Effects of Inhibitors of Polyamine Biosynthesis on the Growth and Melanogenesis of Murine Melanoma Cells

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ABSTRACT

Both 2-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (EC 4.1.1.17), and methylyglyoxal bis(guanidinyl hydrazone) (MGBG), a competitive inhibitor of S-adenosylmethionine decarboxylase (EC 4.1.1.50), strikingly stimulated melanotic expression of murine Cloudman S91 melanoma cells. The stimulation of tyrosinase (EC 1.10.3.1) activity and melanin formation by DFMO was closely associated with intracellular depletion of putrescine and spermidine developed in response to the drug. However, little or no evidence was obtained indicating that enhanced melanogenesis in response to MGBG was mediated through an inhibition of polyamine biosynthesis. Indirect inhibitors of ornithine decarboxylase, such as 1,3-diaminopropane and 1,3-diaminopropan-2-ol, but not putrescine, likewise inhibited the growth of the melanoma cells and stimulated their melanin production. The stimulation of melanogenesis by polyamine antimetabolites was not mediated by cyclic adenosine 3'5'-monophosphate, in contrast to the effect elicited by α-melanotropin. It is also unlikely that MGBG or the diamines acted as lysosomotropic agents capable of stimulating tyrosinase activity in situ, since the enzyme activity was stimulated by the drugs irrespective of whether assayed in cultured cells or using cell-free homogenates. None of the agents stimulated tyrosinase activity in vitro. The effect of DFMO and MGBG on melanoma cell proliferation was reversible, but the restoration of normal growth and melanin formation, especially in cells exposed to DFMO, was remarkably slow. The present results represent a further experimental model, in which the inhibition of polyamine accumulation is accompanied by signs of terminal differentiation.

INTRODUCTION

With the widespread use of polyamine antimetabolites, i.e., inhibitors of polyamine biosynthesis, it has become increasingly apparent that an undisturbed formation of these substances is required for cell proliferation to occur. The putative roles of polyamines in cell differentiation undoubtedly are much more complex. On the basis of the reasonable assumption that cell growth and differentiation are reciprocally regulated, one would expect that an inhibition of cellular proliferation by blocking the formation of the polyamines leads to the expression of a more differentiated phenotype. However, initial experiments performed with polyamine antimetabolites (mainly with DFMO) seemed to indicate quite the opposite; i.e., polyamine biosynthesis appears to be required for differentiation. Thus, the differentiation of Friend erythroleukemia cells is inhibited by various inhibitors of polyamine biosynthesis (6). Similarly, the conversion of 3T3-L1 preadipocytes to mature adipose cells is blocked by DFMO (2) as is the insulin-induced differentiation of the L6 myoblast cell line (4) and the colony-stimulating factor-induced differentiation of human granulocyte progenitor cells (33). DFMO also inhibits the differentiation of human promyelocytic leukemia cells (18, 30). Inhibitors of ornithine decarboxylase likewise inhibit germ cell differentiation and embryonic development (5, 7). In some experimental models, such as phorbol diester-induced terminal differentiation of human promyelocytic leukemia cells, polyamine antimetabolites apparently have no effect on differentiation (10).

The whole picture is, however, becoming more complex in light of a number of recent reports indicating that an inhibition of polyamine biosynthesis in certain cells is in fact accompanied by enhanced expression of various differentiation markers. Exposure of cultured human keratinocytes to MGBG leads to growth inhibition that is accompanied by induction of keratinization (24). Similarly, DFMO stimulates erythroid differentiation of hematopoietic precursor cells (20) and promotes the differentiation of malignant teratocarcinoma cells (27). 2-Fluoromethylornithine, another catalytic irreversible inhibitor of ornithine decarboxylase, induces morphological differentiation of neuroblastoma cells (3). In line with the above observations, we recently found that exposure of murine Cloudman S91 melanoma cells to DFMO or MGBG strikingly enhanced the melanotic expression of the cells as reflected by a stimulation of tyrosinase activity and increased accumulation of melanin (15). By using mouse melanoma B16 cell line, Sunkara et al. (31) reported similar enhancement of melanogenesis in response to DFMO.

