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Divergence in cholesterol biosynthetic rates and 3-hydroxy-3-methylglutaryl-CoA reductase activity as a consequence of granulocyte versus monocyte-macrophage differentiation in HL-60 cells

(phorbol ester/dimethyl sulfoxide/DNA synthesis)

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ABSTRACT **Addition of dimethyl sulfoxide or phorbol myristate acetate (PMA) to HL-60 cell cultures induces granulocytic or monocyte–macrophage differentiation, respectively, in HL-60 cells. Dimethyl sulfoxide-induced granulocyte differentiation in HL-60 cells is associated with a decrease in cellular 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity and with a decrease in the incorporation of [^{14}C]acetate and mevalonate into products of the cholesterol biosynthetic pathway. PMA-induced monocyte–macrophage differentiation in HL-60 cells is associated with a rapid and profound fall in cell proliferation but nonetheless is accompanied by a dose-dependent increase in cellular HMG-CoA reductase activity and [^{14}C]acetate incorporation into the cholesterol biosynthetic pathway. In addition, PMA induces an increase in [^{14}C]mevalonate incorporation into cholesterol and its precursors, suggesting that post-HMG-CoA reductase events in cholesterol biosynthesis are also enhanced. Mature peripheral blood human monocytes possess an active cholesterol biosynthetic pathway, whereas mature human granulocytes are almost entirely lacking in the ability to synthesize post-squalene products. Our results with HL-60 cells indicate that this divergence in sterol-synthesizing ability between two cell lineages, which normally also derive from a common stem cell, can be observed as an early event in the differentiation process.**

The various classes of leukocytes in human peripheral blood differ significantly from each other with respect to cholesterol biosynthesis (1–3). The monocyte displays the greatest capacity to synthesize cholesterol from its precursors, acetate and mevalonate, whereas the mature circulating polymorphonuclear granulocyte has little or no cholesterol-synthesizing ability. The lymphocyte's capacity for cholesterol biosynthesis is intermediate to those of monocytes and granulocytes. Some of these differences in cholesterol biosynthesis amongst the various peripheral blood leukocyte lineages also can be discerned in leukemic cells from patients with acute nonlymphocytic leukemia. Cells from patients with acute promyelocytic leukemia have markedly lower rates of cholesterol biosynthesis than do cells from patients with acute myelomonocytic or acute monocytic leukemia (4).

The HL-60 cell line was originally derived from a patient with acute promyelocytic leukemia (5). Under ordinary conditions of culture, the majority of cells maintain their promyelocytic features. However, exposure of the HL-60 cells to dimethyl sulfoxide (Me_2SO) or certain other agents causes the cells to progress further along the pathway of granulocyte differentiation, and acquire many functional and morphological features of mature granulocytes (6–8). Exposure of the HL-60 cells to phorbol esters, on the other hand, induces differentiation along the monocyte–macrophage differentiation pathway (9–11). In this report, we describe our use of this cell line to examine and compare the changes in the cholesterol biosynthetic pathway associated with these two modes of cellular differentiation.

MATERIALS AND METHODS

(*R*,*S*)-Mevalonic acid lactone, cholesterol, lanosterol, and squalene were all purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA) was from PL-Biochemicals. It was dissolved in acetone at 200 times its desired final concentration in cultures. Me_2SO (distilled in glass) was a product of Burdick and Jackson (Muskegon, MI). [2-^{14}C]Thymidine (56 mCi/mmol; 1 Ci = 37 GBq), 3-hydroxy-3-methyl[3-^{14}C]-glutaryl (HMG)-CoA (56 mCi/mmol), and (*R*)-[2-^{14}C]mevalonic acid lactone (53 mCi/mmol) were obtained from Amersham Searle Corporation. Sodium [1-^{14}C]acetate (57 mCi/mmol), (*R*,*S*)-[2-^{14}C]mevalonic acid lactone (47 mCi/mmol), and (*R*,*S*)-[5-^{3}H]mevalonolactone (13.8 Ci/mmol) were from the New England Nuclear Corporation.

