Oligonucleotides complementary to c-*myb* messenger RNA inhibit growth and induce apoptosis in human Burkitt lymphoma cells

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Abstract. A 24-mer (antisense) phosphorothioate oligonucleotide (ODN) corresponding to the codons 2-9 of the c-*myb* gene was evaluated for its effects on the growth of a human Burkitt lymphoma cell line (Raji) *in vitro*. Raji cells incubated with different concentrations of c-*myb* antisense ODN (5-15 μg/ml) for 24-72 h showed a significant dose-dependent decrease in growth. The same concentrations of control (sense) or scrambled c-*myb* phosphorothioate ODNs did not inhibit Raji cell growth. The c-*myb* antisense ODN, but not the control ODNs, significantly decreased c-*myb* mRNA levels in treated cells as determined by RT-PCR. Additionally, the c-*myb* antisense ODN induced apoptosis of Raji cells as demonstrated by i) flow cytometry to enumerate the A_o (apoptotic cell population) population of propidium iodide stained cells; ii) electron microscopy to evaluate the cell morphology; and iii) DNA fragmentation pattern. Thus, an antisense c-*myb* ODN causes significant growth inhibition of Burkitt lymphoma cells, and one mechanism of growth inhibition is the induction of apoptosis of the lymphoma cells. In addition, antisense c-*myb* ODN did not reduce CFU-GM or BFU-e colony-forming ability of normal hematopoietic stem/progenitor cells. Because the inhibition is sequence-specific and Burkitt lymphoma cell selective, evaluation of the therapeutic effects of c-*myb* antisense ODN against Burkitt lymphoma is warranted.

Introduction

Burkitt lymphoma is an aggressive B-cell tumor occurring predominantly in children. Despite aggressive treatments which can include high dose therapy followed by stem cell transplantation, a significant number of patients relapse, presumably due to the failure to eliminate minimal residual lymphoma cells. Therefore, novel therapeutic approaches to eliminate minimal disease are essential. One of the approaches is use of antisense oligonucleotides complementary to mRNA encoding a gene essential for cellular growth or survival as a therapeutic tool in modulating gene expression (1-5). Our initial approach has been to focus on targeting cell cycle related proto-oncogenes such as c-*myc* and c-*myb* in an attempt to inhibit the growth of lymphoma cells. Such cells have dysfunctional cell cycle check point regulation leading to constitutive and promiscuous progression through the cell cycle. Since translocations involving c-*myc* and immunoglobulin genes occur in many lymphomas, antisense constructs to c-*myc* gene mRNA can be designed which are, in theory, tumor cell specific based on the breakpoint region (6). In many Burkitt lymphoma cells, the c-*myc* gene is not only rearranged but also often overexpressed (Joshi SS *et al*: Proc Am Assn Cancer Res 35: Abst. 617, 1994). We have previously demonstrated a significant growth inhibitory effect of c-*myc* antisense oligonucleotides on OMA-BL-1 human Burkitt lymphoma cells grown *in vitro* (7). However, c-*myc* breakpoint specific/patient specific oligonucleotides may not be practical therapeutic agents, as safety data would not be available for each possible sequence. A rational alternative to antisense c-*myc* is the c-*myb* gene which is also activated in some lymphomas, including Burkitt lymphoma. Studies from the laboratory of Gewirtz and colleagues have demonstrated that antisense oligonucleotides complementary to the c-*myb* gene interfere with this gene's critical function in maintaining cell growth and cell survival characteristics *in vitro* (8-13). Antisense oligonucleotides complementary to c-*myb* proto-oncogene mRNA may be an important therapeutic approach to hematopoietic malignancies *in vitro* (12) and *in vivo* (13). This report describes the effects of a c-*myb* antisense oligonucleotide on the growth of Raji and normal human hematopoietic stem/progenitor cells *in vitro*.

