Novel Liposomes Composed of Dimyristoylphosphatidylcholine and Trehalose Surfactants Inhibit the Growth of Tumor Cells along with Apoptosis

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Novel liposomes composed of α-dimyristoylphosphatidylcholine (DMPC) and trehalose surfactant (DMTre) were produced and inhibitory effects of DMTre on the growth of human colon carcinoma (HCT116) and gastric carcinoma (MKN45) in vitro were examined. The remarkably high inhibitory effects of DMTre on the growth of HCT116 and MKN45 cells were obtained without affecting the growth of normal cells. The thickness of fixed aqueous layer of DMTre was larger than that of DMPC liposomes and increased in a dose-dependent manner. The induction of apoptosis by DMTre was revealed on the basis of flow cytometric analysis. DMTre induced apoptosis through the activation of caspases and mitochondria via Bax. It is noteworthy that remarkable inhibitory effects of DMTre on the growth of human colon and gastric carcinoma cells leading to apoptosis were obtained for the first time.

Key words antitumor effect; trehalose surfactant; apoptosis

Saccharides play important roles in adhering to cells, transmitting information, recognizing molecules on the cell membranes through receptors including lectin. For example, molecular recognition through lactose was found in vivo. The hydration of saccharides with hydrogen bonds provides stability to the structure of water. The hydration of sugar derivatives was discussed in relation to the hydration of the parent sugars. The preparation and characterization of glycoliposomes have been reported.

We have produced hybrid liposomes (HL) composed of vesicular and micellar molecules, which can be prepared by just the sonication of those molecules in a buffer solution. Changing the constituents and compositional ratios of HL can control the physical properties of HL, such as size, shape, the temperature of phase transition, and membrane fluidity.

HL composed of α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenehexadecyl ether without using any drugs inhibited the proliferations of various tumor cells along with apoptosis in vitro and in vivo. The mechanistic details of apoptosis for tumor cells induced by HL and the correlation between antitumor effects and membrane fluidity of HL and membrane fluidity of plasma membranes of tumor cells have been clarified. Successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported without having any adverse effects after the approval of the Bioethics Committee. Specific inhibitory effects of three-component HL composed of DMPC, Tween 20, and sucrose surfactants were obtained on the growth of glioma cells in vitro. In this study, two-component HL (DMTre) composed of DMPC and trehalose surfactant (TreC14) were produced and examined for assessment of antitumor effects using tumor cells and the signal cascade for apoptosis.

DMTre were prepared by using sonication (VELVO VS-N300, 300W) of a mixture containing DMPC (NOF Co., Ltd., Japan) and α-d-glycopyranosyl-α-d-glycopyranoside monomystate (TreC14, Dojindo Ltd., Japan) in a 5% glucose solution at 45°C with 300W, followed by filtration with a 0.45 µm filter.

Physical properties of DMTre composed of DMPC and TreC14 were examined. The thickness of fixed aqueous layer (TFAL) of DMTre was measured from zeta potential (ζ). DMTre was prepared in 5% glucose solution containing various concentrations of NaCl (20, 50, 100, 150, 200 mM). The values of ζ of the sample solutions were measured by laser doppler photometry method using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan) with a He–Ne laser as a light source (633 nm, 10mW) at the scattering angle (θ: 1.0–2.3 × 10−4 m/Volt) applying the smoluchowski equation (ζ = qηU/εc), where η (Pa·s) and ε (N/Volt²) are the viscosity and permittivity of solvent, respectively. The values were measured at 37°C. ζ is defined as the electrophoretic potentials at the position of the slipping plane Δ (nm), which occurs just outside the fixed aqueous layer of DMTre. Then, ζ is expressed as ln(ζ) = ln(Δ−A−Δκ, where κ is the Debye–Hückel parameter (=3.3/εC, C: M for NaCl). If the ζ values are measured in various concentrations of NaCl and plotted against κ, the slope gives the position of the slipping plane or the TFAL in nm units. On the basis of this theory, the TFAL of DMTre was estimated. The results are shown in Fig. 1. TFAL values of DMTre were larger than that of DMPC liposomes. It is noteworthy that TFAL of DMTre increased in a dose-dependent manner.

We examined the inhibitory effects of DMTre on the growth of human colon carcinoma (HCT116) and gastric carcinoma (MKN45) cells 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, Japan). Colon and gastric carcinoma is the common carcinoma worldwide especially in Asia for both of male and female. Cells (5.0×10⁴ cells/mL) were seeded

Fig. 1. Thickness of Fixed Aqueous Layer (TFAL) of DMTre Increased in a Dose-Dependent Manner

[DMPC]=1.0×10⁻⁵ mol%, [TreC14]=1.0–2.3×10⁻⁴ mol%. Data represent the mean±S.D.

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in 96-well plates and cultured in a 5% CO₂ humidified incubator at 37°C for 24 h. Cells were cultured for 48 h after adding DMTre. WST-1 solution was added and incubated for 3 h. Absorbance at wavelength of 450 nm was measured by spectrophotometer (E_max, Molecular Devices Co., U.S.A.). The inhibitory effects of DMTre on the growth of HCT116 and MKN45 cells were evaluated by \((A_{\text{mean}} / A_{\text{control}}) \times 100\), where \(A_{\text{mean}}\) and \(A_{\text{control}}\) denote the absorbance of water-soluble formazan, in the presence and absence of DMTre, respectively. The results are shown in Fig. 2. DMTre inhibited the growth of two tumor cell lines in a dose-dependent manner without affecting the growth of normal (WI-38) cells, though DMPC liposomes had no inhibition against those tumor cells.

