Chemotherapy Using Hybrid Liposomes along with Induction of Apoptosis

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Abstract. *We have produced hybrid liposomes (HL) which can be prepared by ultrasonicating a mixture of dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(23)dodecyl ether in a buffer solution. The fifty-percent inhibitory concentration (IC_{50}) of HL on the growth of human B lymphoma (RAJI) cells in vitro was determined. The IC_{50} of HL on the growth of RAJI cells was one half of that of DMPC liposomes. Induction of apoptosis by HL in RAJI cells was verified on the basis of flow cytometric analysis, agarose gel electrophoresis and fluorescence microscopy. Apoptotic DNA was observed in RAJI cells treated with HL. Fluorescence micrographs of RAJI cells after adding HL indicated the induction of apoptosis. The therapeutic effects of HL in vivo were examined using SCID mice inoculated with RAJI cells. Markedly prolonged survival of mice was obtained after treatment with HL. No adverse effects were observed in normal rats in toxicity tests carried out with HL. Clinical applications of HL for patients were examined after the approval of the Bioethics Committee. Remarkable reduction of a solid tumor and prolonged survival for one patient with advanced lymphoma were attained after treatment using HL. Chemotherapy with drug-free HL was established without any side effects for the first time.*

Non-Hodgkin's lymphoma (NHL) is a tumor of lymphoid tissue in the lymphatic systems. In general, chemotherapy or radiation therapy is used mainly for NHL. However, radiation therapy is often used along with chemotherapy (1). Recently, the addition of rituximab, chimeric monoclonal antibody against the CD 20 antigen, to standard CHOP (cyclophosphamide hydroxydaunomycine (doxorubicin), Oncovin (vincristine) and prednisone) chemotherapy is used widely for NHL patients (2-4). Some patients relapse after therapy with chemotherapy and/or radiation therapy. While chemotherapy drugs and radiation kill tumor cells, they also damage normal cells, causing side-effects. Therefore, a chemotherapy that is effective for NHL without any side-effects is required.

Liposomes are closed vesicles that are formed when phospholipids (constituents of biological membranes) are dispersed in water at relatively low concentrations. Since Bangham discovered liposomes in 1965 (5), they have been studied at both basic and applied levels. Liposomes have contributed significantly to drug delivery, as well as to the analysis of cellular function, due to their mimicry of biological membranes and closed properties (6, 7). Hybrid liposomes, which have been developed by Ueoka, can be prepared by ultrasonication of vesicular and micellar molecules in a buffer solution (8). A schematic representation of hybrid liposomes is shown in Figure 1a. The physical properties of these liposomes such as shape, size, membrane fluidity and the temperature of their phase transition can be controlled by changing the constituents and compositional ratios. Significantly prolonged survival in rats was obtained by using hybrid liposomes as drug carriers in the treatment of brain tumors (9). Hybrid liposomes without drugs were also demonstrated to inhibit the proliferation of various tumor cells *in vitro* (10-13) and *in vivo* (14-16).

In this study, the inhibitory effects of hybrid liposomes composed of phosphatidylcholine and a polyoxyethylene alkyl ether on the growth of lymphoma cells *in vitro*, *in vivo* and for clinical applications were investigated.

Materials and Methods

Hybrid liposomes. Hybrid liposomes were prepared by dissolving both 95 mol% dimyristoylphosphatidylcholine (DMPC, NOF Co., Tokyo, Japan) and 5 mol% polyoxyethylene(23)dodecyl ether ($C_{12}(EO)_{23}$, Sigma-Aldrich Co., MO, USA) in 5% glucose solution with sonication using a sonicator (Velvo, Tokyo, Japan) at 300 W

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Key Words: Hybrid liposomes, antitumor effect, lymphoma, apoptosis, chemotherapy.

