Nanotherapy with Hybrid Liposomes for Colorectal Cancer Along with Apoptosis *In Vitro* and *In Vivo*

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Abstract. *Aim: We examined the therapeutic effects of hybrid liposomes (HL) composed of L-α-dimyristylphosphati-dylcholine (DMPC) and polyoxyethylene (25) dodecyl ether ($C_{12}(EO)_{25}$) on the growth of human colorectal cancer (WiDr) cells in vitro and in vivo. Materials and Methods: HL composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ were prepared by the sonication method and their therapeutic effects in xenograft mouse models of colorectal cancer liver metastases were examined in vivo. Results: The inhibitory effects of HL-25 on the growth of WiDr cells along with apoptosis were assessed in vitro. Remarkable inhibitory effects of HL-25 for the liver metastasis of colorectal cancer cells along with apoptosis were revealed on the basis of histological analysis. Prolonged survival was attained for the xenograft mouse model of colorectal cancer after treatment with HL-25 in vivo. Conclusion: Therapeutic effects of HL-25 without any drugs on the liver metastasis of human colorectal cancer were obtained for the first time in vivo.*

The number of patients with colorectal cancer is increasing each year. Colorectal cancer is the third most common cancer in crude cancer mortality rate in Japan following lung and gastric cancer (1). Colorectal cancer often develops liver metastasis in patients. Surgical resection of the tumor is most effective for achieving a complete cure at an early stage. However, chemotherapy is selected for postoperative metastasis and advanced colorectal cancer, since surgery is not effective for these cancers.

Prolonged survival of patients with progressive colorectal cancer *via* the postoperative adjuvant chemotherapy using 5-fluorouracil (5-FU)/leucovorin (LV) has been reported (2-4). Combination of irinotecan and 5-FU/LV has been shown to be superior to 5-FU/LV alone as a first-line therapy for patients with metastatic colorectal cancer (5). Clinically-meaningful improvement in survival ratio for patients with metastatic colorectal cancer by the addition of bevacizumab to fluorouracil-based combination (irinotecan, 5-FU and LV) chemotherapy has been reported. (6). FOLFOX (5-FU, Folinic acid and Oxaliplatin) and FOLFIRI (5-FU, folinic acid and irinotecan) therapy *via* a combination of new anticancer drugs such as folinic acid and oxaliplatin have also been carried out more recently (7-9). Furthermore, the efficacy of cetuximab and FOLFOX-4 was confirmed in clinical trials, indicating that therapy with cetuximab and FOLFOX-4 was effective in first-line treatment of patients with *KRAS* wild-type in metastatic colorectal cancer (8). Among patients with resected stage III colorectal cancer, however, no improved disease-free survival was observed by the addition of cetuximab to FOLFOX-6 therapy compared with FOLFOX-6 alone (9). Combination chemotherapy is often used for metastatic colorectal cancer. However, side-effects in combination chemotherapy are much more severe than those in chemotherapy alone (2-9). Therefore, the development of a novel chemotherapy treatment without side-effects is desired to improve patients' satisfaction and quality of life.

We have produced hybrid liposomes (HL) (10, 11) that can be prepared by sonication of vesicular and micellar molecules in a buffer solution. The physical properties of HL such as shape, size, membrane fluidity and temperature of phase transition can be controlled by changing the constituents and compositional ratios (10-12). An effective drug delivery system using HL as drug carriers has been obtained in the treatment of brain tumors (13) and duchenne muscular dystrophy (14). High inhibitory effects of HL on the growth of various tumor cells *in vitro* along with the induction of apoptosis have been obtained without using drugs (15-17). Remarkable therapeutic effects of HL were demonstrated on the growth of tumor cells *in vivo* (18-21). No toxicity of HL was observed in normal rats *in vivo* nor were there any side-

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effects (18, 19). After receiving the approval of the Bioethics Committee, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported (18). In addition, a good correlation between the 50% inhibitory concentration (IC_{50}) of HL for the growth of human colorectal cancer (WiDr) cells and membrane fluidity of HL has already been reported (22).

In the present study, we report on the antitumor effects of HL-25 composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene (25) dodecyl ether ($C_{12}(EO)_{25}$) without any drug on the growth of human colorectal cancer (WiDr) cells *in vitro*. We investigated the therapeutic effects of HL-25 using xenograft mouse models of colorectal cancer *in vivo*.

Materials and Methods

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) dodecyl ether ($C_{12}(EO)_{25}$; Nikko Chemicals, Tokyo, Japan,) in 5% glucose solution using bath type sonicater (VS-N300; VELVO-CLEAR, Tokyo, Japan) at 45˚C and filtered through a 0.20 μm cellulose acetate filter (Advantec, Tokyo, Japan).

Dynamic light scattering measurement. The diameter of HL-25 was measured with a light scattering spectrometer (ELSZ-0; Otsuka Electronics, Osaka, Japan) using a He–Ne laser (633 nm) at a 90˚ scattering angle. The hydrodynamic diameter (dhy) 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:

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

Cell culture. Human colorectal cancer (WiDr) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). WiDr cells were maintained in RPMI-1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 1% non-essential amino acids (NEAA), penicillin 100 U/ml, streptomycin 50 μg/ml and 10% fetal bovine serum (FBS, HyClone, Omaha, NE, USA). The cells were cultured in a 5% CO_2 humidified incubator at 37˚C.

