From neoplastic neural development to gene therapy of brain tumors – IGF-I antisense and triple helix approaches

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Summary

IGF-I, insulin – like growth factor I, seems to play a major role in the normal and tumoral development of the nervous system. Glioblastoma is the most frequent brain tumor in man and is usually fatal. Both human and rat glioma cells express high amounts of IGF-I. When rat glioma cells are transfected with vectors expressing either IGF-I antisense RNA or inducing IGF RNA – DNA triple helix, the synthesis of IGF-I was stopped on translation or transcription levels, respectively. Down-regulation in the expression of IGF-I coincides with the reappearance of B-7 and MHC class I antigens at the surface of transfected cells. When injected subcutaneously, the transfected cancer cells initiate an immune reaction involving CD8+ lymphocytes, followed by tumor regression. The «anti-gene» strategy for clinical therapy of glioblastoma, and other tumors expressing IGF-I such hepatomas were introduced in University Hospitals of Cleveland (USA), Shanghai (China), Krakow and Bydgoszcz (Poland).

Key words:
IGF-I, antisense, triple-helix, glioblastoma, gene therapy.

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1. General view

Oncodevelopmental antigens: a-fetoprotein, serum albumin, insulin like-growth factor-I

There is a convergence between ontogenesis and cancerogenesis and the same specific oncodevelopmental antigens, in general oncoproteins, are present in embryo / fetal tissues and in corresponding neoplastic developing tissues. It was demonstrated that alpha-fetoprotein, AFP, an oncoprotein present in different neoplastic or cancer tissues (1), is also present in normal developing tissues (2).

Interest in research on AFP began in 1963, when Abelev and his coworkers discovered the reappearence of this fetal globulin in the sera of mice bearing primary hepatomas (1). Since these data appeared, a great number of studies have been done on the association of AFP, as well as on other oncofetal antigens, with neoplasia. With regard to AFP, not so much attention has been paid to its physiological role in embryonic development. Nevertheless, it is now well established that in early post-implantation embryos of mammals, the ability to synthetize AFP is restricted to the visceral endoderm cells around the embryonic region of the egg cylinder (3). Later in development, AFP is predominantly produced by the yolk sac and the fetal liver (4). In addition, a number of fetal structures that do not synthetize AFP have, a high affinity for the protein though. Thus, Benno and Williams (5) and Trojan and Uriel (6) have drawn attention to the presence of AFP in the developing rat brain, and they (2) have demonstrated the presence of the protein in several embryonic and fetal tissues, including epithelial and/or mesenchymal structures of kidney, pancreas, intestine, brain, skin, gonads, and heart. In order to ascertain whether this AFP- labeling has a specific character and, consequently, some physiological significance, we have undertaken a systematic study, using immunochemical techniques, of the tissue distribution of AFP and other serum proteins in the developing rat, mouse, and monkey as well as in the chicken embryo. The presence of AFP seems to be related to the stage of cell and tissue differentiation. AFP is absent from either undifferentiated or fully differentiated cells (2).

We have compared the localization of AFP with another oncodevelopmental antigen, an oncoprotein – serum albumin, SA. The distribution of SA and AFP and their mRNAs was investigated in primitive neuroectoblastic structures of rat and mouse embryos , and of the teratocarcinomas presenting comparative neoplastic structures. SA-mRNA gave a strong signal in differentiating structures as well as in undifferentiated cell clusters. AFP-mRNA was observed only in differentiating structures (7). In teratocarcinoma-bearing mice injected intraperitoneally with 125-Iod-radiolabeled SA and AFP, significant accumulations of both SA and AFP were demonstrated in the tumors, SA being about 3-fold higher than that of AFP after normalization to quantity of uptake in liver. In the case of comparatively studied neuroblastoma presenting only neuroblastic components (different from teratocarcinoma containing both neuroectoblastic and neuroblastic elements), the accumulation of

radiolabelled SA and AFP showed relationship 1:1. External *in vivo* photoscanning confirmed this relationship of accumulated radiolabelled proteins in both studied tumors; the last observations were useful for differential diagnosis of tumors (7). We have tried to use this technique – injection of radiolabeled oncoproteins, AFP and SA, to suppress the tumors. Unfortunately, the accumulation of radiolabeled AFP was observed not only in the tumors, but also in some physiological liquids as urine.