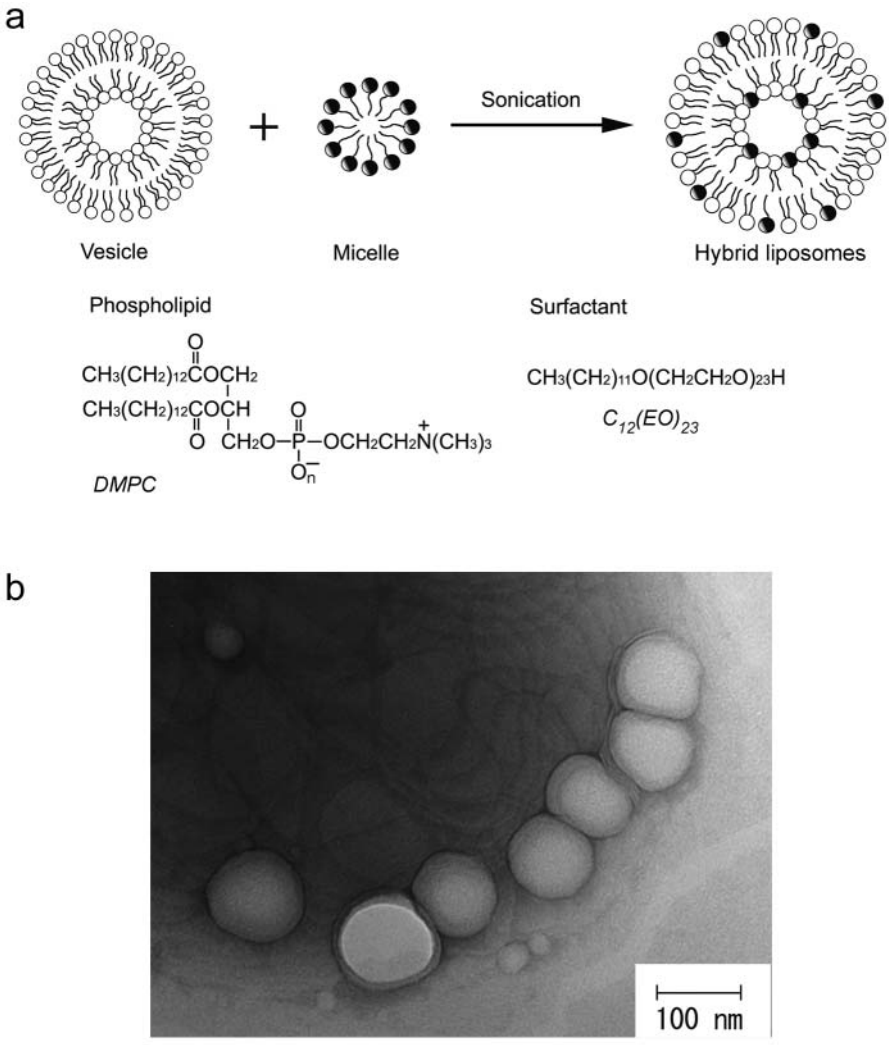

Figure 1. *Hybrid liposomes. (a) Schematic representation of hybrid liposomes. (b) An electron micrograph showing that hybrid liposomes are uniform in size and shape.*

and 45˚C in an atmosphere of nitrogen and filtered in a sterile manner through a 0.20 μm filter.

Dynamic light scattering measurements. The diameter of hybrid liposomes was measured with a light scattering spectrometer (Otsuka Electronic, Osaka, Japan) using a He–Ne laser (633 nm) at a 90˚ scattering angle. The diameter (d_{hy}) was calculated using the Stokes-Einstein formula (*Equation 1*), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient:

$d_{hy}=\kappa T/3\pi\eta D$ (*Equation 1*)

Electron microscopy. An electron micrograph was obtained by means of negative-staining techniques. A sample solution of hybrid liposomes in buffer was mixed with ammonium molybdate. The sample was then applied to a carbon grid and dried overnight at room temperature. Electron micrographs were taken on a JEOL JEM200FX electron microscope.

Cell culture. Human B lymphoma (RAJI) cell lines (17-19) were obtained from Riken Cell Bank (Ibaraki, Japan). RAJI cells were grown in RPMI-1640 medium (Gibco BRL, USA). The media was supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) and antibiotics (100 units/ml penicillin 50 μg/ml streptomycin). These cells were cultured at 37˚C in humidified atmosphere containing 5% CO_2.

