Suppression of *c-myc* oncogene expression by a polyamine-complexed triplex forming oligonucleotide in MCF-7 breast cancer cells

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ABSTRACT

Polyamines are excellent stabilizers of triplex DNA. Recent studies in our laboratory revealed a remarkable structural specificity of polyamines in the induction and stabilization of triplex DNA. 1,3-Diaminopropane (DAP) showed optimum efficacy amongst a series of synthetic diamines in stabilizing triplex DNA. To utilize the potential of this finding in developing an anti-gene strategy for breast cancer, we treated MCF-7 cells with a 37mer oligonucleotide to form triplex DNA in the up-stream regulatory region of the *c-myc* oncogene in the presence of DAP. As individual agents, the oligonucleotide and DAP did not downregulate *c-myc* mRNA in the presence of estradiol. Complexation of the oligonucleotide with 2 mM DAP reduced *c-myc* mRNA signal by 65% at 10 μ M oligonucleotide concentration. In contrast, a control oligonucleotide had no significant effect on *c-myc* mRNA. The expression of *c-fos* oncogene was not significantly altered by the triplex forming oligonucleotide (TFO). DAP was internalized within 1 h of treatment; however, it had no significant effect on the level of natural polyamines. These data indicate that selective utilization of synthetic polyamines and TFOs might be an important strategy to develop anti-gene-based therapeutic modalities for breast cancer.

INTRODUCTION

Blocks of oligopurine.oligopyrimidine sequences are known to form triple helical DNA (triplex DNA) with oligonucleotides capable of binding through the major groove of the duplex DNA (1–5). Therapeutic application of triplex DNA has come to the forefront as an anti-gene strategy recently. Triplex forming oligonucleotides (TFO) have been selectively utilized to suppress the transcription of growth regulatory genes, such as *c-myc* (6), interleukin-2 receptor (7), DNA polymerase α (8), epidermal growth factor receptor (6), dihydrofolatereductase (9), Ha-*ras* (10) and a progesterone responsive gene (11). A major impedi-

ment in advancing this technology has been the low stability of triplex DNA under physiological conditions (4,5). Based on our previous studies on the structural specificity of polyamines in DNA conformational dynamics, including the induction and stabilization of left-handed Z-DNA (12,13) and protein–DNA interactions such as estrogen receptor–DNA recognition (14,15), we undertook a systematic analysis of the ability of natural and synthetic polyamines and their acetylated derivatives in stabilizing triplex DNA (16). Our data showed that polyamines with a trimethylene ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-$) bridging region between the amino and imino groups exerted the maximal efficacy in stabilizing triplex DNA. Among the diamines, DAP increased the melting temperature (T_m) of a poly(dA).2poly(dT) triplex by 10°C compared to that exerted by its nearest lower or higher homologs.

In order to examine whether the selective stabilization of triplex DNA by polyamines could be used in suppressing the transcription of a disease-related gene, we undertook the present study on the transcription of the protooncogene *c-myc* in a hormone-responsive breast cancer cell line, MCF-7. A significantly higher level of *c-myc* gene expression is found in 25% of breast cancer tissues than that of normal tissues (17). The *c-myc* gene is also an ‘immediate early’ gene that is transcriptionally stimulated in response to a variety of growth stimulatory agents, including estradiol, the female hormone that is believed to play a major role in the induction and progression of breast cancer (18). The binding of estradiol with the estrogen receptor (ER) triggers a series of structural and conformational alterations in ER, transforming it to a form that binds to DNA with high affinity (18–20). Recognition of the estrogen response element (ERE) in target genes by ER modulates the transcription of estrogen-responsive genes. The *c-myc* oncogene is stimulated by estradiol within the first 2 h of treatment in MCF-7 cells (18). We previously used this cell line as a model for dissecting the growth regulatory mechanism(s) involving estrogens, anti-estrogens, polyamine homologs and polyamine biosynthetic inhibitors (22–26).

The naturally occurring polyamines, putrescine, spermidine and spermine, are known to play an important role in cell proliferation and differentiation (27,28). Cellular polyamine levels are exquisitely regulated by growth stimulatory and inhibitory agents (29). A major portion of intracellular polyamines is believed to exist in

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Cells ($2-3 \times 10^6$) were harvested from culture, washed with phosphate buffered saline (PBS) and collected by centrifugation. Cells were then treated with 300 μ l of 8% sulfosalicylic acid and sonicated for 15 s in ice. The solution was incubated in ice for 1 h and centrifuged at 10 000 g for 5 min using a microfuge to remove the precipitated protein. Intracellular polyamine levels were determined by HPLC after converting them to their dansyl derivatives by a previously described procedure (24). 1,6-diaminohexane was used as an internal standard. HPLC was performed with a Hewlett Packard HP 1090 Liquid Chromatograph using HP 1046A Programmable Fluorescence Detector.

