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Research Article

In vitro Technical Aspects of Anti-Gene IGF-I Vaccines against Glioma

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Abstract

Research on glioblastoma has demonstrated a significant increase in IGF-I gene expression in this brain tumor. After suppression of IGF-I expression using anti-gene IGF-I technologies, glioma cells become immunogenic, expressing MHC-I. While injected *in vivo*, they induce an immune response mediated by T-CD8 lymphocytes. These cells, applied as autologous anti-cancer vaccines have increased the median survival of glioblastoma patients up to 18 months, but often up to two-three years. These differences in clinical results could be explained by variability in vaccine preparation standards. For this reason, the conditions of *in vitro* preparation of anti-cancer vaccines were explored using antisense, AS, and triple helix, TH technology. The established human glioblastoma primary cell line, and an established rat cell line, C6 glioma, were transfected *in vitro* with IGF-I AS, and with IGF-I TH vectors producing arrest of the synthesis of IGF-I on transduction and transcription levels, respectively. An arrest in IGF-I gene expression was demonstrated in 50-60% of cultured cells. The AS and TH cells expressed MHC-I only in 30-40% of cells. To increase the immunogenic population, the transfected cells were cloned to obtain 100% IGF-I negative population. These cloned cells expressed MHC-I and B7 antigens in 60-70% of cells. The presented *in vitro* results will be applied for preparation of cell vaccines for cancer immunotherapy, with the objective of increasing median survival in glioblastoma treated patients.

Keywords

Glioma cells, IGF-I, Anti-gene technology, Vaccines for cancer immunotherapy

Introduction

Gene expression during the neoplastic development of the brain, and especially during the appearance of malignant glioma, concerns various oncoproteins, as AFP, and growth factors and their receptors (IGF-I, EGF, FGF, VEGF, TGF alpha and beta and their signaling pathways PI3K/AKT including glycogen synthase) [1-4].

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To distinguish between neoplastic glial cells and neuroblasts, we used the IGF-I marker. In this case, glial cells gave positive reaction and neuroblastic cells, negative [5]. This observation suggested that targeting IGF-I gene expression we could stop the glial neoplastic development using anti-gene strategy [6].

The past forty years have accelerated the researches related to the treatment of tumors targeting the proteins using either antibodies i.e. anti AFP, or injection of radiolabelled oncoproteins was proposed [7]. Currently, the anti-gene strategies stopping the protein synthesis either on translation level (antisense, AS) [8] or transcription level (triple-helix, TH) [9] offer new possibilities for cancer therapy.

The antisense sequences are capable of blocking the messenger RNA translation conducting to block of specific protein synthesis. In another anti-gene approach, TH technology, specific oligonucleotide sequences also called triple helix-forming oligonucleotides, TFOs, while introduced into cells are linked to the genomic DNA forming the triple helix structure with the target gene and inhibiting its transcription [2,4,8-10]. A novel development in oligodeoxynucleotide technology is the relatively recent finding that 21-23 mer double-stranded RNA molecules, known as siRNA, can effectively silence gene expression [11]. Whether or not siRNA technology will supplant the classical oligodeoxynucleotide approaches is still in question at the moment, because we do not yet have the final clinical results [10].

Using AS and TH technologies we have obtained in both approaches, similar experimental and clinical results. The transfected AS and TH cells-autologous anti-cancer 'vaccines'-expressed MHC-I and B7.1 and B7.2 antigens, and moreover became apoptotic. These cells induced *in vivo* T-cell mediated immune reaction against their tumorigenic progenitor's cells in syngeneic animals, with tumor regression at distal sites [12,13]. The objective of here presented article was to increase the usefulness of this strategy [6], by making more performant anti IGF-I vaccines. The ultime goal would be to increase of median survival of treated glioblastoma patients.

