Relationship between membrane sterol composition and responsiveness to 12-*O*-tetradecanoylphorbol 13-acetate in HL-60 human promyelocytic leukaemia cell lines

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We have examined the sterol composition and metabolism of promyelocytic leukaemia cell lines (HL-60) after treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). A variant cell line (Blast II cells) which is resistant to TPA was used as control. Analysis of the sterols of TPA-sensitive cells radiolabelled with [³H]leucine, [¹⁴C]acetate or [¹⁴C]pyruvate showed a high incorporation into cholesterol and a low incorporation in lanosterol + dihydrolanosterol. The inverse relationship was observed in TPA-resistant cells. Experiments with other cellular variants representing TPA-sensitive and TPA-resistant classes gave similar results. Analysis of the cellular sterol composition by gas chromatography confirmed that TPA-resistant cells are particularly rich in lanosterol/dihydrolanosterol. TPA treatment enhanced the incorporation of [¹⁴C]pyruvate into the sterol fraction of both cell types. This was accompanied by an alteration of incorporation into several lipids, particularly phospholipids. Pulse-chase studies with [¹⁴C]acetate revealed that TPA induced the release of radioactive lipids into the medium from HL-60 and Blast II cells. However this treatment released phospholipids from the TPA-sensitive cells and sterols and fatty acids from the TPA-resistant cells. We conclude that the sterol composition can regulate specific biochemical processes in the membrane and can be considered as a factor that plays a role in the responsiveness of HL-60 cells to TPA.

INTRODUCTION

The study of cell differentiation is of the upmost importance not only for its fundamental aspects, but also for the understanding of cancer. In certain lymphoblastic cell lines established from leukaemic patients, the cells cannot differentiate, but can be induced to do so by incubation with the tumour-promoting phorbol ester TPA.

Thus, studies of the mode of action of TPA could lead to the better design of anti-leukaemia drugs. In the present study, we have used the human promyelocytic cell line, HL-60, as a model (Collins *et al.*, 1978). After treatment with TPA, the cells express many of the characteristics of macrophages (Huberman & Callahan, 1979). To define the parameters specific to differentiation, a number of variant cell lines resistant to TPA were developed. Previous studies showed that variant HL-60 cell lines resistant to TPA differed from the sensitive cells in the metabolism of their proteins (Anderson *et al.*, 1985) and lipids (Hoffman & Huberman, 1982). Fisher *et al.* (1984) have shown that TPA-resistant variants have decreased lipid fluidity as compared with TPA-sensitive cells. They suggested a possible relationship between the physical state of the membrane lipids of myeloid leukaemic cells and their capacity to respond to induction of differentiation by phorbol esters. On the basis of this observation, we have focused interest on the role of membrane sterols in cell differentiation. It is becoming increasingly evident that sterols may be involved in specific regulatory processes (Demel & De Kruffy, 1976;

Yeagle, 1985). Cholesterol, the most abundant sterol in the cell, together with phospholipids, play a major role in membrane microviscosity. In the present paper, we have studied the sterol composition and metabolism in TPA-sensitive and -resistant cell lines. The effect of TPA on the other cellular lipids was also evaluated in order to correlate any changes with the sterol composition.

EXPERIMENTAL

Chemicals

Lanosterol was purchased from Serva. Commercial 'lanosterol' contained both lanosterol and 24-dihydrolanosterol in the ratio 3:2, respectively, as previously shown (Pinto & Nes, 1983) and confirmed by us by mass spectrometry.

L-[³H-4(n)]Leucine (1 Ci/mmol) was purchased from CEA (Gif-sur-Yvette, France). [¹⁻¹⁴C]Acetic acid, sodium salt (50–60 mCi/mmol) and [³⁻¹⁴C]pyruvic acid were from Amersham International.

