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The Role of Polyamines in the Differentiation of Mouse Neuroblastoma Cells

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Mouse neuroblastoma cells in tissue culture was first established in 1940 (for a review, see ref. 38). Although these murine neuroblastoma cells possess some of the biochemical properties of neurons (e.g. low level of neurotransmitter synthesizing enzymes), they generally remain in a relatively immature state of differentiation (1). Nevertheless, in the presence of cAMP analogs or agents which increase cellular cAMP content, these cells can undergo differentiation, a process defined by the morphological appearance of neurites and the biochemical expression of neurotransmitter metabolizing enzymes (e.g., catechol-O-methyl transferase, acetylcholinesterase, choline acetyltransferase, tyrosine hydroxylase, etc.). Tumorigenicity of neuroblastoma cells has been shown to be abolished after differentiation (37). The ability of cAMP to produce large changes in morphology as well as enzymatic and electrical activities in mouse neuroblastoma cells renders these cells an excellent system for studying the action of cAMP in the regulation of neuronal differentiation. An in depth analysis of the action of cAMP in neuroblastoma cells differentiation may also provide insights into the mechanism of cAMP-induced differentiation of other cell types such as melanoma (34), PC-12 pheochromocytoma (6) and embryonic carcinoma (42).

In studying the cAMP-induced differentiation of mouse neuroblastoma cells, it appears likely that regulation of polyamine metabolism may be a potential target of cAMP action based on the following considerations: (a). The effect of cAMP in the regulation of ornithine decarboxylase (ODC, EC. 4.1.1.17), the rate-controlling enzyme for the biosynthesis of polyamines, has been well documented (for reviews see 18,24,39). (b). Tissue polyamines (putrescine, spermidine and spermine) generally increase under conditions of rapid growth and are considered to be functionally important since their synthesis is sequentially regulated during hypertrophy and hyperplasia (39). (c). The induction of ODC is

one of the early events in growth stimulation (2,8,44). Although there are many studies which suggest a causal relationship of elevated ODC activity/polyamine contents and proliferative/neoplastic growth, there have been until recently relatively few studies on the possible involvement of polyamines and ODC in cell differentiation. In this chapter, we wish to summarize our studies on the role of polyamines in neuroblastoma cell differentiation.

DIFFERENTIATION OF MOUSE NEUROBLASTOMA CELLS

Mouse neuroblastoma cells (NB-15, N-18 or N2a clone) were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4,500 mg glucose per liter) supplemented with 10% fetal calf serum (FCS). To induce the differentiation of neuroblastoma cells, 1mM dibutyryl cAMP (Bt_2cAMP) was added to sparse cultures of neuroblastoma cells at 15 hours after subculture (seeding density $2x10^4$ cells/cm^2). In some experiments 0.5mM 3-isobutyl-1-methylxanthine (IBMX) was added together with Bt_2cAMP. In both cases, greater than 90% of the cells were differentiated.

Cells grown in normal growth medium (i.e. Dulbecco's medium puls 10% FCS) will be designated as NB cells, denoting control neuroblastoma culture, whereas cells grown in the "differentiation" medium (i.e. normal growth medium plus 1mM Bt_2cAMP with or without 0.5mM IBMX) will be designated as ND cells, denoting differentiating neuroblastoma culture.

When seeded at $2x10^4$ cells/cm^2, NB cells reached confluency (saturation density $3\text{~}4x10^5$ cells/cm^2) at 90-100 hours after seeding. In contrast, ND cells reached stationary phase of growth ~70 hours after the addition of Bt_2cAMP (saturation density $0.8\text{~}1.0x10^5$ cells/cm^2). Although neurite outgrowth of the ND cells was detectable 2~6 hours after the addition of Bt_2cAMP, other differentiation phenotypes, such as increases of acetylcholinesterase activity and cAMP-binding activity, were not fully expressed until the ND cells have reached a stationary phase of growth (28). In this article, the term "differentiated neuroblastoma cells" is used to refer to ND cells at the stationary phase of growth.

