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Gene Therapy of Glioblastoma: Anti – Gene Anti IGF-I Strategy

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1. Introduction

One of the most trying pathological conditions of the central nervous system is the malignant glial development of the brain. The major malignancy is the glioblastoma multiforme, GBM, which almost uniformly leads to the patient's demise: mortality is still close to 100 %, and median survival 8 – 11 months. All currently used therapies - surgery, radiotherapy, chemotherapy and pharmacology – have not given satisfactory results; the median survival, using an immune- or chemotherapy is 15 months, rarely 18 months (Stupp *et al.*, 2006). The search for new approaches based on gene molecular biology/immunology techniques, is therefore a necessary step. In the presented chapter we highlight to biomedical researchers and physicians not only the importance of the glioblastoma problem but the fact that we are currently successfully progressing in the studies and the treatment of this pathology. The most recent approaches for the treatment of malignant tumors and especially of gliomas are now focusing on the use of different types of inhibitors. More specifically gene therapy approach using anti - gene technology including antisense strategy is considered to target as well growth factors (IGF-I, EGF, VEGF, TGF) as their receptors and related downstream steps of signal transduction pathways (IRS-1, PI3K, AKT, PKC, Bcl-2, GSK3, glycogen synthase GS) (Helene, 1994; Beckner *et al.*, 2005; Trojan *et al.*, 2007a). Among growth factors, IGF-I plays a principal role during development of the brain reappearing in malignant glial differentiation (Pollak *et al.*, 2004). This hypothesis has strongly underlined the usefulness of techniques permitting to target and stop the expression of growth factors present in tumoral development by anti – gene strategies, particularly antisense approach.

The "discovery" of the antisense approach, AS, was made in 1984/1985 (Rubenstein *et al.*, 1984; Weintraub *et al.*, 1985). The AS approach, as a concept, was created to study basic problems of gene regulation, particularly useful in developmental biology investigations, bypassing inherent limitations of functional studies dependent upon natural mutant cells or artificially mutagenized cells (Izant & Weintraub, 1985). Antisense technique was particularly used to target tumor antigens, which arrest of expression was not efficiently stopped using antibodies or other inhibitors (Dias & Stein, 2002). The demonstration of AS technology as a efficient gene therapy tool, simultaneously suppressing the targeted protein

expression, changing a morphologic phenotype of cultured neoplastic cells, and stopping *in vivo* a growth of experimentally established tumors, was done for the first time using AS anti IGF-I approach for glioma treatment (Trojan *et al*., 1993). We can also consider the 1992/3 years as the beginning of gene therapy of gliomas exploring either strategy of AS anti IGF-I using episomal vector (Trojan *et al*., 1992), or strategy of retroviral vector with gene encoding TK -HSV (Culver *et al*., 1992) (only the first approach – AS anti IGF-I - has shown valuable results in ulterior clinical trials). The first clinical cases of glioblastoma were treated with AS strategies - anti IGF-I and anti IGF-I receptor - in 2000/1 (Wongkojornsilp *et al*., 2001; Andrews *et al.,* 2001). Since the past decade, we observe a significant increase of AS approach for the treatment of tumors and especially of gliomas. Recently, other targets than IGF-I, as TGFbeta and their downstream signal transduction pathway elements as GS among others were proposed for treatment of malignant gliomas using AS technology (Schlingensiepen, R. *et al*., 2005; Ardourel *et al*., 2007). The approach of AS TGF-beta, similarly to that of AS IGF-I, has given satisfactory clinical results.

2. Anti - genes

Since twenty years different approaches of the treatment of tumours, including glioblastoma, were considered. For example, the treatment of liver cancer with antibodies to AFP was widely used. Unfortunately these techniques were not specific for the treated tissues. Actually, the "anti-gene" strategies offer new possibilities for cancer therapy. The anti-genes can be classified into three categories, as follows: 1) the antisense molecules (Rubenstein *et al*., 1984; Weintraub *et al*., 1985; Galderisi *et al*., 1999; Stein, 2001; Dias & Stein, 2002; Biroccio *et al*., 2003; Kalota *et al*., 2004) targeted to the complementary sequence in mRNA, including antisense RNA, antisense oligodeoxynucleotides and ribozymes; 2) the triple helix-forming oligomers (Dervan, 1992; Helene, 1994; Shevelev *et al*., 1997) targeted to the double stranded DNA gene; and 3) the sense oligodeoxynucleotides designed to act as decoys to trap regulatory proteins (Morishita *et al*., 1998). The "antisense" and "triple helix" techniques seem very promising, stopping the protein synthesis at transcription level (Green *et al.,* 1986), and translation level (Derwan, 1992), respectively.

Other recently introduced technologies include those of triple helix, TH (Dervan, 1992; Helene, 1994), as well as potentially useful siRNA (Boado, 2005; Pai *et al*., 2006) and miRNA (microRNA) (Berezikov *et al*., 2006). The role of 21-23 mer double-stranded RNA (si RNA) in the silencing of genes is strongly similar to that of the TH DNA mechanism, which also involves 23 mer RNA (Helene, 1994). As to miRNAs, they are noncoding RNA molecules of 21 to 24 nucleotides that can regulate gene expression at the post-transcriptional level. Moreover, miRNA may play a fundamental role in tumorigenesis, controlling cell proliferation and apoptosis; in gliomas, the miRNA (microRNA-21) level has been reported to be elevated (Corsten *et al*., 2007). Whether or not siRNA technology or miRNA knockdown will supplant the AS oligodeoxynucleotide approaches remains in question at this time, because we do not yet have final clinical results (Dias & Stein, 2002; Pai *et al*., 2006; Corsten *et al*., 2007).

2.1 Antisense approach

The "discovery" of antisense approach was done by the groups of F. Jacob and R.M. Harland (Rubinstein *et al*., 1984; Weintraub *et al*., 1985). This event has been suggested to physiologically occur as the regulation mechanism of gene expression in cells. Some years

ago, regulating activities of untranscribed DNA strand ("antisense" strand) has been suggested (Ring & Roberts, 1994). It has also been widely proven that a lot of genes present an open reading frame on the antisense strand (Merino *et al.,* 1994; Yomo & Urabe, 1994; Campbell *et al.,* 1994). The role of this natural antisense RNA is not yet understood. More recently, it was found that mouse thymidine kinase (Tk) gene expression is regulated by antisense transcription: a putative promoter in intron 3 of the murine Tk will transcribe this antisense RNA. However, concerning natural antisense RNA in prokaryotes, it has been shown that they could play a regulatory role in replication, transcription or translation steps of some genes; it was demonstrated that the translation of the bacterial enzyme transposase was controlled by an antisense RNA (Weintraub *et al.,* 1985).