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Materials and methods

Cell culture and in vitro treatment with oligonucleotides. Raji cells were obtained from American Type Culture

Collection and grown *in vitro* in growth medium consisting of RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics, penicillin (100 U/ml) and streptomycin (100 µg/ml). Two times 10^4 cells/well in 0.2 ml of the above-described growth medium containing different concentrations of c-*myb* antisense or control oligonucleotides (5 µg, 10 µg and 15 µg/ml) were incubated for 72 h *in vitro* at 37°C with 5% CO_2 and 95% air. Triplicate wells were used for each concentration. The cells were counted after 24, 48 and 72 h of culture using a hemocytometer. These experiments were repeated three times.

Oligonucleotides. Phosphorothioate oligodeoxynucleotides corresponding to c-*myb* codons 2-9 were generously provided by Lynx Therapeutics, Inc. (Hayward, CA). The sequences of phosphorothioate 24 mer antisense c-*myb* oligonucleotide and control oligonucleotide, a 24-mer phosphorothioate oligodeoxynucleotide with 'scrambled' base sequence are given below. In addition, in some experiments we also used an unrelated phosphorothioate 21 mer oligonucleotide ISIS-1082 as control. The ISIS-1082 sequence is complementary to herpes simplex virus type I T (14). 5'-d(TATGCTGTGCCGGGGTCTTCGGGC)-3' (antisense-c-*myb*); 5'-d(GCACGCAGCTGAAGCACAAGC ACC)-3' (Scrambled-c-*myb*); 5'-d(GCCGAGGTCCATGTC GTACGC)-3' (ISIS-1082).

CFU-GM assay. The number of *in vitro* hematopoietic colony-forming cells in the c-*myb* ODN-treated normal human donor peripheral blood stem/progenitor cells (PBSC) was determined using an *in vitro* CFU-GM colony assay (15). After incubation with c-*myb* oligomers at a concentration of 80 µg/ml at 37°C for 36 h, $3x10^5$ mononuclear cells from control and treated cultures were mixed with Iscove's modified Dulbecco medium (Gibco, Grand Island, NY) containing 0.3% agar, supplemented with recombinant human GM-CSF, G-CSF and Interleukin-3 (all three at a concentration of 200 U/ml) for 14 days at 37°C in a fully humidified atmosphere of 7% CO_2 in air. Aggregates larger than 50 cells were counted as colonies using a Bellco colony counter.

BFU-e assay. The effects of c-*myb* ODN treatment on erythroid cell populations of normal human peripheral stem cell harvests as described above were evaluated using an *in vitro* BFU-e colony assay (15). $2x10^5$ mononuclear cells and c-*myb* in ODN-treated peripheral blood stem cell harvests were mixed with Iscove's modified Dulbecco medium (Gibco, Grand Island, NY) containing 1% methyl cellulose, supplemented with recombinant human IL-3 (200 U/ml), recombinant erythropoietin (2 U/ml), and incubated for 14 days at 37°C in a fully humidified atmosphere of 7% CO_2 in air. Aggregates larger than 50 cells were counted as colonies using a Bellco colony counter.

Reverse transcripase-polymerase chain reaction (RT-PCR) for c-myb mRNA. RT-PCR for detection of c-*myb* was carried out as described by Majello *et al* (17). The c-*myb* specific primers were obtained from Applied BioSystems, Inc. (Foster City, CA). After 35 cycles of amplification, 10 µl of the PCR product was electrophoresed on a 1.5% agarose gel. The PCR product was stained with ethidium bromide and loaded onto the gel. After electrophoresis, the gel was visualized on a UV transilluminator and the DNA bands were photographed.

