblastoma multiforme cancers demonstrated morphological characteristics similar to those previously described [36]. Each 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%). Analysis of RNA of human glioma cells four weeks after transfection was described earlier [36].

The phenotypic changes in peripheral blood lymphocytes (PBL) of the vaccinated glioblastoma patients were as presented in the *Tab. 3*. In the group of five glioma patients, each of those injected with autologous cells presenting both MHC-I and B-7 (*Fig. 9*), the significant changes were observed mostly after the first vaccination. There was increase in CD 8 percentage with characteristic switching from CD8+CD11b+ to CD8+CD11b-phenotype. Additionally, there was an increased percent of lymphocytes positive for the superficial interleukine-2 receptor (CD25).

Discussion

These data demonstrate that in IGF-I «triple helix» transfected CNS-1 rat glioma and in primary human glioma cells, changes in immunogenic properties and apoptosis occur. These properties were used for selection of human glioma cells that were used in IGF-I triple-helix immunogene therapy.

In the triple helix strategies, the oligonucleotides are

Figure 8. Survival curves of Lewis rats inoculated subcutaneously with CNS-1 rat glioma cells nad injected above the hind leg with 5000000 CNS-1 cells. The following groups of animals (every group, n=8) were considered according to the type of injection: group 1, PBS; group 2, IGF-I "triple helix" cells expressing only MHC-I; group 3, IGF-I "triple helix" cells expressing both MHC-I and B-7. 0 point = 5 days after primary inoculation of CNS-1 cells. The % survival was significant at the p<0.01 level. (1.0 corresponds to 100%).

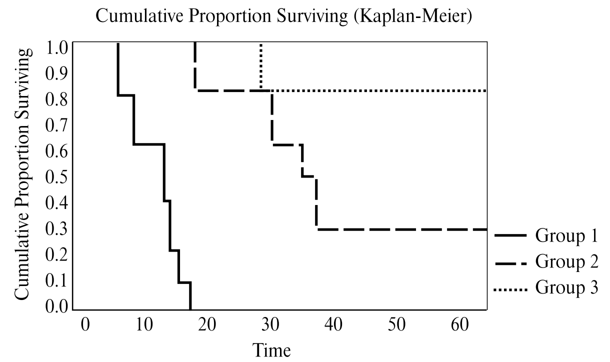
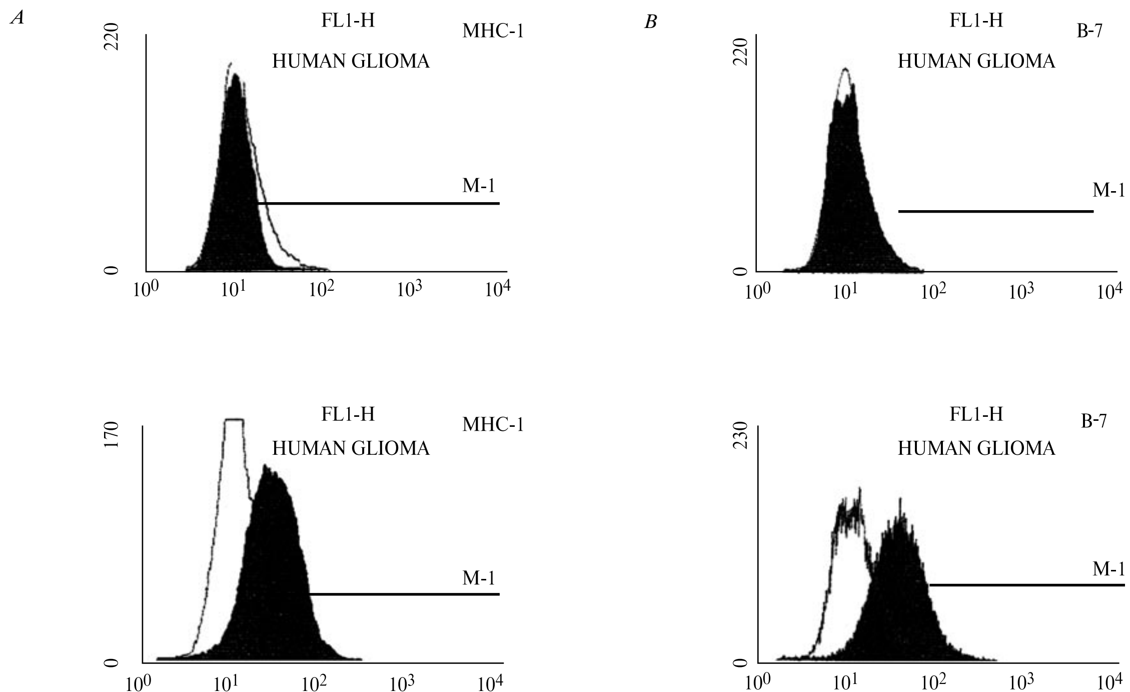


