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Chemotherapy with hybrid liposomes for human breast tumors along with apoptosis *in vivo*

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ABSTRACT

Hybrid liposomes (HL-*n*) can be prepared by dissolving both vesicular and micellar molecules in buffer solution with ultrasonication. A clear solution of HL-*n* composed of 95 mol% dimyristoylphosphatidylcholine (DMPC) and 5 mol% polyoxyethylenedodecyl ether ($C_{12}(EO)_n$, $n = 21$ and 25) having hydrodynamic diameter of 100 nm could be kept over 1 month on the basis of dynamic light scattering measurements was obtained. (1) Increases of fusion and accumulation of HL-*n* including NBDPC as a fluorescence probe into human breast tumor (MDA-MB-453) cell membranes were observed. (2) Reduction of mitochondrial membrane potential and activation of caspase-8, caspase-9, and caspase-3 were observed, indicating that apoptotic signal by HL-*n* should pass through mitochondria and these caspases. (3) Remarkable reduction of tumor volume in a xenograft mice model intravenously treated with HL-*n* without drugs after the subcutaneously inoculation of MDA-MB-453 cells was verified *in vivo*. Induction of apoptosis in tumor of xenograft mice treated with HL-*n* was observed in micrographs on the basis of TUNEL method. It was noteworthy that the therapeutic effects of HL-*n* along with apoptosis were obtained for xenograft mice model of human breast tumor *in vivo*.

1. Introduction

Chemotherapy with anticancer drugs for the whole body is one of the most widely used treatments for breast cancer. However, most anticancer drugs are accompanied with severe side effects (Postma et al., 1995). So, drugs without side effects are necessary to lead to high quality of life (QOL) for patients. Molecular targeted therapy for tumors has recently attracted attention in connection with reducing the toxicity of anticancer drugs. For example, human epidermal growth factor receptor (HER-2) is overexpressed in 25–30% of patients with breast cancer (Slamon et al., 1987, 1989), and therefore anti-HER-2 antibody targeting HER-2 has been developed (Baselga et al., 1996; Cobleigh et al., 1999; Slamon et al., 2001). However, an anti-HER-2 antibody is effective for only the patients expressing HER-2 (Slamon et al., 1987, 1989), but this has also side effects (Slamon et al., 2001).

Liposomes are closed vesicles that are formed when phospholipids (constituents of biological membranes) are dispersed in water at relatively low concentrations. Since Bangham et al. discovered liposomes in 1965 (Bangham et al., 1965), these liposomes have been studied for chemical and medical applications. Especially, liposomes have contributed significantly to drug delivery, and for analyzing the cellular function, due to their mimicry of biological membranes and closed properties (Graybill et al., 1982; Gabizon et al., 1994). The drugs include antitumor agents, hormones, and immunomodulation (Papahadjopoulos and Vail, 1978; Lopez-Berestein and Finder, 1989). We have produced hybrid liposomes (HL) composed of vesicular and micellar molecules (Ueoka et al., 1985). The physical properties of HL such as shape, size, membrane fluidity, and the temperature of phase transition can be controlled by changing the constituents and compositional ratios. We have reported that HL was effective as the reaction fields for steric control in the enantioselective hydrolysis of amino acid esters (Ueoka et al., 1986, 1988).

Abbreviations: TUNEL, terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick end labeling.

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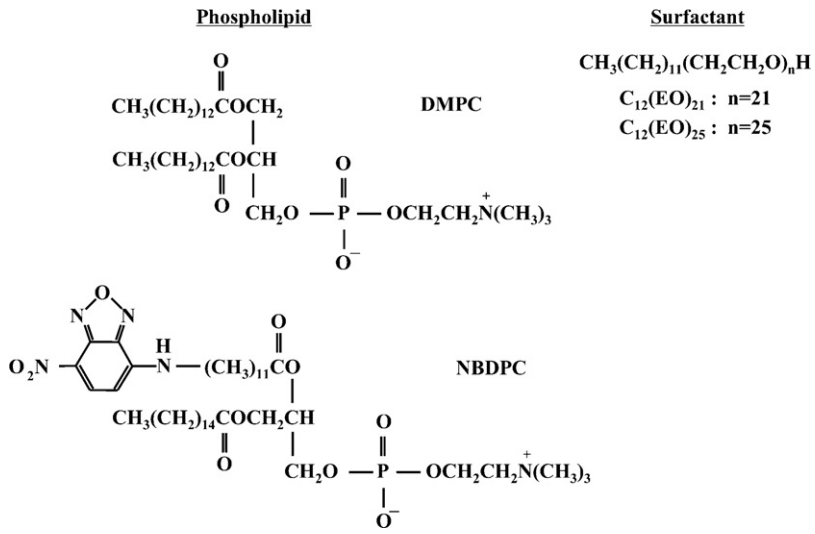

