Effects of Inhibitors of Ornithine Decarboxylase on the Differentiation of Mouse Neuroblastoma Cells

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ABSTRACT

(R)-(S)-Fluoromethylornithine [(S)-FMO], a catalytically irreversible inhibitor of ornithine decarboxylase (L-ornithine carbamoyltransferase, EC 4.1.1.17), induced the differentiation of N2a mouse neuroblastoma cells. The effect of μM FMO was concentration dependent; approximately 50% of the cell population exhibited neurite outgrowth in the presence of 1 μM FMO, while higher concentrations caused severe growth inhibition and cell death. The effect of 1 μM FMO on neuroblastoma differentiation was potentiated greatly by 0.1 to 0.2 mM K⁺ (lô) dibutyryl adenosine cyclic 3',5'-monophosphate (dBcAMP) causing more than 90% of the cell population to differentiate morphologically with thick and long processes; 0.1 to 0.2 mM dBcAMP, by itself, had no effect on cell growth and did not induce neurite outgrowth. The effect of μM FMO, either by itself or in combination with 0.1 to 0.2 mM dBcAMP, on the morphological differentiation of mouse neuroblastoma cell was reversed by the addition of exogenous putrescine or spermidine. The morphological differentiation of mouse neuroblastoma cells induced by 1 μM FMO plus 0.2 mM dBcAMP was accompanied by increases in the regulatory subunit of the type I cAMP-binding protein and as yet uncharacterized activity. These results indicate that the modulation of cellular polyamine contents may be important in neuroblastoma cell differentiation.

INTRODUCTION

The decarboxylation of l-ornithine by ODC leads to the formation of putrescine and is the rate-controlling step in the biosynthesis of polyamines, spermidine and spermine (1, 4, 25). Polyamines are ubiquitous organic cations in prokaryotic and eukaryotic cells and are implicated in many growth-regulatory processes (1, 9, 31). Although the involvement of ODC and polyamines in proliferative and neoplastic growth of mammalian cells is well documented, there have been relatively few studies on the possible roles of ODC/polyamines in cell differentiation. Ruth and Weiss (29) demonstrated an increase of ODC activity during matrix-induced sequential differentiation of cartilage, bone, and bone marrow in vivo. Belz et al. and Pegh (2) reported an increase in spermidine content when confluent ST3-L1 fibroblasts are stimulated to differentiate into adipocytes. On the other hand, Stoecker et al. (35) observed a decrease in ODC activity of L6 myoblasts upon fusion to myotubes. Recently, we have found that the differentiation of mouse neuroblastoma cells in tissue culture, induced by 1 μM dBcAMP plus 0.5 mM 8-BKBN, is accompanied by an early inhibition of putrescine uptake (8) and marked increases in cellular ODC activity and polyamine contents (7). These studies indicate that, in addition to their involvement in cell growth and proliferation, ODC/polyamines may have important roles in cell differentiation.

In attempting to delineate the type(s) of polyamines in the differentiation of mouse neuroblastoma cells, one possible approach is to either inhibit or augment the cellular polyamine contents and to observe the consequence of this effect. Recently, highly specific irreversible inhibitors of ODC, such as μM FMO and μM DIFMO, have been developed (17, 23). Studies have shown that these inhibitors are potent and effective inhibitors of the enzyme, ODC, in various cultured cell systems (5, 20, 22, 27) and tissues (10-12, 25). The availability of these irreversible inhibitors enabled us to study the role of polyamine metabolism in the differentiation of mouse neuroblastoma cells. We report here that 1.0 μM FMO could induce the differentiation of N2a mouse neuroblastoma cells and that 0.1 to 0.2 mM dBcAMP further potentiated the effects of μM FMO on cell differentiation. Our findings provide a direct evidence that changes in polyamine metabolism may be an integral component in the differentiation of neuroblastoma cells.

MATERIALS AND METHODS

Materials. Dwlcoo's modified Eagle's medium and vital cell serum were obtained from Grand Island Biological Co., Grand Island, N. Y. BbcAMP, putrescine, spermidine, spermine, putrescine-8-N-oxide, and L-ornithine-1-naphthylamine chloride (p-nitrochloro) were purchased from Sigma Chemical Co., St. Louis, Mo. (1',3'-)Cinchona (59 mM/cm2) and (l',3'-)Cinchonine (59 mM/cm2) were from Jenk- en Corp., Kirtland, N. M. (8-BKBN) was from Chron Chemical Radiosotope Division, Dallas, Tex. C, DIFMO was kindly provided by Dr. J. Kollisch of Merck, Sharp & Dohme Research Laboratories, Rahway, N. J. (7) was generously provided by the Merrell Research Center, Cincinnati, Ohio. High-potency liquid chloro- phospho- tropy grade solvents were purchased from Baker Chemical Co., Phila- delphia, N. J.

