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ets-1 mRNA as target for antisense radio-oligonucleotide therapy in melanoma cells

Research Article

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Summary

Angiogenesis provides a novel target for anticancer therapy, in particular radiochemo-therapy as endothelial cells in the vascular wall are sensitive to radiation. Antisense phosphorothioate oligodeoxynucleotides (AS-PODNs) may serve as vehicles for carrying cytotoxic or radioactive agents into a particular intracellular location. Radiolabelled AS-PODNs have the potential of having both antisense and radiation effects. Recently, vascular endothelial growth factor (VEGF)-induced invasiveness was shown to be specifically inhibited by AS-PODN directed against *ets-1.* Previous studies have shown that radio-oligonucleotide therapy may be effective with AS-PODNs internally labelled with ^{32}P, ^{33}P or ^{35}S. Theoretically, ^{35}S gave the smallest variation in nuclear dose in the different cell dimensions studied (Kairemo et al., Cancer Gene Ther 1998; 5: 408-12). This means that cell nuclear targets should be treated with the short range β-emitters ^{35}S or ^{33}P for optimal radio-oligonucleotide therapy. Here we explore this possibility using ^{33}P labeled 17-mer AS-PODNs directed against *ets-1* in human melanoma cells in vitro. Inhibition of cell growth was observed in the following order: labeled AS-PODN > nonlabeled AS-PODN > labeled sense PODN > nonlabeled sense PODN > transfection agent. Even with a single ^{33}P at the 5´-end of the AS-PODN melanoma cell uptake of label was approximately 0.5 mBq/cell. The nuclear doses in this experiment varied from 1.9 to 3.7 cGy. Thus, *in vitro* and *in vivo* use of radio-oligonucleotide therapy utilizing ^{33}P radionanotargeting, e.g. in angiogenesis through *ets-1,* are highly recommended.

I. Introduction

The oncogene v-ets was originally discovered as a component of a chimeric genome, along with a truncated v-*myb* gene, present in the genome of E26, an avian leukosis virus (LePrince et al. 1983; Nunn et al. 1983). Since then, a family of transcription factors, known as the *ets* family, involved in a wide variety of biological processes including growth control and development, transformation, and T-cell activation, have been cloned and sequenced from a variety of species ranging from human to Drosophila. The common feature of *ets* proteins is a well-conserved 85 amino acid domain that binds specifically to DNA containing a (G/C)(A/C)GGAAGT consensus sequence (Macleod et al. 1992; Wasylyk et al. 1993; Timms and Kola 1994).

The *ets* gene family includes, in addition to *ets-1* and *ets-2*, also *erg*, *elk-1*, *elk-2*, *pu-1*, *fli-1* and E74 (Fisher et al. 1992). *ets-1* encodes a set of phosphoproteins ranging in size from 39 to 51 kD (Fisher et al. 1992). However, whereas the chicken *ets* protein, which contains both the *ets-1* and *ets-2* domains,

distributes equally between the cytoplasm and nucleus, in the human and other mammals, the *ets-1* protein is cytoplasmic and the *ets-2* protein nuclear. This, together with their noncoordinate expression, suggests that *ets-1* and *ets-2* have different biologic functions (Fujiwara et al. 1988).

Ets-1 is preferentially expressed at high levels in B and T cells and is regulated during both thymocyte development and T cell activation (Chen 1985; Bhat et al. 1989). Studies in mice have shown that Ets-1 is essential for normal maintainance, survival and activation of B- and T-lineage cells (Bories et al. 1995; Muthusamy et al. 1995). Bhat et al. (1990) found that, following T-cell activation, *ets-2* mRNA and proteins are induced, while *ets-1* gene expression decreases to very low levels.

Amplification and rearrangement of *ets-1* has also been implicated in human leukemia (Goyns et al. 1987; Rovigatti et al. 1986). *ets-1* is also expressed in endothelial cells during blood vessel development and in fibroblasts adjacent to tumor cells in various invasive human carcinomas (Wernert et al. 1992; Wernert et al. 1994), suggesting that the *ets-1* gene could be involved in angiogenesis associated with tumor growth and normal development.

