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Induction of apoptosis of human lung carcinoma cells by hybrid liposomes containing polyoxyethylenedodecyl ether

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Abstract

Hybrid liposomes can be prepared by simply ultrasonicating a mixture of vesicular and micellar molecules in aqueous solution. A clear solution of hybrid liposomes composed of 90 mol% dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(23)dodecyl ether ($C_{12}(EO)_{23}$) having a hydrodynamic diameter of 100–120 nm was obtained. Highly inhibitory effects of hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ on the growth of human lung carcinoma (RERF-LC-OK and A549) cells without any drugs were obtained. Induction of apoptosis by hybrid liposomes in RERF-LC-OK and A549 cells was verified on the basis of fluorescence microscopy, agarose gel electrophoresis of DNA and flow cytometry. We elucidated the pathway of apoptosis induced by hybrid liposomes as follows: (a) accumulation of hybrid liposomes in tumor cell membrane was revealed using microphysiometer. (b) Reduction of mitochodrial membrane potential and activation of caspase-9, -3 and -8 were obtained, indicating that apoptotic signal by hybrid liposomes should pass through mitochondria and these caspases. It is worthy to note that such a novel mechanism of apoptosis induced by hybrid liposomes without any drugs was performed for the first time in human lung carcinoma cells.

Keywords: Hybrid liposomes; Lung carcinoma; Apoptosis; Caspase; Antitumor effect

1. Introduction

It is well known that apoptosis is essential in many aspects of normal development and is required for maintaining tissue homeostasis. For instance, inappropriate activation or suppression of apoptosis lead to degenerative pathologies (AIDS, Alzheimer's disease and Parkinson's disease) or tumorigenesis, respectively

Abbreviations: PI, propidium iodide; RNase, RNA catabolic enzyme; Ac-DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-H (7-amino-4-trifluoromethylcoumarin); Ac-IETD-CHO, *N*-acetyl-Ile-Glu-Thr-Asp-H (aldehyde); Ac-LEHD-CHO, *N*-acetyl-Leu-Glu-His-Asp-H (aldehyde)

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(Bellamy et al., 1995; Thompson, 1995; Graeber et al., 1996). Consequently, control of apoptosis is an important potential target for therapeutic intervention. In particular, induction of apoptosis for solid tumor cells by chemotherapeutic drugs is known to be difficult as compared with floating cells such as leukemia. So, there have been a few reports on the mechanism of apoptosis for RERF-LC-OK and A549 cells derived from human lung carcinoma.

We recently produced hybrid liposomes prepared by ultrasonication of vesicular and micellar molecules in aqueous solution (Ueoka et al., 1985, 1988). The physical properties of these liposomes such as shape, size, and the temperatures of phase transition can be controlled by changing the constituents and compositional ratios. Hybrid liposomes have been effective for inhibiting the growth of various tumor cells in vitro (Matsumoto et al., 1995, 1999) and in vivo using animal model of carcinoma (Kanno et al., 1999). In addition, there have been no side effects of hybrid liposomes in the experiment using normal animals in vivo (Kitamura et al., 1996; Ueoka et al., 2000). After passing the bioethics committee, a prolonged survival was attained in the patient with malignant lymphoma after the intravenous injection of hybrid liposomes without drugs, and remarkable reduction of solid tumors was obtained after the local administration of hybrid liposomes (Ueoka et al., 2002). In this study, we attempted to investigate the induction of apoptosis by hybrid liposomes composed of DMPC and ($C_{12}(EO)_{23}$) without any drugs for solid tumor cells like lung carcinoma (RERF-LC-OK and A549) to promote the development of new therapeutic strategies in future.

2. Materials and methods

2.1. Preparation of hybrid liposomes

Hybrid liposomes without any drugs were prepared by sonication of a mixture containing vesicular molecules:L-α-dimyristoylphosphatidylcholine (DMPC, Nippon Oil and Fats Co. Ltd., purity > 99%) and micellar molecules:polyoxyethylene(23)dodecyl ether ($C_{12}(EO)_{23}$, SIGMA) using a bath type sonicator (VELVO VS-N300) in 5% glucose solution at 300 W. $C_{12}(EO)_{23}$ was purified by recrystallization from diethyl ether (Elworthy and Macfarlene, 1965).

