Anti-tumor Effects of Cationic Hybrid Liposomes against Colon Carcinoma along with Apoptosis in Vitro

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New-type three-component cationic hybrid liposomes (HLs) composed of dimyristoylphosphatidylcholine (DMPC), polyoxyethylene(21)dodecyl ether (C12(EO)21) and O,O’-ditetradecanoyl-N-(a-trimethylammonioacetyl) diethanolamine chloride (2C14ECl) were produced. Cationic HLs were smaller and more stable than pure DMPC liposomes. It is noteworthy that cationic HLs could remarkably inhibit the growth of human colon cancer (HCT116) cells along with apoptosis in vitro for the first time in this study.

Key words hybrid liposome; colon carcinoma; apoptosis

Colon carcinoma is a malignant disease formed in the tissues of the colon. The advanced-colon carcinoma spreads through the blood and lymph nodes to other parts of the body, such as the lung, liver, abdominal wall, or ovaries. Standard treatment such as surgery, chemotherapy, and radiation therapy are used for colon carcinoma. However, surgical applications are limited to patients of early stages without metastasis and there are often severe side effects in the treatment of colon carcinoma. The advanced-colon carcinoma spreads through induction of cell cycle arrest. However, the effects of cationic HLs on the growth of colon carcinoma cells in view of the negatively charged characteristics of tumor cell membrane have not yet been elucidated.

To aim at selective chemotherapy against colon carcinoma having anionic rich cell membranes, we produced new-type three-component cationic HLs (Chart 1) composed of dimyristoyl-phosphatidylcholine (DMPC), polyoxyethylene(21)dodecyl ether (C12(EO)21) and O,O’-ditetradecanoyl-N-(a-trimethyl-ammonioacetyl) diethanolamine chloride (2C14ECl) and investigated the inhibitory effects of cationic HLs on the growth of human colon carcinoma (HCT116) cells in vitro.

MATERIALS AND METHODS

Preparation of Hybrid Liposomes Cationic HLs were prepared by using sonication (VS-N300; VELVO, Tokyo, Japan) with drug-free HLs to patients with lymphoma has also reported that HLs 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. More recently, it was observed that HLs could inhibit growth of human colon adenocarcinoma HCT116 through induction of cell cycle arrest.22) However, the effects of cationic HLs on the growth of colon carcinoma cells in vitro.

Chart 1. Schematic Representation of Cationic HLs Composed of DMPC, C12(EO)21, and 2C14ECl

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Japan) of a mixture containing DMPC (purity >99%; NOF Co., Ltd., Tokyo, Japan), micellar molecules: C_{12}(EO)_{21} (Nikko Chemicals Co., Ltd., Tokyo, Japan) and 2C_{14}ECI (DC-6-14; Sogo Pharmaceutical Co., Ltd., Tokyo, Japan) in 5% glucose solution at 45°C with 300W, followed by filtration with a 0.20 μm filter.

**Dynamic Light Scattering Measurements** The diameter of HLs was measured with a light scattering spectrometer (ELS-8000, Otsuka Electronics, Japan) using a He–Ne laser (633 nm) at a 90° scattering angle. The diameter (d_{hy}) was calculated using the Stokes–Einstein formula (Eq. 1), where \( \kappa \) is the Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is the viscosity and \( D \) is the diffusion coefficient:

\[
d_{hy} = \frac{\kappa T}{3\pi \eta D}
\]  

(Eq. 1)

**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 Growth Inhibition Caused by HLs** The 50% inhibitory concentration (IC₅₀) on the growth of tumor cells was determined on the basis of WST-1 [2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-1, Dojindo Laboratories, Kumamoto, Japan). Cells (5.0×10⁵ cells/mL) were seeded in 96-well plates and cultured in a 5% CO₂ humidified incubator at 37°C for 24h. Cells were cultured for a further 48h after adding DMPC (0.1–5 μm), HLs and cationic HLs (0.1–2 μm) on the basis of DMPC concentration. WST-1 solution was added and the cells again incubated for 3h. The absorbance at a wavelength of 450 nm was measured by spectrophotometer (E_max, Molecular Devices Co., California, U.S.A.). The inhibitory effects of HLs on the growth of 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 HLs, respectively.