Cell cultures

The human promyelocytic leukaemia cell line HL-60 was originally provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD, U.S.A.) and the HL-60 Blast II cell line was obtained from Dr. P. Major (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, U.S.A.) (Major *et al.*, 1981). Clones HL-60/TPAres and R-99/TPAsens were isolated by limiting dilution in our laboratory from HL-60 cells. HL-

60/TPAres cells were irreversibly resistant to the induction of differentiation by TPA and could be cultured in the presence of high doses of TPA. R-99/TPAsens cells have a similar response to TPA as the parental HL-60 cells and stop growing in the presence of low doses of TPA.

Cells were cultured at 37 °C in RPMI-1640 medium (Flow Laboratories) with 10% (v/v) heat-inactivated fetal calf serum supplemented with penicillin (1 unit/ml) and streptomycin (100 µg/ml) and glutamine (2 mM) in a humidified atmosphere of 5% CO₂ in air.

The cells were subcultured every 3–5 days to maintain a density of $(1-10) \times 10^5$ cells/ml in 25 cm² tissue culture flasks (Corning Plastic, Corning, NY, U.S.A.).

Separation of lipids

Cells were washed twice with saline. If adherent, the cells were removed by scraping the plastic surface with a rubber policeman. The lipids were extracted with chloroform/methanol (2:1, v/v) as previously described (Anderton *et al.*, 1983).

Neutral lipids were separated by two-dimensional t.l.c. according to a procedure developed in our laboratory. The lipids were spotted on 10 cm × 5 cm precoated silica gel 60 plates (Merck) along with unlabelled neutral lipids as carriers. The plate was run in the shorter dimension in the solvent system chloroform/methanol (50:3.6, v/v) until the solvent front ascended to 0.5 cm from the top of the plate. The plate was then dried and run in the second dimension with the solvent system heptane/di-isopropyl ether/glacial acetic acid (15:10:1, by vol.) up to the bottom edge of the plate. The lipids were visualized by spraying with vanillin/H₂SO₄. The plates were briefly heated at 120 °C to reveal the spots.

Spots were scraped off and counted in scintillation vials with 3 ml of ethanol/water (1:1, v/v) and 9 ml of Picofluor (Packard). The counting efficiency was determined by the external standard channel ratio and the results were expressed in d.p.m. To determine the recovery of lipids after extraction (93–97%), a known amount of a mixture of radioactive lipids was added to non-radioactive cells which were extracted and analysed in the same conditions as the experimental samples.

To confirm the radioactive purity of sterol spots separated on the two-dimensional chromatography, total lipids from labelled cells were saponified for 30 min at 80 °C with 1 M-KOH in methanol/benzene (4:1, v/v) (Sexton *et al.*, 1983) and nonsaponifiable lipids were extracted with hexane after dilution of the saponified mixture with water. The hexane extract was separated by two-dimensional t.l.c. and the distribution of the radioactivity was determined as described above. In all the cases examined, a similar distribution of the radioactivity between the cholesterol and the lanosterol fraction was observed by the separation of total lipids and non-saponifiable lipids.

G.l.c. of the trimethylsilyl derivatives of sterols

After separation by two-dimensional t.l.c., the neutral lipids were visualized under u.v. light by spraying the plate with primuline reagent (1% in acetone/water, 1:1 v/v). The areas corresponding to cholesterol or lanosterol/dihydrolanosterol were scraped into glass tubes containing perhydosqualene added as internal standard and were extracted twice with 2 ml of benzene/methanol (2:1). The solvent was then evaporated under

nitrogen and the residue was treated with 20 µl of acetonitrile and 20 µl of *NO*-bis(trimethylsilyl)trifluoroacetamide ('BSTFA') with 1% trimethylchlorosilane. After 1 h in a sealed tube at room temperature, 2–4 µl were applied to a fused silic column (CP Sil 5, 50 m × 0.32 mm) operating at 320 °C and equipped with a flame ionization detector.