Figure 9. Flow cytometry analysis (FACScan Becton Dickinson). Expression of MHC-I (*A*) and B-7 (*B*) in primary human glioblastoma cell line. *A*) and *B*) upper panels: non transfected cells; lower panels: transfected cells (upregulation of MHC-I and B-7). The glioblastoma cell line presented here corresponds to the patient "P" of *Tab. 3*.



targeted to double stranded DNA containing polypurine-polypyrimidine sequences that readily form triple helixes. The results of the triple helix strategy presented in this study show that an RNA strand containing a 23-nucleotide (nt) oligopurine sequence [15,17,38] may be capable of forming triple-helix structures with an oligopurine-oligopyrimidine sequence of the IGF-I gene as well in cultured rat C6 glioma [15] as in CNS-1 rat glioma. Although we cannot exclude other mechanisms [15], triple helix formation remains the most plausible possibility for the inhibition of IGF-I gene expression. The suppression in IGF-I RNA level (*Fig. 5*) is accompanied by a reduction of cellular IGF-I. This suggests that the RNA strand, which forms the triple helix (*Fig. 3*), has inhibited gene transcription in glioma cells.

The induction of IGF-I triple-helix forming structure, similarly to IGF-I antisense approach, was followed by enhanced expression of MHC-I and B-7 (*Tab. 2*), and loss of in vivo tumorigenicity. Moreover, our previous studies on the IGF-I antisense strategy demonstrated an extensive lymphocytic CD8 positive infiltrate 4-5 days after injection of transfected glioma, teratocarcinoma and hepatoma in the respective animal bearing tumor model systems [6,10,16]. Recently it was demonstrated that a subject with glioblastoma multiforme was recruited for an ex vivo IGF-I antisense gene therapy protocol [39]. After subcutaneous injection, the subject developed peritumor necrosis; the tissue section bordering the necrotic tumor tissue showed the infiltration of lymphocytes consisted of both CD4 and CD8 T-cells. In the results presented here concerning peripheral blood lymphocytes (PBL) of glioblastoma patients treated by IGF-I triple helix immuno-gene therapy the eminent changes were observed after vaccinations (*Tab. 3*). There was increase in percentage of CD 8+ lyphocytes with characteristic switching from CD8+CD11b+ to CD8+CD11b- phenotype. This alteration may reflect the enhanced activation of cyto-

Table 3. IGF-I triple helix gene therapy of glioblastoma patients. Expression of CD molecules in peripheral blood lymphocytes (PBL) derived from "vaccinated " persons*.

Molecule	Before vaccination					After 1st vaccination					After 2nd vaccination				
	P	Q	R	S	T	P	Q	R	S	T	P	Q	R	S	T
CD3	84	71	78	66	80	88	77	79	71	90	85	84	79	71	91
CD4	34	32	42	42	56	41	23	47	46	45	37	40	47	39	48
CD8	54	46	34	30	27	53	61	36	32	45	50	48	40	42	44
CD4/CD8	0.63	0.70	0.62	1.4	2.07	0,77	0.38	0.85	1.44	1.0	0.74	0.83	1.18	0.93	1.09
CD19	3	7	10	9	4	3	2	12	17	2	2	6	11	9	2
CD3-(16+56)+	9	11	9	23	10	6	15	10	14	6	11	9	12	22	7
CD25	11	9	13	18	15	20	17	19	27	26	33	24	21	24	27
CD4+25+	4.8	6.5	9.4	12.4	10.4	8.1	9.5	13	11	14.1	19	17	15	16.5	18
CD8+25+	3.4	0.9	2.0	1.9	3.2	3.6	4.0	3.7	10	1.7	5.2	1.0	2.9	0.8	1.7
CD4+45RO+	nt	nt	nt	41	26	12	nt	nt	40	32	22	24	26	36	30
CD8+45RO+	nt	nt	nt	18	6	17	nt	nt	13	25	14	18	17	19	22
CD8+11b+	26	43	21	20	14	27	46	21	15	19	20	31	24	26	27
CD8+11b-	28	3	13	10	13	26	15	15	17	26	30	17	16	16	17
CD5+19-	81	76	75	67	78	79	77	77	60	89	84	79	77	65	88
CD19+5+	1	0.7	2.1	1.1	0.7	1.1	0.7	2.9	3.2	0.6	0,7	0.4	4	5.7	1