Assessment of cell growth in vitro. Inhibitory effects *in vitro* were examined on the basis of the WST-1 assay (20). The cells were incubated in a 96-well plate and incubated in a humidified 5% CO_2 incubator at 37˚C. The cells were cultured for 2 days after adding the hybrid liposomes. Inhibitory effects of hybrid liposomes on the growth of tumor cells were evaluated by A_{Mean} /$A_{Control}$, where A_{Mean} and

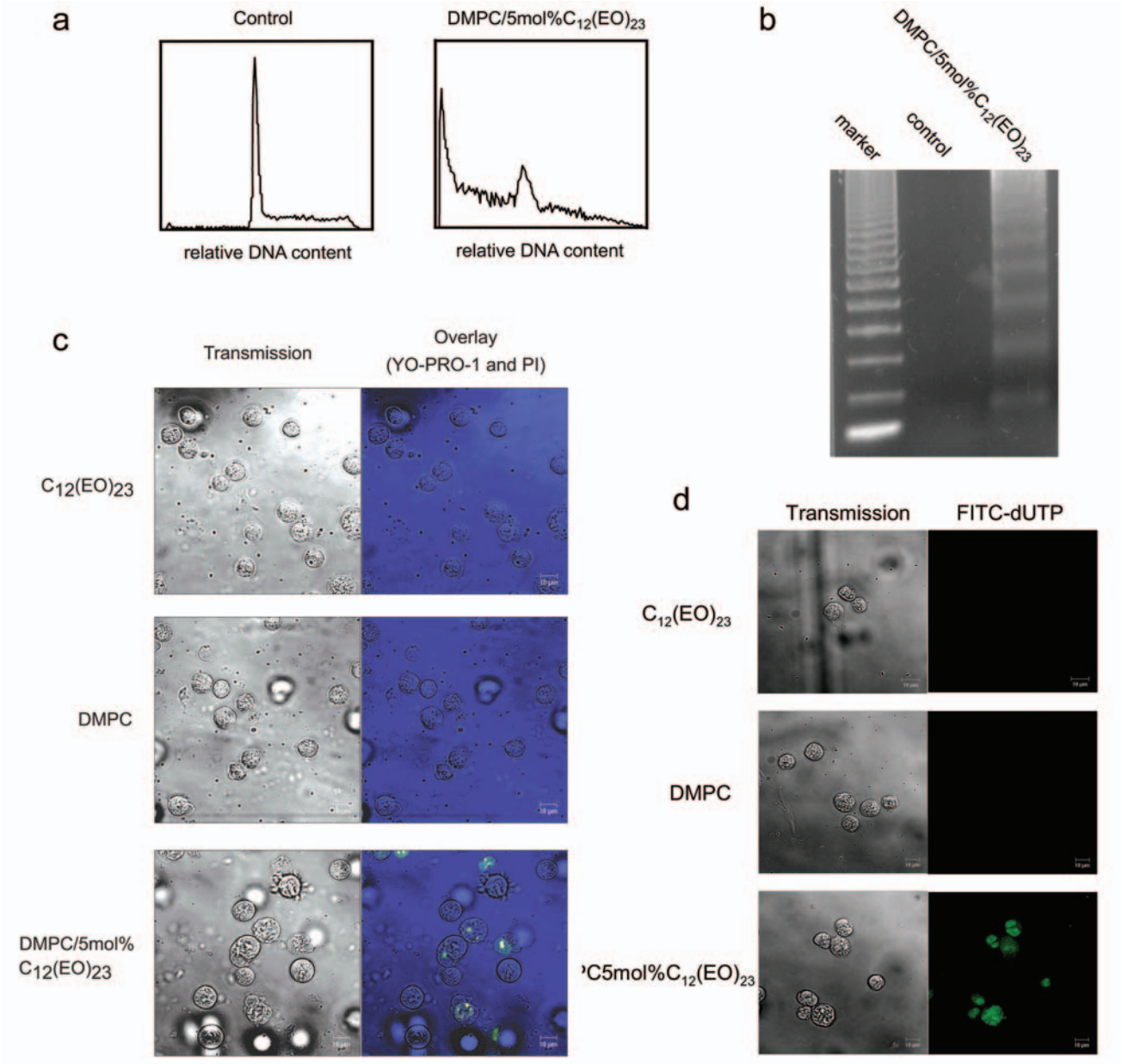

Figure 2. *Induction of apoptosis for RAJI cells by hybrid liposomes in vitro. (a) Flow cytometric analysis for RAJI cells. Fragmented DNA was observed in RAJI cells after the treatment with hybrid liposomes. (b) Agarose gel electrophoresis of DNA from RAJI cells. DNA ladder formation was observed after the treatment with hybrid liposomes. (c) Fluorescence micrographs of RAJI cells using staining method. After treatment with hybrid liposomes, cells were observed to be stained. (d) Fluorescence micrographs of RAJI cells using TUNEL method. Stained RAJI cells were observed after treatment with hybrid liposomes.*

$A_{Control}$ denote the absorbance of water-soluble formazan at 450 nm in the presence and absence of hybrid liposomes, respectively.