2.2. Electron microscopy

Electron micrographs were obtained by means of negative-staining method. A sample solution of liposomes in 5% glucose solution was mixed with a 2% (w/w) aqueous solution of phosphatidic acid. The sample was then applied to a carbon grid and dried overnight in a vacuum desiccator at room temperature. Electron micrographs were taken on a Hitachi 300 electron microscope.

2.3. Dynamic light scattering measurement

The diameter of hybrid liposomes was measured using dynamic light scattering spectrometer (Otsuka ELS-8000) with He–Ne laser light source (633 nm). The diameter was calculated by Stokes–Einstein equation ($d_{hy} = kT/3\pi\eta D$), where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient.

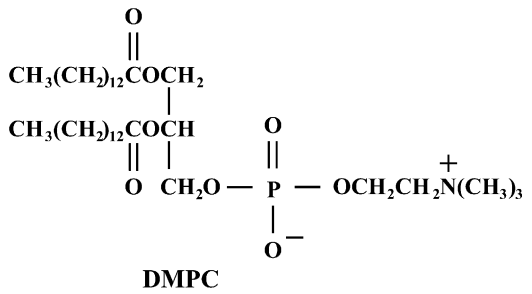
2.4. Cell culture

Human lung carcinoma cell lines (RERF-LC-OK and A549) were obtained from Riken Cell Bank. RERF-LC-OK cells were cultured in RPMI 1640 medium and A549 cells were cultured in DMEM medium containing penicillin (100 units ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C.

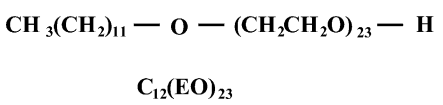
2.5. Assessment of inhibition of hybrid liposomes

WST-1 (2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay was performed by the method in the literature (Ishiyama et al., 1993). RERF-LC-OK and A549 cells were seeded at a density of 1×10^4 cells ml^{-1} in 96-well plate and cultured overnight in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were incubated for 48 h in the medium that contained hybrid liposomes composed of DMPC and $C_{12}(EO)_{23}$. DMPC and $C_{12}(EO)_{23}$ concentration in the medium was 0.3 and 0.03 mM, respectively. WST-1 solution was added and cultured for 3 h, and then the absorbance at wavelength of 450 nm was measured by spectrophotometor (Molecular Device). The inhibitory effects of hybrid liposomes on the

growth of tumor cells were evaluated by the equation of $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote absorbance of water-soluble formazan in the presence and absence of hybrid liposomes, respectively.



DMPC



$C_{12}(EO)_{23}$

2.6. Fluorescent microscopy

Microscopic analysis of cell death was performed as follows. Tumor cells were treated with the hybrid liposomes for 48 h. Nuclear DNA condensation and fragmentation (YO-PRO-1 staining) (Idziorek et al., 1995) were analyzed using both an optical and a fluorescent microscope (TCS NT; Leica, Germany). The cells were stained with YO-PRO-1 (excitation/emission (nm) = 491/509, green color) and PI (excitation/emission (nm) = 493/635, red color) when bound to DNA, using 75 mW argon laser of excitation 488 nm for YO-PRO-1 and excitation 543 nm for PI, respectively.

2.7. Agarose gel electrophoresis

The cells were incubated with the medium contained hybrid liposomes composed of DMPC and $C_{12}(EO)_{23}$ for 0.5, 1, 2, 3 and 6 h, centrifuged at $1000 \times g$ for 5 min, and resuspended in 100 μl of chilled cell lysis buffer. The cytosolic extract was microcentrifuged at $15{,}000 \times g$ for 5 min. The supernatants containing nucleic acid were treated with RNase and proteinase for 1 h. Electrophoresis of DNA was performed on 1.5% agarose gels in TBE buffer (0.45 M Tris–Borate, 0.01 M EDTA) at 100 V/cm by Mini-Gel Electrophoresis System (Mupid-2, COSMO BIO). After electrophoresis, the gels were stained with ethidium bromides (1 mg ml^{-1}), and photographed under UV transilluminator (Lumi PRO TAITEC).