CHANGES OF ODC ACTIVITY AND POLYAMINES DURING NEUROBLASTOMA CELL DIFFERENTIATION

As an initial effort to understand the role of polyamines in neuroblastoma differentiation we carried out time-course studies of the changes of ODC activity and polyamine contents in both the NB and ND cells during a 5 to 6 days culture period (17). ODC activity was determined according to procedure described previously (8). One unit of ODC activity is defined as 1 nmole CO_2 evolved per hour. Fig. 1 shows that the differentiation was accompanied by an attenuation, both in magnitude and in duration, of cellular ODC activity. At t=70-80 hours the ODC activity of

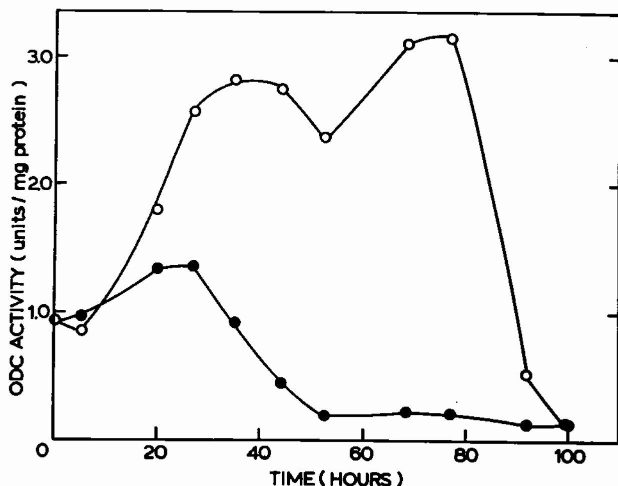

FIG. 1., Changes of ODC activity in NB(-o-) and ND(-•-) cells as a function of time in tissue culture. Time zero refers to the time when Bt_2cAMP (1mM) and IBMX (0.5mM) were added to the ND culture 15 hours after plating. (Data from ref. 17)

ND cells was less than 5% of that of the NB cells. It should be noted that our finding of an early attenuation of ODC activity after the addition of Bt_2cAMP, either with or without IBMX, to sparse cultures of mouse neuroblastoma cells is in apparent contradiction to previous observations that cAMP induces ODC activity in confluent neuroblastoma cultures (3, 4). This apparent dichotomy of cAMP action appears to be related to cell growth and cell density. Thus, in rapidly dividing sparse culture (cell density ~$2x10^4$ cells/cm^2), the addition of Bt_2cAMP results in, in chronological order, neurite outgrowth, attenuation of ODC activity, slowing of growth, and maximal expression of various differentiated phenotypes (17). In contrast, the addition of Bt_2cAMP to confluent culture of neuroblastoma cells (cell density ~$6x10^5$ cells/cm^2) causes a greater than 100-fold stimulation of ODC activity (13) but was ineffective in inducing differentiation of these cells.

For the quantitation of polyamines, we adopted the procedure of Seiler and Wiechman (40) to prepare dansyl derivatives of polyamines which were then quantitatively separated by reverse-phase liquid chromatography (17). Fig. 2 is a representative chromatogram. Under the experimental condition used, complete separation of putrescine, spermidine and spermine was achieved within 8 min. Using this quantitation method, the individual polyamine contents of NB and ND cells were determined at various time points over a 5-day culture period. Fig. 3 illustrates the results obtained using the NB-15 cells. Similar data were also obtained with N-18 and N2a mouse neuroblastoma clonal cells (D. Nau and K.Y. Chen, unpublished). Among three polyamines, the difference in spermidine content between the NB and ND cells is most notable. The spermidine content of the NB cells increased

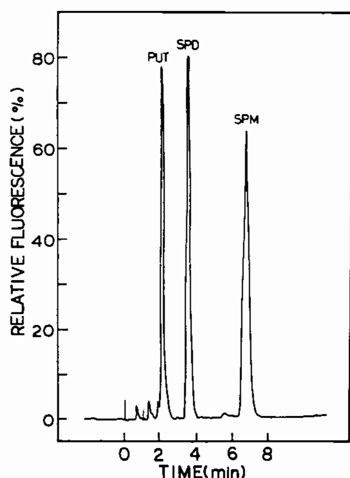

FIG. 2., Elution pattern of dansylated putrescine (PUT), spermidine (SPD), and spermine (SPM). Samples were applied to a reverse-phase column (RP-18, 7µm ODS) connected to a Beckman model 110A pump. Each peak corresponds to 50 pmoles of sample. Solvent system: CH_3CN: H O (85:15 v/v), flow rate 2.3 ml/min.

