Cell membrane dynamics and the induction of apoptosis by lipid compounds

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Received 4 December 1998; received in revised form 1 February 1999

Abstract To investigate the induction of apoptosis by some lipid compounds which are a potent inducer of apoptosis, the plasma membrane fluidity of U937 cells was measured using the fluorescent probe, pyrene. The increase of the membrane fluidity was observed immediately after the treatment of cells with lipid inducers. We also found that the trigger of apoptosis was pulled within 30 min after treatment. Data from the dynamic light scattering experiment indicated that lipid inducers were dissolved to form the emulsion. At the very early stage of apoptosis, possibly, the well-controlled transfer of lipid inducers from the emulsion to the lipid layer of cells can bring about the increase of membrane dynamics which might lead to the induction of apoptosis.

Key words: Apoptosis; Membrane fluidity; Lipid compound; Isoprenoid; Apoptosis inducer; Emulsion

1. Introduction

Apoptosis is induced by various extracellular factors, such as tumor necrosis factor alpha (TNF-α) [1], Fas ligand [2], UV, gamma-ray irradiation [3] or temperature [4]. Recently, many researchers found that apoptosis can be induced by various lipid compounds, such as ceramide [5], sphingosine [6], ether lipid [7], retinoic acid [8], farnesol [9], geranylgeraniol [10], geranylgeranyl acid [11], palmitate and stearate [12]. We also found that dihydroprenyl phosphates with more than seven isoprene units were a potent inducer of apoptosis in rat glioma C6 cells [13] and in human monoblastic leukemia U937 cells [14]. These compounds could stimulate adenylate cyclase and activate CPP32 [15].

It is surprising that various lipid compounds can induce apoptosis. It seems that induced apoptosis is not unique. It is clear that the first event of apoptosis is the partition of added chemical compounds to the plasma membrane of cells due to their amphipathic properties. These compounds significantly perturb the lipid layer and alter its fluidity. It is well known that various cellular functions are modulated by the physical properties of the cell membrane [16]. Tong et al. stated that insulin resistance in non-insulin-dependent diabetes mellitus could be related to changes in cell membrane properties [17]. They measured the membrane fluidity by fluorescence polarization to clarify the relation between the insulin sensitivity and membrane dynamics. We hypothesized that the trigger of apoptosis is related to cell membrane dynamics altered by lipid compounds. We examined the membrane fluidity of U937 cells and assessed the relation between membrane fluidity and apoptotic activity. We also observed the solubilized states of lipid compounds in medium to obtain information about their actions to be transferred to the plasma membrane. These relation between the action of added compounds and the induction of apoptosis will provide significant information regarding the very early stage of apoptosis.

2. Materials and methods

2.1. Materials

Dolichol, dolichyl monophosphate (dol-P), alpha-dihydrodecaprenol (C50-OH), α-dihydrodecaprenyl monophosphate (diammonium salt) (C50-P), alpha-dihydroheptaprenol (C35-OH), α-dihydroheptaprenyl monophosphate (diammonium salt) (C35-P), farnesyl monophosphate (diammonium salt) (F-P), geranylgeranyl monophosphate (diammonium salt) (GG-P), C16 ceramide and C16 ceramide phosphate were donated by Tsukuba Research Laboratories, Eisai Company (Ibaraki, Japan). Farnesol (F-OH) and geranylgeraniol (GG-OH) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Stearic acid and palmitic acid were obtained from Sigma (St. Louis, MO, USA). Benzyl alcohol (BzOH) was purchased from Junsei Chemical (Tokyo, Japan).

2.2. Methods

2.2.1. Dynamic light scattering. Chemical compounds were dissolved in various solvents as shown in Table 1. The hydrodynamic size of each emulsion formed in Hank's balanced salt solution (HBSS) was determined by the dynamic light scattering measurement (Otsuka LPA-600).

