Effects of phosphodiester and phosphorothioate antisense oligodeoxynucleotides on cell lines which overexpress c-myc: Implications for the treatment of Burkitt’s lymphoma

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Summary

The product of the c-myc proto-oncogene is a highly conserved nuclear phosphoprotein whose expression is closely linked to cellular proliferation and differentiation. We have been interested in developing an antisense oligodeoxynucleotide (ODN) strategy to inhibit the proliferation of c-myc-dependent malignancies for use in future specific therapies and bone marrow purging regimens. Our experimental approach was to incubate either antisense or sense ODNs, spanning the S’ cap region of the c-myc gene, with c-myc overexpressing cell lines (HL-60, Raji, MBL, CA-46) for up to seven days. Proliferation assay to test the inhibitory effect of an unmodified antisense ODN 15-mer (GCACGGTCCGGGTT) showed that concentrations as low as 50 μg/ml significantly decreased proliferation of HL-60 cells by approximately 40% \( (P < 0.0001; n = 6) \) compared to controls. Clonogenic assays showed that the same antisense ODN inhibited colony formation by MBL (40%) and Raji (52%) cells. Subsequent experiments to study the effect of a more nuclease-stable, phosphorothioate-modified antisense ODN 18-mer (GCAGCACAGCTCGGGGTT) revealed 66% inhibition of HL-60 cell proliferation at 96 and 120 hours at 50 μg/ml, whereas sense ODN control had no effect. However, tenfold less of the modified antisense ODN (1 μg/ml) was required to inhibit proliferation of HL-60 cells by 50% compared to the unmodified antisense ODN. A decrease in the HL-60 native c-myc protein level was also observed with 100 μg/ml of modified antisense ODN, but not with the sense ODN control, by immunoblot analysis. Additionally, concentrations up to 10 μg/ml of either modified antisense or sense ODN did not decrease CPU-GM formation \( (145 \pm 35\%; P = 0.27) \) in human bone marrow, suggesting that these levels of ODN would have a negligible effect on normal hematopoietic cells. These pilot data suggest that modified antisense ODN directed at the cap region of the c-myc gene could specifically inhibit c-myc expression at a single, lower dose than unmodified ODN and may play a future role in inhibiting the growth of c-myc-dependent malignant cells.

Key words: antisense, Burkitt’s lymphoma, HL-60, phosphorothioate, Raji

Introduction

Several chromosomal translocations have been identified in lymphoproliferative disorders, such as non-Hodgkin’s lymphoma, which have breakpoints associated with proto-oncogenes and immunoglobulin genes. Childhood nonlymphoblastic non-Hodgkin’s lymphoma consists of a majority of small noncleaved-cell lymphomas, either Burkitt’s type, non-Burkitt’s type, or large cell lymphomas [1-4]. A number of leukemias and lymphomas have undergone extensive investigation to determine the presence of oncogenes and chromosomal translocations. Specifically, patients with altered c-myc and bcl-2 expression in adult lymphomas have been found to require alternate treatment strategies [5, 6]. Inhibiting expression of oncogenes involved in tumorogenesis could potentially lead to the development of new therapeutic approaches to these malignancies.

The majority of childhood small noncleaved-cell lymphomas, including most sporadic (North American) and endemic (African) Burkitt’s lymphoma, have been characterized by specific chromosomal translocations that involve the c-myc proto-oncogene on chromosome 8 and an immunoglobulin heavy chain gene on chromosome 14 (90%) or light chain genes on either chromosome 2 or 22 [1-7]. This translocation of c-myc on chromosome 8 and the immunoglobulin gene results in the dysregulation of c-myc and is likely to be associated with oncogenic transformation [8, 9].