HL-60 cells were maintained in tissue culture (humidified 95% air/5% CO_2 incubator at 37°C) in RPMI medium (GIBCO) supplemented with antibiotics, glutamine, and 10% fetal calf serum. These cultures were subdivided and replenished with fresh medium every 5 days. Prior to use in any experiment, cells in logarithmic growth phase were centrifuged and washed once in RPMI.

Human blood type AB serum was heat-inactivated for 30 min at 56°C and stored at −80°C. DNA synthesis in HL-60 cells was measured in triplicate samples by culturing 0.5×10^6 cells in 1 ml of RPMI medium containing 12.5% AB serum, with or without appropriate amounts of various test materials. Cells were harvested at 24-hr intervals, 16 hr after addition of 0.2 μCi of [2-^{14}C]thymidine, by suction filtration onto glass fiber discs. After being washed with phosphate-buffered saline (0.01 M phosphate/0.15 M NaCl, pH 7.4), 5% trichloracetic acid, and methanol, the dried discs were immersed in 10 ml of scintillation fluid for assay in a Packard Tricarb liquid scintillation apparatus.

For experiments in which changes in HMG CoA reductase activity were followed, 3.0×10^6 HL-60 cells were cultured in RPMI containing 20% (vol/vol) AB serum in 25-cm^2 tissue culture flasks (Falcon, no. 3013) at a cell density of 0.6 or 1.0×10^6/ml. After 24 hr of incubation, various experimental substances were added (zero time). At the conclusion of the appropriate incubation period, adherent cells were scraped loose with a rubber policeman, and the cells were washed four times in cold phosphate-buffered saline, drained clear of excess fluid, and frozen at −80°C for later assay. The use of

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; NS, nonsaponifiable; Me_2SO, dimethyl sulfoxide; PMA, phorbol myristate acetate.

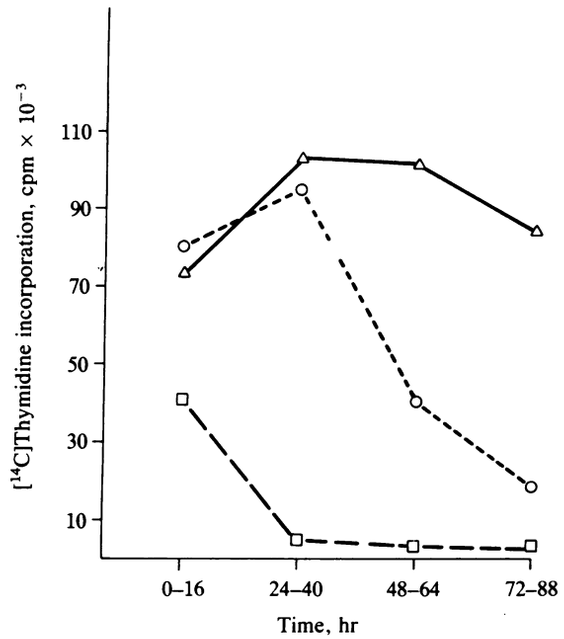

FIG. 1. Time-dependent inhibition of DNA synthesis in HL-60 cells by 1.6 μM PMA (□) and 1.25% Me_2SO (○). △, Control. Me_2SO and PMA were added at the time of culture initiation. [2-^{14}C]Thymidine was added at 24-hr intervals, and the cells were harvested for assay 16 hr later.

20% fetal calf serum in place of human serum gave similar results.

HMG CoA reductase was measured in duplicate by the method described by Kayden *et al.* (12), with [^{3}H]mevalonolactone as an internal standard to correct for losses of the [^{14}C]-mevalonolactone product during the extraction and TLC procedures used for its isolation. The final concentration of HMG CoA (specific activity, 10 μCi/μmol) in the assay mixture was 30 μM (6.6 cpm/pmol). HMG CoA reductase activity is expressed as pmol of [^{14}C]mevalonolactone formed per min/mg of protein. Preliminary studies revealed that HMG CoA reductase activity in frozen cells was stable for at least 4 weeks. Enzyme assays for any given experiment were performed within that period of time.

