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INDUCTION OF DIFFERENTIATION IN MURINE NEUROBLASTOMA CELLS BY MEVINOLIN, A COMPETITIVE INHIBITOR OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

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Mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, stimulated outgrowth of neurites and increased acetylcholinesterase activity in C1300-N2A murine neuroblastoma cells cultured in medium containing 10% fetal calf serum. Changes in cell morphology and enzyme activity were concentration-dependent in the range of 0.25-25 μM mevinolin, and were accompanied by decreased incorporation of [^{3}H]thymidine into DNA. The expression of differentiated characteristics induced by 25 μM mevinolin was blocked by simultaneous addition of 100 μM mevalonate to the culture medium. The data suggest that changes in intracellular levels of mevalonate or one of its isoprenoid derivatives may play a role in the regulation of cell differentiation.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [EC 1.1.1.34] catalyzes the conversion of HMG-CoA to mevalonate, which serves as a precursor in the branched pathway for the biosynthesis of sterols, ubiquinone (coenzyme Q), dolichol and isopentenyl-tRNA in mammalian cells (1). Blocking mevalonate synthesis with competitive inhibitors of HMG-CoA reductase results in inhibition of DNA synthesis and arrest of cell cycling (2-7). Recent studies also have shown that mevalonate or one of its derivatives within the cell can stimulate transformation of peripheral lymphocytes (8). Thus, a role for mevalonate in the regulation of cell proliferation has been documented. In certain tissues, such as the developing brain, a marked decline in the activity of HMG-CoA reductase occurs as maturation progresses and cell division ends (9,10). However, it is not yet known whether changes in the synthesis of mevalonate or specific end products of the isoprenoid pathways are involved in the initiation of cell differentiation. This report provides the first evidence that mevinolin, a competitive inhibitor of HMG-CoA

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reductase (11), induces morphological and biochemical differentiation in a cultured cell line.

MATERIALS AND METHODS

Materials: [methyl-^{3}H]Thymidine (5 Ci/mmol), [5-^{3}H]uridine (5 Ci/mmol) and L-[4,5-^{3}H]leucine (131 Ci/mmol) were purchased from Amersham. Tissue culture medium (Gibco), fetal calf serum (Kansas City Biological) and plastic tissue culture flasks (Corning) were obtained from the designated sources. Mevinolin was a gift from Dr. Alfred W. Alberts of the Merck, Sharp and Dohme Institute for Therapeutic Research. Prior to adding mevinolin to tissue culture medium, the lactone was converted to the sodium salt as described by Kita et al. (12). All other chemicals were from Sigma Chemical Co.

Cell Culture: Monolayer cultures of murine neuroblastoma cells (C1300, N2A) were maintained at 37°C in Dulbecco's modified Eagle medium, supplemented with 10% (v/v) fetal calf serum and equilibrated with 5% CO_2 in air. Stock cultures were subcultured by trypsinization (0.25% trypsin in Dulbecco's phosphate-buffered saline, pH 7.5) and cells were seeded in 25 cm^2 flasks at a density of 12,500 cells/cm^2. All cultures were grown for 24 h before being used for experiments.

Morphological Differentiation: Cells with neurites longer than the diameter of the cell body were considered to be morphologically differentiated. The percentage of differentiated cells in a culture was determined by counting 150-200 cells in three photomicrographs of areas where the cell density was representative of most of the culture. Photomicrographs were taken with a Nikon Diaphot inverted phase contrast microscope equipped with a Nikon PFX Polaroid photographic system.

Acetylcholinesterase Activity: Cells were removed from monolayer cultures by a 5 min exposure to phosphate-buffered saline (pH 7.5) containing 0.02% EDTA. Cells were pelleted by centrifugation, washed three times with phosphate-buffered saline to remove traces of serum, and stored at -80°C. Frozen cell pellets were thawed in 10 mM potassium phosphate (pH 8.0), and aliquots of cell lysate containing 100-200 µg protein were assayed for acetylcholinesterase [EC 3.1.1.7] by means of the colorimetric assay of Ellman et al. (13), modified as described (14). One unit of enzyme activity equaled one nmol of acetylcholine cleaved per min at 25°C.

Protein Determination: Quantitation of protein in cell lysates was accomplished by a microbiuret method (15), using bovine serum albumin as a standard.

