

Hybrid Liposomes inhibit the Growth and Angiogenesis in Human Breast Cancer Model

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Abstract

Therapeutic effects of hybrid liposomes (HL-25) composed of L- α -dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(25)dodecyl ether ($C_{12}(EO)_{25}$) against breast tumor due to anti-angiogenic activity were examined *in vitro* and *in vivo*. Inhibitory effects of HL-25 on the formation of capillary tubes in the human umbilical vascular endothelial cells (HUVEC) were obtained *in vitro*. Remarkable reduction of tumor volume in mouse models of human breast cancer (HBC) was verified after the intravenous treatment with HL-25 without drugs *in vivo*. Anti-angiogenic activity in mouse models of HBC treated with HL-25 was observed on the basis of immunostaining method using CD34. Therapeutic effects along with anti-angiogenic activity of HL-25 without any drugs on the mouse models of HBC were revealed for the first time *in vivo*.

Keywords: Hybrid liposome; Breast cancer; Human umbilical vascular endothelial cells; Angiogenic; Vascular endothelial growth factor

Abbreviations

$C_{12}(EO)_{25}$: Polyoxyethylene(25) dodecyl ether; HBC: Human Breast Cancer; DMPC: L- α -Dimyristoylphosphatidylcholine; HL-25: Hybrid Liposomes composed of DMPC and $C_{12}(EO)_{25}$; HUVEC: Human Umbilical Vascular Endothelial Cells; RES: Reticular Endothelial System; VEGF: Vascular Endothelial Growth Factor

Introduction

Human breast cancer (HBC) is one of the leading causes of cancer-related mortality. Screenings for early diagnosis as well as improvements in surgery and therapy method have prolonged the survival of patients with HBC. However, 5-year survival rate in highly advanced metastatic HBC with progression and angiogenesis was low [1-3]. Activated EGF receptor (EGFR) signaling and vascular endothelial growth factor (VEGF) mediated angiogenesis play a critical role in the progression of HBC [4]. Bevacizumab is an angiogenesis inhibitor targeting VEGF and is one of a group of cancer drugs known as monoclonal antibodies [5-7]. Bevacizumab is used to treat HBC in combination with chemotherapy drugs such as docetaxel [8] or capecitabine [8,9]. However, side effects of Bevacizumab have been reported [10-13]. To lead to high quality of life for patients, drugs without side effects are necessary.

Hybrid liposomes (HL), first developed by Ueoka et al. [14], can be prepared by simply ultrasonating a mixture of vesicular and micellar molecules in buffer solutions, and contain no organic solvent unlike conventional liposomes. The therapeutic effects of HL composed of L- α -dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene (20) sorbitan monolaurate (Tween 20) including antitumor drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) have been observed on the growth of glioma cells *in vivo* [15].

