International Journal of Pharmaceutics 406 (2011) 173–178

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Chemotherapy with hybrid liposomes for acute lymphatic leukemia leading to apoptosis *in vivo*

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ARTICLE INFO

Article history:
Received 19 October 2010
Received in revised form
15 December 2010
Accepted 31 December 2010
Available online 8 January 2011

Keywords:
Hybrid liposomes
Chemotherapy
Acute lymphatic leukemia
Apoptosis
In vivo

ABSTRACT

Hybrid liposomes (HL) composed of 95 mol% L-α-dimyristoylphosphatidylcholine (DMPC) and 5 mol% polyoxyethylene (25) dodecyl ether ($C_{12}(EO)_{25}$) were prepared by sonication. A clear solutions of HL-25 having hydrodynamic diameter of about 50 nm could be maintained over 3 weeks. Remarkable reduction of tumor volume in model mice of acute lymphatic leukemia (ALL) intravenously treated with HL-25 without drugs after the subcutaneously inoculation of human ALL (MOLT-4) cells was verified *in vivo*. Induction of apoptosis in tumor of model mice of ALL treated with HL-25 was observed in micrographs on the basis of TUNEL method. Remarkable decrease of the ascites in ALL model mice treated with HL-25 was observed. It is noteworthy that prolonged survival (>400%) was obtained in model mice of ALL after the treatment with HL-25 without drugs.

1. Introduction

Malignant acute lymphatic leukemia (ALL) (Apostolidou et al., 2007) cells with high proliferation potency spread to lymph node of liver, lung, and abdomen and then metastasizes to the other organs. A complete remission rate (CR) of patients with ALL by chemotherapy or radiation reaches 80%. However, long-term disease-free survival rate is extremely low at 30–50% because some patients relapse (Yavuz et al., 2006; Fielding et al., 2007; Pulte et al., 2009). Furthermore, while chemotherapy drugs and radiation kill tumor cells, they also damage normal cells, causing side-effects. For example, methotrexate (MTX) of an integral component of treatment for ALL can be associated with neurotoxicities and neuropsychologic side effects (Rubnitz et al., 1998; Shuper et al., 2000; Brugnoletti et al., 2009). Therefore, a chemotherapy that could be effective for ALL without any side-effects is required.

We have produced hybrid liposomes (HL) composed of vesicular and micellar molecules (Ueoka et al., 1985, 1988). 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. Inhibitory effects of HL including antitumor drugs have been observed on the growth of glioma cells in vitro and *in vivo* (Kitamura et al., 1996). HL have been effective for inhibiting the growth of various tumor cells in vitro (Matsumoto et al., 1999a,b; Nagami et al., 2006a,b) and *in vivo* using animal model of carcinoma (Kanno et al., 1999). No toxicity of HL was observed in normal rats *in vivo* without any side effects (Nagami et al., 2006a,b) Successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported after passing the committee of bioethics (Ichihara et al., 2008). We examined the mechanistic details for apoptosis of tumor cells induced by HL (Matsumoto et al., 2005) and the correlation between antitumor effects and membrane fluidity of HL had been obtained (Komizu et al., 2006). However, apoptotic pathway and therapeutic effects of HL for human ALL (MOLT-4) cells *in vivo* have not yet been elucidated.

In this study, we investigated the therapeutic effects of HL-25 composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(25)dodecyl ether ($C_{12}(EO)_{25}$) using model mice of carcinoma after the inoculation of human ALL (MOLT-4) cells *in vivo*.

2. Materials and methods

2.1. Preparation of HL-25

HL-25 were prepared by sonication of a mixture containing 95 mol% DMPC (NOF Co. Ltd., Japan) and 5 mol% $C_{12}(EO)_{25}$ (Nikko Chemicals Co. Ltd., Tokyo, Japan) which is known to be a fusion accelerator (Skelley et al., 2009) 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).