Tumor growth, progression and metastasis are dependent on the formation of new capillary blood vessels from existing vessel, a process termed angiogenesis (Folkman and Shing 1992); rapidly growing tumors are often hypoxic due to insufficient vascularization. Angiogenesis is a cascade of processes involving both soluble angiogenic factors and insoluble extracellular matrix factors (Jekunen and Kairemo 1997). Multiple soluble molecules that stimulate angiogenesis are released by tumor cells as well as host cells such as endothelial, epithelial and mesothelial cells and leukocytes. In SK-MEL-2 human melanoma cells cultured under hypoxic conditions the synthesis of Vascular Endothelial Growth Factor (VEGF) is stimulated (Claffey et al. 1996). In endothelial cells VEGF induces the expression of the proto-oncogene *ets-1* and stimulates endothelial cell migration. On the other hand, antisense P-ODNs directed against *ets-1* mRNA inhibit the ability of endothelial cells to migrate (Chen et al. 1997). In fact, induction of *ets-1* expression appears to be a common phenomenon in endothelial cells stimulated by angiogenic growth factors (Iwasaka et al. 1996). Thus, the *ets-1* gene apparently plays a direct role in angiogenesis.

Antisense oligodeoxynucleotides (ODNs) are short (typically 15 bases) stretches of synthetic DNA that are complementary to specific regions of cellular mRNA or DNA. This complementarity allows them to hybridize to specific parts of cellular mRNA or DNA, forming mRNA-DNA or DNA-DNA duplexes. The duplex formation disrupts the function of that particular gene either at the translational or transcriptional level. Due to their specificity antisense ODNs have become attractive potential tools for specific therapeutic applications, e.g. as specific inhibitors of malignant cell growth. In order to be effective the antisense ODNs must first enter the cell and then escape degradation by intracellular nucleases to achieve adequate concentrations in the correct intracellular compartment. As cellular nucleases effectively degrade phosphodiester ODNs, several more nuclease resistant ODNs have been developed. Of these the phosphorothioate ODNs (P-ODNs) in which a non-bridging oxygen atom has been replaced by a sulphur atom, are the most common.

Antisense ODNs may also function as specific carriers of cytotoxic drugs into cells, provided that the target sequence is specifically expressed in that particular cell type. Similarly, by attaching an appropriate radionuclide to the ODN, the selective delivery of radiation to a particular cell may be achieved. The primary target for ionizing radiation is the nuclear DNA and if the radiation source is in close proximity to the DNA, molecular damage would ensue (Kairemo et al. 1999).

Theoretical studies evaluating Auger and gamma-emitting radionuclides as well as beta-emitters (Kairemo et al. 1998) have suggested that short range beta-emitters such as ^{33}P and ^{35}S may be best suited for delivery of radiation confined to the cell nucleus. ODNs are easily labeled with ^{33}P or ^{35}S at their 5´- or 3´-end. Furthermore, since antisense P-ODNs contains both sulphur and phosphorus atoms that could be exchanged with ^{33}P and/or ^{35}S as part of their structure, these radionuclides offer benefits over e.g. transition metal nuclides since they do not require any extra coupling techniques for incorporation of the nuclide into the P-ODN.

The aim of this study was to investigate the potential of radiolabeled antisense oligodeoxynucleotides to specifically inhibit the growth and to destroy melanoma cells utilizing *ets-1* mRNA as target (**Figure 1**).

II. Results

During the first 24 h of incubation with 100 nM ^{33}P-labelled antisense or sense oligonucleotides the cells accumulated ^{33}P to an activity of approximately 0.15-0.25 mBq/cell. No further cellular accumulation of label was observed during the next 24 h. Incubation of cells with 200 nM ^{33}P-labeled oligonucleotides increased cellular accumulation of label to approximately 0.4-0.5 mBq/cell at 24 h incubation, with no further accumulation of label during the next 24 h (**Table 1**). Thus, cellular accumulation of ^{33}P-labeled oligonucleotides apparently increases linearly with extracellular oligonucleotide concentration. The cellular accumulation of ^{33}P at 24 h with 200 nM oligonucleotides corresponds to a cellular oligonucleotide uptake of approximately 2.5 pmol DNA per million cells.