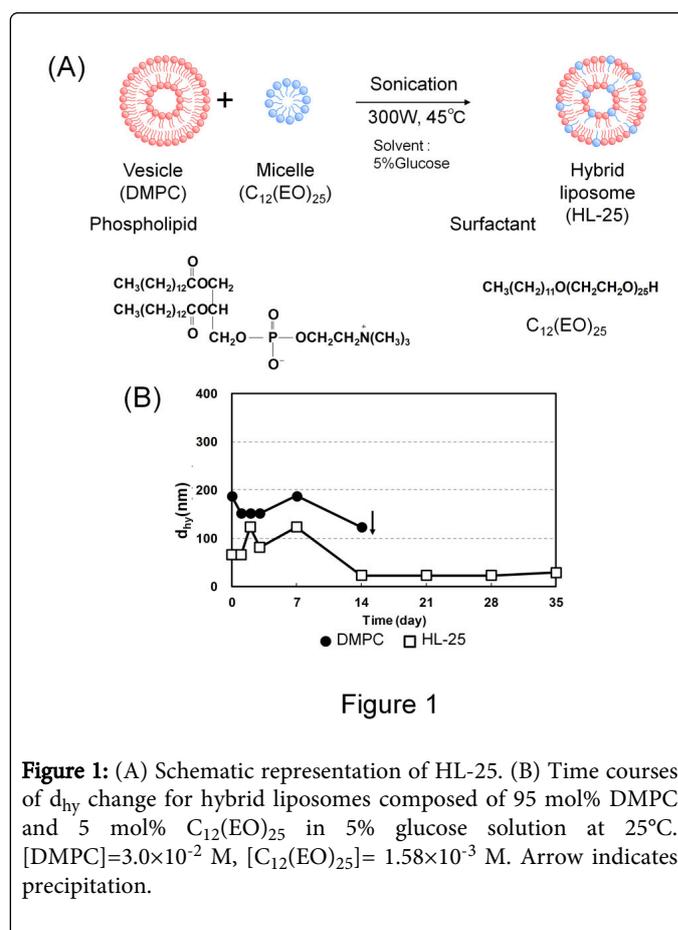


Figure 1

Figure 1: (A) Schematic representation of HL-25. (B) Time courses of d_{hy} change for hybrid liposomes composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ in 5% glucose solution at 25°C. $[DMPC]=3.0 \times 10^{-2}$ M, $[C_{12}(EO)_{25}]=1.58 \times 10^{-3}$ M. Arrow indicates precipitation.

On the other hand, remarkably high inhibitory effects of HL composed of DMPC and polyoxyethylene(n) dodecyl ethers ($C_{12}(EO)_n$) on the growth of tumor cells *in vitro* [16-20] and *in vivo*

[20-24] have been obtained without drugs. Furthermore, successful clinical chemotherapy with HL without any drug to patients with lymphoma has been reported [25-27]. In addition, it has been demonstrated that HL could induce apoptotic cell death in tumor cells [18-27].

In this study, we investigated the therapeutic effects of hybrid liposomes (HL-25, Figure 1A) composed of L- α -dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(25)dodecyl ether (C₁₂(EO)₂₅) due to anti-angiogenic activity for breast tumor.

Experimental Section

Preparation of hybrid liposomes

Hybrid liposomes (HL) were prepared by sonication of a mixture containing 95 mol% L- α -dimyristoylphosphatidylcholine (DMPC, NOF, Tokyo, Japan) and 5 mol% polyoxyethylene(25) dodecylether (C₁₂(EO)₂₅, Nikko Chemicals, Tokyo, Japan,) in 5% glucose solution using bath type sonicator (VS-N300, VELVO-CLEAR, Tokyo, Japan) at 45°C with 300W as shown in Figure 1A. The sample solutions were filtered using a membrane filter (Advantec, Tokyo, Japan) with 0.20 μ m pore size and stored at room temperature (25°C). The DMPC liposomes were prepared in the same manner as described above.

Dynamic light scattering measurements

The diameter of HL-25 was measured using an electrophoretic light scattering spectrophotometer (ELS-ZO, Otsuka Electronics, Osaka, Japan). 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:

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

Cell culture

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

Human umbilical vein endothelial cells (HUVEC) were obtained from Kurabo (Osaka, Japan). HUVEC were cultured in basal medium (Kurabo, HuMedia-EB2, Osaka, Japan) involved breeding additive (Kurabo, HuMedia-EG, Osaka, Japan) in the atmosphere of 5% CO₂ and 37°C until they became confluent according to the protocol of the Kurabo cell culture Kit [28,29]. The HuMedia-EG2 medium consisted of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum (FBS), 10 ng/ml recombinant epidermal growth factor (rEGF), 5 ng/ml recombinant basic fibroblast growth factor (r-bFGF), 10 μ g/ml heparin, and 1 μ g/ml hydrocortisone .

Assessment of cell growth *in vitro*

Inhibitory effects on the growth of HUVEC *in vitro* were examined on the basis of the WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] proliferation assay (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) [30,31]. HUVEC (2.0 \times 10⁴ viable cells/ml) preincubated in HuMedia-EG2

medium for 24 h. HUVEC were cultured for 48 h in CO₂ incubator at 37°C after adding the medium only or to medium containing VEGF (10 μ g/L) and HL-25 at various concentrations. WST-1 solutions were added and the absorbance at wavelength of 450 nm was measured by spectrophotometer (Molecular Devices, CA, USA). The inhibitory effects of HL-25 on the growth of HUVEC 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.

