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Clustering of Lipid Rafts in Plasma Membranes by Hybrid Liposomes for Leukemia Cells along with Apoptosis

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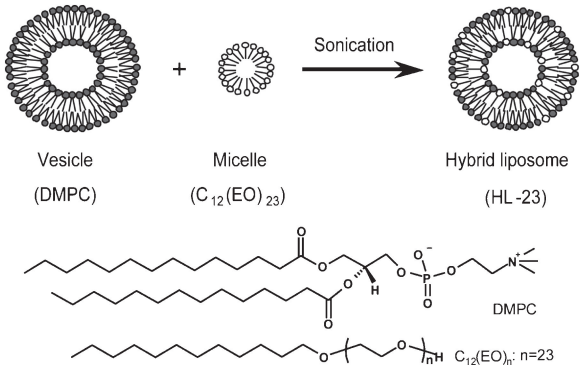
Clustering of lipid rafts in plasma membranes by hybrid liposomes (HL-23) composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(23) dodecyl ether ($C_{12}(EO)_{23}$) for human leukemia HL-60 cells was observed for the first time. It was suggested that the induction of apoptosis by HL-23 could be related to an increase of membrane fluidity and the formation of lipid rafts in the plasma membranes of HL-60 cells.

Apoptosis, or programmed cell death, is a fundamental process in the development of multicellular organisms and it is distinguished from exogenous cell death necrosis. Although it is initiated by many physiologic and pathologic stimuli, all apoptotic cells undergo a similar sequence of morphological and biochemical events.[1] On the other hand, it is well known that many cancer cells are resistant to apoptosis due to the gene mutation of tumor suppressor protein p53,[2] the expression of antiapoptotic proteins such as Bcl-2,[3] and so on. Recently, some studies have reported that apoptosis were induced by antitumor drugs in cancer cells through the death receptor and/or mitochondrial pathways in which the cell membranes (plasma membranes) play an important role as a mediator of apoptotic processes in the initial stage.[4] These studies suggest that membrane targeted chemotherapy for the regulation of apoptosis pathways would be a novel approach to cancer therapy.[5]

Hybrid liposomes, first developed by Ueoka et al.,[6] can be prepared by simply sonicating a mixture of vesicular and micellar molecules in buffer solutions. We have reported the inhibitory effects of hybrid liposomes composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(20) sorbitan monolaurate (Tween 20) including bioactive substances such as antitumor drugs,[7a] sugar surfactants,[7b] and fatty acids[7c] on the growth of tumor cells in drug delivery systems. On the other hand, hybrid liposomes (HL-*n*) composed of DMPC and polyoxyethylene(*n*) alkyl ethers demonstrated remarkable inhibitory effects on the growth of various tumor cells in vitro[8] and in vivo.[8e,8f,9] Furthermore, the successful clinical chemotherapy with drug-free HL-*n* for patients with lymphoma has been reported.[9,10]

In the course of our study on the inhibitory effects of HL-*n* on the growth of human promyelocytic leukemia HL-60 cells, we have elucidated that HL-*n* can fuse and accumulate into the plasma membranes of HL-60 cells, activate the caspase cascades, and induce apoptosis in the cells.[11] It has been also demonstrated that the apoptotic signal first passes through mitochondria, caspase-9, and caspase-3, second through Fas, caspase-8, and caspase-3, and then reaches the nucleus.[11c] However, there have been few studies on the early processes for the plasma membranes of HL-60 cells leading to apoptosis induced by HL-*n*.



Scheme 1. Schematic representation of hybrid liposomes (HL-23) composed of DMPC and $C_{12}(EO)_{23}$.

In this study, we examined the inhibitory effects of hybrid liposomes (HL-23) composed of 90 mol % DMPC and 10 mol % polyoxyethylene(23) dodecyl ether ($C_{12}(EO)_{23}$) (Scheme 1) and the induction of apoptosis in HL-60 cells by HL-23 in relation to the increase of membrane fluidity and the formation of lipid rafts in the plasma membranes in vitro.

