Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 18-23

STIMULATION OF MELANOTIC EXPRESSION IN MURINE MELANOMA CELLS EXPOSED TO POLYAMINE ANTIMETABOLITES

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Received March 23, 1983

SUMMARY: An exposure of cultured Cloudman S91 melanoma cells to inhibitors of polyamine biosynthesis, 2-difluoromethylornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG), distinctly promoted the expression of differentiated biochemical functions of the tumor cells. Slight to moderate growth inhibition produced by the compounds was associated with a stimulation of melanogenesis, as reflected by a striking enhancement of tyrosinase (EC 1.10.3.1) activity and an increase in cellular melanin content. Both antimetabolites acted synergistically with α-melanotropin (MSH), as regards the stimulation of melanogenesis. Exposure of the melanoma cells to MSH resulted in most experiments in a marked decrease of the intracellular polyamine pools, usually involving all three polyamines (putrescine, spermidine and spermine). The DFMO-induced stimulation of melanogenesis was totally suppressed by the administration of putrescine, whereas the MSH-stimulated tyrosinase activity was not influenced by the diamine. Although many recent reports indicate that terminal differentiation is accompanied by a distinct stimulation of polyamine biosynthesis, our results suggest that in certain cells polyamine deprivation may lead to an enhanced expression of differentiated phenotype.

During recent years major emphasis of polyamine research has been centered on the roles of putrescine, spermidine and spermine in cell proliferation and on the use of inhibitors of polyamine biosynthesis (polyamine antimetabolites) as antiproliferative agents (for ref. see 1). Review of current literature indicates that in many cell types terminal differentiation is accompanied by, and possibly also requires, an enhanced biosynthesis of polyamines. The differentiation of Friend erythroleukemia cells induced by a variety of compounds can be blocked by inhibitors of ornithine decarboxylase (EC 4.7.1.17) or by MGBG (2). Similarly DFMO blocks the differentiation of 3T3-L1 cells (3), L6 myoblast cells (4) and human granulocyte-macrophage progenitor cells (5).

On the other hand, an exposure of primary cultures of human keratocytes to MGBG reversibly inhibits cell proliferation and promotes differentiation as indicated by enhanced keratinization of the cells (6). Similarly, inhibition of putrescine and spermidine biosynthesis by DFMO in

0006-291X/83 $1.50

hematopoietic precursor cells triggers the stem cells to enter into cycle and stimulates their erythroid differentiation (7).

The differentiated functions of murine Cloudman S91 melanoma cells can be stimulated by a variety of agents. These include MSH, which acts through an elevation of intracellular cyclic AMP levels and stimulates the formation of melanin pigment as reflected by enhanced tyrosinase activity (8,9). Tyrosinase is an enzyme required for the oxidation of tyrosine to melanin precursors. The expression of biochemical (melanogenesis) and morphological (dendrite outgrowth) signs of differentiation in S91 cells can also be enhanced via cyclic AMP-independent mechanisms, most notably by retinoids (10).

Here we show that murine S91 melanoma cells exposed to inhibitors of polyamine biosynthesis not only slowed down their proliferation rate but began to express differentiated functions. DFMO-induced enhanced melanogenesis was apparently attributable to intracellular polyamine deprivation as any stimulation of tyrosinase activity by DFMO was prevented in tumor cells supplied with exogenous putrescine.

MATERIALS AND METHODS

Cell Cultures: Murine Cloudman S91 melanoma cells (clone M3) were obtained from Flow Laboratories Ltd (U.K.) and cultured in Ham's F-10 medium (Flow Laboratories) supplemented with 15% horse serum and 2% fetal calf serum (attempts to grow the cells in the absence of fetal calf serum were unsuccessful). The medium also contained glutamine (2 mM), penicillin (50 μg/ml) and streptomycin (50 μg/ml). Incubations were carried out in 5 cm diameter tissue culture dishes (Sterilin, U.K.) at 37°C in 5% CO_2/95% air atmosphere. The cells were detached with 1 mM EDTA in Dulbecco's modified phosphate buffered saline (Flow Laboratories) and counted with an electronic particle counter (Coulter Electronics).

Materials: 2-Difluoromethylornithine was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France) as was α-melanotropin from Ciba-Geigy Ltd (Basle, Switzerland). Methylglyoxal bis(guanylhydrazone) was synthesized by and obtained from Orion Pharmaceutical Company (Espoo, Finland). L-[3,5-^{3}H]Tyrosine (spec. radioactivity 58 μCi/mol) was purchased from Amersham International (Amersham, U.K.), and synthetic melanin was obtained from Sigma Chemical Co. (St. Louis, MO).