Flow cytometric analysis. The apoptotic DNA rate in tumor cells by hybrid liposomes was verified using fragmented DNA-staining method with flow cytometer (Beckman Coulter, FL, USA). The RAJI cell was incubated for 6 h after adding hybrid liposomes. The cells were centrifuged at 3000 r.p.m. for 5 min and washed with PBS(–) and fixed in chilled ethanol. The cells were washed again with PBS(–), treated with RNase (0.25 mg/ml, Amresco, OH, USA) and then stained with propidium iodide (PI, 0.5 mg/ml, Invitrogen Co., FL, USA). The samples were analyzed by flow cytometry with single excitation (488 nm) using a 15 mW argon laser. The PI signals were detected by a FL3 sensor at 605-635 nm. Apoptotic DNA rates were calculated by the following equation:

Apoptotic DNA rate = (apoptotic DNA content)/
(DNA content) x100 (*Equation 2*)

Agarose gel electrophoresis. The fragmented DNA produced by incubation with the hybrid liposomes was detected using agarose gel electrophoresis. After the incubation with the hybrid liposomes for 2h, the cells were centrifuged 3000 at r.p.m. for 5 min and resuspended in 100 μl of chilled cell lysis buffer. The cytosolic extract was microcentrifuged at 16,000 r.p.m. for 5 min. The supernatants containing nucleic acid were treated with RNase (0.25 mg/ml, Amresco, OH, USA) and proteinase K (0.25 mg/ml, Roche, Mannheim, Germany) for 1 h. Electrophoresis of the samples was performed on 1.5% agarose gel in TBE buffer (0.45 M Tris-borate, 0.01 M EDTA) at 100 V/cm by a Mini-Gel Electrophoresis System (Cosmo Bio, Tokyo, Japan). After the electrophoresis, the gels were stained with ethidium bromide (1 mg/ml, Invitrogen Co., FL, USA) and photographed under a UV transilluminator (Taitec, Saitama, Japan).

Confocal laser microscopy. Fluorescence micrographs were taken on a Leica TCS SP conforcal scanning laser microscope (Wetzler, Germany). Apoptosis was detected using Vybrant Apoptosis Assay Kit #4 (Invitrogen Co., FL, USA). This kit contains ready-to-use solutions of both the YO-PRO-1 and propidium iodide (PI) nucleic acid stains (21). YO-PRO-1 nucleic acid stain selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence. Necrotic cells are stained with the red-fluorescent PI, a DNA-selective dye that is membrane impermeant but that easily passes through the compromised plasma membranes of necrotic cells. Furthermore, apoptosis was detected additionally by the TUNEL method which labeled the 3'OH-DNA terminal of fluorescent dUTP (22).

Assessment of antitumor activity in vivo. The therapeutic effects of hybrid liposomes were examined *in vivo* using a mouse model of lymphoma. Female SCID (severe combined immunodeficiency) mice were purchased from Clea (Tokyo, Japan). The animals were handled during the study in accordance with the guidelines for animals experimentation of Japanese law. Mice were randomly grouped (n=6) on the basis of body weight on the day of tumor cell inoculation using the stratified randomization method. RAJI cells ($5.0x10^6$ cells) were inoculated intraperitoneally. Liposomes (dose for DMPC of DMPC liposomes was 136 mg/kg and that of DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes was 67.8 mg/kg, 136 mg/kg and 203 mg/kg.) were intraperitoneally administered once each day for 21 days after RAJI cells were intraperitoneally inoculated. The median lifespan was calculated using following the equation:

Median lifespan=(median survival days after treatment) / (median survival days of control group) x100 *(Equation 3)*

Assessment of toxicity in vivo. The safety of hybrid liposomes was examined using normal rats. Male Wistar rats were purchased from Kyudo (Saga, Japan). Six rats were assigned to each group by the stratified continuous randomization method and hybrid liposomes (dose for DMPC of DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes was 67.8 mg/kg, 136 mg/kg and 203 mg/kg) were intravenously administered *via* a vein once a day for six months. The rats were weighed during the experimental period. Blood was collected from the abdominal vena cava under anesthesia with ether after six months. White blood cells, red blood cells and hemoglobin were determined using a Sysmex F-500 multiple automatic blood cell county device (Hyogo, Japan). Alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), albumin, urea nitrogen, creatinine, glucose, total protein, calcium, inorganic phosphorus, sodium, potassium and chloride were measured using dry chemistry systems (DRICHEM 3500; Fujifilm Co. Ltd., Japan). Organs (heart, thymus, lung, liver, spleen and kidney) were weighed after anatomizing the rats.