HL-60, a promyelocytic leukemia cell line with c-myc gene amplification and overexpression of c-myc mRNA, has been used in early studies to examine the effect of antisense ODNs [10, 11]. Inhibition of cell proliferation, c-myc mRNA expression, and protein production has been reported [12-13]. The essential role of c-myc expression for normal cell cycle progression has also been demonstrated using c-myc antisense ODNs [14]. This study showed that an antisense ODN 15-mer was able to block mitogen induced c-myc expression and entry into S phase in human T lymphocytes. A series of unmodified, phosphodiester, antisense ODN 15-mers targeted against a variety of sequences between the S’ cap and initiation codon region of c-myc mRNA have been tested for inhibitory activity [15]. C-myc protein expression in HL-
60 cells was much more sensitive to inhibition by 5' cap region antisense ODNs than by other sequences in the c-myc transcript.

There is some evidence that antisense ODNs can inhibit oncogene expression and selectively affect the proliferation of leukemic cells relative to normal hematopoietic progenitors. An \textit{ex vivo} delivery system has been assessed for the therapeutic ability of oncogene-targeted antisense ODNs to inhibit cell growth [16]. BCR/ABL antisense ODNs were shown to suppress proliferation of clonogenic cells from patients with CML in blast crisis [17]. Leukemic cells have also been shown to be more sensitive to inhibition of c-myc gene function \textit{in vitro} by antisense ODNs than normal mononuclear cells [18]. Therefore, perturbation of oncogene function with antisense ODNs may form the basis for a targeted molecular approach to leukemia therapy.

Despite successful \textit{in vitro} studies utilizing antisense ODNs to inhibit oncogene expression and function, the relatively short half-life of normal phosphodiester ODNs in serum and in cells, due to the presence of nucleases, may limit their potential usefulness \textit{in vivo}. ODNs with a modified backbone, e.g., phosphorothioates and methylphosphonates, are more resistant to nucleases [19, 20] and therefore may be more stable \textit{in vivo} [21, 22].

The aim of this study is to determine the inhibitory effect of unmodified versus modified c-myc antisense ODN on Burkitt's lymphoma cell lines \textit{in vitro}. The inhibition of tumorigenesis could lead to the development of an \textit{ex vivo} molecular method for purging tumor cells in bone marrow.

Materials and methods

Cell culture

The cell lines, Raji, CA-46, and HL-60, were obtained from the American Type Tissue Collection (ATCC) and cultured in RPMI 1640 medium (Sigma) supplemented with 15% heat inactivated fetal bovine serum (FCS; Gemini Products), glutamax (Gibco BRL), and penicillin/streptomycin (Gibco BRL) at 37°C, 95% air/5% CO2. The cells were passed twice a week. Experiments were begun 18-20 hours after passage.

A patient cell line, MJBL, was isolated by Ficoll-Hypaque centrifugation of a diagnostic bone marrow aspirate before treatment. Immunophenotypic and cytogenetic analyses confirmed diagnosis as an undifferentiated lymphoma. Burkitt-type, with a bone marrow aspirate containing >25% L3 (French-American-British classification). Lymphoma cells were cultured as described for those obtained from the ATCC. The cellular phenotype of the established tissue culture was found to be identical to the initial phenotype.

Southern blot hybridization

Genomic DNA was isolated from a Burkitt's lymphoma patient-derived cell line (MJBL). Following restriction enzyme digestion with either Eco RI, Bam HI or Hind III, DNA was subjected to agarose gel electrophoresis and transferred to nylon membranes. The membranes were serially hybridized with 32P-labeled DNA probes for exon 1 (0.9 Kb Pvu II fragment) and exon/intron (1.26 Kb Pvu II fragment) of cmyc, and for the JH (3.8 Kb Bgl II fragment), C\mu (1.3 Kb Eco RI fragment), and C\alpha 1 (0.9 Pst I fragment) regions of the immunoglobulin heavy chain (IgH). Membranes were then washed in 0.3 × SSC at 65°C, and subjected to autoradiography. Rearrangements were detected as different sized fragments compared to genomic DNA from normal donor peripheral blood mononuclear cell (GL) controls.

ODNs

Unmodified phosphodiester antisense ODN 15-mers (Operon) had the sequence, 5'-GCA-CAG-CTC-GGG-GGT3'. Modified phosphorothioate ODN 18-mers (Oligo's Etc.) had the sequences: antisense, 5'-GCA-GCA-CAG-CTC-GGG-GGT3'; and sense, 5'-ACC-CCC-GAG-CTG-TGC-G3'.