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doi:10.1016/j.ijpharm.2010.12.041

2.2. Dynamic light scattering measurements

The diameter of HL-25 was measured with a light scattering spectrometer (Otsuka Electronic, Japan) using a He–Ne laser (633 nm) at a 90° scattering angle. The diameter (d_{hy}) was calculated using the Stokes–Einstein formula (Eq. (1)), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient:

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

2.3. Cell culture

Human ALL (MOLT-4) cell lines (Minowada et al., 1972; Honma et al., 1992; Scuto et al., 2008) were obtained from RIKEN Cell Bank (Ibaraki, Japan). MOLT-4 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 and 50 μg/ml streptomycin). The cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2.

2.4. Caspase fluorometric protease assay

Activation of caspases was measured on the basis of caspase fluorometric protease assay. MOLT-4 cells (6.0×10^6 cells) were treated with HL-25 ([DMPC] = 11.5 mM, [$C_{12}(EO)_{25}$] = 0.61 mM) for 1–48 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 = $I_{treatment} - I_{control}$, where $I_{treatment}$ and $I_{control}$ was fluorescence intensity of AFC with and without HL, respectively.

2.5. Mitochondrial membrane potential

MOLT-4 cells (6.0×10^6 cells) were treated with HL-25 ([DMPC] = 11.5 mM, [$C_{12}(EO)_{25}$] = 0.61 mM) for 48 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. 2-6. Assessment of antitumor activity in vivo

The animals were handled in accordance with the guidelines for animal experimentation of Japanese law during the study. Female SCID mice (C.B-17/Icr-scid) were obtained from CLEA Japan, Inc. MOLT-4 cells (5.0×10^6 cells) suspended into matrigel (BD Co., USA) were subcutaneously inoculated to dorsal of mice. Number of mice was five in each group. The tumor volume reached 100–300 mm^3 at day 7 after the inoculation of MOLT-4 cells, and then HL-25 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 (Banciu et al., 2008). Reduction rate of tumor volume was calculated using the equation of {1 − (median tumor volume of treated group/median tumor volume of control group) × 100}. Furthermore, the volume of ascites was measured.

2.7. 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, USA) according to manufacturer's directions. MOLT-4 cells (5.0×10^6 cells) suspended into matrigel (BD Co., USA) were subcutaneously inoculated to dorsal of SCID mice. HL-25 was administered into the caudal vein of mice once each for 14 days after the MOLT-4 cells were inoculated to mice. Tumor was removed from anaesthetized mice after the treatment with HL-25 and fixed in 10% formalin solution. Paraffin-embedded sections were made, and the detection of apoptosis of a solid tumor was performed on the basis of TUNEL assay according to the conventional method.

2.8. Immunostaining with anti-AIF antibody

Paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanol to water. Tumor sections were heated at 120 °C for 10 min for antigen activation and were blocked with a solution PBS and 1% H_2O_2 for 5 min. The sections were washed with PBS(−) and incubated with anti human/rat/mouse AIF (Apoptosis Inducting Factor) antibody (R&D Systems, USA) in a humidified box at 4 °C for over night. The sections were washed twice with PBS, immunostained with rabbit anti-goat immunoglobulins polyclonal antibody (HRP, USA) for over night at 4 °C. Finally, the detection of the antigen–antibody link was made through immunoperoxydase followed by 3,3′-DAB chromogen. The sections were counterstained with hematoxylin, rinsed in distilled water and mounted.

2.9. Assessment of survival rate in vivo

Female SCID mice (C.B-17/Icr-scid) were obtained from CLEA Japan Inc. SCID mice were randomly grouped ($n = 10$) on the basis of body weight on the day of tumor cells inoculation using the stratified randomization method. MOLT-4 cells (5.0×10^6 cells) were intraperitoneally injected into the SCID mice. HL-25 was intraperitoneally administered once each for 21 days after the inoculation of tumor cells. The median lifespan was calculated using following the equation, median lifespan = (median survival days after the treatment)/(median survival days of control group) × 100. Furthermore, the mice were sacrificed under anesthesia with ether at 82 days after inoculation of tumor cells, and tumor burdens were evaluated by measuring ascites collected from mice ($n = 3$).

2.10. Statistical analysis

Results are presented as 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.