* Five cases indicated by P, Q, R, S, T; nt = not tested. PBL were analyzed by flow cytometry (FACScan Becton Dickinson). Double direct immunotyping with pairs of monoclonal antibodies conjugated with FITC and PE, respectively. Lymphocyte gate was defined according to the CD45 backgating. The data are expressed as percent of positive cells as compared to the isotype control.

toxic T-cells in blood. Additionally, an increased percentage of lymphocytes positive for superficial interleukine-2 receptor (CD25) was found, especially in the context of CD4 molecules. Generally, this has been raported as a specific features of activation for both Th and Tc populations in our results. This finding is compatible with the previous observation of T lymphocytes in IGF-I antisense gene therapy of gliomas [6,10,39].

The simulateous increase in the presence and role of B-7 and MHC-I antigens in the induction of T-cell immunity against tumors has been extensively investigated [26,33,34]. In the current paper we demonstrate that the injection of IGF-I triple helix transfected cells presenting both MHC-I and B-7 molecules stopped effectively the established rat glioma tumors. This was not the case for cells expressing MHC-I only (*Fig. 8*). In in vivo experiments using IGF-I «antisense» vector in place of the IGF-I «triple-helix» vector gave similar results; data not shown.

In this context, antigenic peptides presented by class I MHC molecules were necessary but, in general, not sufficient to stimulate T cell response. In the absence of B-7 molecule, MHC-peptide complexes could selectively inactivate T-cells [40]. (Although "triple helix" cells as compared to "antisense" cells show slightly higher expression of MHC-I and B7 there are no qualitive immunogenic and apoptotic differences between IGF I "antisense" and "triple helix" approaches.)

The costimulatory molecule B-7 of antigen presenting cells (APCs) bound to the counter-receptor CD28 and/or CTLA4 expressed on the T-cells [41-43]. In our work, the absence of IGF-I synthesis in «triple-helix» transfected cells, could lead to a higher level of IGF-I receptor and hence to greater tyrosine kinase content; IGF-I and IGF-II present in fetal calf serum of culture medium, as well as intracellular IGF-II can act via the type I receptor [37]. There is a relation between the signal transduction pathway of tyrosine kinase and induction of B-7 molecules: enhancement in B-7 co-stimulation through a cAMP mechanism linked to tyrosine kinase of the CD 28 receptor has been previously reported [44]. Similar signaling through the tyrosine kinase activity of the IGF-I receptor shows that: tyrosine kinase activates IRS-1 (Insulin receptor substrate-1), and then IRS-1 activates PI3K (phosphatidilinositol 3 kinase) [45,46]. This mechanism could be considered in the cytokine induced B7-1 expression demonstrated in fetal human microglia in culture [47].

Using CNS-1 glioma cells we have confirmed the relation between the immunogenicity and apoptosis found in IGF-I transfected cells [36]. The appearance of apoptotic cells demonstrated that cell death by apoptosis for «triple-helix» cells, is directly related to the suppression of IGF-I expression. IGF-I can block the apoptosis pathway leading to a proliferation of the cell population that is normally programmed to die [14,48]. On the other hand, when function of IGF-I receptor (IGF-I-R) is depressed, certain cancer cells i.e. glioma cells undergo massive apoptosis [49]. It was concluded that IGF-I-R activated by its ligand plays a very protective role in programmed cell death, and that this protection is even more striking in vivo than in vitro [50]. Apoptosis could play a specific role in our strategies; 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 [10,37]. Apoptotic cells, in the context of MHC-I,

are recognized by dendritic cells activating lymphocytes T-CD8 [51,52]. B-7 molecules can be included in this mechanism, because both MHC-I and B-7 molecules are necessary for T cell activation [32,33].

The IGF-I antisense approach for treatement of human gliomas and hepatomas was recently published [39,53,54]. The phenotypic changes in PBL found in IGF-I triple helix gene therapy of patients with glioblastoma multiforme, and establishment of the immune characteristics necessary to produce an effective vaccine, constitute the start point for a Phase II clinical protocol of triple-helix cellular therapy for cancers highly expressing IGF-I.