The cumulative activity per cell was calculated for the two different oligonucleotide concentrations at 48 h incubation. At 100 nM oligonucleotide the cumulative activity was somewhat higher for the antisense compared to the sense oligonucleotide. At 200 nM oligonucleotide concentration there was no difference in cumulative activity between the antisense and the sense

oligonucleotide (**Table 1**). Based on the data shown in Table 1 and assuming a cell diameter of 14 µm, radiation doses per cell were calculated. The radiation doses varied between 1.2 and 4.1 cGy.

In cells that were allowed to accumulate ^{33}P -labelled antisense oligonucleotide directed against ets-1 mRNA for 24 h cell growth was inhibited approximately 25% (**Table 2**). With other oligonucleotides (i.e. labeled sense, unlabeled antisense or sense) the effect on cell growth was less pronounced, irrespective of whether the oligo was labelled or not. The transfection reagent alone inhibited cell growth by approximately 15%. Incubation of cells with 100 or 200 nM oligonucleotide apparently had no effect on cell growth.

After 48 h of incubation no effects on cell growth were observed.

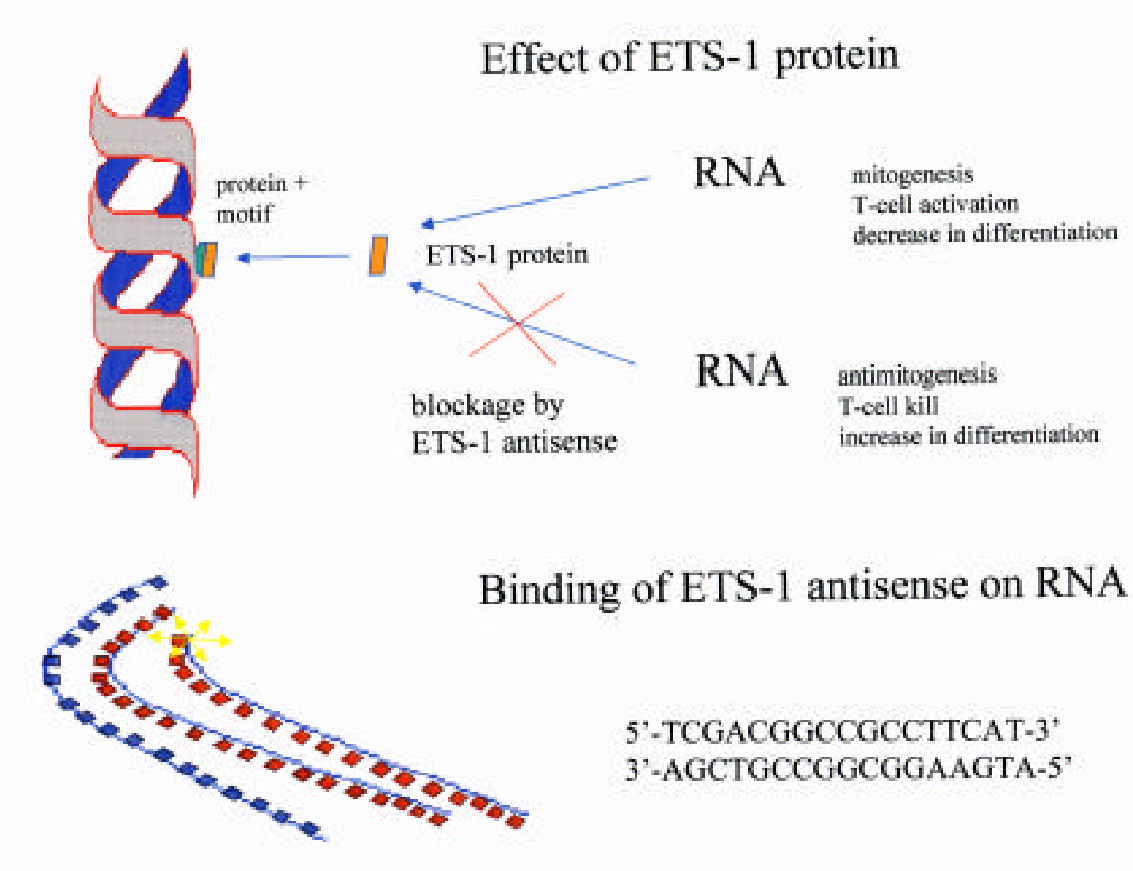

Figure 1. Effects of Ets-1 protein and antisense approach for cell killing.

Table 1: Accumulation of ^{33}P during incubation of cells with labelled oligonucleotides. Activities, cumulative activities and doses per cell are shown.

		Antisense			**Sense**		
Oligo concentration (nM)	Time (hours)	Activity (mBq/cell)	Cumulated activity (Bqs/cell)	Nuclear dose (cGy)	Activity (mBq/cell)	Cumulated activity (Bqs/cell)	Nuclear dose (cGy)
100	24	0.26			0.15		
100	48	0.18	30.2	1.9	0.14	19.0	1.2
200	24	0.47			0.50		
200	48	0.43	59.1	3.7	0.49	64.4	4.1

III. Discussion

In this first, to our knowledge, experiment designing internal labeling characteristics for ODNs we used single 5´-end labeling with ^{33}P to obtain a sufficient specific activity for subcellular dosimetric and cell killing experiments. In order to achieve high specific activity, many ^{33}P-atoms may be incorporated as part of the ODN backbone during synthesis. We have shown here that doubling the ODN concentration in the cell may increase cellular radiation doses more than three-fold (**Table 1**). Theoretically, it is possible to introduce 17 more ^{33}P-atoms in this *ets-1* P-ODN system.