DNA, RNA and Protein Synthesis: Synthesis of DNA, RNA or protein in cell cultures was evaluated by measuring the incorporation of radioactivity from [^{3}H]thymidine (0.2 µCi/ml medium), [^{3}H]uridine (0.2 µCi/ml medium) or [^{3}H]leucine (1.0 µC/ml medium), respectively, into trichloroacetic acid (TCA)-insoluble material as described previously (16).

RESULTS

When C1300 neuroblastoma cells were cultured in medium supplemented with 10% fetal calf serum, most of the cells exhibited an undifferentiated morphology (Fig. 1A). However, addition of mevinolin to the serum-supplemented cultures promoted extensive neurite outgrowth within 24 hours

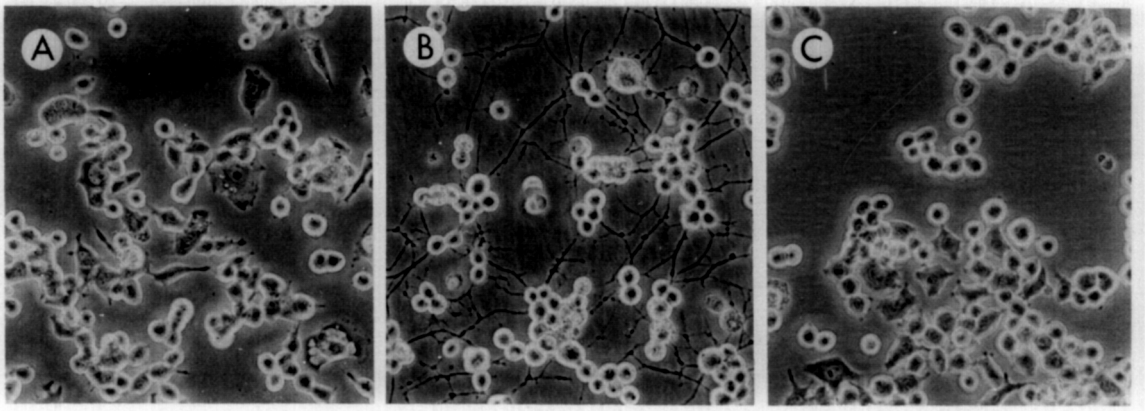

Figure 1. Morphological appearance of C1300-N2A neuroblastoma cultures after growth for 24 h under the following conditions: (A) medium + 10% fetal calf serum; (B) medium + 10% fetal calf serum + 25 μM mevinolin; (C) medium +10% fetal calf serum + 25 μM mevinolin +100 μM mevalonolactone. The bar represents 100 microns.

(Fig. 1B). The percentage of cells with neurites was maximal at a mevinolin concentration of 25 μM (Fig. 2), and the extent of morphological differentiation under these conditions was comparable to that previously observed in neuroblastoma cells induced to differentiate by serum deprivation (17, 18). As shown in Fig. 2, addition of increasing amounts of mevinolin to the culture medium also produced corresponding increases in the activity of acetylcholinesterase, an enzyme used as a biochemical marker for neuronal differentiation in neuroblastoma cells (19,20). Both the morphological differen-

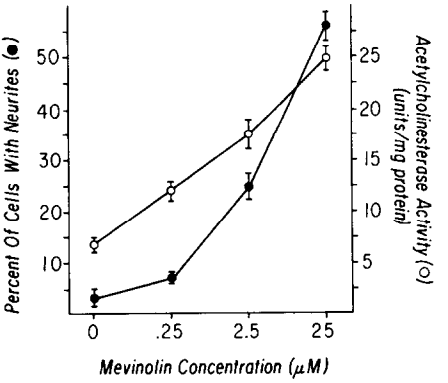

Figure 2. Morphological differentiation and acetylcholinesterase activity in cultured neuroblastoma cells exposed to increasing concentrations of mevinolin for 24 hours. All values are means (±S.E.M.) derived from separate determinations performed on 3 cultures.