Quantitation of apoptotic cells and cell cycle parameters. Viable and apoptotic cell quantitations, as well as cell cycle parameters, were carried out essentially according to the procedure of Telford *et al* (18). Briefly, $2x10^5$ cells from each well were washed twice with 1 ml cold PBS and then fixed in 1 ml cold 70% ethanol for 1 h at 4°C. The cells were centrifuged and resuspended in 1 ml PBS containing 0.1% Triton X-100, 0.1 mM EDTA, 50 µg/ml RNase (50 units/mg) and 50 µg/ml propidium iodide (Sigma, St. Louis, MO). Cells were incubated overnight at 25°C in the dark prior to analysis. Cells were analyzed using an EPICS Elite flow cytometer using Coulter software. Apoptotic cell (A_o) percentages were calculated from the forward angle light scatter versus linear red fluorescence histogram by gating on the A_o population. Apoptotic cell numbers were expressed as a percentage of total cells incorporating dye after exclusion of non-staining viable cells and debris. 10^4 cell events were evaluated for each sample. Data are presented as mean percent apoptotic versus viable cells ± standard error (S.E.) of the mean for triplicate wells. To calculate cell cycle parameters, a single parameter linear red fluorescence histogram was generated for each sample. Cell cycle parameters were quantitated using the Multicycle software program (Coulter, Hialeah, FL). Data are presented as mean S + G2M values ± S.E. of the mean for the triplicate wells.

DNA ladder techniques. The apoptotic cell population in control and c-*myb* antisense ODN-treated Raji cells was evaluated using DNA ladder techniques to demonstrate DNA fragmentation (16). Briefly, 125 ml of 2% agarose in Tris borate buffer (TBE) was poured into a gel support. The stacking gel was made with 16 ml of 0.8% agarose, 2% dodecyl sulphate in TBE with 20 µl of proteinase K. Each cell pellet was suspended in 10 µl of sample buffer (containing 10 mg RNase) and transferred onto the gel and electrophoresed at 20V for 1 h and 90V for 3 h. The gel was then washed overnight in 500 ml of TE (containing 10 mg of RNase). The gel was rinsed in water and stained for 30 min in 500 ml of water containing 1.25 µg/ml of ethidium bromide. Finally, the gel was destained with water, visualized on a UV transilluminator, and photographed.

Electron microscopic analysis. Apoptosis induction of Raji cells by c-*myb* antisense oligonucleotides was confirmed morphologically using transmission electron microscopic techniques. Briefly, 10^6 treated and control Raji cells were pelleted into a micro tube, and fixed in 2-1-para-formaldehyde, 2.5% glutaraldehyde, 0.3% picric acid in 0.1 M Sorensen's phosphate buffer, pH 7.4 for 15 minutes. The cells were subsequently fixed in 4% paraformaldehyde, 5% glutaraldehyde, 0.3% picric acid in Sorensen's buffer for 1 h. The cells were then dehydrated in 100% ethanol and embedded into EMBED 812 (EMS Science) in a gelatin capsule. Sections were prepared using an ultra cut

Ultramicrotope and examined using a Phillips 400 microscope at the magnification stated.

Statistical analysis. All experiments were performed at least 3 times and the statistical significance of the results was determined using the Student's t-test.

Results

The effects of c-*myb* antisense and control oligonucleotides on the *in vitro* growth of Raji cells are shown in Fig. 1. All three concentrations of c-*myb* AS-ODN significantly inhibited growth of Raji cells when compared to control oligonucleotides ($p<0.05$). The control ODN did not show a significant growth inhibition of Raji cells compared to the media only control. The growth rate of Raji cells treated with c-*myb* AS-ODN remained unchanged after 3 days while the growth of control cells reached a plateau.

The inhibition of Raji cell growth caused by the c-*myb* antisense oligonucleotide correlated with the downregulation of c-*myb* gene mRNA expression in surviving cells as determined by RT-PCR analysis of c-*myb* mRNA using human c-*myb* gene specific primers. Fig. 2 shows the results of these studies on Raji cells treated with c-*myb* antisense oligonucleotides, media-alone, or control oligonucleotides. c-*myb* mRNA in Raji cells treated with c-*myb* antisense oligonucleotides significantly decreased ($p<0.05$) both on day 2 and day 3 after initiation of treatment. Control cell mRNA levels did not decrease. In fact, the Raji cells treated with unrelated oligonucleotides as control showed an increase in c-*myb* mRNA levels. This control oligonucleotide is complementary to human herpes simplex virus type I and reason for its enhancing effects on c-*myb* expression are still unclear.