Replicate groups of three flasks were also cultured in certain experiments, which were used for the assessment of cholesterol biosynthesis from [^{14}C]acetate (5 μCi/ml; 0.5 mM) and (*R*)-[^{14}C]-mevalonate (5 μCi/ml; 0.15 mM) during the final 6 hr of culture. These cultures were not washed; rather, the cells and medium were transferred to glass tubes and cold carriers were added, followed by 2 volumes of 1 M KOH in 10% water/90% methanol (vol/vol). After saponification at 60°C for 2 hr, half the original culture volume of ethanol was added. The samples were extracted three times with petroleum ether and analyzed by TLC as described (13, 14). Protein measurements were made by a modified Folin technique (15), with bovine serum albumin as a standard.

RESULTS

As other workers have noted, the induction of granulocyte differentiation by Me_2SO and the induction of monocyte–macrophage differentiation by PMA in HL-60 cells are associated with a progressive decline in the rates of DNA synthesis and cell proliferation (6, 9, 16); the effect of PMA is more rapid and more profound than that of Me_2SO (Fig. 1).

When HL-60 cells were exposed to Me_2SO, they experienced a decrease both in cholesterol biosynthesis from acetate and in HMG CoA reductase activity within 24 hr (Table 1). In contrast, exposure of HL-60 cells to 0.16 μM PMA for 24 hr caused a coordinate 3-fold stimulation of both cholesterol biosynthesis from acetate and HMG CoA reductase activity, which progressed even further (5- to 6-fold stimulation) at 48 hr of exposure (Table 1). Peak HMG CoA reductase stimulation by PMA was noted at 48 hr; after 96 hr of PMA exposure, HMG CoA reductase activity was only 25% greater than in control cells (Fig. 2). Maximal stimulation of HMG CoA reductase activity in HL-60 cells was elicited by 0.16–1.6 μM PMA but was no longer discernible at 1.6 nM PMA (Fig. 3, Table 2).

When HL-60 cells were exposed to various concentrations of PMA for 72 hr, both cholesterol biosynthesis from [^{14}C]acetate and cellular HMG CoA reductase activity were stimulated maximally by 0.16–1.6 μM PMA (Table 2). Despite the failure of 1.6 nM PMA to stimulate HMG CoA reductase activity, this concentration of PMA caused a discernible increase in cholesterol biosynthesis. In addition to the absolute increase in cholesterol, lanosterol, and squalene synthesis induced by PMA, a greater proportion of isotopic acetate entering the nonsaponifiable lipid pool found its way into cholesterol in PMA-exposed cells (48–51%) than in control HL-60 cells (33%). Because the maximal increase elicited in HL-60 cell HMG CoA reductase activity by PMA was 2-fold, while cholesterol synthesis increased 6- to 7-fold, the data suggest that in addition to stimulating HMG CoA reductase activity, PMA induction of monocyte–macrophage differentiation in HL-60 cells is also accompanied by stimulation of post-HMG CoA reductase steps in the cholesterol biosynthetic pathway.

Table 3 confirms that monocyte–macrophage differentiation in HL-60 cells is associated not only with increased HMG CoA reductase activity but also with stimulation of post-HMG CoA reductase steps in the cholesterol biosynthetic pathway. In this experiment cholesterol biosynthesis from [^{14}C]mevalonate was approximately doubled by expo-

Table 1. Comparison of the effect of Me_2SO and PMA on cholesterol, lanosterol, and squalene biosynthesis from [1-^{14}C]acetate and on HMG-CoA reductase activity in HL-60 cells

Time, hr	Cells	Incorporation of [1-^{14}C]acetate during synthesis,* pmol/mg of protein			HMG-CoA reductase,† pmol/min/mg
		Cholesterol	Lanosterol	Squalene	
24	HL-60	2,718 ± 278	1026 ± 28	454 ± 39	14.1 ± 1.2
	HL-60 + Me_2SO	1,839 ± 204	503 ± 12	129 ± 15	5.8 ± 0.1
	HL-60 + PMA	8,652 ± 367	1527 ± 146	202 ± 67	41.7 ± 1.1
48	HL-60	2,113 ± 138	929 ± 103	204 ± 17	11.8 ± 1.5
	HL-60 + Me_2SO	1,001 ± 128	458 ± 77	106 ± 5	4.1 ± 0.4
	HL-60 + PMA	11,583 ± 587	1226 ± 253	518 ± 100	57.4 ± 0.5

All values shown with Me_2SO (1.25%) and PMA (0.16 μM) differ significantly from their respective HL-60 control value ($P < 0.02$) with the exception of lanosterol (48 hr; HL-60 + PMA).
*Mean ± SD of triplicate determinations.
†Mean ± SD of duplicate assays.

