

## ALTERED LIPID MICROVISCOSITY IN LYMPHOBLASTOID CELLS TREATED WITH 12-*O*-TETRADECANOYL PHORBOL 13-ACETATE, A TUMOR PROMOTER

M. CASTAGNA, C. ROCHETTE-EGLY, C. ROSENFELD<sup>+</sup> and Z. MISHAL<sup>+</sup>

*Institut de Recherche Scientifique sur le Cancer 7, rue Guy Mocquet 94800 Villejuif and <sup>+</sup>Institut de Cancérologie et d'Immunogénétique 16 avenue Paul Vaillant Couturier 94800 Villejuif, France*

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### 1. Introduction

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) which was isolated from croton oil [1] together with several other diesters of the tetracyclic diterpene phorbol, has emerged as the most potent tumor promoter. The tumor-promoting activity of TPA is related to its ability to enhance the expression of the tumor phenotype in mouse skin [2,3], in virally-transformed cells [4,5] as well as in various types of cultured cells pre-treated with chemical or physical carcinogenic agents [6–9].

The cell membrane is generally considered to be the target site for the tumor promoter which is known to cause alterations in several membrane-associated processes. TPA alters ion fluxes [10], decreases (Na<sup>+</sup>, K<sup>+</sup>) and (Mg<sup>2+</sup>, Ca<sup>2+</sup>) ATPase activities [11], induces specific changes of adenylate and guanylate cyclase activities [12], modifies glucose uptake [13] and cell permeability to trypan blue [14]. In addition, TPA has been shown to alter the protein composition of the outer side of the membrane [15]; TPA has been shown to induce surface structural changes in the surface of lymphoblastoid cells and to cause adhesion of cells to the substratum [16].

The fluidity of the lipid matrix regulates the dynamic state of the cell membrane. It influences lateral diffusion and rotation of membrane proteins [17], controls their degree of exposure [18] and modulates the activities of membrane-bound enzymes [19,20]. These observations suggested that TPA could affect the fluidity of the lipid core of the cell membranes. The biological lipid complex can be

quantitatively monitored by fluorescence polarization analysis with the aid of the fluorescent hydrocarbon probe 1,6-diphenyl 1,3,5-hexatriene (DPH) [21]. From the recorded degree of fluorescence polarization, the degree of microviscosity of the analyzed sample can be estimated [22].

To study the effect of TPA on the degree of microviscosity, the response of several lymphoblastoid cell lines to treatment with the tumor promoter was investigated. For purposes of comparison, the effect of 4-*O*-methyl-phorbol 12, 13-didecanoate (MePDD), a TPA derivative, which is inactive as tumor promoter, was also examined. We report here a decrease in the degree of lipid microviscosity in all cell lines exhibiting altered adhesion properties in response to TPA treatment. These alterations were accompanied by a marked stimulation of cholesterol and phospholipid metabolism.

### 2. Materials and methods

Murine (L1210 line) and human lymphoblastoid cell lines from normal (LHN 13) and malignant origin (Namalwa, Raji, Reh 6) were used. Cells were treated with phorbol esters or acetone (phorbol ester, solvent), 12 h after plating, and collected at the following times: the cells in suspension were withdrawn with the culture medium and the cells which had adhered to the plastic dish were detached with trypsin (3 min incubation at 37°C in PBS containing 0.06% trypsin plus 0.1% EDTA) before being centrifuged at 400 × *g* for 10 min and resuspended in phosphate-buffered saline (PBS) containing 30 µg/ml of soy bean

trypsin inhibitor. Pooled suspended plus attached cells were pelleted at  $400 \times g$  for 10 min and resuspended in PBS. The cells were washed twice before undergoing fluorescence measurements and biochemical determinations. The two cell populations were kept apart and washed separately in order to either compare the lipid microviscosity of suspended cells to that of attached cells or to carry out the attachment assay [16]. Care was taken that trypsin treatment did not affect the fluorescence measurements.