Analytical Methods: Polyamines were determined by the method of Seiler (11) as modified by Hölttä et al. (12). Tyrosinase (EC 1.10.3.1) activity and melanin content were determined according to Lotan and Lotan (13). MGBG was determined by the enzyme-inhibition assay of Seppänen et al. (14). Two-tailed t-test was used for statistical analyses.

RESULTS

As shown in Table 1, murine melanoma cells exposed to 2 mM DFMO for 4 days were severely depleted of putrescine and spermidine, grew slightly slower

Table 1. Effect of DFMO and MGBG on the growth, polyamine content and melanogenic activity of S91 melanoma cells. 0.2 x 10^6 Cells were grown for three days, whereafter the medium was changed and DFMO and MGBG were added. The cells were grown with the additions for four days. 24 Hours before the termination of the culture, 2.5 µCi of ^{3}H-tyrosine was added to the medium. Each group consists of three culture dishes, and the results are expressed as the mean S.D. For polyamine determinations, samples from all three dishes were combined.

Treatment	Cells x 10^{-6} (per dish)	Polyamines (pmol/10^6cells)			Tyrosinase activity (cpm/10^6 cells/h)	Melanin content (ng/10^6 cells)	MGBG content (pmol/10^6 cells)
		Put	Spd	Spm			
None	2.50 ± 0.13	30	691	1920	347 ± 34	363 ± 32	-
DFMO (2 mM)	2.26 ± 0.13	26	26	1230	1980 ± 475**	663 ± 87**	-
MGBG (0.2 µM)	2.52 ± 0.10	53	583	2740	419 ± 8 *	494 ± 83	13 ± 2
DFMO (2 mM)+ MGBG (0.2 µM)	2.14 ± 0.10*	62	49	1690	3260 ± 285***	964 ± 236*	148 ± 48***

than the untreated cells but exhibited tyrosinase activity which was almost six times higher than that found in the control cells. The enhanced melanogenesis was also reflected by the significantly increased accumulation of melanin in the tumor cells. Low concentrations (0.2 µM) of MGBG, which did not influence cell growth or intracellular polyamine pattern (except by increasing the content of spermine) had alone little effect on melanogenesis, but when combined with DFMO resulted in a synergistic stimulation of tyrosinase activity and of melanin formation (Table 1). The synergism between these two inhibitors was probably attributable to the DFMO-induced enhanced accumulation of MGBG (15), as also shown in Table 1. In fact, an increase in MGBG concentration from 0.2 µM to 2 µM produced a dose-dependent stimulation of melanogenesis in the tumor cells (results not shown).

The results depicted in Table 2 indicate that the extent of DFMO-induced enhancement of melanogenesis was comparable to that achieved with MSH and, when the hormone was combined with either of the polyamine antimetabolites, a further stimulation of tyrosinase activity and melanin accumulation occurred. It is interesting to note that MSH alone markedly reduced the concentrations of all three polyamines (Table 2). DFMO, MGBG or MSH alone had only slight effect on the proliferation rate of the melanoma cells, whereas combined regimens, especially those containing MGBG, reduced the cell number more markedly (Table 2). In spite of the decreased polyamine levels in the MSH-treated cells, the uptake of MGBG remained virtually unchanged as contrasted by the 3-fold increase in the accumulation of MGBG when combined with DFMO (Table 2).

Table 2. The combined effects of DFMO, MGBG, and MSH on S91 melanoma cells. The experimental procedure was as in Table 1, except that the cultures were terminated after three days' treatment with DFMO, MGBG or MSH. Polyamines and melanin were determined after combination of the samples from three identical cultures.

Treatment	Cells x 10^{-6} (per dish)	Polyamines (pmol/10^6 cells)			Tyrosinase activity (cpm/10^6 cells/h)	Melanin content (ng/10^6 cells)	MGBG content (pmol/10^6 cells)
		Put	Spd	Spm			
None	1.92 ± 0.17	330	2250	4350	313 ± 58	287	-
DFMO (2 mM)	1.73 ± 0.08	95	41	1220	1180 ± 725	319	-
MGBG (1 μM)	1.81 ± 0.07	764	3799	2120	663 ± 347	327	290 ± 72
MSH (1 μM)	1.74 ± 0.07	81	593	1350	1590 ± 104***	395	-
DFMO (2 mM)+ MSH (1 μM)	1.62 ± 0.10	72	116	1820	3550 ± 708**	850	-
MGBG (1 μM)+ MSH (1 μM)	1.37 ± 0.11**	137	1370	1300	2600 ± 341***	590	366 ± 198
DFMO (2 mM)+ MGBG (1 μM)	1.45 ± 0.05*	65	145	2520	3150 ± 177***	732	1030 ± 139**

The view that the DFMO-induced stimulation of melanogenesis was mediated by intracellular polyamine depletion was strongly supported by the experimental findings presented in Table 3. An addition of putrescine to the culture medium together with DFMO not only prevented the development of polyamine deprivation but likewise abolished the enhancement of tyrosinase activity (Table 3). Putrescine alone did not have any effect on melanogenesis nor did it influence the MSH-induced stimulation of tyrosinase activity to any appreciable extent (Table 3).