3. Results

3.1. Physical properties of HL-25

We examined the morphology of HL-25 composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ on the basis of dynamic light scattering measurements. As shown in Fig. 1, hydrodynamic diameter (d_{hy}) of HL-25 was about 50 nm, which was preserved for a period of remained stable for more than one month. On the other hand, DMPC liposomes were unstable and precipitated after 14 days. HL were kept at room temperature (25 °C) due to convenience of stock

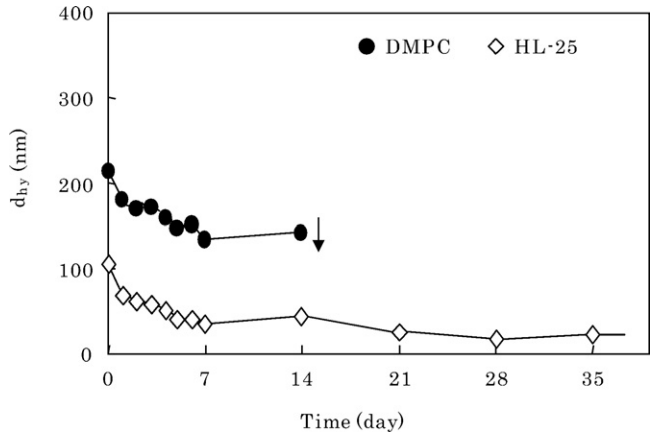

Fig. 1. Time courses of d_{hy} change for HL-25. DMPC: [DMPC] = 2.0×10^{-2} M, HL-25: [DMPC] = 2.0×10^{-2} M, [$C_{12}(EO)_{25}$] = 1.05×10^{-3} M, in 5%glucose solution. Stored at 25 °C. Allow: precipitation.

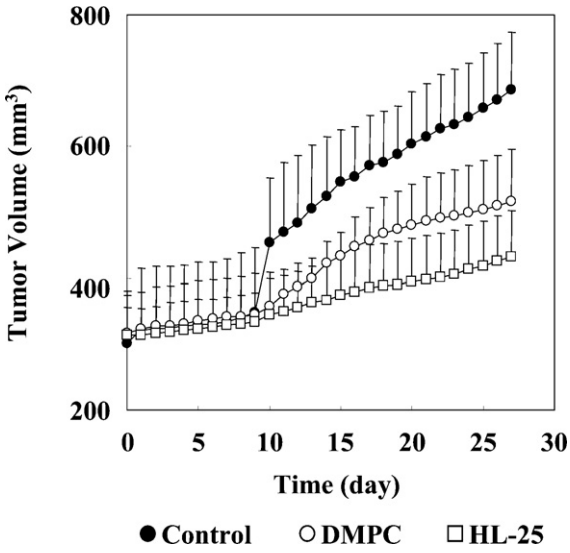

Fig. 2. Tumor volume of model mice of ALL intravenously treatment with HL-25 after subcutaneous inoculating MOLT-4 cells. Dose for DMPC; 136 mg/kg. Data presented are mean ± S.D. Remarkable reduction of ALL tumor volume was obtained in the group treated with HL-25.

for a long term period for clinical application. It is suggested that diameter of HL gradually decreased, since membrane fluidity of HL that kept at room temperature near phase transition temperature (21 °C) (Matsumoto et al., 1999a,b) were gradually stabilized after a preparation at 45 °C.

It is worthy to note that HL-25 having 50 nm in diameter could avoid the reticular endothelial system (RES) (Allen et al., 1991) and should be appropriate for *in vivo* and clinical applications after the intravenous administration.

3.2. Inhibitory effects of HL-25 on the growth of ALL in vivo

We examined inhibitory effect of intravenous treatment with HL-25 on the growth of tumor in subcutaneous xenograft model mice of ALL. The results are shown in Fig. 2. The more remarkable reduction rate of 40% ($p < 0.01$) in tumor volume was obtained in mice treated in HL-25, in contrast with that of 25% ($p < 0.05$) in mice treated with DMPC liposomes. Furthermore, statistical significance between DMPC liposomes and HL-25 was obtained ($p < 0.05$). It is noteworthy that a remarkable reduction of tumor volume was obtained in model mice of ALL intravenously treated with HL-25 without drugs after subcutaneously inoculating MOLT-4 cells.

