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MODULATION OF GROWTH GENE EXPRESSION BY SELECTIVE ALTERATION OF POLYAMINES IN HUMAN COLON CARCINOMA CELLS

Paul Celano, Craig M. Berchtold,
Francis M. Giardiello and Robert A. Casero, Jr.

The Johns Hopkins Oncology Center
and The Johns Hopkins University School of Medicine
Baltimore, Maryland 21231

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SUMMARY: The biosynthesis of the polyamines, putrescine, spermidine and spermine, is temporally linked with expression of many growth related genes. Our previous studies have shown that generalized polyamine depletion of the human colon cancer cell line COLO 320 by 2-difluoromethylornithine is associated with decreased transcription of the c-myc, c-fos, and ornithine decarboxylase (ODC) genes. In the current study, the role of individual polyamines was further defined by the use of a specific inhibitor of spermidine synthase, S-adenosyl-1,8, diamino-3-thio-octane (AdoDATO), and a spermine analogue, N^1,N^{12} bis(ethyl)spermine. Our data demonstrate that depletion of spermidine results in a 60-90% decrease in c-myc mRNA steady state levels. In contrast, c-fos mRNA levels are decreased only when both spermidine and spermine are diminshed. Furthermore, ODC mRNA levels are increased when all polyamines are decreased by DFMO, but are unaffected by a selective reduction in intracellular spermidine levels by AdoDATO. These studies suggest that individual polyamines may have a selective role in the expression of specific growth related genes.

INTRODUCTION: Stimulation of quiescent cells to grow is associated with a nearly simultaneous increase in new polyamine biosynthesis and expression of c-myc, and c-fos, as well as numerous other genes (1-5). Our previous studies have demonstrated that polyamine depletion of the human colon cancer cell line COLO 320 with 2-difluoromethylornithine (DFMO), a suicide inhibitor (6) of ornithine decarboxylase (ODC), results in a decrease in the transcription of the c-myc and c-fos protooncogenes (7,8). Since DFMO results in a decrease all three polyamines, putrescine (Put), spermidine (Spd), and spermine (Spm), the role of specific polyamines in the the regulation of the expression of these genes is unclear. This is important because individual polyamines have a structure-function relationship in overall cell growth (9,10) and in alteration of DNA conformation (11-15). In this study, we analyze the role of individual polyamines by comparing the effects of generalized decrease in polyamines by either DFMO or a spermine analogue, N^1,N^{12} bis(ethyl)spermine (BESpm) (16). This compound has been shown to decrease intracellular polyamines by down regulation of ODC and S-adenosyl methionine decarboxylase, increased

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polyamine catabolism, and export of natural polyamines from the cell. In addition, we examine the effects of a selective decrease in spermidine by the use of a highly specific transition state inhibitor of spermidine synthase, S-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) (17) in human colon cancer cells. These studies suggest that spermidine and spermine may have a differential role in the modulation of the expression of the c-myc and c-fos protooncogenes.

MATERIALS AND METHODS

Chemicals: DFMO was a gift from Merrell-Dow Research Institute (Cincinnati, OH). N^1,N^{12} bis(ethyl)spermine was synthesized (16) and provided by the laboratory of Dr. R. Bergeron (Univ. Florida, Gainsville, FL) and S-adenosyl-1,8,diamino-3-thio-octane synthesized and provided by Dr. J. Coward (Univ. Michigan, Ann Arbor, MI) (17). All other compounds were reagent grade or better.

Cell culture conditions: The human colon carcinoma cell COLO 320 HSR (18) was obtained from the American Type Tissue Culture (Rockville, MD). These cells were propagated continuously in RPMI 1640 (GIBCO) and 9% fetal bovine serum (Sigma, St. Louis, MO). Time course studies were initiated at concentrations of 1 x 10^5 cells/ml in 75 cm^2 flasks. Treated cells were exposed to DFMO (5 mM), BESpm (10-200 uM), or AdoDATO (50-200 uM). Experiments that included spermidine (5 uM) were performed in the prescence of 1 mM aminoguanidine as an inhibitor of serum amine oxidase. Cells were fed every 4 days. Cell viability was determined by trypan blue exclusion.

Polyamine analysis: Total cellular polyamine concentrations were determined by reverse-phase high performance liquid chromatography method of Kabra et al. (19) using total cellular perchloric acid extracts as previously reported from our laboratory (7,8).

Northern blot analysis: Total cellular RNA isolation, electrophoresis and Northern hybridizations were performed as previously reported (7). Filters were serially hybridized to nick-translated [α-^{32}P]-dCTP labeled DNA probes for the human c-myc gene (ClaI-EcoRI fragment), cfos (NcoI-XhoI), ornithine decarboxylase (Dr. O. Janne, The Population Council, New York, NY) and β-actin (Dr. D. Cleveland, Johns Hopkins University).