On the other hand, inhibitory effects of HL composed of L-α-dimyristoylphosphatidyl choline (DMPC) and polyoxyethylene (20) sorbitan monolaurate (Tween 20) including antitumor drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Kitamura et al., 1996) have been observed on the growth of glioma cells *in vitro* and *in vivo*. Furthermore, HL composed of DMPC and polyoxyethylenealkyl ethers without any drugs have remarkable inhibitory effects on the growth of tumor cells *in vitro* (Matsumoto et al., 1999) and *in vivo* (Ueoka et al., 2002). Successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported after passing the committee of bioethics (Ichihara et al., 2008). In addition, we elucidated the mechanistic details of apoptosis of tumor cells induced by HL (Matsumoto et al., 2005) and the correlation between antitumor effects and membrane fluidity of HL (Komizu et al., 2006). However, apoptotic pathway *in vitro* and therapeutic effects *in vivo* of HL for human breast tumor (MDA-MB-453) cells have not yet been elucidated. In this study, we investigated the induction of apoptosis by HL composed of DMPC and polyoxyethylenedodecyl ether ($C_{12}(EO)_n$, $n = 21$ and 25) without any drugs for MDA-MB-453 cells *in vitro* and therapeutic effects of HL using xenograft mice model of human breast cancer *in vivo*. Furthermore, the toxicity of HL was examined using normal mice *in vivo*.

2. Materials and methods

2.1. Preparation of hybrid liposomes

Hybrid liposomes (HL-*n*) were prepared by sonication of a mixture containing 95 mol% L-α-dimyristoylphosphatidylcholine (DMPC, Nippon oil and Fats Co. Ltd., Japan) and 5 mol% polyoxyethylenedodecyl ether ($C_{12}(EO)_{21}$ and $C_{12}(EO)_{25}$, Nikko chemicals Co. Ltd., Japan) using bath type sonicater in 5% glucose solution at 45 °C with 300 W, and filtered with a 0.20-μm cellulose acetate filter (Advantec, Japan).

2.2. Cell culture

Human breast tumor (MDA-MB-453) cell lines were obtained from Riken Cell Banc. Cells were cultured in L-15 medium (GIBCO, U.S.A.) supplement with penicillin (100 unit/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS, HyClone Laboratories Inc., U.S.A.) in humidified atmosphere at 37 °C.

2.3. Observation of fusion and accumulation

MDA-MB-453 cells (6.0×10^6 cells) were treated with HL-*n* including 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBDPC, Avanti Polar Lipid, U.S.A., [DMPC] = 0.61 mM, [$C_{12}(EO)_n$] = 0.024 mM, [NBDPC] = 0.020 mM) and were observed with confocal laser microscope (TCS-SP, Leica Microsystem, Germany) using a 488 nm Ar laser (detection, 505–555 nm).

2.4. Caspase fluorometric protease assay

Activation of caspases was measured on the basis of caspase fluorometric protease assay (Medical and Biological Laboratories, Nagoya, Japan, Casciola-Rosen et al., 1996). MDA-MB-453 cells (6.0×10^6 cells) were treated with HL-*n* ([DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM) for 0.5–6 h. The cells were centrifuged at 3000 rpm for 5 min, and resuspended in 50 μl of chilled cell lysis buffer. The cell lysates were incubated with reaction buffer (50 μl) and respective caspase substrate (50 μl) 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, Helsinki, Finland). Caspase activity was calculated by the following equation, Caspase activity = $I_{\text{treatment}} - I_{\text{control}}$, where $I_{\text{treatment}}$ and I_{control} was fluorescence intensity of AFC with and without HL, respectively.

2.5. Mitochondrial membrane potential

MDA-MB-453 cells (6.0×10^6 cells) were treated with HL-*n* ([DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM) for 0.5 h. 3,3′-dihexyloxacarbocyanine iodide (40 nM, $DiOC_6(3)$, Molecular Probe) were added to evaluate mitochondrial membrane potential ($\Delta\Psi_m$) and incubated at 37 °C for 20 min. The cells were centrifuged at 3000 rpm for 5 min, suspended with 500 μl of PBS(-) and were used for flow cytometric analysis using flow cytometer (Epics XL system II, Beckman Coulter). 15 mW 488 nm air-cooling Ar laser and FL1 (505–545 nm) were used.