3.3. Induction of apoptosis by HL-25 in vivo

We examined the induction of apoptosis by HL-25 for solid tumor in model mice of ALL using the TUNEL method. The results are shown in Fig. 3. Brown color was observed in the tumor cells of mice after the treatment with HL-25, although the apoptosis cells were not observed in the group treated with DMPC liposomes. These results indicate that HL-25 have remarkable inhibitory effects along with apoptosis on the growth of MOLT-4 cells.

Signal transduction of apoptosis by HL-25 for MOLT-4 cells *in vivo* was examined. Fairly more numerous apoptotic positive cells for MOLT-4 cells treated with HL-25 (52.4%) were observed as compared with the case of DMPC liposomes (18.9%) in Annexin-V binding assay as well as TUNEL method. Activation of caspases and mitochondria is an indispensable process in the execution phase of apoptosis. No activity of caspase-8, caspase-9 or caspase-3 by HL-25 was obtained on the basis of caspase fluorometric protease assay. These results suggest that the apoptotic signal could not be transferred through the caspase cascade pathway. Interestingly, mitochondorial transmembrane potential was decreased after the treatment with HL-25 for 48 h using the flow cytometry (data not shown). To elucidate the apoptotic pathway which pass directly through mitochondria with independent caspase cascade, we examined participation of apoptosis inducing factor (AIF). The results are shown in Fig. 4. Many AIF positive cells (brown color) for MOLT-4 cells treated with HL-25 were observed apart from the cases of DMPC liposomes and control group. These results suggest the mitochondorial pathway could be implicated in the apoptosis induced by the HL-25 for MOLT-4 cells.

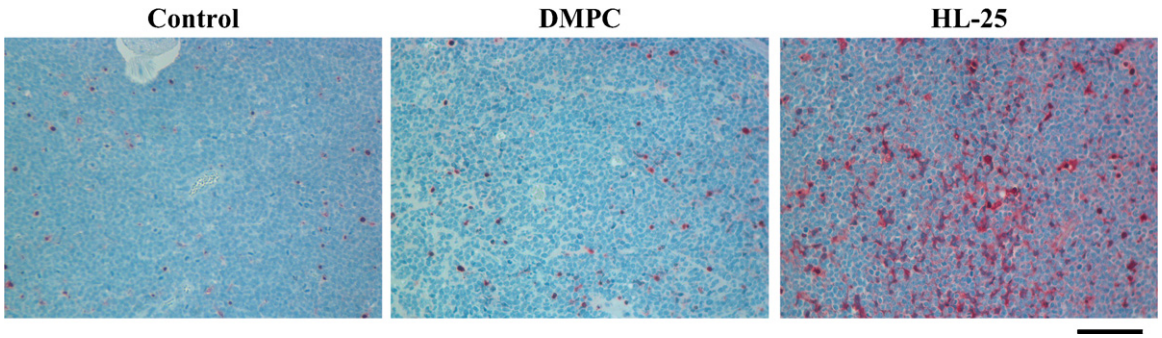

Fig. 3. Micrographs of solid tumor in model mice of ALL treated with HL-25 after the subcutaneous inoculation of MOLT-4 cells using TUNEL method. Dose for DMPC; 136 mg/kg. Arrows indicate apoptotic cells. Scale bar: 100 μm, magnification: ×400. Apoptotic cells (arrows) were observed in the tumor cells of mice after the treatment with HL-25.