2.8. Flow cytometry

Apoptotic DNA rate in tumor cells were measured by the method of flow cytometry. The cells were treated with hybrid liposomes for 0.5, 1, 2, 3, 4, 5 and 6 h, centrifuged at $1000 \times g$ for 5 min, washed with PBS(−) and fixed in chilled-ethanol. The cells were washed again, treated with RNase (0.25 mg ml^{-1}) and then stained with PI (0.5 mg ml^{-1}) that has 493 nm excitation and 635 nm emission wavelength. The samples were analyzed using flow cytometer (Epics XL system II, Beckman Coulter) with a single excitation 488 nm of 15 mW argon laser. The PI signals were detected by FL3 sensor in 605–635 nm.

2.9. Cytosensor microphysiometer analysis

Acidification-rate responses in tumor cells were detected by silicon biosensor in cytosensor microphysiometer (Molecular Devices). The silicon biosensor detects a wide variety of chemical and physical stimuli in the cell membranes (Parce et al., 1989). The cells (RERF-LC-OK and A549) on cover slips in the silicon biosensor were continuously treated with hybrid liposomes composed of DMPC and $C_{12}(EO)_{23}$. Acidification rate of the cells after the treatment with hybrid liposomes was measured every 2 min.

2.10. Caspase fluorometric protease assay

Activation of caspases was measured on the bases of caspase fluorometric protease assay (Medical & Biological Laboratories) (Casciola-Rosen et al., 1996; Lazebnik et al., 1994). Tumor cells were treated with hybrid liposomes for 0.5, 1, 2, 3 and 6 h. The cells were centrifuged at $1000 \times g$ for 5 min, and resuspended in 50 μl of chilled cell lysis buffer(10% Triton X-100, 1 M Tris–HCl, 0.5 M EDTA, pH 8.0). The cell lysates were incubated with Ac-DEVD-AFC (50 μM; caspase-3 substrate), IETD-AFC (50 μM;

caspase-8 substrate) or LEHD-AFC (50 μM; caspase-9 substrate) at 37 °C for 2 h. The specific activities were determined fluorometrically at 400 nm excitation and 505 nm emission for AFC of Fluoroskan Ascent CF Fluorometer (Thermo Labsystems).

2.11. Confirmation of caspase cascade using caspase inhibitor

Cells were pretreated with caspase-8 (300 μM; Ac-IETD-CHO) and caspase-9 (300 μM; Ac-LEHD-CHO) inhibitors (Peptide Institute Inc.) for 2 h (Han et al., 1997; Garcia-Calvo et al., 1998; Gastman et al., 2000). The cells were treated with the hybrid liposomes for 30 min. The inhibition of apoptosis by the caspase-8 and caspase-9 inhibitors was evaluated by measurement of the above-mentioned caspase-3 activities.

2.12. Mitochondrial membrane potential

Cells were incubated with the hybrid liposomes for 30 min, and then 2.5 μl of 3,3-dihexyloxacarbocyanine iodide [$DiOC_6$ (3)] (Molecular Probes) were added to evaluate mitochondrial transmembrane potential ($\Delta\Psi_m$) and incubated at 37 °C for 20 min. The cells were centrifuged, suspended with 500 μl of PBS and were used for flow cytometric analysis. 15 mW 488 nm air-cooling Ar laser and FL1 sensor (505–545 nm) were used.

