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Selective accumulation and growth inhibition of hybrid liposomes to human hepatocellular carcinoma cells in relation to fluidity of plasma membranes

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ABSTRACT

Hybrid liposomes (HLs), composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(23) dodecyl ether, have selectively inhibited the growth of human hepatocellular carcinoma (HCC) cells without affecting normal hepatocytes to trigger apoptosis via caspase-3 activation. Furthermore, HLs distinguished between the HCC and normal cells which had higher and lower membrane fluidities respectively, then fused and accumulated preferentially into the membranes of HCC cells. It is noteworthy that the anti-cancer activity of HLs correlated well with the fluidity of cell membranes for HCC and other cancer cells. These results suggest that HLs could target cancer cell-membranes in relation to their lipid fluidity that provide the possibility of novel nanotherapy for intractable cancer.

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1. Introduction

Cancer cells differ from normal cells in terms of cell growth, motility, invasion, morphology, lipid metabolism, cell stiffness, brush layer on the cell surface, and more [1–5]. In particular, the dynamics of cell membranes is one of the fundamental functions of cellular biophysical characteristics of cancer cells. Cancer cell membranes show changes in the composition and construction, and the cell membranes are more fluid than those of normal cells [6–8]. Some authors have also reported that the cell membranes from patients with malignant leukemia and lymphoma, which have less cholesterol and more unsaturated lipid contents in the cell membranes, showed higher fluidity as compared with those of normal lymphocytes [6,8–10]. Similarly, higher fluidity of cancer cell-membranes was also obtained in other solid tumor cells such as rat or human hepatoma and human lung cancer [11–16]. In addition, the membrane dynamics with higher fluidity should be a fundamental property of cancer cells, and closely related to their biological features of the proliferative potential, invasive potential, and metastatic abilities [17–19]. In recent years, molecular targeted therapeutics have attracted much attention as an efficient therapy for cancers on the basis of molecular level studies on human cells [20,21]. However, there is no report on the cancer chemotherapy targeting the membrane dynamics from the viewpoint of biophysical characteristics of cancer cells vs. normal ones.

Hybrid liposomes (HLs) are nano-sized liposomal particles and can be prepared by sonication of vesicular and micellar molecules in a buffer solution [22]. The physical properties of HLs such as shape, size, membrane fluidity, and the temperature of phase transition can be controlled by changing the constituents and compositional ratios [23]. In the drug delivery system (DDS) study, the therapeutic effects of an anti-cancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) encapsulated into HLs composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(20) sorbitan monolaurate (Tween 20) have been observed for the meningeal gliomatosis model rats *in vivo* [24]. On the other hand, it has been reported that HLs composed of DMPC and polyoxyethylene(23) dodecyl ethers ($C_{12}(EO)_{23}$) without any other anti-cancer drugs inhibited the growth of various cancer cells *in vitro* [25–27], *in vivo* [28,29] and clinical applications [29,30]. Significantly, we have revealed a good correlation between membrane fluidity of HLs composed of DMPC and $C_{12}(EO)_n$ ($n = 4, 8, 10, 21, 23, 25$) and their growth-inhibitions for colorectal cancer cells *in vitro* [27]. We have also demonstrated a good correlation between fluidity of plasma membranes on various cancer cells and anti-cancer effects of HLs *in vitro* [31]. Interestingly, HLs distinguished cancer cells and normal cells which have higher and lower membrane

Abbreviations: BCNU, 3-bis(2-chloroethyl)-1-nitrosourea; $C_{12}(EO)_{23}$, polyoxyethylene(23) dodecyl ether; DDS, drug delivery system; DMPC, L-α-dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; HCC, hepatocellular carcinoma; HLs, hybrid liposomes composed of DMPC and $C_{12}(EO)_{23}$; IC_{50}, fifty percent inhibitory concentration; NBDPC, 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; *P*, fluorescence polarization; PBS (−), phosphate buffered-saline; TIRF, total internal reflection fluorescence; Tween 20, polyoxyethylene(20) sorbitan monolaurate; WST-1, 2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt.