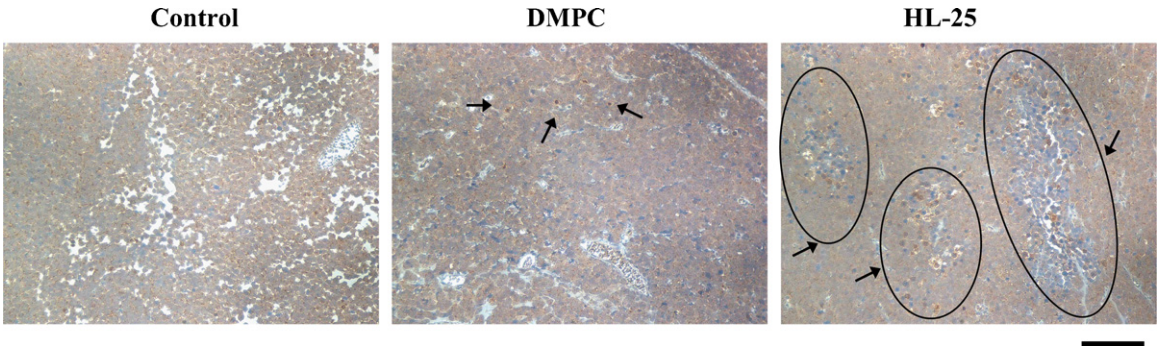

Fig. 4. Micrographs of solid tumor in model mice of ALL treated with HL-25 after the subcutaneous inoculation of MOLT-4 cells using immunostaining with anti-AIF antibody. Dose for DMPC; 136 mg/kg. Arrows indicate AIF positive cells. Scale bar: 100 μm, magnification: ×400. AIF positive cells (arrows) were observed in the tumor cells of mice after the treatment with HL-25.

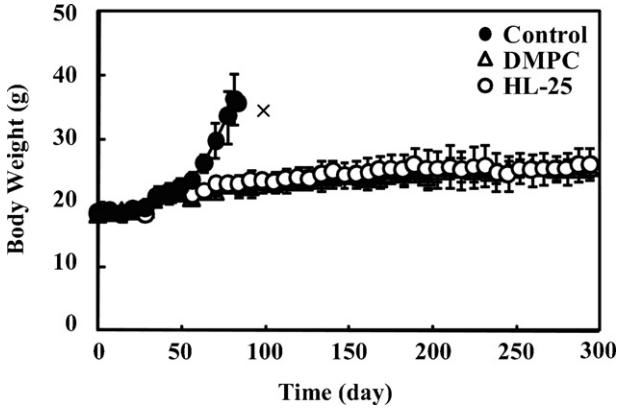

Fig. 5. Body weight change of model mice of ALL treated with HL-25 after the intraperitoneal inoculation of MOLT-4 cells. Dose for DMPC; 136 mg/kg.

3.4. 3-4. Therapeutic effects of HL for model mice of ALL in vivo

We examined the therapeutic effects of HL-25 using mice of ALL with peritoneal dissemination after the intraperitoneal treatment with HL-25. The results are shown in Figs. 5 and 6. The body weight of the mice treated with DMPC and HL-25 gradually increased, whereas that of the mice in the control group drastically increased and reached about 36 g for 82 days as shown in Fig. 5. The photographs show that the mice of control group had distended abdominal regions (Fig. 6a). Interestingly, massive ascites was obtained in peritoneal cavity of the mice of control group after dissection. The results are shown in Fig. 6b. The mice treated with DMPC (0.45 ± 0.16 g, $p < 0.01$) and HL-25 (0.62 ± 0.08 g, $p < 0.01$) had a significantly lower volume of ascites compared with the mice in control group (2.74 ± 0.46 g). Statistical significance between DMPC liposomes and HL-25 was not obtained. Furthermore, survival rates are shown in Fig. 7. The median survival time for mice treated with HL-25 was higher than 420 days without death, although that for mice in the control group was 84.7 ± 2.2. On the other hand, the median survival time for mice treated with DMPC was 385 ± 62, and statistical significance between DMPC liposomes and HL-25 was obtained ($p < 0.05$). It is noteworthy that a significantly prolonged survival (>400%, $p < 0.01$) was obtained in the mice treated with HL-25. These results indicate that HL-25 could strongly inhibit the growth of MOLT-4 cells *in vivo*.