RESULTS

Effect of DFMO, BESpm, and AdoDATO on cell growth and intracellular polyamine concentrations - Treatment of COLO 320 cells with DFMO or BESpm resulted in a significant inhibition of cell growth (Figure 1). Dose response experiments revealed no increased growth inhibition or cytoxocity with dosages up to 5 mM DFMO and 200 uM BESpm (Figure 1). In contrast, AdoDATO had no detectable effect on the growth of COLO 320 cells up to 200 uM concentration (Figure 1). In addition, no loss of cell viability or changes in morphology with any of the tested compounds were noted up to 12 days of treatment.

DFMO and BESpm result in a decrease of Put to below detectable levels significant decreases in Spm levels were also observed, 50% and 80% respectively for DFMO and BESpm (Table 1). DFMO treatment results in complete decrease in Spd, however COLO 320 cells treated with BESpm maintain significant Spd levels. Treatment with AdoDATO results in a selective decrease in

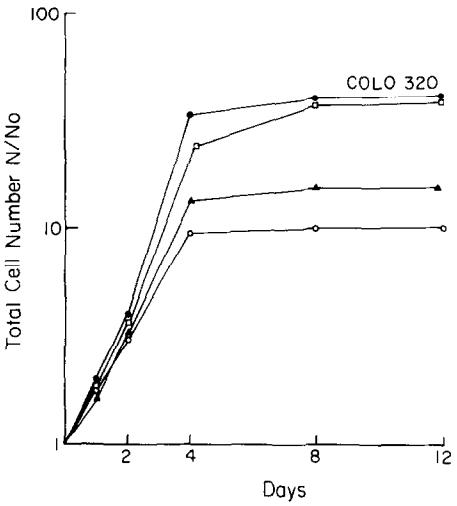

FIGURE 1: Growth kinetics of human colon carcinoma cells COLO 320 (●) treated with 5 mM DFMO (○), 100 uM AdoDATO (□) and 100 uM BESpm (△). In each case cells were refed (with fresh compound where indicated) every 3 days. Each point represents the mean of at least 3 separate determinations.

spermidine to less than 20% of control levels and a 1-2 fold increase in putrescine and spermine levels.

Effect of selective polyamine depletion in c-myc, c-fos, and ODC mRNA levels- Northern blot analysis of mRNA from COLO 320 cells treated with DFMO, BESpm, and AdoDATO revealed differential effects on the mRNA steady state levels of the genes examined (Figures 2A). All three compounds resulted in a

Table 1. Effect of 5 mM DFMO, 100 uM AdoDATO and 100 uM BESpm on the intracellular polyamine concentration in COLO 320 cells

	Polyamines (nmol/mg protein)[a]			
Treatment	Putrescine	Spermidine	Spermine	BESpm
Control	2.00	8.40	13.40	
DFMO	---[b]	---[b]	6.50	
AdoDATO	5.20	1.20	20.00	
BESpm	---[b]	0.90	2.40	19.90

[a] Polyamine values represent the mean of three determinations after 8 days in culture with a standard error of less than 15%.

[b] <0.05 nmol/mg protein.

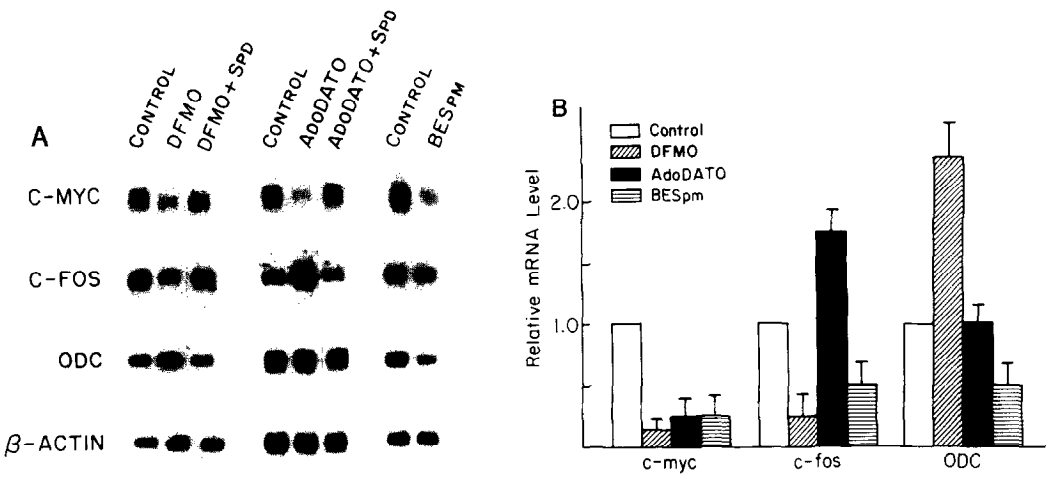

FIGURE 2: Northern blot analysis of growth associated genes in 5 mM DFMO ± 5 uM spermidine (Spd), 100 uM AdoDATO ± Spd, and 100 uM BESpm treated COLO 320 cells.
(A) Total cytoplasmic RNA was isolated from COLO 320 cells after 8 days of treatment, electrophoresed (10 ug/lane), transfered to nylon membranes and hybridized with nick-translated probes (1 x 10^6 cpm/ml). For each compound, the figure represents an autoradiograph of a single filter that has been successively reprobed with the genes indicated.
(B) Quantitative analysis of Northern blot analysis by densitometry.