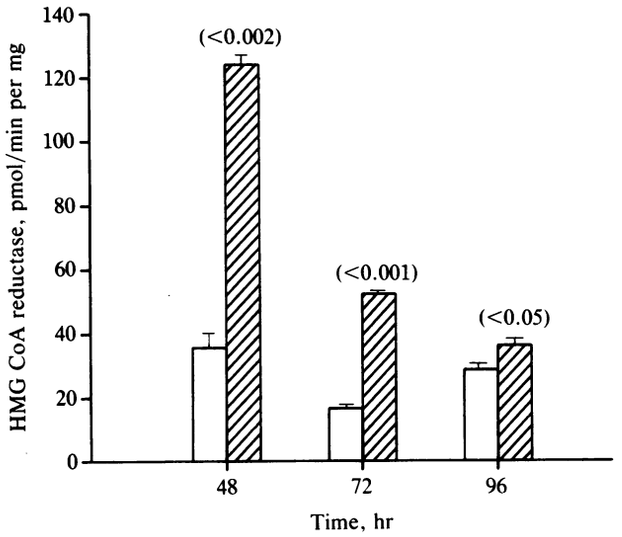

FIG. 2. Stimulation of HMG-CoA reductase activity in HL-60 cells at various times after addition of 0.16 μM PMA (▨). □, Control. Prior to incubation, HL-60 cells contained 25.1 ± 0.2 units of HMG-CoA reductase activity. The numbers shown in parentheses represent the *P* values comparing enzyme activity in PMA-exposed cells with their respective control.

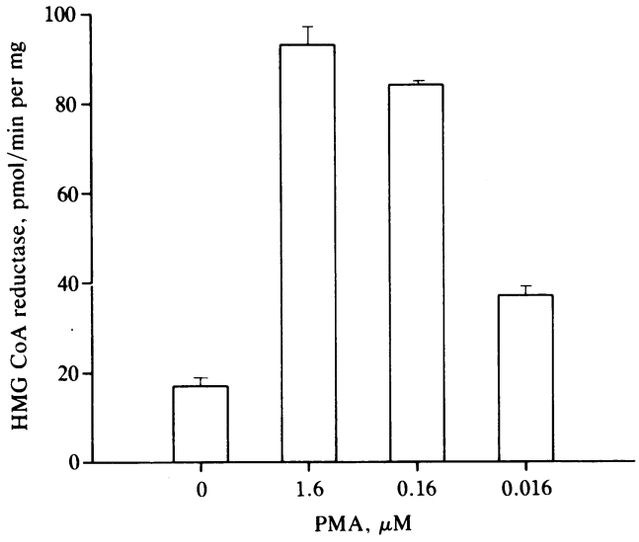

FIG. 3. Stimulation of HMG-CoA reductase activity in HL-60 cells at 48 hr by various concentrations of PMA. Prior to incubation, HL-60 cells contained 27.8 ± 1.3 units of HMG-CoA reductase activity. There is no significant difference in HMG-CoA reductase activity between 1.6 and 0.16 μM PMA. Enzyme activity in all PMA-exposed cells differed from the control value ($P < 0.01$); 1.6 nM PMA did not elicit an increase in HMG-CoA reductase activity (not shown).

sure of HL-60 cells to PMA, while it decreased to approximately half the control value as a consequence of HL-60 cell differentiation along the granulocyte pathway induced by Me_2SO.