Clinical application. Patients with lymphoma were selected for the clinical application of hybrid liposomes without any drugs after the approval of the Bioethics Committee at the National Kumamoto Hospital. The hybrid liposomes (dose for DMPC of DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes was 11.0-16.5 mg/kg) were administered *via* a vein intravenously every day for 10 months. Hybrid liposomes were locally administered (2 times/week, dose for DMPC of DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes was 0.5 mg/kg) to solid tumor (neoplasm) for two months and photographs were taken using ultra sonical echo.

Results

Physical properties of hybrid liposomes. We examined the morphology of hybrid liposomes composed of DMPC and 5mol%$C_{12}(EO)_{23}$ on the basis of electron microscopy. Figure 1b shows the presence of uniform vesicles for the hybrid liposomes with a diameter of 100 nm. A clear solution of hybrid liposomes having a hydrodynamic diameter of 100 nm could be kept for over one month on the basis of dynamic light-scattering measurements.

Inhibitory effects of hybrid liposomes on the growth of RAJI cells. The IC_{50} of hybrid liposomes was determined for human B lymphoma cells (RAJI) *in vitro*. The IC_{50} values were 0.16 mM for hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$ and 0.34 mM for liposomes on the basis of the DMPC concentration, respectively. These results indicate that hybrid liposomes of DMPC/5mol% $C_{12}(EO)_{23}$ should be of greater effect as compared with single-component DMPC liposomes.

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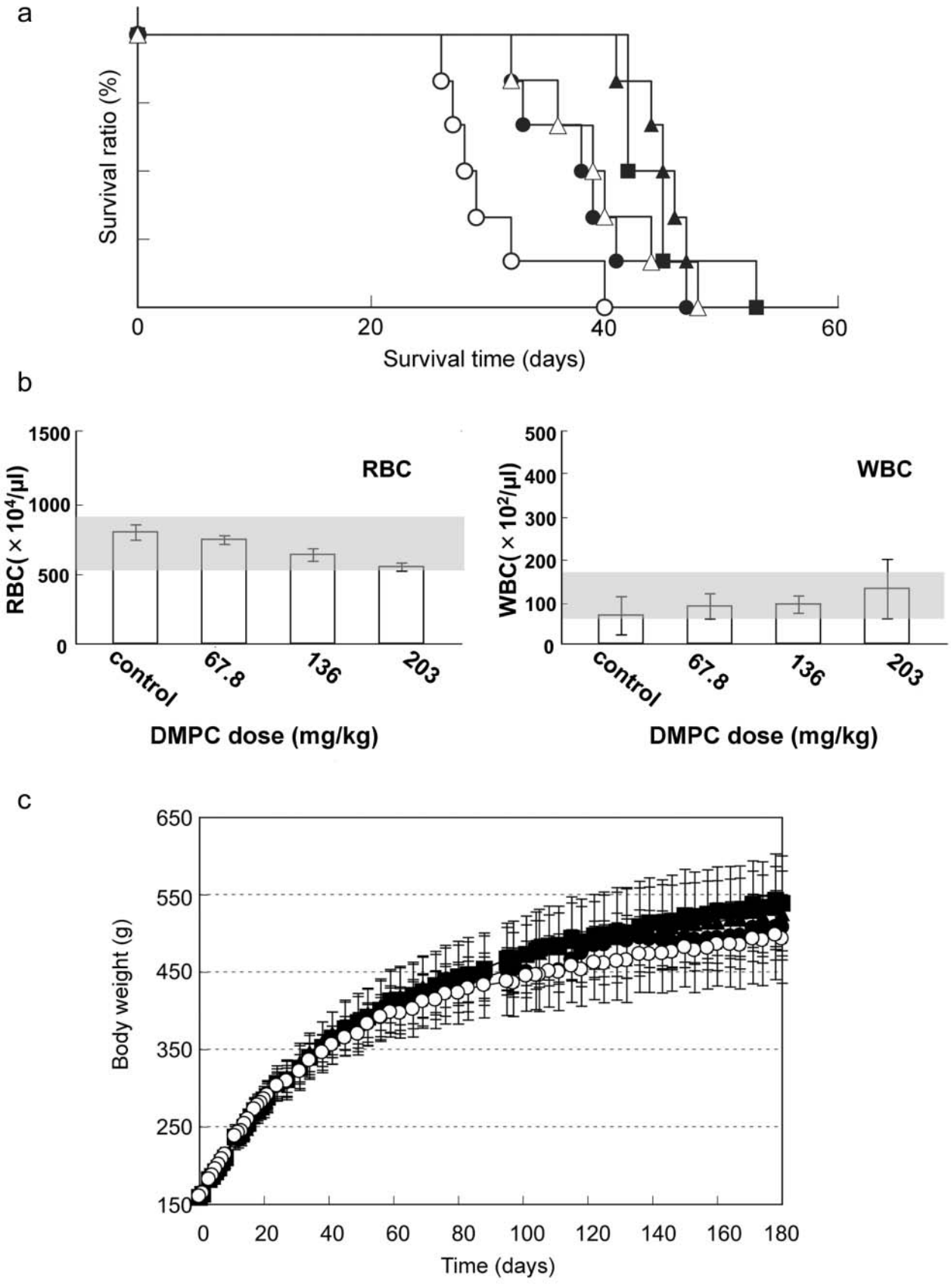
Figure 3. *Therapeutic effects and safety of hybrid liposomes in vivo (n=6). (a) Survival curves of mice treated with hybrid liposomes after the intraperitoneal inoculation of RAJI lymphoma. Prolonged survival was obtained in mice treated with hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$. Control (○), DMPC liposome (136 mg/kg) (△), Dose for DMPC of hybrid liposomes was 67.8mg/kg (●), 136 mg/kg (▲) and 203 mg/kg (■), respectively. (b) Hematological findings in rats treated intravenously with hybrid liposomes. Normal limits are indicated by the shaded areas in the two Figures. There were no abnormal findings in rats treated intravenously with hybrid liposomes. (c) Body weight of rats treated intravenously with hybrid liposomes for 180 days. The body weight normally increased in rats treated intravenously with hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$. Control (○), dose for DMPC of hybrid liposomes was 67.8 mg/kg (●), 136 mg/kg (▲) and 203 mg/kg (■).*