Assessment of growth inhibition in vitro. The IC_{50} on the growth of tumor cells was determined on the basis of the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] proliferation assay (Cell Counting Kit-1, Dojindo Laboratories, Kumamoto, Japan) (23-24). Cells (5.0×10^4 cells/ml) were seeded in 96-well plates and cultured in a 5% CO_2 humidified incubator at 37˚C for 24 h. Cells were cultured for a further 48 h after adding DMPC (0.1-5 mM), and HL-25 (0.1-2 mM on the basis of DMPC concentration). WST-8 solution was added and the cells incubated for 3 h. The absorbance at a wavelength of 450 nm was measured by a spectrophotometer (Emax; Molecular Devices Co., Sunnyvale, CA, USA). The inhibitory effects of HL-25 on the growth of tumor cells were evaluated by $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote the absorbance of water-soluble formazan in the presence and absence of HL-25, respectively.

Annexin-V binding assay. Detection of early apoptotic cells was performed by the method of Annexin-V binding assay using the Annexin-V-FLUOS Staining kit (Roche Diagnostics K.K., Basel, Switzerland). Cells were cultured for 6 h after adding HL-25 ([DMPC]=0.3 mM, $[C_{12}(EO)_{25}]$=0.017 mM). The cells were washed with phosphate buffered saline without Ca and Mg (PBS(–)) and stained with Annexin-V-fluorescein and propidium iodide (PI). The stained cells were observed using a confocal laser microscope (TCS-SP; Leica Microsystems, Wetzlar, Germany) with a 488 nm Ar laser. The Annexin-V-fluorescein signals were detected at 500-562 nm and the PI signals were detected at 638-693 nm.

Terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick-end labeling (TUNEL) method in vitro. Detection of apoptotic cells was performed by the TUNEL method using an In Situ Cell Death Detection Kit (Roche Diagnostics K.K.). The WiDr cells (5×10^4 cells/ml) were seeded in flasks and were cultured for 24 h. HL-25 ([DMPC]=0.3 mM, $[C_{12}(EO)_{25}]$=0.017 mM) or DMPC liposomes were added and cultured for 48 h. Supernatants were transfered in a centrifuge tube and cells were harvested using 0.05% Trypsin-EDTA. Paraformaldehyde solution (4%) was added and the cells were fixed after centrifugal separation. After formalin was removed, the cells were washed in PBS (–). The impregnated solution was added and the cells were stored in shade condition at 4 ˚C for 2 min. After the cells were washed, the TUNEL reaction mixed-solution was added and incubated at 37˚C for 60 min within a humectant box. TOPRO-3 blue stain (Molecular Probes, Inc., Eugene, OR, USA) was added and the cells were stored in room temperature for 20 min under the shade condition. The stained cells were observed using a confocal laser microscope with a 488 nm Ar laser line for TUNEL (detection at 515-565 nm) and 633 nm He/Ne laser line for TOPRO-3 (detection at 640-700 nm).

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 SCID (severe combined immunodeficiency) mice (C.B-17/Icr-scid) were obtained from CLEA (Tokyo, Japan), caged and bred by 100% freshly ventilatory conditions every 1 h for 14 times at room temperature (25±1˚C), humidity 50±10% and illumination cycle for every 12 h. The mice were randomly grouped on the basis of body weight by a stratified randomization method. The number of mice was five in each group. WiDr cells (5.0×10^6 cells) were intrasplenically transplanted into the SCID mice (25). HL-25 (Dose: 136 mg/kg for DMPC) was intravenously administered once each day for 14 days after the inoculation of WiDr cells. Then, the livers were isolated, weighed and fixed in 10% formalin solution. The livers were embedded in paraffin and sectioned at 5 μm of thickness. Sections were stained with hematoxylin and eosin (HE) and observed using an optical microscope (Nikon TS-100, Tokyo, Japan).

Immunostaining with anti-carcinoembryonic antigen (CEA) 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 then blocked with a solution of PBS and 1 % H_2O_2 for 5 min. The sections were washed with PBS(–) and incubated overnight with anti-human CEA antibody (R&D Systems, Minneapolis, MN, USA) in a humidified box at 4˚C. The sections were washed twice with PBS and

immunostained with rabbit anti-goat immunoglobulin polyclonal antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, MA, USA) for overnight at 4˚C. Finally, the detection of the antigen-antibody link was made through immunoperoxydase followed by 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen and observed using optical microscope.

TUNEL method in vivo. Detection of apoptotic cells was performed on the basis of the TUNEL method using an *in situ* apoptosis detection kit (ApopTag Plus Peroxidase, Intergen, Purchase, NY, USA) according to manufacturer's directions. Paraffin-embedded sections were made and detection of apoptosis of tumor cells in liver was performed on the basis of the TUNEL assay according to the conventional method.

Assessment of survival rate in vivo. HL-25 (Dose: 136 mg/kg for DMPC) was intravenously administered once each day for 14 days for the xenograft mouse model of colorectal cancer after the inoculation of WiDr cells. The median lifespan was calculated using the following equation:

Median lifespan=(median survival days after the treatment)/(median survival days of control group) ×100. (Equation 2)

Statistical analysis. Results are presented as mean±Standard Deviation (SD). Data were statistically analyzed using the Student's *t*-test and the log-rank test. A *p*-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Physical properties of HL-25. We examined the physical properties of HL-25 composed of DMPC and 5 mol% $C_{12}(EO)_{25}$. The diameter of HL-25 was measured by dynamic light scattering measurement. The results are shown in Figure 1. A clear solution of HL-25 having a hydrodynamic diameter under 100 nm was preserved for a period of one month. In contrast, DMPC liposomes were unstable and precipitated after 14 days.