Acknowledgments

We thank Dr M. Sanson for providing CNS-1 cells (INSERM), Prof. J.-Y. Delattre, Dr. J-C Francois (INSERM) and Prof. R. Przewlocki (Polish Academy of Sciences) for helpful suggestions concerning in vitro and in vivo experiments. We are very thankful to Prof. J. Ilan (CWRU, Cleveland) for generously facilitating the molecular biology technology and Prof. Claude Bouchaud (University of Paris) for help in microscopy studies. We are also indebted to Mr Piotr Ulanowski and Marcin Szymkowiak for graphic computer assistance. This work was supported by grants of KBN (Kraków and Bydgoszcz), N° 6 P05C 016 20 and 3 P05B 089 23.

References

1. Daughaday WH, Hall K, Raben MS, Salmon WD, Van den Brande JL, Wyk JI. Somatomedin: proposed designation for sulphation factor. Nature, 1972; 235: 107.

2. Froesch CS, Schwander J, Zapf J. Actions of insulin-like growth factors. Ann Rev Physiol, 1985; 47: 443-67.

3. Holthuizen E, Le Roith D, Lund PK, Roberts CT Jr, Rotwein P, Spencer EM. Modern Concepts in Insulin-like Growth Factors. New York: Elsevier, 1991; p. 736.

4. Merimee TJ, Laron Z. Growth hormone, IGF-I and growth – new views of old concepts. London-Tel Aviv: Freund Publishing House Ltd, 1996; p. 266.

5. Baserga R. Oncogenes and strategy of growth factors. Cell, 1994; 79: 927-30.

6. Trojan J, Johnson T, Rudin S, Blossey B, Kelley K, Shevelev A, Abdul-Karim F, Anthony D, Tykocinski M, Ilan Ju, Ilan J. Gene therapy of murine teratocarcinoma: separate functions for insulin-like growth factors I and II in immunogenicity and differentiation. Proc Natl Acad Sci USA, 1994; 91: 6088-92.

7. Rubin R, Baserga R. Biology of disease. Insulin-like growth factor I receptor. Its role in cell proliferation, apoptosis and tumorigenicity. Lab Inves, 1995; 73: 311-31.

8. Ayer-le Lievre C, Stahlbom PA, Sara VR. Expression of IGF-I and -II mRNA in the brain and craniofacial region of the rat fetus. Development, 1991; 111: 105-15.

9. Heldin CH, Westermark B. Growth factors as transforming proteins. Eur J Biochem, 1989; 184: 487-96.

10. Trojan J, Johnson TR, Rudin SD, Ilan Ju, Tykocinski ML, Ilan J. Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. Science, 1993; 259: 94-7.

11. Antoniades HN, Galanopoulis T, Nevile-Golden J Maxwell M. Expression of insulin-like growth factors I and II and their receplorm RNAs in primary human astrocytomas and meningiomas: In vivo studies using in situ hybridization and immunocytochemistry. Int J Cancer, 1992; 50: 215-22.

12. Kiess W, Lee L, Graham, DL, Greenstein L, Tseng LY, Richler MM, Nissley SP. Rat C6 glial cells synthesize insulin-like growth factor I (IGF-I) and express IGF-I receptors and IGF-II/inannose-6-phosphale receptors. Endocrinology, 1989; 124: 1727-36.

13. Rodriguez-Tarduchy G, Collins MKL, Garcia I, Lopez-Rivas A. Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoietic cells. Eur J Immunol, 1992; 149: 535-40.

14. Upegui-Gonzalez LC, Duc HT, Buisson Y, Arborio M, Lafarge-Frayssinet C, Jasmin C, Guo Y, Trojan J. Use of the IGF-I antisense strategy in the treatment of the hepatocarcinoma. Adv Exp Med Biol, 1998; 451: 35-42.

15. Shevelev A, Burfeind P, Schulze E, Trojan J, Helene C, Ilan J. Potential triple helix mediated inhibition of IGF-I gene expression significantly reduces tumorigenicity of glioblastoma in an animal model. Cancer Gene Ther, 1997; 4: 105-12.