2.2.2. Cell membrane fluidity. The cell membrane fluidity was detected by pyrene excimer fluorescence. The human promonocytic leukemia cell line U937 was grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2. 2 ml cell suspension (1.25×10^6 cells/ml) in HBSS was placed to a cuvette for the fluorescence measurement. Cells were incubated in HBSS containing 5 μM pyrene (Funakoshi, Tokyo, Japan) and 0.1% bovine serum albumin (Sigma) at 37°C. After each compound was added to a cell suspension, the intensities of monomer fluorescence of pyrene at 386 nm (IM) and of excimer fluorescence at 480 nm (I_E) were collected with excitation at 340 nm, respectively. The ratio of intensities, I_E/I_M, is increased by pyrene collision related to the fluidity of the plasma membrane. Therefore, the membrane fluidity was evaluated by I_E/I_M [18].

2.2.3. Cell morphology. For morphological assessment, cells treated with different agents were pelleted by centrifugation.

After fixed in methanol and stained with Giemsa, they were examined under an optical microscope. The occurrence of apoptosis in each group was determined based on the expression of cytoarchitectural characteristics of apoptosis. Also, nuclear morphological observation of cells stained with DAPI (4,6-diamidino-2-phenylindole, Sigma) was carried out using a fluorescent microscopy.

2.2.4. Detection of DNA fragmentation. Agarose gel electrophoresis was used to detect internucleosomal DNA cleavage as described previously [13]. For U937 cells, 100 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 0.5% Triton X-100 was added to the cell pellet and the preparation was left at 4°C for 20 min. After centrifugation at $14\,000\times g$ for 20 min, the supernatant was incubated for 20 min with 2 μl of 20 mg/ml RNaseA (Sigma) aqueous solution, followed by an additional incubation for 30 min

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PII: S0014-5793(99)00204-5

with 2 μl of 20 mg/ml proteinase K (Boehringer-Mannheim, Germany) aqueous solution. The fragmented DNA was then precipitated adding 20 μl of 5 M NaCl and 120 μl of 2-isopropanol overnight at −20°C. After centrifugation at 14 000×*g* for 15 min, DNA was dissolved in TE buffer and analyzed on 1.7% of Nusieve 3:1 agarose gels (FMC BioProducts, Rockland, ME, USA).

3. Results and discussion

We could observe shrinkage of size and condensation of nucleoplasm and nuclear fragmentation in U937 cells within 4 h after the addition of dolichyl monophosphate (dol-P) of 6 μM or more, which was dissolved in the ethanol:*n*-dodecane mixture (98:2, by volume). When cells were centrifugated to remove free dol-P and resuspended in HBSS after 30 min of incubation with dol-P (6 μM), we could also observe apoptosis of cells within 4 h. On the other hand, cells treated with dol-P (6 μM), only for 5 min, showed virtually no apoptosis even after longer than 8 h (data not shown). These results suggest that apoptosis of a cell is triggered at least within 30 min after the addition of lipid compounds. During this period, it is thought that added compounds are transferred and situated to the lipid region of the plasma membrane. To obtain the information about the perturbation upon the transfer to the membrane, we carried out the membrane fluidity experiment using pyrene. Fig. 1 shows the time course of the membrane fluidity of U937 with dol-P. The membrane fluidity increased immediately after adding dol-P. On the other hand, cells treated with dolichol showed a little decrease in the fluidity. Addition of the same amount of the solvent mixture had no effect on the membrane fluidity. Except for the solvent mixture (98:2 by volume), treatment with dol-P could not cause apoptosis. It was seen from Fig. 2 that treatment with C50-P or C35-P led to an increase in the fluidity and then the fluidity for C50-P gradually decreased with incubation time. Both chemicals were an inducer of apoptosis [13]. It could be observed in Fig. 2 that the membrane fluidity of cells treated with C50-OH or C35-OH increased to a less extent than that with C50-P or C35-P. It is noted that apoptosis is induced whenever the membrane fluidity increases within several minutes after the treatment with lipid compounds.