RESULTS

Distribution of different radioactive precursors among the neutral lipids of HL-60 and Blast II cells

HL-60 (TPA-sensitive) and Blast II (TPA-resistant) cells were incubated with [³H]leucine, [¹⁴C]pyruvate or [¹⁴C]acetate for 45, 96 and 27 h respectively (Table 1). In the TPA-resistant Blast II cells, the percentage of radiolabel incorporated into free cholesterol was 2–4-fold lower than in TPA-sensitive HL-60 cells. Inversely, the radioactivity incorporated into lanosterol + dihydrolanosterol of TPA-resistant cells was 5–6-fold higher than in the sensitive cells (Table 1). In TPA-resistant cells, the percentage of the radioactivity incorporated into triacylglycerols was lower than in sensitive cells.

Chemical characterization of the cell sterols

After extraction, purification and derivatization as described under 'Experimental' we have confirmed by combined gas chromatography–mass spectrometry that the two peaks separated by gas chromatography from the lanosterol spot correspond respectively to dihydrolanosterol and lanosterol (results not shown). The presence of lanosterol in wild-type HL-60 cells has been previously reported (Cooper *et al.*, 1981). The amount of cholesterol (139 ± 9 nmol/mg of protein) was the same in all the cell lines examined, but the amounts of lanosterol and dihydrolanosterol were much higher in the resistant cells (respectively 17 ± 2 and 20 ± 2 nmol/mg of protein in HL-60/TPAres and 6 ± 1 and 5 ± 1 nmol/mg of protein in Blast II cell lines). In the sensitive cells the amount (< 0.5 nmol/mg of protein) was too low to be measured precisely.

Effect of TPA on the metabolism of cell lipids labelled with [¹⁴C]pyruvate or [³H]leucine

To determine if the differences observed for the distribution of radioactivity among the sterol fractions depended on the cell sterol composition, and was not restricted to the two cell lines examined, incorporation of radioactive pyruvate was studied in the two other cell lines, R-99/TPAsens and HL-60/TPAres. The results of the incorporation of [¹⁴C]pyruvate in absence or in presence of TPA are summarized in Table 2. These results show that the repartition of radioactivity between the sterols could be used as a basis to separate the sensitive and resistant variants. TPA stimulated the incorporation of [¹⁴C]pyruvate into sterols in all cell types. The effect of TPA on the different phospholipid species was rather minor and varied with the different cell lines (results not shown). The incorporation into the total neutral lipid fraction increased in both resistant and sensitive cells following TPA treatment.

To determine the effect of TPA on the metabolism of pre-existing radiolabelled sterols, cells were radiolabelled with [³H]leucine for 42 h prior to exposure to TPA. The

Table 1. Distribution of different radioactive precursors into the neutral lipids of HL-60 and Blast II cells

Exponentially growing cells were labelled with [^3H]leucine (10 $\mu\text{Ci/ml}$ of RPMI) for 45 h, with [^{14}C]pyruvate (1.5 $\mu\text{Ci/ml}$ of RPMI) for 96 h and with [^{14}C]acetate (2.5 $\mu\text{Ci/ml}$ of RPMI) for 27 h. The values are the means of two determinations from a single experiment, but are typical of several. For all the values, the s.d. is smaller than 10 % of the mean. Results are expressed as % of neutral lipid radioactivity. Values statistically different between sensitive and TPA-resistant cells (paired t test): $^aP < 0.001$, $^bP < 0.05$.