Tube formation assay *in vitro*

The inhibitory effects of HL-25 on the formation of capillary tubes in the human umbilical vascular endothelial cells (HUVEC) were examined on the basis of tube formation assay *in vitro* [28,29]. An angiogenesis assay kit (Kurabo, Osaka, Japan) was used according to the manufacturer's instructions. The HuMedia-EG2 medium containing VEGF (final 10 μ g/L; Kurabo, Osaka, Japan) with concentrations of under IC₂₀ (200 μ M) of HL-25 and 5% glucose (control) was exchanged on d 4, 7, and 9. After 11 days, the cells were washed and directly fixed with 70% ice-cold ethanol for 30 min in the wells. The fixed cells were serially incubated with 1% bovine serum albumin in buffer, mouse monoclonal antibody against human CD31 (Kurabo, Osaka, Japan), alkaline phosphatase-conjugated goat anti-mouse IgG (Kurabo, Osaka, Japan), and nitro-blue tetrazolium chloride (NBT)/5-Bromo-4-Chloro-3'-Indolylphosphatase p-Toluidine salt (BCIP) (violet, Kurabo, Osaka, Japan), washed and photographed. The images were analyzed using Angiogenesis Image Analyzer software (Kurabo, Osaka, Japan) to measure the gross area of CD31-positive tubes (the area of endothelial tubes) in culture. The percentages of the area of endothelial tubes were calculated. Tube length area was quantified using image analysis software ImageJ (Version 1.46r, National Institutes of Health, Bethesda, MD, USA).

ELISA

Supernatant medium from the MDA-MB-453 cells cultures were assayed for cytokine concentrations using Human VEGF Quantikine ELISA Kit as described by the manufacturer (R&D Systems, MN, U.S.A.). MDA-MB-453 cells were cultured on 12-well culture plates (5 \times 10⁵ cells/well) in L-15 medium containing HL-25 (under IC₅₀ (150 μ M) [19]). The supernatants were added in duplicate to appropriate pre-coated plates. After the plates were washed, horseradish peroxidase-conjugated detection antibody was added. The substrate used for color development was tetramethylbenzidine. The optical density was measured at 450 nm with a microplate reader (Molecular Devices, CA, U.S.A.). We deducted quantity of the concentration of VEGF in the serum and evaluated quantity of VEGF of the cells treated with HL-25.

Assessment of antitumor effects *in vivo*

The mice were handled in accordance with the guidelines for animal experimentation set out in Japanese law. The animal studies were approved by the Committee on Animal Research of Sojo University. Female nude mice (BALB/cA Jcl-nu/nu) were obtained from CLEA (Tokyo, Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was five in each group. MDA-MB-453 cells (5.0 \times 10⁶ cells) suspended into matrigel (BD Co., NJ, 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 100-300 mm³ at day 7 after the inoculation of MDA-MB-453 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 $V = 0.5a^2b$, where a and b denote the smallest and longest superficial diameter, respectively [32, 33]. Reduction rate of tumor volume was calculated using the equation of $1 - (\text{median tumor volume of treated group} / \text{median tumor volume of control group}) \times 100$. Tumors were resected from mice anaesthetized with ketamine and weighted.

Immunostaining with anti-CD34 antibody

Tumors were resected from anaesthetized mice after the treatment with HL-25 and fixed in 10 % formalin solution. Paraffin-embedded sections were made, 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 % H₂O₂ for 5 min. The sections were washed with PBS(-) and incubated with anti-human/rat/mouse CD34 antibody (R&D Systems, MN, U.S.A.) in a humidified box at 4°C for overnight. The sections were washed twice with PBS, immunostained with rabbit anti-goat immunoglobulins polyclonal antibody (horseradish peroxidase (HRP) conjugate, Abcam, Cambridge, UK) for overnight at 4°C. Finally, the detection of the antigen-antibody link was made through immunoperoxidase followed by 3,3'-diaminobenzidine (DAB) chromogen. The sections were counterstained with hematoxylin, rinsed in distilled water and mounted. The sections were observed with an optical microscope (Nikon TS-100, Tokyo, Japan). The angiogenesis dimensions (CD34 positive area) were estimated using image analysis software ImageJ (Version 1.46r, National Institutes of Health, Bethesda, MD, U.S.A.).

Statistical analysis

Results are presented as mean \pm S.D. Data were statistically analyzed using Student's T-test and Dunnett's test. A p value of less than 0.05 was considered to represent a statistically significant difference.

