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Review Article

Glioblastoma - Application of Gene Therapy During A Quarter of A Century: Anti - Gene IGF-I Strategy

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Abstract

This review gives an example of current recommended post-surgery treatment of malignant brain tumor – glioblastoma whose prognostic is always fatal. In this striking area of neuro-oncology, despite the use of modern approaches of surgery, radiotherapy, hormonotherapy and especially chemotherapy, the results are still far from our expectations. Two years ago, the government of USA has approved the Moonshot program which recommends the introduction in oncology, as an obligatory challenge, of immunotherapy – the domain based on basic science of molecular biology, genetics and immunology. In this article we have described general ideas and clinical results of cancer gene therapy, being also cancer immunogen therapy, which has showed the promising results as follows. In cancer cells provided from surgical biopsy, the expression of IGF-I, principal growth factor responsible of neoplastic development, was suppressed using anti – gene technology. These genetically modified cancer cells, playing the role of vaccines, while injected subcutaneously in cancer patients, induced an immune anti – response, mediated by lymphocytes CD8. During last twenty-five years, the malignant tumors, principally glioblastoma, were treated using anti - gene IGF-I therapy. The median survival of treated patients has reached 20 months, and in some case 3 and 4 years. (Using classical treatment, including chemotherapy, the median survival is maximum 14 month, rarely 18 months).

Keywords: Cancer Gene Therapy; Glioblastoma; Antisense; Triple Helix; IGF-I; Lymphocytes TCD 8 And 28

Abbreviations

IGF-I: Insulin Like Growth Factor I; AFP: Alpha-Fetoprotein; AS: Antisense; TH: Triple Helix; PBL: Peripheral Blood Lymphocytes

Introduction

Immunogenic therapy that induces an immune anti-tumour response is one of the latest strategies for the treatment of many forms of cancer. Starting in 2016, cellular immunotherapy or immunogenic cell therapy as a cancer treatment became a mandatory complementary therapy in the United States with the program "The Cancer Moonshot" overseen by the US government [1] and the Parker Institute of Los Angeles in relation with the best university hospitals in the United States.

As far as immune response mediated by T lymphocytes is considered as therapy, William Coley was recognized as the precursor of immunotherapy thanks to his targeted therapy towards coley toxins [2]. In 1992, the 'creation' of gene therapy approach by Anderson., *et al.* [3] was followed the same year 1992/93 by the 'creation' of cancer gene therapy by Trojan., *et al.* the last therapy being *sensu stricto* cancer immunogen therapy [4]. The cancer gene therapy was applied in clinical trial in parallel with cancer immunotherapy "created" in 1993/94 by groups of Townsend and Allison, and of Guo [5,6] and followed by others [7,8].

Development of the cancer disease

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States [9]. Normally,

human cells grow and divide to form new cells, when they get old or damaged, they die and new cells, often stem cells, take their place. However sometimes this process breaks down and results in a process of uncontrolled growth (including IGF-I – principal growth factor responsible of neoplastic development) and spread of cells, forming a mass called tumor. It can occur almost anywhere in the body, often invading surrounding tissue and can cause metastases at distant points in the body [10].

Cancer is an epigenetic disease: epigenetic changes are inheritable changes that alter gene expression without changing the primary sequence of DNA. The implications of these changes are broad and impact many aspects of normal development, the pathophysiology of the disease, and the development of cancer therapies [11]. Genetic changes that contribute to cancer tend to affect three main types of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes [2,10,11].

As to proto-oncogene, the reference is generally related to the growth factor genes (example: IGF-I, EGF, TGF beta) present in normal cell division. However, these genes can be altered, becoming more active and be transformed in oncogenes. These phenomena play an important role in the cancer development leading cells to stimulate cell growth and division [2,11,12].

Tumor suppressor genes play an important role in the development of cancer. Their normal main function is to instruct cells in the production of proteins that prevent cell growth and cell division in order to produce programmed cell death. So, the absence of their activity leads to a disorderly growth and division of cells [2,11].

DNA repair genes code for proteins that correct errors occurred during DNA replication. Mutations of these genes lead to the inability to perform such correction, allowing mutations in tumor suppressor genes and the multiplication of oncogenes [2,11,13].