One of the profound effects we observed in Raji cells was that the c-*myb* antisense oligonucleotides induced apoptosis as demonstrated by flow cytometry, electron microscopy and DNA ladder techniques. Fig. 3 shows the apoptotic cell population in Raji cells treated with control and antisense oligonucleotides as determined by flow cytometry. The number of viable cells in antisense ODN-treated cultures were significantly decreased when compared to cells treated with control ODNs, with significantly more cells dying by apoptosis in antisense ODN-treated culture than in control cultures treated with an unrelated oligonucleotide (Fig. 3). In addition, the proportion of cells remaining in the proliferative phases of cell cycle, particularly S, G2 and M, was greater in control cultures than in cultures treated with c-*myb* antisense ODN (Fig. 3).

The percentage of viable cells shown in Fig. 3 were calculated and expressed as percentage of media control. Fig. 4 shows the number of dying/apoptotic cells in control and c-*myb* antisense ODN-treated Raji cells. A significantly ($p<0.05$) higher number of cells underwent apoptosis in c-*myb* antisense ODN-treated cultures than in control cultures.

Because DNA fragmentation is a good apoptosis indicator, control and treated Raji cells were also analyzed using DNA ladder techniques. The results of DNA ladder analysis are shown in Fig. 5. Our results showed the DNA fragmentation induced by c-*myb* antisense ODN in Raji cells when compared to media alone control cells or control ODN

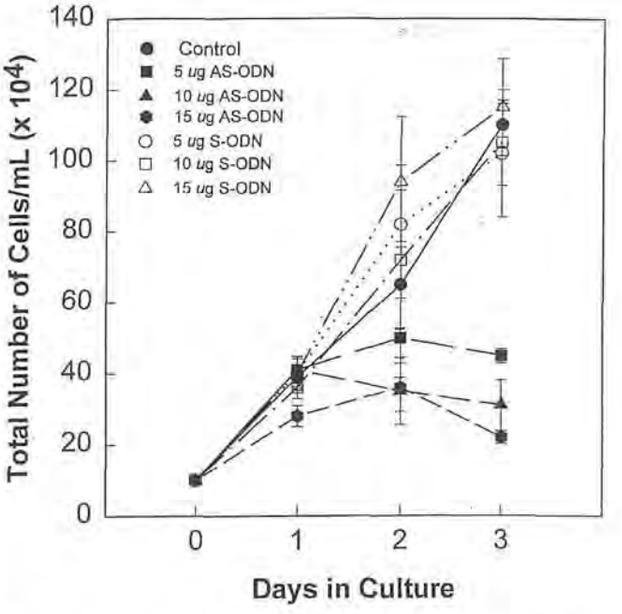

Figure 1. *In vitro* growth rate of Raji human Burkitt's lymphoma cells in the presence of different concentrations of c-*myb* antisense and antisense-scrambled oligonucleotides. Cells were grown *in vitro* for 3 days with or without oligonucleotides and number of cells were counted on different days. Triplicate samples were used for each time point and experiments were repeated three times.