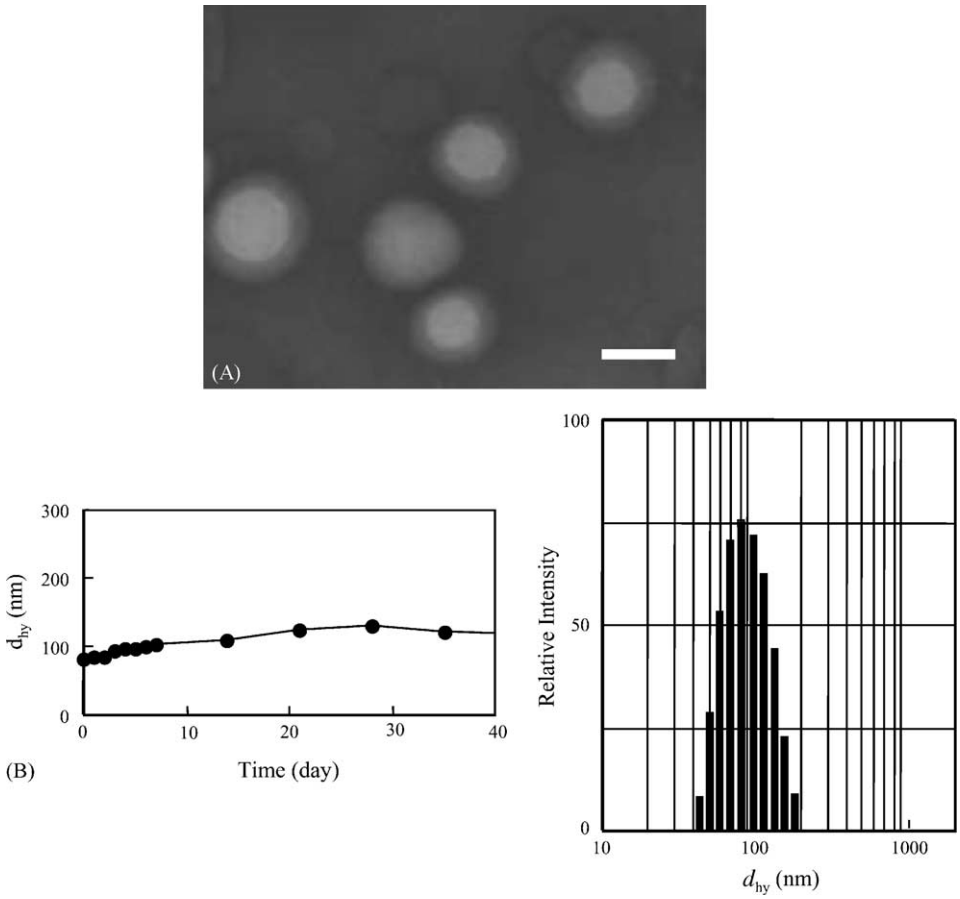


Fig. 1. (A) An electron micrograph of hybrid liposomes composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ using negative staining method. Scale bar: 100 nm. (B) Time course of d_{hy} change and distribution of d_{hy} for the hybrid liposomes.

3. Results and discussion

3.1. Morphology of hybrid liposomes

Morphology of hybrid liposomes composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ was examined on the bases of electron microscopy. An electron micrograph of hybrid liposomes showed the presence of spherical vesicles with a diameter of 100–120 nm as shown in Fig. 1(A). A clear solution of hybrid liposomes having hydrodynamic diameter of 120 nm with single and narrow distribution could be kept over one month with the method of dynamic light scattering measurement as shown in Fig. 1(B).

3.2. Inhibitory effects of hybrid liposomes on the growth of RERF-LC-OK and A549 cells

RERF-LC-OK and A549 cells were used for the evaluation of inhibitory effects of hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ on the growth of lung carcinoma cells. The results are shown in Fig. 2 No significant inhibitory effects of individual component (DMPC vesicles or $C_{12}(EO)_{23}$ micelles) on the growth of RERF-LC-OK and A549 cells were obtained. Interestingly, remarkably high inhibitory effects of hybrid liposomes were obtained. These results indicate that hybrid liposomes themselves without any antitumor drugs should be effective for inhibiting the growth of tumor cells.