DISCUSSION

The present studies were designed to investigate the relationship between cholesterol metabolism and the program of granulocyte or monocyte–macrophage differentiation in HL-60 cells and are based on the unique opportunity these cells present to induce selectively these alternative pathways in a common cell precursor (11). Other workers have shown that granulocyte differentiation in HL-60 cells is accompanied by a 40% decrease in acetate incorporation into cholesterol within 16 hr after the addition of Me_2SO and an 80% decrease at 40 hr. The decreases in cholesterol biosynthesis noted were reversible at 16 hr but irreversible at the later time and were accompanied by decreases in phospholipid synthesis of a similar magnitude; synthesis of triglycerides was not affected (16). Our experiments confirm the inhibition of cholesterol biosynthesis from acetate as a consequence of Me_2SO-induced granulocyte differentiation in HL-60 cells and further show that it is accompanied by appropriate decreases in cellular HMG CoA reductase activity and by inhibition of [^{14}C]mevalonate incorporation into cholesterol and its precursors, indicating concomitant suppression of post-HMG CoA reductase events in cholesterol biosynthesis.

PMA can induce monocyte–macrophage differentiation when added to HL-60 cells at concentrations ranging from 1.6 nM to 1.6 μM (9). Depending on the concentration of PMA to which the cells are exposed, differentiation proceeds despite repeated cell washing after 20 min to 24 hr of exposure to the phorbol ester (9). Within 1–2 hr of exposure of HL-60 cells to PMA (1–0.01 μM), a dose-related increase in the synthesis of phosphatidylcholine from endogenous choline occurs, associated with a simultaneous decrease in the rate of synthesis of phosphatidylcholine via *N*-methylation of phosphatidylethanolamine (17). Our studies indicate that monocyte–macrophage differentiation in HL-60 cells also is accompanied by an increase in HMG CoA reductase activity, which is apparent after 24 hr of exposure to PMA. The increase in HMG CoA reductase activity induced by PMA can be discerned even after 72 hr of exposure to the phorbol ester, is dose-related, and is accompanied by marked stimulation of [^{14}C]acetate into cholesterol and its precursors. By 72 hr, the degree to which cholesterol synthesis from acetate is stimulated by PMA exceeds by far the simultaneously de-

Table 2. The effect of various concentrations of PMA on cholesterol biosynthesis and HMG-CoA reductase activity in HL-60 cells

HL-60 cells	Incorporation of [^{14}C]acetate during synthesis,* pmol/mg of protein				HMG-CoA reductase,† pmol/min/mg
	Cholesterol‡	Lanosterol	Squalene	NS lipids	
Control	7,887 ± 507 (32.7)	2824 ± 184	91 ± 3	24,112 ± 2,699	28.8 ± 3.2
+ PMA, 1.6 μM	54,456 ± 5,488 (48.1)	8712 ± 336	144 ± 9	113,256 ± 8,112	50.3 ± 0.1
+ PMA, 0.16 μM	45,926 ± 1,693 (46.0)	6240 ± 433	133 ± 15	98,480 ± 24,673	61.4 ± 8.2
+ PMA, 0.016 μM	37,319 ± 3,546 (53.8)	4822 ± 411	104 ± 16	69,398 ± 4,006	43 ± 3.3
+ PMA, 1.6 nM	16,137 ± 2,680 (36.6)	2382 ± 417	97 ± 9	44,091 ± 13,754	30.3 ± 0.2

Cholesterol biosynthesis was determined at 72 hr, 6 hr after addition of isotope. HMG-CoA reductase activity was also determined at 72 hr. Even at a concentration of 1.6 nM, PMA causes a significant increase in cholesterol biosynthesis ($P < 0.02$). All PMA HMG-CoA reductase values are significantly different from control ($P < 0.05$) with the exception of that at 1.6 nM. Prior to incubation, HL-60 contained 23.7 ± 2.4 units of HMG-CoA reductase activity. NS lipids, nonsaponifiable lipids.

*Mean ± SD of triplicate determinations.

†Mean ± SD of duplicate assays.

‡Values in parentheses = $\frac{\text{pmol of [}^{14}\text{C]acetate} \rightarrow \text{cholesterol}}{\text{pmol of [}^{14}\text{C]acetate} \rightarrow \text{NS lipids}} \times 100$.