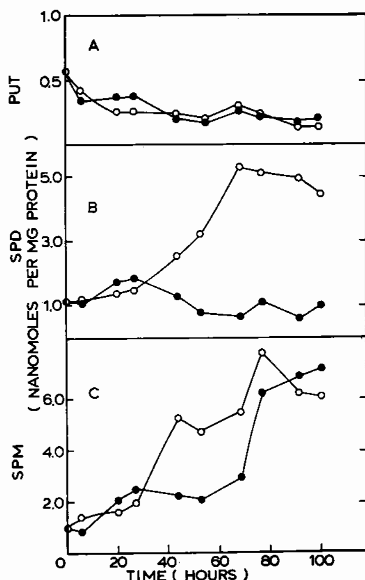

FIG. 3., Changes of polyamine contents in NB(-o-) and ND(-•-) cells as a function of time in tissue culture. Time zero refers to the time when Bt_2cAMP and IBMX were added to the ND culture 15 hours after plating. (Data from ref. 17)

steadily from t=0 reaching a maximal value of 5.3 nmoles/mg protein at t=~70 hours. The elevated level of spermidine in the NB cells was sustained for the remainder of the 5-day culture period. The pattern of change in spermidine content of the ND cells was distinct from that of the NB cells; except a small peak of spermidine (1.8 nmoles/mg protein) at t=~20 hours the spermidine level of ND cells hovered at the basal level (1.0 nmole/mg protein) throughtout the 5-day culture period.

In both NB and ND cells, the putrescine level decreased steadily as a function of time in culture. There was no significant difference in the putrescine content of the NB and ND cells Quantitation of the spermine content in the NB and ND cells. showed a progressive time-dependent increase in both the NB and ND cells. However, the increase in spermine content of the NB cells was more pronounced than that of the ND cells. The significance of the late increase of spermine content in the ND cells as illustrated in Fig. 3 is not clear. It should be noted that the magnitude of such increase varied from experiment to experi-

ment (D. Nau and K.Y. Chen, unpublished).

PUTRESCINE TRANSPORT SYSTEM

Studies have shown that extracellular polyamines can be transported into cells and can regulate the activities of polyamine biosynthetic enzymes (7, 23). While the physiological significance of polyamine transport system is not clear, several studies have suggested that it may play some role in the regulation of growth. For example, Pohjanpelto (35) has found that putrescine transport is greatly increased in human fibroblasts which are stimulated to grow. Kano and Oka (25) have reported that the active transport system for polyamines in mouse mammary explants can be stimulated by insulin and prolactin.

Our studies with the mouse neuroblastoma cells have demonstrated a common transport system for spermidine, spermine and putrescine in mouse neuroblastoma cells (14). The time course of the rate of putrescine uptake in both NB and ND cells (NB-15 clone) over a 5-day culture period is shown in Fig. 4A. The results clearly indicate that the cAMP-induced neuroblastoma differentiation was associated with a significant decrease of

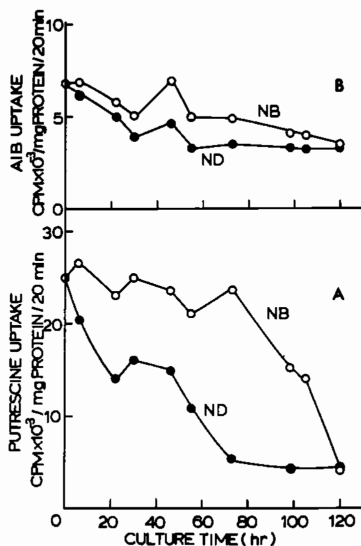

FIG. 4., Initial rate of transport of putrescine (A) and aminoisobutyrate (B) into NB and ND cells as a function of time in tissue culture. (Data from ref. 14)

putrescine transport. Six hours after the addition of Bt_2cAMP and IBMX, the putrescine transport rate of the ND cells was reduced by 27% when compared to that of the NB cells. At the same time, the transport of aminoisobutyric acid (AIB) in the ND cells was only slightly inhibited (Fig. 4B). Kinetic analysis indicated that the decrease in the rate of putrescine transport in the ND cells may be attributable to a change of apparent Km value of the putrescine transport system. We found the the Vmax values of the NB and ND cells at day-4 were identical (5.3 mnoles/mg

protein/hr) but the Km values were different; the Km's were 2.75 μM for the NB and 28.5 μM for the ND cells. Differentiation-associated changes of plasma membrane structure of mouse neuroblastoma cells have been previously reported (16, 27, 42), it appears that the change of Km value of putrescine transport system in the ND cells may represent one of earlier detectable membrane changes associated with the differentiation of mouse neuroblastoma cells.