Cancer does not usually occur as a result of a single mutation. It is admitted that this disease results from an accumulation of mutations involving these three types of genes. These mutations are the result of a mixture of different factors: endogenous (genetic mutations) and exogenous (drugs, chemical carcinogens, biological carcinogens, radiation and immunological) [2,11,13].

As mentioned above, biomedical research over the last thirty years has had two main challenges: cancer diseases and diseases of the nervous system [14]. The treatment of malignant tumors, especially of central nervous system, is one among important challenges of modern medicine [2,15].

Growth factors and their role in the development of cancer

This Review focuses on alterations in the expression of IGF-I growth factor, an oncoprotein also called somatomedine, leading to human tumor development, and in consequence on therapeutic proposals related to IGF-I expression.

The investigations on normal and neoplastic development have demonstrated that two oncoproteins, AFP – alphafetoprotein, and IGF-I play the principal role in both processes. It was shown that AFP and IGF-I and also IGF-II being present in the normal tissue development particularly in the central nervous system (CNS) [14, 16,17] participate also in its neoplastic processes, including the most malignant tumor, glioblastoma multiforme [15].

As to AFP, the liver and yolk sac of the fetuses were identified as the main sites of synthesis of this oncoprotein in ontogenesis [16,17]. This oncoprotein, being also serum protein, is always present in fetal development. During adulthood it is expected to be absent; however, in some non-neoplastic and neoplastic conditions, a high concentration of AFP may be found [16]. This evidence underlines the existence of convergence between ontogenesis and oncogenesis, and especially brain oncogenesis [17].

Concerning IGF-I, this growth factor is a polypeptide of 70 amino acids, expressed in many tissues, including the brain. The presence of IGF-I is essential for normal growth according the physiological and clinical studies mentioned in literature [18]. IGF-I, similarly to AFP, is involved in tissue development and differentiation, especially in the development of the nervous system [14,17] as a mediator of Growth Hormone, TSH, glucose metabolism, acting locally with autocrine/paracrine, with a predominant role in cancer development compared to other growth factors [14,19-22]. It acts through its union to a specific IGF-I-Receptor [19,22].

In the developing fetal brain, glial cells express relatively little IGF-I-R mRNA, unlike the expression of IGF-I that is mainly located in the cerebellum, sensory transmission systems and the cortex [15]. During postnatal development, IGF-I mRNA is located in the formation of the hippocampus and in the subventricular area of the anterolateral ventricle, suggesting a potential role in promoting the proliferation of glial cells originating in these areas [15].

IGF-I has been reported to block the apoptosis by signal transduction pathway (IRS/ / PI3K / AKT / Bcl2 or GSK3 or caspases) (Figure 1). Such blocking occurs at the cytoplasmic and nuclear levels in a variety of cell lines, including neuronal and glial cells [20,23,24].

The over-expression of IGF-I, accompanied by its high serum concentration, is an anticipatory signal of malignant brain development, especially of glioblastoma [15,25]. On the contrary, the blocking of IGF-I synthesis induces apoptotic and immunogenic phenomena (i.e., expression of MHC-I) in different cancer cell lines including glioma cells [26].

Different studies have shown that the interaction between IGF-I and its receptor (IGF-IR) initiates a malignant process [19,20,27], and the deregulated expression of IGF-1 is associated with the development of 17 different tumors [4]. With regard to the oncoproteins mentioned above, AFP and IGF-I, engaged in brain normal and neoplastic development, there is one important observation: AFP is

Figure 1: Signal transduction pathway of IGF-I (RTK / PI3K / AKT / GS); RTK (tyrosine kinase receptor); IRS1-4 (IRS); PI3K (phosphatidylinositol 3 kinase); PTEN (phosphatase and tensin homologue removed on chromosome 10); PDK1 (phosphooinositide-dependent kinase 1); Bcl 2 (key apoptosis molecules); mTOR (mammalian rapamycin target); RAP-FKBP12 (rapamycin-protein complex FKBP-12); GSK3 (glycogen synthase kinase 3); GS (glycogen synthase); MAPK (MAP kinases); MEK (MAP kinase-mitogen-activated protein kinase); ERK (MAP kinases Erk1 and 2); RAS, RAF (kinases); c-myc (oncogene); PKC (protein kinase kinase) [14].

present in both glial and neuronal cells, while IGF-I is only present in glial cells. This difference has oriented research on glioblastoma towards IGF-I; glioblastoma expresses high concentrations of IGF-I [28].

