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Review

IGF-I: from diagnostic to triple-helix gene therapy of solid tumors✪

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Alterations in the expression of growth factors and their receptors are associated with the growth and development of human tumors. One such growth factor is IGF-I (insulin-like growth factor I), a 70-amino-acid polypeptide expressed in many tissues, including brain. IGF-I is also expressed at high levels in some nervous system-derived tumors, especially in glioblastoma. When using IGF-I as a diagnostic marker, 17 different tumors are considered as expressing the IGF-I gene.

Malignant glioma, the most common human brain cancer, is usually fatal. Average survival is less than one year. Our strategy of gene therapy for the treatment of gliomas and other solid tumors is based on: 1) diagnostic using IGF-I gene expression as a differential marker, and 2) application of "triple-helix anti-IGF-I " therapy. In the latter approach, tumor cells are transfected with a vector, which encodes an oligoribonucleotide — an RNA strand containing oligopurine sequence which might be capable of forming a triple helix with an oligopurine and/or oligopyrimidine sequence

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Abbreviations: AFP, alpha-fetoprotein; CNS, central nervous system; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF-I, insulin-like growth factor I; TAP, transporter antigen protein; TFO, triple-helix forming oligonucleotide; TH, triple helix; TNF, tumor necrosis factor.

of the promotor of IGF-I gene (RNA-IGF-I DNA triple helix).

Human tumor cells transfected *in vitro* become down-regulated in the production of IGF-I and present immunogenic (MHC-I and B7 expression) and apoptotic characteristics. Similar results were obtained when IGF-I antisense strategy was applied. In both strategies the transfected cells reimplanted *in vivo* lose tumorigenicity and elicit tumor specific immunity which leads to elimination of established tumors.

IGF-I AND DIAGNOSTIC

There is a convergence between ontogenesis and cancerogenesis and the same specific antigens (oncoproteins) like α-fetoprotein (AFP), growth hormone (GH), and growth factors, such as IGF, FGF or EGF are present in embryo / fetal tissues and in neoplastic developing tissues. It was demonstrated that AFP, an oncoprotein present in different cancer tissues, especially in liver cancer (Abelev, 1971) is also present in normal developing tissues, and particularly in the central nervous system (CNS) (Trojan & Uriel, 1979). Similarly it was demonstrated that IGF-I, insulin-like growth factor I, is present both in normal developing CNS and in neoplastic glial cells (Kiess *et al.,* 1989; Ayer-le-Lievre *et al.,* 1991; Trojan *et al.,* 1992; Sandberg *et al.,* 1998). The presence of IGF-I was confirmed in different neoplastic derivatives, including hepatic tissues, and also using the model of murine teratocarcinoma (Trojan *et al.,* 1994).

IGF-I is a 70-amino-acid polypeptide involved in cell and tissue differentiation (Daughaday *et al.,* 1972; Froesch *et al.,* 1985; Han *et al.,* 1987; Baserga, 1994; Trojan *et al.,* 1994). IGF-I plays an important role in growth as a mediator of growth hormone (Froesch *et al.,* 1985; Johnson *et al.,* 1991; 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 a receptor (Jones & Clemmons, 1995; Collet & Candy, 1998; Rosen, 1999; Hva *et al.,* 1999). Binding to a specific IGF-I receptor and subsequent activation of a protein tyrosine kinase signal transduction cascade, is similar to that of insulin (Werner & Le Roith, 2000; Adams *et al.,* 2000). IGF-I, mediated by IGF-I receptor, has been reported to block the apoptosis pathway in a variety of cell lines (Rodriguez-Tarduchy *et al.,*1992; D'Mello *et al.,* 1993; Muta & Krantz, 1993; Baserga, 1994). Conversely, blocking IGF-I synthesis induces apoptotic and immunogenic phenomena (Upegui-Gonzalez *et al.,* 1998). However in general, the molecular mechanism of control of IGF-I expression is poorly defined. Although the IGF-I gene consists of six exons (Daughaday & Rotwein, 1989; Sussenbach *et al.,* 1992), the mature human IGF-I peptide is encoded only by exons 3 and 4, and a similar situation was