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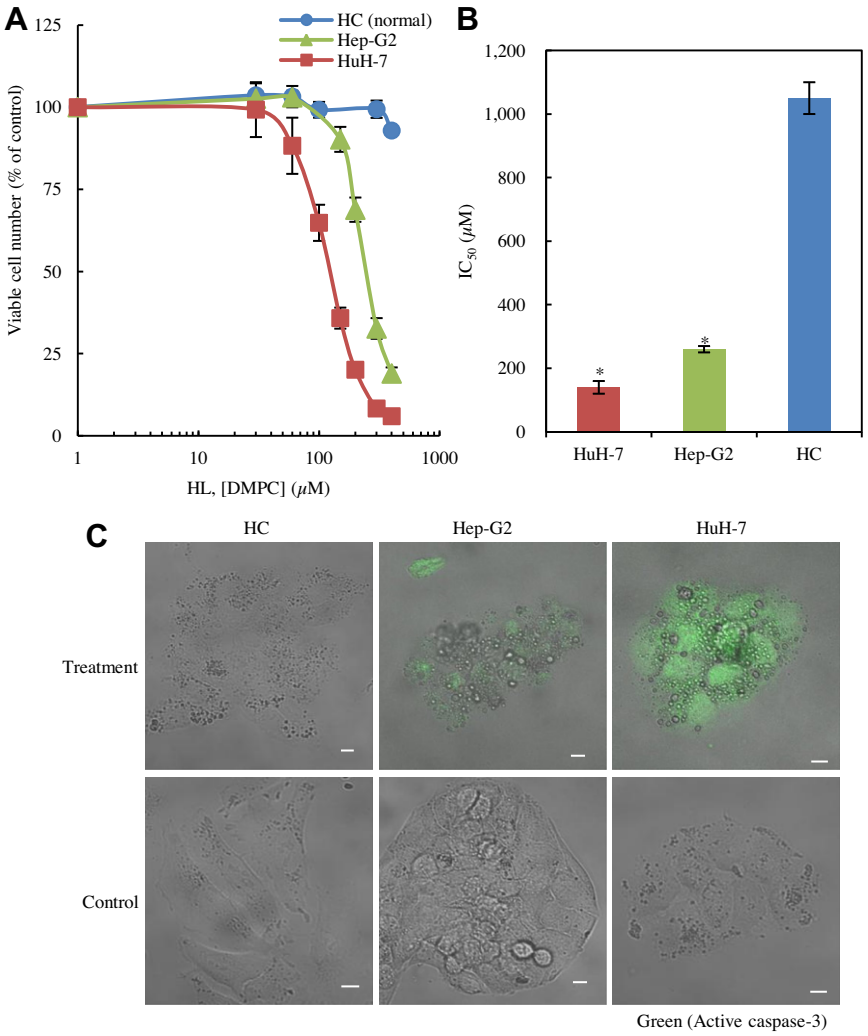

Fig. 1. HLs selectively inhibit the growth of HCC cells leading to apoptosis. (A) The inhibitory effects of HLs on the growth of HCC cells were determined from the concentration-dependence of the viable cell number. (B) Fifty percent inhibitory concentration (IC_{50}) of HLs on the growth of HCC cells for 48 h. Data are means ± S.E. from two independent experiments, where each determination was made in quadruplicate. *Significant difference from the normal HC cells, $p < 0.05$. (C) Microscopic imaging of caspase-3 activation (apoptotic cell death) in HCC cells treated with HLs. After 48 h of incubation in the presence of HLs at the 250 μM, the cells stained with PhiPhiLuxG_1D_2 (green fluorescence) were observed with a confocal laser microscope. Excitation/detection = PhiPhiLuxG_1D_2: 488 nm/505 – 555 nm. Scale bar, 10 μm.

fluidities respectively, and fused and accumulated preferentially into cancer cells for human lung cancer [14] and human adult T-cell leukemia cells [32]. However, there is still no report on the correlation between anti-cancer activity of HLs and membrane fluidity of cancer cells vs. normal cells.

We have already shown that HLs inhibit the growth of Hep-G2 and HuH-7 of human hepatocellular carcinoma (HCC) cells leading to apoptosis *in vitro* [26,31]. In this study, we examined the inhibitory effects of HLs on the growth of HCC cells and normal hepatocytes in relation to the fluidity of plasma membranes, through the specific accumulation of HLs into the membranes of HCC cells.

2. Materials and methods

2.1. Preparation of hybrid liposomes

Hybrid liposomes (HLs) were prepared by the following methods [25]. L-α-Dimyristoylphosphatidylcholine (DMPC) (NOF, Japan) and polyoxyethylene(23) dodecyl ether ($C_{12}(EO)_{23}$) (Sigma Chemical, USA) were mixed in 5% glucose solution and sonicated with a sonicator (VS-N300, VELVO, Japan) at 45 °C with 300 W, followed by filtration with 0.20 μm filter.