Tyrosinase activity is used widely as a biochemical differentiation marker in melanoma cells, and several substances are known to stimulate the enzyme activity via elevation of intracellular cyclic AMP content (16, 34) or through mechanisms not involving changes of adenylyl cyclase activity (17). However, caution must be exercised before the stimulation of tyrosinase activity can be taken as an indicator of terminal differentiation in melanoma cells, since the enzyme appears to be subject to regulation by its immediate microenvironment. Saeki and Oikawa (26) recently found that tyrosinase activity was stimulated by various lysosomotropic agents and by changes of culture pH values to a more alkaline one. The latter authors (26) assumed that the phenomenon could be attributed to changes of intramelanosomal environment to one more optimal for tyrosinase. Thus, stimulation of tyrosinase activity may occur under conditions that have nothing to do with terminal differentiation.

We have now extended our previous work on the effect of polyamine antimetabolites on cultured melanoma cells by addressing the critical question of whether the stimulation of melanotic expression produced by polyamine antimetabolites is...
really mediated by intracellular polyamine deprivation. In the case of DFMO, dose-response curves and the fact that exogenous putrescine abolishes the stimulation suggested that the observed effects were based on polyamine depletion. The stimulation exerted by MGBG on melanogenesis was apparently not based on a prevention of polyamine accumulation, but the effect was mediated neither by cyclic AMP nor by a mechanism involving a lysosomotropic-type of action.

MATERIALS AND METHODS

Murine Cloudman S91 melanoma cells (clone M3; Flow Laboratories, Ltd.) were cultured in Ham's F-10 medium (Flow Laboratories) supplemented with 15% horse serum and 2% fetal calf serum as described earlier (12). In all experiments, the cells were first allowed to attach overnight, after which the drugs were added. The growth medium was changed every second day.

Tyrosinase activity was assayed directly from the cell culture by the method of Lotan and Lotan (17). In some experiments, the cells were homogenized in water (all glass homogenizer) and frozen and thawed once to disintegrate the melanosomes (19, 26). Tyrosinase activity was assayed from the homogenates essentially as described by Pomerantz (22). Polyamines were determined from perchloric acid extracts by the method of Seiler (28) as modified by Hölttä et al. (8), MGBG was assayed by the method of Seppänen et al. (29), and melanin was assayed according to the method of Lotan and Lotan (17). Cyclic AMP was assayed with the Amersham International kit (Amersham, United Kingdom).

RESULTS

As depicted in Chart 1A, both DFMO and MGBG inhibited the growth of murine melanoma cells at rather low concentrations (2 mM and 2 μM, respectively). It is likewise obvious from Chart 1 that, in contrast to DFMO, MGBG hardly influenced cell growth through an inhibition of polyamine accumulation. In response to the drug, the putrescine level (Chart 1B) was strikingly elevated, spermidine contents (Chart 1C) fluctuated well above the control level, and it is difficult to believe that the moderate decrease in spermine concentration (Chart 1D) was alone sufficient to reduce the growth rate. The increases in putrescine and especially spermidine concentrations in response to MGBG was most probably related to an enhanced uptake of serum-derived polyamines (melanoma cells require a peculiarly high serum concentration for their growth, 15% horse serum plus 2% fetal calf serum) in the absence of any diamine oxidase activity (inhibited by MGBG), as demonstrated recently (1, 13, 14). Treatment with DFMO, on the other hand, rapidly produced a profound intracellular depletion of putrescine (Chart 1B) and spermidine (Chart 1C).

Irrespective of their effects on polyamine accumulation, both drugs strikingly stimulated melanogenesis, which apparently is one of the best biochemical indicators of the differentiation of melanoma cells. As shown in Chart 2, DFMO enhanced tyrosinase activity and the accumulation of melanin pigment over a broad concentration range. A concentration of DFMO as low as 0.1 mM stimulated the activity of tyrosinase more than 10-fold and likewise enhanced the production of melanin content (Chart 2). Worth noting was the finding that this low drug concentration had no effect on cell proliferation (Chart 2) but reduced the cellular concentration of spermidine to one-third of control level (not shown). Upon increasing the concentration of DFMO to 1

\[ \text{L-[3,5-}^3\text{H]Tyrosine (specific radioactivity, 58 Ci/mol) was purchased from Amersham International. DFMO was a gift from Centre de Recherche Merrell International, Strasbourg, France. MGBG was obtained from Orion Pharmaceutical Company, Espoo, Finland. α-Melanotropin was a gift from Ciba-Geigy, Ltd., Basel, Switzerland.} \]
POLYAMINES AND MELANOMA DIFFERENTIATION

Charts. Effect of increasing concentrations of MGBG on the growth and melanogenesis of melanoma cells. The cells (initial density, 0.6 x 10^6 cells/dish) were grown in the absence or presence of MGBG (0.1 to 5 µM) for 3 days, after which cell density, tyrosinase activity, melanin content, and intracellular MGBG concentration were determined. Each point represents a mean of 2 separate cultures.