In 1992, Trojan and his co-workers have demonstrated that another oncodevelopmental antigen, an insulin like-growth factor, IGF-I (8-12), is present in glioma cells but absent in neuroblastoma cells (13). IGF-I is a 70-amino acid polypeptide involved in cell and tissue differentiation (8). 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 (14). These observations permitted to study separately, using IGF-I and IGF-II as the oncoprotein markers, different groups of diseases: of glial, neural and digestive tube or hepatocyte origin. IGF-I is present in early stages of embryonal development (8-10,15,16). Since the late 80's, the presence of IGF-I in the blood has been considered as a potential marker of neuropathological diseases (11,15,17-19). According to Baserga (11), IGF-I is actually recognized as the most important growth factor related to the differentiation and maturation of developing tissues (as it was suggested earlier (19)). IGF-I and -II are expressed in 17 different tumors (for references see 20).

2. Gene therapy
Insulin like-growth factor-I and IGF-I antisense and IGF-I triple-helix approaches

The past twenty years have accelerated the researches related to the treatment of tumors. For example, the treatment of liver cancer with antibodies to AFP was widely used. Unfortunately, these techniques were non specific for the treated tissues. Actually, the "anti-gene" strategies offer new possibilities for cancer therapy and among them «antisense» technique seems very promising, stopping the protein synthesis at translation level (2,22); (Fig. 1). Between 1989-1994 Trojan and his co-workers demonstrated the usefulness of this strategy, in *in vitro* and in *in vivo* experiments, for gene therapy purpose (13,14,20). These results were obtained in normal animals and not nude mice models or transgenic mice model (23). Other strategy concerns the treatment of tumors using tumor vaccines composed of hybridomas: cancer cells conjugated to B lymphocytes. This strategy was explored in the treatment of hepatomas (24). Another approach of gene therapy of gliomas and hepatomas was proposed using retroviral vector containing gene encoding thymidine kinase of herpes simplex virus (25,26). This technique did not give valuable clinical results. Also another approach, using the transfer of IL-2, was not suc-

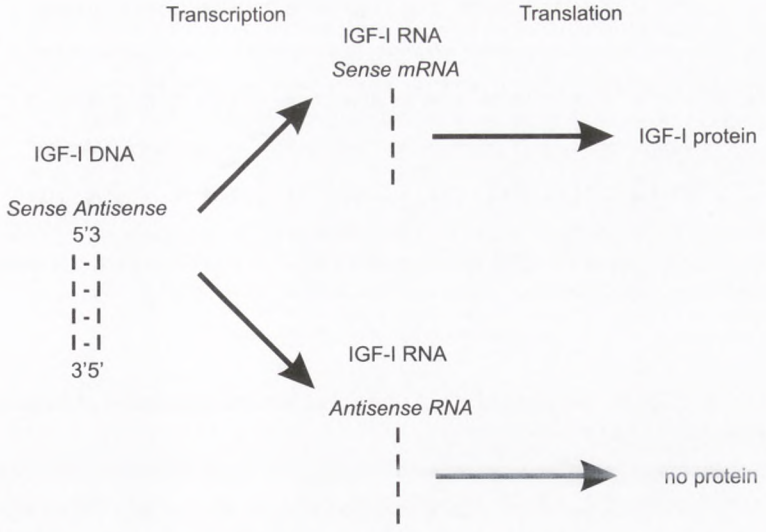

Fig. 1. The transcription – translation procedure used by cells during normal IGF-I synthesis. In IGF-I antisense technology, the antisense RNA with the antisense sequence are expressed by an episomal vector, encoding IGF-I cDNA inserted in antisense orientation, introduced into the cells. The final result is the inhibition of IGF-I mRNA (sense RNA) activity by binding to the antisense RNA (13).