16. Upegui-Gonzalez LC, Ly A, Sierzega M, Jarocki P, Trojan LA, Duc HT, Pan Y, Shevelev A, Henin D, Anthony D, Nowak W, Popiela T, Trojan J. IGF-I triple helix strategy in hepatoma treatement. Hepato/Gastroentero, 2001; 48: 660-6.

17. Derwan P. Reagents for the site-specific cleavage of megabase DNA. Nature, 1992; 359: 87-8.

18. Helene C. Control of oncogene expression by antisense nucleic acids. Eur J Cancer, 1994; 30: 1721-6.

19. Rubinstein JL, Nicolas JF, Jacob F. L'ARN non sens (nsARN): un outil pour inactiver spécifiquement l'expression d'un gène donné in vivo. Comptes Rendus Académie des Sciences Paris, 1984; 299: 271-4.

20. Weintraub H, Izant G, Harland RM. Anti-sense RNA as a molecular tool for genetic analysis. Trends in Genetics, 1985; 1: 23-5.

21. Green PJ, Pines O, Inouye M. The role of antisense RNA in gene regulation. Annual Review of Biochemistry, 1986; 55: 569-97.

22. Izant JG, Weintraub H. Constitutive and conditional suppression of exogenous and endogenous genes by antisense RNA. Science, 1985; 229: 345-52.

23. Morrison RS. Suppressinn of basic fibroblast growth factor expression by antisense deoxyribonucleotides inhibits the growth of transformed human astrocytes. J Biol Chem, 1991; 266: 728-34.

24. Selinfreund RH, Barger SR, Welsh MJ, van Eldik LJ. Antisense inhibition of glial S100 beta production results in alterations in cell morphology, cytoskeletal organisation, and cell proliferation. J Cell Biol, 1990; 111: 2021-8.

25. Kim SK, Wold BJ. Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. Cell, 1985; 42: 129-38.

26. Trojan J, Blossey BK, Johnson TR, Rudin SD, Tykocinski M, Ilan Ju, llan J. Loss of tumorigenicity of rat glioblastoma directed by episome-based antisense cDNA transcription of insulin-like growth factor 1. Proc Natl Acad Sci USA, 1992; 89: 4874-8.

27. Hambor JER, Hauer CA, Shu HK, Groger RK, Kaplan DR and Tykocinski ML. Use of an Epstein-Bair virus episomal replicon for anti-sense RNA-mediated gene inhibition in a human cytotoxic T-cell clone. Proc Natl Acad Sci USA, 1988; 85: 4010-4.

28. Whitesell L, Rosolen S, Neckers LM. Episome-generated N-myc antisense RNA restricts the differentiation potential of primitive neuroectodermal cell lines. Mol Cell Biol, 1991; 11: 1360-71.

29. Johnson TR, Biossey BK, Rudin SD and Ilan J. Regulation of IGF-I RNA transcript levels in C6 glial cells. J Cell Biol, 1990; lll: 505a.

30. Blanchet O, Bourge JF, Zinszner H, Israel A, Kourilsky P, Dausset J, Degos L, Paul P. Altered binding of regulatory factors to HLA class I enhancer sequence in human tumor cell lines lacking class I antigen expression. Proc Natl Acad Sci USA, 1992; 89: 3488-92.

31. Plautz GE, Yang ZY, Wu BY, Cao X, Huang L, Nabel GE. Immunotherapy of malignancy by in vivo gene transfer into tumors. Proc Natl Acad Sci USA, 1993; 90: 4645-9.

32. Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter IA, Mc Growan P, Linsley PS. Costimulation of anti-tumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA-4. Cell, 1992; 71: 1093-102.

33. Townsend SE, Allison JA. Tumor rejection after direct costimulation of CD8+ T cell transfected melanoma cells. Science, 1993; 259: 368-70.

34. Guo Y, Wu M, Chen H, Wang XN, Liu GL, Ma J, Sy MS. Effective tumor vaccine generated by fusion of hepatoma cells with lymphocytes B cells. Science, 1994; 263: 518-20.

35. Trojan J, Duc H, Upegui-Gonzalez L, Hor F, Guo Y, Anthony D, Ilan J. Presence of MHC-I and B-7 molecules in rat and human glioma cells expressing antisense IGF-I mRNA. Neuroscience Letters, 1996; 212: 9-12.

36. Ly A, Duc HT, Kalamarides M, Trojan LA, Pan Y, Shevelev A, François J-C, Noël T, Kane A, Henin D, Anthony D, and Trojan J. Human glioma cells transformed by IGF-I triple-helix technology show immune and apoptotic characteristics determining cell selection for gene therapy of glioblastoma. J Clin Pathol, 2001; 54: 230-9.

