Therapeutic Effects of Hybrid Liposomes on Gastric Carcinoma Involve Apoptosis

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Abstract. *Hybrid liposomes (HLs) composed of 90 mol% dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(23)dodecyl ether ($C_{12}(EO)_{23}$), having a hydrodynamic diameter under 100 nm, were preserved for a period of one month. The inhibitory effects of HLs on the growth of gastric carcinoma (CoRa 622 G6) cells in vitro were investigated. Decrease in mitochondrial membrane potential and activation of caspase-8, caspase-9 and caspase-3 were observed, indicating that apoptotic signals induced by HLs should pass through mitochondria and these caspases. Remarkable inhibitory effects on the metastasis of gastric carcinoma along with apoptosis and prolonged survival were obtained for mouse models of gastric carcinoma with peritoneal dissemination after the treatment with HLs in vivo.*

Gastric cancer metastasizes to lymph nodes, the liver and peritoneum after invasion through stratum submucosum, muscularis propia, stratum subserosum and serosa. In the therapy of gastric carcinoma, the effectiveness of anticancer drugs as neoadjuvant chemotherapy before curative resection has been reported (1). 5-Fluorouracil (5-FU) is universally used as an anticancer agent for gastric cancer. However, therapy with 5-FU is accompanied with severe side-effects (2). Anticancer drugs without side-effects are necessary to attain high quality of life (QOL) for patients with gastric carcinoma.

Liposomes are closed vesicles that are formed when phospholipids are dispersed in water at relatively low concentrations. Liposomes have contributed significantly to delivery, for example, of antitumor agents, hormones, and in immunomodulation (3, 4). We have produced hybrid liposomes (HLs) composed of vesicular and micellar molecules. The physical properties of HLs, such as shape, size and membrane fluidity, and the temperature of phase transition can be controlled by changing the constituents and compositional ratios (5). Remarkable inhibitory effects of HLs without any drugs were demonstrated on the growth of tumor cells *in vitro* (6-9) and *in vivo* (10-12). It has been reported that HLs fused and accumulated into tumor cell membranes (8) and induced apoptosis through the activation of caspases (7). Recently, successful clinical chemotherapy with drug-free HLs of patients with lymphoma was reported after passing a committee of bioethics (12). However, the therapeutic effects of HLs for gastric carcinoma *in vivo* have not yet been elucidated.

In this study, we investigated the therapeutic effects of HLs composed of L-α-dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(23)dodecyl ether ($C_{12}(EO)_{23}$) on gastric carcinoma with peritoneal dissemination *in vivo*.

Materials and Methods

Preparation of HLs. HLs were prepared by sonication of a mixture containing 90 mol% DMPC (NOF Co. Ltd., Tokyo, Japan) and 10 mol% $C_{12}(EO)_{23}$ (Sigma-Aldrich Co. Ltd., Missouri, USA) in 5% glucose solution using a bath type sonicater at 45˚C with 300 W for 5 min (1 min/ml), and were then filtered with a 0.20 μm cellulose acetate filter (Advantec, Tokyo, Japan).

Dynamic light scattering measurements. The diameter of HLs 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:

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

Electron microscopy. An electron micrograph was obtained by means of negative-staining techniques. A sample solution of HLs 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 an electron microscope (JEM100FX, JEOL Co. Ltd., Tokyo, Japan).

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Key Words: Hybrid liposomes, chemotherapy, gastric carcinoma, apoptosis, cotton rat.

Cell culture. Gastric carcinoma (CoRa 622 G6) cell line (13) was obtained from Hokkaido Institute of Public Health (Hokkaido, Japan). CoRa 622 G6 cells were grown in RPMI-1640 medium (Gibco BRL, MD, USA). The medium was supplemented with 10% fetal bovine serum (FBS; Hyclone, Nebraska, USA) and antibiotics (100 units/ml penicillin and 50 μg/ml streptomycin). These cells were cultured at 37˚C in a 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 (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) (14). CoRa 622 G6 cells were seeded at a density of 1.0×10^4 cells/ml in a 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 after adding HLs or single-component liposomes of DMPC. WST-1 solution was added and cells cultured for a further 2 h, and then the absorbance at 450 nm was measured by spectrophotometer (Molecular Devices, California, USA). The inhibitory effects of HLs on the growth of tumor cells was evaluated by $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote the absorbance of water-soluble formazan, which was useful as an indicator of cell viability, in the presence and absence of sample solutions, respectively.