cessful even when using animal model (27); the tumors regressed but did not disappeared completely. Finally, the IGF-I antisense approach and recently introduced another "anti gene strategy" – IGF-I triple- helix approach (28), appear to be among rare strategies giving the possibilities of success in clinical trial. The clinical trial based on IGFI «antisense» approach was introduced in 1997 in USA (for the treatment of glioma) and in China (for the treatment of hepatoma) (29). The IGF-I antisense and triple-helix technologies were introduced for clinical trial in Poland in 2001 (Collegium Medicum of Jagiellonian University, Kraków, and Ludwik Rydygier Medical University, Bydgoszcz).

IGF-I expressing mouse teratocarcinoma containing neuroglial and muscle tissue derivatives is widely used as a model system to study regulation of cell determination and differentiation (30). Using antisense strategies (21,22,31), we have defined a role for IGF-I tumorigenicity of teratocarcinomas, gliomas and hepatomas evasion of immune surveillance (14,20,32). PCC-3 EC embryonal carcinoma cells derived from mouse teratocarcinoma, C6 rat glioma cells and LFC rat hepatomas transfected with a vector producing IGF-I antisense RNA , pAnti-IGF-I, expressed MHC-I and B7 antigens (33). These cells lost tumorigenicity and induced a T-cell mediated immune reaction both against themselves and against their non transfected tumorigenic progenitor cells in syngeneic animals. The same IGF-I antisense transfectants were shown to elicit a curative anti-tumor immune response with tumor regression at distal sites. Moreover, using IGF-I antisense approach we have demonstrated that

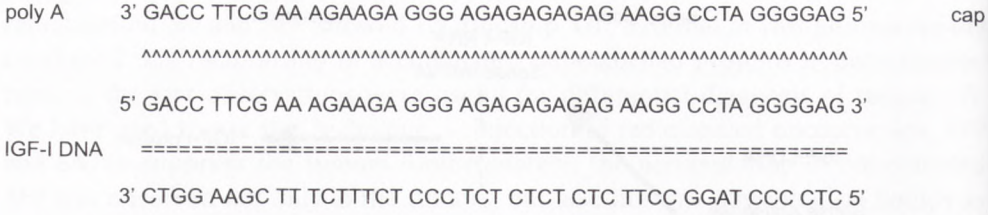

Fig. 2. Homopurine triple helix formed in *in vitro* experiment by oligopurine third strand (poly A 3' GAG...GAG 5' cap) and DNA of IGF-I (13). We admit that similar mechanism occurs in *in vivo* studies (28); ^ ^ ^ Hoogsteen hydrogen bonds; = Watson-Crick bonds.

transfected rat glioma, mouse teratocarcinoma, and mouse and rat hepatoma cells become apoptotic (34).

Recently, using triple-helix approach (35,36), we have obtained the results similar to those produced by IGF-I antisense approach. C6 rat glioma, PCC-3 mouse teratocarcinoma and AT1F1 mouse hepatoma cells were transfected with a pMT-AG-Triple vector which encodes an oligoribonucleotide that forms RNA – IGF-I DNA triple-helix structure (37); (Fig. 2). As far as the mechanism is considered, the IGF-I triple-helix approach stops the protein synthesis most likely at transcriptional level (38), while IGF-I antisense approach stops it at translation level, as it was demonstrated earlier (13). The IGF-I triple helix and antisense transfected cells presented the identical immunogenicity (MHC-I and B7 expression) and apoptotic characteristics also and showed an anti-tumor effect *in vivo* (38,39). We have concluded that IGF-I triple helix strategy (IGF-I TH) can parallel antisense approach and would be very useful in anti-tumor therapy.