a
Survival ratio (%)
0
20
40
60
Survival time (days)
b
RBC
RBC(×10⁴/μl)
1500
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203
DMPC dose (mg/kg)
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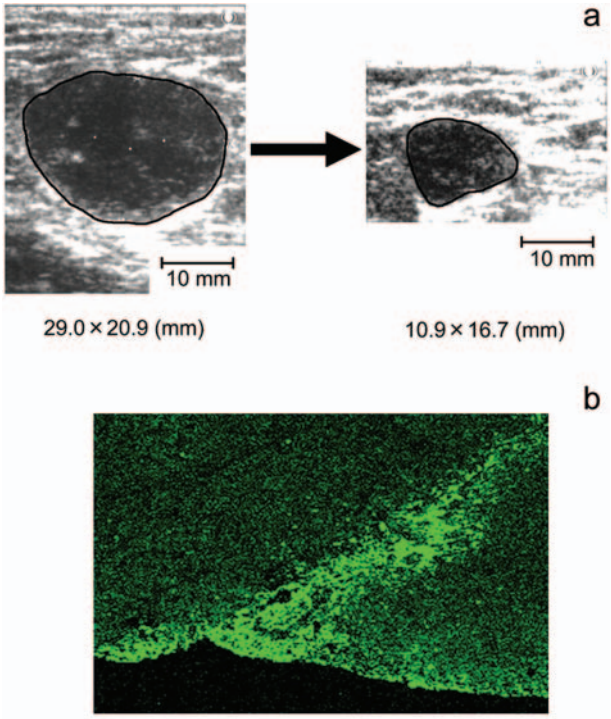

Figure 4. *A pilot clinical test of hybrid liposomes in a patient with recurrent malignant lymphoma. (a) Lymph node neoplasm (solid tumor) after local administration of hybrid liposomes of DMPC/3mol%$C_{12}(EO)_{23}$ for two months. A remarkable reduction of the neoplasm (solid tumor) was obtained. (b) A fluorescence micrograph of the lymph node neoplasm (solid tumor) using the TUNEL method after treatment with hybrid liposomes. The green color due to apoptosis was observed.*