Antisense ODN treatment

Antisense ODN was incubated with 5000 cells in 100\mu l media per well in a 96-well microtiter plate. ODN was added at concentrations from 1 to 500 \mu g/ml. Incubations were performed in RPMI 1640 medium with 15% fetal bovine serum when modified ODN was used, and in serum-free RPMI 1640 medium supplemented with 15% fetal bovine serum after three hours incubation at 37°C when unmodified antisense ODN was used. When unmodified antisense ODN was used, 25% of the original ODN dose was also added to the cultures at 24 and 48 hours.

Proliferation and viability of cells

For trypan blue staining, twenty microliter aliquots of cells were removed at the indicated time intervals after incubation for staining with an equal volume of 0.4% trypan blue. Titors of viable cells were determined by counting trypan blue-excluding cells in a hemacytometer [23].

For \textit{in vitro} clonal assays, cell suspensions were plated in methylcellulose medium (Stem Cell Technologies) containing 0.5 U/ml erythropoietin (Amgen) and 5% PHA-lymphocyte conditioned media (Stem Cell Technologies). The plates were incubated at 37°C in a humidified atmosphere containing 5% CO2, and colonies were counted after 14 days using an inverted phase-contrast microscope.

Treatment of bone marrow

Human bone marrow was obtained from normal donors after informed consent. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque centrifugation. Cells (2 × 10^6/ml) were treated with c-myc phosphorothioate antisense or sense ODN at 0.1, 1.0, and 10 \mu g/ml for 96 hours. Cells were then washed with fresh media and 5 × 10^6 cells/ml were seeded immediately into methylcellulose media for \textit{in vitro} clonal assays, as described above.

Immunoblot assay

HL-60 cells (5 × 10^6/ml) were incubated in control media and media containing either phosphorothioate antisense or sense ODN for 96 hours. Cells were washed once in phosphate-buffered saline, and proteins were extracted by NP-40 detergent lysis for 30 min on ice [25]. The supernatant was collected and assayed for total protein using the BCA protein assay (Pierce). Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), loading 50 \mu g protein per lane. Proteins were transferred to a nitrocellulose membrane and blocked with 5% nonfat dry milk for 1 hour at room temperature. Blots were then incubated with 2% (v/v) of a mouse monoclonal antibody directed against c-myc (Ab-1: Oncogene Science) overnight at 4°C, followed by incubation with 0.2% (v/v) rabbit anti-mouse IgG (Sigma). Bands were visualized using an alkaline phosphatase conjugated goat anti-rabbit antibody (0.2% v/v; BRL) with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP)/p-nitro blue tetrazolium chloride (NBT; Bio-Rad) as the substrate.
Results

Southern blot analysis revealed that MJBL had a c-myc translocation in the region upstream of the 5' cap region since additional c-myc hybridizing fragments were not detected (Figure 1). Raji also has a c-myc translocation in the region upstream of the 5' cap region (Figure 2). Therefore, both of these cell lines retain the c-myc 5' cap region, including exons 1, 2, and 3, on the translocated chromosome. The translocation of CA-46 separates the c-myc 5' cap region and c-myc coding region onto different chromosomes (Figure 2).

Incubation with the unmodified phosphodiester 5' cap antisense ODN was found to significantly inhibit proliferation of MJBL (40%) and Raji (52%) Burkitt’s lymphoma cells, at initial concentrations of 100 µg/ml (P = 0.004 and P = 0.01, respectively), as well as HL-60 (40%) cells, at initial concentrations of 50 and 100 µg/ml (P = 0.03 and P = 0.003, respectively) compared to control, whereas 10 µg/ml did not show significant difference from control (Figure 3). However, these experiments required addition of 25% of the initial dose at 24 and 48 hours to produce an effect, presumably because of their instability.