Inhibitory effects of HL-25 on the growth of WiDr cells in vitro. We examined the inhibitory effects of HL-25 on the growth of human colorectal cancer (WiDr) cells on the basis of the WST-8 proliferation assay. The results are shown in Figure 2. Fifty percent inhibitory concentration (IC_{50}) values of HL-25 and single-component liposomes of DMPC on the growth of WiDr cells were 146 μm±14 μM and 192±22 μM, respectively. There was a significant difference (p=0.0004) in IC_{50} values between the DMPC liposomes and HL-25. These results indicate that higher inhibitory effects of HL-25, compared to DMPC liposomes, were obtained.

Induction of apoptosis for WiDr cells treated with HL-25 in vitro. Detection of early apoptotic cells was performed on the basis of the Annexin-V binding assay. The results are shown in Figure 3A. Green color, indicating early-stage apoptosis, was observed in fluorescence micrograph of WiDr cells using Annexin-V binding assay after treatment with HL-25 for 6 h. However, lower-intensity green color was observed in fluorescence micrograph of WiDr cells treated with DMPC liposomes. Red color, indicating necrosis in cells treated with DMPC and HL-25, was not observed on the basis of PI staining.

As a next step, we examined induction of apoptosis of WiDr cells after treatment with HL-25 on the basis of the TUNEL method. The results are shown in Figure 3B. Interestingly, WiDr cells were dyed green at 48 h after adding HL-25, indicating that HL-25 induced apoptosis for WiDr cells, although cells were not dyed using the DMPC liposomes. These results indicate that HL-25 induces apoptosis in WiDr cells.

Autopsy analysis for a xenograft mouse model of colorectal cancer in vivo. We examined the therapeutic effects of HL-25 using a xenograft mouse model of colorectal cancer with liver metastasis *in vivo*. HL-25 was administered into the caudal vein of mice once each day for 14 days after the inoculation of WiDr cells. The liver was removed from anaesthetized mice immediately after treatment with HL-25. We observed the therapeutic effects of HL-25 on the xenograft mouse model of colorectal cancer on the basis of autopsy. The results are shown in Figure 4. A reduction of tumor size in the liver of HL-25-treated group was observed; however, enlargement of WiDr metastatic tumor-nodes into the liver of the control and DMPC treated group was confirmed. These results indicate that the therapeutic effects of HL-25 could be obtained on the xenograft mouse model of colorectal cancer *in vivo*.

Histological analysis for a xenograft mouse model of colorectal cancer in vivo. We evaluated histologically the therapeutic effects of HL-25 using the liver tissues of the xenograft mouse model of colorectal cancer of WiDr cells *in vivo*. After HL-25 administration and animal handling as described above, we observed the liver tissues using a microscope and HE staining. As shown in Figure 5A, large metastatic nodules were observed in the liver of the control and DMPC groups, indicating a malignant transformation by metastasis of WiDr cells to the liver of our mouse model. On the other hand, the reduction of metastatic nodules was observed in the liver of the groups treated with HL-25. HL-25 remarkably inhibited metastasis of WiDr cells to the liver.

To establish the therapeutic effects of HL-25, we carried out immunostaining using CEA as histochemical marker of metastatic colorectal cancer. As shown in Figure 5B, many CEA-positive cells (brown in color) in the control and DMPC groups were observed. In contrast, no CEA-positive cells were observed in the livers of the group treated with HL-25, which were fairly similar to normal livers. These results indicate that HL-25 could have an anti-metastatic effect on the liver metastasis of our xenograft mouse model of colorectal cancer *in vivo*.

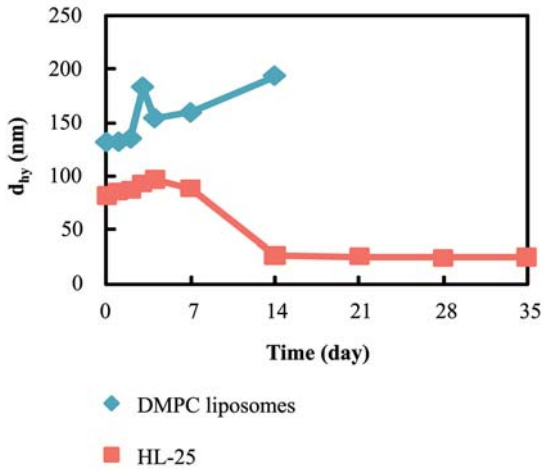

Figure 1. *Time course of d_{hy} change for hybrid liposomes (HL) composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ in 5% glucose solution at 25°C, [DMPC]=10mM, [$C_{12}(EO)_{25}$]=0.5mM.*

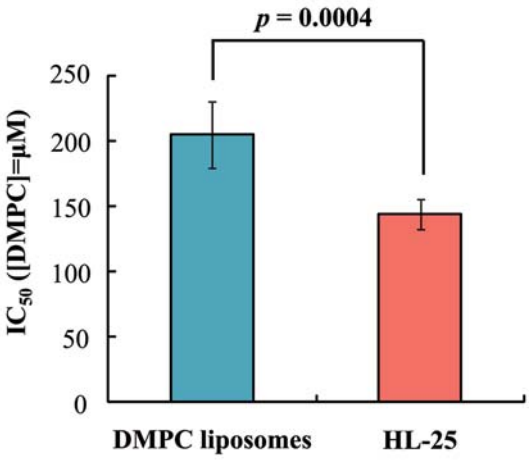

Figure 2. *Inhibitory effects of HL-25 on the growth of WiDr cells. Data presented are mean±SD.*