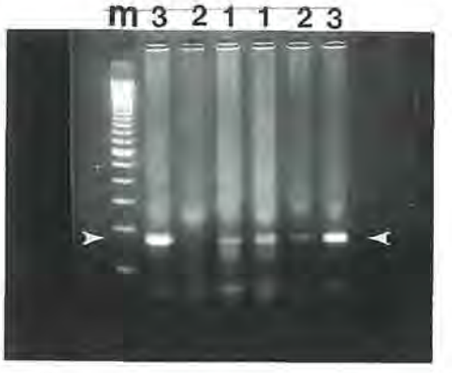

Figure 2. RT-PCR analysis of Raji cells treated with control and c-*myb* antisense oligonucleotides for c-*myb* RNA levels using c-*myb* specific primers. Lane m, Marker DNA; Lane 1, Media only control; Lane 2, c-*myb* antisense oligonucleotide treated cells; Lane 3, Cells treated with scrambled oligonucleotides. Arrows indicate the PCR product specific for c-*myb* gene.

treated Raji cells. The flow cytometric analysis as well the DNA ladder analysis confirmed the c-*myb* antisense ODN induced apoptosis in Raji cells.

Since the cytomorphological changes in the c-*myb* treated Raji cells were so drastic we further analysed the treated and control Raji cells for their morphological changes using transmission electron microscopy techniques. Fig. 6 shows the electron micrographs of Raji cells treated for 72 h with c-*myb* antisense ODN or control ODN treated cells as compared to control cells. Significant numbers of apoptotic cells were evident in c-*myb* antisense ODN-treated cultures as shown in Fig. 6C-E, whereas the Raji cells treated with

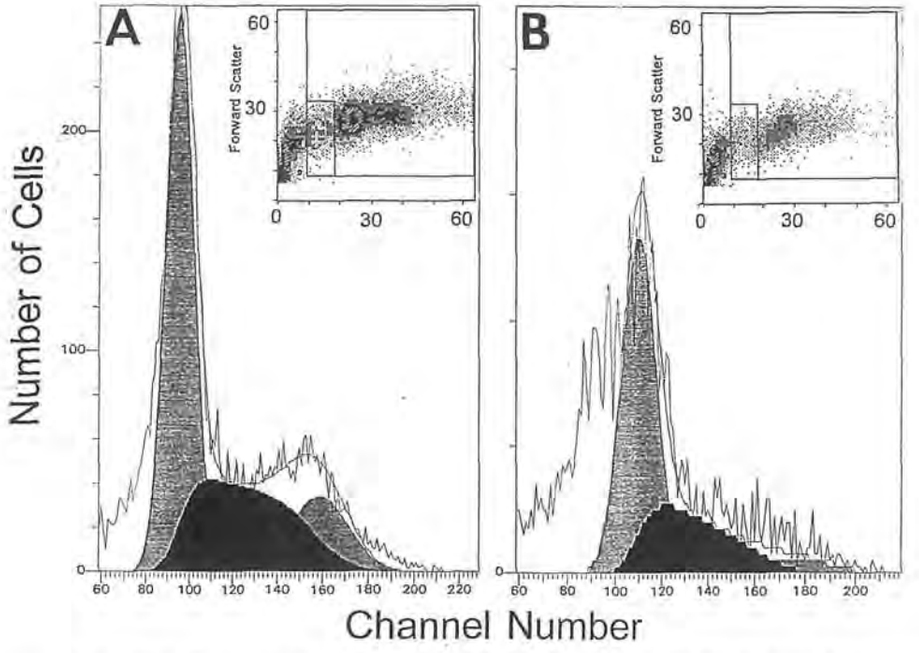

Figure 3. Flow cytometric analysis of Raji cells treated with control or c-*myb* antisense oligonucleotides for 72 h for their cell cycle analysis and apoptotic cell populations (A_0). A, Cells treated with c-*myb* antisense oligonucleotide; B, Cells treated with control oligonucleotides. The number of cells undergoing apoptosis are shown in the inner box present in the cell scattergram shown in each panel.