Since these phosphodiester ODNs were apparently sensitive to nuclease present in serum, we tested the effect of phosphorothioate modified 5' cap antisense ODNs. In order to compensate for the weaker DNA-RNA hybrid formation, due to the bulkier phosphorothioate linkage, the length of the ODN was increased to an 18-mer [25]. A three-fold inhibition of proliferation was observed at 96 and 120 hours (Figure 4) using the same concentration that caused a decrease in proliferation with the phosphodiester antisense ODN (50 µg/ml). Therefore, subsequent experiments utilized 96-hour incubation times.

To determine the minimum amount of phosphorothioated antisense ODN necessary to observe an inhibitory effect, 1, 10, 50, and 500 µg/ml antisense were added to 5 x 10⁴ HL-60 cells/ml. Phosphorothioated antisense ODN at a concentration as low as 1 µg/ml was sufficient to significantly (P = 0.05) inhibit proliferation (Figure 5) compared to the control. The sense ODN (50 µg/ml) did not show a significant inhibitory effect.

When the inhibitory effect of the phosphodiester and phosphorothioate antisense ODN were compared directly, a tenfold lower (one log) concentration of the phosphorothioate antisense ODN (100 µg/ml vs. 10 µg/ml) was
required to achieve the same 50% inhibition of HL-60 proliferation (Figure 6).

Immunoblot analysis revealed twofold decreased c-myc protein expression in HL-60 cells treated with phosphorothioate antisense compared to sense ODN or control (Figure 7).

There was no inhibitory effect by either phosphorothioate antisense or sense ODNs on bone marrow mononuclear cell colony formation at any of the concentrations tested (Figure 8).

Additionally, the effect of the 5' cap phosphorothioate antisense and sense ODNs on CA-46, a negative control cell line for this oligodeoxyxynucleotide, was studied by adding 10, 50, 100, and 250 μg/ml of ODN to 5 × 10^5 CA-46 cells/ml. After 96 hours of incubation, no significant inhibitory effect was observed (Figure 9).

Discussion

Constitutive c-myc expression is associated with Burkitt's lymphoma and selected forms of B-cell acute lymphoblastic leukemia (B-ALL), resulting from reciprocal chromosome translocations between c-myc on chromosome 8 and immunoglobulin loci on chromosomes 14, 22, or 2. HL-60, a human promyelocytic leukemia cell line, also overexpresses c-myc and has become the model system for our current study. C-myc expression is linked to cellular growth, being low in quiescent cells and in-

Figure 3. Proliferation of MBL, Raji, and HL-60 after exposure to various concentrations of unmodified phosphodiester antisense ODN. Cells were treated with unmodified phosphodiester antisense ODN as described in 'Materials and methods' for 96 hours. 5000 cells/well were plated in RPMI 1640 + 15% FCS medium and viable cells were counted after 96 hours. Values are expressed as mean ± SEM for three experiments (triplicate determinations). MBL: control vs. 100 μg/ml, P = 0.004, Raji: control vs. 100 μg/ml, P = 0.01, HL-60: control vs. 50 μg/ml, P = 0.03, control vs. 100 μg/ml, P = 0.003.

Figure 4. Time course of HL-60 cell proliferation upon exposure to modified phosphorothioate ODN. Cells were treated with 50 μg/ml of phosphorothioated antisense and sense ODN as described in 'Materials and methods'. 5000 cells/well were plated in RPMI 1640 + 15% FCS medium and viable cells were counted 72, 96, and 120 hours after incubation of the ODN. Values are expressed as percentage of the initial number of cells plated.

Figure 5. Proliferation of HL-60 after exposure to various concentrations of modified phosphorothioate ODN. Cells were treated with modified phosphorothioate antisense ODN as described in 'Materials and methods' for 96 hours. 5000 cells/well were plated in RPMI 1640 + 15% FCS medium and viable cells were counted after 96 hours. Values are expressed as mean ± SEM for three experiments (triplicate determinations). Control vs. 1 μg/ml, P = 0.05, Raji: control vs. 10 μg/ml, P = 0.04; control vs. 50 μg/ml, P = 0.04.