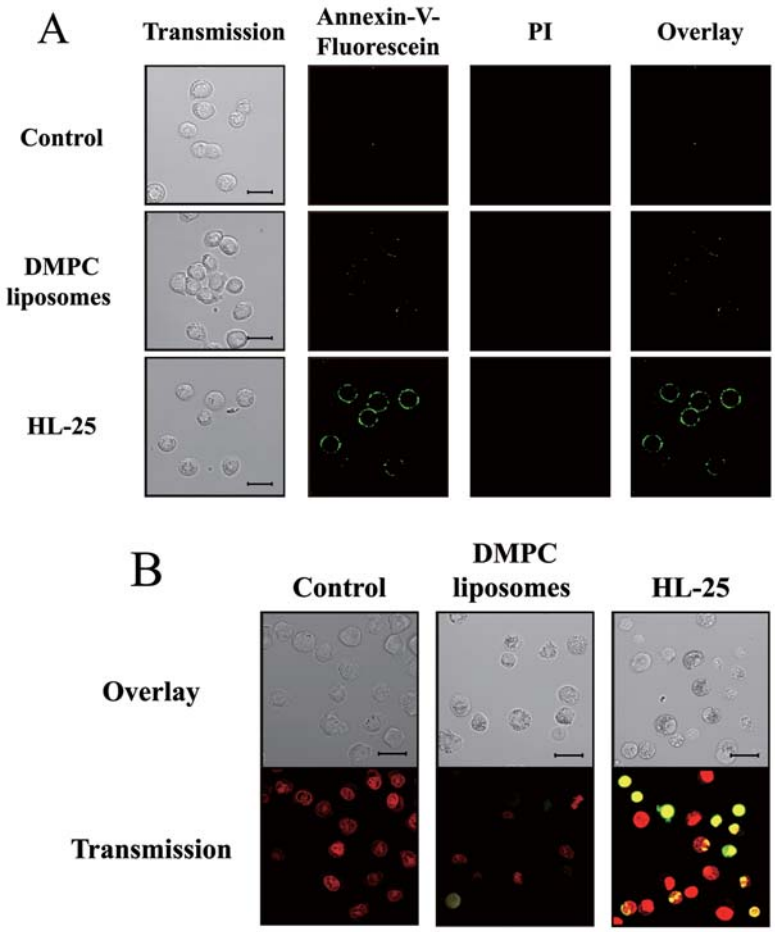

Figure 3. *Fluorescence micrographs of WiDr cells treated with HL-25. (A) Annexin-V binding assay, [DMPC]=300 μM, reaction time: 6h, scale bar: 20 μm. (B) TUNEL method, [DMPC]=300 μM, reaction time: 48h, scale bar: 20 μm.*

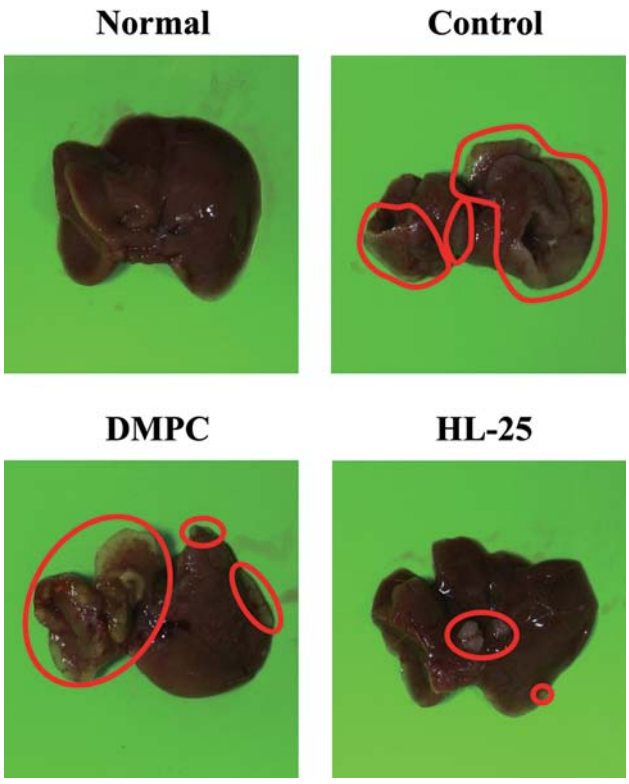

Figure 4. *Therapeutic effects for a xenograft mouse model of colorectal cancer treated with HL-25 on the basis of autopsy analysis. The red circles indicate tumors.*

Induction of apoptosis by HL-25 for the xenograft mouse model of colorectal cancer in vivo. We examined the mechanism of the therapeutic effects of HL-25 on the liver metastasis of WiDr cells *in vivo* on the basis of the TUNEL method. HL-25 was administered as described above and animals were handled as in previous experiments. As shown in Figure 6, only few apoptotic cells were observed in the tumor cells in the liver tissue of the group treated with DMPC. Interestingly, many apoptotic cells were observed in tumor cells in the liver tissue of the group treated with HL-25. On the other hand, no apoptotic cells were observed in the normal and control groups. These results indicate that HL-25 has therapeutic effects on the xenograft mouse model of colorectal cancer of WiDr cells along with apoptosis *in vivo*.