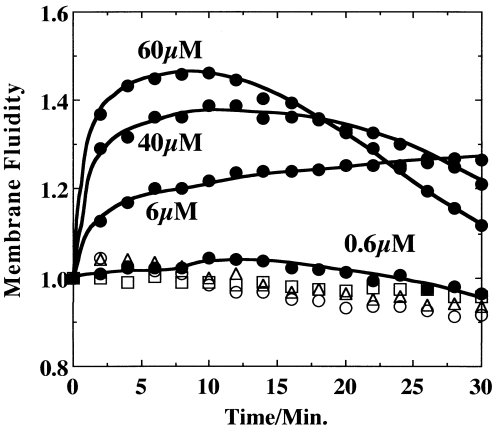

Fig. 1. Time courses of the membrane fluidity of U937 cells after treatment with the solvent mixture of ethanol and *n*-dodecane (98:2 by volume) (triangle), dolichol dissolved in the solvent mixture (open circle), dolichyl phosphate dissolved in the solvent mixture (closed circle) and no additives (square). U937 cells were dispersed in HBSS including BSA (0.01 weight%). Final concentrations of dol-P were shown in the figure.

On the other hand, treatment of cells with GG-P or F-P could not lead to the increase in the membrane fluidity and treatment with GG-OH or F-OH could do so. We previously described that neither GG-P nor F-P induced apoptosis. However, both GG-OH and F-OH could induce apoptosis [13]. C16 ceramide and C16 ceramide phosphate showed the same tendency in both the fluidity and the induction of apoptosis as GG-OH and GG-P. When cells were treated with stearic acid and palmitic acid, respectively, apoptosis was induced and at the same time the fluidity of the plasma membrane increased (Fig. 3).

These results strongly suggest that the induction of apoptosis did not depend on the presence of the phosphate moiety and that the membrane fluidity is an important physical prop-

Table 1
Membrane fluidity, solubilized states and induction of apoptosis by lipid compounds

Lipid (final concentration)		Solvent (by volume)	Emulsion size (nm)	Fluidity[a]	Apoptosis[b]
dol-O	(6 μM)	Ethanol	Not detected	−	−
	(6 μM)	Ethanol/n-hexane (98:2)	15.4 ± 1.5	No datum	−
	(6 μM)	Ethanol/n-octane (98:2)	330.5 ± 3.0	No datum	−
	(6 μM)	Ethanol/n-dodecane (98:2)	196.7 ± 11.1	+	+
	(0.6 μM)	Ethanol/n-dodecane (98:2)	263.7 ± 2.7	−	−
	(60 μM)	Ethanol/n-dodecane (98:2)	178.2 ± 6.9	+	+
gG-OH	(25 μM)	Ethanol/n-dodecane (98:2)	157.4 ± 25.2	+	+
	(50 μM)	Ethanol/n-dodecane (98:2)	180.8 ± 8.2	+	+
	(100 μM)	Ethanol/n-dodecane (98:2)	272.3 ± 4.5	+	+
	(25 μM)	Ethanol	181.8 ± 1.5	+	+
	(50 μM)	Ethanol	208.0 ± 21.2	+	+
	(100 μM)	Ethanol	214.9 ± 14.7	+	+
gG-P	(25 μM)	Ethanol/n-dodecane (98:2)	201.2 ± 16.9	−	−
	(50 μM)	Ethanol/n-dodecane (98:2)	201.2 ± 20.7	−	−
	(100 μM)	Ethanol/n-dodecane (98:2)	237.8 ± 3.0	−	−
f-OH	(25 μM)	Ethanol/n-dodecane (98:2)	Not detected	+	+
	(50 μM)	Ethanol/n-dodecane (98:2)	186.3 ± 14.0	+	+
	(100 μM)	Ethanol/n-dodecane (98:2)	202.5 ± 6.1	+	+
f-P	(25 μM)	Ethanol/n-dodecane (98:2)	186.3 ± 4.6	−	−
	(50 μM)	Ethanol/n-dodecane (98:2)	162.3 ± 9.9	−	−
	(100 μM)	Ethanol/n-dodecane (98:2)	166.8 ± 1.9	−	−