2.6. Detection of cytosolic cytochrome c

Mitochondrial separation was performed using ApoAlert Cell Fractionation Kit (Clontech Laboratories Inc, U.S.A.). MDA-MB-453 cells (6.0×10^6 cells) were treated with HL-*n* ([DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM) for 0.5–1 h. The cells were harvested using cell scraper and washed with cell wash buffer. The cell pellet was

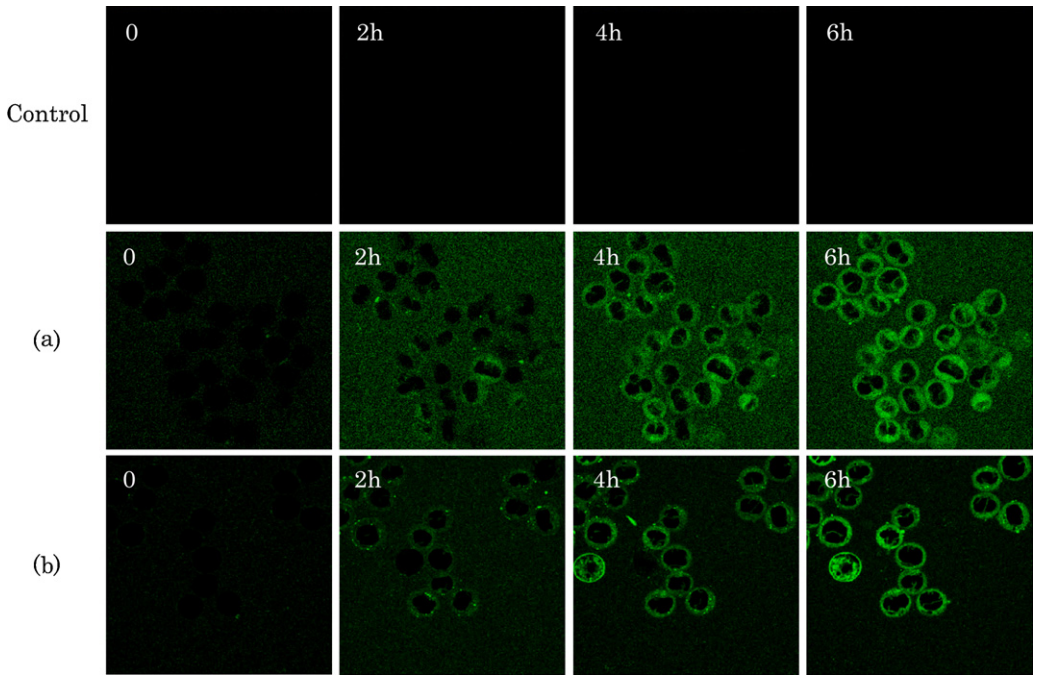

Fig. 1. Fluorescence micrographs of MDA-MB-453 cells after the treatment with HL-21 (a) and HL-25 (b) including fluorescence probe using confocal laser microscopy. [DMPC] = 0.45 mM, [$C_{12}(EO)_n$] = 0.025 mM, [NBDPC] = 0.020 mM, HL-*n*:91 mol% DMPC/5 mol% $C_{12}(EO)_n$/4 mol% NBDPC. Increases in accumulation of HL-*n* including fluorescence probe into MDA-MB-453 cell membrane were observed for 6 h.

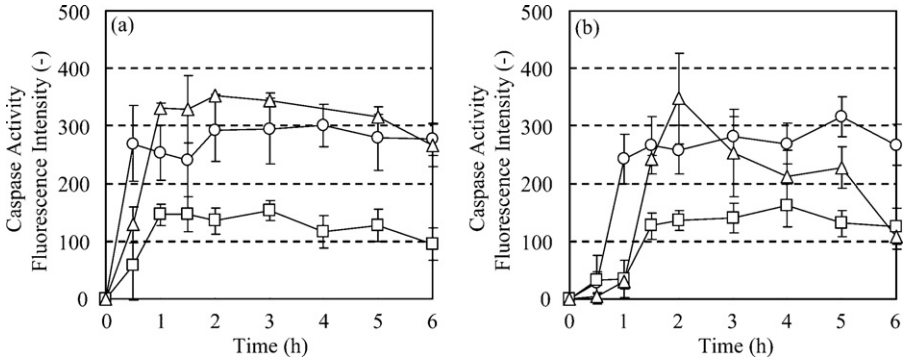

Fig. 2. Activation of caspase of MDA-MB-453 cells after the treatment with HL-21 (a) and HL-25 (b). (○) Caspase-3, (□) Caspase-8, (△) Caspase-9. [DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM, data presented are mean (n = 3) ± S.D. The activity of caspase-3 in MDA-MB-453 cells drastically increased at 30 min and 1 h after the treatment with HL-21 and HL-25, respectively. In the case of caspase-9, drastic increases were observed at 1 and 1.5 h after the treatment with HL-21 and HL-25, respectively, although moderate activation of caspase-8 was obtained.

