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-dimyristylphosphatidylcholine (DMPC) and polyoxyethylene (25) dodecyl ether (C12(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% C12(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 (IC50) 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 (C12(EO)25; Nikko Chemicals, Tokyo, Japan) in 5% glucose solution using bath type sonicator (VS-N300; VELVO-CLEAR, Tokyo, Japan) at 45˚C and filtered through a 0.20 μm cellulose acetate filter (Advantec, Tokyo, Japan). The hydrodynamic diameter (dhy) was calculated 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 k is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient:

\[ d_{hy} = kT/3\eta D \]  

(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). WiDr cells were cultured for 24 h. 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 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). Paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanol to water. Tumor sections were 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, dehydrated 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% H2O2 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 immunoperoxidase 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:

\[
\text{Median lifespan} = \frac{\text{median survival days after the treatment}}{\text{median survival days of control group}} \times 100.
\]

**Statistical analysis.** Results are presented as mean±Standard Deviation (SD). Data were statistically analyzed using the Student’s *t*-test and 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% C12(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 (IC50) 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 IC50 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 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 $d_{4,4}$ 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.
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 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
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 IC50 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 \textit{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\textsubscript{12}(EO)	extsubscript{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. 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. In pathway A, the apoptotic signal first passed through the mitochondria, then caspase-9 and caspase-3, and then reached the nucleus following these two pathways \textit{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 \textit{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 \textit{in vivo}. In other research, HL have also been shown to have no side-effects using healthy rats \textit{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.


dition that HL-25 administration were obtained for the first time in colorectal cancer \textit{in vitro} and \textit{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 \textit{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 \textit{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**


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