Figure 1. Effect of estradiol on *c-myc* mRNA levels in G1 arrested MCF-7 cells. Cells were synchronized by isoleucine starvation for 30 h. Estradiol was added to G1-synchronized cells and the medium was changed to that containing isoleucine. Control cells were treated with an equivalent volume of ethanol as used in the preparation of estradiol. Cells were harvested in TRI-Reagent and RNA was analyzed by Northern blotting. A typical autoradiogram, showing the *c-myc* hybridization signal, is presented in (A) and the corresponding ethidium bromide-stained RNA gel is shown in (B).

T_m measurements

Oligo I, Oligo III and Oligo IV were dissolved separately in water. The three strands were then mixed in 1:1:1 molar ratio in 10 mM cacodylate buffer (pH 7.5) or 10 mM Tris-HCl buffer (pH 7.5) in the presence or absence of DAP. The solutions were boiled for 10 min and allowed to attain equilibrium at 22°C for 16 h.

Absorbance versus temperature profiles were recorded using a Perkin Elmer Lambda 2 spectrophotometer (16). The temperature of the five-cell holder was controlled by a thermoelectrically regulated programmer interfaced to an IBM PS2 computer. Melting profiles were obtained by increasing the temperature at a rate of 0.5°C per min with the absorbance and temperature being recorded every 30 s. Melting temperature was taken as the temperatures corresponding to half dissociation of the complexes and the reproducibility was within $\pm 0.5^\circ\text{C}$. The first derivative, dA/dT (where A is absorbance and T is temperature), of the melting curve was computer generated and was also used for determining melting temperature of the triplex (T_{m1}) and duplex (T_{m2}) forms of the oligonucleotide and these two methods were consistent within 0.5°C .

RESULTS

Figure 1 shows the effect of 4 nM estradiol on *c-myc* mRNA levels in MCF-7 cells progressing in cell cycle. We used 4 nM estradiol in these experiments because previous cell growth and gene regulation studies were conducted under these conditions (22,23,25). There was no significant increase in cell proliferative response at higher concentrations of estradiol. At the 0 h time point, the medium was changed to that containing isoleucine and estradiol (in ethanol) and was added to triplicate dishes. Equivalent volume of ethanol was added to control cells. As is evident from Figure 1A, estradiol stimulated *c-myc* mRNA at 30 min after its addition. Increased *c-myc* mRNA was also observed at the 60 and 90 min time points. RNA gel picture (Fig. 1B) showed that equivalent amount of RNA was loaded in all lanes. Further analysis of the membrane with the GAPDH probe also showed that the observed differences in *c-myc* mRNA levels were not due to differences in transfer efficiency (results not shown). Densitometric scanning and normalization with GAPDH showed

Figure 2. Effect of Oligo I and DAP as individual agents on the expression of *c-myc* mRNA in MCF-7 cells. (A) G1 arrested cells were allowed to progress in cell cycle for 30 min (lanes 1–3) or 1 h (lanes 4–6) in the presence (4 nM) (lanes 2,3,5 and 6) or absence (lanes 1 and 4) of estradiol. Lanes 3 and 6 represent *c-myc* mRNA in Oligo I (10 μM)-treated cells. (B) G1 arrested cells were allowed to progress in cell cycle in the presence of estradiol alone (lanes 1 and 4), estradiol + 2 mM DAP (lane 2 and 5), estradiol + 5 mM DAP (lanes 3 and 6). Cells were harvested 30 min (lanes 1–3) or 1 h (lanes 4–6) after the addition of estradiol or estradiol + DAP.

2- to 4-fold increase in the intensity of the *c-myc* mRNA band in estradiol-treated cells compared to that in untreated control.

We next examined the effect of Oligo I on the expression of *c-myc* mRNA at various time points (Fig. 2A). G1 synchronized cells were treated with estradiol or estradiol + Oligo I. *c-myc* mRNA induction was observed at the 30 and 60 min time points. After normalization of the blot intensity with that of GAPDH, estradiol induction of *c-myc* mRNA was in the 1.5- to 2-fold range. Addition of 10 μM TFO did not suppress the *c-myc* mRNA level at 30 and 60 min after its addition. In contrast, a 30% increase in the level of *c-myc* mRNA was observed. The reason for this increase is not clear, but it is possible that the *c-myc* mRNA stability might have changed in the presence of the TFO.