THE EFFECTS OF ASPARAGINE ON ODC INDUCTION IN UNDIFFERENTIATED AND DIFFERENTIATED MOUSE NEUROBLASTOMA CELLS

It has been previously suggested that plasma membrane/cytoskeletal structure may be involved in the regulation of ODC activity in mammalian cells (12, 21, 32). Work from many laboratories has also indicated changes of plasma membrane/cytoskeletal structure upon differentiation of the mouse neuroblastoma cells (9, 16, 27, 42); our work on the putrescine transport also supports differences in membrane structure of the undifferentiated and differentiated cells. In view of these considerations, we studied and compared the induction of ODC of the undifferentiated and differentiated mouse neuroblastoma cells.

Our previous studies have shown that FCS, Bt_2cAMP, and asparagine can elicit maximal increases of ODC activity of confluent neuroblastoma cells maintained in fresh Dulbecco's medium. We have also shown that when neuroblastoma cells are maintained in a salts/glucose medium (e.g. Earle's balanced slats solution), only asparagine can elicit a maximal increase of ODC activity; serum factors, hormones or agents which increase intracellular cAMP content are completely ineffective (13). These results suggest a possible primary function of asparagine in the induction of ODC activity in cultured cells.

Using the salts/glucose solution as an induction medium, we have compared the effects of asparagine on the induction of ODC activity in the undifferentiated and differentiated mouse neuroblastoma cells (10). The results shown in Fig. 5 demonstrated that 10mM asparagine alone produced a marked increase in ODC activity of the undifferentiated neuroblastoma cells; an effect that was not dependent on or potentiated by the addition of FCS or Bt_2cAMP. This is to be contrasted with results obtained with the differentiated neuroblastoma cells. In the differentiated neuroblastoma cells, 10 mM asparagine only produced a slight increase in ODC activity, which was, however, potentiated by the simultaneous addition of either 10% FCS or 1 mM Bt_2cAMP. The molecular basis for the differences in regulation of ODC activity in the undifferentiated and differentiated neuroblastoma cells, and particularly the response to asparagine as an inducer of ODC activity; is not clear. It is possible that the sensitivity towards asparagine may be related to the dynamic state of the plasma membrane/cytoskeletal structure.

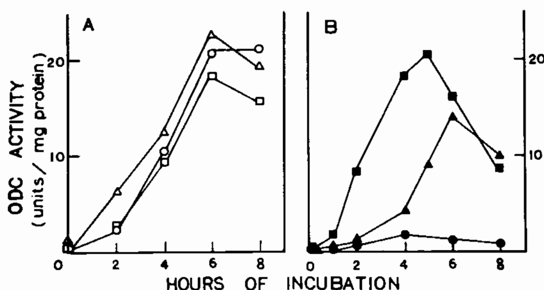

FIG. 5., (A) ODC induction in NB cells (stationary phase) by 10mM asparagine (o), 10mM asparagine plus 10% FCS (□) or 10mM asparagine plus 1mM Bt_2cAMP (Δ). (B) ODC induction in ND cells (stationary phase) by 10mM asparagine (●), 10mM asparagine plus 10% FCS (■) or 10mM asparagine plus 1mM Bt_2cAMP (▲). (Data from ref. 10)

METABOLIC LABELING OF AN 18,000 Dalton PROTEIN BY [^{14}C]PUTRESCINE AND THE CONVERSION OF PUTRESCINE TO GABA

In the previous sections of this chapter we have discussed differences in the intracellular concentration and transport of polyamines as well as the regulation of ODC activity of the NB and ND cells. To further define the precise function(s) of polyamines in growth regulation, it is desirable to identify and characterize the specific biochemical reactions involving polyamines. In this connection, we have investigated the incorporation of radioactive polyamines into cellular proteins. Two specific biochemical events associated with polyamines have thus been identified in mouse neuroblastoma cells, namely: (a) Specific labeling of an 18,000 dalton protein, and (b) Conversion of putrescine to amino acids via GABA (15). Preliminary studies of these two biochemical events in both undifferentiated and differentiated mouse neuroblastoma cells have been made. The result of a representative experiment is shown in the form of an autoradiogram in Fig. 6. The addition of [^{14}C]putrescine to cultures of neuroblastoma cells resulted in the labeling of an 18,000-dalton protein band and other cellular proteins. The labeling of the 18,000-dalton protein appears to be a specific metabolic incorporation reaction of putrescine and spermidine (11, 15). Folk and co-workers (20, 33) have reported a [^{3}H]putrescine-labeled 18,000 -dalton protein in human lymphocytes.