decrease of 60-90% in c-myc mRNA levels. Similarly, steady state levels of c-fos were decreased by DFMO and BESpm treatment. In contrast, AdoDATO, which results in a selective decrease in Spd, is associated with a 2 fold increase in c-fos mRNA levels. ODC mRNA levels varied with each compound tested with DFMO resulting in an increase in mRNA levels, AdoDATO had no effect, and BESpm resulted in a decrease in ODC levels.

The observed alterations in mRNA steady state levels in experiments with DFMO and AdoDATO could be prevented by the addition of exogenous spermidine (Figure 2A). Similar repletion experiments are not possible in studies using BESpm because of uptake competition between naturally occurring polyamines, spermidine, or spermine, and this analogue (20,22).

DISCUSSION: Polyamines are critical for the proliferation of normal and neoplastic cells (23,24). The temporal association between increased polyamine synthesis following a growth stimulus and the expression of the c-myc and c-fos protooncogenes has suggested that these events are mechanistically linked (1-6). Our previous studies have demonstrated that generalized depletion of intracellular polyamines with DFMO results in a decrease in c-myc and c-fos mRNA steady state levels (6,7). Further studies in isolated nuclei have also demonstrated that spermidine can selectively increase the transcription of

c-myc and c-fos in vitro (7). In the current study, we now demonstrate that selective modulation of intracellular polyamines with a specific inhibitor of spermidine synthesis, AdoDATO, and with the spermine analogue, BESpm, can differentially modulate the steady-state levels of these genes.

The decreased expression of the c-myc protooncogene appears to be associated with decreased levels of intracellular spermidine. All compounds utilized reduced intracellular spermidine (Table 1), and reduced c-myc mRNA steady state levels (Figure 2). Most importantly, experiments utilizing AdoDATO confirm our previous studies which have demonstrated that reduction of c-myc expression in COLO 320 cells is not simply the result of decreased growth rate. Cells treated with AdoDATO have virtually identical growth rate to control cells (Figure 1), but have a significantly reduced c-myc expression (Figure 2A). Furthermore, maintenance of intracellular spermidine by addition of spermidine to the culture media prevents the decrease in the expression of c-myc produced by AdoDATO treatment.

The expression of the c-fos and ODC genes is also selectively altered in response to alteration in intracellular polyamines. In contrast to c-myc, c-fos mRNA levels are decreased in COLO 320 cells only when both spermidine and spermine are reduced. A selective decrease of spermidine by AdoDATO results in a slight increase in c-fos expression. Steady-state mRNA levels of ODC are also unaltered by selective decreases in spermidine. Depletion of intracellular polyamines by DFMO results in an increase in ODC mRNA levels consistent with our previous findings (6,7), and those of others (25-28). Interestingly, treatment of COLO 320 cells with BESpm results in a decrease in ODC mRNA levels. Since appreciable intracellular levels of this spermine analogue are achieved, we speculate that this may be decreasing ODC mRNA levels by a feedback process operative at either the post transcriptional or transcriptional levels. It should be emphasized that despite the high intracellular concentration of BESpm, it is unable to support cell growth of COLO 320 cells (Figure 1) or cannot sufficiently substitute in the normal expression of the c-myc and c-fos genes (Figure 2). Additionally, recent studies suggest that BESpm treatment can result in a greater than 1000-fold induction of the spermidine/spermine N^2-acetyltransferase protein which may, in part, be mediated at the transcriptional level (29).

Several investigators have determined that natural and synthetic polyamines have specific structural determinants which can influence overall cell growth (9,10), B to Z conformational changes of DNA (11-14) and the structure of nucleosomes (15). The current study provides evidence that spermidine and spermine may have a differential role in the expression of specific genes. These studies support our previous studies using DFMO alone to demonstrate that polyamines may directly influence the transcription of the c-myc and c-fos

genes and may also influence both transcriptional and post-transcriptional stability of ODC mRNA (7). Polyamines also have a selective role in the expression of human chorionic gonadotropin mRNA in JEG-3 choriocarcinoma cells, (30) as well as the transcriptional induction of globin mRNA in differentiating mouse erythroleukemia cells (31). Further study will be necessary to determine the role of individual polyamines in the regulation of the expression of these genes by examining the chromatin structure, or the interaction and/or synthesis of key transcriptional regulatory factors.

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