Therapeutic Effects of Cationic Hybrid Liposomes on the Hepatic Metastasis of Colon Carcinoma Along with Apoptosis in Vivo

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Therapeutic effects of cationic hybrid liposomes (HL) composed of 87 mol% dimyristoyl-phosphatidylcholine (DMPC), 5 mol% polyoxyethylene (21) dodecyl ether (C12(EO)21) and 8 mol% O,O'-ditetradecanoyl-N-(α-trimethyl-ammonioacetyl) diethanolamine chloride (2C14ECl) on the metastasis of human colon carcinoma (HCT116) cells were examined in vivo. Cationic HL having a hydrodynamic diameter less than 150 nm were preserved for one month. Therapeutic effects were obtained in the hepatic metastasis mouse models of HCT116 cells after the intravenous injection of cationic HL. The histological analysis indicated the induction of apoptosis in the liver section of the hepatic metastasis mouse models treated with cationic HL in vivo. Therapeutic effects of cationic HL without any drugs on the hepatic metastasis were revealed for the first time on the basis of histological analyses in vivo.

Key words hybrid liposome; hepatic metastasis; colon carcinoma; apoptosis; chemotherapy

Colorectal cancer (CRC) is the third most common cancer in the world with more than 600000 deaths every year.1) About 40% of patients with CRC develop metastases. Because the venous drainage of the colon is through the portal vein, which goes directly to the liver, more than 70% of the CRC metastases are located in the hepatic tissue. Metastatic diffusion results in a very poor prognosis with a median survival of about two years in treated patients. However, long term survival is possible in the 15% of patients that can benefit from metastasis surgery, usually after induction chemotherapy.2) Induction chemotherapy is a first-line treatment for cancer in which a patient is given doses of chemotherapy first. These doses may be high, with the goal of attempting to quickly attack the cancer, and after induction chemotherapy, additional treatment options such as surgical resection can be selected.3) While the chemotherapy drugs kill tumor cells, they also damage normal cells, causing severe side-effects such as gastrointestinal dysfunction and bone marrow toxicity. Therefore, chemotherapy that is effective for the metastasis of cancers without any side-effects is required.

On the other hand, we have produced hybrid liposomes (HL) which can be prepared by just the sonication of vesicular and micellar molecules in a buffer solution.4,5) HL are free from any contamination with organic solvents and remain stable for longer periods. The physical properties of these liposomes such as size, membrane fluidity, phase transition temperature, and hydrophobicity can be controlled by changing the constituents and compositional ratios of the HL. In the course of our study for HL, the following interesting results have been obtained. (a) Stereochemical control of the enantiomeric purity of amino acid esters could be established by temperature regulation and changing the composition of the HL.4,5) (b) Inhibitory effects of HL including antitumor drugs,6) sugar surfactants,7) or polyunsaturated fatty acids8) and cationic lipids9) have been observed on the growth of tumor cells in vitro and in vivo. (c) High inhibitory effects of HL on the growth of human leukemia,10) lung carcinoma11) and breast tumor12) cells in vitro along with the induction of apoptosis have been obtained without using drugs. (d) A good correlation between membrane fluidity and antitumor effects of HL on human colon tumor cells have been observed in vitro.13) In addition, we elucidated the remarkably inhibitory effects on the growth of human colon cancer (HCT116) cells along with apoptosis by cationic HL including cationic lipid that targeted negatively charged characteristics of tumor cell membranes in vitro.14) However, therapeutic effects of cationic HL in vivo have not yet been examined.

In this study, we investigated the therapeutic effects of three-component cationic HL composed of 87 mol% dimyristoyl-phosphatidylcholine (DMPC), 5 mol% polyoxyethylene(21) dodecyl ether (C12(EO)21) and 8 mol% O,O'-ditetradecanoyl-N-(α-trimethyl-ammonioacetyl) diethanolamine chloride (2C14ECl) on the hepatic metastasis mouse models of human colon carcinoma (HCT116) cells in vivo. Furthermore, the therapeutic effects of cationic HL on the hepatic metastasis mouse models were revealed on the basis of histological analysis of liver tissues.

MATERIALS AND METHODS

Preparation of Hybrid Liposomes Cationic HL were prepared by sonication (VS-N300; VELVO, Tokyo, Japan) of a mixture containing DMPC (purity >99%; NOF Co., Ltd., Tokyo, Japan), micellar molecules: C12(EO)21 (Nikkol Chemicals Co., Ltd., Tokyo, Japan) and 2C14ECl (DC-6-14; Sogo Pharmaceutical Co., Ltd. Tokyo, Japan) in 5% glucose solution at 45°C with 300 W, followed by filtration with a 0.20 μm filter.