Tumors of CNS. *Glioblastoma multiforme*

Brain tumors result from alterations in the different cell lines derivated from neuroectodermal germ layer. They are divided into primary and secondary injuries. Primary brain tumors are classified in four degrees according to the World Health Organization (WHO), being considered of low-grade (grade I and II), and high-grade (grade III and IV) tumors. This classification makes it possible to establish a mortality prognosis with, in general, the highest surviving grade I and the lowest surviving grade IV [29,30].

According to its histology, WHO classifies primary brain tumors as astrocytic, oligodendroglia, ependymal, choroid plexus, neuroepithelial of other origin, neuronal, pineal, embryonal, cranial and paraspinal nerve tumors, and meningiomas [29,30]. According the CBTRUS [31], survival rates of one year, for the primary brain tumors, is between 50% (lymphoma) and 90% (oligodendroglioma), with the striking exception for glioblastoma - rarely 30%. In the last case, the survival of 14 months rarely 18 months was observed using chemotherapy.

Brain malignant tumors originating from glial cells are divided into: astrocytoma's, oligodendrogliomas and ependymomas. Glioblastoma multiforme (GBM) is the most common among astrocytoma's. It may develop from a diffuse astrocytoma (grade II) or an anaplastic astrocytoma (grade III), but most frequently occurs de novo (primary tumor). Its incidence is between 3 - 8 cases per 100,000 people in Europe and North America. Glioblastoma is more frequent in men than in women, concerning age 45 -70 years. Prognosis is always fatal - average survival of these patients is approximately 9 months. Some optimistic statistics has signaled survival up to 5 years in 5% cases... [2,15,29-31].

The objective of this review was to summarize the results of glioblastoma treatment obtained in clinical trials using immunogen anti IGF-I therapy. The main goal of this therapy was to evaluate the implementation of immunotherapy in this pathology, concomitant with radio and chemotherapy, improving the quality of life on the assumption of increased survival. Clinical research on the application of anti – gene IGF-I approach is based on the molecular biology techniques permitting the correction of genetic defects responsible for the development of neoplastic processes, and inducing an immune anti – tumor response.

Anti – gene anti – IGF-I strategy

Current treatment options for patients with advanced malignancy such as glioblastoma, including surgery, radiation, or chemotherapy, are limited. For this reason, using the knowledge of molecular biology, genetics and immunnology domains related to neoplastic development, the anti – gene strategy was established permitting to target IGF-I gene, and to stop the synthesis of IGF-I considered as the most important factor of neoplastic development [19,20,22,32]. The preclinical studies have demonstrated in animals bearing glioma and also other tumors (teratocarcinomas containing neuroglial tissues) the efficiency of anti – gene IGF-I technology either of antisense or triple helix types [33,34]. In these studies, a significant immune response was observed towards tumor proliferation in animals vaccinated with modified genetically cancer cells of IGF-I antisense / triple helix type [4,33,34].

Anti - gene strategies represent a new possibility for patients requiring cancer treatment and, among them, "antisense" and "triple helix" techniques seem very promising; its main purpose is to stop protein synthesis of principal growth factors responsible for neoplastic development like IGF-I, EGF, TGF beta, during transcription [13,35] and translation [36-38], respectively. The gene therapies for the treatment of gliomas, and also other cancers, have been proposed since the 1990s [28,39,40]. The first successful cancer gene therapy was realized using the introduction in cancer cells of vectors expressing IGF-I antisense RNA, which have stopped IGF-I mRNA linked to the synthesis of IGF-I [4].

Materials and Methods

Clinical laboratory

First step

Histopathology

Figure 2: Immunocytochemical labelling (immunoperoxidase technic) of IGF-I [14,41]. Note the brown color of cytoplasm confirming the presence of IGF-I.