The effect of DAP was also studied as a single agent on the transcription of *c-myc* oncogene in MCF-7 cells (Fig. 2B). We used 2 and 5 mM concentrations of the DAP in these experiments. Previous physical chemical studies showed that these concentrations were effective in stabilizing poly(dA).2poly(dT) triplex (16). At 2 mM concentration, DAP had no marked effect on *c-myc* mRNA; however, in the presence of 5 mM DAP, a 2- to 3-fold increase was found, compared to the control. This increase might be due to enhanced transcription of *c-myc* mRNA since natural polyamines, such as spermidine and spermine, enhanced *c-myc* mRNA level (36). To optimize conditions of reduced *c-myc* expression, we chose 2 mM DAP in subsequent experiments.

Figure 3A shows our results on the effect of a combination of DAP and Oligo I on *c-myc* mRNA level in MCF-7 cells in the presence of estradiol. In contrast to the stimulatory effect of Oligo I as an individual agent, the combination of Oligo I and DAP suppressed the transcription of *c-myc* oncogene at the 1 h time point (lane 5). Densitometric scanning showed a 65% decrease in *c-myc* mRNA level compared to that in estradiol-treated (lane 2) cells. As shown in Figure 3B, addition of 10 μM control oligonucleotide (Oligo II) and 2 mM DAP showed only a minor change (15%) in *c-myc* mRNA level. These results indicate that suppression of *c-myc* gene expression by polyamine-complexed Oligo I is specific to the triplex forming nucleotide sequences and is not a non-specific effect of the oligonucleotide.

In the next series of experiments, we examined the dose-dependent effect of Oligo I on *c-myc* expression (Fig. 4). In the presence of 2 mM DAP, increasing concentrations of Oligo I exerted a dose-dependent suppression of *c-myc* gene expression. After normalization with GAPDH signal, *c-myc* mRNA levels

Figure 3. Suppression of *c-myc* mRNA in the presence of DAP complexed Oligo I. G1 arrested cells were allowed to progress in cell cycle for 1 h in the presence of various agents as follows. (A) Lanes represent cells treated with ethanol (lane 1), 4 nM estradiol (lane 2), 4 nM estradiol + 2 mM DAP (lane 3), 4 nM estradiol + 10 μ M Oligo I (lane 4) and 4 nM estradiol + 10 μ M Oligo I + 2 mM DAP (lane 5). (B) Cells were treated with ethanol vehicle (lane 1), 4 nM estradiol (lane 2) and 4 nM estradiol + 10 μ M Oligo II + 2 mM DAP (lane 3). Similar results were obtained in two triplicate experiments. Standard deviations in intensity of the hybridization signal, quantified by a laser densitometric scanner, were within 10%.

were 22, 50 and 84%, respectively, lower than that of untreated control in the presence of 5, 10 and 20 μ M concentrations of Oligo I. We also determined the expression of another estradiol-induced gene, *c-fos* in the presence of oligo I and DAP (Fig. 4). The normalized *c-fos* hybridization signal was only reduced by 5, 13 and 18%, respectively, in the presence of 5, 10 and 20 μ M Oligo I and 2 mM DAP.

We conducted the majority of our experiments using synchronized cells in order document the sequential changes in cell growth- and cell cycle-regulatory genes as a part of our ongoing studies on estrogenic regulation of cell cycle in breast cancer. We have, however, determined the effect of DAP (2 mM) and Oligo I (10 μ M) in logarithmically growing cells and found a similar effect of a combination of Oligo I and DAP on *c-myc* mRNA.

Quantification of the yield of total RNA in different treatment protocols varied from 42 to 63 μ g/dish. There was no pattern suggestive of increased degradation after Oligo I treatment, but a general increase in total RNA (10–30%) occurred in the presence of DAP and Oligo I, compared to controls. These results suggest that the effect of Oligo I + DAP on the expression of *c-myc* gene is not due to non-specific activation of ribonuclease.

A facile transportation of polyamines within MCF-7 cells has been found by us and others (25). To examine whether the internalization of DAP is affected by Oligo I, we determined intracellular polyamine levels using a sensitive HPLC technique. We harvested cells treated with DAP in the presence and absence of Oligo I at 0, 0.5, 1 and 4 h for polyamine assay. As shown in Figure 5, DAP was internalized within 0.5 h of its addition to cell culture medium in the absence of Oligo I. DAP levels peaked within 1 h of treatment. However, there was a delay in the internalization of DAP in cells treated with this polyamine in combination with Oligo I. Measurable levels of intracellular DAP was found at 1 h of treatment. At this point, DAP concentration was 50% lower than that found in cells treated with this diamine alone. There was no marked increase in DAP levels beyond the 1 h time point in both cases.