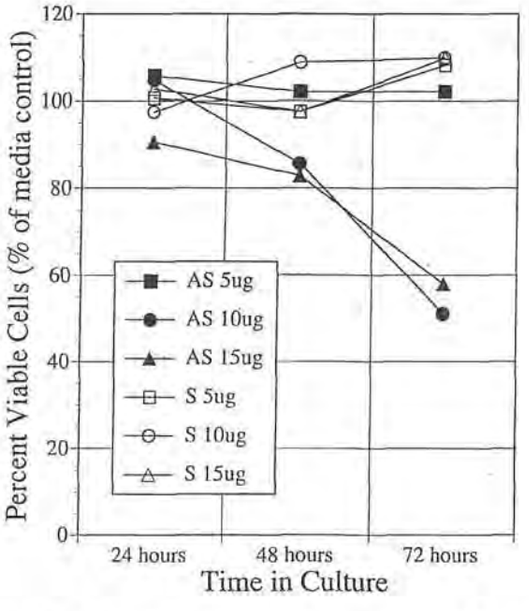

Figure 4. c-*myb* antisense oligonucleotide induced apoptosis in Raji cells as determined by flow cytometric analysis described in Fig. 3. Percent viable cells were calculated from Fig. 3 data from panel A and C and expressed as percentage of media alone control.

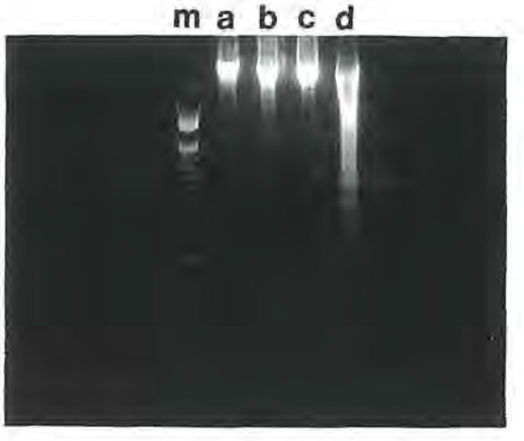

Figure 5. DNA ladder analysis of Raji cells treated with control and c-*myb* antisense oligonucleotides *in vitro* for 72 h. m, Marker DNA; A, Control cells; B, Cells treated with control oligonucleotides (scrambled c-*myb* sequences); C, Cells treated with ISIS-182 oligonucleotides; D, Cells treated with antisense c-*myb* oligonucleotides.

media only or control oligonucleotides did not show such significant number of cells in apoptosis.

In order to determine the effects of c-*myb* antisense ODN on normal human hematopoietic stem/progenitor cells, the normal human volunteer donors peripheral blood stem cell products were treated with c-*myb* antisense ODN for 36 h at 37°C at a concentration of 50 µg/ml. The CFU-GM and BFU-e content in the treated cells as wel as control cells were determined using *in vitro* clonogenecity assay. Fig. 7 shows the CFU-GM and BFU-e colonies formed by the control and c-*myb* treated cells before and after incubation with or without c-*myb* antisense oligonucleotides. No significant difference between control and c-*myb* treated cells was observed with regard to their hematopoietic stem/progenitor cell colony forming ability as determined by their ability to form CFU-GM or BFU-e hematopoietic progenitor cell colony formation *in vitro*. These results indicate that the c-*myb* antisense ODNs do not have any harmful effect on normal hematopoietic stem progenitor cells.