Prolonged survival of HL-25 for a xenograft mouse model of colorectal cancer in vivo. We examined the therapeutic effects of HL-25 using a xenograft mouse model of colorectal cancer after intravenous treatment with HL-25. HL-25 was intravenously administered *via* the caudal vein once each day for 14 days after the inoculation of WiDr cells. After the dosing period, the survival rate of the inoculated mice was

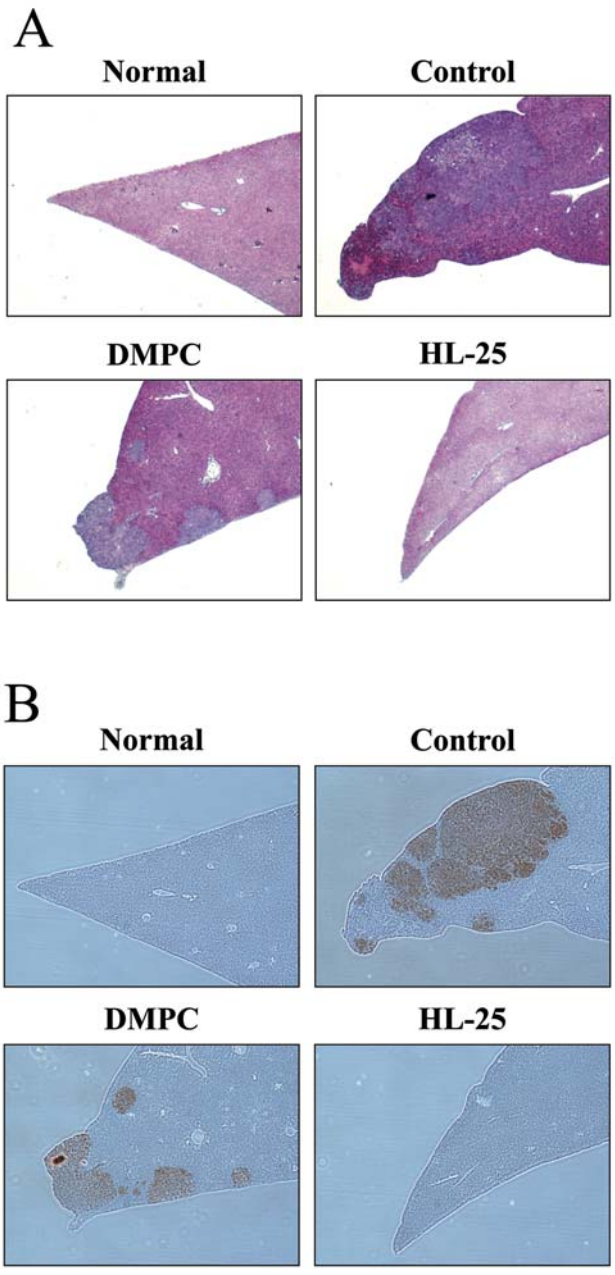

Figure 5. *Assessment of the therapeutic effects of a xenograft mouse model of colorectal cancer treated with HL-25 on the basis of histological analysis. (A) HE staining of liver tissue of a xenograft mouse model of colorectal cancer treated with HL-25. (B) CEA immunostaining of liver tissue of a xenograft mouse model of colorectal cancer treated with HL-25.*

observed. The results are shown in Figure 7. The median survival time for the group treated with HL-25 (53±13) was longer than that of the control group (35±2). However, the median survival time for the group treated with DMPC was

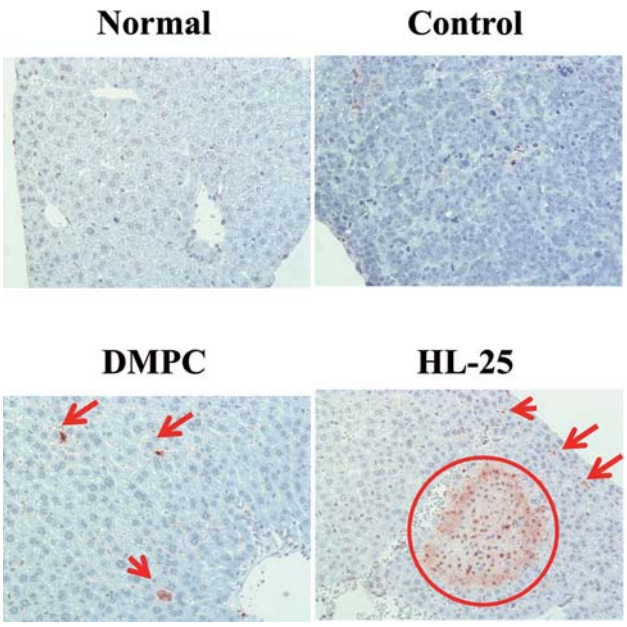

Figure 6. *Micrographs of tumor in a xenograft mouse model of colorectal cancer treated with HL-25 using the TUNEL method. The arrows and circles indicate apoptotic cells.*