The labeling of various cellular proteins other than the 18,000-dalton protein may be attributable to general protein synthesis using radioactive amino acids derived from [^{14}C]putrescine through its conversion to GABA, the GABA shunt and Krebs cycle. This notion is supported by the findings that (a) aminoguanidine, a diamine oxidase inhibitor which inhibits the conversion of

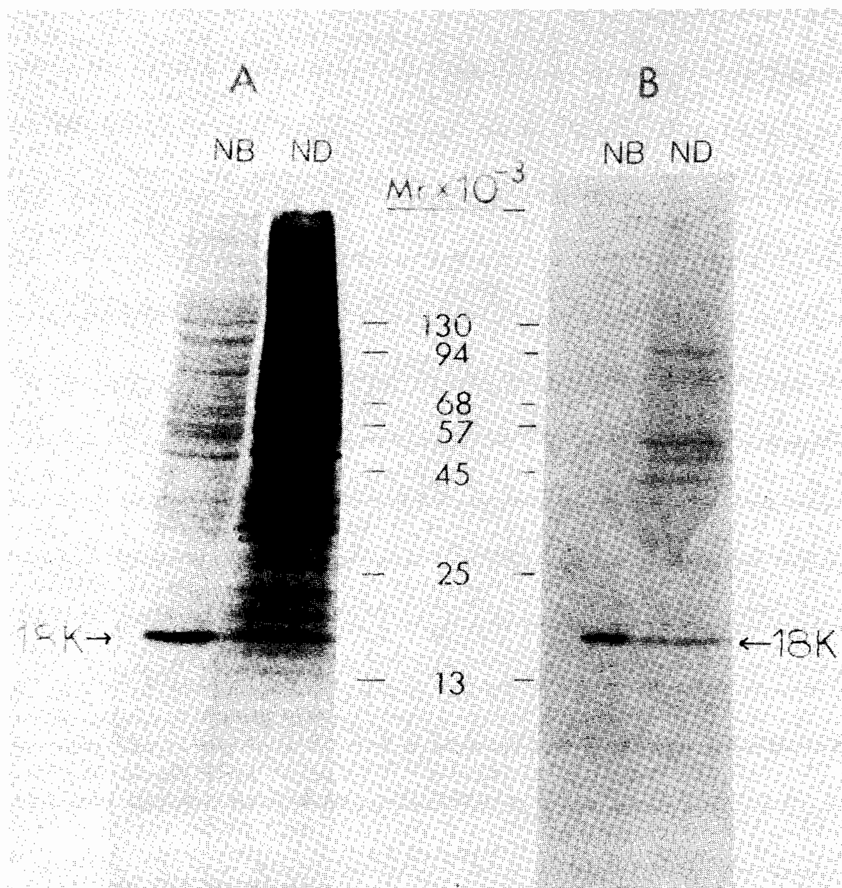

FIG. 6., Fluorogram of the radioactively labeled proteins in the undifferentiated and differentiated neuroblastoma cells exposed to 1μCi/ml [^{14}C]putrescine in fresh Dulbecco's medium with (A) or without (B) 10% FCS for 20 hours. Each lane contained 50μg protein. (Data from ref. 15)

putrescine to GABA, completely abolishes the labeling of all cellular proteins except the 18,000-dalton protein, and (b) with the exception of the 18,000-dalton protein, the labeling pattern of the undifferentiated or differentiated cells can be reproduced by using [^{14}C]GABA as precursor (15). The data shown in Fig. 6 indicate that the labeling of the 18,000-dalton protein is more prominent in the undifferentiated cells than in the differentiated cells whereas the conversion of putrescine to amino acids via GABA is more prominent in the differentiated cells than in the undifferentiated cells. The physiological significance of the changes of these two polyamine-associated biochemical events upon cell differentiation is currently under investigation.