The hybrid liposomes (HL-23) were prepared by sonication (VS-N300, VELVO-CLEAR, Japan) of a mixture containing 90 mol % DMPC (NOF, Japan) and 10 mol % $C_{12}(EO)_{23}$ (Sigma Chemical, U.S.A.) in 5% glucose solution as described previously.[11c] The sample solutions were sterilized using a membrane filter with 0.20 μm pore size. Dynamic light-scattering measurements with an Otsuka Electronics ELS-8000 apparatus (Japan) showed that the size of HL-23 was less than 100 nm in diameter and remained stable for more than one month (data not shown).

We have reported previously that HL-*n* markedly inhibited the growth of human promyelocytic leukemia HL-60 cells in vitro.[11a,11c,11d] In addition, it was also indicated that the inhibitory effects of HL-*n* were attained through the induction of apoptosis in HL-60 cells on the basis of confocal laser microscopic observations, agarose gel electrophoresis, and flow cytometry analysis.[11] In this study, we confirmed the effects of HL-23 on the induction of apoptosis in HL-60 cells using flow cytometry. HL-60 cells (RIKEN Cell Bank, Japan) (1.0×10^5 cells/mL) were incubated with HL-23 ([DMPC] = 5.0×10^{-4} M) in a humidified atmosphere of 5% CO_2 at 37 °C, washed with phosphate buffered-saline (PBS(−)), resuspended in PBS(−) containing 500 μg mL^{-1} propidium iodide (PI), 0.25 μg mL^{-1} RNase, and 0.1% Triton X-100. Then, the content of apoptotic DNA fragments in HL-60 cells was analyzed using a flow cytometer (Epics XL system II, Beckman Coulter, U.S.A.) (excitation/detection = 488 nm/605–635 nm).[11a,11b] The results are shown in Figure 1. The rate increased rapidly at 2–4 h and

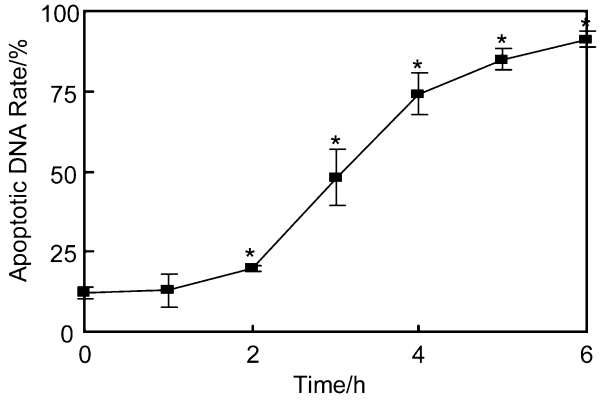


Figure 1. Apoptotic DNA rate of HL-60 cells after the treatment with HL-23. [DMPC] = 5.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 5.6×10^{-5} M. Error bars indicate SE for 2 individual experiments. *: Significant difference ($p < 0.05$) compared with the control (Student's t-test).

reached maximum at 6 h after the treatment with HL-23. This result indicates that HL-23 could effectively induce apoptosis in HL-60 cells.

It was also elucidated that the first step of inducing apoptosis could be related to the fusion and accumulation of HL-*n* into the plasma membranes of HL-60 cells as shown in microphysiometry analysis.[11a,11b] In this study, we examined the initial events of HL-23-indued apoptosis with a fluorescent lipid 1-palmitoyl-2-{12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-dodecanoyl}-*sn*-glycero-3-phosphocholine (NBDPC) (Avanti Polar Lipids, U.S.A.). HL-60 cells (2.5×10^5 cells/mL) were treated with HL-23 ([DMPC] = 1.0×10^{-4} M) containing NBDPC (4.7×10^{-6} M) and the fluorescence intensity was measured with a flow cytometer (excitation/detection = 488 nm/500–530 nm). The fluorescence intensity per cell increased rapidly at 1 min after treatment with HL-23 including NBDPC, followed by a gradual increase for 30 min as shown in Figure 2. This indicates that HL-23 could fuse with HL-60 cells within a few minutes and accumulate gradually for more than 30 min.