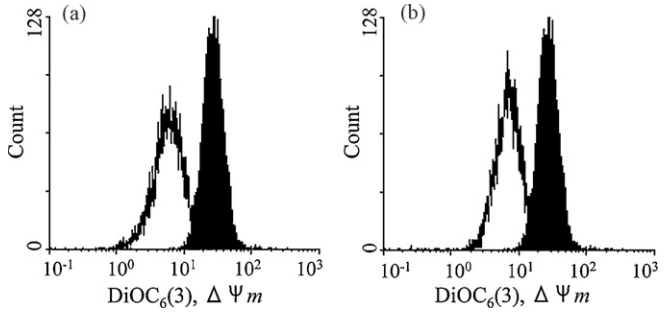

Fig. 3. Decrease of mitochondrial membrane potential for MDA-MB-453 cells after the treatment with HL-21 (a) and HL-25 (b) for 30 min. Control (shaded histogram), HL-*n* (unshaded histogram), [DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM. Mitochondorial transmembrane potential ($\Delta\Psi_m$) was decreased after the treatment with HL-*n* for 30 min.

resuspended in fractionation buffer (800 μl) treated with 1 mM DTT and protease inhibitor cocktail, and then incubated on ice for 10 min. Cells were homogenized by dounce tissue grinder on ice and centrifuged at 2800 rpm for 10 min at 4 °C. The super-

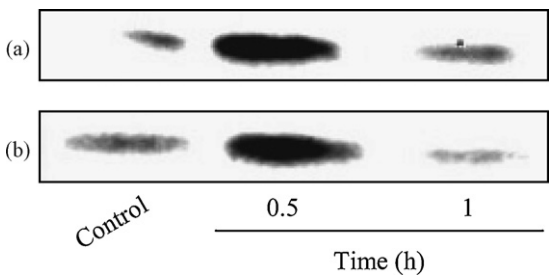

Fig. 4. Release of mitochondrial cytochrome c in MDA-MB-453 cells after the treatment with HL-21 (a) and HL-25 (b). [DMPC] = 11.5 mM, [$C_{12}(EO)_n$] = 0.61 mM. The cytochrome *c* released from mitochondria was detected 30 min after the treatment with HL-*n*.

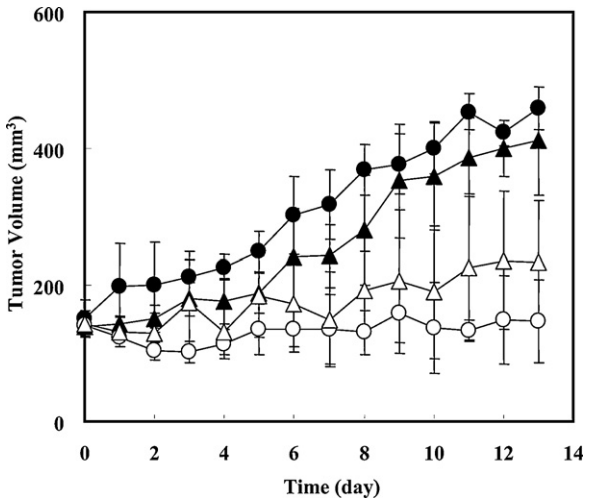

Fig. 5. Reduction of tumor volume in xenograft model mice intravenously treated with hybrid liposomes (HL-*n*) after subcutaneous inoculation of MDA-MB-453 cells. (●) Control, (▲) DMPC Liposome, (○) HL-21, (△) HL-25. HL-21: $P < 0.01$, HL-25: $P < 0.05$. Dose for DMPC = 203 mg/kg, data presented are mean ± S.D. Remarkable reduction of tumor volume (50% in HL-25 and 70% in HL-21, respectively) was obtained in xenograft mice models intravenously treated with HL-*n* without drugs after the subcutaneously inoculating MDA-MB-453 cells.