Caspase fluorometric protease assay. Activation of caspases was measured on the basis of caspase fluorometric protease assay (Medical and Biological Laboratories, Nagoya, Japan) (15). CoRa 622 G6 cells were seeded at a density of 2.5×10^5 cells/ml in flask and cultured for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C. The cells were treated with HLs ([DMPC]=3.42 mM, $[C_{12}(EO)_n]$=0.38 mM) for 10, 20, 30, 60 and 120 min. The cells were centrifuged at 3,000 rpm for 5 min, and resuspended in 50 μl of chilled cell lysis buffer. The cell lysates were incubated with *N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin) (Ac-DEVD-AFC; 50 μM; caspase-3 substrate), *N*-acetyl- Ile-Glu-Thr-Asp-(7-amino-4-trifluoromethylcoumarin) (Ac-IETD-AFC; 50 μM; caspase-8 substrate) or N-acetyl-Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin) (LEHD-AFC; 50 μM; caspase-9 substrate) at 37˚C for 2 h. The specific activities were determined fluorometrically at 390 nm excitation and 500 nm emission for AFC using Fluoroskan Ascent CF Fluorometer (Thermo Labsystems, Helsinki, Finland). Caspase activity was calculated by the following equation, Caspase activity=$I_{treatment}-I_{control}$, where $I_{treatment}$ and $I_{control}$ were the fluorescence intensity of AFC with and without HL, respectively.

Mitochondrial membrane potential. CoRa 622 G6 cells were seeded at a density of 2.5×10^5 cells/ml in flask and cultured for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C. The cells were treated with HLs ([DMPC]=3.42 mM, $[C_{12}(EO)_n]$=0.38 mM) for 10-20 min. After centrifugation at 3,000 rpm for 5 min, cell pellets were incubated with JC-1 (16) staining solution (Cayman Chemical Company, Michigan, USA) for 20 min at 37˚C, and then washed with wash buffer after discarding the medium. Pellets were suspended to culture medium, were seeded to a glass-bottom uncoated dish and were observed with a confocal microscope (TCS-SP Leica, Heidelberg, Germany). At low membrane potential, JC-1 continues to exist as monomer and produces a green fluorescence (emission at 527 nm); at high membrane potential, JC-1 forms J aggregates (emission at 590 nm) and produces red fluorescence.

Assessment of antitumor activity in vivo. The animals were handled in accordance with the guidelines for animal experimentation under Japanese law during the study. Female nude mice (BALB/cA Jcl-*nu*/*nu*, CLEA Japan Inc., Tokyo, Japan) were randomly grouped (n=5) on the basis of body weight on the day of tumor cell inoculation using the stratified randomization method. CoRa 622 G6 cells (5.0×10^6 cells) were inoculated intraperitoneally. HLs (doses for DMPC of HLs were 136 mg/kg and 203 mg/kg) were intraperitoneally administered once each day for 14 days after 1 day of the peritoneal inoculation of CoRa 622 G6 cells. The mice were sacrificed 43 days after inoculation of tumor cells. The tumor nodules were counted macroscopically, and the volume of ascites was weighted.

Survival rate in vivo. Female nude mice (BALB/cA Jcl-*nu*/*nu*) were randomly grouped (n=5) on the basis of body weight on the day of tumor cells inoculation using the stratified randomization method and were treated with HLs as described above. The median lifespan was calculated using the following equation, median lifespan=(median survival days after treatment)/(median survival days of control group)×100.

TUNEL method. Detection of apoptotic cells was performed on the basis of the TUNEL method using an *in situ* apoptosis detection kit (ApopTag Plus Peroxidase; Intergen, New York, U.S.A). Tumors were removed from HL-treated sacrificed mice 43 days after the inoculation of tumor cells and were 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 of digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 30˚C for 60 min. Subsequently, anti-digoxigenin antibody was applied and sections were incubated for 30 min at room temperature. Detection of the antigen-antibody link was made through immunoperoxidase followed by 3,3'-diaminobenzidine chromogen. The sections were counterstained with 5% methyl green, rinsed in distilled water, mounted and were observed on the basis of TUNEL method using optical microscopy (Nikon TS-100; Tokyo, Japan).

Statistical analysis. Results are presented as the mean±S.D. Data were statistically analyzed using Student's *t*-test. A *p*-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Physical properties of HLs. We examined the morphology of HLs composed of DMPC and 10 mol% $C_{12}(EO)_{23}$ on the basis of electron microscopy. As shown in Figure 1, uniform vesicles for the HLs with a diameter about 100 nm were obtained. A clear solution of HLs having a hydrodynamic diameter under 100 nm were preserved for a period of one month without change on the basis of dynamic light-scattering measurements.