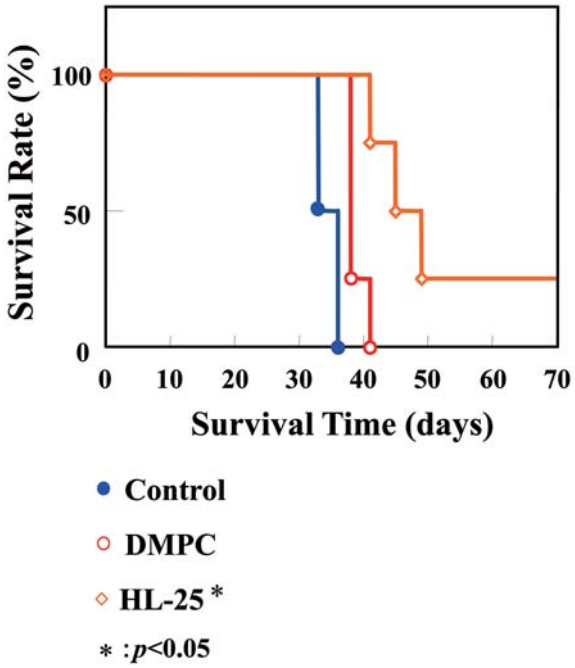

Figure 7. *Survival curves of a xenograft mouse model of colorectal cancer treated with HL-25. There was a significant difference (*p<0.05) compared with the control group.*

only 39±1, thus a statistically significant difference between the control and DMPC groups alone was not recorded. It is noteworthy that a prolonged survival rate (>150%, $p<0.05$) was obtained in the group treated with HL-25. These results indicate that HL-25 prolongs the survival of the mouse model with colorectal cancer.

Discussion

Surgical resection of tumor tissue is the most effective approach for the treatment for colorectal cancer. Preoperative combination chemotherapy is selected for advanced colorectal cancer with metastasis in the case of unresectable metastases tissues (26-29). However, combination chemotherapy has more severe side-effects compared to chemotherapy alone due to the lack of tumor-specific treatments. General combination chemotherapy has very little or no specificity, causing side effects such as hair loss and damage to the liver, kidney and bone marrow. Therefore, a novel chemotherapy scheme without any side-effects is desirable. Recently, nanotherapy with nanoparticles has been reported to be an effective cancer treatment using zirconium phosphate nanoplatelets (30), functionalized polymeric nanoparticles loaded with paclitaxel (31) or cyclodextrin-based polymer particles containing the DNA topoisomerase I inhibitor camptothecin (32). Targeted nanotherapies have been developed to improve drug delivery and enhance therapeutic response.

Fluctuation of tumor cell membranes is very different from that of normal cells. The membranes of tumor cells are generally more fluid compared to normal ones. HL showed remarkably higher inhibitory effects on the growth of human colorectal cancer cells compared to DMPC liposomes, in which membrane fluidity was smaller than that of HL (22). Furthermore, a good correlation between the IC_{50} values of HL for the growth of human colorectal cancer (WiDr) cells and membrane fluidity of HL has been already reported (22). It is also noteworthy that total internal reflection fluorescence micrographs showed that HL distinguished between tumor (WiDr) cells and normal (CCD33Co) cells (22). Specific accumulation of HL into the WiDr cell membranes was observed (22). These results suggest that the inhibitory effects of HL on the growth of tumor cells should be related to membrane fluidity.

We examined the morphology of HL-25. HL-25 under 100 nm in diameter could avoid the reticular endothelial system (RES) (33) and could be appropriate for the intravenous administration *in vivo* and clinical applications.

It is well-known that apoptosis plays an important role in many aspects of normal development and is required for maintaining homeostasis in tissues. Inappropriate activation

and suppression of apoptosis leads to degenerative pathologies and tumorigenesis, respectively. Consequently, control of apoptosis is an important potential target for cancer therapeutics.

We examined the antitumor effects of HL-25 alone on the growth of human colorectal cancer (WiDr) cells *in vitro*. The induction of apoptosis by HL-25 was verified for WiDr cells on the basis of Annexin-V binding assay and the TUNEL method. The pathways of apoptosis induced by HL-25 in WiDr cells were clarified. The pathways of apoptosis induced by HL of DMPC/10 mol% $C_{12}(EO)_{10}$ in human promyelocytic leukemia (HL-60) cells has already been reported (17). HL fused and accumulated in HL-60 cell membranes. Two apoptotic pathways by HL for HL-60 cells have been reported. In pathway A, the apoptotic signal first passed through the mitochondria, then caspase-9 and caspase-3, and then reached the nucleus. In pathway B, the apoptotic signal first passed through FAS, then caspase-8 and caspase-3, and then reached the nucleus (17). Activation of caspase-8, -9 and -3 after treatment with HL-23 for WiDr cells *in vitro* has been reported (22). Therefore, the apoptotic signal by HL-25 for WiDr cells could pass through mitochondria and activation of caspase-9, -8 and -3, and then reach the nucleus following these two pathways *in vivo*. On the other hand, no induction of apoptosis was observed in WiDr cells treated with DMPC liposomes.

We examined the therapeutic effects of HL-25 using xenograft mouse models of colorectal cancer with liver metastasis *in vivo*. Reduction of tumor in the livers of the group treated with HL-25 was observed, although enlargement of tumor-nodes by metastasis of WiDr cells into the liver of control and DMPC groups was confirmed on the basis of autopsy. Anti-metastatic effects of HL-25 on the liver metastasis of our xenograft mouse model of colorectal cancer were obtained on the basis of histological analysis using HE and CEA immunostaining. Many apoptotic cells were observed in the tumor cells in the liver tissue of the group treated with HL-25 on the basis of TUNEL method. Interestingly, a prolonged survival rate (>150 %) was obtained in the group treated with HL-25. These results indicate that HL-25 has therapeutic effects on the xenograft mouse model of colorectal cancer of WiDr cells along with apoptosis *in vivo*. In other research, HL have also been shown to have no side-effects using healthy rats *in vivo* (18). HL were metabolized in the livers after intravenous administration to healthy mice as already described (20).