natant was removed to a 1.5 ml microcentrifuge tube, and then centrifuged at 10,500 rpm for 10 min at 4 °C. The supernatant was designated the cytosolic fraction whereas the pellet, which contains the mitochondria, was designated the mitochondrial fraction. The concentration of protein in the collected supernatant was determined using -Proteostain- Protein Quantification Kit-Wide Range (Dojindo Laboratories, Japan). Protein of cytosolic fraction (40 μg) was electrophoresed on 12.5% SDS-polyacrylamidegels (Bio-Rad, Richmond, CA) and transferred polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA). The PVDF membrane was washed with TBS-T (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20) and treated with 5% ECL Blocking Agent (dried milk powder, GE Healthcare Bio-Science Corp., USA) in TBS-T for 1 h at room temperature. The PVDF membrane was washed twice with TBS-T, immunoblotted with rabbit anti-human cytochrome *c* polyclonal antibody (Clontech Laboratories Inc., USA) for over night at 4 °C. The PVDF membrane washed three times with TBS-T, and incubated with the peroxidase-conjugated affinity-purified secondary antibodies (ECL Plus Western Blotting Reagent Pack, GE Healthcare Bio-Science Corp., U.S.A.) for 1 h at room temperature. After washing three times with TBS-T, the specific signals were detected by AISIN SEIKI LumiVisionPRO (Japan) with ECL Plus Western Blotting Detection Reagents.

2.7. Therapeutic effects of hybrid liposomes in vivo

Female nude mice (BALB/cA Jcl-*nu*/*nu*) were obtained from CLEA Japan, Inc. The animals were handled in accordance with the guidelines for animal experimentation of Japanese law during the study. MDA-MB-453 cells (5.0×10^6 cells) suspended into matrigel (BD Co., U.S.A.) were subcutaneously inoculated to dorsal of mice. The mice were randomly grouped on the basis of the tumor volume of mice using the stratified randomization method after confirming increase of tumor volume in mice. Number of mice was three in each group. The tumor volume reached 100–200 mm^3 at day 7 after the inoculation of MDA-MB-453 cells, and then HL-*n* were intravenously administered once each day for 14 days from day 7. The tumor volume was measured using Vernier caliper and calculated using the equation of $V = 0.5 \times a^2 \times b$, where a and b denote the smallest and longest superficial diameter, respectively (Inaba et al., 1992; Tsuruo et al., 1994; Banciu et al., 2008). Reduction rate of tumor volume was calculated using the equation of 1-(median

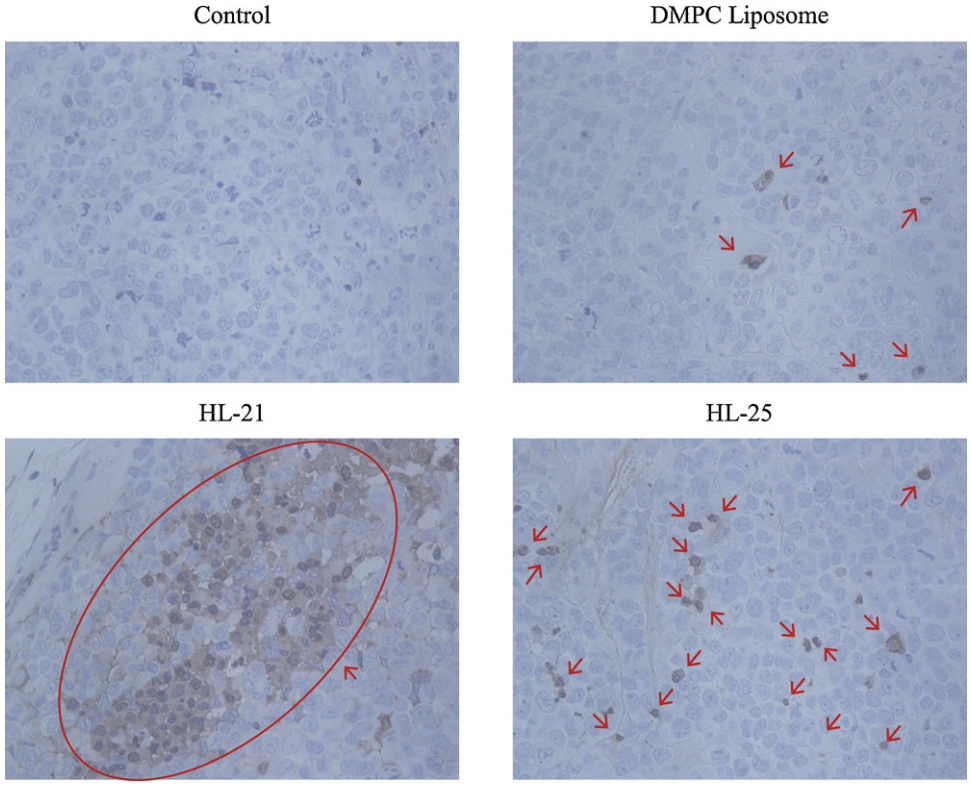

Fig. 6. Micrographs of tumor in xenograft model mice using TUNEL method after the treatment with hybrid liposomes (HL-*n*). Apoptotic cells (arrows) were observed in the tumor cells of xenograft mice after the treatment with HL-*n*.

