Changes in Cell Membrane and Cellular Lipids in Apoptotic Cells Induced by Dolichyl Phosphate Differ from Findings in Cells Induced by Etoposide

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Abstract: Cell membrane fluidity and changes of cellular lipids of U937 cells during apoptosis are compared between the cells treated with dolichyl phosphate (dol-P) and etoposide. Dol-P induced lateral cell membrane perturbation immediately after addition and continued for at least 40 min. However, etoposide induced apoptosis without increase in cell membrane fluidity. Phosphatidylethanolamine was translocated together with phosphatidylserine after 4 hr of treatment in both dol-P and etoposide treated cells. The concentration of lipid levels changed in dol-P-induced cells and the cholesterol/phospholipid ratio almost doubled. Conversely, none of the phospholipid and cholesterol levels changed in etoposide treated cells. Although both dol-P and etoposide induce apoptosis, dol-P, but not etoposide, appeared to function as a modulator of membrane fluidity and to change membrane constituents and membrane physiology during the course of apoptosis.


Key words: apoptosis, membrane fluidity, dolichyl phosphate, etoposide, phospholipid, cholesterol

1 Introduction

Although apoptosis is triggered by a variety of inducers, the common characteristics are maintained: cell shrinkage and nuclear condensation frequently accompanied by DNA laddering, followed by disruption of the cytoskeleton (1, 2). We have reported that dolichyl phosphate (dol-P) induces apoptosis in rat glioma C6 cells (3), and in human monoblastic leukemia U937 cells (4). Although dol-P is an essential carrier lipid in the biosynthesis of N-linked glycoprotein, the apoptosis inducing activity of dol-P is apparently not related to N-linked glycoprotein synthesis, but rather is mediated by reduction in mitochondrial transmembrane potential 1–3 hr after induction, followed by activation of caspases-3, and -8 (2–4 hr) (4–7). When the cell progresses to apoptotic cell death, the membrane undergoes various changes, starting with inducer contact and ending with membrane disruption into apoptotic bodies. In this study, membrane alterations of U937 cells during apoptosis were investigated and compared using two different types of apoptosis inducers, dol-P and etoposide. Dol-P, naturally present in cell membranes, is expected

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to cause changes in membranes when added. On the other hand, etoposide, an inhibitor of DNA topoisomerase-II, is known to work on the nucleus (8). We recently found that dol-P and other structurally related isoprenoid phosphates induced apoptosis of U937 cells, accompanied by increases in membrane fluidity at very early stages of induction (9). In this research, the initial membrane fluidity was compared with the time required for inducers in contact with the cell to trigger apoptosis. The relation between the increase in membrane fluidity and flip-flop action of aminophospholipids was determined by sequentially quantifying the cell surface phosphatidylserine (PS) and phosphatidylethanolamine (PE). The lipid compositions of the membrane, which is strongly related to the shape and properties of the cells (10), were investigated after fluidity changes and dynamic translocations of aminophospholipids had occurred (4 hr). Although similar loss of aminophospholipid asymmetry was found, only dol-P caused membrane perturbation and induced various changes in membrane lipids including increase of cholesterol during the course of apoptotic cell death. Alternatively, etoposide seemed to react directly to the nucleus without perturbing plasma membrane and without largely altering lipid composition.

2 Experimental

2.1 Materials

Dolichyl phosphate was synthesized in and provided by Dr. Isao Yamatzu, Serendip Research Institute, Tokyo Japan. FL-SA-Ro, as a probe of PE, was kindly provided by Dr. M. Umeda and Dr. K. Emoto of The Tokyo Metropolitan Institute of Medical Science, Department of Inflammation Research. Etoposide and Annexin-V-FLUOS were obtained from Sigma Chemical Company, Missouri, USA, and Boehringer Mannheim, Germany, respectively.