Lymphocytes were isolated from blood obtained from healthy donors by Ficoll-hypaque gradient centrifugation [23]. T lymphocytes were isolated from thymus obtained from myasthenic patients by gently pressing and filtration through multiple layers of cheese-cloth: the cell suspension contained  $>95\%$  of lymphocytes as shown by microscope examination. Lymphoblasts were isolated from the peripheral blood of leukemic patients in clinical relapse, as in [23]. T and B lymphocytes and leukaemic cells were seeded at  $3 \times 10^6$  cells/ml in RPMI medium 1640 (Gibco Paisley, Scotland) containing 20% fetal calf serum before being treated by phorbol esters under conditions similar to those used for cell lines.

Liposomes were prepared from the whole cells as in [24].

The fluorescence measurements were conducted as in [25]. The cells were labelled with DPH by incubation for 30 min at  $25^\circ\text{C}$  with 3 ml  $2 \mu\text{M}$  DPH dispersed in PBS. The fluorescent labelled samples were subjected to fluorescence polarization analysis with an Elscint microviscosimeter. From the recorded  $p$  values, the degree of microviscosity was calculated as in [26].

Lipid was extracted from the whole cells pre-labelled with both DL-[2- $^3\text{H}$ ]mevalonic acid lactone obtained from the Radiochemical Centre (Amersham) and [ $^{32}\text{P}$ ]phosphoric acid from CEA (Saclay). Extracts were then assayed for total cholesterol [27], total phospholipids [28] and counted for  $^3\text{H}$  and  $^{32}\text{P}$  radioactivities using an intertechnique spectrometer.

Phorbol esters were purchased from Consolidated Midland Co.

### 3. Results

TPA decreased the lipid microviscosity of various

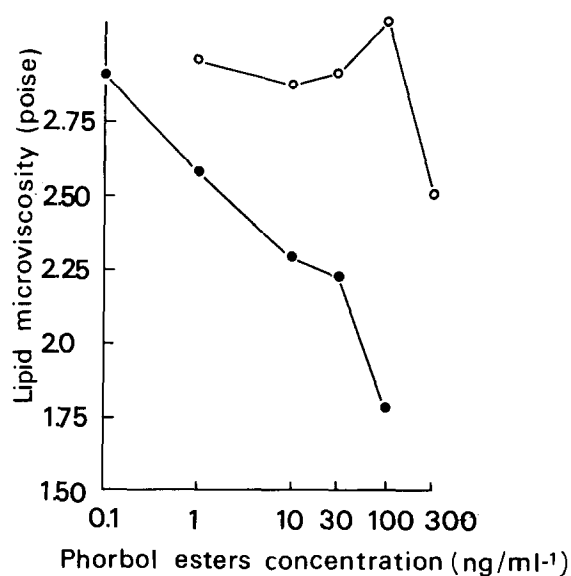


Fig.1. Namalwa cells  $6 \times 10^5/\text{ml}$  were re-fed with RPMI medium 1640 supplemented with 20% fetal calf serum. Cells were treated 12 h after seeding with various amounts of TPA (●—●) or MePDD (○—○) and underwent fluorescence measurements 2 h later. Control cultures were treated with the same volume of acetone (phorbol ester solvent) as that added to cells treated with phorbol esters (usually  $1 \mu\text{l}/\text{ml}$  medium). Acetone did not impair either cell proliferation or fluorescence measurements. The values were means of two individual experiments.