Precursor ... Cell ...	Radioactivity (% of total neutral lipid)					
	[^3H]Leucine		[^{14}C]Pyruvate		[^{14}C]Acetate	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Monoacylglycerol	6.0	8.9	1.5	2.2	2.2	3.7
1,2-Diacylglycerol	4.2	4.1	1.0	4.0 ^b	6.6	7.2
1,3-Diacylglycerol	2.5	4.4 ^b	0.9	5.4 ^a	3.5	8.9 ^a
Free cholesterol	19.3	11.5 ^a	13.7	5.2 ^a	22.4	5.3 ^a
Lanosterol + dihydrolanosterol	4.3	23.6 ^a	2.2	13.4 ^a	3.6	16.9 ^a
Fatty acids	1.5	4.0 ^a	0.5	0.5	1.4	1.0
Ubiquinone	3.0	5.8 ^b	1.9	2.2	1.7	1.9
Triacylglycerol	49.1	20.2 ^a	65.0	31.7 ^a	47.1	19.9 ^a
Fatty acid methyl ester	6.7	11.2 ^b	11.8	25.0 ^b	9.9	22.9 ^b
Cholesterol ester	3.3	6.5 ^b	2.6	10.4 ^a	1.6	12.5 ^a
Cholesterol/lanosterol	4.49	0.49 ^a	6.23	0.39 ^a	6.22	0.31 ^a

Table 2. Effect of TPA on the incorporation of [^{14}C]pyruvate into the lipids of TPA-sensitive and TPA-resistant HL-60 cells

The cells were incubated in the absence or presence of TPA (10 ng/ml) for 10.5 h. Radioactive pyruvate was added to the cells 3.5 h before the end of the incubation (1.5 $\mu\text{Ci/ml}$ of RPMI). Values are the means of duplicate determinations from one representative experiment. For all the data the s.d. is less than 8 % of the mean. Results are expressed in d.p.m. % of total lipid radioactivity. Values statistically different between control and TPA-treated cells (paired t test): $^aP < 0.01$, $^bP < 0.05$.

Cell line ...	Radioactivity (% of total lipid)							
	HL-60		R-99/TPAsens		Blast II		HL-60/TPAres	
	Control	+ TPA ^a	Control	+ TPA	Control	+ TPA	Control	+ TPA
Monoacylglycerol	0.7	0.9	1.8	1.8	1.3	1.2	1.0	1.4
1,2-Diacylglycerol	2.4	2.1	3.5	2.2 ^b	3.4	3.0	1.7	1.5
1,3-Diacylglycerol	1.2	1.7	2.8	2.8	5.0	4.4	1.9	2.3
Free cholesterol	5.0	11.4 ^a	11.0	15.0 ^b	0.8	1.2 ^a	0.4	0.8 ^a
Lanosterol + dihydrolanosterol	2.1	6.0 ^a	2.2	3.2 ^a	6.2	10.0 ^a	15.8	21.6 ^a
Fatty acids	1.1	0.8	0.8	0.6	0.7	1.3 ^a	0.9	0.8
Ubiquinone	0.4	0.8 ^b	0.3	0.3	0.4	0.6 ^b	0.6	0.6
Triacylglycerol	8.1	7.5	7.5	7.3	6.1	6.0	4.1	3.3
Fatty acid methyl ester	1.3	1.3	1.1	2.2 ^a	4.0	3.9	2.0	2.8
Cholesterol ester	0.4	0.6 ^b	1.2	2.2 ^a	1.3	1.4	0.4	0.4
Phospholipid	77.3	67.1 ^a	67.9	62.3 ^b	70.8	67.1	71.8	64.7 ^a
D.p.m. in lipid/ μg of protein	22.1	24.7	19.8	21.6	21.4	19.6	19.9	16.3

radiolabelled sterols were then examined after a chase period of 3, 6 and 19 h (with or without TPA treatment).

We observed that the ratio (d.p.m. in free cholesterol)/(d.p.m. in lanosterol + dihydrolanosterol) increased regularly during the incubation time and was unaffected by TPA in both cell lines (Fig. 1).