TABLE 1

Effects of Addition of Mevalonate on Mevinolin-Induced Differentiation in Neuroblastoma Cells

	Cell Morphology (% cells with neurites)	Acetylcholinesterase (units/mg protein)	[^{3}H]Thymidine Incorporation (c.p.m./ug protein)
No Addition	3.4 ± 1.0	10.4 ± 2.2	268 ±10
25 µM Mevinolin	52.7 ± 2.5	30.5 ± 1.4	140 ± 4
25 µM Mevinolin + 100 µM Mevalonolactone	5.1 ± 0.2	15.1 ± 2.3	264 ± 7

Cells were plated in 25 cm^2 flasks and grown for 24 h. The medium then was changed so that cultures contained the supplements described above. After an additional 24 h incubation, the cultures were assessed for morphological differentiation, acetylcholinesterase activity or DNA synthesis. Values are means (± S.E.M.) of separate determinations performed on 3 cultures.

tiation and the increase in acetylcholinesterase activity in cells exposed to 25 µM mevinolin were prevented by simultaneous addition of 100 µM mevalonate to the culture medium. (Fig. 1C, Table 1).

The effects of mevinolin on macromolecular synthesis in neuroblastoma cells are depicted in Figure 3. After 24 hours, mevinolin produced a

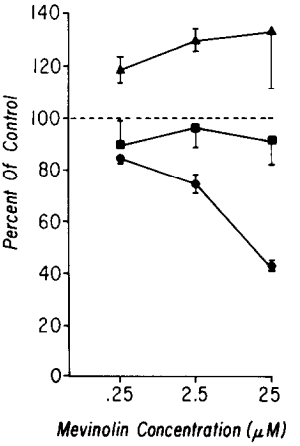

Figure 3. Effects of mevinolin on incorporation of [^{3}H]thymidine (●), [^{3}H]uridine (▲), and [^{3}H]leucine (■) into TCA-insoluble material. Cells were grown in medium supplemented with the indicated concentrations of mevinolin for 24 h. Values are expressed as percent of the incorporation of radioactivity in control cultures incubated without mevinolin for 24 h. Each point represents the mean (±S.E.M.) derived from separate determinations performed on 3 cultures.

concentration-dependent decrease in the ability of the cells to incorporate [^{3}H]thymidine into the TCA-precipitable fraction. In contrast, mevinolin had little or no effect on incorporation of [^{3}H]leucine and a slight stimulatory effect on incorporation of [^{3}H]uridine. Supplementation of the mevinolin-treated cultures with enough mevalonate to block the induction of neurite outgrowth and the increase in acetylcholinesterase activity, also prevented the decline in DNA synthesis (Table 1).

DISCUSSION

The data demonstrate that mevinolin can act as a potent inducer of neuroblastoma differentiation. The ability of a small amount of mevalonate to block the extension of neurites and the induction of acetylcholinesterase activity in cells exposed to mevinolin suggests that these phenomena are related to inhibition of HMG-CoA reductase and a consequent reduction in the intracellular mevalonate concentration, rather than to nonspecific perturbation of cellular metabolism by mevinolin.

Several agents that inhibit cell proliferation (e.g., cytosine arabinoside, 5-bromodeoxyuridine, dimethyl sulfoxide) are known to induce differentiation in neuroblastoma cells (21-23). Therefore, it will be of interest to determine whether the differentiation induced by mevinolin is linked to suppression of DNA synthesis by this compound (Fig. 3), or instead proceeds via a mechanism that is independent of the cell cycle. Recent studies have demonstrated that mevalonate or one of its intracellular derivatives is important for the maintenance of cell shape (24), and that the activity of HMG-CoA reductase can respond to changes in the physical state of the microtubular and microfilament networks (25, 26). Thus, the possibility that the influence of mevinolin on neuroblastoma cell morphology is mediated through an effect of mevalonate deficiency on the organization of the cytoskeleton appears to be a promising area for future exploration.

An important question raised by these studies concerns the chemical identity of the compound whose depletion triggers the expression of differentiated characteristics in neuroblastoma cells. High concentrations of

mevinolin could suppress the synthesis of non-sterol isoprenoid derivatives of mevalonate (e.g., dolichol, ubiquinone), as well as cholesterol. In this regard, two lines of evidence suggest that depletion of cholesterol may not be the precipitating event in the induction of differentiation by mevinolin. The first is that morphological differentiation of neuroblastoma cells is inhibited, rather than stimulated, by prior sterol depletion of the cells (18). The second is that the changes induced by mevinolin in the present study occurred in cells that were cultured with fetal calf serum, which should supply sufficient exogenous lipoprotein-bound cholesterol to prevent sterol depletion (18). Nevertheless, detailed studies of the complex interrelationships among isoprenoid biosynthesis, membrane cholesterol content, cell proliferation and cell differentiation are needed to clarify this issue. The data presented in this report suggest that the N2A neuroblastoma cell line will be a useful system in which to undertake such studies.

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