2.2 Cell Culture and Viability

The human promonoblastic leukemia cell line U937 was grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and placed to each well of a 24-well plate. Dolichyl phosphate dissolved in ethanol and n-dodecane (98:2 by volume) (ED) was applied to induce apoptosis, as described (5). The final concentration of dol-P was 6 μM, and ED was 0.8% by volume. Cells cultured with the same concentrations of ED were prepared as a reference for dol-P treated cells. Etoposide dissolved in dimethylsulfoxide (DMSO) was similarly applied to the culture medium, at a final concentration of 17 μM. Cells cultured with the same concentration of DMSO (0.5% by volume) were prepared as the reference for etoposide treated cells. The cells were incubated for 4 hr and pelleted by centrifugation.

Cell viability was measured by counting living cells under trypan blue staining.

All measurements obtained in this research were taken in triplicate.

2.3 Cell Membrane Fluidity

The cell membrane fluidity was measured, as previously described (6). In short, cells were incubated in Hanks’ balanced salt solution (HBSS) containing 5 μM pyrene and 0.1 wt% BSA at 37°C for 1 hr. Intensities of the monomer fluorescence of pyrene (I_m) (386 nm) and of excimer fluorescence (I_e) (480 nm) were determined by dynamic light scattering measurements (Hitachi F-4041, Japan). The membrane fluidity was evaluated based on I_e/I_m.

2.4 Quantification of External Aminophospholipids

Cultured cells (1 × 10^6) were recovered and suspended in 100 μL Annexin-V-FLUOS for 15 min at room temperature for PS probe, or in 100 μL FL-SA-Ro for 15 min in ice for PE probe, as previously described (11). Cell suspensions were analyzed using FACScan (Nippon Becton Dickinson Co., Tokyo, Japan).

2.5 Cholesterol Analysis

Cholesterol was quantified chemically using the Phthalaldehyde-sulfuric acid Method (12), revised for microanalysis, and the absorbance was measured at 595 nm by a microplate reader (Bio-Rad Model 3550, Nippon Bio-Rad Laboratories Co., Tokyo, Japan).

Visualization of cholesterol was performed using filipin as a fluorescence probe. Cells were fixed with 1% paraformaldehyde, 0.5 μg/mL filipin in ethanol was added and were analyzed using a fluorescent microscope (Olympus BX-50). Quantification of filipin stained cholesterol was performed by FACS, as previously described (13).

2.6 Chemical Analysis of Phospholipid

The amount and composition of phospholipids were
determined, as previously described (14). To summarize, total lipid was extracted from a cell pellet and subjected to two-dimensional thin-layer chromatography (TLC) (Whatman HPK Silicagel 60, layer thickness: 200 μm). Plates were first developed in tetrahydrofuran: acetone:methanol:water (50:20:40:8, by volume), and then in trichloromethane:acetone:methanol:acetic acid:water (50:20:10:15:5, by volume). PS, PE, phosphatidylethanolamine (PC), phosphatidylinositol (PI), sphingomyelin (SM), and cardiolipin (CL) were scraped off separately and quantified as phosphorous content (wt%), following revised Bartlett Method (15).

2.7 Protein Determination
Protein contents were measured using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL USA).

3 Results and Discussions

3.1 Cell Viability
In order to discover what is occurring to the membrane upon contact with apoptosis inducers, we have compared the time required to trigger apoptosis with the change of cell membrane fluidity between dol-P and etoposide treatments. The cells were washed 5, 10, 20 and 30 min after addition of inducers, and incubated a further 4 hr without inducers, at which time, cell viability was measured. As one of the indications for progression of apoptosis, the cell viability was measured using trypan blue staining. In dol-P treated cells, the cell viability began to reduce for cells stimulated for 10 min, and further decreased to about two thirds of the non-treated cells for cells stimulated for 30 min as shown in Fig. 1(A). Therefore, the initiation of apoptosis seemed to be a relatively slow process, induced gradually only after 10 min of stimulation. In etoposide treated cells, viability dropped to about one half for cells stimulated for 5 min but viability remained unchanged until 30 min. Accordingly, it was speculated that dol-P required about 30 min of contact time whereas etoposide required only 5 min to proceed to apoptosis execution.

