Membrane Lipid Dynamics in Human Promyelocytic Leukemia Cells Sensitive and Resistant to 12-O-Tetradecanoylphorbol-13-acetate Induction of Differentiation

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ABSTRACT

A series of fluorescent probes was used to analyze membrane lipid dynamics in promyelocytic leukemia cells sensitive (HL-60) or resistant (R-55) to phorbol diester induction of cell differentiation. When examined with the probe 1,6-diphenyl-1,3,5-hexatriene, which can penetrate the plasma membrane and intercalate in the lipids of both leaflets of the plasma membrane, as well as in organelar membranes, R-55 cells were found to have higher fluorescence anisotropy values, indicative of decreased lipid fluidity, as compared to HL-60 cells. In contrast, when HL-60 and R-55 cells were compared using a series of membrane-impermeant fluorophores (stachyose derivatives of anthroyloxyystearate and pyrenebutoxyhydrizide) that incorporate only into the outer hemileaflet of the plasma membrane, no difference was observed in membrane lipid fluidity. Exposure to 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml) for 24 hr decreased the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in both HL-60 and R-55 cells, whereas by 48 hr only the HL-60 cells displayed the reduction. No effect on the fluorescence anisotropy of 1-(4'-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene, which is believed to localize in the plasma membrane, was observed in R-55 cells exposed to 12-O-tetradecanoylphorbol-13-acetate (10 or 100 ng/ml), whereas HL-60 cells treated with 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml) showed a marked reduction in the fluorescence anisotropy. These observations suggest that the ability of HL-60 cells to respond to 12-O-tetradecanoylphorbol-13-acetate may be affected by the physical state of the plasma membrane lipids and that the resistant phenotype is associated with decreased fluidity of either the inner leaflet of the plasma membrane and/or of the cytosolic organelar membranes.

INTRODUCTION

Diterpene phorbol ester tumor promoters, such as TPA, are not carcinogenic by themselves but can enhance tumorigenesis in vivo (3, 22), as well as cell transformation in vitro (20, 28, 29). When applied to cells in culture at concentrations as low as $10^{-8}$ to $10^{-11}$ M, TPA induces a spectrum of changes including mimicry of cell transformation in normal cells and enhancement in expression of the transformed phenotype in cells previously transformed by chemical carcinogens or viruses, inhibition or induction of differentiation depending on the target cell, and alterations in cell membrane structure and function (for review, see Refs. 13, 16, 35, and 38). Although the critical biochemical changes involved in tumor promoter action are not known, numerous studies have suggested that an initial target for TPA is the plasma membrane (35, 38). Indeed, recent studies using the tumor promoter PDBU, which is less lipophylic than TPA, indicates that a wide variety of cell types contain high-affinity and saturable membrane receptors for the phorbol esters (10, 14, 18, 24, 34, 36, 39).

The ability to analyze the molecular basis for the action of tumor promoters has been aided by the isolation of cells resistant to various tumor-promoter-induced cellular alterations (5, 8, 15, 17, 23, 25, 26, 36). TPA-resistant Friend erythroblast leukemia cell lines, which are refractory to inhibition of differentiation, induction of cell adhesion, and release of arachidonic acid and prostaglandins E2 and F2α, all induced by phorbol esters, have been isolated (15, 40). Similarly, nonresponsive HL-60 cells, which are not induced to differentiate and do not display an increase in polyamine levels or phospholipid methylation after TPA treatment (23, 25, 26, 36), have been isolated. In both cell types, resistance to the phorbol esters does not appear to result from an alteration in either the number or binding affinity of the phorbol ester receptors (18, 36, 39). Since receptor mobility, which is profoundly affected by membrane lipid fluidity (2, 27, 31), appears to play an important physiological role in a number of biological systems, we have explored the dynamics of membrane lipids in TPA-responsive and TPA-resistant HL-60 cells using a series of membrane-permeant and membrane-impermeant fluorophores (7, 33).

MATERIALS AND METHODS

Culture Conditions and Assessment of Cell Differentiation. The HL-60 leukemia cell line was provided by Robert C. Gallo (National Cancer Institute, Bethesda, MD). An HL-60 cell variant, R-55, resistant to the inhibitory growth effect of TPA, was isolated by culturing HL-60 cells for 55 subcultures (5 to 8-day intervals) in the presence of increasing concentrations of TPA (25, 36). The size of HL-60 and R-55 cells varied by less than 10%. The mean diameters of HL-60 and R-55 cells were $830 \pm 30$ and $870 \pm 20$ μm, respectively (S.D.). Both cell types were grown in tissue culture plates or flasks at 37° in RPMI 1640 supplemented with 20% fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). For experiments, cells in the logarithmic phase of growth were seeded at $5 \times 10^5$ cells/100-mm plate and treated with TPA 24 hr after cell seeding.