Results and discussions

Physical properties of HL-25

We examined the morphology of HL-25 composed of 95 mol% DMPC and 5 mol% C₁₂(EO)₂₅ on the basis of dynamic light scattering measurements. The time course of the hydrodynamic diameter (d_{hy}) change for HL-25 using an electrophoretic light scattering spectrophotometer is shown in Figure 1B. The mean d_{hy} of HL-25 stored at room temperature (25°C) was under 100 nm with a single and narrow distribution and remained stable for more than one month. In contrast, DMPC liposomes were unstable and precipitated after 14 days. HL-25 were kept at room temperature due to the convenience of stock for a long term period for clinical application. It is suggested that the diameter of HL-25 gradually decreased, since the membrane fluidity of HL-25 that was kept at room temperature near phase transition temperature (21°C) [17] gradually stabilized after a preparation at 45°C. It is worthy to note that HL-25 having under the

100 nm in diameter could avoid the reticular endothelial system (RES) [34] and thus should be appropriate for the intravenous administration in vivo and clinical applications.

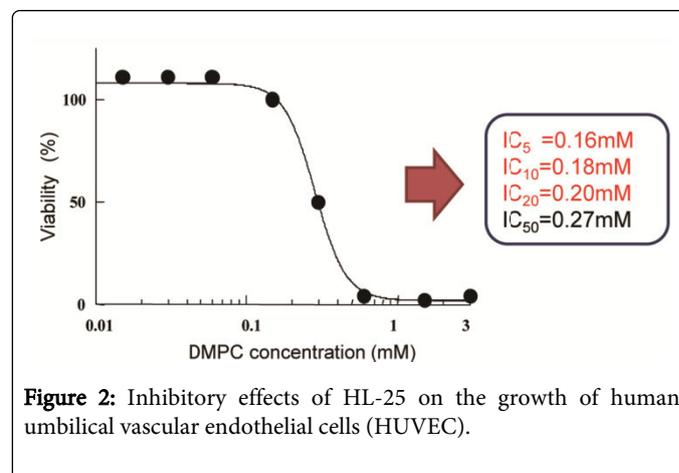


Figure 2: Inhibitory effects of HL-25 on the growth of human umbilical vascular endothelial cells (HUVEC).

Inhibitory effects of HL-25 on the growth of HUVEC *in vitro*

We examined the inhibitory effects of HL-25 on the growth of human umbilical vascular endothelial cells (HUVEC) on the basis of WST-1 assay *in vitro*. The results are shown in Figure 2. HL-25 inhibited the growth of HUVEC cells in a dose-dependent manner. 50% inhibitory concentration (IC₅₀) value of 270 μ M for DMPC was obtained for HUVEC. IC₅, IC₁₀ and IC₂₀ of HL-25 for HUVEC were 160, 180 and 200 μ M, respectively. Based on these results, a concentration of under 200 μ M was used for *in vitro* studies to ensure an observable response without affecting cellular viability. IC₅₀ value of HL-25 on the growth of MDA-MB-453 cells was 150 μ M [19]. That is, HL-25 inhibits on the growth of MDA-MB-453 cells in 150 μ M, although HL-25 does not affect the growth of normal HUVEC cells in 150 μ M.

Anti-angiogenic Activity of HL-25 for the tubule formation of HUVEC *in vitro*

We examined the activity of HL-25 for the tubule formation of HUVEC using an angiogenesis assay kit *in vitro*. The results are shown in Figure 3. Microphotographs using immunostaining with CD-31 show that the area of endothelial tubes in a co-culture of HUVEC with human fibroblasts incubated with 10 mg/L VEGF and 5% glucose solution (control) increase (Figure 3A). The area of tubule formation of HUVEC treated with HL-25 was decreased, although the area of tubule formation of HUVEC after adding DMPC liposomes was the same as those of control. It is suggested that HL-25 could suppress the VEGF-induced migration of HUVEC at low concentration. Next, the dimensions of tubule area in the co-culture of HUVEC with human fibroblasts were estimated using image analysis software ImageJ. The results are shown in Figure 3b. The dimensions of tubule area of the control and DMPC liposomes-treated HUVEC were 0.406 mm² and 0.362-0.481 mm², respectively, whereas that of HUVEC treated with HL-25 was 0.097-0.136 mm² (p<0.05 vs. control and DMPC liposomes). These results indicate that HL-25 could strongly inhibit the tubule formation of HUVEC at low concentration *in vitro*.