37. Lafarge-Frayssinet C, Sarasin A, Duc HT, et al. Gene therapy for hepatocarcinoma: antisense IGF-I transfer into a rat hepatoma cell line inhibits tumorigenesis into syngeneic animal. Cancer Gene Therapy, 1997; 4: 276-85.

38. Rininsland F, Johnson T, Chernicky C, Schulze E, Burfeind P, Ilan J. Suppression of insulin-like growth factor type-I receptor by a triple-helix strategy inhibits IGF-I transcription and tumorigenic potential of rat C6 glioblastoma cells. Proceedings of Proc Natl Acad Sci USA, 1997; 94: 5854-9.

39. Wongkajornslip A, Ouyprasertkul M, Sangruchi T, Huabprasert S, Pan Y, Anthony D. The analysis of peri-tumor necrosis following the subcutaneous implantation of autologous tumor cells transfected with an episome transcribing an antisense insulin-like growth factor I RNA in a glioblastoma multiforme subject. J Med Assoc Thai, 2001; 4: 740-7.

40. Steiman RM, Turkey S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exper Med, 2000; 191: 411-6.

41. Linsley PS, Clark EA, Ledbetter JA T-cell antigen CD28 mediates adhesion with B cells by interacting with ativation antigen B7/13. Proc Natl Acad Sci USA, 1990; 87: 5031-5.

42. Freeman GB, Gray GS, Gimmi CD, Lombard DB, Zhou L-I, White M, Fingeroth JD, Gribben JG, Nadler LM. Structure, expression and T cell costimulatory activity of murine homologue of the human B lymphocyte activation antigen B7. J Exper Med, 1991; 174: 625-31.

43. Harding F, Mc Arthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated and signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones. Nature, 1992; 356: 607-9.

44. Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4 and B7/BBI in interleukin-2 production and immunotherapy. Cell, 1992; 71: 1065-8.

45. D'Ambrosio C, Ferber A, Resnicoff M, Baserga R. A soluble insulin-like growth factor receptor that induces apoptosis of tumor cells in vivo and inhibits tumorigenesis. Cancer Research, 1996; 56: 4013- 20.

46. Dudek H, Datta SR, Franke TS, Birbaun MG, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal servival by the serine-threonine protein kinase Akt. Science, 1997; 275, 661-5.

47. Satoh J, Lee YB, Kim SU. T-cell costimulatory molecules B7-1 (CD80) and B7-2 (CD86) are expressed in human microglia but not in astrocytes in culture. Brain Research, 1995; 704: 95-6.

48. Ellouk-Achard S, Djenabi S, De Oliveira G, Desauty G, Duc HT, Zohair M, Trojan J, Claude JR, Sarasin AL, Lafarge-Frayssinet C. Induction of apoptosis in rat hepatocarcinoma cells by expresssion of IGF-I antisense c-DNA. J Hepatol, 1998; 29: 807-18.

49. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Research, 1995; 55: 249-52.

50. Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman HL, Kajustora J, Rubin R, Zoltick P, Baserga R. The insulin-like growth factor I receptor protects tumor cells from apoptosis in vitro. Cancer Research, 1995; 55: 2463-9.

51. Sotomayor E, Fu Y, Lopez-Cepero M, Herbert L, Jimenez J, Albarracin C, Lopez D. Role of tumor derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. J Immunol, 1991; 147: 2816-23.

52. Matthew L, Saiter B, Bhardwag N. Dendritic cells acquire antigen from apoptotic cells and induce class I restricted CTL. Nature, 1998; 392: 86-9.

53. Anthony D, Pan Y, Wu S, Shen F, Guo Y. Ex vivo and in vivo IGF-I antisense RNA strategies for treatment of cancer in humans. Advances in Experimental Medicine and Biology, 1998; 451: 27-34.

54. Pan YX, Anthony D. IGF-I antisense strategies for cancer treatement. In: Walter and Stein "Methods in molecular medicine", Ed. Humana Press, 200, 35: 189-204.

55. Carpentior AF, Xie J, Moklitan K, Delatle JY, Succesful treatement in intraozanial glimes in rat by oligodeoxymolecules otides containing CpG motifs. Clin Cancer Res, 200; 6(6): 2469-73.