An antisense RNA, hybridized on its complementary sequence in a mRNA blocks the ribosome progression during the translation of the mRNA. This observation constitutes the "starting point" of the antisense or non-sense approach (Rubinstein *et al.,* 1984) based on antisense RNA or antisense oligonucleotides to modulate artificially and specifically the expression of genes involved in important cellular processes. The mRNA complementary sequence is introduced in the cell either by a plasmid vector (dsDNA) coding for an antisense RNA or by a single stranded oligonucleotide form. The plasmid vector allows the intracellular transcription of antisense RNA which can strongly hybridize to the mRNA and stop the translation. Generally, an effective inhibition demands a high copy number of antisense RNA relative to mRNA . The antisense oligodeoxynucleotides, once in the cell, can stimulate the ribonuclease H after hybridization with target RNA. This enzyme, which is implicated in DNA replication, damages RNA moiety of the hybrids formed in the cell. On the other hand, the antisense oligonucleotide can remain as nondegraded, hybridizing to another messenger and inducing the degradation of this mRNA. In this way, in the presence of RNAse H, the antisense oligonucleotide acts in a catalytic marrow, with the enzyme potentiating the antisense effect (Hélène, 1990).

The chemical stability of plasmid-derived antisense RNA seems much more efficient than that of antisense oligonucleotides delivered directly into cells. The antisense oligonucleotides are exposed to intra- and extracellular nuclease actvity. Antisense oligomers action can be reinforced by association with polycations like polyethyleneimin (PEI), polylysine or cationic lipids (DOTMA, DOTAP) facilitating endocytosis of oligomers (Galderisi *et al.,* 1999). These positively charged molecules are also used for transfection of cells with plasmids encoding antisense RNA.

The first antisense oligonucleotide used in clinical pharmacology was as anti-cytomegalovirus therapy (Vitravene™) (Vitravene Study Group, 2002). The antisense strategy was then largely used in order to analyze gene expression and intron splicing. The phosphorothioates are the most widely studied oligonucleotides, because of their nuclease stability are highly soluble and have excellent antisense activity. These data have led to the introduction of phosphorothioate oligonucleotides into clinical therapeutic trials (melanoma, chronic lymphocytic leukemia, lung cancer and other tumors) (Jansen *et al*., 2000; Geiger *et al.,* 1998).

A good example of a new generation oligonucleotide is the N3' P5' PN. The PN exhibits highly selective and specific antisense activity *in vitro* and *in vivo*. An 11-mer PN, complementary to junction region of the *bcr-abl* mRNA (thought to be a determinant of the chronic myelogenous leukemia phenotype) efficiently inhibited the growth of treated BV173 cells (Gryaznow *et al*., 1996). Another exemple of new generation oligonucleotides concerns antisense survivine oligonucleotides, ASODN, which were transfected into gastric cancer

cell line SGC 7901 (Yang *et al*., 2004). ASODN caused a statistically significant reduction of cell viability and the cell growth was significantly inhibited. A significant loss of survivin mRNA was also presented, and the protein level was significantly decreased. ASODN may provide a novel approach to therapy of gastric cancer.

The antisense technology was used to study several protein actions: the alpha subunit of human chorionic gonadotrophin in choriocarcinoma cells (Cao *et al.,* 1995); the regulating protein E2F-1, in S cellular cycle phase, and its action on genes linked to proliferation (Sala *et al.,* 1994); nerve growth factor (NGF) in skin of transgenic mice, and its relationship with response to mechanical stimuli (Davis *et al.,* 1993). Lately, the antisense strategy is "classically" used to analyze gene expression and intron splicing. The same technology was employed to study the function of the heat shock protein hsp70, overexpressed in mouse fibrosarcoma cells; a direct correlation was found between hsp70 overexpression and tumorigenicity of cells. Cells which express high rates of hsp70 are resistant *in vitro* to cytotoxic cells and macrophages (Jaattela, 1995).

The action of IGF-I - BP-4, insulin-like growth factor I – binding protein 4, has also been studied using antisense strategy. The IGF-I – BP-4 was shown to inhibit the mitogenic effect of exogenous IGF on IIT29 tumor cells (Singh *et al.,* 1994). The same antisense strategy was applied to study p27kipl protein. The quiescent state of cells needs the p27. The inhibition of p27 expression induces the progression of cell cycle and the cyclin D1 promoter activity. Hamster fibroblasts transformed in this way grow faster than non-treated cells, even in serum free medium (Rivard *et al.,* 1996).

In "antisense" anti-tumor experimental therapy different strategies were applied coming from 1992. Among them were strategies based on :

- antisense oncogenes (i.e. Okabe *et al.,* 1993);
- antisense of genes encoding enzymes (i.e. Ahmad *et al.,* 1994);
- antisense of protein related to MHC expression (i.e. Lichtenstein *et al.,* 1992) and
- antisense of genes encoding growth factors (i.e. Trojan *et al*, 1992); the last antisense strategy seems to constitute the most promoting approach in clinical trials. Some examples of gliomas experimental studies are done in Table 1.

microRNA 21 (miR-21)	Antisense oligonucleotide Experimental therapy	Shi et al. Zhonghua Yi 2008;25(5):497
TGF beta and specific. immun. activation	Antisense oligonucleotide (NPs) Experimental therapy	Schneider et al. J Neuroimmun 2008; 195(1-2): 21.
TGF beta and immun. activation	Antisense oligonucleotide Experimental therapy	Vega et al. Future oncol 2008; 4(3): 433
heat shock protein 27 (Hsp27)	Antisense oligonucleotide Experimental therapy	Aloy et al. Int J Radiat Oncol Biol Phys 2008; 70(2): 543.
VEGF	Antisense (vector) Experimental therapy	Lin et al. Cancer Sci 2008; 99(12): 2540
TGF beta 2	Antisense oligodeoxynucleotide Clinical trial	Schlingensiepen et al. Rec Res Cancer Res 2008; 177: 137.
IGF-I	Antisense (vector) Clinical trial	Trojan et al. JAC 2008/09; 1: 1.

MiR221/222	Antisense oligonucleotide Experimental therapy	Zhang et al. Zhonghua Zhong Liu Za Zhi. 2009; 31(10): 72
TGF beta 2	Antisense oligodeoxynucleotide Clinical trial	Hau et al. Expert Rev Anticancer Ther 2009; 9(11):1663.
IGF BP2	Antisense (vector) Experimental therapy	Moore et al. Proc Natl Acad Sci USA 2009; 106(39): 16675
CD133/prominin-1	Antisense oligonucleotide Experimental therapy	Yao et al. Oncol Rep 2009; 22(4): 781.
EGFR	Antisense oligonucleotide Experimental therapy	Loew et al. Anticancrer Agents Med Chem 2009; 9(6): 703.
TGF beta	Antisense oligodeoxynucleotide Clinical trial	Vallieres IDrugs 2009; 12(7): 445.
microRNA-21	Antisense oligonucleotide Experimental therapy	Li et al. Brain Res 2009; 25(1286): 13.
miR221/222	Antisense oligonucleotide Experimental therapy	Zhang et al. Int J Oncol 2009; 34(6): 1653.
VEGF	Antisense (vector) Experimental therapy	Yang et al. J Neurooncol 2010; Aug 26 Epub
miR-21	Antisense oligonucleotide Experimental therapy	Zhou et al. Oncol Rep 2010; 24(1):195.
c-Met	Antisense oligonucleotide Experimental therapy	Chu et al. Oncol Rep 2010; 24(1):189.
AKT2	Antisense oligonucleotide Experimental therapy	Zhang et al. Oncol Rep 2010; 24(1):65.
EGFR	Antisense oligonucleotide Experimental therapy	Li et al. Oncol Rep 2010; 23(6): 1585.
PED/PEA-15 (ERK1/2-interacting protein)	Antisense oligonucleotide Experimental therapy	Botta et al. Hum Gene Ther 2010; 21(9): 1067.
miR-21 & 5FU	Antisense oligonucleotide Experimental therapy	Ren et al. J Biomater Sci Polym Ed 2010; 21(3): 303.
miR-21	Antisense oligonucleotide Experimental therapy	Zhou et al. Lab Invest. 2010; 90(2): 144.
EGFR	Antisense oligonucleotide Experimental therapy	Kang et al. J Biomed Mater Res A 2010; 93(2): 585
TGFbeta & T cell therapy	Antisense oligodeoxynucleotide Clinical trial	Dietrich et al. Curr Opin Oncol 2010; 22(6):604
IGF-I	Antisense (vector) Clinical trial	Trojan et al. Biomed & Pharmacother 2010; 64(8): 576.