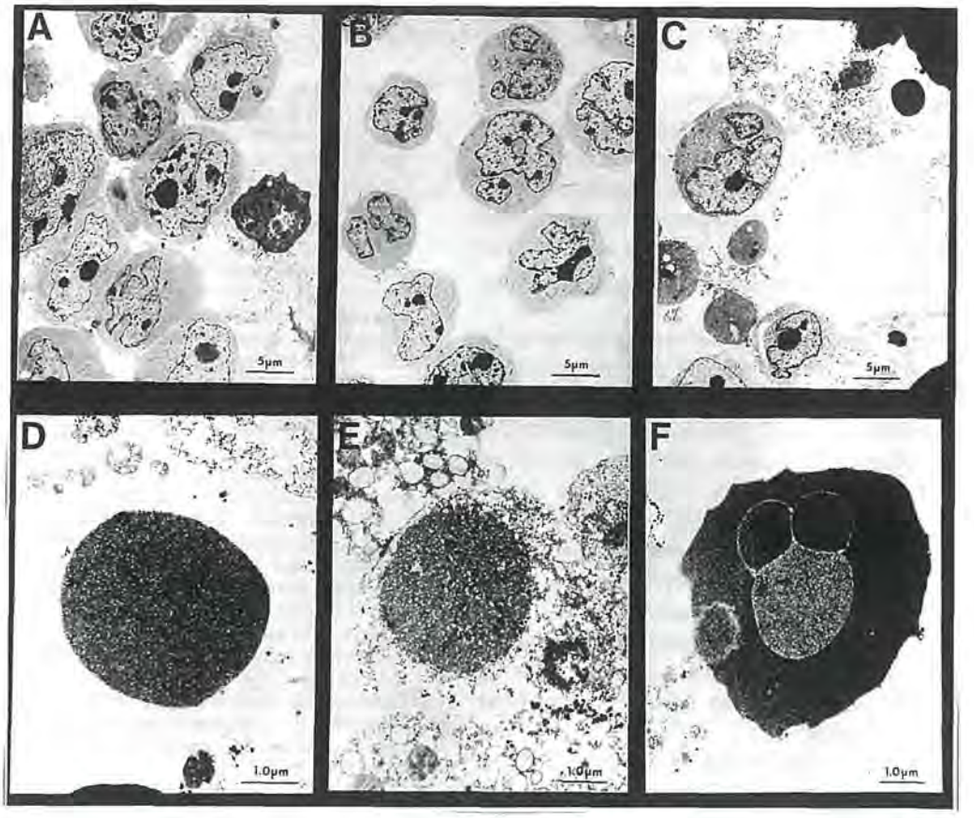

Figure 6. Electron microscopic analysis of Raji cells treated with control and c-*myb* antisense oligonucleotides *in vitro* for 72 h. A, control cells; B, Cells treated with control oligonucleotides; C, Cells treated c-*myb* antisense oligonucleotides; D, E and F, Higher magnification of apoptotic Raji cells treated with c-*myb* antisense oligonucleotides.

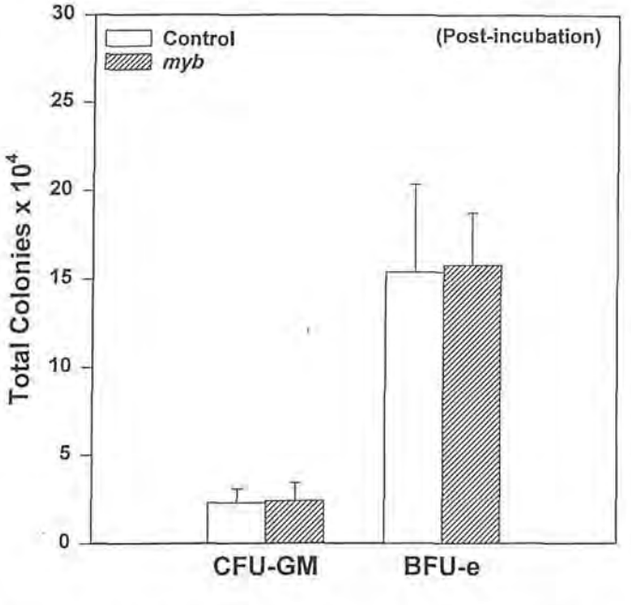

Figure 7. Effects of c-*myb* antisense ODN on CFU-GM and BFU-e colony formation by normal human peripheral blood stem/progenitor cells. The cells were incubated at 37°C for 36 h with 50 μg/ml oligonucleotides. After the incubation period, the cells were washed and assayed for CFU-GM and BFU-e content as described in the text.