[a]Increase(+); no change(−).
[b]Evaluated by both cell morphological observation and DNA fragmentation.

erty related to the induction of apoptosis. To examine whether such increases of the membrane fluidity are specific to apoptosis, we studied the relation between the membrane fluidity and apoptosis of cells treated with a fluidizer (BzOH). Although the fluidity was suddenly and largely increased by the treatment with BzOH as shown in Fig. 3, no apoptosis was observed regardless of the concentration of BzOH. The high concentration led to membrane rupture. This large and temporary increase is significantly different from those of apoptosis inducers described above.

Probably, this difference in the fluidity change is attributable to the mode of the transfer of chemicals to the membrane. Some lipids are amphipathic and behave like a surfactant. The emulsifying efficiency of a surfactant is related to the polarity of the molecules, which is defined in terms of the hydrophile-lipophile balance (HLB). The least hydrophilic materials have low HLB numbers and increasing HLB corresponds to an increasingly hydrophilic character. Fig. 4 shows the relation between apoptosis and the HLB number of lipids. Apoptosis could be induced by treatment with lipids in the window between 5.3 and 6.2 of HLB. It is difficult for lipids with a low HLB to form the emulsion in an aqueous medium and if formed, it seems to be unstable. When HLB is high, the formed emulsion seems to be highly stable. Solubilized states of added lipids would play an important role in transferring lipids to the plasma membrane. Thus, we detected the hydrodynamic size by the dynamic light scattering measurement to understand the solubilization behavior of lipids in an aqueous medium. Table 1 shows the results of dol-P, GG-OH and F-OH. We found that these apoptosis inducers dissolved in the mixture of ethanol and *n*-dodecane (98:2) could form the emulsion of which sizes were dependent on the concentration of lipid compounds. The size of the emulsion for dol-P (6 μM) was approximately 200 nm. Droplets of the sub-micron size were also observed for GG-OH and F-OH. On the contrary, dol-P dissolved in ethanol could not form the emulsion. Furthermore, neither change in the membrane fluidity nor induction of apoptosis could be observed. These findings strongly suggest that the transfer of lipid compounds to the plasma membrane is facilitated by the formation of the emulsion.

On the other hand, dol-P dissolved in the mixture of ethanol and hexane or *n*-octane could form the emulsion, however, no apoptosis was observed. This indicates that the transfer efficiency is governed by the emulsion stability. Such a well-controlled transfer can probably lead to the increase in the membrane fluidity at the very early stage of apoptosis, which is significantly related to the induction of apoptosis. Although whether the chemical structure of lipid compounds is responsible for the induction of apoptosis is not clear, apoptosis inducers such as dol-P might regulate the membrane dynamics so that lateral packing pressure on membrane proteins can induce their more functional conformations [19] or inducers might bind directly to protein targets so as to modulate the specific receptors as well as anaesthetics which exert a specific effect on the ion-channel proteins [20].

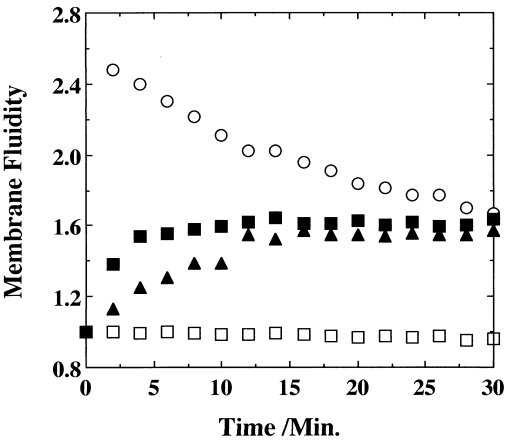

Fig. 3. Time courses of the membrane fluidity of U937 cells after treatment with stearic acid (closed square), palmitic acid (closed triangle), no additives (square) and BzOH (open circle). U937 cells were dispersed in HBSS including BSA (0.01 weight%). Final concentrations of stearic acid, palmitic acid and BzOH were 15 μM, 15 μM and 50 mM, respectively.