Cell Culture and Drug Treatment. N2a mouse neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (with 1000 mg glucose per liter) supplemented with 10% fetal calf serum. Cells were maintained at 37°C in a water-jacketed perfusion incubator (90% air and 5% CO₂) in a humidified atmosphere under sterile conditions before use and added to cell culture immediately after subculture. The seeding cell density was approximately 1 x 10⁴ cells/cm². Occasionally, drugs were added 5 to 10 hr after subculture. No difference in results was
observed. Cell growth was measured by the number of viable cells attached to the growth surface. To study the effect of exogenous polyamines on neuroblastoma differentiation, putrescine or spermidine was added to the culture medium immediately after drug withdrawal, when spermidine was used, the culture medium also contained 0.05 mM antimycin A, a potent inhibitor of the mitochondrial respiratory chain (10), to prevent the formation of toxic oxidized products.

Biochemical Assays. ODC activity was determined, using a 12,000 r.p.m., 0.1 ml supernatant obtained from cell homogenate, according to procedures described previously (4, 6). For the quantitation of putrescine, spermidine, and spermine, the method of Seiler and Schramm (33) was adopted to prepare the dansyl derivatives of polyamines. The analysis of the dansylated polyamines was carried out by high-pressure liquid chromatography using a 7.5-cm aminopropylsilica reverse-phase column. The solvent system used was acetonitrile/H2O (85/15, v/v) as described elsewhere (17). Quantitation of the regulatory subunit of the type I cAMP-binding protein was carried out by a photofluorimetry labeling technique with B-N[3H]cAMP as described previously (18, 19). Briefly, neuroblastoma cell extracts were incubated with 1 μM B-N[3H]cAMP (1 to 2 Ci/mmol) at 4°C for 1 hr. The samples were then phenylated and subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography to determine the amount of radioactive incorporat-

RESULTS

In this study, both o-FMO and a-FMO were used to study the effects of inhibition of ODC and depletion of cellular poly-

amines on neuroblastoma cell differentiation. The results obtained with a-FMO were similar to those of o-FMO, and only those results obtained using a-FMO are presented.

Chart 1 shows the effect of a-FMO on the growth of N2A mouse neuroblastoma cells. A reduced rate of cell proliferation was observed with increasing concentrations of a-FMO. The growth-inhibitory effect of a-FMO on mouse neuroblastoma cells is similar to that of o-FMO reported in other cell culture systems (5, 22).

In addition to the inhibitory effect of a-FMO on cell growth, we observed a decrease in the proportion of the N2A mouse neuroblas-
toma cells in the a-FMO-treated culture became morphologically differentiated 5 to 6 days after initiation of the drug treatment. The extent of morphological differentiation, as characterized by extensive neurite outgrowth (>50 μm long), was dependent on the concentration of a-FMO used (Chart 2). The optimal concentration for neurite outgrowth was 0.5 to 1.5 mM. Under these conditions, about 50% of the cell population extended neurites. However, at higher concentrations of a-FMO (1–3 mM), we observed not only growth inhibition but also an inhibition of neurite outgrowth; dead cells and cell debris were present in those cultures treated with high concentrations of a-FMO.

In an effort to search for a means to maximize the differentia-
tion of N2A cells in the a-FMO-treated culture, we found that 0.1 to 0.2 mM BuCAMP increased the percentage of neurite-bearing cells in the a-FMO-treated culture from 50 to

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It is noteworthy that the time course of changes in ODC activity and polyamine contents in the control H2A cell culture was similar to that described for the N15 mouse neuroblastoma cells reported previously (7). In addition, 0.1 to 0.2 mM BucAMP had no effect on ODC activity or polyamine contents either by itself or when added together with 1 mM α-FMO. This result suggests that the potentiating effect of suboptimal concentrations of BucAMP on α-FMO-induced neuroblastoma differentiation may not be related directly to effects of BucAMP on ODC/polyamines.