Previous results have shown that tumor cells, glioma and hepatoma, transfected with IGF-I antisense expression vector, had no longer induced tumor formation when injected into host recipients as compared to unmanipulated cells (20,32). The following mechanisms leading to this tumor inhibition in host animals could be proposed:

1. Tumor cells treated by IGF-I antisense become immunogenic to the isogenic recipients whose the immune system of which was triggered *via de novo* expression of MHC-I presenting antigen as well as B7 costimulation molecule (33);

2. Expression of MHC-I could also have non immunological effects on the inhibition of tumor growth and metastasis as reported by several authors using MHC-I transfected tumor cells (40);

3. Effects of antisense or triple-helix IGF-I and targeting to IGF-I on tumor growth could also be discussed at the molecular basis in considering the balance between survival versus death signals. Thus also the role of IGF-I must be analyzed for its inhibitory effects on prototypical proinflammatory cytokine tumor necrosis factor alpha, TNF-alpha (39); TNF-alpha is a pleiotropic cytokine that promotes inflammation and signals of death. TNF-alpha – mediated activation of apoptosis is regulated at

the level of the p55 receptor (TNFR1) through a 60 Kda protein, the silencer of death domains (SODD). The response to ligand binding, the SODD is released, allowing the activation of the p55 death domain that leads to an increase of JUN amino-terminal kinase/ stress activated proteine kinases (INK / SAPK), sphigomyelinase, caspases and NO;

4. IGF-I is known as a factor protecting cells from apoptosis in different cells and tissues including neurons (41,42). The mechanism of apoptosis is related to the receptor of IGF-1 (tyrosine kinase), it is self – related to phosphorylation of IRS-1 (Insulin Receptor Substrate) (43). For this reason different researchers have tried to stop the apoptotic effect using the antisense IGF-I receptor approach (44,45);

5. Traditional characterizations of IGF-I as an important growth factor have been broadened with the recent discovery that it acts as a survival signal. The anti-inflammatory effects IGF-I are established through an increase of phosphatidylinosotol 3' kinase (PI3 kinase) activity and a maintain of Bcl-2 survival proteins. Other important response to IGF-I activation include expressison of Fos and Jun as well as mitogen – activated proteine kinase (39).

The induction of IGF-I antisense in transfected cells was followed by the change in cell morphology, increase in apoptosis and enhanced expression of MHC-I and B-7. Transfected cells were long and narrow in shape and frequently string-like in appearance. The change might either be the signal of a reversion of the malignant phenotype or the recovery of some antigenic potential of these cells. The IGF-I antisense or triple-helix transfected cells, when co-transfected with vectors encoding MHC-I and/or and B-7 antisense cDNA, maintained, however, 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% (28). 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 immunogenic cells. The role of both B-7 and MHC-I antigens in the induction of T cell immunity against tumors has been extensively investigated (24,46,47). As far as B-7 appearance 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 (48). Whether or not similar signaling through the tyrosine kinase activity of the IGF-I receptor will need to be investigated (48,49). 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 (50). This would be in agreement with the 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. Nuclear fragmentation is probably due to the