Inhibitory effects of HLs on the growth of CoRa 622 G6 cells. We examined the inhibitory effects of HLs on the

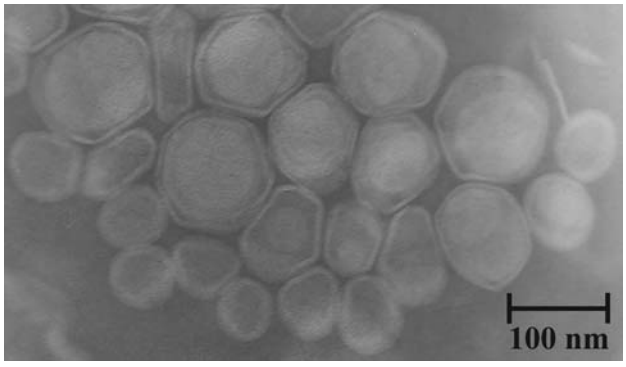

Figure 1. *Electron micrograph of hybrid liposomes composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$. Presence of uniform vesicles for the HLs with a diameter of 100 nm was confined in electron micrograph.*

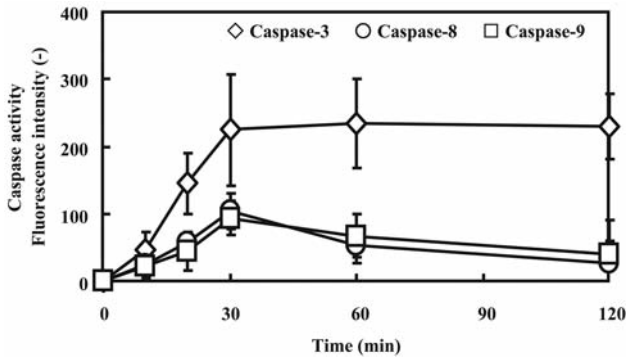

Figure 2. *Time course for the activity of caspases in CoRa 622 G6 cells after treatment with hybrid liposomes. HLs: [DMPC]=3.4 mM, $[C_{12}(EO)_{23}]$=0.38 mM. Data presented are the mean±S.D. The activity of caspase-3, -8 and -9 in CoRa 622 G6 cells drastically increased at 30 min after the treatment with HLs.*

growth of gastric carcinoma (CoRa 622 G6) cells on the basis of WST-1 assay. Fifty percent inhibitory concentrations (IC_{50}) of HLs and single-component liposomes of DMPC were 0.14 mM and 0.24 mM, respectively. These results indicate that HLs should be of greater effect as compared with single-component liposomes of DMPC on the growth of CoRa 622 G6 cells.

Induction of apoptosis by HLs. Activation of caspases is an indispensable process in the execution phase of apoptosis. We examined the mechanism for the inhibitory effects on the growth of tumor cells of HLs *in vitro*. Activation of caspases was measured on the basis of caspase fluorometric protease assay. Time courses for the activity of caspase-8, caspase-9 and caspase-3 induced by HLs are shown in Figure 2. The caspase activity in CoRa 622 G6 cells increased at 30 min after the treatment with HLs. These results indicate that the HLs should induce apoptosis through caspase-8, caspase-9 and caspase-3 in the caspase cascade. We then examined the mitochondrial pathway for apoptotic signal transduction by the HLs using the JC-1 assay. The results are shown in Figure 3. Red fluorescence was observed in control cells, green fluorescence was observed in CoRa 622 G6 cells after the treatment with HLs for 20 min, indicating that mitochondrial potentials decreased. These results indicate that HLs should execute apoptosis through mitochondria.

Therapeutic effects of HLs in vivo. We examined the therapeutic effects of HLs using mouse models of gastric carcinoma with peritoneal dissemination *in vivo*. The results are shown in Figure 4. Few tumor nodules were observed for the mice treated with HLs, although mice in the control group had many tumor nodules (Figure 4A). The number of tumor nodules in mice treated with HLs was significantly lower than those of the control group (p<0.01 at DMPC of 136 mg/kg and 203 mg/kg) (Figure 4B). It is also worth noting that a remarkable decrease of the ascites in mice treated with HLs was observed in comparison with that of mice in the control group (p<0.05 at DMPC of 136 mg/kg and 203 mg/kg) (Figure 4C). These results indicate that HLs could be effective for inhibiting the metastasis of gastric carcinoma cells in the peritoneum.