**Fusion and Accumulation of HLs into the Cell Membrane** The fusion and accumulation of HLs including a fluorescence probe (1-palmitoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol-4-yl]amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBDPC; Avanti Polar Lipids, Alabama, U.S.A.) into the membrane of HCT-116 cells was performed using confocal laser microscopy (TCS-SP; Leica Microsystems, Berlin, Germany). Cells (2.0×10⁶ cells/mL) were cultured in a 5% CO₂ humidified incubator at 37°C for 24h. The cells were treated with HLs ([DMPC]=0.45 mM, [C_{12}(EO)_{21}]=0.027 mM, [2C_{14}ECI]=0.044 mM) including fluorescence-labeled lipid for 4h and were observed using confocal laser microscopy with a 488 nm Ar laser line (detection at 505–555 nm).

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate (dUTP)-Biotin Nick End Labeling (TUNEL) Method** Detection of apoptotic cells was performed by the TUNEL method using an In Situ Cell Death Detection Kit (Roche Diagnostics K.K.). HLs ([DMPC]=0.91 mM, [C_{12}(EO)_{21}]=0.052 mM, [2C_{14}ECI]=0.084 mM) were added to the cell suspension (4.0×10⁵ cells/mL) and cells cultured for 48h. The medium including dead cells was centrifuged and the cells were fixed with 4% paraformaldehyde solution, and then processed for TUNEL assay according to the manufacturer’s instructions. The stained cells were observed using a confocal laser microscopy (TCS-SP; Leica Microsystems) with a 488 nm Ar laser line (detection at 515–565 nm).

**Apoptotic DNA Measurements with Flow Cytometry** The HCT116 cells treated with HLs for 24h were centrifuged at 3000 rpm for 5min and washed with phosphate buffered saline (PBS) (–), then fixed in chilled ethanol. The cells were washed again, treated with RNase (0.25 mg/mL) and then stained with propidium iodide (PI, 0.5 mg/mL) that has 493 nm excitation and 635 nm emission wavelength. The samples were analyzed using flow cytometer (Epics XL system, Beckman Coulter, California, U.S.A.) with a single excitation 488 nm of 15 mW argon laser. The PI signals were detected by FL3 sensor in 605–635 nm. Apoptotic DNA rates were calculated by apoptotic DNA rates=(apoptotic DNA content/DNA content)×100.

**Mitochondrial Membrane Potential** Cells were incubated with HLs ([DMPC]=11.5 mM, [C_{12}(EO)_{21}]=0.66 mM, [2C_{14}ECI]=1.06 mM) for 30min, and then 2.5 μL of 3,3-dihexyloxacarbocyanine iodide [DiOC6(3)] (Molecular Probes, Oregon, U.S.A.) were added to evaluate mitochondrial transmembrane potential (ΔΨ_m) and cells were incubated at 37°C for 20min. The cells were centrifuged, suspended with 500 μL of...
PBS and were used for flow cytometric analysis, with a 15 mW 488 nm air-cooling Ar laser and FL1 sensor (505–545 nm).

**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 AND DISCUSSION

**Physical Properties of Hybrid Liposomes** We examined the physical properties of cationic HLs composed of 87 mol% DMPC, 8 mol% 2C14ECl, and 5 mol% C12(EO)21 on the basis of a dynamic light scattering method. As shown in Fig. 1, the mean *d*_½ of cationic HLs were 100 nm in diameter, which
remained stable for more than one month at 37°C. On the other hand, DMPC liposomes were unstable and precipitated after 28 d. HLs (95 mol% DMPC/5 mol% C12(EO)21) were gradually increased with time. It is worthy to note that threecomponent HLs having 100 nm in diameter could avoid the reticularendothelial system.21)

Inhibitory Effects of Cationic HLs on the Growth of HCT116 Cells With respect to the inhibitory effects of cationic HLs on the growth of human colon cancer (HCT116) (ATCC, U.S.A.) cells, we examined the fifty percent inhibitory concentration (IC50) of cationic HLs on the basis of WST-1 assay in vitro.15) The results are shown in Fig. 2. The IC50 value of cationic HLs was 0.17±0.005 mM, whereas those of HLs and DMPC liposomes were 0.36±0.05 and 0.50±0.04, respectively. It is well known that liposome composed of phospholipids accumulated in the influence regions. We have already reported that inhibitory effects of DMPC liposomes on the growth of tumor cells in vitro and in vivo.17) Moderate inhibitory effect of DMPC liposomes on the growth of human colon tumor cells was obtained in this study due to the characteristics of phospholipids. It was obvious that cationic HLs markedly inhibited the growth of HCT116 cells.