Cell differentiation in HL-60 and R-55 cells was characterized through previously described techniques (25) by an increase in the percentage...
MEMBRANE LIPID FLUIDITY IN HL-60 CELLS

morphologically mature cells and in the amount of lysozyme activity.

Fluorescence Studies. Lipid fluidity was assessed by the steady-state fluorescence polarization of a number of lipid-soluble fluorophores. The compounds used (Chart 1) were DPH; a cationic derivative of DPH, TMA-DPH (Molecular Probes, Inc., Junction City, OR); the stachyose derivative of SPBH (7); and the stachyose adipic dihydrazide derivatives of SADH-2AS and SADH-12AS. The stachyose derivatives do not permeate membranes and therefore report signals from the outer hemileaflet of the plasma membrane (7). The description of the synthesis and purification of the SADH-AS derivative will be published elsewhere. For fluorescence polarization studies, suspensions containing PBS (1 to 2 × 10⁶ cells/ml; 5 mM sodium phosphate, pH 7.4, in 145 mM NaCl, and 5 mM KCl) were incubated with one of the following (final concentrations are in parentheses): DPH (1.5 μM); TMA-DPH (2.0 μM); SPBH (80–100 μM); and SADH-2AS or SADH-12AS (1 to 5 μM). All probes were added as aqueous solutions except for DPH, which was added from a 1.5 mM stock ethanol solution. For DPH studies, the suspensions were incubated with shaking at 37° for 30 to 120 min. The cells were then washed 3 times by centrifugation with 50 volumes of PBS and resuspended for fluorescence estimations. For the TMA-DPH studies, cells were incubated with TMA-DPH for 3 min at 25° and the suspensions used directly (no significant fluorescence was detected in the supernatant solutions on centrifugation of these suspensions). Cells treated with the stachyose derivatives were incubated with shaking for 30 min at 37° and the suspensions washed 3 times with 50 volumes of PBS prior to resuspension. The various incubation times used for the different fluorescent probes are based on their different rates of uptake; water-insoluble molecules like DPH require longer incubation periods to provide a sufficient signal for estimations. The fluorescence anisotropy of the probes was quantified at 25° in an SLM Model 4000 polarization spectrofluorometer, as described previously (7, 19, 33). The results are expressed as the fluorescence anisotropy, r, and as the anisotropy parameter [(r0/r) - 1]⁻¹, where r0 is the maximal limiting anisotropy, taken as 0.390 (DPH and TMA-DPH), 0.290 (SADH-2AS and SADH-12AS), or 0.119 (SPBH). As discussed elsewhere (33), the “fluidity” of the membrane varies inversely with [(r0/r) - 1]⁻¹ or r. In these studies, we did not distinguish between the static component of lipid fluidity (lipid order) as assessed by the maximal hindered anisotropy, r0, and the dynamic component as assessed by the correlation time of the fluorophore.

RESULTS

Effect of TPA on Growth and Differentiation of TPA-sensitive and TPA-resistant HL-60 Cells. After 2 days of exposure, concentrations of TPA as low as 0.1 ng/ml (1.6 × 10⁻¹⁰ M) inhibited the growth of HL-60 cells; at a TPA concentration of 0.3 ng/ml, a 65% reduction in cell number was observed (Chart 2A). In the TPA-resistant variant of HL-60, R-55, a 0.3-ng/ml dose of TPA resulted in only a 15% reduction in cell number, while a 10-ng/ml dose was required for approximately a 50% reduction in cell number. In addition to inhibiting cell growth, TPA caused in the HL-60 cells an increase in the percentage of morphologically mature cells (Chart 2B). After 2 days of treatment with TPA (0.3 ng/ml), >50% of the HL-60 cells were induced to differentiate into more mature cells, while even at a 100-fold higher concentration of TPA, R-55 cells did not exhibit this morphological change (Chart 2B; Table 1). However, when the cultures were analyzed after 6 days, TPA (1 ng/ml) was shown to induce a morphological change in R-55 cells, but the degree of this alteration was less than that observed when HL-60 cells were treated with comparable or lower doses of TPA for only 2 days. Concomitant with the morphological alterations in TPA-treated HL-60 cells was an increase in lysozyme activity (Chart 2C). This increase was observed when HL-60 cells were treated with TPA (0.1 ng/ml) for 2 days and when R-55 cells were treated with TPA (10 ng/ml) for 6 days (Chart 2C; Table 1). Again, the magnitude of the lysozyme response was far greater with HL-
with DPH, the fluorescence anisotropy values of R-55 cells were higher than those of HL-60 cells, implying less motional freedom of the probe and therefore decreased membrane fluidity. In contrast, no difference in fluorescence anisotropy between HL-60 and R-55 cells was observed when they were assayed with the membrane-impermeant stachyose derivatives (Table 2). These findings indicate that the fluidity of lipids in the outer hemileaflet of the plasma membranes of the TPA-resistant and TPA-sensitive cells is similar, whereas the more internal components (inner hemileaflet of the plasma membrane and/or the organelar membranes) are less fluid in the TPA-resistant R-55 cells.