On the other hand, an increase in cell membrane fluidity has been observed along with the induction of apoptosis in cancer cells.[12] Therefore, we evaluated the membrane fluidity of HL-60 cells by the fluorescence polarization (*P*) of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Nacalai Tesque, Japan) in the plasma membranes, which reflects the microviscosity of the hydrophobic membranes around the fluorescent probe.[13] HL-60 cells (2.5×10^5 cells/mL) were labeled with DPH (1.0×10^{-6} M) and washed twice with PBS(−). After the incubation of HL-60 cells for 10 min, the *P* value was measured in the presence of HL-23 ([DMPC] = 0–1.0×10^{-4} M) using a Hitachi F-4500 fluorescence spectrophotometer (Japan).[14] The results are shown in Figure 3. The *P* value immediately decreased after the addition of HL-23, indicating the increase in membrane fluidity of HL-60 cells, in dose- and time-dependent manners. The rapid response of *P* value to HL-23 was in agreement with that of the fluorescence intensity of HL-60 cells to HL-23 including NBDPC shown in Figure 2. The increase in membrane fluidity could be related to the early fusion of HL-23 with HL-60 cells.[15]

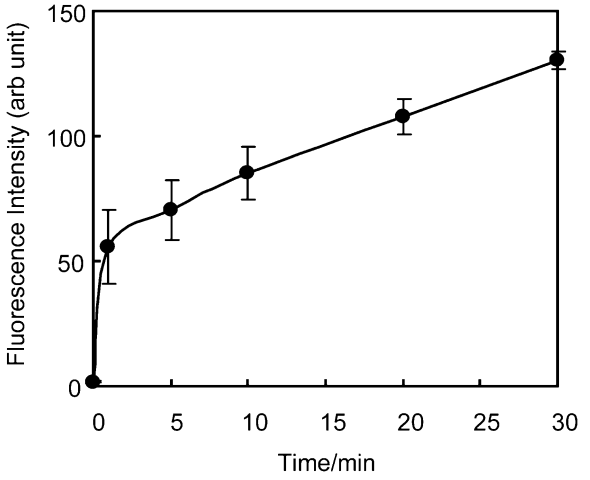


Figure 2. Fluorescence intensity of HL-60 cells after the treatment with HL-23 including NBDPC. [DMPC] = 1.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 1.1×10^{-5} M, [NBDPC] = 4.7×10^{-6} M. Error bars indicate SE for 3 individual experiments.

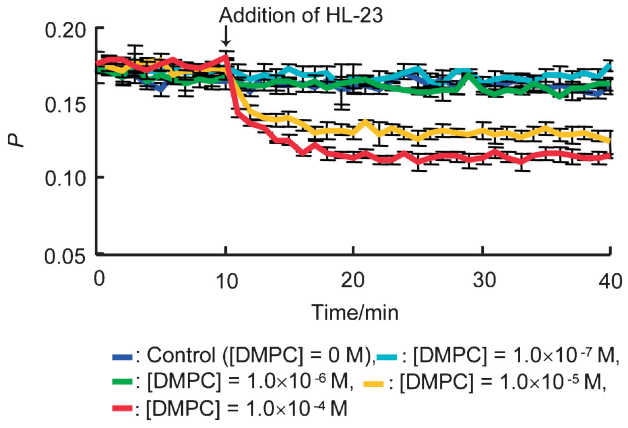


Figure 3. Fluorescence polarization (*P*) of DPH-labeled HL-60 cells after the treatment with HL-23. [DPH] = 1.0×10^{-6} M, [DMPC] = 0–1.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 0–1.1×10^{-5} M. Error bars indicate SE for 3 individual experiments.