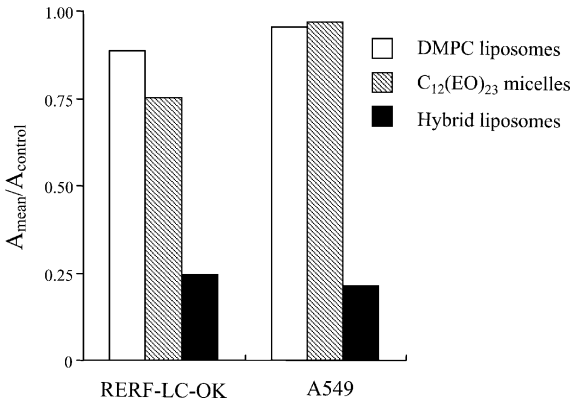


Fig. 2. Inhibitory effects of hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ on the growth of RERF-LC-OK and A549 cells. The cells were seeded at a density of 1×10^4 cells ml^{-1} in tissue culture dish and incubated in the medium that contained the hybrid liposomes in a 5% CO_2 humidified incubator at 37 °C for 48 h. The inhibitory effects of hybrid liposomes on the growth of tumor cells were evaluated by the equation of $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote absorbance of water-soluble formazan in the presence and absence of hybrid liposomes, respectively. [DMPC] = 0.3 mM, [$C_{12}(EO)_{23}$] = 0.03 mM.

3.3. Induction of apoptosis by hybrid liposomes

Fluorescent micrographs of RERF-LC-OK and A549 cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ are shown in Fig. 3. The green color (YO-PRO-1) was observed in RERF-LC-OK and A549 cells that could be characterized nuclear DNA fragmentation in apoptosis cells. Conversely, no red or orange (YO-PRO-1 or PI) color of necrosis cells was observed. These results indicate that hybrid liposomes should induce apoptosis for RERF-LC-OK and A549 cells.

We examined the nuclear DNA fragmentation with the hybrid liposomes using agarose gel electrophoresis. The results are shown in Fig. 4. It is noteworthy that exposure of RERF-LC-OK and A549 cells to the hybrid liposomes caused DNA fragmentation

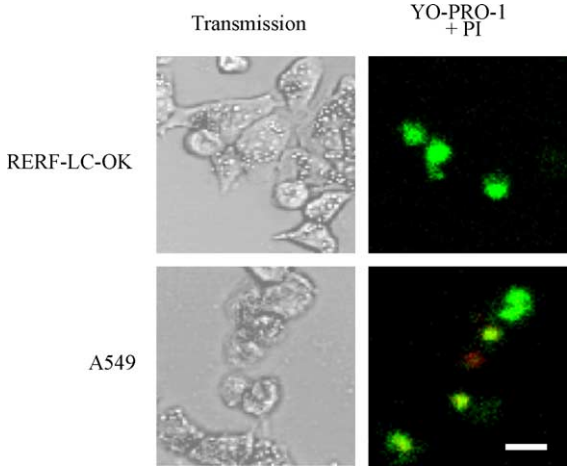


Fig. 3. Fluorescent micrographs of RERF-LC-OK and A549 cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. Scale bar: 40 μm. The cells were stained with YO-PRO-1 (excitation/emission (nm) = 491/509, green color) and PI (excitation/emission (nm) = 493/635, red color) when bound to DNA, using 75 mW argon laser of excitation 488 nm for YO-PRO-1 and excitation 543 nm for PI, respectively after the cells (1×10^5 cells ml^{-1}) were incubated in the medium that contained the hybrid liposomes at 37 °C for 48 h. [DMPC] = 0.3 mM, [$C_{12}(EO)_{23}$] = 0.03 mM.