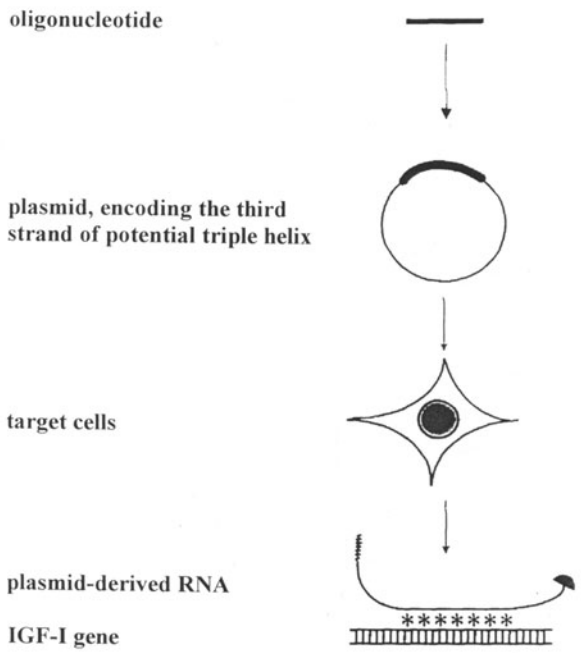

Figure 1. A schema of triple-helix approach with a 23 bp purine homooligonucleotide inserted into episomal vector (plasmid).

The vector transfected to targed tumor cells encodes RNA forming triplex with DNA of the IGF-I gene – for details see Fig. 2.

found also in the widely studied rat model (Adamo *et al.,* 1994). Deregulated expression of growth factors and/or their receptors, and especially of IGF-I, is associated with growth as well as with different diseases, including tumors (Heldin & Westermark, 1989; Antoniades *et al.*, 1992; Trojan *et al.*, 1993; Baserga, 1994; Rubin & Baserga, 1995).

In the past few years, both laboratory investigations and population studies have provided strong circumstantial evidence that IGF physiology influences cancer risk (Yu & Robin, 2000; Pollak, 2000). Evidence further suggests that certain lifestyles, such as one involving a high-energy diet, may increase IGF-I levels, a finding that is supported by animal experiments indicating that IGFs may abolish the inhibitory effect of energy restriction on cancer growth (Yu & Robin, 2000).

According to Baserga (1995), IGF-I is one of the most important growth factors related to normal and neoplastic differentiation. IGF-I is expressed in 17 different tumors (for references see Trojan *et al.,* 1993). Since the 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; Zumkeller, 2002).

The IGF system consists of IGF-I and IGF-II, the type I and type II IGF receptors, and specific IGF binding proteins (IGFBP-1 to -6). These factors regulate both normal and malignant brain growth. Enhanced expression of IGF-I and IGF-II mRNA transcripts has been demonstrated in gliomas, meningiomas, and other tumors. Abnormal imprinting of IGF-II occurs in gliomas, medulloblastomas, and meningiomas (Zumkeller & Westphal, 2001). Both types of IGF receptor are expressed in gliomas and, in particular, the type I IGF receptor appears to be upregulated in malignant brain tissue. Meningiomas and, to a lesser degree, malignant gliomas were found to synthesise IGFBP-1, supporting the notion that IGFBPs contribute towards the growth of CNS tumors in humans; glioblastoma cell lines were found to express mRNA for IGFBP-1 (42% of cell lines), IGFBP-2 (65%), IGFBP-3 (97%), IGFBP-4 (3%), IGFBP-5 (74%) and IGFBP-6 (94%) as determined by polymerase chain reaction (Zumkeller, 2002). The relationship between IGF-I and IGFBP is starting to be introduced in clinical diagnostics as one of the indications of precancerous development.

Among the numerous examples of cancer growth and metastasis monitored by the IGF-I marker (serum level) one should mention colorectal cancers (Mishra *et al.,* 1998; Manousos *et al.,* 1999; Giovanucci *et al*, 2000; Wu *et al.,* 2002; Bustin *et al.,* 2002), of breast (Vadgama *et al.,* 1999; Campbell *et al.,* 2001; Helle *et al.,* 2001; Eppler *et al.,* 2002; Bajetta *et al.,* 2002), prostate cancer (Wolk *et al.,* 2000; Mita *et al.,* 2000) and of lung (Lee et *al.*, 1999; Yu *et al.,* 1999; Olchovsky *et al.,* 2002). The role of IGFs in cancer is supported by epidemiologic studies which have found that high levels of circulating IGF-I and low levels of IGFBP-3 are associated with increased risk of several common cancers, including those of the prostate, breast, colorectum, liver and lung (Giovanucci, 1999; Yu & Robin, 2000).

