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MODULATION OF POLYAMINE BIOSYNTHESIS AND TRANSPORT BY ONCOGENE TRANSFECTION

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Summary: The effects of oncogene expression on phenomena related to polyamine metabolism were examined in Rat-1 cells stably transfected with EJ2-ras or N-myc oncogenes. In ras-transfected cells, ornithine decarboxylase activity was about 12-times higher than in either the parent or N-myc-transfected cell lines. By contrast, polyamine uptake was markedly increased in N-myc-transfected cells, as indicated by their enhanced sensitivity to the antiproliferative and enzyme regulatory effects of the polyamine analog, N^1, N^{12}-bis(ethyl)spermine (BESm), their intracellular accumulation of BESm and by their increased sensitivity to the growth inhibitory effects of methylglyoxalbis(guanylhydrazone)--another analog which utilizes the polyamine transport mechanism. These associations between N-myc and ras expression and critical aspects of polyamine metabolism suggest a possible role for the latter in facilitating the growth promoting properties of these oncogenes.

By virtue of their close association with cell growth, the polyamine biosynthetic enzymes, ornithine decarboxylase (ODC, EC 4.1.1.17) and S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), exhibit interesting similarities to cellular oncogene and proto-oncogene products. Exposure of resting cells to growth promoting stimuli is typically accompanied by a rapid rise in ODC and SAMDC activities which thereafter parallel the proliferative response. The role of these enzymes is obligatory since highly specific inhibitors inhibit cell growth (1,2) and since enzyme deficient mutants are auxotrophic for polyamines (3). In the case of neoplasia, ODC, like certain oncogenes, seems to play a critical role in cell transformation as evidenced in tumor promotion (4) and carcinogenesis systems (5).

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Abbreviations: BESm, N^1,N^{12}-bis(ethyl)spermine; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; MGBG, methylglyoxal-bis(guanylhydrazone); SAMDC, S-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N-acetyltransferase.

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Similarities can also be extended to the proteins themselves since, in both cases, the gene products tend to be highly inducible, short-lived and critically regulated by sensitive control mechanisms (6,7).

We have undertaken to determine whether oncogene expression alters polyamine metabolism in N-myc or EJ2-ras-transfected Rat-1 cells--oncogenes with products of distinctly different intracellular functions and distributions (reviewed in 7). The parameters studied relate to polyamine biosynthesis and its regulation, polyamine catabolism and polyamine uptake. All are known to be under regulatory control and generally linked directly or indirectly to cell proliferation.

Materials and Methods

Materials. The spermine analog, N^1,N^{12}-bis(ethyl)spermine (BESm) was synthesized as described elsewhere (8). Methylglyoxal-bis(guanylhydrazone) (MGBG) was obtained from Aldrich Chemicals (Milwaukee, WI).

Cell culture. Cell lines of parent Rat-1 fibroblasts and Rat-1 cells stably transfected with EJ2-ras (using pUC EJ-6.6 and pSV2-gpt) or with N-myc (using pMp34.1 and SV2-hygro) were provided courtesy of Dr. Robert A. Weinberg (Whitehead Institute, Cambridge, MA). The method of transfection has been previously described (9). Cells were seeded into T25 flasks with RPMI-1640 medium containing 10% fetal bovine serum (Hyclone), penicillin/streptomycin and glutamine. Cultures of N-myc cells also contained 0.1 mg/ml hygromycin B as the selection marker with no effect on cell growth or drug sensitivity. Cells were harvested following short exposure to trypsin/EDTA and counted with an automated cell counter.

Enzyme Assays. ODC, SAMDC and spermidine/spermine N-acetyltransferase (SSAT) activities were determined on an enzyme-containing extract obtained by sonication of cells using enzyme assays described previously (10,11).

Polyamine pools. Polyamine levels were determined by high-pressure liquid chromatography using a modification (E. Kelly and C.W. Porter, unpublished data) of the method of Kabra *et al*. (12), whereby acid extracts and reference solutions were derivatized with dansyl chloride and extracted by elution from a Bond-ELUT C18 column with methanol on the Vac-ELUT apparatus (Analytichem International, Harbor City, CA). Methanol extracts were then injected onto a phenol column using a mobile phase methanol gradient (30 to 72% in phosphate buffer) with fluorescence detection at an excitation wavelength of 340 nm and an emission wavelength of 515 nm. Poly- amine levels were then calculated based upon external standard curves run within 48 hrs of the sample chromatograms.

Results and Discussion

Growth characteristics of the parent and oncogene-transfected Rat-1 cells are depicted in Fig. 1. All three cell lines grew at approximately the same rates during logarithmic growth with doubling times of 16 to 18 hr. Differences in growth kinetics became apparent at day 3 when the Rat-1 cells began to plateau due to contact inhibition while the transfected lines continued to grow until, presumably, media nutrients became limiting.