Table 1
Hematological findings of normal mice after the intravenous administration of HL-*n* for 2 weeks.

Sample	Dose (mg/kg)	RBC ($\times 10^4/\mu l$)	WBC ($\times 10^2/\mu l$)	Hgb (g/dl)	HCT (%)
Control	–	495 ± 68	29 ± 10	15.3 ± 4.0	30.0 ± 1.8
DMPC	DMPC 203	445 ± 30	27 ± 4	13.5 ± 0.7	27.9 ± 1.2
DMPC/5 mol%$C_{12}(EO)_{21}$	DMPC 203 $C_{12}(EO_{21}$ 17.5	454 ± 44	33 ± 26	13.4 ± 1.0	29.0 ± 0.8
DMPC/5 mol%$C_{12}(EO)_{25}$	DMPC 203 $C_{12}(EO_{25}$ 20.3	458 ± 36	33 ± 14	13.4 ± 0.3	29.0 ± 1.2

Data represented are the mean ± S.D. Number of mice was three in each group. The DMPC dose of the hybrid liposomes was 48.5 mg/kg for clinical application.

tumor volume of treated group/median tumor volume of control group) × 100. The data were expressed as the mean ± S.D. and analyzed using Dunnett's test (Kasai et al., 2002).

2.8. TUNEL method

Detection of apoptotic cells was performed on the basis of TUNEL method using an in situ apoptosis detection kit (ApopTag Plus Peroxidase, Intergen, U.S.A.). Tumor was removed from anaesthetized xenograft mice after intravenously administration of HL once a day for 14 days and fixed in 10% formalin solution. Paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanol to water. The sections were incubated with proteinase K for 15 min at room temperature and endogenous peroxidase was blocked with a solution PBS and 2% H_2O_2 for 5 min. The slides were then incubated with a solution of digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 30 °C for 60 min. Subsequently, the anti-digoxigenin antibody was applied and incubated for 30 min at room temperature. Detection of the antigen–antibody link was made through immunoperoxydase followed by 3,3′-DAB chromogen. The sections were counterstained with 5% methyl green, rinsed in distilled water and mounted.

2.9. Assessment of toxicity in vivo

Female mice (BALB/cA Jcl) were obtained from CLEA Japan, Inc. Animals were handled in accordance with the guidelines for animal experimentation of Japanese law during the study. The mice were randomly grouped on the basis of the body weight using the stratified randomization method. Number of mice was three in each group. Hybrid liposomes were intravenously administered into the caudal vein of mice once each day for 2 weeks. The mice were weighed during the experimental period. The blood was collected from the heart in mice under anesthesia with ether after fasting for 24 h as previously reported (Kurata et al., 2003; Senoh et al., 2004; Lee et al., 2004; Aiso et al., 2005). White blood cells (WBC), red blood cells (RBC), hemoglobin (Hgb) and hematocrit (HCT) were counted using multiple automatic blood cell county device (F-500, Sysmex Co., Japan). Glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), blood urea nitrogen (BUN) and creatinine (CRE) were measured using dry chemistry systems (DRICHEM 3500 V, FUJIFILM Co. Ltd., Japan). Organs (heart, lung, liver, spleen and kidney) were weighed after anatomizing the mice. The data were expressed as the mean ± S.D. and analyzed using Dunnett's test (Kasai et al., 2002).

3. Results and discussion

3.1. Fusion and accumulation of HL-n (n = 21, 25) into tumor cell membranes

We examined fusion and accumulation of hybrid liposomes (HL-*n*/NBDPC (*n* = 21, 25)) including NBDPC as a fluorescence probe into MDA-MB-453 cell membrane using a confocal laser scanning microscope. The results are shown in Fig. 1. Increases in accumulation of HL including NBDPC into MDA-MB-453 cell membrane were observed for 6 h. These results suggest that HL could fuse and accumulate into MDA-MB-453 cell membranes.

