Invited Review

Pediatric Neurosurgery

Pediatr Neurosurg 1998;28:279–285

Use of Antisense Vectors and Oligodeoxynucleotides in Neuro-Oncology

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Introduction

The discovery that complementary fragments of DNA can cause the transcription arrest of selected genes [1, 2] has launched a new field of drug development in which early clinical trials are now proceeding [3–7]. The idea of using antisense-mediated gene inhibition as an alternative to conventional chemotherapy is particularly exciting for malignant brain tumors, since results with standard chemotherapy have been disappointing. The term 'antisense' refers to the fact that the nucleic acids synthesized are complementary (in an antiparallel orientation) to the coding (i.e. 'sense') genetic sequence of the target mRNA [4, 6, 8]. Two main types of antisense treatment have been employed to date: (1) transfection of cells with antisense cDNA, and (2) treatment of cells with antisense oligodeoxynucleotides (ODNs). Antisense constructs are also used in the laboratory as probes for the detection of specific mRNA sequences in cells or tissue specimens.

In order to be useful therapeutically, an antisense construct must: (1) exhibit stability in the physiologic environment; (2) be taken up and retained by the target cells; (3) specifically bind target mRNA; (4) successfully block expression of the target gene; (5) be free of unwanted toxic and nonspecific side effects, and (6) be easily synthesized in sufficient quantities to facilitate clinical use [4, 9–12]. Antisense therapy is attractive due to its theoretical specificity [12–15], and (to date) relative lack of known adverse effects, particularly when the vector or ODN is administered directly into the CNS [7, 16–19].

Antisense cDNA versus ODNs: Background and Considerations for Use in Neuro-Oncology

Antisense mRNA control was first demonstrated for ColE1, a bacterial DNA plasmid [8, 20]. Posttranscriptional regulation of gene expression using the antisense approach has now been extensively studied. Typically, exogenous antisense cDNA constructs are introduced into cultured cells by plasmid transfection or microinjection. The antisense sequence is then transiently transcribed within the cell from the inserted DNA expression vector. The antisense vector strategy has been successfully used in vitro against glioblastoma cells for gene targets including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), protein kinase C, isotype α (PKCα), the urokinase receptor, transforming growth factor-β1, calmodulin and E2F-1 [21–29].

Often in such studies, antisense-treated and control tumor cells are then implanted subcutaneously or intracerebrally into experimental animals and the growth of antisense-treated tumors is compared to control tumor growth. In this way, antisense-treated cells have been shown to be less tumorigenic than control glioblastoma cells. For true in vivo studies of tumor treatment using an antisense vector, however, the target tumor cells would have to be infected with a replication-defective virus administered to the host animal. In comparison with the cDNA approach, antisense ODNs do not require a viral vector delivery system; they are also easier to synthesize

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1016–2291/98/0286–0279$15.00/0

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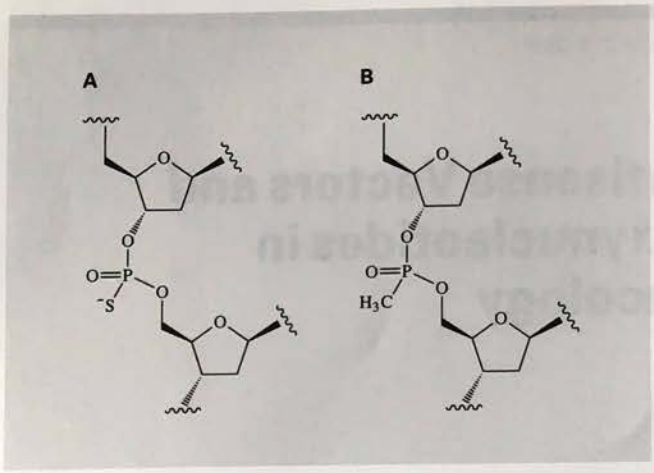

Fig. 1. Backbone structure of phosphorothioate (**A**) and methylphosphonate (**B**) ODNs.

and modify [10, 12, 30, 31]. Therefore, the antisense ODN approach has been much more widely used.