IGF-I is an important mitogen required by some cell types to progress from the G1 to the S phase of the cell cycle. IGFBPs can have opposing actions, in part by binding IGF-I, but also by direct inhibitory effects on target cells. Because the tissue determinants of IGF bioactivity appear to be regulated in parallel with circulating IGF-I level, it is reasonable to hypothesize that the substantial intra-individual variability in circulating levels of IGF-I and IGFBP-3 may be important in determining risk of some cancers. In general, two- to four-fold elevated risk has been observed for prostate cancer in men in the top quartile of IGF-I relative to those in the bottom quartile, and low levels of IGFBP-3 were associated with an approximate doubling of risk. For colorectal neoplasia, four-fold elevated risk was observed in men and women with low

IGFBP-3, whereas high IGF-I was associated with a doubling of risk (Giovanucci, 1999).

IGF-I AND ANTI-GENE THERAPIES

As far as the role of oncoproteins in tumorigenesis is concerned, different approaches of anti-tumor treatment have been considered. The most classical was treatment using antibodies, i.e. treatment of liver cancer with injection of antibodies directed toward oncoproteins like AFP. Unfortunately, this type of technique was not specific enough for the treated tissues (AFP like other oncoproteins is present in different types of differentiating cells). Following the hypothesis that neoplastic differentiation is related to the presence of oncoproteins, the arrest of oncoprotein synthesis at the gene level seemed to be the best way to stop tumor development. "Anti-gene" strategies offer new possibilities for cancer therapy: antisense technique (arresting protein synthesis at the transcription level) (Rubinstein *et al.,* 1984; Weintraub *et al.,* 1985; Green *et al.,* 1986) or triple helix technique (stopping the synthesis at translation level) (Dervan, 1992; Hélène, 1994).

In the past twenty years, it has been shown that natural antisense RNA which is transcribed from one strand of DNA could hybridize to the sense RNA. This natural physiological regulation represents the basics of the antisense approach to inhibit artificially the expression of particular genes involved in human diseases. Using this antisense strategy, the translation of messenger RNA (sense RNA) can be blocked by binding a complementary strand to this mRNA. The achievement of this artificial regulation could be done using either short oligonucleotides delivered to cells *via* appropriate carriers or by using plasmid constructs transcribing intracellular antisense RNA (Trojan *et al.,* 1992).

IGF-I antisense gene therapy (Trojan *et al.,* 1993) was introduced in clinical trials to treat hepatoma (Shanghaï, China) and glioblastoma (Cleveland, U.S.A., and Bydgoszcz, Poland) (Anthony *et al.,* 1998). Glioblastoma is the most frequent and usually fatal tumor. Recently some interesting results concerning glioblastoma treatement were published by American-Asian cooperation (Wongkajornslip *et al.,* 2001). A subject inflicted with glioblastoma who underwent partial tumor resection and radiotherapy, after subcutaneous injections of IGF-I antisense transfected glioma cells for 8 weeks, developed peritumor necrosis. The latter lesion was infiltrated by lymphocytes containing both CD8 and CD4 cells. The functional activity of these lymphocytes was demonstrated by the active production of interferon gamma and tumor necrosis factor alpha.

In the triple helix (TH) technology the oligonucleotides that block gene expression are triple-helix forming oligonucleotides

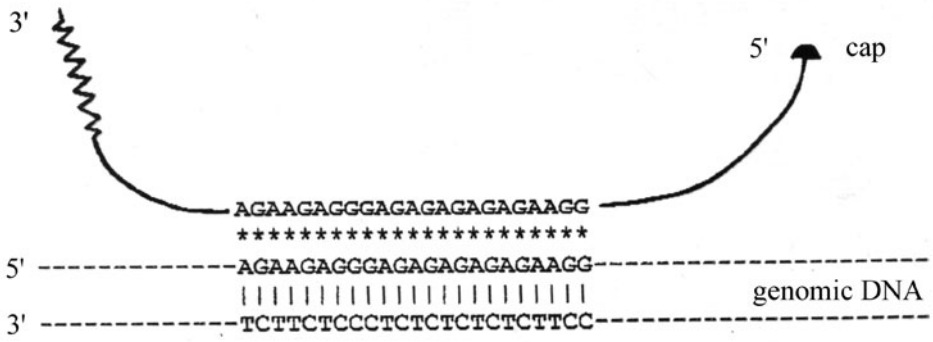

Figure 2. Potential structure of antiparallel RNA–DNA–DNA triple helix complex.