3.2. 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 for leading to the nuclear DNA fragmentation and apoptotic cell death (Zou et al., 1997). Time courses for activating caspase-8, caspase-9 and caspase-3 by HL-*n* (*n* = 21, 25) are shown in Fig. 2. Generally, the activation of caspase-8 and 9 is observed prior to that of caspase-3. However, in this study the activation of caspase-3 was observed prior to the activation of caspase-8 and 9 as shown in Fig. 2. This result suggests that the apoptosis signal might be carried out through the other pathway. We are now under consideration including the other pathway. Next, we examined the mitochondrial pathway for apoptotic signal transduction by HL-*n* (*n* = 21, 25) using flow cytometry. The results are shown in Fig. 3. Interestingly, mitochondorial transmembrane potential was decreased after the treatment with HL-*n* (*n* = 21, 25). Furthermore, the cytochrome c released from mitochondria was detected after the treatment with HL as shown in Fig. 4. These results suggest that the mitochondorial pathway could be implicated in the apoptosis induced by the HL-*n* (*n* = 21, 25) for MDA-MB-453 cells.

Table 2
Biochemical findings of normal mice after the intravenous administration of HL-*n* for 2 weeks.

Sample	Dose (mg/kg)	GOT (IU/l)	GPT (IU/l)	CRE (mg/dl)	BUN (mg/dl)
Control	–	119 ± 6	36 ± 11	0.4 ± 0.1	34.3 ± 1.5
DMPC	DMPC 203	108 ± 21	29 ± 2	0.3 ± 0.1	28.8 ± 8.5
DMPC/5 mol%$C_{12}(EO)_{21}$	DMPC 203 $C_{12}(EO_{21}$ 17.5	113 ± 51	24 ± 2	0.3 ± 0.0	25.3 ± 2.9
DMPC/5 mol%$C_{12}(EO)_{25}$	DMPC 203 $C_{12}(EO_{25}$ 20.3	154 ± 44	27 ± 4	0.3 ± 0.0	32.6 ± 7.6

Data represented are the mean ± S.D. Number of mice was three in each group. The DMPC dose of the hybrid liposomes was 48.5 mg/kg for clinical application.

Table 3
Relative organ weight of normal mice after the intravenous administration of HL-*n* for 2 weeks.

Sample	Dose (mg/kg)	Relative organ weight (g/100 g B.W.)				
		Heart	Lung	Liver	Spleen	Kidney
Control	–	0.48 ± 0.04	0.54 ± 0.03	5.07 ± 0.37	0.44 ± 0.06	1.32 ± 0.04
DMPC	DMPC 203	0.46 ± 0.07	0.60 ± 0.09	4.84 ± 0.31	0.92 ± 0.09*	1.33 ± 0.12
DMPC/5 mol%$C_{12}(EO)_{21}$	DMPC 203 $C_{12}(EO_{21}$ 17.5	0.47 ± 0.15	0.69 ± 0.12	5.19 ± 0.15	0.97 ± 0.09*	1.26 ± 0.05
DMPC/5 mol%$C_{12}(EO)_{25}$	DMPC 203 $C_{12}(EO_{25}$ 20.3	0.43 ± 0.05	0.69 ± 0.01	4.37 ± 0.42	0.79 ± 0.14*	1.30 ± 0.03

Data represented are the mean ± S.D. Number of mice was three in each group. The DMPC dose of the hybrid liposomes was 48.5 mg/kg for clinical application.
* $P < 0.05$.

3.3. Therapeutic effects of HL-n (n = 21, 25) in vivo

We examined the therapeutic effects of HL-*n* (*n* = 21, 25) using xenograft mice models after subcutaneously inoculating MDA-MB-453 cells *in vivo*. The results are shown in Fig. 5. The median of tumor volume was 458.23 ± 31.71 mm^3 in the control group and 146.66 ± 61.01 mm^3 and 233.60 ± 90.88 mm^3 in the group treated with HL-21 and HL-25, respectively. It is noteworthy that a remarkable reduction of tumor volume (70% in HL-21 and 50% in HL-25, respectively) was obtained in xenograft mice models intravenously treated with HL-*n* without drugs after subcutaneously inoculating MDA-MB-453 cells. Especially, the tumor volume of mice treated with HL-21 was almost constant, and remarkably high inhibitory effects of HL-21 on the growth of tumor cells in xenograft mice were obtained. 50% inhibitory concentration (IC_{50}) of HL-21 for the growth of MDA-MB-453 cells was lower than that of HL-25 (Nagami et al., 2006).

Moreover, we examined the induction of apoptosis by HL-*n* for breast tumor in xenograft mice using the TUNEL method. The results are shown in Fig. 6. Brown color was observed in the tumor cells of xenograft mice after the treatment with HL-*n*, although the apoptotic cells were slightly observed after adding DMPC liposomes. Interestingly, the number of apoptotic cells was HL-21 > HL-25. These results indicate that HL-21 have remarkable reduction effects along with apoptosis on the growth of breast tumor *in vivo* as compared with HL-25.