Table 1. Examples of experimental and clinical gene therapies of gliomas using antisense technology (selection from the 2008s).

2.2 Triple helix approach

Since the 1990's, in parallel with antisense strategy, another approach – triple helix strategy is starting to be successfully introduced in experimental and clinical gene therapy trials (Scaggiante *et al.,* 1994; Postel *et al.,* 1991; Thomas *et al.,* 1995). The triple helix (TH) technology is the new approach, which belongs together with antisense approach to anti-gene strategies *sensu lato*, i.e. the techniques targeting the expression of respective up-regulated gene. The TH technology was "discovered" by groups of P.B. Derwan (Derwan, 1992) and of C. Helene (Helene, 1994). Its action is well defined by gene inhibition at the translation level. In brief, the short specific oligonucleotides (so called triple-helix forming oligonucleotides, TFOs) are delivered to cells both by cell transfection with chemical carriers and via vector plasmid that can drive the synthesis of TFO RNA. TFOs link to genomic double-strand DNA, form triple-helix structure with target gene and strongly inhibit its expression at transcriptional level. A triple-helical structure on DNA is considered to block transit of RNA polymerase. TFOs are usually targeted against polypurine/polypyrimidine sequences located in control regions (promoters) of the genes of interest (Derwan, 1992).

The examples of the inhibitory activity of triplex-forming oligonucleotides on target genes involved in tumorigenesis are now available (i.e. Giovannangeli & Hélène, 1997; Vasquez & Wilson, 1998). Most of the TFOs are targeted to polypurine-polypyrimidine sequences located in control regions of the gene of interest and are cell delivered via transfection with various chemical carriers. An alternative way to introduce TFOs in cells is to use a plasmid vector that can drive the synthesis of an RNA triplex-forming oligonucleotide inside the cells. This TFO generated in situ is therefore protected from degradation by nucleases and could reach its DNA target without being trapped in lysosomal vesicles. Obviously, it could be transfected in cells via either standard cell transfection procedures or via ways similarly used in virus-based gene therapy. An application of this triplex-based approach has been used for the inhibition of the IGF-I which plays a major role in tumorigenesis (Shevelev *et al.,* 1997).

Triple helix strategy was also applied to the ras oncogenes which are the most frequently activated oncogenes in human cancer. In vitro transcription of human Ha-ras was inhibited by triplex-forming oligonucleotides targeted to sequences recognized by the Sp I transcription factor (Mayfield *et al.,* 1994). Growth factors are known to play a role in tumorigenesis, and thereby represent relevant targets for antigene therapies. The synthesis of human tumor necrosis factor (TNF), which acts as an autocrine growth factor in various tumor cell lines including neuroblastoma and glioblastoma, has been blocked by triplex-forming oligonucleotide treatment (Aggarwal *et al.,* 1996

2.3 Biotechnological limitations

Human gene therapy is defined as a medical intervention based on the administration of genetic material in order to modify or manipulate the expression of a gene product or to alter the biological properties of living cells. Cells may be modified *ex vivo* for subsequent administration or altered *in vivo* by gene therapy products given directly to the subject. Example that falls under this definition includes use of antisense oligonucleotides to block gene transcription or use of sequence-specific oligonucleotides to correct a genetic mutation (Miller & Simek, 2000).

The specificity of antisense mechanism of action should be verified by: a proof of cellular uptake, the use of multiple control oligonucleotide sequences and direct measurement of

target mRNA or protein levels. Anyway, phosphorothioate oligonucleotides are in general able to produce a wide spectrum of nonspecific effects, especially at high concentration. Fortunatelly non-antisense effects can be therapeutically useful although their unpredictability can confound research applications of these biologically active molecules in human gene therapy (Lebedeva & Stein, 2002).

Antisense oligonucleotides have been widely employed as a method to decrease tumor cell viability and chemoresistance and to induce apoptosis *in vitro* and *in vivo.* The "weakness" of oligonucleotides is not only their sensitivity to nuclease digestion, which affects their half-life in culture and *in vivo,* but also their inappropriate intracellular compartmentalization. It seems that the most reliable way to choose an antisense sequence is the "mRNA walking" method (i.e. in bcl-2 antisense) (Lebedeva & Stein, 2002).

Undesirable properties have been identified for phosphorothioate oligodeoxynucleotides. When dosed at high levels it is possible to identify toxicities in rodents and primates. However, at doses currently under evaluation in the clinic, phosphorothioate oligodeoxynucleotides have been well tolerated. Extensive medicinal chemistry efforts have been successfully focused on identifying improved antisense oligonucleotides. Oligonucleotide modifications have been identified that exhibit increased resistance to serum and cellular nucleases, enabling use of oligonucleotides that do not have phosphorothioate linkages (Benett *et* al., 2000). The tissue distribution of oligonucleotides may be altered with either chemical modifications or formulations. The modified oligonucleotides have been described that potentially exhibited less toxicity than first-generation phosphorothioate oligodeoxynucleotides. Because experience with these modified oligonucleotides is rather limited, it remains to be seen whether they will have a distinct toxicity profile. The data also suggest that oral delivery of antisense oligonucleotides may be feasible, which would increase the utility of the technology. Identification of second- and third-generation oligonucleotides should ameliorate therapies for patients (Benett *et al*., 2000).

3. IGF-I and tumorigenicity

There is a convergence between ontogenesis and cancerogenesis and the same specific antigens (oncoproteins) are present in embryo/fetal tissues and in corresponding neoplastic developing tissues. The development of the brain is related to appearance of specific antigens. These disappear in mature brain and reappear in the development of neoplastic nervous tissue development. Gene expression during neoplastic brain development concerns oncoproteins (such as alpha-fetoptrotein, as well as serum albumin) (Trojan *et al*., 1984), growth factors and their respective receptors (i.e. IGF-I, EGF, FGF, VEGF, TGF-alpha and -beta) (Baserga, 1994). Their down stream proteins and glycogen signalling elements including glycogen synthase (GS), are also involved (Patel *et al*., 2004; Trojan *et al*., 2007). In 1992 Trojan and his co-workers have demonstrated that an Insulin-like growth factor 1, IGF-I, is present in glioma cells but absent in neuroblastoma cells (Trojan *et al*., 1992). Using teratocarcinoma model, Trojan and his co-workers have shown that neoplastic hepatocytes express IGF-I and IGF-II, and neuroblastic cells express IGF-II (Trojan *et al*., 1994). These observations permitted to study separetly, using IGF-I and IGF-II as the oncoprotein markers, different groups of diseases: of glial, neural and digestive tube and hepatocyte origin.