THE EFFECT OF α-FLUOROMETHYLORNITHINE ON THE DIFFERENTIATION OF MOUSE NEUROBLASTOMA CELLS

While the data presented above may suggest that polyamines play some role in the differentiation of mouse neuroblastoma cells, they do not provide direct evidence of a cause-effect relationship. Recently, highly specific irreversible inhibitors of ODC such as D,L-α difluoromethylornithine (α-DFMO) and (R,S)-α-fluoromethylornithine (α-FMO) have been developed (26, 31). Studies have shown these compounds are potent and effective inhibitors of ODC in many cultured cell systems and animal tissues (19, 29, 30, 36). The availability of these inhibitors enabled us to examine directly the effect of inhibition of polyamine biosynthesis on the differentiation of mouse neuroblastoma cells. We have recently found that the presence of either α-DFMO or α-FMO at 0.5 ~ 1.5 mM can reduce the amount of Bt_2cAMP needed for maximal induction of neuroblastoma differentiation by 5 to 10 fold. Thus, 1 mM Bt_2cAMP was needed to induce maximal differentiation of the neuroblastoma cells in the absence of α-DFMO or α-FMO, while only 0.1 ~ 0.2 mM Bt_2cAMP was required to elicit a similar if not greater degree of differentiation of neuroblastoma cells in the presence of 0.5 ~ 1.5 mM α-DFMO or α-FMO. It should be noted that Bt_2cAMP at 0.1 ~ 0.2 mM neither affect the cell growth nor induce cell differentiation.

Fig. 7 shows the photomicrographs of N2a mouse neuroblastoma cells grown in the presence of 0.2 mM Bt_2cAMP alone (Fig. 7A) and cells grown in the presence of a combination of α-FMO and Bt_2cAMP at various concentrations (Fig. 7 B-D). It can be seen that cells grown in the presence of 0.5 - 1.5 mM α-FMO plus 0.1 - 0.2 mM Bt_2cAMP exhibited thick and long processes, sometimes greater than 600 μm in length, and forming extensive neurite webs. Exogenous putrescine or spermidine at 0.1 mM completely blocked the action of 1 mM α-FMO, either alone or with 0.1 - 0.2 mM Bt_2cAMP, on neuroblastoma cell differentiation (unpublished observation). These preliminary studies suggest that modulation of intracellular ODC activity, and thus polyamine levels may bear a causal relationship to neuroblastoma differentiation. However, the fact that in the presence of 0.5 ~ 1.5 mM α-FMO, a suboptimal concentration of Bt_2cAMP (0.1 ~ 0.2 mM) is needed to bring about maximal neuroblastoma differentiation, also indicates that changes of polyamine metabolism alone is not sufficient for neuroblastoma differentiation.

The mechanism through which suboptimal concentrations of Bt_2cAMP potentiate the effect of α-FMO on neuroblastoma cell differentiation is not clear. Recently Gunning et al. (22) have reported a synergistic effect of Bt_2cAMP and β-nerve growth factor on the neurite outgrowth of PC-12 pheochromocytoma cells and suggested that Bt_2cAMP is responsible for the initiation of neurite outgrowth. In light of their study, the potentiating role of Bt_2cAMP on the α-FMO-induced neuroblastoma differentiation may be related to microtubule assembly. The possible involvement