3.2 Membrane Fluidity Changes
The encounter of cells with the reagents, was monitored by lateral cell membrane fluidity, measured as the ratio of pyrene excimer fluorescence (Ie) / monomer fluorescence (Im). Membrane fluidity of U973 cells treated with dol-P, dissolved in ED, increased immediately after addition, became almost constant after 3 min and remained until 40 min as shown in Fig. 1(B). Together with cell viability studies, initiation of apoptosis seemed to occur after membrane perturbation, which continued for about 10 min, and the gradual execution process was performed during persistent perturbation. This initial increase and continuation of membrane perturbation indicates that dol-P partitioned to the membrane. Furthermore, alteration of the membrane dynamics caused by partitioned dol-P seemed to be one of the key events for the execution of apoptosis, The membrane fluidity of etoposide (in DMSO) treated cells remained unchanged, close to that of the cells treated with DMSO. Thus, etoposide is speculated to induce apoptosis without physically stimulating the membrane.

3.3 Translocations of Aminophospholipids
Aminophospholipid translocations were measured to determine the relation between membrane fluidity and flip flop action of aminophospholipids. When translocations of aminophospholipids from the inner to outer membrane were quantified, there were no significant accumulations on the outer surface of the cell membrane after 1 hr of induction. However, after 4 hr, about 80% of dol-P treated and 40% of etoposide treated cells showed accumulation of PS at the cell surface, as shown in Fig. 2 (A). As PS was translocated, another aminophospholipid, PE, was also translocated (11). After 4 hr, about 65% of dol-P treated and 30% of etoposide treated cells showed exposure of PE on the cell surface (Fig. 2 (B)). Accordingly, the membrane fluidity detected at the initial stage of apoptosis induction by dol-P was not the result of translocation of phospholipids, but the result of interaction of the cell membrane with the reagent. The translocation of PS to the outer membrane, which was observed for both dol-P and etoposide treated cells, is expected to change the mode of recognition by phagocytes (16). On the other hand, the translocation of PE to the outer surface of the cell is closely related to actin filament disassembly (17). Although the significance of PE accumulation on the cell surface during apoptosis is beyond the scope of this study, translocation of PE is undoubtedly one of the key events of apoptosis.
3.4 Changes of Lipid Compositions

Following aminophospholipid externalization, changes in lipid compositions were investigated. Quantitative changes of total lipid phosphorus, cholesterol and phospholipids were determined after a 4 hr treatment when translocation of phospholipids was observed. Cholesterol is located mostly in the cell membrane (18) while phospholipids are distributed throughout various membranes of cell components. By the time DNA fragmentation is apparent, not only cell membrane but also dynamic membrane changes are occurring simultaneously in organelia such as mitochondria (19). The changes in lipid compositions of mitochondria have been reported in apoptotic villus tip cells (20). To investigate the macroscopic changes of the cell as one system, phospholipids of the total cells were studied. The results of dol-P treated cells were compared with that of cells treated with ED used to dissolve dol-P, and etoposide treated cells were compared with that of DMSO, the solvent of etoposide, as summarized in Table 1.

3.4.1 Cholesterol levels
Fig. 2 Translocations of Phosphatidylserine (PS) and Phosphatidylethanolamine (PE).
The externalization of aminophospholipids was determined by FACS. [A]: The externalization of PS was measured as Annexin-V–FLUOS photolabeled PS. [B]: The externalization of PE was measured as FL–SA–Ro photolabeled PE. The Y-axis indicated the percentage of stained cells.