Unmodified ODNs are polyanions with a phosphodiester backbone. They are very rapidly degraded under physiological conditions primarily by 3′-exonucleases [10, 11, 32, 33]. ODN modifications are used to retard degradation, and to improve entry into cells and mRNA binding [34]. The phosphorothioate modification of the oligonucleotide backbone (fig. 1A), in which a sulphur atom replaces one of the oxygen atoms in the phosphate group, produces an oligonucleotide which is more resistant to nuclease digestion. Another variation of the backbone produces the methylphosphonate modification (fig. 1B) [35, 36]. This produces an uncharged molecule, which is less susceptible to nuclease digestion, and less soluble in water [4, 32, 37]. A phosphoramidate modification has also been described [38, 39].

Uptake of ODNs by cells is believed to occur by fluid-phase pinocytosis and/or receptor-mediated endocytosis [30, 40]. Cellular entry is dependent upon ODN structure, cell type, and treatment conditions [41]. Enhanced delivery of ODNs to cells has been achieved through coadministration of cationic lipids or by linking them to peptides or hydrophobic moieties, among other methods [7, 37, 40, 42, 43]. In considering access to the CNS, use of the relatively lipophilic methylphosphonates – or a liposome delivery system [44, 45] – could be advantageous.

Once inside the cell, antisense ODNs must (1) leak out or be released from the vesicles, then (2) bind (i.e. hybridize) to the target mRNA template, in order to block successful translation of the corresponding protein [17, 41, 46, 47]. Stable hybridization usually requires an ODN of 15 bases or longer. The bound ODN-mRNA complex is termed the 'heteroduplex' (fig. 2) because it contains ribonucleic and deoxyribonucleic acid. The more specific part of the mRNA targeted is often at the 5′ end of the transcript, spanning the translation initiation codon [31, 37]. mRNA inactivation occurs either through steric blocking of the ribosome complex, or by triggering mRNA cleavage by RNase H [6, 13, 33, 41, 48–51]. RNase H sensitivity is dependent upon the backbone modification [41, 51]. Antisense ODNs can also interfere with gene expression by triple-helix formation, in which the ODN binds double-stranded DNA in the nucleus [13, 17, 40, 52–55].

Ingenious antisense agents called 'ribozymes' have also been designed. Ribozymes induce catalytic cleavage of target RNA by adding a sequence which has natural self-splicing activity [17, 36, 40, 56]. In carrying out the mRNA cleavage, the ribozyme itself is not altered, and can therefore bind to and cleave additional mRNA molecules [40, 56]. The cellular uptake and subcellular distribution of a ribozyme targeted to epidermal growth factor receptor mRNA has been studied in U87-MG glioma cells [57].

ODN Targeting of Brain Tumors

Studies of intravenous injection of phosphorothioate ODNs have shown a plasma half-life of $^1/_2$–1 h. Steady-state plasma levels can be achieved with repeated daily intravenous injections [7, 32, 58]. Animal studies of ODN biodistribution have shown that ODNs administered systemically (as negatively charged molecules of approximately 5 kDa) enter the brain only in extremely small quantities [5, 32, 37, 58, 59]. Because of this, direct injection (or osmotic minipump infusion) into the CSF, brain parenchyma or tumor bed has been advocated [4, 10, 16, 18, 60, 61]. Figure 3 shows a nude rat being implanted subcutaneously with an Alizet™ osmotic minipump, for the purpose of delivering antisense ODNs directly into the bed of an implanted brain tumor.

Animal studies of intraventricular administration of ODNs have shown that (as with systemic administration) phosphodiester ODNs are rapidly degraded, whereas phosphorothioate ODNs are resistant to degradation and cleared in a manner consistent with bulk flow [60, 62]. Studies of intraventricular phosphorothioate ODN infusion lasting 1 week did not show any evidence of toxicity, yet the ODNs permeated the brain extensively and were

Fig. 2. Three-dimensional representation of the bound ODN-mRNA complex, the 'heteroduplex'. The mRNA template is represented in gray, the ODN in white (arrows). The 3′ mRNA end is at the top of the figure. Formation of the heteroduplex blocks translation either through steric hindrance or activation of RNase H.