2.2. WST-1 assay

Two HCC (HuH-7 [33] and Hep-G2) cells were purchased from Riken Cell Bank (Japan). Human primary hepatocyte (HC) cells were from Dainippon Pharmaceutical (Japan). The cells were seeded in 96 well plates (1.0×10^3 cells/well) in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, HLs were added into each well and the plates were incubated for 48 h. The viable cell number was measured by WST-1 (2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) assay with a Cell Counting Kit (Dojindo Laboratories, Japan) according to the manufacturer's instructions [31]. The fifty percent inhibitory concentration (IC_{50}) of HLs was determined from the concentration-dependence of the viable cell number.

2.3. Caspase activity measurements with confocal microscopy

The active caspase-3 activity was measured using PhiPhiLux-G_2D_2 (OncoImmunin, Gaitherssburg, MD) according to the manufacturer's instructions. The cells (2×10^4 cells/ml) were treated with 250 μM HLs for 48 h, incubated with 5 μM PhiPhiLux-G_2D_2 substrate for 30 min, and then the caspase-3 activity was visualized by a confocal laser scanning microscope (TCS-SP, Leica, Germany).

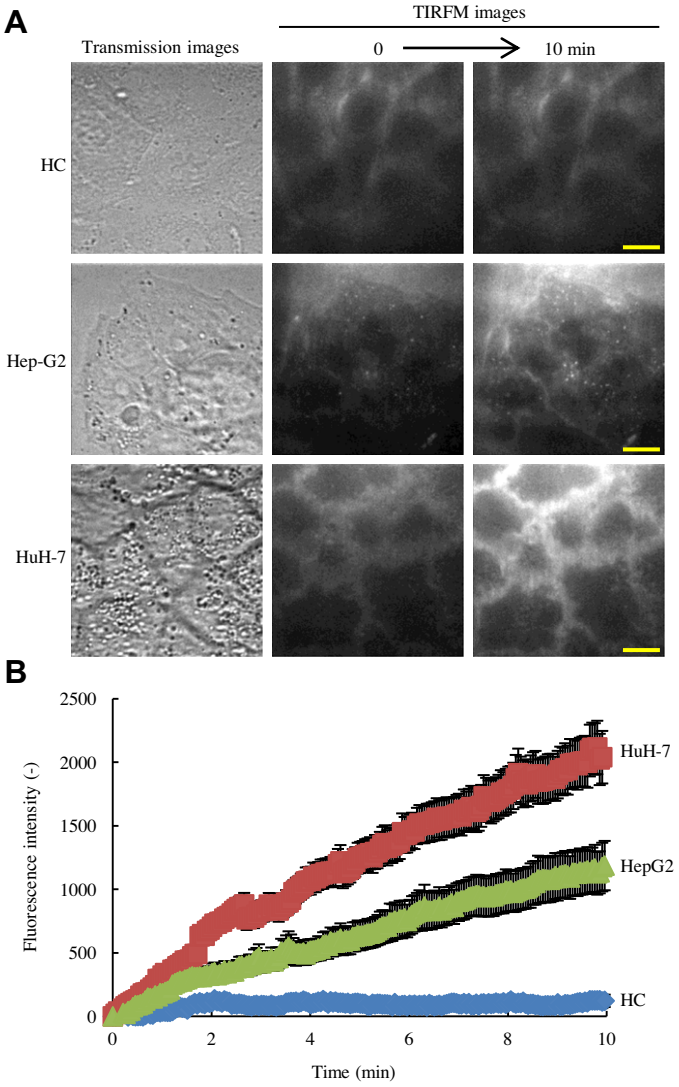

Fig. 2. HLs/NBDPC selectively fuse and accumulate into the plasma membranes of HCC cells. (A) TIRF images of HCC (HuH-7, Hep-G2) and normal (HC) cells treated with HLs/NBDPC. Scale bar, 10 μm. (B) Time course of mean-fluorescent intensity of NBDPC embedded into the cell membranes (HuH-7, red; Hep-G2, green; HC, blue). The data shown are the mean ± S.E. ($n = 3$). After addition of HLs/NBDPC into the culture solutions, the cells were observed using TIRF system equipped with an air-cooled CCD camera.