IGF-I is a 70-amino acid polypeptide involved in cell and tissue differentiation (Daughaday *et al.*, 1972; Froesch *et al.*, 1985; Baserga, 1994; Trojan *et al.*, 1994) coded by IGF-I gene (Sussenbach *et al.,* 1992). IGF-I plays an important role in growth as a mediator of growth hormone, GH, and a locally acting stimulator (Froesch *et al.,* 1985; Le Roith *et al.,* 2001). The action of IGF-I on cellular metabolism depends on binding proteins, IGFBP, which prolong the half life of this factor and modify its interaction with receptor (i.e. Rosen, 1999). IGF-I acts via specific IGF-I receptor and subsequent activation of a protein tyrosine phosphorylic signal transduction cascade, similar to that of insulin action (Werner and Le Roith, 2000). Through its binding to IGF-I-R, which activates a protein tyrosine phosphorylic signal transduction cascade, PI3K/AKT/GSK3, similar to that of insulin action (Adams et al., 2000), IGF-I has been reported to block the apoptosis pathway (IRS/PI3K/AKT/Bcl or GSK3 or Ca^{++} or caspases). Such a blockade occurs at the cytoplasmic and nuclear levels in a variety of cell lines, including neuronal and glial cells (D'Mello *et al.,* 1993; Baserga, 1994; Mason *et al.*, 2000, Chrysis *et al.*, 2001). The anti-inflammatory and anti-apoptotic effects of IGF-I are established through an increase of phosphatidylinosotol 3' kinase (PI3 kinase) activity and a maintain of Bcl-2 survival proteins. PI3 kinase is directly related to insulin receptor substrate (IRS-1), the latter following the tyrosine kinase (IGF-I receptor) (D'Ambrosio *et al.,* 1996). IGF-I being known as a factor protecting cells from apoptosis, different researchers have tried to stop apoptotic effect using the approach of antisense IGF-I receptor (Resnicoff *et al.,* 1994). The block of IGF-I synthesis, induces apoptotic and also immunogenic phenomenons (Upegui-Gonzalez *et al.,* 1998).
The human IGF-I gene is located within a region of over 85 kb on the chromosome 12 - 12p22 (Daughaday and Rotwein, 1989; Sussenbach *et al.,* 1992). Deregulated expression of growth factors and/or their receptors, and especially of IGF-I, is associated as well with growth as with pathology of different diseases, including tumors (Trojan *et al.*, 1993; Baserga, 1994; Rubin and Baserga, 1995). Since last Symposium "IGFs and Cancer", held in Halle in Germany (15-17.09.2000), IGF-I is considered as a diagnostic marker and a biological modulator in different types of tumors, especially in brain tumors (Zumkeller & Westphal, 2001).

4. Methodology

Described methodology established for an experimental preclinical research was applied for clinical research.

4.1 Plasmids

The episome based plasmid pMT-Anti IGF-I was constructed as previously described (Trojan *et al.,* 1992). The vector pMT-EP, under the control of the metallothionein, MT-I, inducible promotor was used as its base. 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. Comparatively, the same plasmid was prepared containing either CMV or HS (heat shock) promotors. The vector expressing IGF-I triple helix (pMT-AG triple helix) was constructed as previously described (Shevelev *et al.,* 1997). This cassette consists of a 23 bp DNA fragment cloned into the vector pMT-EP which transcribes 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 with either the lac-Z reporter gene, or cDNA expressing IGF-II antisense RNA as insert was used in control experiments (Trojan *et al.*, 1994).
Vectors encoding MHC-I or B-7 antisense cDNA were constructed in the laboratory of J. Ilan (CWRU, Cleveland) using pMT-EP containing the neomycine (G418) resistance gene instead of the hygromycin B resistance gene, and the MHC-I or B7 insert in antisense orientation in place of the IGF-I gene sequence.

4.2 Cell culture

Human primary glioma cell lines (Anthony *et al.*, 1998; Trojan *et al.*, 2003), were cultured in DMEM+F12 (v/v) (GIBCO-BRL) supplemented with 10 % FCS, 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37° C and 5 % C02. Hygromycin B (Boehringer Mannheim) at a concentration of 0,005 mg/ml was added 48 hours after transfection to select for transfected cells. Then the concentration of hygromycin for cell culture was determined as previously described (Anthony *et al.*, 1998). B-104 rat neuroblastoma cell line (obtained from ATCC) was used as a negative control (Trojan *et al.*, 1992).
Primary cell cultures of human glioma were derived from tumors of glioblastoma patients during surgical resection in the University Hospital of Cleveland, OH, Hopital Val-de-Grace, Paris and the Medical University Hospital of Bydgoszcz (5 to 6 cases from every hospital). Surgical sections approximately 3x3 mm X 1-2 cm in length were placed in DMEM containing high glucose concentration, 100 U/ml penicillin and 100 U/ml streptomycin. Specimens were then transferred to phosphate buffered saline (PBS) containing no Ca2+ or Mg2+ and dissected into 1-2 mm fragments. The tissue was then centrifuged at 1500 rpm x 5 min. The pellet was resuspended in DMEM containing 20 % FCS supplemented with 2mM glutamine, 100 U/ml penicillin, 100 microg/ml streptomycin and 10 ng/ml EGF. Cell suspensions were adjusted to a concentration of 2 million cells / well in 6-well plates and incubated at 37 C and 5 % C02 in culture medium containing 10 ng/ml EGF (Sigma) (GIBCO). After two days, dead cells were removed and incubation was continued in DMEM containing 10% FCS, and no EGF, for three additional days. The medium was then changed to DMEM minus FCS and incubation was continued x 48 hours. Following this first week, cells were maintained in 5% FCS / DMEM / 10% C02 / 37° C until stable transfection was established (approximately 4 weeks).

4.3 Transfection

Cultures of cells, 60-80 % confluent, were transfected in 6-well plates utilizing a ratio of 1 µg plasmid DNA per 400 000 cells. The FuGENE 6 Transfection Reagent (Boehringer Mannheim) was used according to the supplier's instructions. To determine the efficiency of transfection, the process was carried out using the 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 5mM K3Fe(CN)6, 2mM MgCl2, 0,8 mg/ml X-gal made in PBS. The selected IGF-I « antisense » or « triple helix » cell clones (expressing MHC-I and B7) were co-transfected with vectors either encoding MHC-I or B7 antisense cDNA , in the presence of 0,4 mg/ml of G-418 .

4.4 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 ; 60 µM Zn S04

(Sigma) was then added x 5 hours to induce the MTI promoter. The cells were then prepared for Northern blot. Labeling of human IGF-I cDNA and chicken beta actin cDNA and hybridizations were done according to Maniatis and procedures previously described (Trojan *et al,* 1992, 2003); the 770 bp human IGF-I cDNA and 500 bp rat IGF-I cDNA used as probes were a gift from J. llan (CWRU, Cleveland). The Northern blot was used also to verify expression of IGF-I in solid glioblastomas.