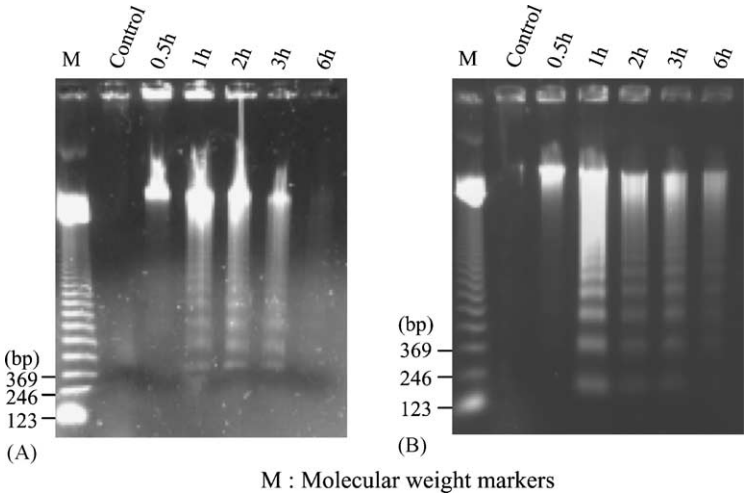


Fig. 4. Agarose gel electrophoresis of DNA from RERF-LC-OK (A) and A549 (B) cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. The cells were seeded at a density of 5×10^5 cells ml^{-1} in tissue culture dish and incubated in the medium that contained the hybrid liposomes for 0.5, 1, 2, 3 and 6 h. The supernatants containing nucleic acid were treated with RNase and proteinase for 1 h. Electrophoresis of DNA was performed on 1.5% agarose gels in TBE buffer (0.45 M Tris-Borate, 0.01 M EDTA) at 100 V/cm by Mini-Gel Electrophoresis System (Mupid-2, COSMO BIO). After electrophoresis, the gels were stained with ethidium bromides (1 mg ml^{-1}), and photographed under UV transilluminator (Lumi PRO TAITEC). [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

characteristic of apoptosis. After the treatment with DMPC vesicles or $C_{12}(EO)_{23}$ micelles, no DNA ladder pattern was observed (results not shown). Furthermore, the time course of DNA fragmentation in RERF-LC-OK and A549 cells treated with the hybrid liposomes was detected using flow cytometer as shown in Fig. 5. Apoptotic DNA reached a constant state at 3 h after the treatment with the hybrid liposomes. These results indicate that the hybrid liposomes certainly induced apoptosis in RERF-LC-OK and A549 cells.

3.4. Intracellular responses by hybrid liposomes

The accumulation of hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ in the tumor cell membranes were investigated using cytosensor microphysiometer. Cytosensor microphysiometer measures the change in extracellular acidification rate resulting either from alterations in the energy demand made on the cells as they respond to the effector agents or from alterations in sodium–hydrogen exchange across the cell membrane (Parce et al., 1989). The results are shown in Fig. 6. The acidification rate gradually increased to reach a maximum value, and thereafter it decreased. It is presumed that the increase in acidification was

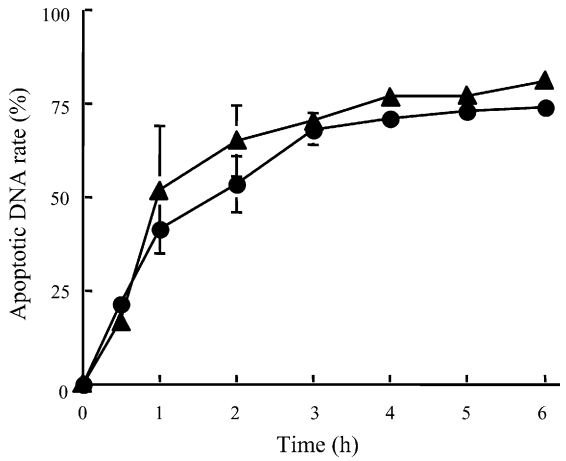


Fig. 5. Apoptotic DNA rate for RERF-LC-OK (●) and A549 (▲) cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. The cells were seeded at a density of 5×10^5 cells ml^{-1} in tissue culture dish and incubated in the medium that contained the hybrid liposomes at 37 °C for 0.5, 1, 2, 3, 4, 5 and 6 h. The cells were washed, treated with RNase (0.25 mg ml^{-1}) and then stained with PI (0.5 mg ml^{-1}) that has 493 nm excitation and 635 nm emission wavelength. [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