Next, we examined the survival rate of mice with gastric carcinoma with peritoneal dissemination after the treatment with HLs. The results are shown in Figure 5. The median survival time for the controls, mice treated with HLs at DMPC of 136 mg/kg, and these in the group treated with HLs at DMPC of 203 mg/kg was 52.8, 72.0 and 77.8 days, respectively. It is of interest that a significantly prolonged survival was (DMPC: at 136 mg/kg, >135%, p<0.05 and at 203 mg/kg, >145%, p<0.01) obtained in the groups treated with HLs (dose for DMPC, 136 mg/kg) and in the group treated with HLs. Furthermore, we examined the mechanism of the therapeutic effects of HLs on the mouse model of gastric carcinoma after the peritoneal dissemination on the basis of histological analysis using the TUNEL method. Many apoptotic cells were observed in the tumor tissue of the group treated with HLs as shown in Figure. 6. These results indicate that HLs induced apoptosis of CoRa 622 G6 cells.

Discussion

Chemotherapy with 5-FU is effective for the treatment of patients with gastric cancer (2). 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 is desirable.

It is well known that apoptosis is essential in many aspects of normal development and is required for maintaining tissue

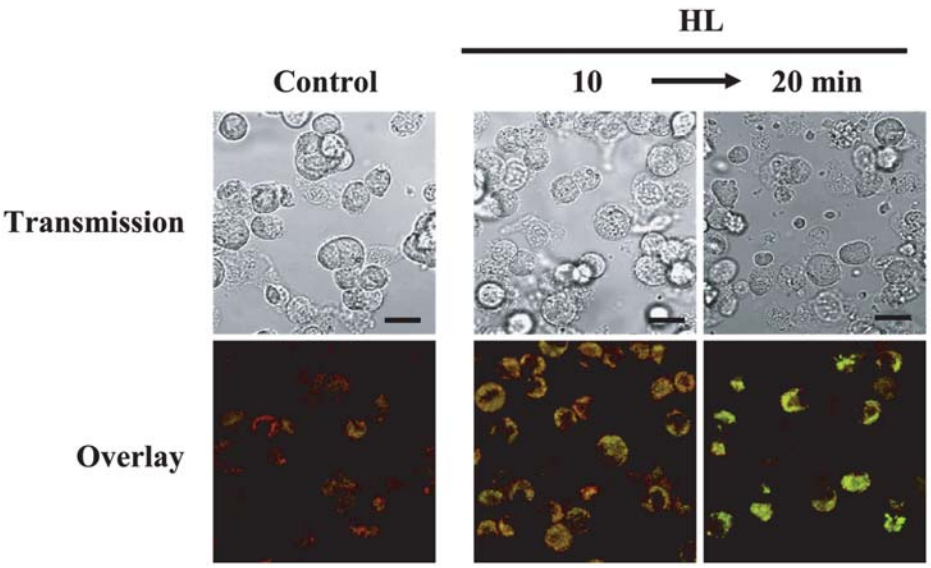

Figure 3. *Decrease in mitochondrial transmembrane potential for CoRa 622 G6 cells after treatment with hybrid liposomes. HLs: [DMPC]=3.4 mM, [$C_{12}(EO)_{23}$]=0.38 mM. Mitochondorial transmembrane potential decreased after the treatment with HLs for 20 min.*