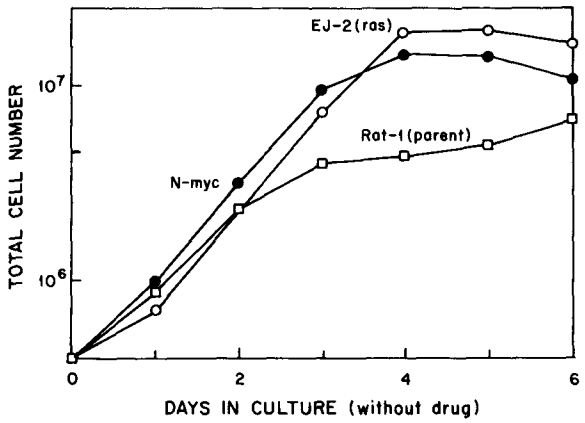

Figure 1. Growth of untreated Rat-1 cells and oncogene-transfected Rat-1 cells during a 6-day period without change of medium.

As shown by the values for untreated cells in Table 1, polyamine biosynthesis of the three cell lines was differentially affected in transfected cells. Whereas both transfected cell lines showed a marginal (2- to 3-fold) increase in SAMDC activity, the line transfected with ras showed an impressive 12-fold rise in ODC activity under steady-state growth conditions. Unexpectedly, these changes were not reflected in polyamine pools. The increase in ODC activity with ras transfection may be a general finding since Holtta et al. (13) have demonstrated a similar phenomenon in NIH 3T3 cells and Guerrero et al. (14) observed that ODC-mRNA was hyper-inducible in rat pheochromocytoma cells transfected with a mouse N-ras oncogene. In accompanying biochemical studies, Holtta et al. (13)

Table 1. Effect of BESm on Polyamine Enzymes and Pools in Rat 1 Cells and Oncogene-Transfected Rat 1 Cells*

Cell Line	Treatment (48 hr)	Enzyme Activity (nmol/hr/mg)			Polyamine Pools** (pmol/10^6 cells)			
		ODC	AdoMetDC	SSAT+	PUT	SPD	SPM	BESm
Rat 1	None	0.67	0.48	3.72	21	103	131	-
	10 μM BESm	0.66	0.45	5.82	11	59	83	8
ras	None	8.45	1.22	8.16	12	92	107	-
	10 μM BESm	8.35	1.17	7.98	11	90	90	9
N-myc	None	0.99	1.38	1.44	27	147	157	-
	10 μM BESm	0.07	0.24	70.92	0	5	29	260

*Based on data from three experiments performed in duplicate.
**PUT, putrescine; SPD, spermidine; SPM, spermine.
+SSAT activity measured after 24 hr treatment.

determined that the observed de-regulation of ODC activity in NIH 3T3 cells was largely due to an enhanced accumulation of ODC mRNA although other mechanisms also seem to be involved. While this increased ODC activity could afford cells a greater growth potential (15), it did not alter their sensitivity to the antiproliferative effects of the irreversible ODC inhibitor (1), α-difluoromethylornithine (DFMO, Fig 2A).

In contrast to results with DFMO, the cell lines were differentially sensitive to a regulator of polyamine biosynthesis, BESm (Fig. 2B). The latter is a spermine analog which interferes dramatically with polyamine biosynthesis by mimicking natural polyamines in suppressing ODC and SAMDC activities via regulatory mechanisms inherent to the pathway (10). In so far as these are understood, they include selective interference with enzyme protein translation and induction of a protein inhibitor of the enzyme (reviewed in 16). Whereas the parent Rat-1 cells and ras-transfected