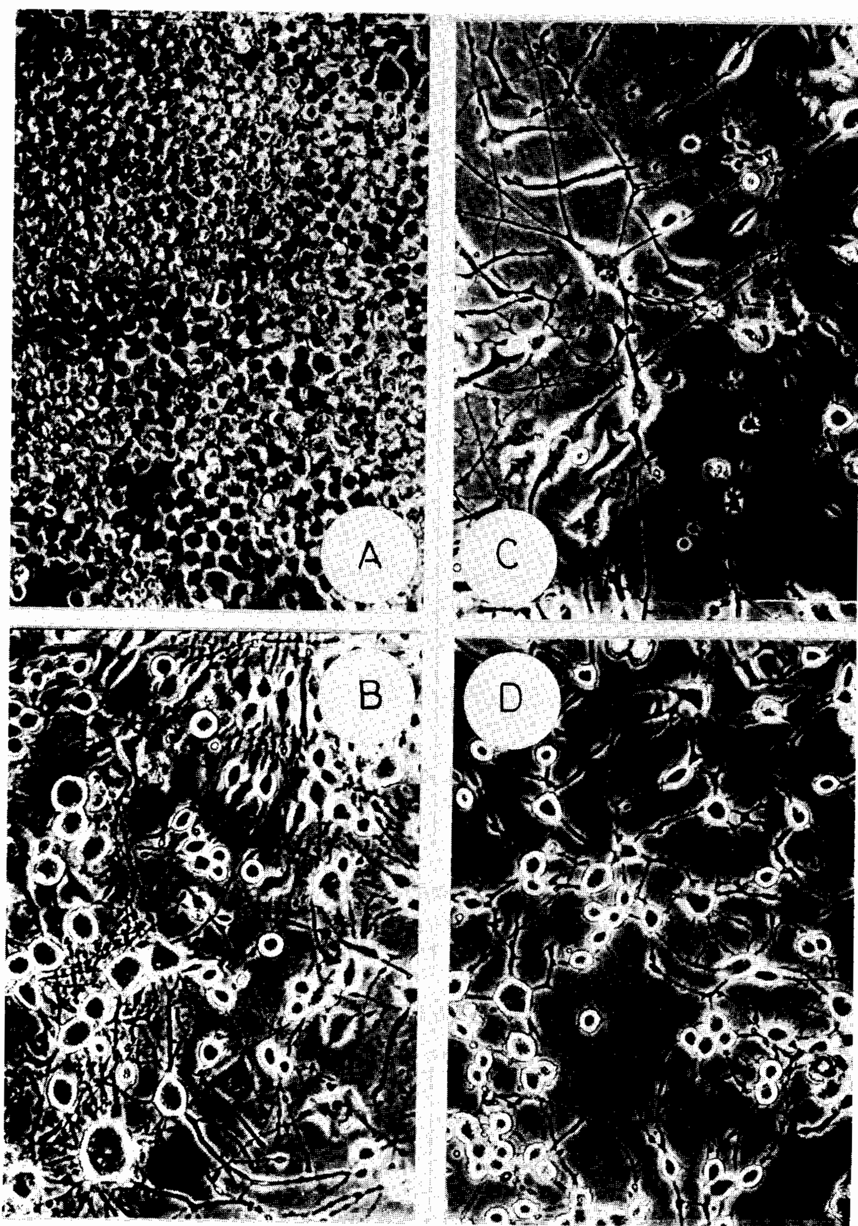

FIG. 7. N2a mouse neuroblastoma cells treated with (A) 0.2mM Bt_2cAMP, (B) 1 mM -FMO plus 0.2 mM Bt_2cAMP, (C) 1.5 mM -FMO plus 0.1 mM Bt_2cAMP, and (D) 0.5 mM -FMO plus 0.2 mM Bt_2cAMP. Phase contrast photomicrographs of cells were taken 8 days after initiation of treatment. (Chen, K.Y., unpublished data.)

of cAMP and protein phosphorylation in microtubule polymerization has been proposed (41). Alternatively, it is possible that low concentrations of Bt_2cAMP may modify certain biochemical event(s) which by itself has no effect on tumor growth or differentiation but can "prime" or prepare cells for maximal differentiation when cellular ODC activity and polyamine contents are reduced by α-FMO treatment.

CONCLUSION

The data presented in this chapter have demonstrated that the differentiation of mouse neuroblastoma cells is accompanied by significant changes of polyamine metabolism. These changes include (a) a decrease of cellular ODC activity and spermidine level (17), (b) a decrease in putrescine uptake (14), (c) a decrease in the asparagine-dependent ODC induction (10), (d) a decrease in the metabolic labeling of the 18,000-dalton protein by $[^{14}C]$putrescine (15), and (e) an increase of the conversion of putrescine to amino acids via GABA (15). The essential role of polyamines and ODC in the differentiation of mouse neuroblastoma cells is suggested by the findings that α-FMO, a suicidal enzyme inhibitor of ODC, plus suboptimal concentration of Bt_2cAMP (0.1 - 0.2 mM) can cause maximal differentiation of neuroblastoma cells and the differentiation can be blocked by exogenously added putrescine or spermidine.

Tumor differentiation is undoubtedly a complex phenomenon. Our data emphasize that the modulation of ODC/polyamines may be essential for the differentiation of mouse neuroblastoma cells. Further studies are needed to elucidate the precise mechanisms involved. Recently, Bethell and Pegg (5) showed that α-DFMO inhibits the differentiation of 3T3-L1 fibroblasts into adipose cells. Their findings suggest the involvement of ODC/polyamines in normal cell differentiation. In view of these considerations, it is plausible that modulation of cellular ODC activity and polyamine centents is of general significance in the differentiation of both normal and tumor cells.

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