Table 1 Levels of Various Lipids in U937 Cells Treated with Dolichyl Phosphate or Etoposide.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Dol-P in ED</th>
<th>ED</th>
<th>Etoposide in DMSO</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>total lipid phosphorus (nmole/mg protein)</td>
<td>156.1 ± 2.4**4</td>
<td>189.5 ± 5.6**4</td>
<td>202.8 ± 7.0</td>
<td>200.7 ± 1.7</td>
</tr>
<tr>
<td>cholesterol</td>
<td>60.9 ± 3.2**4</td>
<td>39.4 ± 0.4**4</td>
<td>36.9 ± 0.2</td>
<td>30.6 ± 0.5</td>
</tr>
<tr>
<td>cholesterol/phospholipid ratio</td>
<td>0.39 ± 0.02**4</td>
<td>0.21 ± 0.01**4</td>
<td>0.18 ± 0.01</td>
<td>0.15 ± 0.00</td>
</tr>
</tbody>
</table>

phospholipid composition (%)

- phosphatidylcholine (PC) 53.1 ± 0.9**1 46.1 ± 4.2*1 51.8 ± 1.3 51.1 ± 5.0
- phosphatidylethanolamine (PE) 16.3 ± 0.9 16.0 ± 1.6 16.8 ± 0.7 18.8 ± 1.6
- phosphatidylinositol (PI) 9.4 ± 0.5**3 5.6 ± 0.8**3 6.3 ± 0.4 7.2 ± 1.4
- phosphatidylserine (PS) 5.2 ± 0.4**2 7.9 ± 1.1**2 6.5 ± 0.6 6.5 ± 0.9
- sphingomyelin (SM) 7.1 ± 1.2 9.0 ± 0.5 6.4 ± 0.6 7.0 ± 0.1
- cardiolipin (CL) 3.6 ± 1.1 5.0 ± 1.5 4.0 ± 0.6 3.7 ± 1.1
- lysophosphatidylcholine (LPC) 2.2 ± 0.0 3.4 ± 0.2 2.7 ± 0.8 1.7 ± 0.3
- unknown 3.3 ± 0.2 8.0 ± 1.1 6.1 ± 0.6 4.6 ± 0.5

phospholipid ratios

- PC/PE ratio 3.2 ± 0.2 2.9 ± 0.4 3.1 ± 0.2 2.7 ± 0.3
- PC/SM ratio 7.4 ± 1.3 5.1 ± 0.5 8.1 ± 0.8 7.2 ± 0.7
- PC/PS ratio 10.1 ± 0.8**3 5.9 ± 1.0**4 7.9 ± 0.8 7.8 ± 1.5
- PC/CL ratio 14.8 ± 4.5 10.0 ± 3.1 13.8 ± 2.1 13.9 ± 4.3
- PC/PI ratio 5.7 ± 0.3**3 8.3 ± 1.4**3 8.3 ± 0.6 7.1 ± 1.5
- PE/PS ratio 3.1 ± 0.3 2.0 ± 0.3 2.6 ± 0.3 2.9 ± 0.5
- (PC/SM)/(PE/PS) ratio 2.4 ± 0.5 2.5 ± 0.5 3.1 ± 0.4 2.5 ± 0.5