Human melanoma G361 cells were utilized to establish the *in vitro* model system. These cells are well characterized, simple to maintain in culture, and they express *ets-1*. They are rather resistant to radiation. These cells are well suited for use in xenograft models in mice, because they grow subcutaneously and they may develop new vessels (angiogenesis).

In this preliminary experiment, melanoma cells were incubated with two concentrations of ^{33}P-labeled ODNs for 24 and 48 hours. The accumulation and elimination of ODNs by the cells seemed to be low. Total cellular uptake was less than 1% of added ODNs in the cell culture media. Furthermore, very little events between 24 and 48 hours, this is because influx and efflux are in balance (**Table 1**). It is important to find the optimal time and concentration for antisense treatment. Actually, in our experiments the lower ODN concentrations gave clearly different doses both for antisense ODN and sense ODN, respectively. The significance of this, if any, is unknown. At higher concentration the doses both for antisense ODN and sense ODN were rather similar. This demonstrates that it is important to find the optimal mode of ODN delivery.

During these experiments we did not yet find the optimal amount of transfection reagent, because very small differences on cell growth in various conditions were observed (**Table 2**). However it was clear that the best effect was obtained using labeled antisense-ODN. This finding has not been shown earlier in the literature. In fact, this to our knowledge, the first report of cell killing utilizing endocytotic therapy with ^{33}P-radionuclide.

We calculated internal radiation doses as described previously (Kairemo et al. 1998) as D (target ← target). This means that cell-to-cell-interactions as well as activities in the cell media were neglected (negligible?). This should be the case in optimal radionanotargeting. Following loading of the cells with radiolabeled ODNs a simple subcellular fractionation into nuclear, membrane and cytosolic fractions can be performed. This will yield data on the nuclear, cytoplasmic, and cell surface distribution of label (data not shown). Using this approach internal radiation doses were calculated as previously described (Kairemo et al. 1999). Using cell diameter of 14 μm, a nuclear dose of 3 cGy was obtained which is in accordance with the cell doses shown in Table 1.