Discussion

This report describes a significant inhibitory effect by a phosphorothioate ODN complementary to c-*myb* mRNA on the growth of Raji human Burkitt lymphoma cells. Similar antiproliferative effects of the same c-*myb* antisense ODN have been reported for HL-60 myeloid leukemic cells (6) as well as chronic myelogenous leukemia cells (7,8). However, the concentrations of oligonucleotides required to significantly inhibit the growth of Raji cells was considerably lower than that of the concentration used in other studies (20 μg/ml versus 50-100 μg/ml). Raji cells are sensitive to the inhibitory effects of c-*myb* antisense ODN at 10-20 μg/ml when compared to other cells, such as K562, which required 80-100 μg/ml for inhibition in our laboratory. The growth inhibitory effects of Raji cells by c-*myb* antisense ODN appear to be specific because other sequences such as scrambled c-*myb* or ISIS-1082 oligonucleotides were ineffective and appear to exert an antisense effect because we see a decreased message for c-*myb* gene in antisense ODN-treated Raji cells (Fig. 2). One of the prominent features of the effects of the c-*myb* antisense ODN on Raji cells was induction of apoptosis. However, about 48 h of exposure of the cells to antisense ODN were required to see a significant induction of apoptosis. This is a much longer time span than the 4-6 h required for induction of thymocyte apoptosis by dexamethasone (18). It is, however, shorter than the 5-10 days required for induction of apoptosis of leukemia cells treated with an ODN complementary to the mRNA of the

p53 gene (Copple B, *et al*: Proc Am Assn Cancer Res 35: Abst 307, 1994; Iversen PL *et al*: Proc Am Assn Cancer Res 35: Abst 307, 1994). In the latter instance, superinduction of p53 mRNA might be a consequence of ODN treatment, and this is the most likely cause of late apoptosis. Because of the timing of the apoptosis induction of Raji cells by the anti-c-*myb* ODN, this is possibly a consequence of interference with cell cycle progression and not a direct effect. Other genes may be induced during this period, which is the subject of further investigation.

Both c-*myc* and c-*myb* proto-oncogenes are known to be involved in regulation of cell proliferation and differentiation. In the case of Raji cells, c-*myb* but not c-*myc* appears to be important in regulation of cell growth. Antisense ODNs targeting c-*myc* did not significantly inhibit the growth of Raji cells *in vitro* (Joshi and Wu, unpublished data). Therefore, downregulation of the c-*myb* proto-oncogene, preferentially expressed in hematopoietic tissues by antisense ODNs complementary to an appropriate segment of the c-*myb* mRNA, might have therapeutic potential against some hematological malignancies, including Burkitt lymphoma. However, the malignancies which might respond must be defined, since not all tumors may have dysfunctional regulation of the c-*myb* gene.

Normal organ and tissue toxicity is one of the major concerns in using an antisense ODN complementary to an essential gene, such as these proto-oncogenes. However, at least in the case of the c-*myb* antisense ODN, no significant toxicities to normal tissues have been observed *in vivo* (12), although the consequences of c-*myb* expression by the most primitive hematopoietic precursor cells is yet to be fully explored. If the majority of such cells are quiescent and only express c-*myb* when cycling, this may have implications for c-*myb* administration scheduling. Since our studies indicated that the c-*myb* antisense ODNs are cytotoxic to malignant cells such as Raji cells and not cytotoxic to normal hematopoietic stem progenitor cells, the c-*myb* antisense ODNs can also be used to purge malignant cells contaminated in the stem cell products. In this regard, c-*myb* antisense ODN has been used to purge leukemic cells in acute myelogenous and chronic myelogenous leukemia patients (Bishop *et al*, unpublished results). Our results, therefore, warrant further *in vitro* studies to define the mechanism(s) of action of Raji cell growth inhibition induced by antisense to c-*myb*. Preclinical *in vivo* studies are also warranted to determine the therapeutic efficacy of c-*myb* antisense ODNs against Burkitt lymphoma as an essential step in the development of this novel clinical application technology.

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