4.5 Histology

The removed human samples of tumours 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).

4.6 Immunocytochemistry and flow cytometry analysis (FACS)

Immunocytochemical localization of IGF-I protein was done by the immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Cells were fixed in 4 % paraformaldehyde. Polyclonal antibodies against rat and mouse IGF-I and against human IGF-I were purchased from Valbiotech (Paris, France).

For FACS, paraformaldehyde-fixed cells were treated as described earlier (Trojan *et al*., 1996). Stained cells were analyzed for MHC-I, MHC-II and B7 antigens, as well as for CD antigens of PBL cells, in a FACSCAN flow cytometer (Becton Dickinson).

4.7 Fluorescein cell - death detection

Apoptosis was determined by dUTP-fluorescein terminal transferase-labeling of nicked DNA (TUNEL apoptosis assay). The « In situ Cell Death Detection Kit, fluorescein » (Boehringer Mannheim) was used according to supplier's instructions.

4.8 Preparation of cell membranes

Human glioma cells membranes were prepared according to the method of M.A. Matlib with modifications (Matlib *et al.,* 1988). The Na+-Ca2+ exchange system in vascular smooth muscle cell membrane vesicles isolated from cultured cells and from tissue is similar. Homogenization of tissues was performed on ice by Polytron homogenizer in 20 mM MOPS, 250 mM sucrose, 0.05% BSA, 0.25 mM PMSF, pH 7.5. Homogenates were centrifuged for 10 min at 1000 x g, and the supernatant was recentrifuged for 15 min at 10000 x g. Microsome membranes were sedimented from the supernatant by centrifugation for 60 min at 100000 x g. The pellet was resuspended in 20 mM MOPS, pH 7.5, layered on top of 0.8 M sucrose in 20 mM MOPS and centrifuged in SW-27 bucket rotor (60 min x 24000 rpm). The pellet was collected from the interphase and recentrifuged under the same conditions. Finally, the membrane pellet was resuspended in 20 mM MOPS, pH 7.5, frozen in liquid nitrogen and stored at -70^{o}C. For treatment of glioblastoma patients, the membrane pellet resuspended in MOPS, was, one hour befor vaccination, ressuspended in PBS (0.9% NaCl, pH 7.5) in ratio 1: 100.

4.9 Vaccination of glioblastoma patients

Human glioma cell lines were transfected with the “triple helix” pMT–AG TH plasmid vector. Clones of transfected cells (down-regulated for IGF-I and expressing MHC-I and B7 molecules) were selected after two months - coming from a day of transfection. Before

injection the cells were irradiated. The first injection was done using the membranes only of so prepared 1 mln cells - injected subcutaneously into the left arm of operated glioblastoma patients (The next 3 weeks permitted to prepare a sufficient number of 5 million cells for the second injection, and then for the third injection). The blood was collected before the first vaccination, and then 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.
The samples of monoclonal antibodies were used for flow cytometer analysis as follows: conjugated to FITC - (a) CD45, (b) CD 4, (c) CD3, (d) CD25, (e) CD45RO, (f) CD19, (g) CD8, (h) CD8CD11b+, (i) control antibody IgG1, and those conjugated to PE – (a) CD14, (b) CD8, (c) CD16+CD6, (d) CD4(CD8), (e) CD4(CD8), (f)CD5, (g) CD8CD11b-, (h) CD8CD28, (i) IgG2.

5. Results

The approval for the gene therapy clinical trial (based on NIH clinical study n°1602, Bethesda, Maryland, 24. 11. 1993) was administrated by the Bioethical Commission of the L. Rydygier Medical University, Bromberg (Bydgoszcz), Poland (n° KB/176/2001, 28. 06. 2002) and registered by international Wiley Gene Therapy Clinical Trial database n° 635 and 636 (J. Gene Med., updated 2002), and by NATO Science program (LST 980517).
Primary glioma cell cultures were established from biopsies of human GBM (Trojan *et al.*, 1996). The established cell lines were transfected with "antisense" or "triple helix" IGF-I vector. The cells were down regulated in IGF-I and presented both MHC-I and B7.1 molecules. The IGF-I antisense cells or "vaccine" were irradiated before injection into status post-surgically resected glioblastoma patients. The significant changes observed were primarily after the first vaccination (Fig. 1). The phenotypic changes in peripheral blood lymphocytes were as follows. There was an increase in the percentage of CD8+T cells with a characteristic CD8+CD11b- and CD8CD28+ phenotype after each of three vaccinations, the alteration that may reflect the enhanced activation of T cytotoxic cells in blood (Fig. 2). Additionally, an increased percentage of the lymphocytes positive for superficial interleukine-2 receptor (CD25) was observed. No changes in other CD molecules were demonstrated (Trojan *et al.*, 2003, 2007). In our work in progress (new protocol) 4th and 5th injections of IGF-I TH cells in glioblastoma patients have been introduced. After the 4th injection the blood of treated patients showed a progressive increase in CD8 and NK cells, as compared with the 1st and 2nd injections, which underlines the *in vivo* immune effect of injected IGF-I TH cells. An increase in CD25 after the 2nd and 3rd injections was also observed. Then, after the 4th and 5th injections this progression slowed down. The only side effect observed was a post-vaccination fever of 38°C, corresponding probably to a cellular immune response (induction of T lymphocytes). These alterations may reflect the enhanced activation of cytotoxic T cells (Trojan *et al.*, 2007a).
The promising results were obtained in six Phase-I patients at University Hospitals of Cleveland, USA, in two patients in Bangkock Thailand and in four patients at the University Hospital of Bromberg (Bydgoszcz), Poland. In these Phase I trials, no unacceptable complications in patients were observed from the treatment. The only complicating finding was transit increase in temperature to 38-38.5 ^{0}C lasting 24-48 hours (confirming the presence of immune anti-tumour response). This usually occurred by 12 hours and in 8 of the 12 GBM patients that were treated. In the patients treated in the United States study, and in those investigated in Bromberg, Poland, tumour burden at time of treatment was advanced. One patient who was treated at University Hospitals of Cleveland, had lived 24