Effect of TPA on Fluorescence Anisotropy in TPA-resistant and TPA-sensitive HL-60 Cells. The effects of 24 and 48 hr of exposure to TPA (0.01 to 100 ng/ml) on DPH [(r0/r) - 1]-1 in HL-60 and R-55 cells is shown in Chart 3, A and B. A 1-ng/ml dose of TPA was shown to increase the fluidity in both cell types after 24 hr of treatment. However, the maximal decrease in the DPH anisotropy parameter was noted after treatment with TPA (10 ng/ml). At a 10-ng/ml dose of TPA, the anisotropy parameter of the R-55 cells was similar to the value of the untreated HL-60 cells or HL-60 cells treated with a TPA dose of either 0.01 or 0.1 ng/ml. When compared after 48 hr of treatment, the values of the R-55 cells had returned almost to the original level, whereas the HL-60 cells treated with TPA at 1, 10, or 100 ng/ml still displayed increased fluidity as compared to the untreated controls (Chart 3). Parallel observations with TMA-DPH are also illustrated in Chart 3, C and D. In the absence of TPA, the [(r0/r) - 1]-1 values of the HL-60 and R-55 cells did not differ significantly, confirming the preceding results with the impermeant stachyose fluorophores (Table 2). Relatively small changes were observed in the fluorescence anisotropy parameter of the R-55 cells after 24 or 48 hr treatment with TPA. In the HL-60 cells, by contrast, TPA at 10 or 100 ng/ml resulted in a major decrease in TMA-DPH [(r0/r) - 1]-1, which was still apparent after 48 hr of treatment. After shorter periods of exposure, i.e., 3 or 4 hr, TPA-induced only minor changes in fluidity of either HL-60 or R-55 cells.


Table 1

Effect of TPA on cell growth, morphology, and lysozyme activity in TPA-resistant HL-60 cells

<table>
<thead>
<tr>
<th>TPA concentration (ng/ml)</th>
<th>0.0</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
<th>3.0</th>
<th>10.0</th>
<th>30.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td>2.7</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>% of morphologically mature cells</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Lysozyme activity (µg equivalents/10^7 cells)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Effect of TPA on cell growth, morphology, and lysozyme activity in TPA-resistant HL-60 cells. TPA-resistant HL-60 cells, R-55, were seeded at 5 x 10^5/100-mm plate, and 24 hr later received the indicated concentrations of TPA. Cell number, morphological cell maturation, and lysozyme activity were determined 2 and 6 days following TPA treatment.

60 than R-55 cells and a lower level of TPA was required to elicit a response in HL-60 cells.

Fluorescence Polarization Studies of TPA-sensitive and TPA-resistant HL-60 Cells. The structures of the fluorescent probes used to investigate the fluidity of lipid domains of HL-60 and R-55 membranes are shown in Chart 1. DPH permeates the cell, and its signal reflects lipid domains of both the plasma and intracellular membranes. The cationic TMA-DPH is believed to localize mainly in the plasma membrane (30). The stachyose derivatives are impermeant (7) because of the bulky oligosaccharide substitutent, and the lipid-soluble fluorophore substituents report from the outer hemileaflet of the intact cell. When tested...
MEMBRANE LIPID FLUIDITY IN HL-60 CELLS

Table 2
Fluorescence anisotropy of various probes in TPA-resistant and TPA-sensitive HL-60 cells

<table>
<thead>
<tr>
<th>Fluorescent probe</th>
<th>TPA-resistant HL-60 (R-55)</th>
<th>TPA-sensitive HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\ell_0/\ell_\infty$</td>
<td>$r$</td>
</tr>
<tr>
<td>DPH (30 min)</td>
<td>1.980 ± 0.009c</td>
<td>0.162</td>
</tr>
<tr>
<td>DPH (2 hr)</td>
<td>1.994 ± 0.002</td>
<td>0.165</td>
</tr>
<tr>
<td>SADH-2AS</td>
<td>1.340 ± 0.015</td>
<td>0.102</td>
</tr>
<tr>
<td>SADH-12AS</td>
<td>1.394 ± 0.013</td>
<td>0.116</td>
</tr>
<tr>
<td>SPBH</td>
<td>1.017 ± 0.009</td>
<td>0.010</td>
</tr>
</tbody>
</table>

* The probes used were DPH, the stachyose derivatives of adipic acid dihydrazide-anthroyloxy stearic acid (SADH-2AS and SADH-12AS), and the stachyose derivative of pyrenebutyl hydrazide (SPBH).