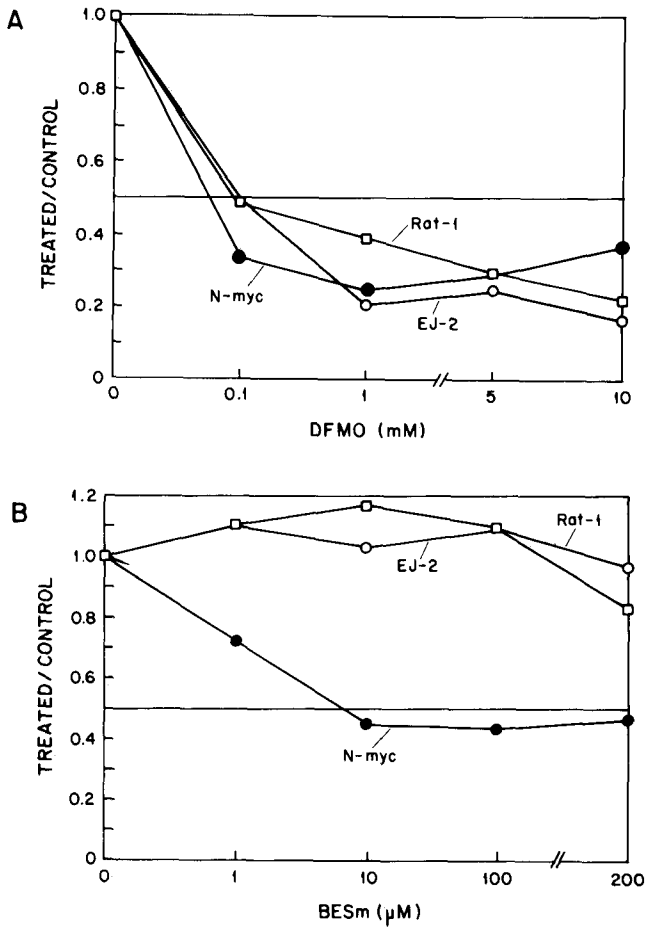

Figure 2. Effect of increasing concentrations of DFMO (panel A) or BESm (panel B) on the growth of Rat 1 and oncogene-transfected Rat 1 cells following 4 days treatment.

cells were quite resistant to the antiproliferative effects of BESm at concentrations up to 200 μM, cells transfected with N-myc were relatively sensitive to the analog with an IC_{50} of 10 μM.

To determine the basis for the greater sensitivity of N-myc-transfected cells to BESm, the relative effects of the analog on polyamine biosynthetic activity was examined in all three cell lines (Table 1). In both the Rat-1 parent line and in the ras-transfected cells, 10 μM BESm had little effect on either decarboxylase activities or polyamine pools. In the N-myc transfected cells, however, ODC and SAMDC activities were decreased by 99% and 85%, respectively, and polyamine pools (including those of spermine) were almost completely depleted. In addition, the activity of SSAT, a rate-limiting enzyme involved in polyamine catabolism, was elevated nearly 50-fold in the BESm-treated cells but essentially unaltered in the parent or ras-transfected cells (Table 1). The effects of BESm on growth and enzyme activities in N-myc cells are entirely consistent with observations made in other cell lines (10,11,17) and indicate that the expression of this oncogene, at least, did not seem to alter the typical regulatory responses of the polyamine pathway.

Measurement of the intracellular analog concentration in BESm-treated cells strongly suggested that the differential sensitivity of the N-myc-transfected cells was due to an increased accumulation of the analog (Table 1). N-myc-transfected cells took up about 26 times more analog than either of the other two lines. Further indication that increased uptake was responsible for N-myc sensitivity to BESm became apparent in growth inhibition studies with another polyamine analog, methylglyoxal-bis(guanylhydrazone) (MGBG). As with BESm, the N-myc transfected cells were differentially inhibited by the drug (Table 2). It seems probable that the high inducibility of SSAT activity to BESm in N-myc transfected cells is a reflection of intracellular drug levels rather than of alterations in enzyme regulation.

Table 2. Relative Effects of BESm and MGBG on Growth of Rat-1 and Oncogene-Transfected Rat-1 Cells

Treatment (4 days)	Percent of Control Growth		
	Rat-1	ras	N-myc
10 μM BESm	89	110	46
1 μM MGBG	118	105	48
10 μM MGBG	80	110	10

Both BESm and MGBG are believed to enter cells by a transport mechanism (18,19) which, among naturally occurring molecules, seems to be specific for polyamines. The carrier is subject to regulatory control since its activity increases with cell proliferation (20) or depletion of intracellular polyamine pools with DFMO (21). Although polyamine transport could be influenced by regulatory disturbances produced by N-myc expression, this is probably not directly related to the gene product itself since the N-myc protein is located in the nuclear matrix (22).

The above findings indicate that oncogene expression in Rat-1 cells has distinct consequences to polyamine biosynthesis and transport which appear to be dependent on the transfecting oncogene. Transfection with EJ2-ras leads to a dramatic increase in ODC activity while transfection with N-myc results in an apparent increase in polyamine uptake. Importantly, neither of these effects were attributable to differential growth rates of the cells since all three lines double at approximately the same rates (16-18 hr).

Both increased ODC activity and enhanced polyamine uptake could contribute to the stability and availability of intracellular polyamine pools. As a lead-in enzyme to the polyamine pathway, ODC is considered to be key, if not rate-limiting, in controlling pathway flux. Interestingly, Sistonen et al. (15) found that the growth rate of ras-transfected NIH-3T3 cells correlated with ODC activity and this, in turn, with the number of gene copies in the cell. Although exogenous polyamines occur at extremely low concentrations in whole animals, an enhanced ability to scavenge and concentrate them intracellularly by increased uptake could reinforce intracellular pools during cell growth. Since polyamines are critically important in initiating and sustaining cell growth, oncogene-associated effects on polyamine metabolism and accrual may contribute to the enhanced growth potential of the transformed phenotype. The general nature of this possibility requires further confirmation in other oncogene-transfected cell lines.

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