It was reported that the anticancer drug cisplatin could induce the clustering of lipid microdomains "lipid rafts" enriched with cholesterol, sphingolipids, and ganglioside GM_1 in plasma membranes and could mediate apoptosis toward human colon carcinoma cells along with the increase in membrane fluidity.[4b,12b] Thus, we investigated the clustering of lipid rafts in plasma membranes of HL-60 cells with a marker Cholera toxin subunit B conjugates Alexa Fluor 647 (CTB) (Molecular Probes, U.S.A.), which binds to the pentasaccharide chains of ganglioside GM_1 on the cellular surfaces.[16] HL-60 cells (1.0×10^5 cells/mL) were treated with HL-23 ([DMPC] = 5.0×10^{-4} M) for 10 min, washed with cold PBS(−), stained with CTB (10 μg mL^{-1}) for 30 min on ice, and observed using a confocal laser microscope (excitation/detection = 633 nm/650–670 nm). The results are shown in Figure 4. The red fluorescence was concentrated to the distinct patches on the plasma membrane of HL-60 cells treated with HL-23, indicating that HL-23 could induce the clustering of lipid rafts in plasma

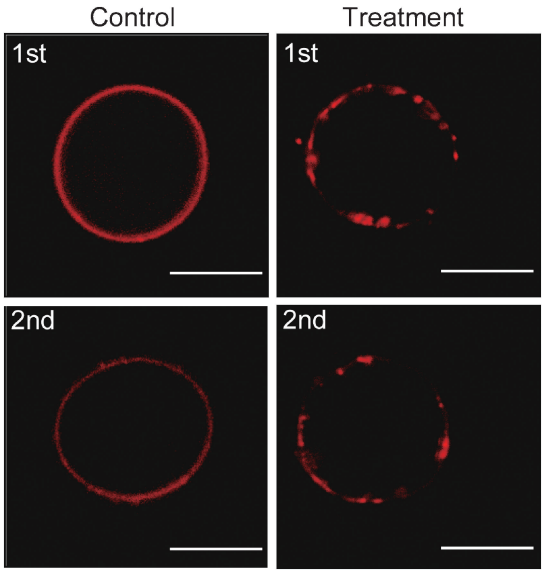


Figure 4. Fluorescence micrographs of CTB-labeled HL-60 cells treated with HL-23 for 10 min. [DMPC] = 5.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 5.6×10^{-5} M. The micrographs were obtained from 2 individual experiments. Scale bar, 10 μm.

membranes. On the other hand, the even red fluorescence from CTB was exhibited at the plasma membrane of HL-60 cells (control) untreated with HL-23, indicating the homogeneous distribution of lipid rafts in the plasma membranes. It is of interest that the clustering of lipid rafts could occur within 10 min after the treatment with HL-23. It is well known that the localized lipid rafts serve as platforms into which the apoptotic signaling molecules are recruited and are involved in the production of apoptosis inducer ceramides.[4,12b,17] Although the mechanistic details are not yet clear, the clustering of lipid rafts must be an important event of HL-23-induced apoptosis in the early stage. The findings in this study suggest that first HL-23 could fuse and accumulate into HL-60 cells, and next the apoptotic signals might be transferred through the localized lipid rafts in plasma membranes with high fluidity, and finally apoptosis should be induced.

In conclusion, we demonstrated for the first time that the hybrid liposomes (HL-23) composed of 90 mol % DMPC and 10 mol % polyoxyethylene(23) dodecyl ether ($C_{12}(EO)_{23}$) could induce the clustering of lipid rafts in the plasma membranes for HL-60 cells leading to apoptosis.

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14 The emission at 430 nm originating from DPH was monitored upon excitation at 360 nm using a fluorescence spectrophotometer. The fluorescence polarization (P) of DPH-labeled HL-60 was calculated by eq 1

$$P = (I_{vv} - C_f I_{vh})/(I_{vv} + C_f I_{vh}) \quad (1)$$

where I is the fluorescence intensity and the subscripts v and h refer to the orientations, vertical and horizontal, respectively, for the excitation and analyzer polarizers in this sequence: e.g., I_{vh} indicates the fluorescence intensity measured with a vertical excitation polarizer and a horizontal analyzer polarizer. C_f is the grating correction factor, given by I_{hv}/I_{hh}.

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