Each result is the mean value from triplicate experiments ±S.D. *P < 0.20; **P < 0.10; ***P < 0.05; ****P < 0.001
Levels of various lipids were determined after 4 hr treatment.
In dol-P treated cells, total lipid phosphorus decreased from 190 to 156 nmoles/mg protein while total cholesterol increased from 39 to 61 nmoles/mg protein. As a result, there was a two-fold increase in the ratio of cholesterol to phospholipid from 0.21 to 0.39. However, the cholesterol to phospholipid ratio remained constant in etoposide treated cells. To confirm the cholesterol change, the free cholesterol was visualized using filipin as a fluorophore. Filipin bound cholesterol was accumulated as granules inside cytoplasm close to the nucleus of dol-P treated cells as shown in Fig. 3 (B), but in etoposide treated cells, only faint filipin bound cholesterol was observed at the cell membrane (Fig. 3 (C)). FACS analysis of filipin stained cells indicated that free cholesterol increased in dol-P treated cells 1.0 to 1.5 hr after induction, as shown in Fig. 3 (A). The stage of cholesterol increase was concurrent with an increase in activation of caspases-3 and -8 (7). Cholesterol is closely related to apoptosis and membrane properties. The accumulation of intracellular cholesterol is consistent with observations during phorbol ester-induced apoptosis in human peripheral blood monocyte (21) or apoptosis induced by transforming growth factor β1 in human erythroleukemia K562 cells (22). On the other hand, externally added cholesterol acted as apoptosis inducer in K562 cells (23). Our studies imply that the increase of cholesterol observed in dol-P treated cells was not a cause but a result of apoptosis induction, since apoptosis was induced within 10 min of stimulation but the increase of cholesterol began only after 1 hr from induction. Moreover, increase in cholesterol was expected to lead to a reduction in fluidity of the cell membrane (24). However, increase in cell membrane fluidity observed at initial treatment with dol-P must be an independent phenomenon from cholesterol change, since the cholesterol increase began at a much later stage of induction. The increase of cholesterol level, only observed in dol-P treated cells, suggests that this increase is one of the consequences of persistent membrane stimulation during and after apoptosis is initiated.

3.4.2 Phospholipid levels

Changes in phospholipid levels are closely related to the physical properties of cell membranes. When PC is
increased, ion permeability and susceptibility to osmotic shock change (25). Phospholipid levels are also closely related to the activity of important protein kinases required for cell survival. For example, PI is the substrate of PI 3-kinase, which is needed to generate a membrane-restricted second messenger that activates several protein kinases required for cell survival (26, 27). In dol-P treated cells, PC and PI slightly increased while PS decreased. The phospholipid ratio such as PC/PS almost doubled from 5.9 to 10.1, and PC/PI decreased to about two thirds from 8.3 to 5.7. Surprisingly, there were no changes in any of the lipid levels in the etoposide treated cells, including the cholesterol and phospholipid ratios. Increase of the PC/PS ratio observed in dol-P treated cells must be independent of PS translocation since the ratio remained constant in etoposide treated cells. There were no significant changes in other phospholipid ratios such as PC/PE, PC/SM, or (PC/SM)/(PE/PS) that may demonstrate the influence of aminophospholipid translocations.

4 Conclusions

Two apoptosis inducers, dol-P and etoposide, both mediated translocation of well-known PS to the outer cell surface. At the same time, another aminophospholipid, PE, was found to localize at the outer surface of the dol-P and etoposide treated cells. In spite of the similarities observed in aminophospholipid translocations, membrane dynamics and lipid changes differed greatly between the two inducers. Dol-P, naturally present in cell membranes, perturbed cell membranes immediately after contact (in 2 min) but initiation of apoptosis did not start until 10 min after induction, while perturbation continued for at least 40 min. Changes in phospholipid levels were observed and cholesterol level almost doubled. On the other hand, etoposide initiated apoptosis within 5 minutes without significantly increasing the membrane fluidity and produced minimal changes in lipid components. We have reported that apoptosis-inducing ability is reflected by an increase of membrane dynamics for lipid apoptosis inducers (9). The present observations indicate that reagents such as etoposide, although lipophilic, do not require a change of membrane dynamics upon contact with the cells in order to initiate the signal transduction of apoptotic cells. The biophysical changes, such as initial increase and persistent membrane fluidity, are distinguished features observed in dol-P induced apoptotic cells. Since physical changes (lateral packing pressure) of the membrane has been suggested to alter conformation of receptor protein (28), dynamic biophysical changes observed in dol-P treated cells are implied to result in biochemical changes of the membrane. Further investigations of the behavior of added dol-P within the cells are necessary to provide clearer understanding among dol-P, increase in membrane fluidity, changes in membrane constituents and physiologically.

Acknowledgments

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