The first and second strands are genomic DNA (IGF-I); the third strand is the homopurine RNA. *****Hoogsteen hydrogen bonds; IIIIIIIWatson-Crick bonds.

(TFOs). They block RNA polymerase transit by forming a triple-helical structure on DNA (Dervan, 1992; Hélène, 1994).

The TH strategy was 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 TFOs targeted to sequences recognized by the Sp1 transcription factor (Mayfield *et al.,* 1994). Using transient transfection assays, it was demonstrated that a purine-rich TFO could also inhibit the transcription of murine c-Ki-*ras* gene in NIH 3T3 cells (Alluni-Fabroni *et al,* 1996).

Growth factors are known to play a role in tumorigenesis and thereby represent convenient targets for anti-gene 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, could be blocked by TFO treatment (Aggarwal *et al.,* 1996). TFOs were also shown to bind *in vitro* to human EGF receptor promoter (Durland *et al.*, 1991), and to inhibit *in vitro* transcription of the *HER2/neu* gene (Ebbinghaus *et al.,* 1993). The transcription of endogenous human *HER2/neu* oncogene, which is overexpressed in breast cancer and other human malignancies, was inhibited by TFO treatment of a breast carcinoma MCF-7 cell line (Porumb *et al.,* 1996).

More examples of the inhibitory activity of TFO on target genes involved in tumorigenesis are now available (Maher, 1996; Chan & Glazer, 1997; Giovannangeli & Hélène, 1997; Vasquez & Wilson, 1998). Most of the TFOs are targeted to polypurine and/or 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 the TFOs in the cells is to use a plasmid vector that can drive the synthesis of the TFO RNA 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 into cells *via* either standard cell transfection procedures or *via* ways used in virus-based gene therapy. An application of this triplex-based approach was used for the inhibition of the IGF-I protein in tumorigenesis of glioblastoma and hepatocarcinoma (Shevelev *et al.,* 1997; Upegui-Gonzalez *et al.,* 2001; Ly *et al.,* 2001).

The IGF-I triple helix (IGF-I TH) strategy shows that an RNA strand containing a 23-nucleotide (nt) oligopurine sequence may be capable of forming a triple helix in cultured human primary glioma or rat C6 cells with an oligopurine and/or oligopyrimidine sequence of the IGF-I gene. Although we can not exclude other mechanisms, triple helix formation remains the most plausible explanation for the inhibition in expression of the IGF-I gene (Shevelev *et al.,* 1997). The 23-nucleotide target regions are also present in other growth factors, such as IGF-II or FGF. These target regions are composed only of A and G bases, but their sequence is different in different growth factors. For example, the IGF-I gene expressed in glioblastoma has a 23-nucleotide target region different from the IGF-II gene in neuroblastoma (Trojan *et al.,* non published data).

Another way of using TFO delivered inside the cells *via* transcription of a plasmid vector was recently described with the IGF-I receptor gene in glioblastoma cells (Rininsland *et al,* 1997).

IGF-I TRIPLE-HELIX THERAPY: APOPTOTIC AND IMMUNE MECHANISMS

To demonstrate that IGF-I, and not another factor, plays a really important role in neoplastic diseases, gene therapy based on IGF-I TH approach was applied for experimental gliomas (Shevelev *et al.,* 1997; Ly *et al.,* 2001). This method gave as good results as the IGF-I antisense strategy applied previously for ex-

perimental glioma, teratocarcinoma and hepatoma treatement (Trojan *et al.,* 1993; 1994; 1996; Lafarge-Frayssinet *et al.,* 1997; Upegui-Gonzalez *et al.,* 1998; Ellouk- Achard *et al.,* 1998).

In the IGF-I antisense strategy, the transfectants lost tumorigenicity and induced a T-cell mediated immune reaction both against themselves and against their non transfected tumorigenic progenitor cells in syngeneic animals. Consequently, these cells were shown to elicit a curative anti-tumor immune response with tumor regression at distal sites.