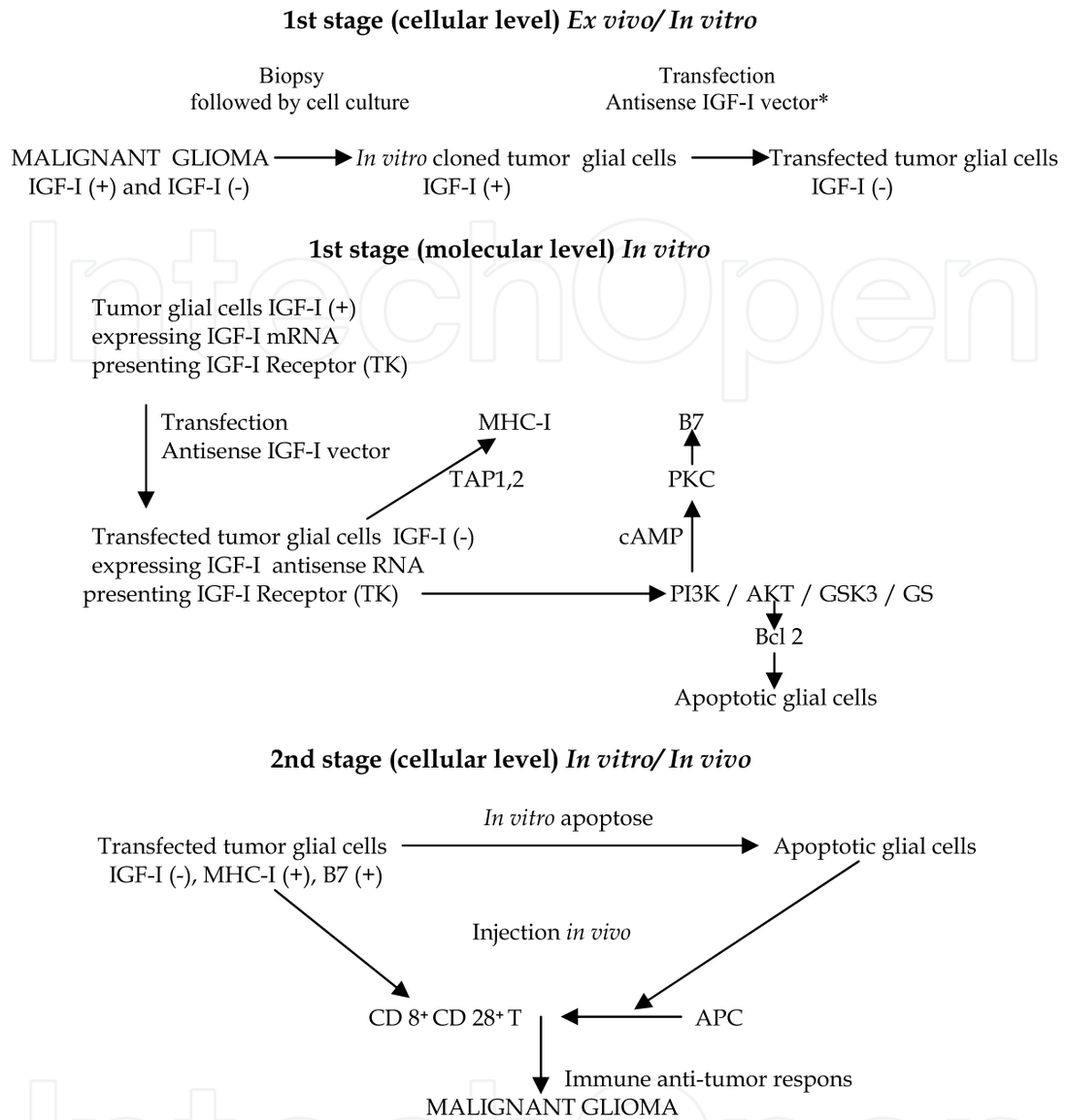

Fig. 1. Schema of IGF-I antisense therapy. The tumor cells are cloned *in vitro* to obtain a cell line positive for IGF-I. *After transfection of the cell line with a vector containing IGF-I cDNA in antisens orientation, the cells express IGF-I antisens RNA, and become negatively stained for IGF-I and positively for MHC-I and B7. Moreover they become apoptotic. Both phenomenons, immune and apoptotic, are related to signal transduction pathway (*the presented pathway is common for different growth factors as EGF, VEGF, TGF-beta or PDGF).* The injected transfected cells including apoptotic cells, together with APC cells induced *in vivo*, activate T lymphocytes (CTL $CD8^+CD28^+$); activated CTL produce immune anti tumot response (Beckner *et al.*, 2005; Fontenau *et al.*, 2002; Ly *et al.*, 2001; Trojan *et al.*, 2007a, 2010). Abréviations : 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); MAPK (MAP kinase – mitogen activated protein kinase); PKC (protein kinase C).