Second step

1. Tissue culture of cancer cells provided from biopsy of tumor expressing IGF-I.

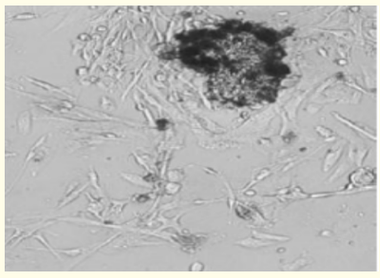

Figure 3: Note cancer glial cells proliferating from glioblastoma biopsy - black compact tissue (up).

2. *In vitro* transfection of glioma cell cultures with IGF-I 'antisense vector'.

Transfection was performed using Fugene kit [14,41].

Figure 4: Antisense vector: the episomal plasmid expressing IGF-I RNA antisense. The cassette contains the origin of replication of the virus Epstein-Barr and the gene that encodes the nuclear antigen I, together, drive extrachromosome replication [28].

Clinical treatment

First of all, the selected patients should always present diagnostically confirmed astrocytoma IV. The selected patients cannot have been previously treated with actinotherapy or chemotherapy (the interference of these therapies could diminish the efficiency of immune therapy; moreover, this could impede the correct evaluation of the role of immune therapy in cancer treatment).

Figure 5: (left) Proliferation of glial cancer cells: cells are labelled for IGF-I using immunoperoxidase (dark cytoplasm) technique [14]. (right) The same glial cells after transfection using the antisense IGF-I vector. The cells have completely changed their morphology: they are longer than the primary glioma cells (left photo). These cells are used as anti-cancer vaccines.

The first obligatory treatment of selected patients was surgery done according to the classical protocol for glioblastoma. The post-surgery treatment was composed of an obligatory radiotherapy (six sessions of radiation). During this period of radiotherapy - two months - the patient was treated also with chemotherapy using a low dose of temozolomide. The radiotherapy was followed by immunogen therapy without chemotherapy. So, two months after surgery, the applied immunotherapy was composed of four vaccination with autologous AS/TH cells performed with interval of one month. 48 hours before every vaccination, the cell pellets were irradiated with 5000 cGy gamma (Co60 or Cs137). The vaccination of the patients was done as a subcutaneous injection (in the higher part of the left arm) of 1 ml of physiologic solution composed of 200 000 – 1 000 000 antisense transfected cells containing both apoptotic and non-apoptotic cells [15,41].

The blood for laboratory exam, including PBL labelling, was collected at first before vaccinations (after applied radiotherapy), and then collected 3 weeks after every of three vaccinations. The following clinical tests were included in the protocol:

1. IRM exam done before the first "vaccination", then 10 days after the first, second and third.
2. Stereotaxic biopsy of tumoral lesion done 4 weeks after the second vaccination. The biopsy will be divided in two parts: for histology and tissue culture exams.

Specific biology exam of the blood included: PBL labelling for CD8, CD8 / CD4, CD28, CD8+11b+ / CD8+11b- (Becton Dickinson Pharmingen, direct immunstaining). Paraformaldehyde-fixed cells were examined in FACSscan BD cytometer. Data were presented as percentage of positive cells [14,41].

Ethical committees

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, no KB/176/2001, 28/06/2002, and no 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, no 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, no LST 980517. Recently the clinical protocol of anti – IGF-I cancer gene therapy was approved by Ethical Committee of CIO – Center of Oncological Investigations, Bogota, Colombia, no AVAL-009-2019, 11.04.2019

Results and Discussion

The first clinical trial protocol using cancer gene therapy of anti IGF-I type, for patients with glioblastoma (1998) (USA, Asia) and liver cancers (2000) (Europe, Asia) consisted of three successive injections as follows. Four patients were divided into two groups. In the first group, patients were treated with cell membranes isolated from IGF-I "antisense" tumor cells (membrane therapy). In the second group, the first membrane injection was continued by two successive injections of cell therapy composed of IGF-I "antisense" tumor cells (containing apoptotic and non-apoptotic cells). The exam of blood removed before and after three injections was analyzed for PBL cells. The molecules CD8, CD28, CD8+11b- and + were tested by cytofluorometer technique comparatively in two laboratories: Villejuf, France, and Bydgoszcz, Poland. The significant increase was observed in the case of CD8+11b- if compared to CD8+11b+ diminishing after every injection (Figure 6) [14,15].