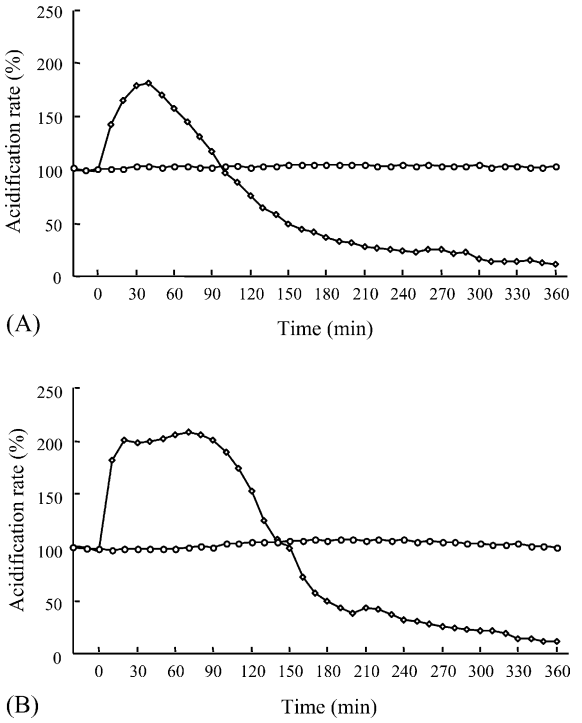


Fig. 6. Acidification rate for RERF-LC-OK (A) and A549 (B) cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. Acidification rate of the cells after the treatment with the hybrid liposomes was measured at 37 °C every 2 min for 6 h. [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

caused by activation of some membrane proteins in the cells and the decrease in acidification was caused by subsequent apoptosis of RERF-LC-OK and A549 cells (Baxter et al., 1999).

3.5. Activation of caspases by hybrid liposomes

Activation of caspases is an indispensable process in the execution phase of apoptosis. In particular, activation of caspase-3 plays an important role in leads to the nuclear DNA fragmentation and apoptotic cell death (Nunez et al., 1998; Zou et al., 1997). Time courses for activating caspase-3 by hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ are shown in Fig. 7. The caspase-3 activity in RERF-LC-OK or A549 cells drastically increased at 30 min after the treatment with the hybrid liposomes. On the other hand, no significant increase of caspase-3 activation was obtained after the treatment with DMPC vesicles or $C_{12}(EO)_{23}$ micelles. Furthermore, we investigated the blocking for caspase-3 inhibitors of caspase-family proteases (Xiang et al., 1996). Inhibitors of caspase-8 (Ac-IETD-CHO) and caspase-9 (Ac-LEHD-CHO) are employed for verifying all of the caspase cascades. Results for RERF-LC-OK and A549 cells are presented (Fig. 8). The activation of caspase-3 (substrate: Ac-DEVD-AFC) induced by the hybrid liposomes was almost completely inhibited by the caspase-8 inhibitor, but was partially inhibited by the caspase-9 inhibitor. These results indicate that the hybrid liposomes should execute apoptosis through caspase-3, caspase-8 and caspase-9 in the caspase cascade.

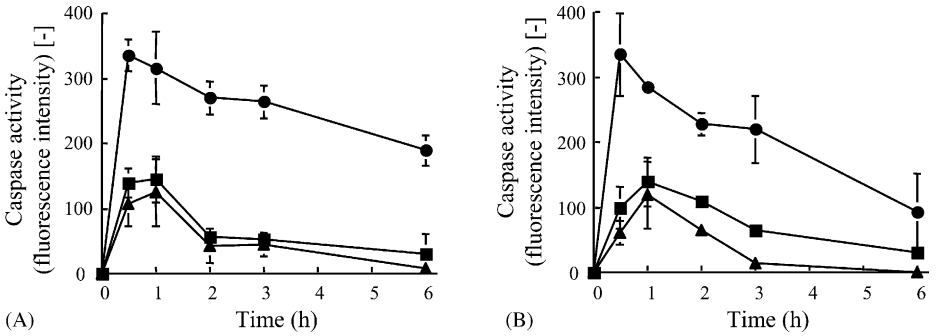


Fig. 7. Time courses for activation of caspases of RERF-LC-OK (A) and A549 (B) cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. Caspase-3 (●), caspase-8 (▲) and caspase-9 (■) were employed in these experiments. The cells were seeded at a density of 5×10^5 cells ml^{-1} in tissue culture dish and incubated in the medium that contained the hybrid liposomes at 37 °C for 48 h. Activation of caspases was measured on the bases of caspase fluorometric protease assay as described in Section 2. [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