Materials and Methods

Vectors

The IGF-I antisense and triple helix 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 (Figure 1). For control experiments, the vectors suppressing MHC-I and B7 molecules, expressing antisense MHC-I and antisense B7 (MHC-I AS and B7 AS), as well as antisense IGF-II RNA expressing vector, were constructed as previously described. To stimulate the MT promotor, the cells were cultivated in presence of 60 uM $ZnSO_4$ [13,14].
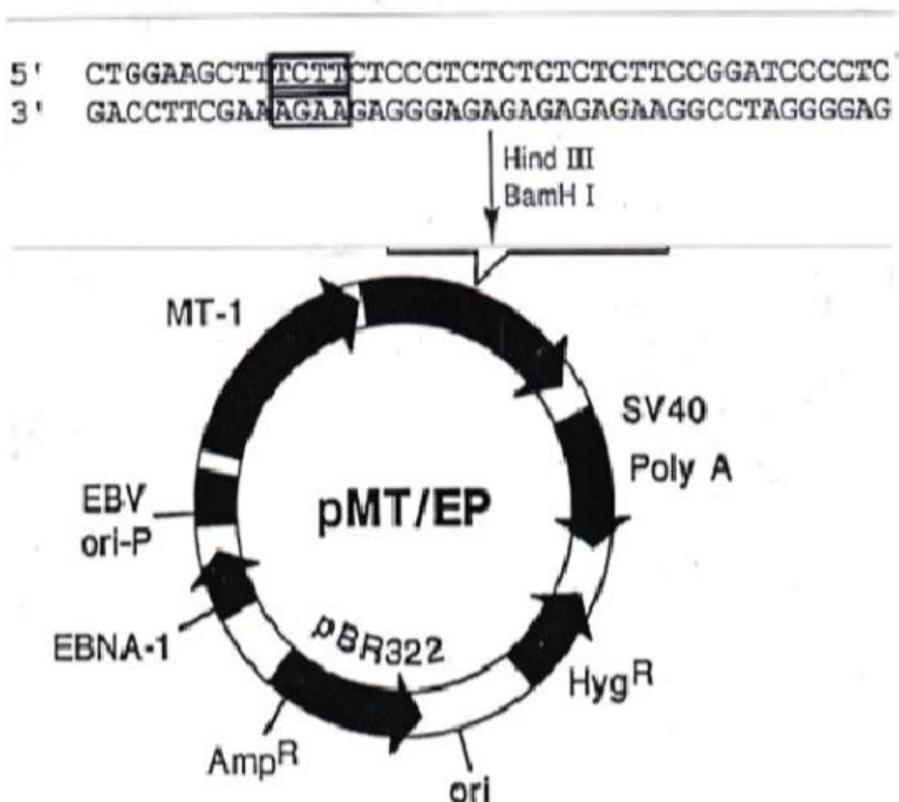
Figure 1: (left) Diagrammatic representation of IGF-I triple helix expression vector, the episomal vector construct, derivatived from pMT/EP (empty vector). In IGF-I antisense expression vector, cDNA of IGF-I (sequence of 536 bp of exon 1) is inserted in antisense orientation [5].

5' CTGGAAGCTTTCTTCTCCCTCTCTCTCTCTTCCGGATCCCCTC

3' GACCTTCGAAAGAAGAGGGAGAGAGAGAGAAGGCCTAGGGGAG

In vitro cells

The rat cell line C6 was used for the experiments [8]. Primary cell cultures of human glioma were derived from tumors of glioblastoma multiforme patients during surgical resection in the INC-National Institute of Cancerology, Bogota. The human cell line, HG, was established according to the technique described previously [13]. As a control Hela cell line was used. In general, rat and human glioma cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin, at 37 C and 5% CO_2.

Cells, 60-80% confluent, were transfected in 6-well plates in a ratio of 1 ug of plasmid per 3-4 × 10^5 cells. The FuGENE 6 Transfection Reagent (Boehringer Mannheim) was used according to the supplier's instructions (3 ul of Reagent per 1 ug DNA). Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml (C6) and 0.005 mg/ml (primary human glioma, HG) 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 (C6) and 0.015 mg/ml (HG) and maintained with each change of fresh medium over the next 2-3 months.

Immunocytochemistry

Parental, non-transfected cells and transfected cells were verified for IGF-I presence by immunoperoxidase technique using anti IGF-I antibodies. Immunocytochemical localization of MHC1 and B7 antigens was done by immunofluorescence technique. Antibodies targeting rat MHC-I (OX-18) and MHC-II (OX-17), were used (Valbiotech, Paris, France). To stain B-7, a fusion protein CTLA4-Ig was used (Bristol Myers Squib, Seattle, WA, USA). Monoclonal antibodies, labeling human MHC-I (HLA ABC), MHC-II, CD80 and CD86 antigens were applied for direct immunostaining (Becton Dickinson Pharmingen).

RT-PCR

RNA from cells was isolated using High Pure RNA Isolation Kit (Roche Diagnostics GmbH nr 1828665), and RT PCR was used according Reverse Transcription System Promega Corporation (nr A3500).