To further examine the role of ODC and polyamines in mouse neuroblastoma differentiation, we investigated whether exogenous polyamines would antagonize the action of α-FMO and BucAMP in neuroblastoma cell differentiation. Fig. 5 shows that putrescine or spermine could reverse the differentiation of H2A cells induced by 1 mM α-FMO plus 0.1 mM BucAMP. Similar results were obtained when 1 mM α-FMO was used by itself to induce neuroblastoma cell differentiation (data not shown). When spermidine was used to study its effect on the α-FMO-induced neuroblastoma cell differentiation, the presence of ammonium chloride was necessary to inhibit the dramatic cell activity present in the cell serum-containing growth medium. In the absence

<table>
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<th>Regulatory amount of the type II (transcription-activating) protein</th>
<th>α-FMO (transcription-activating protein)</th>
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<tr>
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Table 1

![Chart 3](chart3.png)  
**Chart 3.** Effects of various concentrations of α-FMO and BucAMP, either alone or in combination, on the morphological differentiation of N15 mouse neuroblastoma cells. Cells were treated at a density of 1.0 × 10⁶ cells/ml in the presence of various lengths of time period and concentrations of α-FMO. Nontreated control cells (cells with no incubation with α-FMO) were tested on Day 7. At least 100 cells were counted, and the percentage of multinucleated was expressed as a percentage of total viable cells (i.e., typical true negative).

![Chart 4](chart4.png)  
**Chart 4.** Effects of α-FMO in the absence and presence of BucAMP (0.2 mM) alone or in combination. In the absence of polyamines, the differentiation of N15 mouse neuroblastoma cells was stimulated significantly by addition of 0.1 to 1.0 mM α-FMO to the culture medium alone (data not shown). The discrepancy between the findings and ours may be due to different cell systems used, indeed, we noticed that, under their experimental conditions, there is no growth-associated increase of spermine content in both of their control cultures.
to differentiate by the addition of cAMP analogs or agents which increase intracellular cAMP concentration (12, 28). Such differ-
entiation is characterized by the morphological appearance of
neurite outgrowth and the biochemical appearance of enzyme
activities related to neurotransmitter metabolism. The molecular
mechanisms of cAMP-induced neuroblastoma differentiation
may have a broad implication, because cAMP is known to be
involved in the differentiation of a variety of animal cells including
melanoma (15, 24), nephrocytomycymoma (14, 32), granulosa (16),
and muscle cells (35). On the basis of the observations that the
differentiation of mouse neuroblastoma cells induced by 1 μM
Bt2cAMP and 0.5 μM IBMX (a phosphodiesterase inhibitor) is
accompanied by a 10-fold increase in the apparent Km value of
putrescine transport (8) and a 5- and 15-fold decrease, respec-
tively, of spermidine and cellular ODC activity (7), we have
proposed that one of the primary target sites of cAMP action in
neuroblastoma differentiation may be the alteration of polyamine
metabolism (7). Indeed, many studies using cells in tissue culture
and experimental animals have suggested that cAMP can mod-
ulate cellular ODC activity (for reviews, see Refs. 4 and 31).

The present study is aimed at further elucidating the role of
ODC and polyamines in mouse neuroblastoma differentiation.
Our results suggest that: (a) α-FMO, at optimal concentrations
(1 to 1.5 μM), can induce the morphological differentiation of
mouse N2a neuroblastoma cells; (b) the action of α-FMO on
neuroblastoma differentiation appears to be mediated through
inhibition of ODC activity and reduction of polyamine contents;
(c) suboptimal concentrations of Bt2cAMP (0.1 to 0.2 μM) can
potentiate the effect of α-FMO on mouse neuroblastoma differ-
entiation; such a potentiating effect does not appear to be related
to further changes of ODC activity and polyamine contents.

The apparently bell-shaped dose-response curve (Chart 2) of
the effect of α-FMO on neuroblastoma cell differentiation may be
related to toxicity of high concentrations of α-FMO (>3 μM),
since floating cells and cell debris appeared in these cultures 4
to 5 days after initiation of the treatment. Alternatively, the
phenomena observed with high concentrations of α-FMO (inhi-
bition of cell differentiation, cell growth, and ultimately cell death)
may be related to an observation made recently by Pihlapetalo et al. (26). They reported that polyamine starvation causes
disappearance of microtubules and actin filaments in polyamine-
axotrophic Chinese hamster ovary cells. It is possible that α-
FMO at higher concentrations may severely deplete the cellular
polyamine contents to the extent that neurite formation as well
as cell division become impossible.

The observation that exogenous putrescine or spermidine can
block the action of 1 μM α-FMO, either alone or with 0.1 to 0.2
μM Bt2cAMP, on neuroblastoma cell differentiation (Fig. 2) sug-
gests that modulation of ODC activity and polyamine contents
can bear a causal relationship to neuroblastoma cell differenta-
 tion. Since the extensive neurite webs became prominent ap-
proximately 5 days after the addition of α-FMO, whereas the
inhibition of ODC activity and polyamine contents was apparent
on Day 1, its seems likely that the alteration of ODC/polyamine
may trigger a chain of biochemical events that, after a lag period,
may lead to cell differentiation.