4. Discussion

Chemotherapy with anticancer drug such as MTX is effective for the treatment of patients with ALL (Rubnitz et al., 1998; Shuper

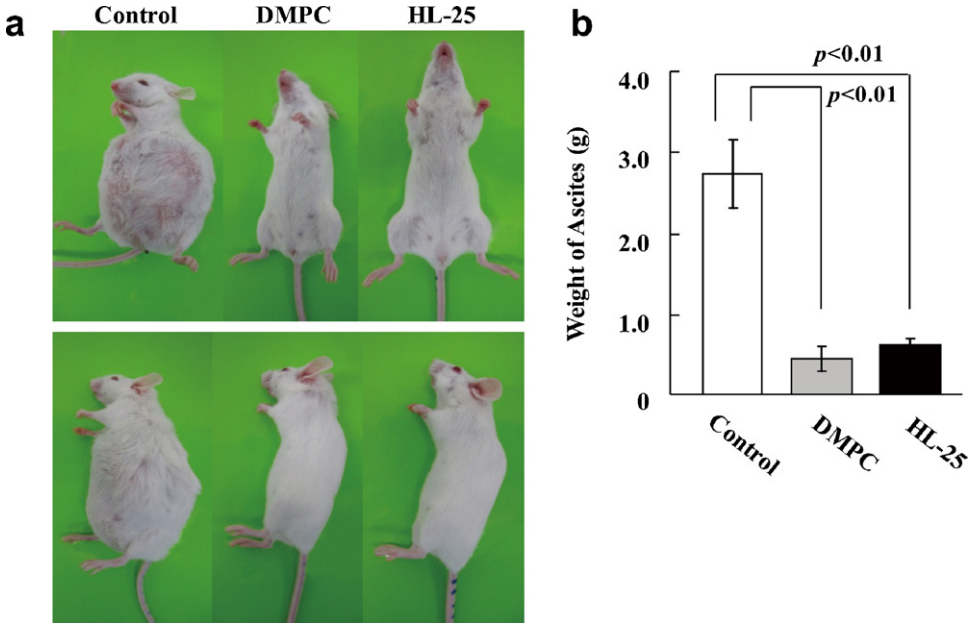

Fig. 6. Therapeutic effects of HL-25 for model mice of ALL with peritoneal dissemination *in vivo*. (a) Photographs of ascites-bearing mice of control group or mice treated with HL-25 for 14 days after being inoculated with MOLT-4 cells intraperitoneally. (b) The weight of ascites in mice inoculated with MOLT-4 cells. Dose for DMPC; 136 mg/kg.

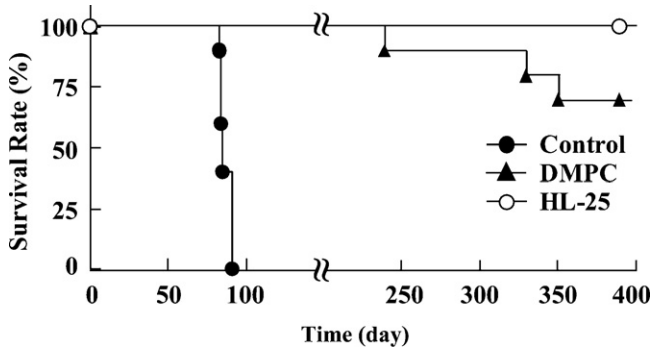

Fig. 7. Survival curves for model mice of ALL with peritoneal dissemination treated with HL-25 after the intraperitoneal inoculation of MOLT-4 cells. Dose for DMPC; 136 mg/kg.

et al., 2000; Brugnoletti et al., 2009). Although anticancer drugs kill tumor cells, they also damage normal cells, causing side-effects. Therefore, chemotherapy along with induction of apoptosis without any side effects should be desirable.

It is well known that apoptosis is essential in many aspects of normal development and is required for maintaining tissue homeostasis. Consequently, control of apoptosis is an important potential target for therapeutic intervention.

Morphological change in human promyelocytic leukemia (HL-60) cells after the treatment with HL composed of phospholipids having the same hydrophilic head group (phosphatidylcholine group) and different hydrophobic alkyl chains (L-α-dilauroylphosphatidy- lcholine (C12: DLPC), DMPC (C14), L-α-dipalmitoylphosphatidylcholine (C16: DPPC)) and $C_{12}(EO)_{23}$ using a time-laps video were examined (Nagami et al., 2006a,b). The formation of bleb and corpuscle indicating characteristic feature of apoptosis was observed for HL of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. On the other hand, swelling of cells and dissolving of cell membrane, that is necrosis, were observed for HL of 90 mol% DLPC/10 mol% $C_{12}(EO)_{23}$. Neither apoptosis nor necrosis was observed for HL of 90 mol% DPPC/10 mol% $C_{12}(EO)_{23}$. Thus, two methylene groups of acyl chains in phosphatidylcholines could distinguish between apoptosis and necrosis has already been reported (Nagami et al., 2006a,b).