In clinical application, a prolonged survival of more than one year was attained in one patient with lymphoma in last stage after intravenous injection of HL without any side-effects. In addition, a remarkable reduction of a lymph node neoplasm (solid tumor) was observed after local administration (2 times/week) of HL (18). Therefore, therapy using hybrid liposomes for colorectal cancer could be important for clinical applications in the future.

Conclusion

Through this study, we have clearly demonstrated that remarkable therapeutic effects along with apoptosis due to HL-25 administration were obtained for the first time in colorectal cancer *in vitro* and *in vivo*. The noteworthy aspects are as follows: (i) HL-25 with a hydrodynamic diameter under 100 nm was successfully preserved for a period of one month; (ii) Significant inhibitory effects of HL-25 on the growth of WiDr cells were obtained; (iii) HL-25 induced apoptosis for WiDr cells *in vitro*; (iv) Remarkably important therapeutic effects of HL-25 were obtained in the xenograft mouse model of colorectal cancer on the basis of autopsy, hematoxylin-eosin staining and CEA immunostaining *in vivo*; (v) Induction of apoptosis was observed in the xenograft mouse model of colorectal cancer after treatment with HL-25 on the basis of the TUNEL method; (vi) Prolonged survival was obtained in our xenograft model of colorectal cancer after treatment with HL-25. The results of this study would contribute to the development of nanotherapy for patients with colorectal cancer in future clinical applications.

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References

1 Center for Cancer Control and Information Services, National Cancer Center, Japan, http://ganjoho.jp/pro/statistics/ en/gdb_ trend.html?1%9

2 Wolmark N, Rockette H, Fisher B, Wickerham DL, Redmond C, Fisher ER, Jones J, Mamounas EP, Ore L, Petrelli NJ, Spurr CL, Dimitrov N, Romond EH, Sutherland CM, Kardinal CG, Defusco PA and Jochimsen P: The benefit of leucovorin-modulated fluorouracil as postoperative adjuvant therapy for primary colon cancer: results from national surgical adjuvant breast and bowel project protocol C-03. J Clin Oncol *11*: 1879-1887, 1993.

3 O'Connell MJ, Mailliard JA, Kahn MJ, Macdonald JS, Haller DG, Mayer RJ and Wieand HS: Controlled trial of fluorouracil and low-dose leucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. J Clin Oncol *15*: 246-50, 1997.

4 Wolmark N, Rockette H, Mamounas E, Jones J, Wieand S, Wickerham DL, Bear HD, Atkins JN, Dimitrov NV, Glass AG, Fisher ER and Fishier B: Clinical trial to assess the relative efficacy of fluorouracil and leucovorin, fluorouracil and levamisole, and fluorouracil, leucovorin, and levamisole in patients with Dukes' B and C carcinoma of the colon: results from national surgical adjvant breast and bowel project C-04. J Clin Oncol *17*: 3553-3559, 1999.

5 Saltz LB, Douillard JY, Pirotta N, Alakl M, Gruia G, Awad L, Elfring GL, Locker PK and Miller LL: Irinotecan plus fluorouracil/Leucovorin for metastatic colorectal cancer: a new survival standaed. The Oncologist *6*: 81-91, 2001.

6 Hurwiz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Eng J Med *350*: 2335-2342, 2004.

7 Peeters M, Strickland AH, Lichinitser M, Suresh AV, Manikhas G, Shapiro J, Rogowski W, Huang X, Wu B, Warner D, Jain R and Tebbutt NC: A randomised, double-blind, placebo-controlled phase 2 study of trebananib (AMG 386) in combination with FOLFIRI in patients with previously treated metastatic colorectal carcinoma. Br J Cancer *108*: 503-511, 2013.

8 Bokemeyer C, Bondarenko I, Hartmann JT, de Braud F, Schuch G, Zubel A, Celik I, Schlichting M and Koralewski P: Efficacy according to biomarker status of cetuximab plus FPLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. Ann Oncol *22*: 1535-1546, 2011.

9 Alberts SR, Sargent DJ, Nair S, Mahoney MR, Mooney M, Thibodeau SN, Smyrk TC, Sinicrope FA, Chan E, Gill S, Kahlenberg MS, Shields AF, Quesenberry JT, Webb TA, Farr GH Jr, Pockaj BA, Grothey A and Goldberg RM: Effect of Oxaliplatin, Fluorouracil, and Leucovorin with or without Cetuximab on survival among patients with resected stage III colon cancer: A randomized trial. JAMA *307(13)*: 1383-1393, 2012.

10 Ueoka R, Moss RA, Swarup S, Matsumoto Y, Strauss G and Murakami Y: Extraordinary micellar enantioselectivity coupled to altered aggregate sturucture. J Am Chem Soc *107*: 2185-2186, 1985.

11 Ueoka R, Matsumoto Y, Robert A. Moss, Shanti Swarup, Sugii A, Harada K, Kikuchi J and Nurakami Y: Membrane matrix for the hydrolysis of amino acid esters with marked enantioselectivity. J Am. Chem Soc *110*: 1588-1595, 1988.