The following primers were used for RT PCR study of rat IGF-I and rat MHC-I:

forward primer IGF-I: TAGTCCCTGCCTCTTAAGAG,

and reverse primer IGF-I: AGGGGCGTTAAAACTTGGGT;

forward primer MHC-I: ACA CTC GCT GCG GTA TTT CT,

and reverse primer MHC-I: CCT TGG CTT TCT GTG TCT CC

(sequence according "rgd" Rat Genome Database).

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

Moreover, to verify in another way the presence or absence of IGF-I in parental and transfected cells, the extracted RNA was transformed in cDNA by retrotranscription, and the cDNA was quantified in spectrophotometer Gene Specl (260 and 280 nm).

Results

The following optimal conditions were established for cell cultures as well of rat C6 glioma as of primary cells originated from glioblastoma biopsy (Figure 2): DMEM medium supplemented with Glutamax, 10% CO_2, 15% NBCS (neonatal bovine calf serum), 200 U/ml of penicillin, 200 ug/ml of streptomycin, 1.2 ug of amphotericin B, 10.000 ng/ml of insulin, 5 ug/ml of transferrin, at 37 C.

As to the transfection of glioma cells, the optimum 'timing' was as follows: One week after beginning of C6 cells culture, and three weeks after establishment of primary HG cell line. The confluence of cells as 85-90% was also the optimum factor. The optimum time to form a complex of transfection was 20 minutes, using transfection reagent: Vector as 1: 1 in the volume 1.5 ul each. The timing of cell transfection was
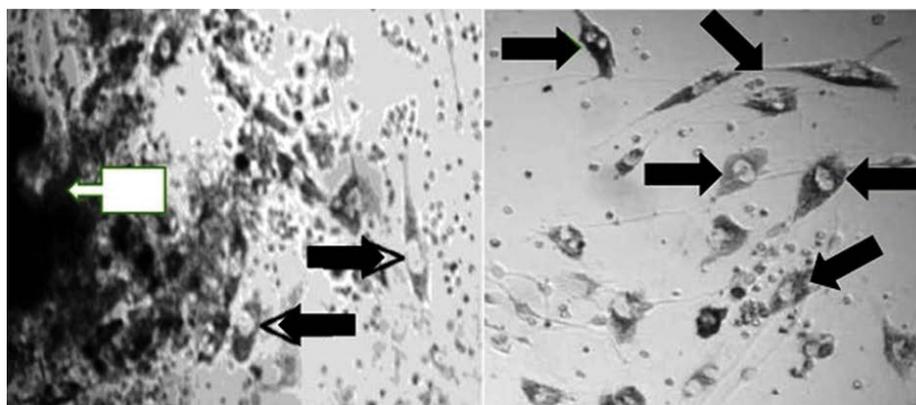
Figure 2: *In vitro* staining of IGF-1 biomarker in glioma cell culture. The tissue and cells are stained for IGF-1 using anti IGF-1 antibodies applied in immunoperoxidase technique. (left) Five days of culture established from human glioblastoma biopsy. Note the first cells (black/white arrows) proliferating from compact tissue of biopsy (white arrow). The cells in this stage of culture present a relatively weak IGF-I staining. (right) Ten days of the same culture. Note the cells (black arrows) showing much more dark cytoplasm of staining (250X).

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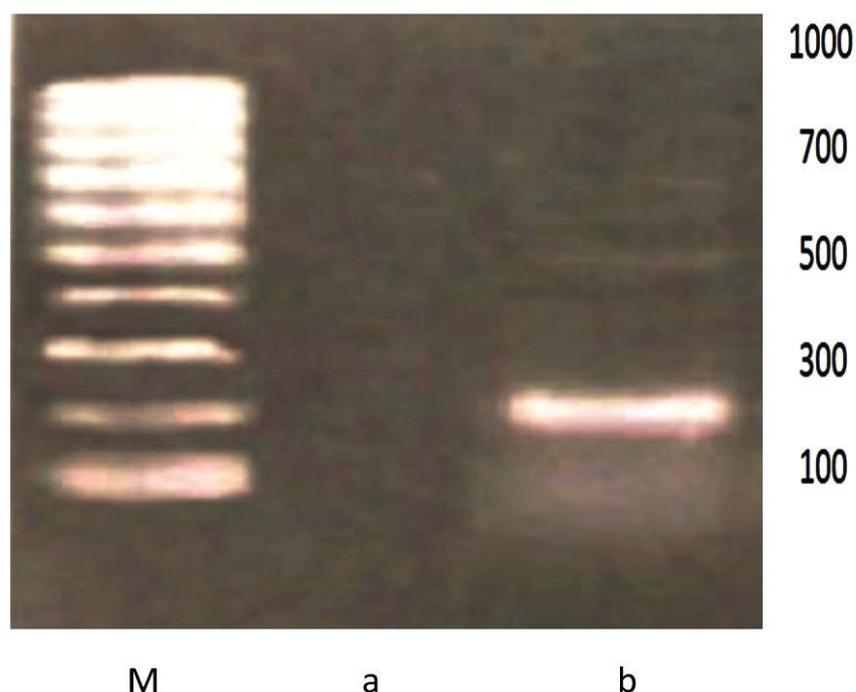
Figure 3: IGF-I expression in C6 glioma cells. RT PCR of "antisense vector" containing MT promoter. a) AS IGF-I cells stimulated with Zn SO$_4$; b) AS IGF-I cells non-stimulated with ZnSO$_4$. M-marker. Note in b) 200 bp bands of amplified DNA using IGF-I primer. The similar results were obtained using human glioma HG cell line.