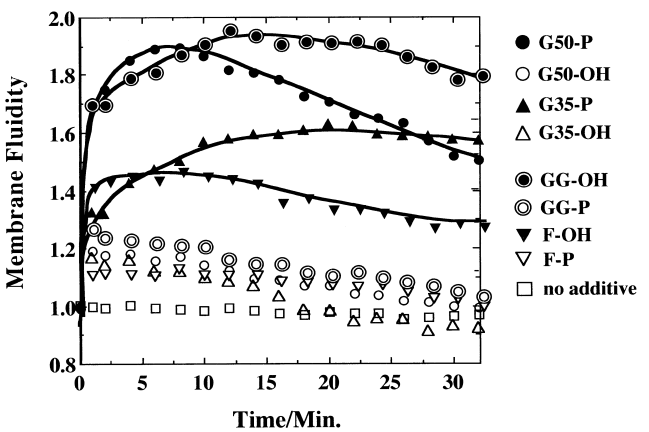

Fig. 2. Effect of the the phosphate moiety on the change in the membrane fluidity. U937 cells were dispersed in HBSS including BSA (0.01 weight%). Final concentrations of C50-OH, C50-P, C35-OH and C35-P were 6 μM, while those of GG-P, GG-OH, F-P and F-OH were 50 μM.

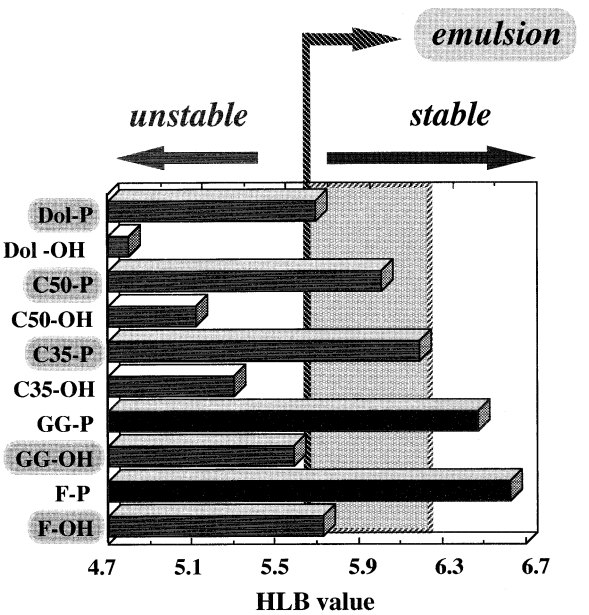

Fig. 4. HLB values of various isoprenoids. The HLB number was calculated by Kawakami's method [22]. Each lipid was dissolved in the mixture of ethanol and *n*-dodecane (98:2) and was then added to a cell suspension.

Detection of conformational and functional changes of membrane proteins might provide more insight into the mechanism of the induction of apoptosis by chemical inducers. In some cells, induction of apoptosis is associated with the leakage of cytochrome *c* from the mitochondria into the cytosol [21]. It is thought that Bcl-2-related proteins or the permeability transition pore are associated with the opening of the channels for the leakage. Therefore, in the future we should also accumulate the information on the actions of lipid inducers on the intracellular membrane and such proteins to clarify the effects of chemical inducers.

Acknowledgements: We thank Dr I. Yamatsu (Tsukuba Research Laboratories, Eisai Company) for providing derivatives of isoprenoids.

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