We next examined whether the gene regulatory effects of DAP in combination with Oligo I is due to depletion of natural polyamines. Our results, presented in Figure 6, showed no

Figure 4. Oligo I dose dependent effects on *c-myc* (left 4 lanes) and *c-fos* (right 4 lanes) mRNA levels in the presence of 2 mM DAP. G1 arrested cells were allowed to progress in cell cycle for 1 h in the presence of the indicated concentrations of Oligo I. Total RNA was extracted and Northern blot analysis was conducted to determine the level of *myc* and *fos* mRNAs. GAPDH levels from the corresponding blots are also given for comparison.

significant difference in polyamine levels in DAP-treated cells in the presence or absence of Oligo I. Thus DAP has no major effect on polyamine biosynthesis or degradation, under conditions at which it could suppress *c-myc* gene expression in association with Oligo I.

We also examined whether DAP could stabilize triplex formation *in vitro* in Oligo I and the sequences of the promoter region of *c-myc* gene (Oligo III and Oligo IV) by T_m measurements. There was only one transition of the oligonucleotide complex in Tris buffer, in the absence of DAP ($T_m = 77^\circ\text{C}$). In contrast, in the presence of 0.5 mM DAP two sharp transitions corresponding to T_{m1} at 80°C and T_{m2} at 97°C were observed. From mixing experiments at different ratios, T_{m1} was assigned to triplex melting whereas T_{m2} represented duplex melting. The increase in duplex T_m from 77 to 97°C is a polyamine effect as reported earlier (16). In experiments conducted in 10 mM cacodylate buffer, however, there was only one transition both in the presence and absence of DAP. The formation of triplex DNA in Tris buffer might be a consequence of the ability of bulky cations such as Tris to disrupt tetraplex formation in G-G rich oligonucleotides (37,38). These experiments clearly demonstrate the ability of diaminopropane to stabilize triplex DNA at the sequences present in the promoter region of the *c-myc* gene.

DISCUSSION

Results presented in this paper demonstrate that a TFO (Oligo I) complexed with DAP has a marked effect on suppressing the steady-state level of *c-myc* mRNA. Oligo I was selected to form triplex DNA at the promoter region of *c-myc* oncogene, as suggested from previous studies by other investigators (3,6,39). Our *in vitro* T_m measurements confirmed the ability of Oligo I to form triplex DNA in the presence of DAP. A control oligonucleotide with the same nucleotide composition, but a sequence distribution not amenable to triplex DNA formation, was unable to alter *c-myc* mRNA level significantly. DAP was the most efficacious diamine in triplex DNA stabilization in our physical chemical studies (16). As individual agents, Oligo I and DAP had a tendency to increase *c-myc* mRNA levels in MCF-7 cells. In contrast, up to 84% decrease in *c-myc* mRNA occurred by combining these two agents. Quantification of polyamine levels showed that the effect of DAP-complexed Oligo I was not a consequence of interference with the milieu of natural poly-

Figure 5. Effect of Oligo I on the uptake of DAP in MCF-7 cells. G1 arrested cells were allowed to progress in cell cycle in the presence of 4 nM estradiol (■), 4 nM estradiol + 2 mM DAP (▨) and 4 nM estradiol + 10 μ M Oligo I + 2 mM DAP (▧). Cells were harvested at 0.5, 1 and 4 h after initiation of cell cycle and intracellular DAP level was quantified by an HPLC technique, after pre-column derivatization with dansyl chloride. Results shown are the mean of triplicate experiments. Error bars $\pm$ SD.

amines within the cell. Thus, selective utilization of synthetic polyamines might be an important strategy to realize the potential of triplex DNA-based gene inactivation.

Several recent studies have attempted to utilize triplex DNA-based anti-gene strategy as a therapeutic approach for treating cancer and other diseases by selective inactivation of disease-related genes. DNase I foot-printing and gel mobility assays have shown triplex formation by Oligo I in the promoter region of *c-myc* (3,6). Down-regulation of *c-myc* by triplex formation has also been shown in HeLa cells (39). In other studies, transcription of IL-2 receptor gene (7,40) was down-regulated by a modified oligonucleotide targeted for triplex formation at the promoter region of this gene. In spite of these encouraging reports on the use of triplex DNA as an anti-gene strategy, oligonucleotide uptake and stability within cells remain highly variable between cell cultures systems and between laboratories (41). The successful suppression of *c-myc* mRNA in our system could be attributed to several factors: (i) the optimization of triplex stabilization for polyamine structure and concentration by physical chemical studies; and (ii) potential enhancement of oligonucleotide uptake in the presence of DAP, although further studies are needed to demonstrate this effect. Since peak induction of *c-myc* expression was observed within 1 h of addition of estradiol and this induction was found to be transient in both logarithmic and synchronized cells, we focused on the effects of Oligo I and DAP at this time point. The dynamics of *c-myc* induction and the stability of *myc* mRNA as well as the up-take and stability of the oligonucleotides might be important in understanding differences observed between this and other systems.