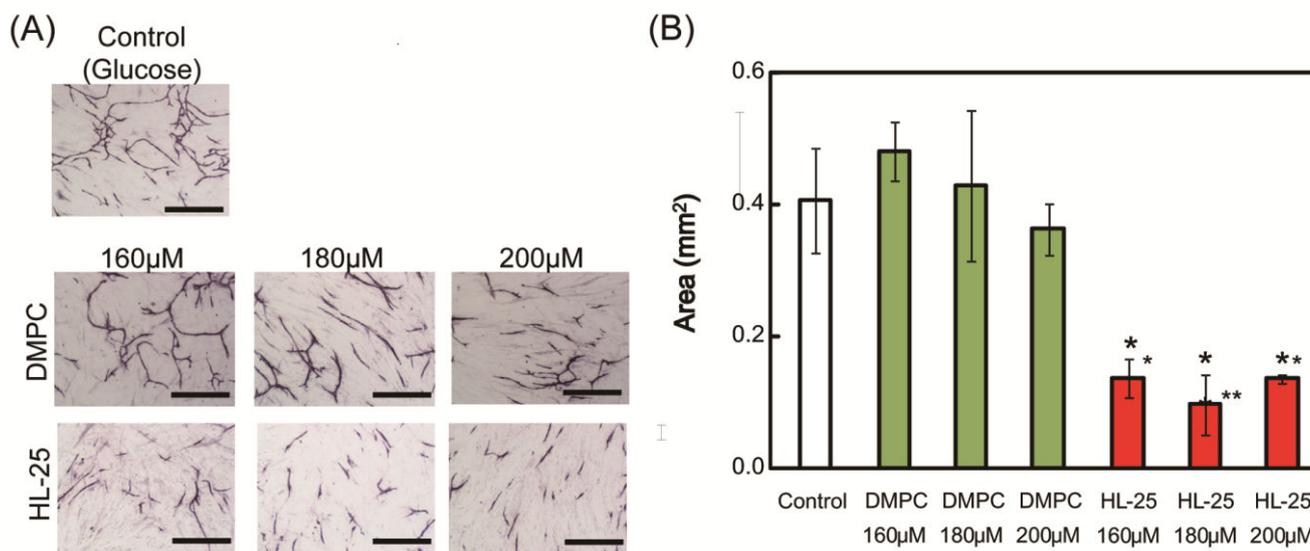


Figure 3: (A) Inhibitory effects of HL-25 for the tubule formation of HUVEC Scale bar, 10 mm. (B) Dimensions of tubule area in HUVEC after the treatment with HL-25. Error bars indicate S.D. * $p < 0.05$ and ** $p < 0.01$ vs. a control group.

Inhibitory effects of HL-25 for VEGF secretion in MDA-MB-453 cells *in vitro*

We examined the mechanism of angiogenesis inhibition of HL-25 *in vitro* by measuring the secretion of angiogenic factors such as VEGF from MDA-MB-453 cells using ELISA. The results are shown in Figure 4. Remarkable decrease of VEGF in MDA-MB-453 cells treated with HL-25 was observed in a dose-dependent manner. Interestingly, HL-25 suppressed VEGF secretion in MDA-MB-453 cells at low concentration of under IC_{50} (150 μ M) [19]. We have already reported that induction of apoptosis of HL at 450 μ M for human breast tumor cells along with activation of caspases [19]. These results suggest that suppression of VEGF secretion by HL-25 of low concentration could be related to anti-angiogenic effects. We will investigate anti-invasion, and migration effects of HL for human breast cancer cells such as MDA-MB-453 and MCF-7 cells *in vitro* in near future.