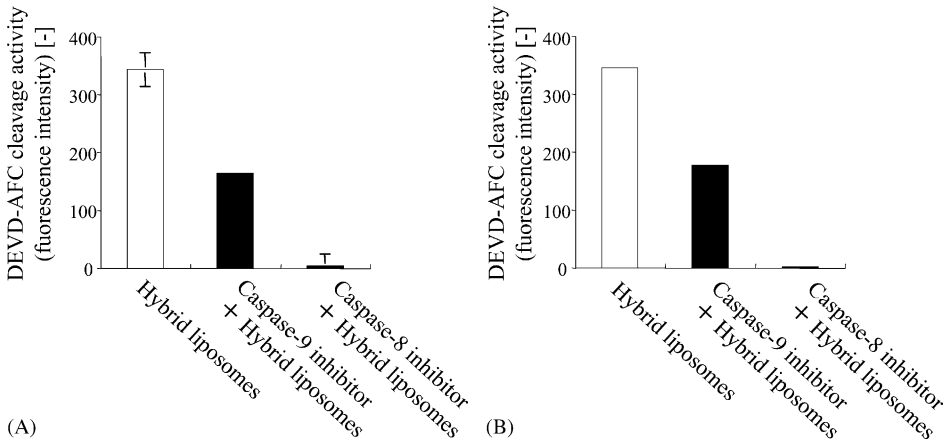


Fig. 8. Blocking of apoptosis by caspase-8 or caspase-9 inhibitors. RERF-LC-OK (A) and A549 (B) cells were treated with the hybrid liposomes at 37 °C for 30 min after the pretreated with 300 μM of caspase-8 or caspase-9 inhibitors. Activation of caspase-3 was measured on the bases of caspase fluorometric protease assay. [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

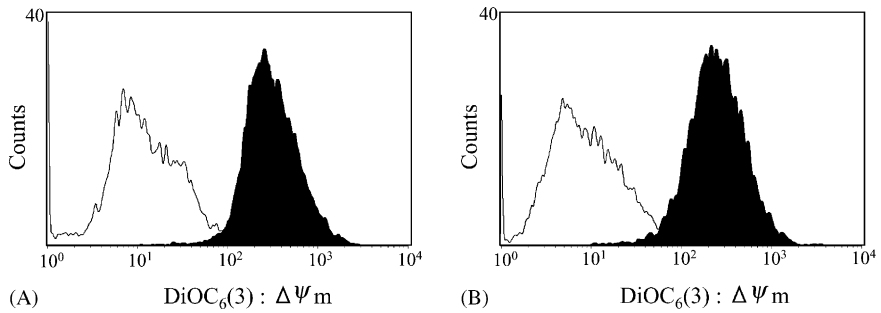


Fig. 9. $\Delta\Psi_m$ disruption of mitochondria in RERF-LC-OK (A) and A549 (B) cells by hybrid liposomes. Cells were incubated with hybrid liposomes at 37 °C for 30 min and then 2.5 μl of 3,3-dihexyloxacarbocyanine iodide [$DiOC_6$ (3)] were added to evaluate mitochondrial transmembrane potential ($\Delta\Psi_m$) and incubated at 37 °C for 20 min. The cells were centrifuged and used for flow cytometric analysis. [DMPC] = 11.5 mM, [$C_{12}(EO)_{23}$] = 1.28 mM.

Next, we examined the mitochondrial pathway for apoptotic signal transduction by the hybrid liposomes using flow cytometry. The results are shown in Fig. 9. Interestingly, mitochondrial transmembrane potential was decreased after the treatment with hybrid liposomes, suggesting that the mitochondrial pathway should be also implicated in apoptosis induced by the hybrid liposome.

4. Conclusion

We clearly demonstrated that hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ induced apoptosis in human lung carcinoma cells in this study. We elucidated a pathway of apoptosis induced by the hybrid liposomes as follows: (a) accumulation of the hybrid liposomes in tumor cell membrane was revealed using microphysiometer. (b) Reduction of mitochondrial membrane potential and activation of caspase-9, -3 and -8 were observed, indicating that apoptotic signal by the hybrid liposomes should pass through mitochondria and these caspases. It is worthy to note that such a novel mechanism of apoptosis induced by the hybrid liposomes without any drugs was performed for the first time in human lung carcinoma cells. We are now in the process of trying to establish a novel approach to chemotherapy with hybrid liposomes in clinical stud-

ies, which should be a promising therapy for patients with carcinoma.

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