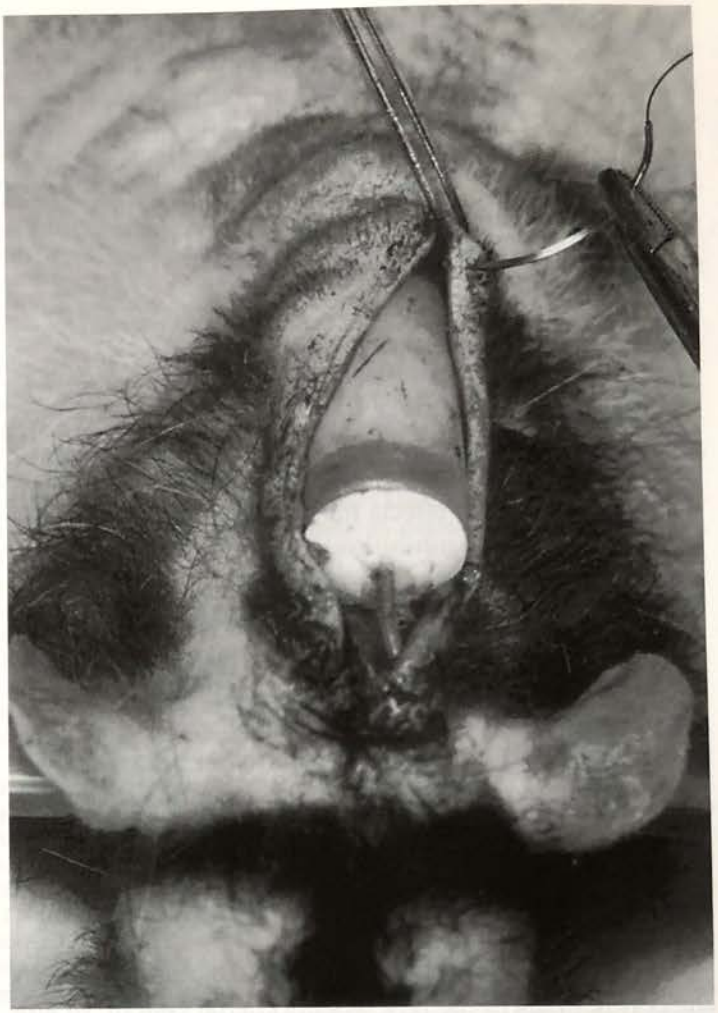

Fig. 3. Photograph of a nude rat being implanted with a subcutaneous Alizet™ osmotic minipump. Such pumps can be used to deliver ODNs to the CSF or tumor bed of experimental animals.

taken up by astrocytes [60, 62]. Other investigators have confirmed the superiority of phosphorothioate ODNs for CNS administration, the cellular uptake and biodistribution of intracranially administered ODNs, and their apparent lack of adverse effects [16, 63–66]. ODNs may be more stable within the CNS than in other bodily compartments [67]. Direct ODN infusion has been widely used to block the transcription of many different genes in nonneoplastic rat brain [see 9]. Some therapeutic effects have been seen with administration of a single ODN dose [67].

Reported target genes for antisense ODN therapy in glioma cells in vitro have included bFGF, c-*erb* B, c-*myb*, c-*myc*, c-*sis*, CD44, p34cdc2, mdm2, IGF-1, PDGF, TGF-β, PKCα, tumor necrosis factor, urokinase, the urokinase receptor and S100β protein [10, 16, 52, 54, 55, 68–83]. For cultured medulloblastoma cells, Liu et al. [84] used antisense ODNs to block expression of leukemia inhibitory factor (LIF). LIF down-modulation was thought to result in a decrease in cellular proliferation. Regarding in vivo brain tumor studies, Yazaki et al. [18] reported the use of a phosphorothioate ODN directed

against PKCα, which, when given intraperitoneally, showed efficacy against U-87 (human glioblastoma) cells grown subcutaneously and intracerebrally, in mice. Interestingly, the administration of antisense-treated tumor cells has been shown to trigger an antitumor response in rats, leading to tumor regression [85].