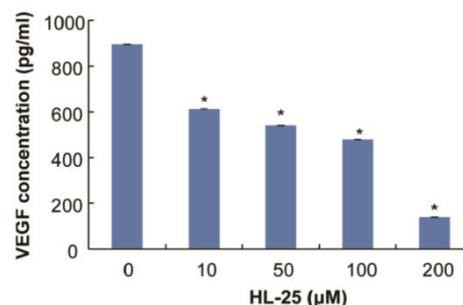
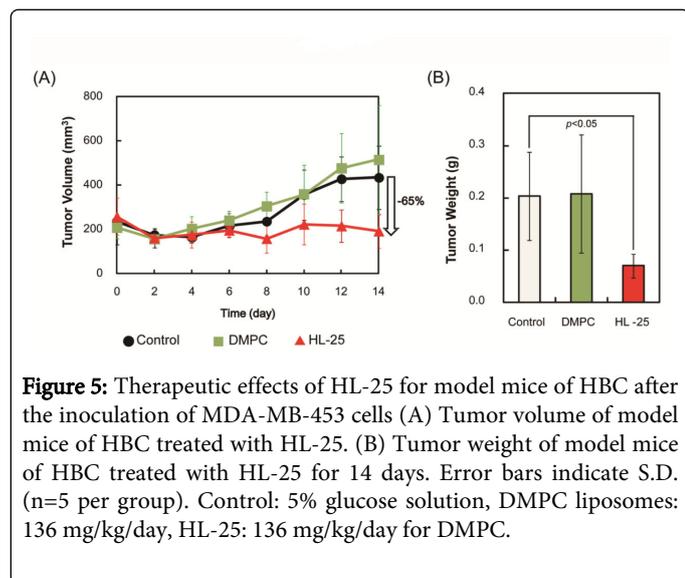


Figure 4: Decrease of VEGF in human breast tumor (MDA-MB-453) cells after the treatment with HL-25. * $p < 0.01$ vs. a control group. IC_{50} value toward MDA-MB-453 cells: 150 μ M.

Therapeutic effects of HL-25 for xenograft mouse models of HBC *in vivo*

We examined the inhibitory effects of HL-25 on the growth of tumors in xenograft mouse models of HBS subcutaneously inoculated with MDA-MB-453 cells. HL-25 was administered into the caudal vein of mice once each for 14 days after from 7 days the MDA-MB-453 cells were inoculated to mice. First, we examined the time course of tumor volume for mouse models of HBC treated with HL-25. The results are shown in Figure 5A. The median of tumor volume was 435.03 ± 142.77 mm^3 in the control group and 516.14 ± 246.82 mm^3 and 191.94 ± 75.83 ($p < 0.05$ vs. control) mm^3 in the group treated with DMPC and HL-25, respectively. It is noteworthy that a remarkable reduction of tumor volume (60 %) was obtained in mouse models of HBC

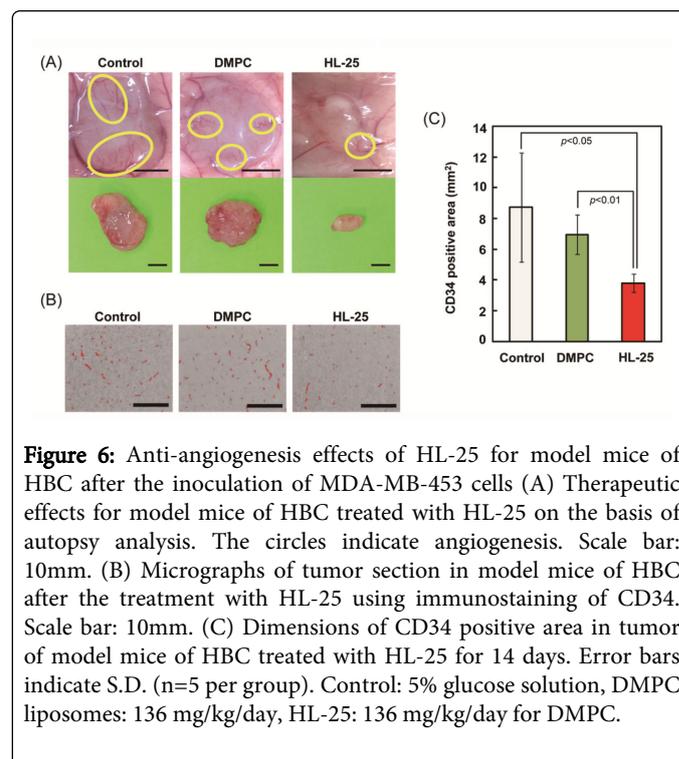
intravenously treated with HL-25 without drugs after subcutaneously inoculating MDA-MB-453 cells. We next examined the tumor weight of mouse models of HBC treated with HL-25. The results are shown in Figure 5B. The mouse models of HBC treated with HL-25 (0.07 ± 0.02 g, $p < 0.05$ vs. control) had a significantly lower weight of tumor compared with the mice in control group (0.20 ± 0.11 g). Furthermore, statistical significance between tumor weight of mice treated with DMPC liposomes (0.21 ± 0.11 g, $p < 0.01$) and HL-25 was obtained. These results indicate that HL-25 could be effective for the mouse models of HBC *in vivo*.