Table 1  
Fluorescence polarization  $p$  and lipid microviscosity  $\bar{\eta}$  at  $25^\circ\text{C}$  of liposomes prepared from phorbol treated LHN 13 cells

Addition Time	Acetone	TPA	MePDD
$p$	0.228	0.202	0.222
$\bar{\eta}$	2.11	1.62	1.98

Accuracy of fluorescence polarization measurement  $p$  is  $\pm 0.005$

LHN 13 line was subcultured by refeeding with RPMI medium 1640 supplemented with 10% calf serum at  $5 \times 10^5$  cells/ml. Cells were treated with phorbol esters at  $100 \text{ ng}/\text{ml}$  and liposomes prepared as in section 2 24 h later. The procedure to measure fluorescence polarization in liposomes was as in the text for the whole cells at various times after the start of the treatment. Figures represent mean of two experiments  $\bar{\eta}$  is expressed in poises

lymphoblastoid cell lines. The effect observed in Namalwa cells lines is shown in fig.1. The effect of TPA on microviscosity occurred rapidly, was dose-dependent and was detectable at a concentration of 1 ng/ml. The cells appeared to be about 100-fold less sensitive to MePDD, the inactive derivative of TPA. The TPA-mediated alterations of microviscosity were also observed when liposomes prepared from the whole cells were subjected to fluorescence polarization analysis and the effect was observed over the 3 day period of the experiment, as illustrated in table 1.

A number of lymphoblastoid cell lines from human and murine origin as well as thymus and peripheral blood lymphocytes were examined for the effects of phorbol esters on lipid microviscosity determined by the measurements of fluorescence polarization. The results reported in table 2, showed that B lymphocytes and 3 out of 5 cell lines exhibited a decreased lipid microviscosity as demonstrated by an increase in fluorescence polarization. The results, reported in table 2, showed that B lymphocytes and 3 out of 5 cell lines exhibited a decreased lipid microviscosity after exposure to TPA, whereas MePDD was not significantly effective upon any of them.

It should be pointed out that those cells which responded to TPA treatment by a decrease of lipid microviscosity also exhibited altered adhesive properties and yielded a positive response to the attachment assay [16]. These data suggested that the lipid microviscosity of suspended and attached cell populations might be affected differently. In order to investigate this possibility three responsive cell lines were tested: LHN13, Namalwa and Reh 6. The results revealed that a decrease of lipid microviscosity was observed in the attached cells whereas that of cells growing in suspension was unaffected or slightly increased (table 3).

The unresponsive lymphoblastoid cell lines such as Raji or L1210 lines neither attached to the culture dish, nor exhibited a decrease of membrane microviscosity regardless of the TPA concentration and the time of the fluorescence polarization measurements. Lipid microviscosity was generally unaffected or slowly increased (data not shown). Since a slight increase of microviscosity occurred in aged cultures it was suggested that this increase of microviscosity may be due to the inhibition of growth which was generally observed in lymphoblastoid cell in response to TPA [16].

Table 2  
Effect of phorbol esters on lipid microviscosity in lymphocytes and lymphoblastoid cell lines

Cells	Source	Lipid microviscosity (poise)			Response to attachment assay
		Controls	TPA-treated cells	MePDD-treated cells	
Lymphocytes peripheral blood	Human normal lymphocytes	2.76	1.62	—	+
Leukaemic lymphocytes	Leukaemic patients in relapse	2.88	2.31	—	+
Thymic lymphocytes	Thymus from myasthenic patients	2.17	2.06	2.11	—
LHN 13 line	Human normal lymphocytes	2.48	2.0	2.38	+
L1210 line	Mouse lymphoma	1.74	1.82	—	—
Namalwa line	Burkitt's lymphoma	2.61	2.17	2.50	+
Raji line	Burkitt's lymphoma	1.86	1.90	1.90	—
Reh 6 line	Human acute lymphoblastic leukemia	2.06	1.72	2.13	+

Lymphocytes were obtained as in section 2. The culture conditions for LHN 13 and Namalwa lines were as in table 1 and fig.1. The three other cell lines were cultivated in RPMI medium 1640 supplemented with either 20% horse serum (L1210), 20% fetal calf serum (Raji) or 10% calf serum (Reh 6). Cells were treated with 30 ng/ml phorbol esters and analyzed for fluorescence polarization 6 h later. Figures are means of 2–6 reproducible experiments