Table 3. A comparison of the effect of Me_2SO and PMA on cholesterol biosynthesis from [^{14}C]mevalonate in HL-60 cells

Cells	Incorporation of [^{14}C]mevalonate during synthesis, pmol/mg of protein			
	Cholesterol	Lanosterol	Squalene	NS lipids
HL-60 control	1047 ± 57	281 ± 13	232 ± 40	2135 ± 23
HL-60 Me_2SO	493 ± 64	129 ± 7	121 ± 17	1179 ± 377
HL-60 PMA	2739 ± 376	1193 ± 36	208 ± 15	6087 ± 42

[^{14}C]Mevalonate incorporation was measured at 72 hr, 6 hr after isotope addition. Values are means ± SD. Me_2SO and PMA were at 1.25% and 1.6 μM, respectively. NS lipids, nonsaponifiable lipids.

termined increase noted in PMA-exposed cellular HMG CoA reductase activity. In addition, the efficiency with which [^{14}C]acetate is incorporated into cholesterol in HL-60 cells after PMA exposure is increased. These observations, suggesting that PMA-induced monocyte–macrophage differentiation in HL-60 cells is accompanied by a stimulation of post-HMG CoA reductase steps in cholesterol biosynthesis, are reinforced by the observation that [^{14}C]-mevalonate incorporation into HL-60 cell cholesterol and lanosterol is enhanced by PMA when measured at 72 hr.

When HMG CoA reductase activity is measured after 96 hr of culture, lesser differences are noted between control and PMA-treated cells. The decline in HMG CoA reductase activity in HL-60 cells during the later stages of exposure to PMA may be related to the fact that their differentiation to macrophages is largely completed at the end of 72 hr (9). Human monocytes also experience both a rise and subsequent fall in HMG CoA reductase activity during their *in vitro* maturation into macrophages, but the peak reductase activity occurs at 5–7 days of culture, followed by a gradual decline over the ensuing week (18). The differentiation of blood monocytes into macrophages is accompanied by a large increase in cell volume and, consequently, by an increase in plasma membrane area (19). The increased cholesterol biosynthesis evoked in HL-60 cells by PMA-induced changes and the elevated rates of choleterol synthesis in the normal monocyte–macrophage may in part be due to the need of such cells to expand their plasma membranes in the course of their differentiation (4, 20). In addition, Rovera *et al.* have described the accumulation of large numbers of lipid droplets in PMA-treated cells (9).

One explanation for the elevated rate of cholesterol biosynthesis seen in leukemic cells is the need for cells in a proliferative state to endow their daughter cells with cholesterol-containing membrane constituents (21). Our earlier studies on rates of cholesterol biosynthesis in leukemic cells clearly demonstrated that the differences observed were not due to differences in their respective rates of cell proliferation as measured by DNA synthesis (4, 13). Our present studies also emphasize that cell proliferation is not a major determinant of cholesterol biosynthesis in HL-60 cells, because even though DNA synthesis and cell proliferation are most acutely and markedly depressed by PMA treatment, it is precisely this maneuver which elicits the greatest increase in the cholesterol biosynthetic pathway. Thus, in the HL-60 cell line, as in human acute nonlymphocytic leukemia (4), cellular differentiation, rather than cell proliferation, plays a more critical role in regulating endogenous cellular cholesterol biosynthesis.

Human monocyte–macrophage cholesterol and lipoprotein metabolism has been the subject of recent intensive investigation because of the possible role of this cell, in the guise of the subintimal foam cell, in the pathogenesis of atherosclerosis (18, 20, 22–25). Although we have emphasized other aspects of the PMA-differentiated HL-60 monocyte–macrophage in our present studies, it also (*i*) may prove to be a useful model for the study of the monocyte–macrophage low density lipoprotein and scavenger pathway lipoprotein receptors and (*ii*) may contribute to our understanding of the respective roles of cholesterol of endogenous and exogenous origin in the accumulation of cholesterol and cholesteryl esters by human monocyte–macrophage.

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