Table 1: Identification of presence or absence of IGF-I in C6 cell samples. Quantification of cDNA obtained by retrotranscription of isolated RNA, using spectrophotometer Gene Specl 1 at 260-280 nM. Values between 100 and 300 ng/ul.

| Sample	Cells	Concentration of IGF-I cDNA (ng/ul)
1	Parental cells	300
2	Hela cells	260
3	AS cells	210
4	AS cells	111
5	TH cells	180
6	TH cells	100

Table 2: Immunocytochemical staining of IGF-I and of immunogenic antigens MHC-I and -II, and B7 in the rat glioma C6 cells. NT: Non transfected parental cells; pMT-EP: Transfection with "empty vector"; IGF-I or -II AS: Transfection with IGF-I or IGF-II antisense expression vectors; IGF-I TH: Transfection with IGF-I triple helix expression vector; IGF-I AS/IGF-I TH: Co-transfection with antisense and triple helix vectors; IGF-I AS/MHC-I AS/B7 AS, triple co-transfection; Transfected cells were cultured in presence of Zn^{2+}, in order to activate the MT-I promotor [5,19], (Figure 3 and Figure 4). The similar results were obtained with HG cells.

Cells	IGF-I	MHC-I	MHC-II	B7
NT	+++*	-	-	-
pMT-EP	+++	-	-	-
IGF-I AS	-	++	-	++
IGF-I TH	-	+++	-	++
IGF-I AS/IGF-I TH	-	+++	-	++
IGF-II AS	+++	-	-	-
IGF-IAS/MHC-IAS/B7AS	-	-	-	-
IGF-ITH/MHC-IAS/B7AS	-	-	-	-

*+++labeling corresponding to maximum intensity of staining (dilution of antibodies 1:200); ++labeling corresponding to intermediate intensity of staining (dilution of antibodies 1:100).

minimum 4 hours, and maximum 6 hours. (The reagent of transfection should be eliminated before 20 hours because of toxicity).

The transfected cells of both antisense and triple helix types showed an inhibition of IGF-I in 50%-60% of cell population (Figure 3 and Table 1). They have expressed MHC-I in 30%-40% of cells (Figure 4 and Table 2). After cloning, the inhibition of IGF-I in both AS and TH cells was demonstrated in 100% of cells. The antisense or triple helix transfected cloned cultures were stained positively for both MHC-I and B7 antigens in 60-70% of cells. Moreover, "triple helix" cells compared with antisense cells showed a slightly higher expression of MHC-I (Table 2).

Discussion

In the current paper we have demonstrated the optimum conditions for cultivation as well rat glioma cells as primary human glioma cell line. Moreover, we have established the criteria for anti-cancer vaccines: the population of IGF-I AS and IGF-TH transfected cells became more immunogenic

thanks to the cloning of cells negative for IGF-I expression. The culture of these transfected cells, totally negative for IGF-I, has permitted to increase two times the number of MHC-I positive cells: From 30% to 60-70% cells. The immunogenic character of the transfected cells expressing both MHC-I and B7 antigens, explain their vaccine effect in *in vivo* tumorigenicity [14-16] (Table 2).