C6 glioma cells transfected with an IGF-I TH vector displayed morphological changes, upregulation of MHC class I antigens and B7 antigen, followed by apoptosis similarly to the IGF-I antisense transfectants (Trojan *et al.,* 1996; Ly *et al.,* 2000). Moreover, they increased expression of the protease nexin I (Shevelev *et al.,* 1997). Dramatic inhibition of tumor growth occurred in nude mice following injection of the transfected C6 cells (Shevelev *et al.,* 1997). Similar results of the IGF-I TH strategy were obtained using a syngeneic model of PCC3 derived mouse teratocarcinoma (Ly *et al.,* 2000). We have concluded that the IGF-I TH strategy can parallel the antisense approach and should be very useful in anti-tumor gene therapy.

The role of both B-7 and MHC-I antigens in the induction of T cell immunity against tumors has been extensively investigated (Linsley *et al.,* 1990; Freeman *et al.,* 1991; Chen *et al.,* 1992; Harding *et al.,* 1992; Guo *et al.,* 1994). The explanation of B-7 appearance in the IGF-I TH transfected cells would be as follows: in our work, the transfected cells were growing in a culture medium containing a high concentration of a fetal calf serum (15–20%), while non transfected cells were maintained in a low concentration (5–8%). This could lead to a higher activation of IGF-I receptor (a tyrosine kinase); IGF-I and -II present in a fetal calf serum, as well as intracellular IGF-II act *via* the type-1 IGF-I receptor (Baserga, 1995; Lafarge-Frayssinet *et al.,* 1997). There is a relation between the signal transduction pathway of a tyrosine kinase and the induction of B7 molecules (Schwartz, 1992; Satoh *et al.,* 1995; Angelisova *et al.,* 1996); the enhancement in B7 co-stimulation through a cAMP mechanism linked to the tyrosine kinase activity of the CD28 receptor has been demonstrated (Schwartz, 1992).

As to the MHC-I expression, down-regulation of MHC-I due to the action of IGF-I has been reported for experiments with rat thyroid cells (Saji *et al.,* 1992). 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. Moreover, using tumor cells transfected with a IGF-I TH vector, we found increased level of TAP-1 and -2 in these cells, explaining also the presence of MHC-I (Ly *et al.,* 2001).

The mechanism of apoptosis is related to the receptor of IGF-I (a tyrosine kinase), itself related to phosphorylation of IRS-1 (insulin receptor substrate) (D'Ambrosio *et al.,* 1996). For this reason different researchers have tried to stop the apoptotic effect using the antisense approach to IGF-I receptor (Sell *et al.,*1993; Baserga *et al.,* 1994; Resnicoff *et al.,* 1994; Valentinis *et al.,* 1994). In glioma cells, the absence of IGF-I, caused by IGF-I antisense or triple-helix technologies, is associated with massive apoptosis (Ly *et al.,* 2000; 2001). There is a relationship between the immune process, related to the MHC-I or the HLA system (Blanchet *et al.,* 1992), and the apoptotic process — both phenomena simultaneously increasing or decreasing in IGF-I TH transfectants (Ly *et al.,* 2001). 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 IGF-I TH technology was also investigated using the model of mouse hepatoma (Upegui-Gonzalez *et al.,* 2001). The IGF-I TH transfected hepatoma cells also stopped pro-

ducing IGF-I, and recovered MHC-I expression accompanied by apoptosis but were down-regulated in the production of IL-10 and TNF-α. Injection of the transfected cells into mice bearing hepatoma at terminal-phase significantly prolonged their survival. The results suggest that injection of hepatoma cells transfected using the TH approach could constitute a vaccine against hepatoma. To our knowledge, we are the first to obtain *in vivo* results with RNA–DNA triple helix produced after plasmid vector transfection *in vitro*. The TH strategy for clinical gene therapy of tumors is currently being introduced in University Hospitals of Krakow (digestive tube cancer and liver cancer) and of Bydgoszcz (glioblastoma), Poland. Our IGF-I TH treatment is registered by the international Wiley Gene Therapy Databases – No. 635 and 636 (Gene Therapy Clinical Trials, updated September, 2001 by the *Journal of Gene Medicine*). The first clinical results obtained with glioblastoma and colon cancer are very promising. The results related to IGF-I TH treatment of colon cancer (five cases to date) are undergoing evaluation and will be published next year. For comparative purposes we have also explored the possibility of using our TH strategy for the therapy of other tumors expressing IGF-I such as breast, prostate and ovary cancers. The latter cancers will be treated at the University Hospital of Bydgoszcz in 2003.

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