Induction of apoptosis by hybrid liposomes. We examined a plausible mechanism for inhibition of the growth of RAJI cells by hybrid liposomes. Firstly, the DNA content of RAJI cells treated with DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes was measured using a flow cytometer. The results are shown in Figure 2a. A marked increase in apoptotic DNA was observed in RAJI cells treated with hybrid liposomes, that is, the apoptotic DNA rate was 69%. Nuclear DNA fragmentation with hybrid liposomes was examined using agarose gel electrophoresis. The results are shown in Figure 2b. It is noteworthy that exposure of RAJI cells to the hybrid liposomes caused DNA fragmentation characteristic of apoptosis. In addition, we examined induction of apoptosis by hybrid liposomes on the basis of confocal microscopy using a staining method. Figure 2c shows fluorescence micrographs of RAJI cells treated with hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$, DMPC liposomes and $C_{12}(EO)_{23}$ micelles by staining with YO-PRO-1 and PI. Green color was observed in the cells after adding hybrid liposomes, although green color in the cells was not observed in the case of treatment with DMPC liposomes or $C_{12}(EO)_{23}$ micelles. The green color was also observed in fluorescence micrographs of RAJI cells treated with hybrid liposomes on the basis of the TUNEL method as shown in Figure 2d. These results indicate that hybrid liposomes should induce apoptosis for RAJI cells.

Therapeutic effects and toxicity of hybrid liposomes in vivo. The therapeutic effects of DMPC/5mol%$C_{12}(EO)_{23}$ hybrid liposomes on a mouse model of carcinoma *in vivo* were examined. The results are shown in Figure 3a. Prolonged survival of mice was obtained by treatment with hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$. It is of interest that a significantly prolonged survival rate (150%) was obtained after the treatment with hybrid liposomes at a dose of 136 and 203 mg/kg for DMPC. Although prolonged survival (140%) was obtained in mice by treatment with the unstable DMPC liposomes.

Assessment of the chronic toxicity of hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$ was carried out using normal healthy rats after the injections of hybrid liposomes for six months. The number of red and white blood cells of rats treated with hybrid liposomes were within normal limits (23) as shown in Figure 3b. In addition, all of the other biochemical parameters, such as ALP, GOT and GPT activities, as well as levels of albumin, urea nitrogen, creatinine, glucose, total protein, calcium, inorganic phosphorus, sodium, potassium and chloride, were not significantly different from those observed in the controls (data not shown). Furthermore, no weight loss was observed in the rats, as shown in Figure 3c. These results indicate that hybrid liposomes should have no side-effects *in vivo*.

Clinical applications of hybrid liposomes. Clinical applications of hybrid liposomes of DMPC/5mol% $C_{12}(EO)_{23}$ without any drug for 10 patients with lymphoma (2 patients), gastric cancer (2 patients), kidney cancer (one patient), mammary cancer (one patient), pharyngeal cancer (one patient), hepatoma (one patient), gallbladder cancer (one patient) and rectal cancer (one patient) were examined after passing the committee of bioethics at the different hospitals. Informed consents were obtained in accordance with the Declaration of Helsinki. We report the pilot study using hybrid liposomes for one patient (treated by MD Kiyokawa, one of authors) with advanced stage B-lymphoma who did not recover by any chemotherapeutics. The hybrid liposomes were administered intravenously once every day at a daily dose of 11.0-16.5 mg/kg for DMPC. There were no abnormal findings post administration on routine blood test and hematochemistry (data not shown). A prolonged survival, more than one year, was attained in this patient after the intravenous injection of hybrid liposomes without any side-effects. It should be noted that a remarkable reduction of the lymph node neoplasm (solid tumor) was

observed after local administration (2 times/week) of hybrid liposomes, as shown in Figure 4a.

Moreover, we examined induction of apoptosis in the lymph node neoplasm (solid tumor) using the TUNEL method. Tissue sections were prepared from lymph nodes and stained by a TUNEL-based method. A fluorescence micrograph is shown in Figure 4b. The green color was obtained in the lymph node neoplasm (solid tumor) after the injection of hybrid liposomes, indicating that hybrid liposomes should induce apoptosis in the lymph node. During the period of treatment, no abnormal findings were obtained on the basis of various hematological and biochemical tests. These results demonstrate that hybrid liposomes should be safe and effective in clinical applications.

Discussion

Chemotherapy with rituximab is effective for the treatment of lymphoid tissue in patients with non-Hodgkin's lymphoma (NHL) (2-4). Although anticancer drugs and radiation kill tumor cells, they also damage normal cells, causing side-effects. Therefore, chemotherapy along with induction of apoptosis without any side-effects for NHL is desirable.