The expression of IGF-I in primary cultures established from glioblastoma biopsies vary between 80-100%. For these reasons, the cultures without total expression were cloned to
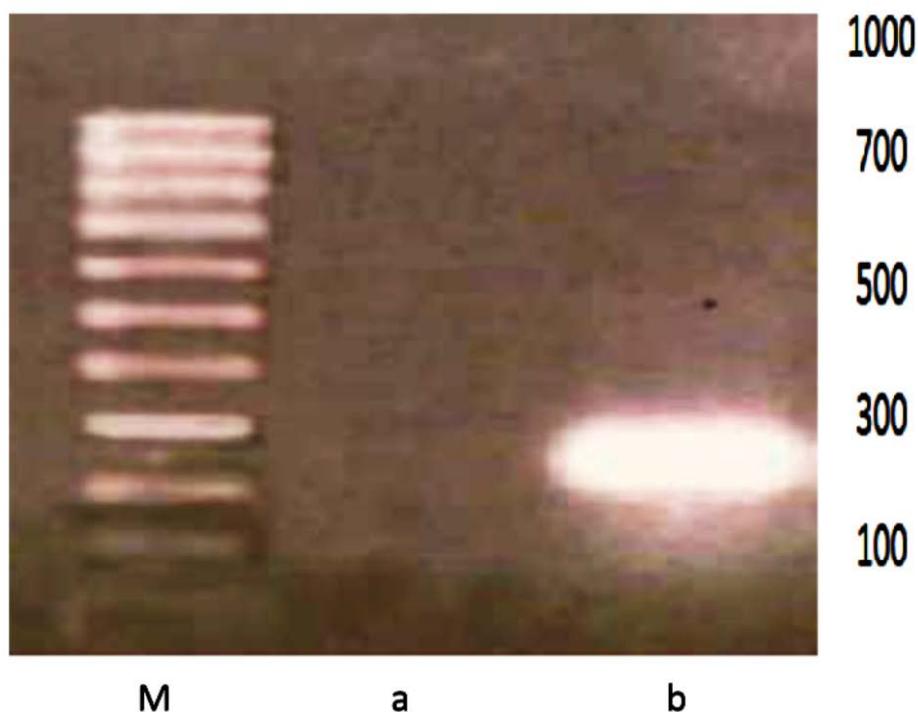
Figure 4: MHC-I expression in C6 glioma cells. RT PCR of "antisense vector" containing MT promoter. a) AS IGFI cells non-stimulated with ZnSO$_4$; b) AS IGF-I cells stimulated with ZnSO$_4$. M-marker. Note in b) 200 bp bands of amplified DNA using MHC-I primer. The similar results were obtained using human glioma HG cell line. Concerning MHC-II and B7 expressions (using MHC-II primer and B7 primer, respectively), the results were identical to those shown for MHC-I.

obtain 100% IGF-I positive cell line. That was important for the transfection of these cultures using anti-gene IGF-I vectors. After transfection, as mentioned in the paper, only 50-60% of cells have showed an inhibition of IGF-I. Once more the cloning of cells was performed to obtain 100% cell population without expression of IGF-I (these transfected cells have also showed an increase in MHC-I expression). The necessity of cloning of cells as well of parental as of transfected cultures was verified in *in vivo* experimental animal model: Only the 'vaccines' prepared from cloned cells have stopped the development of tumors in 90-100% cases. No cloned 'vaccines' have stopped the development of tumors only in half of treated animals. For this reason, as far as prognostic importance in human glioblastoma is considered, we have kept the cloning procedure for preparation of vaccines in clinical trial.

As well the AS and TH vectors preparation, as the *in vitro* experiments, were repeated for statistical purpose using different cell lines. The results were reproduced every time with the same efficiency. The most important detail concerned the transfection of cells with AS or TH vectors: The choose of MT promotor in prepared vectors permitted to perform the transfection in the same conditions-using always 50-60 micro M ZnSO$_4$ concentration in culture medium. The IGF-I inhibition performed in these conditions was efficient: The transfected cells, verified in permanently maintained cultures up to six months, were negative for IGF-I presence.

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 fetal calf serum of culture medium, as well as intracellular IGF-II can interact with the type I receptor. 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 [13,17,18].

The phenotypic modification of transfected cells concerns also the phenomenon of apoptosis [19] (the results not shown). Apoptotic cells, in the context of MHC-I, are recognized by dendritic cell activating lymphocytes T-CD8 [20-22]. B7 molecules may also be included in this mechanism, because both MHC-I and B7 molecules are necessary for T cell activation [15,23].

Different immunotherapies and immunogene therapies including antisense strategy to treat glioblastoma has been recently investigated especially in relation to growth factors [22,24-26]. The immunotherapies therapies of gliomas are in permanent progress [22,27,28].

Acknowledgement

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