There was a decrease in the up-take of DAP in the presence of the oligonucleotide. Breast cancer cells have been reported to have a highly regulated polyamine transporter (42). It is not known whether Oligo I utilizes this pathway for up-take. On the other hand, in the presence of Oligo I, DAP might be entering the cell through a much inefficient pathway associated with the up-take of Oligo I. Studies on changes in the internalization mechanisms of DAP and Oligo I as individual agents and in combination might

Figure 6. Effect of Oligo I and DAP on the level of natural polyamines in MCF-7 cells. G1 arrested cells were allowed to progress in cell cycle in the presence of estradiol (4 nM, control), or estradiol + 10 μ M Oligo I + 2 mM DAP (treatment). Cells were harvested at 0.5, 1 and 4 h after initiation of cell cycle and intracellular polyamine levels were quantified by HPLC. Symbols represent putrescine in control (▨) and treatment (□) groups; spermidine in control (▧) and treatment (■) groups. Results shown are the mean of triplicate experiments. Error bars $\pm$ SD.

provide important new insights on the function of proteins involved in polyamine and oligonucleotide transport.

Recent studies indicate that the effects of TFOs might also include other pathways of gene regulation. For example, specific proteins have been found to bind to triplex DNA/H-DNA and act as a transcriptional insulator (43). Our *in vitro* studies indicated a high affinity binding of estrogen receptor to poly(dA-dT).2poly(dA-dT) triplex in the presence of polyamines (44). Furthermore, the oligonucleotide used in this study is rich in guanosine and the possibility of G.G hydrogen bonding and the formation of tetraplexes also exist (37,38). Tetraplex formation has been reported to be important in the anti-viral activity of certain oligonucleotides (45). Thus further studies are needed to elucidate the precise mechanism of action of TFOs including the one used in these experiments. Our control experiments, especially that showing a lack of Oligo I + DAP effect on *c-fos* expression in MCF-7 cells, indicate that Oligo I might be acting at the *c-myc* promoter region through triplex DNA formation.

Strategies to circumvent problems of oligonucleotide stability include use of intercalating agents such as ethidium and berenil and the substitution of an abasic or polymeric linker (46–49). These modifications are likely to alter the conformational and configurational flexibility of the oligonucleotide and clamp the oligonucleotide within the major groove of double helical DNA. Alternatively, optimization of triplex stability might be achieved partly by variations in oligonucleotide length and sequence (50). Our approach has been to use closely related homologs of the naturally occurring polyamines (16). Addition of exogenous spermidine, spermine or their homologs are likely to be more toxic than putrescine or putrescine homologs as the higher polyamines are better substrates for polyamine oxidases that generate hydrogen peroxide and aldehydes as degradation products (51). In other studies, we found that DAP had no toxic effect on the growth and immune response of normal cells (52). An important advantage of using DAP in stabilizing triplex DNA is that this compound undergoes facile transportation within cancer cells (53) and the

internalization of this polyamine has no significant effect on the well-orchestrated polyamine homeostasis mechanism(s) (Fig. 6). Thus, the natural functions of polyamines are not affected by this synthetic diamine, at least at the early time periods. Since *c-myc* is an 'immediate early gene' activated in the early stages of cell cycle progression, the consequences of suppressing the expression of this gene should include blockade of cell cycle progression. Thus the use of this diamine in stabilizing triplex DNA might provide a good therapeutic window in gene specific therapy.

Breast cancer is a major public health problem in the Western world today, affecting one in eight women in the US. Despite extensive research during the past several decades, the death rate from this malady has shown only a slight increase. Results of our present study, in conjunction with our previous findings on the structural specificity of polyamines in stabilizing triplex DNA (16), indicate a new approach of using selective homologs of the natural polyamines in developing an anti-gene strategy for breast cancer.

In summary, our results show that complexation of a triplex forming oligonucleotide with a polyamine might be a viable approach to realize the potential of triplex DNA-based anti-gene strategy for breast cancer treatment. This approach might have general utility in selective suppression of disease-related genes.

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