Chart 3. Effect of increasing concentrations of MGBG on the growth and melanogenesis of melanoma cells. The cells (initial density, 0.6 x 10^6 cells/dish) were grown in the absence or presence of MGBG (0.1 to 5 µM) for 3 days, after which cell density, tyrosinase activity, melanin content, and intracellular MGBG concentration were determined. Each point represents a mean of 2 separate cultures.

Chart 4. Effect of melanotropin and inhibitors of polyamine synthesis on the accumulation of cyclic AMP (cAMP) in melanoma cells. The cells (initial density, 0.4 x 10^6 cells/dish) were grown for 42 hr. after which 1 × 10^-6 melanotropin (MSH), 2 µM DFMO, or 2 µM MGBG was added, and the cultures were allowed to grow for the times indicated.

mm, the activity of tyrosinase and the content of melanin reached their maxima (Chart 2). At this drug concentration (1 mM), the concentration of cellular spermidine dropped to its nadir (around 10% of control level; not shown). A further increase in the concentration of DFMO (up to 10 mM) resulted in a decrease in tyrosinase activity and melanin content, but the decline was not particularly steep, as illustrated in Chart 2.

MGBG likewise stimulated tyrosinase activity and the accumulation of melanin but, in contrast to DFMO, this occurred only over a narrow concentration range (Chart 3). Enhanced melanogenesis in response to MGBG appeared to be associated with growth inhibition (Chart 3) rather than polyamine depletion, since only spermine content was moderately decreased (less than 50%; not shown) at MGBG concentrations eliciting maximum stimulation of tyrosinase activity. The stimulation of melanogenesis was also closely associated with the build-up of high intracellular concentrations of the drug as illustrated in Chart 3. In general, the activity of tyrosinase varied over a wide range depending on the growth conditions of the culture. However, a longitudinal experiment (not shown) indicated that the enzyme activity was stimulated by the treatments at any time point of the culture.

Whatever the mechanism of enhanced melanogenesis in response to polyamine antimetabolites is, it was clearly not mediated through a stimulation of adenylate cyclase as shown in Chart 4, where melanotropin served as a positive control.

Table 1 shows that the so-called indirect inhibitors of ornithine decarboxylase, such as 1,3-diaminopropane (23) and 1,3-diaminopropan-2-ol (21), but not putrescine (12), inhibited the accumulation of all polyamines in the melanoma cells and exerted a clear-cut antiproliferative action. While putrescine only marginally enhanced the activity of tyrosinase, the unphysiological diamines produced a striking stimulation of melanogenesis (Table 1). L-Ornithine (3 mM) had no effect on tyrosinase activity (not shown).

In all the experiments described above, the activity of tyrosinase was assayed in growing cells, i.e., exposing the cells to radiolabeled tyrosine and measuring tritiated water liberated into the medium (17). However, the possibility remained that polyamine antimetabolites, especially MGBG and the diamines as basic substances, may stimulate the activity of tyrosinase in situ inside melanosomes, a mechanism recently described for the action of some lysosomotropic agents (26). The following experimental approach was used to investigate this possibility. Tyrosinase activity was determined both in intact cells treated with polyamine antimetabolites and using cell free homogenate as the source of the enzyme. As shown in Table 2, diaminopropane produced a comparable stimulation of tyrosinase activity in both systems, whereas the stimulation by MGBG was greater in homogenates than in intact cells. This experiment eventually rules out the possible lysosomotropic action by diaminopropane and MGBG. The fact that DFMO enhanced the enzyme activity more in intact cells than in cell-free extracts is unlikely based on a lysosomotropic-type action. In addition, none of the compounds influenced tyrosinase activity when added into the assay system.

Regardless of the mechanism of action, the effect of DFMO and MGBG on melanoma cell growth and on melanotic expression was a reversible one, as illustrated in Table 3. Interestingly, the recovery of melanoma cells from DFMO-induced growth retardation was remarkably slow even in comparison with MGBG. After the latter compound was omitted, the cells rapidly recovered upon the dilution of high intracellular drug concentrations (Table 3). Upon removal of DFMO, putrescine depletion was fully restored by Day 3, whereas it took 6 to 9 days to reach normal spermidine levels (not shown).