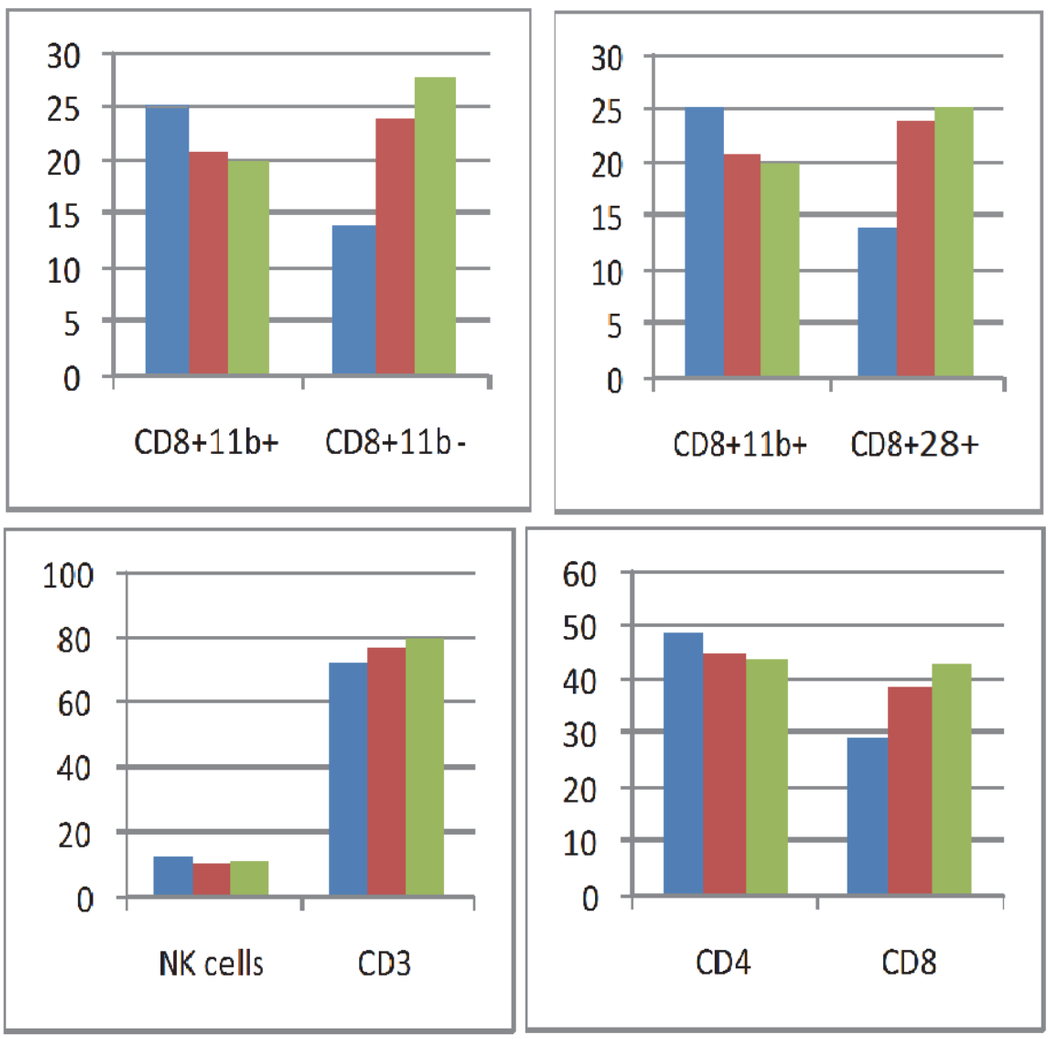

Fig. 2. Flow cytometric peripheral blood lymphocyte CD marker patterns following cellular anti - gene anti IGF-I therapy in glioblastoma multiforme. CD molecules were labelled in peripheral blood lymphocytes (PBL) obtained from prevaccinated and "vaccinated " cancer patients. Each of the first column (blue) corresponds to data obtained before vaccinations; each second and third column corresponds to data obtained after two successive cellular vaccinations. Two cases of glioblastoma were examined (every column represents the median value of two cases). The successive vaccinations consited of injections of $1.5x10^6$ to $2.0x10^6$ transfected cells. Interval between successive injections was four weeks. PBL were analyzed by flow cytometry analysis using FACScan Becton Dickinson cytometer. Double direct immunotyping with pairs of monoclonal antibodies conjugated with FITC and PE were used. Lymphocyte gate was defined according to the CD45 back gating. Data are expressed as percent of positive cells when compared to the isotype control. Difference in percentage of CD8+ CD11b- and CD8+ CD28+ subpopulations before and after vaccination was strongly significant with a range of p from 0.001 to 0.02 according to the Student's t test, and weakly significant concerning the decreasing CD8+ CD11b+ subpopulation from the relevant patients. Difference in percentage of NK, CD3, CD4 sub-populations before and after vaccinations was not significant.

months from time of diagnosis. He had been treated with conventional courses of combination chemotherapy followed by stem cell transplantation, prior to treatment with vaccine. Among five other patients treated in USA (University Hospitals of Cleveland), two of the treated patients forming a group of maximum median OS have both survived 19 months. The therapy done in USA has shown that the number of cell vaccinations (between one and four) was not related to to the median OS. Other group of three patients treated in USA, have not responded as positively to the therapy. The patient had advanced disease with cerebral oedema at the time of first treatment with vaccine, and also were receiving treatment with high dose of decadron or related steroids to reduce the effect of CNS oedema. This of course has caused further jeopardy to the immune system, and can explain the relatively negative results in three treated cases (not published data). In two of the four patients with GBM treated in Bromberg (NATO Science Programme – U.S./France/Poland), life from time of diagnosis to time of demise was 19 and 24 months. In two control treated patients, life was an average of 9,5-10 months (Trojan *et al.,* 2010). The significant clinical results were published in 2006/2007, when it was shown that using AS approach following radio- and chemotherapy, the median survival of patients reached 21 months (Trojan *et al.*, 2007a, 2007b). In 2010 we have communicated, that this relatively high median survival of glioblastoma patients could be explained by immune response related to the increase of CD28 molecules in PBL cells shown after every of two successive "vaccinations". Moreover this phenomenon was observed also in other studied tumours (four cases of liver, colon, ovary, uterus and prostate cancers (Trojan *et al.*, 2010). Histopathologic examination of resected glioblastoma tumours showed that subjects had developed peritumour necrosis and tissue bordering the necrotic tumour showed infiltration by lymphocytes consisting of both $CD8^+$ T and $CD4^+$ T cells (Wongkajornsilp *et al.,* 2011). There was no difference before or after the vaccination in the CD3, CD16+CD56, CD19, CD5, CD45 and CD14 levels.

6. Discussion

The immunosuppression phenomenon was largely described in cancer patients (Brooks *et al.,* 1981; Roszman *et al.,* 1991). TGF-beta was identified as factor suppressing T lymphocytes in tumors (Couldwell *et al.,* 1991). Surgery seems to diminish the immunosuppressive effect (Sawamura & de Tribolet, 1990). Immune response could be increased by different approaches as the injections of interferon, IL-2, activated lymphocytes, monoclonal antibodies or irradiated cells (i.e. Apuzzo & Mitchell, 1981) or using approach of IGF-I antisense treatment (Trojan *et al.,* 1993; Ly *et al.,* 2001).

Previous results have shown that tumor cells of glioma, transfected with IGF-I antisense expression vector had no longer induced tumor formation, when injected into host recipients as compared to unmanipulated cells (Trojan *et al.,* 1993). The mechanisms leading to this tumor inhibition in host animals could be drawn:

Tumor cells treated by IGF-I antisense become immunogenic to the isogenic recipients whose immune system was triggered via the novo expression of MHC-I presenting antigen as well as B7 costimulation molecule (Trojan *et al.,* 1996). The effects of antisense IGF-I and targetting to IGF-I on tumor growth could also be discussed at the molecular basis in considering the balance between survival versus death signals. Thus the role of insulin-like growth factor must also be analyzed for its inhibitotry effects on prototypical proinflammatory cytokine tumor necrosis factor alpha (TNF alpha) (Upegui-Gonzalez *et al.,* 2001). TNF alpha is a pleiotropic cytokine that promotes inflammation and signals of death.

The IGF-I antisense transfected cells, when co-transfected with vectors encoding MHC-I and/or and B-7 antisense cDNA, however maintained their previous IGF-I « antisense » morphology, the number of apoptotic cells in the cultures of the double co-transfected IGF-I antisense glioma cells decreased from 60-70 to 20-30 % (Ly *et al.,* 2001). The observation suggests that a relation could exist between immunogenicity and apoptosis in IGF-I transfected cells. They also indicate that both antigens, B-7 and MHC-I, are necessary to « render » the IGF-I antisense or triple-helix glioma cells immunogenic. The role of both B-7 and MHC-I antigens in the induction of T cell immunity against tumors has been extensively investigated (Chen *et al.,* 1992). As far as B-7 appeararance in IGF-I antisense transfected cells is considered, the absence of IGF-I synthesis would be expected to lead to a higher activation of the receptor of IGF-I (tyrosine kinase). This in turn could lead to induction in the expression of B7 antigen; enhancement in B7 co-stimulation through a cAMP mechanism linked to tyrosine kinase of the CD 28 receptor has been previously reported (Schwartz, 1992). As to the MHC-I expression, down-regulation of MHC-I due to action of IGF-I has been reported for experiments with rat thyroid cells (Saji *et al,* 1992). This would be in agreement with results reported here concerning the inverse correlation between IGF-I and MHC-I protein expression in glioma cells.

In tumor cells, the absence of IGF-I, when induced by IGF-I antisense technology, is associated with massive apoptosis. A qualitative relationship between the level of IGF-I receptor and tumorigenesis in nude mice, which correlates to the extent of apoptosis has been shown (Resnicoff *et al.,* 1996). When the function of IGF-I receptor is decreased, glioma cells undergo massive apoptosis. It was concluded for the IGF-I-R result, that this receptor activated by its ligand plays a protective role against programmed cell death. This protection was even more striking *in vivo* than *in vitro* (Resnicoff *et al.,* 1996). Another possible interpretation could be that an immune response occurring in the animals inhibits tumorigenesis. This is probably because nude mice do have a residual immune system containing both natural killer cells and B lymphocyte. The observation that C6 glioma cells transfected with IGF-I-R anti-gene approach express MHC-I (Szpechcinski *et al*., 2004) seems to confirm that both apoptosis and an immune mechanism occur in the inhibition of tumour genesis. These IGF-I-R antisense and triple helix transfected C6 cells also express protease nexin I, which may reduce the tumourigenic potential of the C6 glioma cells injected into nude mice (Rininsland *et al.*, 1997, Shevelev *et al.*, 1997).