The mechanism through which suboptimal concentrations
of Bt2cAMP potentiate the effect of α-FMO on neuroblastoma cell
differentiation is not clear. Gunning et al. (14) have reported a
synergistic effect of Bt2cAMP and β-nerve growth factor on the

**Polyamines and Neuroblastoma Differentiation**

**Chart 5. Effects of α-FMO (1 μM) and Bt2cAMP (0.2 μM), alone or in combina-
tion, on polyamine contents in N2a mouse neuroblastoma cells as a function of
time in culture.** Cells were subcultured (seeding density, 1.6 × 10^6 cells/ml-mm
2) on Day 0 in the absence (C) or presence (B) of 0.2 μM Bt2cAMP, 1 μM α-
FMO (C), and 1 μM α-FMO plus 0.2 μM Bt2cAMP (D). Polyamines were quantitated
by the high-pressure liquid chromatography method described in the text. PUT, putrescine; SOD, spermidine; SPM, spermine.

**DISCUSSION**

Mouse neuroblastoma cells in tissue culture can be induced

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neurite outgrowth of PC-12 pheochromocytoma cells and suggested that B_{2}AR is responsible for the initiation of neurite outgrowth. In light of their study, the potentiating role of B_{2}AR on the α-FM0-induced neuroblastoma differentiation may be related to microtubule assembly. The possible involvement of αCAMP and protein phosphatidylinositol in microtubule polymerization has been proposed (34, 36). It is possible that low concentrations of B_{2}AR may modify a certain biochemical event(s) by which it has no effect on tumor growth or differentiation but can "prime" or prepare cells for maximal differentiation when cellular ODC activity and polyanine contents are required by α-FM0 treatment. 

Tumor differentiation is undoubtedly a complex phenomenon. Bethel and Pegg (2) have shown that α-FM0 inhibits the differentiation of 3T3-L1 preadipocytes, suggesting the involvement of ODC/polyamines in normal cell differentiation. Similarly, Fozard et al. (12, 12) have demonstrated the need of ODC-polyamines for embryonic development. On the other hand, Luk et al. (25) have found that α-FM0 prevents cell growth but has no effect on the chemically induced differentiation of promyelocytic HL-60 cells, implying that polyanines are only involved in the proliferation but not in the terminal differentiation of these cells.

The relationship between the proliferation and differentiation of mouse neuroblastoma cells is complex as indicated by the following observations: (a) the differentiation of mouse neuroblastoma cells is associated generally with a decrease of cellular growth rate (7, 19, 29); (b) inhibition of cell growth does not necessarily induce neuroblastoma cell differentiation (18); (c) maximal expression of differentiated phenotypes of mouse neuroblastoma cells requires several cycles of cell division after the addition of 1 μM B_{2}AR to the cell culture (7, 19).

In this present data together with our previous findings that differentiation neuroblastoma is accomplished by a decrease of ODC/polyamines (7) suggest that the modulation of ODC/polyamines may be important in the differentiation of mouse neuroblastoma cells; however, the precise role of ODC/polyamines in neuroblastoma differentiation and transformation will remain to be elucidated.

REFERENCES


Fig. 1. Photomicrographs of NDA mouse neuroblastoma cells (untreated) treated with 0.2 mm 8-Br-cAMP (A), 1 mm FMISO plus 0.2 mm 8-Br-cAMP (B), 1.5 mm FMISO plus 0.1 mm 8-Br-cAMP (C), and 0.5 mm FMISO plus 0.2 mm 8-Br-cAMP (D). Phase-contrast photomicrographs of representative fields of the cell cultures were taken 8 days after initiation of the treatment. Bar at upper left, 50 µm.
Fig. 2. Effects of exogenous pyrophosphate in suppressing the action of w-P30 and BSA on neuroblastoma cell differentiation. NR3C1 mouse neuroblastoma cells were seeded at a density of 1.2 x 10⁶ cells/cm². w-P30 (1 μg/ml) and 0.2 μM BSA were added at the time of seeding. 3 μM to inhibit cell differentiation. When 0.1 μM w-P30 was added, B, 0.1 μM 6-bromonicotinamide (BMN) was added. D, 0.1 μM 6-bromonicotinamide and 50 μM clomipramine (CM) added. Photomicrographs of representative fields of the cell cultures (unstained) were taken on Days 7, 15, and 20 for A, B, and C, respectively. GFP at upper pH, 50 μm.