Effect of TPA on the exchange of cell lipids labelled with [^{14}C]acetate

The exchange of sterols and other lipids between the cells and the medium during differentiation was evaluated

by labelling the cells with [^{14}C]acetate for 27 h. They were then reincubated for 14 h in a fresh medium without radioactive label in the presence or absence of TPA and the lipids extracted from both cells and medium were analysed. The results are shown in Table 3. The total incorporation of radioactivity into the lipids of Blast II cells was almost 7-fold less than that in HL-60 cells (0.15×10^6 d.p.m./ μg of protein versus 1.03×10^6 d.p.m./ μg of protein). TPA treatment of HL-60 cells led to a decrease of ^{14}C label from the neutral lipid fraction: from an initial value of 130549 ± 9510 d.p.m./ μg of cell

Fig. 1. Effect of TPA on the distribution of the radioactivity between free cholesterol and lanosterol/dihydrolanosterol in HL-60 and Blast II cells labelled with [^3H]leucine

Exponentially growing cells were labelled with [^3H]leucine (5 $\mu\text{Ci/ml}$ of RPMI) for 42 h and then maintained in the same medium for an additional 3, 6 or 19 h in the absence or presence of TPA (10 ng/ml). Each bar ($\pm$ S.D.) represents the mean of two determinations from one representative experiment.

Table 3. Exchange of sterols and other lipids between the cells and the medium during differentiation

Exponentially growing cells were incubated for 27 h in the presence of 50 μCi of [^{14}C]acetate in a 75 cm^2 culture flask containing 20 ml of complete medium. The cells were harvested by centrifugation, washed twice with fresh medium and reincubated without radioactive label for 14 h in the absence or presence of TPA (8 ng/ml). The lipids were extracted from the cells and from the medium and analysed. The data are the average of two determinations from one experiment, but are typical of several. The S.D. is less than 10% for all the values. The column headed 'Zero time' represents the incorporation of [^{14}C]acetate into individual lipids after 27 h of incubation. Values are expressed in d.p.m./ μg of cell protein. Values statistically significant between control and TPA-treated cells (paired t test): ^a $P < 0.01$; ^b $P < 0.05$.

	Radioactivity (d.p.m./ μg of protein)				
	Cell lipids			Medium lipids	
	Zero time	- TPA	+ TPA	- TPA	+ TPA
HL-60					
Free cholesterol	29 295	22 675	20 762	16 872	16 299
Lanosterol + dihydrolanosterol	4 716	2 449	2 089	1 409	1 564
Fatty acids	1 763	991	931	15 198	17 481
Triacylglycerol	61 535	53 843	34 881 ^a	1 099	2 063 ^a
Cholesterol ester	2 075	2 157	2 354	252	272
Phospholipid	899 977	847 476	754 739	18 631	41 325 ^a
Blast II					
Free cholesterol	1 441	1 402	1 434	615	963 ^a
Lanosterol + dihydrolanosterol	4 597	3 603	3 923	1 465	2 416 ^a
Fatty acids	274	299	251	3 270	7 103 ^a
Triacylglycerol	5 416	6 893	5 084 ^b	320	208 ^b
Cholesterol ester	3 400	2 693	2 447	185	90 ^a
Phospholipid	126 206	128 047	127 982	5 604	5 217

protein, the total radioactivity of the neutral lipid fraction decreased to $111\,797 \pm 8\,975$ in the untreated culture and to $85\,174 \pm 8\,111$ in the TPA-treated. On the contrary, in the Blast II cells, TPA did not affect significantly the loss of radioactivity from the neutral lipid fraction (results not shown).

The radioactivity present in cholesterol after a chase in unlabelled medium showed that 30% of the cholesterol was released from HL-60 cells, but only 3% from Blast cells. Treatment of either of the cell lines did not affect this turnover. During the chase period there was a decrease of lanosterol + dihydrolanosterol in both cell lines (absence or presence of TPA). However, unlike the

HL-60 cells, TPA increased the release of radioactive sterols from Blast II cells into the medium. In HL-60 cells, the total amount of radiolabelled phospholipids released into the medium was more than double that of untreated cells. TPA treatment of the resistant cells had little effect on the release of phospholipids.