Dynamic Light Scattering Measurements The diameter of cationic HL was measured with a light scattering spectrometer (ELS-8000, Otsuka Electronics, Osaka, Japan) using a He–Ne laser (633 nm) at a 90° scattering angle. The diameter (dhy) 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:

\[ d_{hy} = \frac{\kappa T}{3\pi \eta D} \]
**Cell Culture**  Human colon carcinoma (HCT116) cell lines were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). HCT116 cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with penicillin 100 U/mL, streptomycin 50 µg/mL, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.). The cells were cultured in a 5% CO₂ humidified incubator at 37°C.

**Assessment of Antitumor Activity in Vivo**  The mice were handled in accordance with the guidelines for animal experimentation in Japanese law. The animal studies were approved by the Committee on Animal Research of Sojo University. BALB/c-R/J mice were kindly provided by Prof. Okada (Kumamoto University, Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was four in each group. HCT116 cells (5.0×10⁶ cells) were intrasplenically transplanted into the mice. Subsequently, DMPC liposome (Dose: 136 mg/kg/d for DMPC), HL (Dose: 136 mg/kg/d for DMPC, 65.7 mg/kg/d for C₁₂(EO)₂₁, and cationic HL (Dose: 136 mg/kg/d for DMPC, 65.7 mg/kg/d for C₁₂(EO)₂₁, 65.7 mg/kg/d for 2C₁₄ECl) were intravenously administered once each day for 14 d. Safety of 136 mg/kg/d for DMPC in chronic toxicity test using mice and rats has been reported. After 14 d, the liver was removed from anaesthetized mice after the treatment with DMPC liposome, HL, and cationic HL and fixed in 10% formalin solution. The paraffin-embedded sections were made, and the microscopic observations of solid tumors were performed on the basis of TUNEL assay according to the conventional method.

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

**RESULTS**

**Physical Properties of Cationic HL**  We examined the morphology of cationic HL on the basis of dynamic light scattering measurements. Results are shown in Fig. 1. A clear solution of cationic HL having a hydrodynamic diameter less than 150 nm with a narrow range size distribution (Figs. 1A, B) were preserved for one month. Cationic HL and HL were stable in serum-containing medium (20% fetal bovine serum (FBS)), although DMPC liposomes were unstable and precipitated as shown in Fig. 1(C). This suggests that cationic HL could avoid the reticular endothelial system and could be appropriate for intravenous treatment in vivo and clinical applications.

**Therapeutic Effects of Cationic HL on the Hepatic Metastasis**  We investigated the therapeutic effects of cationic HL...
HL on the hepatic metastasis mouse models of colon carcinoma HCT116 cells in vivo. The relative liver weight of hepatic metastasis mouse models is shown in Fig. 2. It is worthy to note that the relative liver weight of the group treated with HL (6.54±0.72) and cationic HL (5.58±0.75) was close to that of the normal group (4.75±0.30), although that of the untreated control (non-treated) group obviously increased. The relative liver weight of the group treated with DMPC liposomes was 8.75±1.64. The relative liver weight of the group treated with cationic HL was lower than those of DMPC liposomes and HL. Interestingly, there was a significant difference (p<0.01) in the relative liver weight between the control group and the cationic HL treated group. The tumor regression effects of cationic HL (p<0.01) had larger significant difference against the control than HL (p<0.05).

**Autopsy Analysis of the Hepatic Metastasis** We histologically evaluated the therapeutic effects of cationic HL using the liver tissues of the hepatic metastasis mouse models of HCT116 cells in vivo. We observed the therapeutic effects of cationic HL on the hepatic metastasis mouse models in an autopsy. As shown in Fig. 3, reduction of tumor in the liver of HL and cationic HL treated group was observed, although enlargement and tumor-nodes by metastasis of HCT116 cells in the liver of the control group were confirmed. These results indicate that the therapeutic effects of cationic HL could be obtained on the hepatic metastasis mouse models in vivo.

**Histological Bioanalysis** We histologically evaluated the therapeutic effects of cationic HL using the liver tissues of the hepatic metastasis mouse models of HCT116 cells in vivo. We observed the liver tissues of mouse models with a microscope by HE staining method. As shown in Fig. 4, the large metastatic nodules were observed in the liver of the control group, indicating a malignant transformation by metastasis of HCT116 cells to the liver of mouse models. On the other hand, the reduction of metastatic nodules was observed in the liver of the groups treated with HL and cationic HL. Especially, cationic HL remarkably inhibited metastasis of HCT116 cells to liver.

**Induction of Apoptosis by Cationic HL** We examined the mechanism of the therapeutic effects of cationic HL on the hepatic metastasis of HCT116 cells in vivo using TUNEL method. The results are shown in Fig. 5. Many apoptotic cells were observed in the tumor cells in the liver tissue of the groups treated with HL and cationic HL. A significant number of apoptotic cells appeared brown color in the liver tissues of the group treated with cationic HL. On the other hand, no apoptotic cells were observed both of normal and control groups. Slight therapeutic effects of DMPC liposomes...
along with apoptosis have been reported for model mice of carcinoma.9,21) These results indicate that cationic HL have therapeutic effects on the hepatic metastasis mouse models of HCT116 cells along with apoptosis in vivo.