12 Goto K, Matsumoto Y and Ueoka R: Hybrid liposomes coupled to steric control with high enantioselectivity. J Org Chem *60*: 3342-3346, 1995.

13 Kitamura I, Kochi M, Matsumoto Y, Ueoka R, Kuratsu J and Ushio Y: Intrathecal chemotherapy with 1,3-bis(2-chloroethyl)-1-nitrosourea encapsulated into hybrid liposomes for meningeal gliomatosis: an experimental study. Cancer Res *56*: 3986-3992, 1996.

14 Yukihara M, Ito K, Tanoue O, Goto K, Matsushita T, Matsumoto Y, Masuda M, Kimura S and Ueoka R: Effective drug delivery system for duchenne muscular dystrophy using hybrid liposomes including gentamicin along with reduced toxicity. Biol Pharm Bull *34*: 712-716, 2011.

15 Matsumoto Y, Kato T, Kemura Y, Tsuchiya M, Yamamoto Y and Ueoka R: Intracellular resuponses of hybrid liposomes against leukemia cells related to apoptosis with antitumor activity. Chem Lett *28*: 53-54, 1999.

16 Iwamoto Y, Matsumoto Y and Ueoka R: Induction of apoptosis of human lung carcinoma cells by hybrid liposomes containing polyoxyethylenedodecyl ether. Int J Pharm *292*: 231-239, 2005.

17 Matsumoto Y, Iwamoto Y, Matsushita T and Ueoka R: Novel mechanism of hybrid liposomes-induced apoptosis in human tumor cells. Int J Cancer *115*: 377-382, 2005.

18 Ichihara H, Nagami H, Kiyokawa T, Matsumoto Y and Ueoka R: Chemotherapy using hybrid liposomes along with induction of apoptosis. Anticancer Res *28*: 1187-1195, 2008.

19 Shimoda S, Ichihara H, Matsumoto Y and Ueoka R: Chemotherapy with hybrid liposomes for human breast tumors along with apoptosis *in vivo*. Int J Pharm *372*: 162-168, 2009.

20 Ichihara H, Matsuoka Y, Matsumoto Y and Ueoka R: Therapeutic effects of hybrid liposomes on the growth of gastric carcinoma involve apoptosis. Anticancer Res *30*: 2011-2016, 2010.

21 Kitajima H, Komizu Y, Ichihara H, Goto K and Ueoka R: Hybrid liposomes inhibit tumor growth and lung metastasis of murine osteosarcoma cells. Cancer Med *2*: 267-276, 2013.

22 Komizu Y, Matsumoto Y and Ueoka R: Membrane targeted chemotherapy with hybrid liposomes for colon tumor cells leading to apoptosis. Bioorg Med Chem Lett *16*: 6131-6134, 2006.

23 Ishiyama M, Miyazono Y, Sasamoto K and Ohkura Y and Ueno K: A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta *44*: 1299-1305, 1997.

24 Tominaga H, Ishiyama M, Ohseto F, Sasamoto K, Hamamoto T, Suzuki K and Watanabe M: A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun *36*: 47-50, 1999.

25 Jeong JS, Lee SW, Hong SH, Lee YJ, Jung HI, Cho KS, Seo HH, Lee SJ, Park S, Song MS, Kim CM and Kim IH: Antitumor effects of systemically delivered adenovirus harboring trans-splicing ribozyme in intrahepatic colon cancer mouse model. Clin Cancer Res *14*: 281-290, 2008.

26 Marshall JL: Managing potentially resectable metastatic colon cancer. Gastrointest Cancer Res *2*: S23-S26, 2008.

27 Bismuth H, Adam R, Lévi F, Farabos C, Waechter F, Castaing D, Majno P and Engerran L: Resection of nonresectable liver merastases from colorectal cancer after neoadjuvant chemotherapy. Ann Surg *224*: 509-520, 1996.

28 Aloia TA, Vauthey JN, Lover EM, Ribero D, Pawlik TM, Wei SH, Curley SA, Zorzi D and Abdalla EK: Solitary colorectal liver metastasis: resection determines outcome. Arch Surg *141*: 460-466, 2006.

29 Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Vos AH, Van Groeningen CJ, Sinnige HA, Richel DJ, Voest EE, Dijkstra JR, Vink-Börger ME, Antonini NF, Mol L, van Krieken JH, Dalesio O and Punt CJ: Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med *360*: 563-572, 2009.

30 Díaz A1, González ML, Pérez RJ, David A, Mukherjee A, Báez A, Clearfield A, Colón JL: Direct intercalation of cisplatin into zirconium phosphate nanoplatelets for potential cancer nanotherapy. Nanoscale *5*: 11456-11463, 2013.

31 Delie F1, Petignat P, Cohen M: GRP78-targeted nanotherapy against castrate-resistant prostate cancer cells expressing membrane GRP78. Target Oncol *8*: 225-230, 2013.

32 Ng TS, Wert D, Sohi H, Procissi D, Colcher D, Raubitschek AA, Jacobs RE: Serial diffusion MRI to monitor and model treatment response of the targeted nanotherapy CRLX101. Clin Cancer Res *19*: 2518-2527, 2013.

33 Huang SK1, Lee KD, Hong K, Friend DS and Papahadjopoulos D: Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. Cancer Res *52*: 5135-5143, 1992.

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