In clinical trials started in 2000s, at first the strategy antisense was used. Then the mixt strategy antisense / triple helix was performed. It means the cultured tumor cells were transfected using fifty: fifty IGF-I antisense and IGF-I triple helix vectors. This mixt strategy was more efficient because IGF-I was suppressed as well on translation (antisense) as on and transcription (triple helix) levels. Established cancer cells originated from biopsies of patients with malignant glioma (glioblastoma multiforme), and also other malignant tumors as colon carcinoma, ovarian carcinoma (cystadenocarcinoma), prostate adenocarcinoma (Bromberg University Hospital, Poland; Case Western Reserve University, Ohio, and NY University Hospital, U.S.A.; University Hospital Paul Brousse, Villejuif, France). This clinical trial was realized as the NATO Science program and then was transferred in 2010 to South America (Colombia) [14,32,41].

These clinical trials yielded encouraging results. First of all, no side effects were evidenced other than a slight increase in temperature, second, an increase in survival was demonstrated: 19-24 months in glioblastoma patients compared to the range of 12-14 months in conventional therapy, The most important objective results concerned the induction of immune anti – tumor response by genetically modified tumor cells ('vaccines' prepared by cancer gene therapy technology). In all types of treated tumors by cancer gene therapy of anti – gene IGF-I approach, the increase of CD8+, CD8+11b- and CD28+ polymorphonuclear cells in vaccinated patients was demonstrated (Figure 7). This increase in immune reactivity could explain the increase of median survival time of the vaccinated patients [32].

Figure 6: Flow cytometric ("FACS") peripheral blood lymphocyte CD marker patterns following cellular gene therapy in glioblastoma multiforme. 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 [14].

Figure 7: 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 transfected tumor cells induce an immune response mediated by T lymphocytes. Moreover, APC cells also participate as shown in the immunogene mechanism (one part of APC cells is induced by apoptotic cells in vitro originated from transfected 'anti – gene'cells; another part of APC is induced by apoptotic cells originated from irradiated in vivo tumor). The activated T lymphocytes destroy as well the vaccine (transfected tumor 'anti – gene' cells) as a tumor.

The gene therapy of glioblastoma (cancer gene therapy) was performed targeting IGF-I [22,42,43] using a vector that express either antisense IGF-I RNA, or triple helix IGF-I [4,34]. This therapy also represents the beginning of clinical immunogenic therapy against cancer, a treatment related to strong apoptotic and immune phenomena; since 2000, the strategy of anti - gene (antisense/ triple helix) anti IGF-I has shown promising results in the treatment of different malignant tumors [14,32,44].

Conclusion

Multiple studies and clinical cancer immunotherapy trials have been carried out focusing on the different alterations that lead to the development of cancer [7]. Cancer immunogen therapy of anti - gene IGF-I approach, after being introduced in USA and Europe, was introduced recently in South America [32].

The treatment options for patients with advanced malignant tumors, including brain tumor glioblastoma (current mortality 100%), being limited in efficacy, therefore the search for new strategies like chemotherapy [45], use of inhibitors, including antibodies (i.e., avastin), antisense oligonucleotides, different types of inhibitors (imatinib, gefitinib), short peptides and other small molecules, or cellular immune therapy constitutes a permanent challenge [7,14,45-47]. Using chemotherapy the median survival has reached in some cases almost a year and a half, but the cancer immunogene therapy offers a better survival [14,32,45]. As far as cancer gene therapy is concerned, the gene delivery permitting higher transgene expression using either viral vectors or synthetic vectors, constitute the permanent study [48].

Our presented work on methodology of anti – gene IGF-I cancer gene therapy has defined the common criteria for selection of anti – tumor vaccines (expression of IGF-I, MHC-I, B7) and for PBL cells markers (CD 8+ related molecules) in patients presenting the arrest of growing tumors [32,41].

Glioblastoma was largely studied in the context of developmental neuro pathology [15,49]. Now the current challenge is focused on the possible solutions of therapy. Among the new strategies in the efforts of treating malignant tumours, especially glioblastoma, targeting different growth factors, and more specifically IGF-I, TGF beta, VEGF or EGF [20,32], the cellular immunogen therapy, particularly anti – gene therapy, and cellular immunotherapy are currently among the most promising approaches for treatment of cancer diseases [7,32].

Conflict of Interest

We declare that any financial interest or any conflict of interest exist.

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