The potentiation of MSH-stimulated melanogenesis by DFMO (and MGBG) as well as the fact that putrescine did not abolish the enhanced tyrosinase activity in response to the hormone suggest that the mechanism of action of the polyamine antimetabolites differs from the cyclic AMP-mediated action exerted by MSH.

Microscopic examination of the cells treated with DFMO and MGBG showed that the biochemical changes evoked by the compounds were also accompanied by signs of morphological differentiation (results not shown). This suggests that the inhibitors not only affect some individual biochemical characteristics, but may even bring about a general differentiation of the melanoma cells.

Table 3. Reversion of the effects of DFMO by putrescine in S91 melanoma cells. The experimental conditions were as in Table 1 (note that the first two groups are identical to those of Table 1).

Treatment	Cells x 10^{-6} (per dish)	Polyamines pmol/10^6 cells			Tyrosinase activity (cpm/10^6 cells/h)	Melanin content (ng/10^6 cells)
		Put	Spd	Spm		
None	2.50 ± 0.13	30	691	1920	347 ± 34	363 ± 32
DFMO (2 mM)	2.26 ± 0.13	26	26	1230	1980 ± 475**	663 ± 87**
MSH (1 μM)	2.02 ± 0.01**	66	551	2030	1810 ± 105***	740 ± 190*
Putrescine (5 μM)	2.45 ± 0.33	30	599	1190	370 ± 80	407 ± 78
DFMO (2 mM)+ putrescine (5 μM)	2.27 ± 0.10	59	1300	2540	406 ± 76	596 ± 140*
MSH (1 μM)+ putrescine (5 μM)	1.98 ± 0.15*	37	435	1440	1780 ± 400**	546 ± 20**

DISCUSSION

Murine melanoma cells offer a convenient model for the study of cell differentiation, since these cells, in addition to showing morphological signs of differentiation, express easily measurable biochemical markers (melanogenesis) when stimulated to differentiate. The best studied stimulus for differentiated behavior is MSH, whose mechanism of action involves the stimulation of adenylate cyclase (for ref. see 9). MSH and theophylline act synergistically in inhibiting cell growth, promoting differentiation and reducing tumorigenicity of S91 melanoma cells (16). In contrast to MSH, various retinoids stimulate differentiation of melanoma cells by a mechanism that does not involve measurable changes in intracellular levels of cyclic AMP or in the formation of prostaglandins (10,17).

Very little is known about the metabolism or roles of the polyamines during the differentiation of murine melanoma cells. Scott et al. (18) reported that retinoids inhibit ornithine decarboxylase activity in MSH-treated S91 melanoma cells, and proposed that the retinoid-induced inhibition of cell growth and and the promotion of differentiation may be attributable to an inhibition of ornithine decarboxylase activity. However, as demonstrated by Lotan et al. (17), the growth inhibition of S91 melanoma cells produced by retinoids could not be reversed by the addition of putrescine, nor did the authors find any inhibition of ornithine decarboxylase activity in melanoma cells grown in the presence of retinoids.

The relation of the slight to moderate growth inhibition produced by the polyamine antimetabolites to the enhanced expression of differentiation markers remains open, as it does also in the case of retinoids (10). However, the growth inhibition, if not a prerequisite, is likely to be a contributing factor to promote differentiation, since an arrest of growth of B16 murine melanoma cells exposed to anti-tumor antibiotics, such as anthracyclines, is also associated with enhanced expression of differentiation markers (19).

Polyamine antimetabolites may, by inhibiting growth and promoting differentiation, offer new approaches for the treatment of melanoma, which is an aggressive tumor not easily manageable with conventional chemotherapy (20).

ACKNOWLEDGEMENTS

The skilful technical assistance of Ms. Riitta Sinervirta and the secretarial work of Ms. Heini Howard are gratefully acknowledged. This work received financial support from the National Research Council for Natural Sciences (Academy of Finland) and from the Finnish Foundation for Cancer Research.

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