Fusion and Accumulation of Cationic HLs into HCT116 Cells How do cationic HLs suppress the growth of HCT-116 cells? We examined the anti-tumor mechanism of cationic HLs in HCT116 cells. The fusion and accumulation of cationic HLs toward HCT116 cells was examined using confocal laser microscope. The results are shown in Fig. 3. An increase in accumulation of cationic HLs including NBDPC as a fluorescence probe was observed, though less accumulation of HLs was obtained. On the other hand, in the case of DMPC liposomes accumulation was undetected. These results suggest that HLs including cationic lipids could have higher inhibitory effects on the growth of colon carcinoma cells as compared with DMPC liposomes and HLs through the selective fusion and accumulation against colon carcinoma cells. HLs induced apoptosis in human colon adenocarcinoma WiDr4) and mouse colon carcinoma Colon26 cells in vitro.21) Furthermore, we also reported that HLs 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.19) More recently, it was observed that HLs could inhibit growth of human colon adenocarcinoma HCT116 through induction of cell cycle arrest and apoptosis.22) However, with respect to cancer cell lines having the negatively charged characteristics in cell membrane arrest such as HCT-116,3,4) cationic HLs could more rapidly fuse to cell membrane than HLs.

Induction of Apoptosis for HCT116 Cells Treated with Cationic HLs We examined the induction of apoptosis in HCT116 cells exposed to cationic HLs by TUNEL method using a confocal laser microscope.20) Fluorescence micrographs of HCT116 cells after the treatment with cationic HLs on the basis of the TUNEL method are shown in Fig. 4. Interestingly, HCT116 cells were dyed in green after adding cationic HLs, indicating that HLs induced apoptosis for HCT116 cells, although the cells were not dyed when using the DMPC liposomes. On the other hand, a little cells were dyed in green after adding HLs as compared in the case of cationic HLs.

To investigate the apoptotic pathways of cationic HLs on HCT116 cells, apoptotic DNA rates in HCT116 cells treated with cationic HLs were measured using flow cytometry.15) The results are shown in Fig. 5A. A high apoptotic DNA rate was obtained after the treatment with cationic HLs, though less induced apoptosis by HLs was obtained. These results indicate that the cationic HLs could induce apoptosis for HCT116 cells. Furthermore, we examined the mitochondrial pathway for apoptotic signal transduction by cationic HLs using flow cytometry. The results are shown in Fig. 5B. Interestingly, mitochondrial transmembrane potential was decreased after the treatment with cationic HLs, suggesting that the mitochondrial pathway should be also implicated in apoptosis induced by cationic HLs.

Tumor cells are negatively charged in general. Anionic lipids (PS) in the outer plasma membrane of tumor cells increase as compared with the normal cells.3,4) In addition, negatively charged sialic acid-containing glycolipid-containing glycolipids overexpress in colon carcinoma.2–8) We believe that cationic HLs selectively fused and accumulated into negatively charged tumor cell membranes, and the apoptotic signal passed through mitochondria and activation of caspase, and then reached the nucleus.

We have already reported that there is no toxicity of cationic HLs for normal human renal proximal tubule epithelial (RPTE) cells (IC50=0.58 mM).4) It is considered that cationic HLs...
HLs can selectively inhibit the growth of colon carcinoma cells without affecting normal colon cells. Furthermore, no toxicity of cationic HLs was revealed using normal mice in vivo without any side-effects. Cationic HLs might be applied in chemotherapy for patients with cancer in the future.

In conclusion, we clearly demonstrated that cationic HLs composed of DMPC, 2C14EC1, and C12(EO)21 showed inhibitory effects on the growth of human colon cancer HCT116 cells in vitro along with apoptosis. The noteworthy aspects are as follows. (a) The IC50 values of cationic HLs on the growth of HCT116 cells were remarkably smaller than those of the DMPC liposomes. (b) Selective fusion and accumulation of cationic HLs against HCT116 cells were obtained. (c) The induction of apoptosis by cationic HLs was verified for HCT116 cells on the basis of flow cytometry and TUNEL method. It is noteworthy that remarkable inhibitory effects of drug-free cationic HLs leading to apoptosis on the growth of human colon cancer HCT116 cells having negatively charged characteristics were obtained for the first time.

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