Table 2. Effect of oligonucleotide treatment on cell growth.

	100 nM oligo	200 nM oligo
Treatment	Cell growth (%)	Cell growth (%)
None	100	100
^{33}P-Antisense	77	76
Antisense	86	79
^{33}P-Sense	87	82
Sense	86	84
SuperFect	84	86

The human melanoma G361 cell line produces adequate levels of Ets-1 protein and mRNA under standard culture conditions. Utilizing known inducers (e.g. VEGF) or inhibitors (e.g. tissue plasminogen activator) of *ets-1* expression the sensitivity of the detection systems may be changed. Following validation of the detection systems the ability of antisense ODNs to down-regulate the cellular expression of *ets-1* can be studied. Our preliminary results indicate that radiolabeled antisense ODNs have to be evaluated with respect to any effects on *ets-1* expression in addition to or synergistically with the pure antisense effect. Further studies are needed to decipher the molecular mechanisms of cell killing by radioactive antisense oligonucleotides .

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IV. Materials and Methods

A. Cell culture

Human melanoma cells G631 were cultured in RPMI1640 medium containing 2 mM L-glutamine, 50 U penicillin/ml 50 μg streptomycin/ml and 10% (v/v) FCS, in an atmosphere of 5% CO_2/95% air in 60 mm dishes. The doubling time of the cells was approximately 24h.

B. Phosphorothioate oligonucleotides

Antisense and sense phosphorothioate oligonucleotides against *ets-1* were obtained from Amersham Pharmacia Biotech. The oligonucleotide sequences were as described by Chen et al. (Chen et al. 1997) as follows: Antisense: 5'-TCGACGGCCGCCTTCAT-3'; Sense: 5'-ATGAAGGCGGCCGTCGA-3'. The oligonucleotides were purified by FPLC from the manufacturer and were reconstituted in sterile 1xTE buffer.

C. Labeling of oligonucleotides

The oligonucleotides were 5'-end labeled with γ-^{33}P-ATP according to the manufacturers instructions, utilizing a kit from Amersham Pharmacia Biotech. Unincorporated γ-^{33}P-ATP was removed by passage through an anion exchange column (Qiaquick oligonucleotide removal kit, Qiagen). Incorporation of ^{33}P into the oligonucleotides was in the range of 60-80% and specific activity was approximately 200 Bq/pmol DNA.

D. Incubation of cells

Cells were seeded in 60 mm dishes at a density of $5x10^5$ cells per dish in culture medium (se above). The next day cells were washed twice in DPBS before fresh culture medium containing labelled or unlabelled oligonucleotides (200 nM) in SuperFect™ (Qiagen) were added. The cells were incubated in the presence of oligonucleotides for a maximum of 48 h.

E. Determination of cell growth

At the designated time points the cells were washed in DPBS, trypsinated and the resulting cell suspension counted in a Coulter Z1 (Coulter Electronics, Ltd.). The effect of treatment on cell growth is expressed relative to the control cells receiving no treatment.

F. Cell dosimetry

The nuclear dose of the internalized oligodeoxynucleotide was estimated using the principles of Medical Internal Radiation Dose (MIRD) schema (Loevinger and Berman 1976). The dose D was calculated as a product of cumulated activity $\tilde{A}$ and specific absorption fraction, S, where all the radiation sources k are summed up together:

$$D = \sum_k \tilde{A}_k \cdot S_k$$

The nuclear dose was calculated using the assumption of uniformly distributed activity and subcellular S-factors of Goddu et al. (Goddu et al. 1993) for the cellular and nuclear diameters of 7 and 4 μm. We assumed that subcellular S-factors of phosphorus-33 are similar to those of sulphur-35, which gives the total S-factor (cell→nucleus) to be 6.3×10^{-4} Gy/Bqs. The dose from the adjacent cells or from the medium was not taken into account. The tracer concentration was assumed to increase from 0 to 24 hrs linearly and remain constant from 24 to 48 hours.

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