**Safety of Cationic HL in Vivo** We examined the safety of cationic HL in vivo using hepatic metastatic mouse models of colon carcinoma. Result are shown in Fig. 6. No weight loss was observed in hepatic metastasis mouse models after the intravenous administration of cationic HL. We have already reported that HL and cationic HL have no side-effects in vivo in the safety tests intravenously administered via a vein using mice9,21) and rats.17–19) These results indicate that cationic HL could not have severe side effects.

**DISCUSSION**

Membrane fluidity of tumor cells differ from that of normal cells. The membranes of tumor cells are generally more fluid as compared with normal ones. We have reported that HL composed of electrically neutral phospholipid being more fluid as compared with DMPC liposomes showed remarkable high inhibitory effects compared with DMPC liposomes on the growth of tumor cells.13) Furthermore, a good correlation between IC₅₀ of HL for the growth of human colon tumor
(WiDr) cells and membrane fluidity of HL was already reported.\(^5\) HL having larger membrane fluidity showed higher inhibitory effects on the growth of various cancer cells in vitro and in vivo.\(^{14,17-19,21,22}\) We have already reported pharmacokinetics of HL using normal mice and rats.\(^{17-19,21,22}\) HL circulated in blood for 3 h after intravenous administration to normal mice, and then metabolized in the liver.\(^{17-19}\) Specific accumulation of HL including fluorescence probe into tumor cells that immunostained using carcinoembryonic antigen (CEA) as a histochemical marker of metastatic colon carcinoma in the liver of hepatic metastasis mouse models of colon carcinoma was observed using a confocal laser scanning microscope.\(^{23}\)

On the other hand, we elucidated the inhibitory effects on the growth of HCT116 cells along with apoptosis by cationic HL including electrically cationic lipid in vitro and in vivo.\(^{4,14}\) Enhanced expression of anionic lipids, phosphatidylserine (PS), in outer plasma membrane of the tumor cells as compared with in the normal cells has been reported.\(^{24,25}\) In addition, negatively charged sialic acid-containing glycolipids overexpress in colon carcinoma.\(^{26-29}\) 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.\(^9\) 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. Cationic HL having positive charge accumulated only into negatively charged tumor cell membranes through electrostatic interaction between cationic HL and anionic surface of tumor cell membranes.\(^{9,14}\) That is, cationic HL distinguished between membranes of negatively charged tumor cells and normal ones. Cationic HL selectively fused and accumulated into negatively charged tumor cell membranes, and the apoptotic signal passed through mitochondria and activation of caspase-9, -8 and -3, and then reached the nucleus. Specific accumulation of fluorescent-labeled cationic HL was observed in the renal tumor tissue of orthotopic xenograft mouse models of human renal cell carcinoma for 6 h after the intravenous injection of intraperitoneal administration of cationic HL including fluorescence probe. In contrast, no accumulation was observed in the renal tissue of normal mice (Supplemental data, Figs. S1, S2). Cationic HL circulated in blood for 3 h after intravenous administration to normal mice, and then metabolized in the liver as in the case of HL (Supplemental data, Fig. S3). There is the problem of potential hemolysis by conventional liposomal drugs.\(^{30}\) No hemolysis was observed in red blood cells after the treatment with HL and cationic HL. On the other hand, hemolysis in the red blood cells treated with DMPC liposomes increased as dose concentration increased.\(^9\) These results indicate that cationic HL could not have severe side effects.

Therapeutic effects of cationic HL on the hepatic metastasis mouse models of human colon carcinoma (HCT116) cells were revealed on the basis of histological analyses in vivo. These results suggest that cationic HL could inhibit the hematogenous metastasis of HCT116 cells to the liver and the growth of HCT116 cells in the liver along with apoptosis.

It was suggested that cationic HL (under 150 nm) stable in the presence of serum could avoid the reticular endothelial system and should be appropriate for clinical application. The successful chemotherapy using cationic HL without any anticancer drug in this study should be important for clinical applications in the future.

In conclusion, we clearly demonstrated for the first time that therapeutic effects of cationic HL were obtained on the hepatic metastasis of human colon carcinoma in vivo. The noteworthy aspects are as follows. (a) Cationic HL having a hydrodynamic diameter less than 150 nm were preserved for one month. (b) Therapeutic effects of cationic HL were obtained in hepatic metastasis mouse models of colon carcinoma in vivo. (c) Induction of apoptosis in metastatic mouse models was observed on the mouse models treated with cationic HL in vivo. It is noteworthy that therapeutic effects of cationic HL on the hepatic metastasis of colon carcinoma along with apoptosis were indicated in vivo.

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REFERENCES