Obstacles to Clinical Use of ODNs in Neuro-Oncology

Nonspecific effects of ODN treatment of cells have been reported, particularly for phosphorothioated ODNs used at concentrations above 20–50 μ*M* [4, 16, 54, 70, 71, 86–89]. Nonspecific effects may in some cases be advantageous, such as the inhibition of the proliferation and/or migration of glioblastoma cells [70]. As polyanions, ODNs have been demonstrated to nonspecifically bind proteins such as VEGF, bFGF, PKC, and protein tyrosine receptors including the epidermal growth factor receptor [6, 14, 90, 91]. Phosphorothioated ODNs have also been reported to cause nonspecific induction of tumor necrosis factor, induction of Sp1 nuclear transcription factor binding activity, and inhibition of transferrin receptor expression [5, 15, 92, 93]. Because of this, treatment controls for experiments and clinical protocols must be carefully designed.

Systemically administered ODNs are accumulated by the components of the reticuloendothelial system. In animal studies, elevation of liver enzymes, splenomegaly, immune stimulation, thrombocytopenia, prolongation of the activated partial thromoplastin time and/or liver failure have been reported [6, 67, 90, 94]. Some of these effects were found to be dependent on ODN base sequence, backbone modification and/or dosage schedule [67]. Direct tumor bed infusion would be expected to allow these effects to be avoided. In one report of a possible adverse effect on the CNS, an ODN injected into rat brain was found to cause an inflammatory response, with induction of interleukin 6 expression [95].

Even with acceptable toxicity, adequate ODN entry into tumor cells, and translation arrest of the target gene, successful treatment of malignant tumors is not likely to be an easy task. Malignant gliomas are known to be heterogeneous; different sets of genes producing the malignant phenotype may be expressed in different patients. Blocking one molecular pathway might simply result in the activation of an alternative pathway, allowing cancer cells to continue to proliferate and invade normal brain [9]. Combination therapy with different ODNs, or use of ODNs in conjunction with conventional chemotherapeutic agents, may be required to achieve therapeutic efficacy [40, 96, 97].

Clinical Studies and Future Prospects

The impressive advances made in molecular biology over the past two decades first led to the identification of potential targets for gene-targeted therapy and have now resulted in automated commercial production of molecules capable of specifically disrupting the activity of these targets. A large amount of experimental data relevant to the therapeutic use of antisense ODNs has been gathered over the past decade [9, 34]. Antisense ODN treatment of cancer cells can certainly be used to block gene expression in vitro. Early results with ODNs administered in animal brain tumor studies have also been encouraging [16, 18].

Clinical trials with ODNs are now proceeding for several different diseases, including cancer [6, 7, 67, 90, 98]. Tumor genes that are being targeted clinically include c-*myb, bcl-2*, Ha-*ras*, PKCα, p53 and c-*raf* kinase [6, 67, 94]. The results of the first phase I trials of a phosphorothioated ODN targeting p53 mRNA have been reported [90, 98, 99]. No toxicity was observed in patients who received 0.05–0.2 mg/kg/h ODN i.v. for 10 days. A phase I study for malignant brain tumors currently underway involves the systemic administration of an anti-PKCα ODN (Isis Pharmaceuticals, Inc., Carlsbad, Calif.). Doubly-modified ODNs are currently under development [6, 11, 41, 100]. The potential for antisense technology to develop antineoplastic agents that are useful clinically has been described as 'vast' [37]. Successful clinical use of antisense ODNs will become increasingly more likely, however, as their pharmacokinetics and potential side effects are more clearly delineated, and the appropriate chemical modifications and gene targets identified.

Acknowledgements

The authors appreciate the assistance of Ms. Pamela Williams in revising the manuscript. Dr. Valya Tereshko performed the three-dimensional modeling (fig. 2). Dr. Engelhard's laboratory is supported by a generous gift from the American Brain Tumor Association in honor of Mr. M. Gitlitz.

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