Anti-angiogenesis activity of HL-25 for mouse models of HBC *in vivo*

We examined the anti-angiogenesis activity of HL-25 on the growth of tumors in xenograft mouse models of HBS subcutaneously inoculated with MDA-MB-453 cells. After the treatment period of HL-25 for 14 days, we examined the anti-angiogenesis activity of HL-25 on the mouse models of HBC in an autopsy. The results are shown in Figure 6A. Remarkable decrease of angiogenesis in tumor of mouse models of HBC treated with HL-25 were observed as compared with control and DMPC. Next, we carried out immunostaining using CD34 as a histochemical marker of angiogenesis to establish the anti-angiogenesis activity of HL-25. The results are shown in Figure 6B. Large number of CD34 positive cells in tumor sections in control and DMPC group were confirmed. In contrast, remarkable decrease of CD34 positive cells were observed in the tumor tissue of the group treated with HL-25. To estimate dimensions of CD34 positive area in tumor sections of mouse models of HBC, we carried out calculation using image analysis software ImageJ. The results are shown in Figure 6c. Remarkable decrease of angiogenesis (CD34 positive area) in mouse models of HBC treated with HL-25 (3.79 ± 0.59 mm², $p < 0.05$ vs. control, $p < 0.01$ vs. DMPC) as compared with Control (8.73 ± 3.55 mm²) and DMPC (6.97 ± 1.29 mm²) was confirmed. It is noteworthy that remarkably high anti-angiogenesis activity of HL-25 for mouse models of HBC was observed. This anti-angiogenic effects might be related to tumor suppression by HL-25, because angiogenesis has been associated with an increase of tumor cells. On the other hand, we have reported that the induction of apoptosis by HL could be related to an increase of membrane fluidity and the clustering of lipid rafts in the

plasma membranes of human leukemia (HL-60) cells [35]. Generally, VEGF are secreted in tumor cells that are not receiving enough oxygen. HIF-1 (hypoxia-inducible factor), a transcription factor of mRNA of VEGF, is activated in oxygen deficiency tumor cells. VEGF then is released through a cell membrane from tumor cells. The results of this study suggest that suppression mechanism of VEGF secretion by HL-25 could be related to destabilization of the cell membrane by clustering of lipid rafts in the plasma membranes and reduction in stability of VEGF or inactivation of HIF-1. Thus, anti-angiogenesis mechanism of HL are under investigation in detail at present. Furthermore, we are now investigating anti-angiogenesis and tumor effects using another breast cancer cell lines such as MCF-7 cells *in vitro* and *in vivo*.



HL having larger membrane fluidity showed higher inhibitory effects on the growth of colorectal cancer cells. Furthermore, we also reported that HL distinguished between normal colon CCD33Co cells and tumor colon WiDr cells, then fused and accumulated into the plasma membranes of tumor cells, leading to apoptosis [36]. In addition, we have already reported that HL have no side-effects *in vivo* in the safety tests intravenously administered via a vein once a day for 14 days (mice) [22] and six months (rats) [25,26,37]. These results suggest that HL-25 could selectively accumulate into tumor cells of mouse models of HBC and inhibit the growth and VEGF secretion in MDA-MB-453 cells.

Conclusion

We clearly demonstrated that the anti-angiogenesis activity of HL-25 along with tumor suppression were obtained for xenograft mouse models carrying HBC. The noteworthy aspects in this study are as follows: (a) Inhibitory effects of HL-25 on the tube formation in the human umbilical vascular endothelial cells (HUVEC) were obtained *in vitro*. (b) Inhibitory effects of HL-25 for VEGF secretion in MDA-MB-453 cells were observed using ELISA *in vitro*. (c) Remarkably high

therapeutic effects of HL-25 were obtained in mouse models of HBC on the basis of tumor volume and weight measurement. (d) Anti-angiogenic activity of HL-25 for mouse models of HBC was observed on the basis of immunostaining method using CD34. The results in this study could be advantageous in the chemotherapy for patients with HBC with angiogenesis in clinical applications.

Acknowledgments

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