Human renal carcinoma cells (OS-RC-2) treated with HL had been positive on the basis of TUNEL method and Annexin-V binding assay, indicating that HL induced apoptosis for tumor cells, although apoptotic cells were not obtained using the DMPC liposomes (Umebayashi et al., 2010). However, the apoptotic cells were not observed in normal human renal proximal tubule epithelial (RPTE) cells after the treatment with HL. That is HL could distinguish a normal cells and a cancer cells and should induce apoptosis only for cancer cells.

The pathways of apoptosis induced by HL of DMPC/10 mol% $C_{12}(EO)_{10}$ in human promyelocytic leukemia (HL-60) cells have already been reported (Matsumoto et al., 2005). Such HL 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.

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. Fluidity of HL of DMPC/10 mol% $C_{12}(EO)_n$ (n = 4–25) is higher than that of DMPC liposomes on the basis of fluorescence polarization measurement. Remarkable high inhibitory effects compared with DMPC liposomes on the growth of human colon tumor (WiDr) were obtained (Komizu et al., 2006). Furthermore, a good correlation between the IC_{50} of HL for the growth of WiDr cells and membrane fluidity of HL was already reported (Komizu et al., 2006). It is also noteworthy that total internal reflection fluorescence micrographs showed that when administered to tumor (WiDr) and normal (CCD33Co) colon cells, HL fused and accumulated into the WiDr cells only (Komizu et al., 2006). That is, membrane fluidity of HL is higher than that of DMPC liposomes (Matsumoto et al., 1999a,b, 2005; Nagami et al., 2006a,b; Komizu et al., 2006), and more rapidly fused to membrane of cancer cell having high fluctuation (Komizu et al., 2006). These results suggest that the inhibitory effects of the HL on the growth of tumor cells should be related to membrane fluidity.

We examined the therapeutic effects of HL-25 composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ using model mice after the inoculation of human ALL (MOLT-4) cells *in vivo*. Remarkable reduction of tumor volume along with apoptosis was obtained in model mice of ALL intravenously treated with HL-25 without drugs after subcutaneously inoculating MOLT-4 cells. The significantly prolonged survival rate (>400%) was obtained in mice model of ALL with peritoneal dissemination after the intraperitoneal treatment with HL-25 *in vivo*. Although prolonged survival was obtained in mice model of ALL with peritoneal dissemination after the local (intraperitoneal) administration with the DMPC liposomes, hydrodynamic diameter of the DMPC liposomes (220–320 nm) was over 100 nm. It was suggested that HL (50 nm) could avoid the reticular endothelial system (Allen et al., 1991) and should be appropriate for clinical applications. Therapeutic effects of DMPC liposomes have been reported for model mice of melanoma (Ueoka et al., 2000). HL demonstrated no side effects using healthy rats *in vivo* (Nagami et al., 2006a,b; Ichihara et al., 2008). HL were metabolized in the liver after intravenous administration to healthy mice as described previously (Ichihara et al., 2008).

Thus, the successful therapy for model mice of ALL using HL-25 without any anticancer drug in this study should be important for clinical applications of patients with ALL in the near future.

5. Conclusion

We clearly demonstrated that therapeutic effects of HL-25 along with apoptosis were obtained for model mice having human acute lymphoblastic leukemia. The noteworthy aspects in this study are as follows: (a) Remarkable reduction of tumor volume in model mice of ALL treated with HL-25 was verified. (b) Induction of apoptosis was observed in model mice of ALL after the treatment with HL-25 on the basis of TUNEL method. (c) Remarkable decrease of the ascites in ALL model mice treated with HL-25 was observed. (d) Significantly prolonged survival (>400%) was obtained in ALL model mice after the treatment with HL-25. The results in this study should be advantageous in the chemotherapy for patients with acute lymphatic leukemia in the near future clinical applications.

Acknowledgements

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, and 20560732).

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