Several other changes due to TPA were observed among the neutral lipids on both cell lines. Following treatment with TPA, a decrease of ^{14}C label from cellular triacylglycerol was observed in both cell lines. A high rate of fatty acid exchange occurs in both cell lines, which is increased by TPA treatment significantly only in the resistant cells.

DISCUSSION

HL-60 cells resistant to differentiation by TPA were shown to differ from the TPA-sensitive cells in having a high content of lanosterol + dihydrolanosterol. These are metabolic precursors of cholesterol. The significance of the high levels of lanosterol + dihydrolanosterol in the resistant cells is not known. However, observations with other systems may suggest possible functions.

In artificial membranes, lanosterol has been shown not to pack as well as cholesterol and is not as effective in reducing membrane permeability as is cholesterol (Yeagle *et al.*, 1977).

In HL-60 cells, the observations made by Fisher *et al.* (1984) that TPA-resistant cells have a decreased membrane fluidity suggests that the membrane rich in lanosterol + dihydrolanosterol will have a decreased membrane fluidity.

However, the sterols play a more specific role than just modulating the physical state of the membrane. Cholesterol has been shown to be associated with the regulation of unsaturated fatty acid biosynthesis in LM fibroblasts (Freter *et al.*, 1979). In cultured myogenic cells, sterol synthesis was coupled to phosphatidylcholine synthesis (Cornell & Goldfine, 1983). In our study lipid-exchange reactions, as measured with [¹⁴C]acetate, differed in HL-60 and Blast II cells. This may suggest a possible involvement of sterols in lipid exchange activity between cells and medium. Recently, we have demonstrated that the pattern of proteins acylated with [³H]-myristate in HL-60 cells differed from that of Blast II cells (Malvoisin *et al.*, 1987). Such a disparity may be explained by the presence of lanosterol + dihydrolanosterol in the membrane. TPA, like cholesterol, has a strong amphipathic character; it may therefore modify differently the membrane, depending upon the cholesterol/lanosterol composition. This in turn may activate differently the enzymes embedded within the membrane and/or modify the mobility of the receptors and the transport of molecules and ions through the membrane.

The lanosterol + dihydrolanosterol may act alone or synergistically with the cholesterol to modify the regulation of biochemical processes involved in the triggering of differentiation.

The presence of a high percentage of lanosterol + dihydrolanosterol in the resistant cells is probably due to a difference in the regulation of cholesterol biosynthesis. Cholesterol might control the demethylation steps from lanosterol to cholesterol by a feedback mechanism.

Our studies complement the results obtained by several authors who found a correlation between sterol and carcinogenesis. Thus, carcinogens provoke a loss of control of cholesterol synthesis in the liver (Siperstein, 1970). Coleman & Lavietes (1981) consider that an uncontrolled cholesterol synthesis can account for many of the metabolic abnormalities observed in tumour tissues. Similarly, cholesterol metabolism of normal fibroblasts differs from that of transformed cells (Ostlund & Yang, 1985).

A relationship has been demonstrated between membrane fluidity and the capacity of cells to differentiate (Inbar & Shinitzky, 1974). The microviscosity of the

plasma membrane of normal lymphocytes is almost twice that of malignant transformed lymphoma cells from mice (Shinitzky & Inbar, 1974).

In addition, Filipovic & Buddecke (1987) have observed a suppression of cholesterol synthesis and an accumulation of desmosterol in hepatoma cells and human fibroblasts treated with calmodulin antagonists. Different calmodulin antagonists have been reported to induce myeloid differentiation of HL-60 cells (Veigl *et al.*, 1986). It is tempting to speculate that these molecules act by interfering with sterol metabolism.

In conclusion, our results reveal that the sterol composition is one of the factors involved in the resistance to the induction of differentiation by TPA. We suggest that the difference in sterol composition could be the basis of a new strategy to reach selectively transformed cells and understanding the mechanism of differentiation.

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