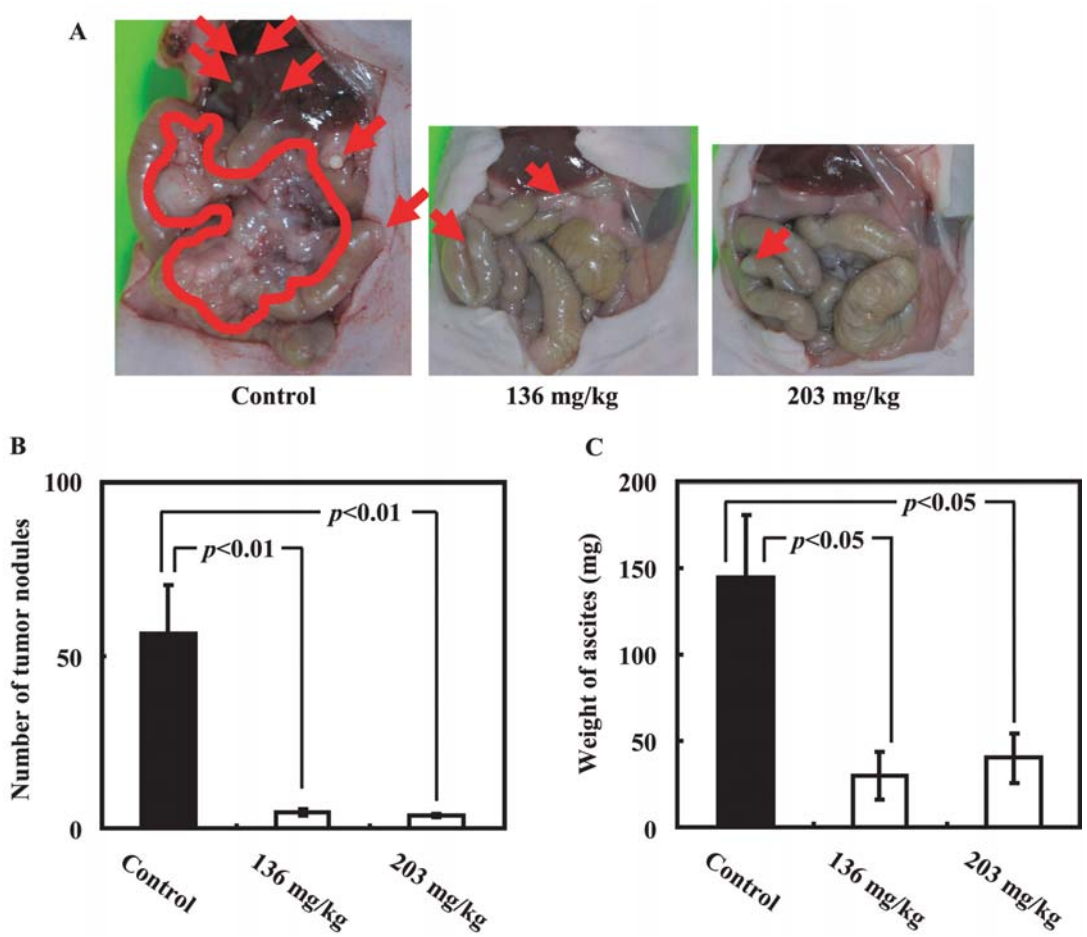

Figure 4. *Therapeutic effects of hybrid liposomes on the peritoneal dissemination of gastric carcinoma in vivo. A: Abdomen; B: tumor nodules; C: ascites. Data presented are the mean±S.D. A remarkable decrease in the number of tumor nodules and the ascites in mice treated with HLs were observed in comparison with mice in the control group.*

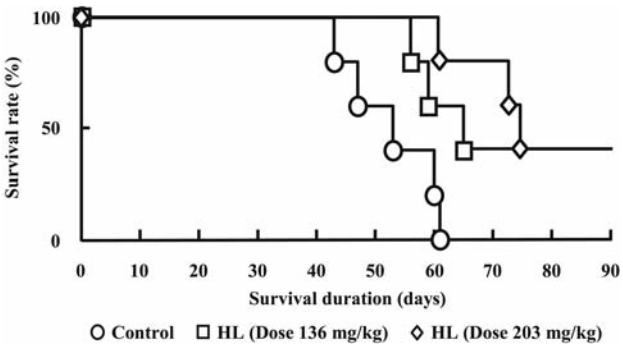

Figure 5. *Survival curves of mice treated with hybrid liposomes after the inoculation of CoRa 622 G6 cells. Prolonged survival was obtained in the group treated with HLs.*