* Mean ± S.D.

** DISCUSSION **

In the present study, we observed that the fluidity of the outer hemileaflet of the plasma membranes of TPA-sensitive and TPA-resistant HL-60 cells is similar, whereas the inner membranes (the inner hemileaflet of the plasma membrane and/or the organelar membranes) of the R-55 cells are less fluid than those in TPA-responsive HL-60 cells. These results suggest a possible relationship between the physical state of the membrane lipids of myeloid leukemic cells and their capacity to respond to phorbol ester induction of differentiation.

An alternative possibility is that the membrane distribution of DFH and TMA-DPH differs in the sensitive and resistant cells. Inasmuch as cytosolic membranes are considerably more fluid than the plasma membrane, which contains more cholesterol, it is possible that a greater fraction of the probe reaches the intracellular membranes of the sensitive cells. We consider this alternative hypothesis less likely, because variations in the time of exposure to DPH or TMA-DPH did not appear to alter the difference between the sensitive and resistant strains.

Phorbol ester tumor promoters are known to induce a wide array of membrane alterations in diverse cell types, including: altered Na/K ATPase activity; increased 2-deoxyglucose, $^{32}$P, $^{86}$Rb, and $^{45}$Ca transport; increased phospholipid turnover; increased arachidonic acid and prostaglandin synthesis; altered cell surface glycoproteins; and altered binding of epidermal growth factor (for review, see Refs. 13, 16, 35, and 38). TPA has also been found to alter membrane lipid fluidity in rat embryo cells (19), human lymphocyte and lymphoblastoid cell lines (6), ascites mouse ovarian tumor cells (4), and mouse C3H10Tf/2 cells (37). In rat embryo cells (19), changes in membrane fluorescence anisotropy resulting from TPA treatment did not require new RNA or protein synthesis, indicating a more direct interaction with existing cellular membrane components. Although high-affinity and saturable cell surface receptors for phorbol esters have been identified in many different cell types (10, 14, 18, 24, 34, 36, 39), recent studies indicate that phorbol esters, because of their lipophilic-hydrophilic composition, can also insert into the phospholipid leaflet and diffuse through biological membranes (11, 12). Studies in which artificial membranes are used suggest that the binding of PDBU to multilamellar liposomes composed of phospholipids is similar to the binding of PDBU to intact fibroblast and epidermal cells (11).

The mechanism by which tumor-promoting phorbol esters induce differentiation in human promyelocytic leukemic cells is not currently known. Recent studies indicate that TPA must remain bound to the surface of HL-60 cells to elicit a biological response, suggesting that a receptor-mediated transmembrane process is involved in TPA induction of differentiation (9, 21). Since the cell surface binding of phorbol esters is not altered in the resistant R-55 cells (36), it is reasonable to propose that the basis of the TPA resistance is a block in the signal transmission that occurs subsequent to receptor occupancy. A decrease in the fluidity of the membrane lipids of the R-55 cells would be expected to impair such a transmission process. Moreover, the findings that the resistance of R-55 cells can be overcome at high concentrations of TPA, >1 ng/ml (Table 1; Chart 3), could be explained by an increase in fluidity, perhaps because of direct intercalation of the phorbol ester in the bilayer lipids (37). In accord with this hypothesis, previous studies have demonstrated that TPA-resistant HL-60 cells are also defective in their ability to down-regulate specific $[^{3}H]$PDBU binding (36); this membrane change, too, could result from decreased fluidity of the inner leaflet of the plasma membrane. A component of the secondary signal may involve translocation of the TPA-receptor into the cytosol (1) and/or transmission of the signal to the nucleus (32).
An alternative hypothesis is that fluidity alterations induced by TPA, which occur after prolonged (24 to 48 hr) incubation with the phorbol ester, are a consequence of secondary alterations, i.e., they result from induction of differentiation by TPA and do not reflect a direct early effect of these compounds on the plasma membrane. Studies are currently in progress to address this issue directly and to determine the relationship between induction of differentiation and fluidity alterations in HL-60 cells using compounds other than TPA, such as trans retinoic acid and dimethyl sulfoxide, to induce differentiation. An understanding of those dynamic changes in membrane lipid composition and physical properties that occur concomitant with TPA resistance and in response to TPA treatment may prove fundamental in understanding how tumor promoters exert their pleiotropic biological effects in diverse cell types.

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