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 and suppression of apoptosis lead to degenerative pathologies (Alzheimer's desease) and tumorigenesis, respectively. Consequently, control of apoptosis is an important potential target for therapeutic intervention.

The hybrid liposomes being more fluid as compared with DMPC liposomes for the basis of fluorescence polarization measurement showed remarkable high inhibitory effects compared with DMPC liposomes on the growth of human lymphoma-human B-lymphocyte hybridoma (24). Furthermore, a good correlation between IC_{50} of hybrid liposomes for the growth of human colon tumor (WiDr) cells and membrane fluidity of hybrid liposomes was already reported (25). It is also worthy to note that TIRF (total internal reflection fluorescence) micrographs showed that hybrid liposomes distinguished between tumor (WiDr) and normal (CCD33Co) colon cells, and then fused and accumulated into the only WiDr cells (25). These results suggest that the inhibitory effects of the hybrid liposomes on the growth of tumor cells should be related to the membrane fluidity.

The induction of apoptosis by hybrid liposomes was verified for RAJI cells on the basis of flow cytometry, gel electrophoresis and staining method. The pathways of apoptosis induced by hybrid liposomes of DMPC/10mol%$C_{12}(EO)_{10}$ in human promyelocytic leukemia (HL-60) cells has already been reported (26). Hybrid liposomes of DMPC/10mol%$C_{12}(EO)_{10}$ fused and accumulated in HL-60 cell membranes, and the apoptosis signal first passed through the mitochondria, caspase-9 and caspase-3 (pathway (A)), second through Fas, caspase-8, caspase-3 (pathway (B)) and then reached the nucleus. A pathway of apoptosis induced by hybrid liposomes for RAJI cells is now under investigation.

The significantly prolonged survival rate (150%) was obtained in the mouse model of carcinoma after the treatment with hybrid liposomes having hydrodynamic diameter of 80-100 nm *in vivo*. Although prolonged survival (140%) was obtained in mice by the treatment with the DMPC liposomes, hydrodynamic diameter of the DMPC liposomes (220-320 nm) was over 100 nm. It was suggested that hybrid liposomes (80-100 nm) could avoid the reticular endothelial system (27) and should be appropriate for clinical application. Hybrid liposomes demonstrated no side-effects using normal rats *in vivo*. Hybrid liposomes were metabolized in the liver after intravenous administration to normal mice as described previously (16).

In clinical application, a prolonged survival, more than one year, was attained in one patient with lympyoma after the intravenous injection of hybrid liposomes without any side-effects. In addition, a remarkable reduction of the lymph node neoplasm (solid tumor) was observed after local administration (2 times/week) of hybrid liposomes. The high γ-GTP values recovered to the stage of normal ones after the administration of hybrid liposomes having a diameter of 100 nm (data not shown). This result indicates that hybrid liposomes could avoid the reticular endothelial system. Although clinical applications of the liposome-encapsulated doxorubicin have been already reported, those were still accompanied with severe side-effects (28-32). Therefore, the successful chemotherapy using liposomes without any anticancer drug in this study should be important for clinical applications in the future.

Conclusion

The noteworthy aspects of this study are as follows: (a) Hybrid liposomes of DMPC/5mol%$C_{12}(EO)_{23}$ having a diameter of 100 nm were able to avoid the clearance by the reticular endothelial system (RES). (b) IC_{50} of hybrid liposomes on the growth of RAJI cells was one half of that of DMPC liposomes. (c) The induction of apoptosis by hybrid liposomes was verified for RAJI cells on the basis of flow cytometry, gel electrophoresis and staining method. (d) The significantly prolonged survival rate (150%) was obtained in the mouse model of carcinoma after treatment with hybrid liposomes *in vivo*. (e) Hybrid liposomes demonstrated no side-effects using normal rats *in vivo*. (f) Remarkable reduction of the lymph node neoplasm (solid tumor) along with apoptosis and the prolonged survival for a patient with advanced lymphoma was attained after the

treatment with hybrid liposomes without any side-effect in clinical applications.

Chemotherapy with drug-free hybrid liposomes could be established without any side-effects. Such successful clinical applications of hybrid liposomes without drugs are reported in this study for the first time.

Acknowledgements

We thank Dr. Taku Matsushita of Sojo University for his technical assistance in confocal laser microscopy. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science, and Culture of Japan (No. 17360403, 17500323, 18031041).

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Received October 29, 2007
Revised January 3, 2008
Accepted February 12, 2008