Figure 6. Proliferation of HL-60 after exposure to modified phosphorothioate and unmodified phosphodiester ODN. Cells were treated with 50 μg/ml modified phosphorothioate sense, 10 μg/ml modified phosphorothioate antisense (SAS), or 100 μg/ml unmodified antisense (AS) ODN as described in 'Materials and methods' for 96 hours. 5000 cells/well were plated in RPMI 1640 + 15% FCS medium and viable cells were counted after 96 hours. Values are expressed as mean ± SEM for three experiments (triplicate determinations). Control vs. SAS, P = 0.05; control vs. AS, P = 0.04.
Figure 7. Immunoblot analysis of c-myc protein levels following incubation with ODN. HL-60 cells were treated with 100 μg/ml phosphorothioate antisense (P[S]AS) and sense (P[S]S) ODN for 96 hours. Cell lysates from HL-60 were electrophoresed, blotted, and probed with a c-myc antibody as described in Materials and methods. Scanning densitometry was utilized to quantify the levels of c-myc protein. Results shown are representative of three different experiments.

Figure 8. Proliferation of bone marrow mononuclear cells after exposure to modified phosphorothioate ODN. Cells were treated with 0, 1, or 10 μg/ml modified phosphorothioate antisense or sense ODN as described in Materials and methods for 96 hours. 5000 cells/well were plated in methylcellulose medium and colonies were counted after 14 days. Values are expressed as mean ± SEM for three experiments (triplicate determinations).

Figure 9. Proliferation of CA-46 cells after exposure to modified phosphorothioate ODN. Cells were treated with 10, 50, 100, or 250 μg/ml modified phosphorothioate antisense or sense ODN as described in Materials and methods for 96 hours. 5000 cells/well were plated in RPMI 1640 + 15% FCS medium and viable cells were counted after 96 hours. Values are expressed as mean ± SEM for three experiments (triplicate determinations).

creased in cells proceeding through the cell cycle [26]. The c-myc protein itself has a relatively short half-life of approximately 30 minutes, which is convenient for observing changes in its expression [27]. We have been interested in developing an antisense ODN strategy to inhibit the proliferation of c-myc-dependent lymphomas for use as molecular purging agents in bone marrow transplant therapy.

Phosphorothioate-modified ODNs are more resistant to nuclease attacks than phosphodiester ODNs [28]. Although uptake of phosphorothioated ODNs by HL-60 cells was shown to be slower than phosphodiester ODNs, the affinity of phosphorothioate ODNs for cell surface receptors is higher than that of unmodified ODNs [29]. Our experiments showed that a single, lower dose of phosphorothioated antisense ODN (Figure 5) gave the same inhibitory effect as multiple, higher doses of unmodified phosphodiester ODN (Figure 6).

Although phosphorothioated ODNs have exhibited nonspecificity in some cases [30], this cell line was used to determine the tumor specificity of the antisense ODN. The cell line, CA-46, has a c-myc translocation within the first intron of the gene which separates the 5' cap region of the gene from the coding region. This explains the unresponsiveness of CA-46 to the 5' cap antisense ODN (Figure 9) and allows CA-46 to be used as a negative control for inhibition by 5' cap antisense ODN. Effective antisense for CA-46 has been identified within the first intron of the c-myc gene [31]. Thus, we have a translocation dependent antisense therapy to test more extensively.

In order to use antisense ODN as a therapeutic drug, it must enter cells effectively as well as remain stable in serum. An alternative approach to increase the stability of ODNs as well as to enhance cellular penetration would be to conceal the ODN from nucleases using liposomes, poly (L-lysine) conjugates, cholesterol, or other vehicles of drug delivery. ODNs within the size range of 10–15 nucleotides conjugated to a polylysine carrier were shown to be most effectively transported into cells and did not require elimination of serum from culture medium, unlike the unconjugated ODN [32]. However, cytotoxicity and incomplete protection from nucleases are still problematic and may require modification of the ODN backbone. Synthetic cationic lipids have been used to transfect cultured cells [33]. Lipid-mediated transfer has been subsequently applied to cystic fibrosis gene therapy [34] as well as to the delivery of a number of ODNs [35–38]. Therefore, we are currently investigating the delivery of these phosphorothioated antisense ODNs using cationic lipids as a vehicle for transport into the cell.
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