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Effect of diamines on the accumulation of polyamines and melanin in melanoma cells

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell density (×10^4 cells/dish)</th>
<th>Putrescine (pmol/10^6 cells)</th>
<th>Spermidine (pmol/10^6 cells)</th>
<th>Tyrosinase activity (cpm/10^6 cells)</th>
<th>Melanin (μg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.82</td>
<td>2060</td>
<td>1970</td>
<td>902</td>
<td>0.88</td>
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<td>Putrescine</td>
<td>2.36</td>
<td>2650</td>
<td>1560</td>
<td>1110</td>
<td>1.21</td>
</tr>
<tr>
<td>Diaminopropane</td>
<td>1.20</td>
<td>481</td>
<td>682</td>
<td>5730</td>
<td>1.65</td>
</tr>
<tr>
<td>Diaminopropanol</td>
<td>1.41</td>
<td>80</td>
<td>272</td>
<td>762</td>
<td>1.43</td>
</tr>
</tbody>
</table>

* Not determined (putrescine not separable from diaminopropane).

Table 2

Effect of inhibitors of polyamine biosynthesis on tyrosinase activity in melanoma cells as measured in cell cultures and cell-free homogenates

The melanoma cells were grown in the absence or presence of 2 μM MGBG or 3 μM diaminopropane for 3 days or of 2 μM DFMO for 4 days. Tyrosinase activity was measured either directly in the cell cultures or in cell-free homogenates as described under "Materials and Methods." Each value represents a mean of 2 separate cultures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tyrosinase activity (cpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>239</td>
</tr>
<tr>
<td>MGBG</td>
<td>424</td>
</tr>
<tr>
<td>Diaminopropane</td>
<td>1100</td>
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<tr>
<td>Experiment 2</td>
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<tr>
<td>None</td>
<td>56</td>
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<tr>
<td>DFMO</td>
<td>483</td>
</tr>
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</table>

Recovery of the melanoma cells from DFMO- and MGBG-induced growth inhibition upon removal of the drugs

Mouse melanoma cells were grown in the presence of 2 μM DFMO or 2 μM MGBG for 5 days, after which the cells were detached and subcultured in drug-free medium for the times indicated. The cells grown for 9 days were again subcultured by Day 5. The calculation of generation times was based on a time period of 3 days. Each time point represents a mean of 3 separate cultures.

<table>
<thead>
<tr>
<th>Previous treatment</th>
<th>Time after drug removal (days)</th>
<th>Generation time (hr)</th>
<th>Melanin (μg/10^6 cells)</th>
<th>MGBG (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>36</td>
<td>0.85</td>
<td>0.65</td>
</tr>
<tr>
<td>DFMO</td>
<td>3</td>
<td>108</td>
<td>1.35</td>
<td>7.65</td>
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<tr>
<td>MGBG</td>
<td>3</td>
<td>167</td>
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<td>0.74</td>
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<td></td>
<td>6</td>
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<tr>
<td></td>
<td>9</td>
<td>36</td>
<td>0.66</td>
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</tr>
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</table>

* ND, not detectable.

DISCUSSION

Our present results clearly indicate that compounds considered as polyamine antimetabolites, even though acting in greatly different ways, bring about a striking stimulation of melanotic expression in murine melanoma cells. However, the mechanism whereby the formation of melanin pigment is enhanced remains fully open. The stimulation of tyrosinase activity and melanin formation apparently does not involve cyclic AMP as a second messenger, as does the action of melanotropin, for instance (16, 34). In contrast to DFMO, the effect of MGBG was in all likelihood not mediated through prevention of polyamine accumulation, since under our experimental conditions (serum-rich medium) this drug, if anything, increased total polyamine concentrations in the exposed cells. Similarly, unphysiological diamines, although producing a distinct polyamine depletion, may have direct inhibitory effects on protein synthesis (9, 32), and these agents also promote the excretion of polyamines (11). However, it is likewise unlikely that MGBG or diamines exert a lysosomotropic type of action, such as proposed for certain monoamines (26) bringing about a stimulation of tyrosinase activity by merely optimizing the melanosomal microenvironment (pH changes, etc.) for the enzyme.

It is tempting to attribute the stimulation of melanotic expression by DFMO to putrescine and spermidine depletion per se. In favor of this view are the experimental findings indicating that enhanced melanin formation can be abolished by putrescine (etc.) for the enzyme.

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