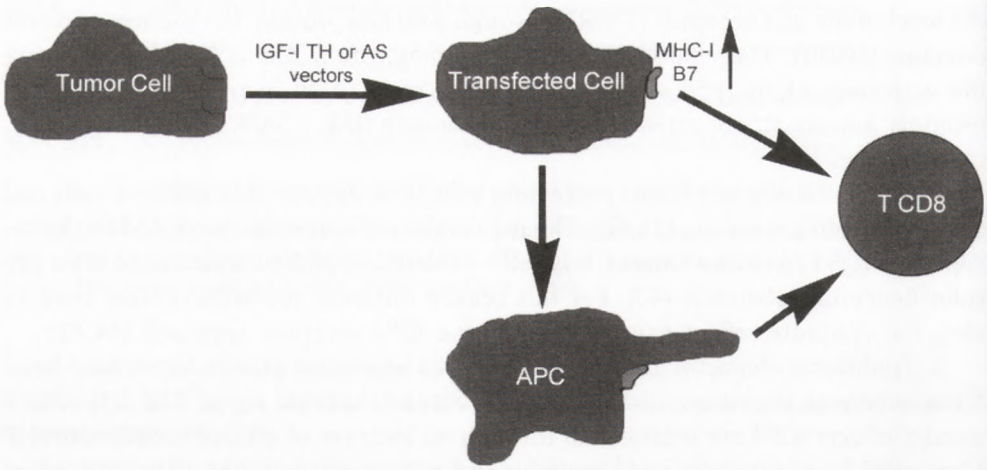

Fig. 3. Mechanism of IGF-I antisense or IGF-I triple-helix immuno-gene therapy. Tumor cells transfected with a vector of IGF-I antisense (AS) or triple-helix (TH) type express MHC-I and B7 molecules. These immunogenic cells become also apoptotic. Lymphocytes T CD8 can be activated either directly by TH or AS cells or indirectly by APC-antigen presenting cells which are involved in tumor-immunogenicity mechanisms.

effect of Ca^{2+} dependent endonuclease activity in the linker region between nucleosomes (51,52). Another characteristic of the apoptotic pathway is that integrity of the plasma membrane was preserved. Most functions of the membrane remained unchanged (52).

The presented results show that inhibition of IGF-I up-regulates B-7 and MHC-I expression in transfected "antisense" glioma and hepatoma cells. It is necessary to add that increased expression of protease nexin I which may reduce the tumorigenic potential of the C-6 glioma cells was also observed when the IGF-I «triple-helix» cells or IGF-I receptor «triple helix» cells were injected into nude mice (37,53). On the other hand, in our work concerning mouse hepatoma cells transfected by IGF-I antisense or triple-helix approach (39), we have observed the decrease of cytokines like Il-10 which is a strong immunosuppressor (54), and TNF-alpha which can act as a factor stimulating a tumoral growth (55). Moreover, we have found the increased level of TAP 1 and 2 related to MHC-I in these cells. 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 (56). The last data could suggest the following mechanism of IGF-I antisene or triple-helix therapy: suppression of IGF-I – induction of MHC-I and B7 – induction of apoptosis – involving of APC cells – induction of CD8 T cells (28) (Fig. 3).

A further elucidation of the relationship between the immune process and the apoptotic process existing in IGF-I antisense or triple-helix strategies is under investigation. One of the other basic questions concern the molecular mechanisms re-

lated to IGF-I antisense and triple-helix technologies. In the work in progress, we have already excluded RNAi phenomenon (57) in antisense approach. Using IGF-I antisense technology, only the expression of IGF-I, and never other proteins like i.e. IGF-II, were stopped (14). Yet, in anti –gene strategy, RNAi mechanism and RNA-DNA triple-helix mechanism, though superficially look alike (as far as 22 nucleotides are concerned), are to be compared. To discuss the possibility of RNAi mechanism involving in IGF-I triple-helix technolology, we add some information concerning RNAi. RNAi is the process of sequence-specific, post transcriptional gene silencing initiated by double stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The mediators of sequence–specific messenger RNA degradation are 21 and 22 nucleotide duplexes (generated in different organisms by ribonuclease III cleavage from longer dsRNA, or prepared experimentally). The results of our work in progress actually excluded this mechanism in IGF-I triple helix approach. Among other questions, we have also excluded the role of non-coding RNA (using i.e. sense and antisense RNA controls (13,37)). As to DNA methylation, in our approach based on episomal plasmid transfection (13), we cannot completely eliminate this possibility classically considered in oligonucleotides transfections. Nevertheless, we have not found the example of DNA methylation in triple-helix studies (58).

Taking together, the clinical trial of IGF-I anti – gene therapy, showing the promising results (29,59), the studies of basic molecular mechanism of triple-helix could allow to increase the efficiency of this strategy (58).

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