A further elucidation of the relationship between the immune process, related to MHC-I or HLA system (Blanchet *et al.,* 1996), and the apoptotic process is under investigation. Recently it was demonstrated that dendritic cells which are involved in tumor-immunogenicity mechanisms by activation of lymphocytes CD8 in the context of MHC-I, recognize apoptotic cells (Matthew *et al,* 1998). The last data could suggest the following mechanism of IGF-I anti - gene therapy : suppression of IGF-I – induction of MHC-I and B7 – Induction of apoptosis – involving of APC cells – induction of CD8 T cells. The relationship between two phenomenons, immunogenicity and apoptosis is crucial for the discussion of mechanism of IGF-I antisense gene therapy. Moreover, this point is capital for the selection of cell clones used in gene therapy of glioblastoma in clinical trial.

The first clinical results obtained with glioblastoma using anti – gene anti IGF-I therapy are very promising. Comparatively, the most recent chemotherapy, proposing temozolomid combined with radiotherapy, has shown in recurrent glioma patients the median progression-free survival as 10 weeks, and median overall survival as 30 weeks, respectively (Stupp *et al*., 2005). However, median survival is the most important consideration to be

taken into account (Stupp reported a 14.6 months median survival). In some cases of glioblastoma a strong association between methylation of the promoter region of the gene for 06-methylguanin-DNA methyltransferase (MGMT) and a benefit from temozolomid has been demonstrated (Hegi *et al.*, 2005). Patients whose tumours had methylation of the MGMT gene, and who received chemo-radiation, had a 2-year survival rate of 46% compared with a 2-year survival rate of less than 2% in patients whose tumours had an actively unmethylated MGMT gene. On the contrary, other studies do not support the correlation of MGMT promoter methylation. Both temozolomide/MGMT and IGF-I anti-gene approaches strongly support the strategy of individualized therapy. In the case of the IGF-I anti-gene approach the verification of MHC-I and B7 in the "vaccine" of every patient is the *sine qua non* condition for success in obtaining maximum survival.

Obviously, IGF-I was not the only growth factor target as an anti-gene approach for glioblastoma treatment. The recently studied TGF-β2 antisense compound (AP 12009) gave satisfactory results in preclinical investigations, and was introduced in a clinical phase I/II study in malignant tumours, including glioblastoma (Kaminska et al., 2005, Schlingensiepen et al., 2006). The important data have been presented in international trial since 2004: in three phase I/II dose escalation studies of GBM patients, the median overall survival time (mOS) from start of the first chemotherapy after recurrence was 44 weeks. The mOS for a patient subgroup that received temozolomide as chemotherapy before AP-12009 was 46.1 weeks. In 2007 the mOS group was 28.6 months (and 75% were still alive), and in the control group, survival was 20.2 months (and 42% remained alive). In another clinical AS TGF-beta study, a phase I clinical trial of GBM was performed using autologous tumor cells modified by a AS TGF-beta2 vector (Fakhrai et al., 1996). Six patients with progressive GBM were enrolled in the trial. Patients received 2-7 subcutaneous injections of transfected tumor cells. There were indications of humoral and cellular immunity induced by the vaccine. Two patients had partial regressions and two had stable disease following therapy. The oMS was 68 weeks. mOS of the responding patients was 78 weeks (Fakhrai et al., 1996).

The *in vitro* and *in vivo* "antisense" results obtained with IGF-I and its receptor seem more significant than those obtained with other growth factors. That is probably due to a special role playing by IGF-I among other growth factors (Pollak *et al.*, 2004; Trojan *et al.*, 2007a) – thus IGF-I via IGF-I-R, not only increases cell proliferation but "supervises" mitogenic action of other growth factors (EGF, PDGF etc.) by its autocrine-paracrine stimulation, becoming somewhat of growth factors director. As to a clinical trial of glioblastoma using the antisense IGF-I-R strategy (Andrews *et al.*, 2001) 12 patients with recurrent glioblastoma and anaplastic astrocytoma were treated using an antisense oligonucleotide directed against IGF-I-R (implantation into the rectus sheath of irradiated autologous glioma cells encapsulated in diffusion chambers, after incubation with antisense IGF-I-R). Three patients were re-treated later using the same dose of oligodeoxynucleotides. Treatment was associated with incidences of vein thrombosis, but also with a rather high rate of clinical and radiological improvements. Two complete responses and four partial responses were achieved. Two patients were alive at 168 and 134 weeks after antisense therapy. Histological analysis of tumours resected from patients with disease progression revealed lymphocytic infiltration and necrosis (Andrews *et al.*, 2001). It seems that this therapy could be more efficient if the cell "vaccines" used were prepared after cloning of IGF-I-R antisense cells for MHC-I expression (Szpechcinski *et al.*, 2004). Currently, regarding IGF-I, about 400 articles are published a year, and since 2001, more than 2000 publications deal with a relationship existing between growth factors and gliomas. In this context, the treatment of gliomas using

different technologies targeting growth factors and their down stream elements, has produced a burst in use of the antisense approach, presenting almost 100 publications a year since 2005.

7. Conclusion

The presented chapter on gene therapy of GBM draws attention to the latest studies in the area of antisense cancer therapy (in relation with apoptotic and immune phenomena as well as signal transduction pathway) being among the most promising strategy of treatment of this malignant brain tumour. Although the number of "antisense" clinical trials is much lower than that of experimental preclinical therapies (Table 1), we would like to underline that every experimental therapy is a potential clinical trial, the later often depending on hospital/administrative logistics. The current clinical strategies of glioma treatment are generally a combination of chemotherapy with therapies using different types of inhibitors (imatinib, gefitinb) including antibodies (i.e. avastin) targeting growth factors and their receptors (i.e. Stupp *et al*. 2005; Reardon *et al*. 2006; Wen *et al*. 2006). The most recent therapies are now focusing on antisense technology used alone or combined also with pharmacological treatment (Dietrich *et al*. 2010). A pharmacologic strategy – the use of temozolomide, introduced by Dr R. Stupp, has offered a new hope for the treatment of this tumour. However, even though the median survival has reached almost two years, we are still far from victory (Hegi *et al.,* 2005; Gorlia *et al.,* 2008). Among the new strategies in efforts to successfully treat GBM, the use of AS approach targeting IGF-I, TGF-beta or VEGF, their receptors and their down stream transduction signalling elements (Trojan *et al.,* 2007a; Pan et al., 2007; Hau *et al*., 2009), appears to offer hope for a promising solution.

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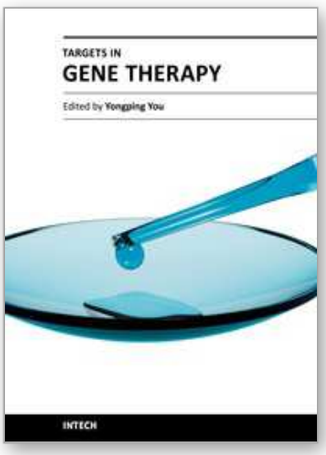

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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