Table 3  
Differential effects of TPA on lipid microviscosity  $\bar{\eta}$  of suspended and substrate-attached lymphoblastoid cells

Treatment \ Cell lines	LHN 13		Namalwa		Reh 6	
	Suspension	Attached	Suspension	Attached	Suspension	Attached
Acetone	2.56	2.56	2.52	2.52	2.10	2.10
TPA	2.63	1.83	2.61	1.98	2.14	1.64

$\bar{\eta}$  was expressed in poises

The three cell lines were treated with TPA at 100 ng/ml TPA. Then attached and suspension type cells were separately examined for fluorescence polarization 24 h later. Figures are means of 2 or 3 reproducible experiments

Some experiments have been carried out in order to determine whether the observed effect of TPA on membrane microviscosity was associated with alterations of the main lipid components which are known to influence lipid microviscosity [29]. The metabolism and cellular content of cholesterol and phospholipid were examined within 24 h after the addition of phorbol esters. Both phospholipid and cholesterol synthesis were markedly stimulated from 6 h after the start of treatment with the tumor promoter. The inactive TPA derivative caused a much lesser effect on phospholipid and cholesterol synthesis. The cholesterol to phospholipid molar ratio was slightly lower than that of controls (table 4).

#### 4. Discussion

A small but significant effect of TPA on the native

fluorescence of membrane protein has been reported [30] suggesting that the tumour promoter may affect structural and dynamic properties of the cell membranes. The present experiments show a decrease in the degree of lipid microviscosity of human lymphocytes and lymphoblastoid cells in response to TPA and provide further evidence for such alterations.

The effect of TPA on the degree of fluidity of the cell membrane occurred rapidly and was associated with surface structural changes and altered adhesion properties [16]. The decreased microviscosity was observed in several cell lines but some of them appeared to be unresponsive in regard to lipid microviscosity as well as adhesion effect.

The decreased microviscosity of the lipid membrane was found to be associated with a marked stimulation of both cholesterol and phospholipid metabolism. Such a TPA-mediated activation of phospholipid synthesis has been described [31] in

Table 4  
Phorbol ester-induced alterations in cholesterol and phospholipid metabolism

Treatment \ Time after treatment	Cholesterol : phospholipid (mol/mol)			Spec. act. cholesterol (dpm/ $\mu$ g)			Spec. act. phospholipid (dpm/ $\mu$ g phospholipid-phosphate)		
	Acetone	TPA	MePDD	Acetone	TPA	MePDD	Acetone	TPA	MePDD
6 h	0.52	0.49	0.51	27.7 $\pm$ 5.1	58.1 $\pm$ 12.8	15.7 $\pm$ 6.4	886 $\pm$ 40	1451 $\pm$ 52	695 $\pm$ 25
24 h	0.57	0.48	—	19.1 $\pm$ 4.6	123 $\pm$ 24	—	550 $\pm$ 19	1977 $\pm$ 61	526 $\pm$ 12

LHN 13 cells, which were phorbol ester-treated 12 h after plating and received, simultaneously, 2.5  $\mu$ Ci/ml [ $^{32}$ P]phosphoric acid (2 Ci/mg) and 3  $\mu$ Ci/ml [ $^3$ H]mevalonic acid lactone (168 Ci/mol), 4 h later. Over a 2 h period the cells were incubated then harvested before undergoing biochemical determinations. Lipids were extracted from the pooled suspended plus attached cells. For experimental details see text

mouse skin and the effect suggested to result in the initiation of cell proliferation observed in such a tissue after TPA treatment. The results presented here, which were obtained on growth-inhibited lymphoblastoid cells, favour the possibility that TPA specifically acts upon lipid metabolism. MePDD, a TPA derivative inactive as tumour promoter, was found to be much less effective, both on lipid metabolism and the fluidity of the lipid membrane, suggesting that these TPA-mediated alterations may be relevant to tumor promotion.

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