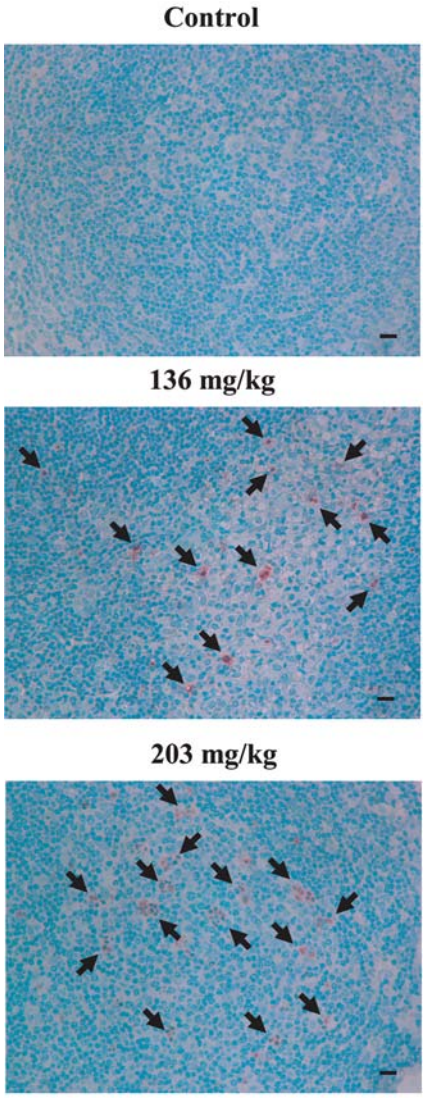

Figure 6. *TUNEL staining of tumor tissue from mice with peritoneal dissemination of gastric carcinoma treated with hybrid liposomes. Arrows indicate apoptotic cells. Scale bar: 10 μm, magnification: ×400. Apoptotic cells (arrows) were observed in the tumor cells of mice after the treatment with HLs.*

homeostasis. Consequently, control of apoptosis is an important potential target for therapeutic intervention.

Fluctuation of tumor cell membranes is very different from that of normal cells. The membranes of tumor cells are generally more fluid as compared with normal ones. HLs being more fluid as compared with single-component liposomes of DMPC for the basis of fluorescence polarization measurement showed remarkable high inhibitory effects compared with single-component liposomes of DMPC on the growth of human colon tumor (8). Furthermore, a good correlation between the IC_{50} of HLs for the growth of human colon tumor (WiDr) cells and membrane fluidity of hybrid liposomes was already reported (8). It is also noteworthy that total internal reflection fluorescence micrographs showed that when administered to tumor (WiDr) and normal (CCD33Co) colon cells, HLs fused and accumulated into the WiDr cells only (8). These results suggest that the inhibitory effects of the HLs on the growth of tumor cells should be related to membrane fluidity.

We examined the mechanism for the inhibitory effects of HLs on the growth of tumor cells of *in vitro*. Activation of caspases was verified for CoRa 622 G6 cells on the basis of caspase fluorometric protease assay. The pathways of apoptosis induced by HLs of DMPC/10 mol% $C_{12}(EO)_{10}$ in human promyelocytic leukemia (HL-60) cells has already been reported (7). Such HLs fused and accumulated in HL-60 cell membranes, and the apoptosis signal first passed through the mitochondria, then caspase-9 and caspase-3 (pathway (A)), second by through FAS, caspase-8 and caspase-3 (pathway (B)) and then reached the nucleus. The apoptotic signal by HLs for CoRa 622 G6 cells passed through mitochondria and activation of caspase-9, -8 and -3, and then reached the nucleus.

Remarkable therapeutic effects along with apoptosis and the significantly prolonged survival rate (>145%) were obtained in the mouse model of gastric carcinoma after the treatment with HLs *in vivo*. HLs demonstrated no side-effects using healthy rats *in vivo* (12). HLs were metabolized in the liver after intravenous administration to healthy mice as described previously (12).

In clinical application, a prolonged survival, more than one year, was attained in one patient with lymphoma after the intravenous injection of HLs 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 HLs (12). Therefore, successful therapy using HLs without any anticancer drug in this study should be important for clinical applications in the future.

Conclusion

We clearly demonstrate that remarkable therapeutic effects of drug-free HLs were obtained for the first time in a mouse model of gastric carcinoma with peritoneal dissemination *in vivo*. The noteworthy aspects are as follows: (a) HLs having a hydrodynamic diameter under 100 nm were successfully preserved for a period of one month; (b) A decrease of mitochondrial membrane potential and activation of caspase-8, -9 and -3 were observed, indicating that apoptotic signals by HLs should pass through mitochondria and these caspases; (c) Remarkable therapeutic effects along with apoptosis and prolonged survival were obtained in this mouse model of gastric carcinoma with peritoneal dissemination after the treatment with HLs *in vivo*. The results in this study could contribute to the development of chemotherapy for patients with gastric carcinoma in future clinical applications.

Acknowledgements

We are grateful to Mr. Shiro Kawase formerly of Hokkaido Institute of Public Health for his helpful discussion. 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. 20107007, 20360377, 20560732).

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Received December 11, 2009
Revised April 20, 2010
Accepted April 23, 2010