Therefore, inhibitory effects of DMTre could be related to hydration in tumor cells. Tumor cell membranes are more fluid than those of normal ones. DMTre selectively fused and accumulated into carcinoma (HCT116 and MKN45) cells but not into normal (WI-38) ones (data not shown), suggesting that DMTre could distinguish between carcinoma and normal cell membrane.

The induction of apoptosis by DMTre for HCT116 and MKN45 cells was examined using flow cytometric analysis. Apoptotic DNA rates in tumor cells after the treatment with DMTre were measured on the basis of flow cytometry. The cells treated with HL for 24 h were centrifuged at 3000 rpm for 5 min, washed with phosphate buffered saline (PBS(−)), and resuspended in PBS(−). The cells were washed again, treated with RNase (0.25 mg/mL) and then stained with propidium iodide (PI, 40 µg/mL) that has 493 nm excitation and 635 nm emission wavelength. The samples were analyzed using a flow cytometer (Epics XL system, Beckman Coulter, U.S.A.) with a single excitation 488 nm of 15 mW Ar laser. The PI signals were detected by FL3 sensor in 605–635 nm. Apoptotic DNA rates were calculated by apoptotic DNA rate = (apoptotic DNA content/DNA content)×100. The results are shown in Fig. 3. Apoptotic DNA rate increased after the treatment with DMTre as the dose of DMTre increased and reached a high apoptotic DNA rate (90%).

To investigate the apoptotic pathways induced by DMTre on HCT116 and MKN45 cells, activation of caspases by DMTre was examined. The results are shown in Fig. 4. It is note-
worthy that fluorescent intensity increased and tumor cells were dyed in green after the treatment with DMTre, indicating that DMTre induced apoptosis for tumor cells through the activation of caspases. Moreover, we examined the mitochondrial pathway in the apoptosis induced by DMTre for HCT116 and MKN45 cells using flow cytometric analysis and cytochrome c release assay. The mitochondrial transmembrane potential for the tumor cells after the treatment with DMTre was the
same as that for the control ones. However, as shown in Fig. 5, fluorescent intensity for HCT116 and MKN45 cells after the treatment with DMTre decreased, indicating that cytochrome c of mitochondria could release and then caspase-9 could be activated by DMTre. We examined Bax assay for HCT116 and MKN cells after the treatment with DMTre. Cells were seeded at a density of $2.0 \times 10^4$ cells per well in 6-well plates and incubated for 24 h. DMTre were added into each well and the plates were incubated for 48 h. The cells were centrifuged at 3000 rpm for 5 min, fixed with a 4% paraformaldehyde solution and permeabilized with 70% ethanol for 30 min. The cell lysates were washed with 1 mL wash buffer and gently resuspended. Fifty microliters anti-Bax was added to each tube and gently mixed for 30 min. The cells were washed with wash buffer and 30 µL anti-immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) was added. The Bax protein of the cells was analyzed using a confocal laser microscope (TCS-SP, Leica, Germany) with a single excitation 488 nm of 15 mW Ar laser. The results are shown in Fig. 6. The tumor cells were dyed in green after the treatment with DMTre, indicating that the cytochrome c release was related to the activation of Bax.

In conclusion, we clearly demonstrated that novel liposomes, DMTre, composed of DMPC and TreC14 inhibitory effects on the growth of HCT116 and MKN45 cells in vitro along with apoptosis. The noteworthy aspects are as follows: (a) The remarkably high inhibitory effects of DMTre on the growth of HCT116 and MKN45 cells were obtained without affecting the growth of normal cells. (b) The thickness of fixed aqueous layer of DMTre was twice that of DMPC liposomes. (c) The induction of apoptosis by DMTre was revealed on the basis of flow cytometric analysis. Furthermore, apoptotic pathway induced by DMTre was examined. (d) DMTre induced apoptosis through the activation of caspases and mitochondria via Bax. It is noteworthy that the remarkable inhibitory effects of DMTre on the growth of human colon and gastric carcinoma cells leading to apoptosis were obtained for the first time.

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REFERENCES AND NOTES


22) Cells were seeded at a density of 2.0×10^4 cells per well in 6-well plates and incubated for 24h. DMTrE were added into each well and the plates were incubated for 48h. The cells were centrifuged at 3000rpm for 5min and resuspended in 50μL of chilled cell lysis buffer. Each substrate solution (PhiPhiLux-G1D1, CaspaLux 8-L1D2, CaspaLux 9-M1D2 for caspase-3, caspase-8, and caspase-9, respectively) was added to the cell pellets and cells were incubated for 1h. After washing twice with 1mL of ice-cold PBS(−), the cells were resuspended in 1mL fresh PBS(−). Activation of caspases was determined and analyzed using a flow cytometer according to the manufacturer’s instructions. The stained cells were observed using a confocal laser microscope with a single excitation 488 nm of 15mW Ar laser.

23) The cell lysates treated with DMTrE were washed with 1mL wash buffer and resuspended with 250μL blocking buffer. Anti-cytochrome c was added to each tube and gently mixed for 1h. The cells were washed with wash buffer and 500μL anti-IgG FITC was added. Releasing cytochrome c of mitochondria was analyzed by using flow cytometer.