3.4. Toxicity of HL-n (n = 21, 25) in vivo

Safety tests were carried out using normal mice after the treatment intravenously with HL-*n* (*n* = 21, 25) for 14 days. No weight loss was observed in the mice as shown in Fig. 7. Hematological tests were examined and the results were shown in Table 1. In general, the average number of red blood cells (RBC) in female BALB/c mice is approximately 1000 × 10^4/μl. On the other hand, the average number of RBC in this study was 495 ± 68 × 10^4/μl, however the number of RBC, Hgb and Hct for mice treated with HL-*n* was almost the same as that of control group, suggesting that HL-*n* have no side effects in RBC tests. The number of white blood cells (WBC) increased in mice after the treatment with HL-21. Remarkable immunostimulation effects of HL on human peripheral blood mononuclear cells *in vitro* have been reported (Komizu et al., 2007). Furthermore, an increase in lymphocytes was observed under microscope without any increase in the neutrophils count, which normally indicated the presence of some inflammatory process in normal rats after administering the DMPC liposomes or hybrid liposomes (Ueoka et al., 2000; Ichihara et al., 2003). Therefore, it was considered that white blood cell counts increased due to an immunostimulation effect of HL. Biochemical findings in mice treated intravenously with HL-*n* are shown in Table 2. All the biochemical parameters such as creatinine, BUN, and GPT activities were not significantly

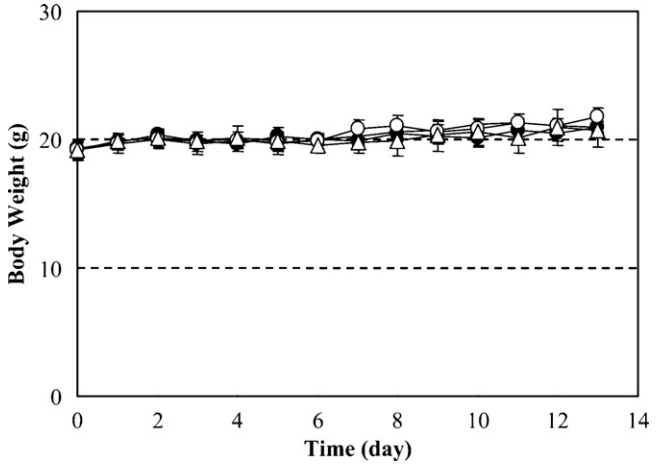

Fig. 7. Body weight change of normal mice after the intravenous administration of hybrid liposomes (HL-*n*) for 2 weeks. (●) Control, (▲) DMPC Liposome, (○) HL-21, (△) HL-25. Dose for DMPC = 203 mg/kg, Data presented are mean (*n* = 3) ± S.D. No weight loss was observed in normal mice after the intravenous administration of HL-*n*.

different from those obtained in the controls. Although an increase of GOT was obtained, immediate metabolism of HL through liver was confirmed in a pharmacokinetics test using normal mice as reported previously (Ichihara et al., 2003). Furthermore, relative organ weights in mice treated intravenously with HL-*n* are shown in Table 3. No abnormal finding was observed in the heart, lung, liver and kidney, although an increase in the weight of the spleen after the treatment with HL-*n* was obtained. These results indicate that HL-*n* might have slight side effects in spleen. The same observation for spleen was obtained after the treatment with a fat emulsion and the recover was confirmed (Reimold, 1979), so the mice could recover in a short time span after the intravenous injection of HL-*n*.

4. Conclusion

We clearly demonstrated that therapeutic effects of HL-*n* along with apoptosis without side effects were obtained for xenograft mice model of human breast tumor *in vivo*. The noteworthy aspects in this study are as follows: (a) Increases of fusion and accumulation of HL-*n* including NBDPC as a fluorescence probe into human breast tumor (MDA-MB-453) cell membranes were observed. (b) Reduction of mitochondrial membrane potential and activation of caspase-8, caspase-9, and caspase-3 were observed, indicating that apoptotic signal by HL-*n* should pass through mitochondria and these caspases. (c) Remarkable reduction of tumor volume in a xenograft mice model intravenously treated with HL-*n* without drugs after the subcutaneously inoculation of MDA-MB-453 cells was verified *in vivo*. Induction of apoptosis in tumor of xenograft mice treated with HL-*n* was observed in micrograph on the basis of

TUNEL method. (d) No severe side effects were observed in toxicity tests of HL-*n* using normal mice, although increases of GOT and in the weight of the spleen were obtained. Clinical applications of HL for patients with lymphoma were performed after being passed by the committee of bioethics (Ichihara et al., 2008). So, the results in this study could contribute to the chemotherapy for patients with breast cancer in future clinical applications.

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