2.4. TIRF microscopy

Accumulation of HLs into the plasma membranes of cancer cells was observed by total internal reflection fluorescence (TIRF) microscopy [34]. After the pre-incubation of cancer cells (1.0×10^5 cells/ml) for 24 h, HLs (100 μM DMPC) containing 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-*sn*-glycero-3-phosphocholine (NBDPC) (Avanti Polar Lipid, USA) (4.6 μM) was added into the culture solutions, and then the cancer cells were observed with a TIRF microscope system (IX71, Olympus, Japan) equipped with an air-cooled CCD camera (EM-CCD C9100-13, Hamamatsu Photonics, Japan) as described previously [27,31].

2.5. Fluorescence depolarization method

Membrane fluidity of intact cells was evaluated on the basis of fluorescence depolarization method with a fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Nacalai Tesque, Japan). After the pre-incubation of cells for 24 h, the cells were treated with 0.05% Trypsin/EDTA and suspended in phosphate buffered-saline (PBS (−)), and then DPH (0.1 μM) was added into the cell suspension (2.0×10^4 cells/ml). The cell suspension was allowed to stand for 15 min at 37 °C, and the fluorescence polarization (P) of DPH was measured using a fluorescence spectrophotometer (F-2000, Hitachi, Japan) as described previously [31].

3. Results and discussion

The hybrid liposomes (HLs) used in this study were prepared by sonication of a mixture containing 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ in 5% glucose solution as described previously [25]. Dynamic light-scattering measurements done with the Otsuka Electronics ELS-8000 apparatus (Japan) showed that the size of HLs was less than 100 nm in diameter and remained stable for more than 1 month.

Here, we examined the inhibitory effects of HLs on the growth of HCC (HuH-7 and Hep-G2 cells) and normal hepatocytes (HC) *in vitro* on the basis of WST-1 assay. As shown in Fig. 1A, HLs inhibited the growth of both HCC cells in a dose-dependent manner. However, HLs do not exert the growth-inhibition of normal HC cells in the concentration range of 0–400 μM. It is noteworthy that greater inhibitory effects of HLs on the growth of HuH-7 (IC_{50} = 140 μM) and Hep-G2 (IC_{50} = 250 μM) cells compared with HC (IC_{50} = 1050 μM) cells were obtained on the basis of IC_{50} (Fig. 1B, $p < 0.05$). These results indicate that HLs should be selective in inhibiting the growth of HCC cells. We further demonstrated that the growth-inhibitions of HLs for HCC cells were caused by apoptotic cell death and performed caspase-3 intracellular activity assay to detect the caspase-3 activation using confocal laser scanning microscopy. The results are shown in Fig. 1C. Both HCC cells employed in this study were dyed in green after the treatment with 250 μM HLs for 48 h, indicating that the apoptotic cell death via activation of caspase-3 could be induced by HLs. However, caspase-3 activation was not observed in HC cells. Thus, the selective inhibitory effects of HLs on the growth of HCC cells should be attained through the induction of apoptosis via caspase-3 activation.

Specific accumulations of HLs including fluorescent lipids NBDPC (HLs/NBDPC) into cancer cells have been observed for human colorectal cancer cells [27], human primary effusion lymphoma cells [35] and human cholangiocarcinoma cells as compared to these normal cells [36], on the basis of TIRF microscope and flow cytometry. To investigate the accumulation of HLs into the plasma membranes of HCC and normal hepatocytes, we used fluorescent lipids NBDPC (HLs/NBDPC) and observed then using the TIRF microscope as described previously [27,31]. The results are shown in Fig. 2A. Also, the mean-fluorescence intensity of NBDPC in the plasma membranes of the HCC cells was plotted with time in Fig. 2B. The fluorescence intensities for HuH-7 and Hep-G2 cells drastically increased for the 10 min after the treatment with HLs/NBDPC, though those for normal HC cells were low and almost constant. The fluorescence intensity for Hep-G2 and HuH-7 cells was about 9- and 17-fold higher than that for HC cells at 10 min after adding HLs/NBDPC. These results indicate that HLs/NBDPC could accumulate more in the plasma membranes of both HCC cells as compared with those in the HC cells. These results suggest that HLs could selectively fuse and accumulate into HCC cells but not so much into normal hepatocyte cells. Further, the possibility exists that HLs/NBDPC, having the property of specific accumulation into cancer cells, may serve as a diagnostic tool for an early detection of HCC and other cancer cells.

Steady-state fluorescence polarization studies with the fluorescent lipid probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) are utilized

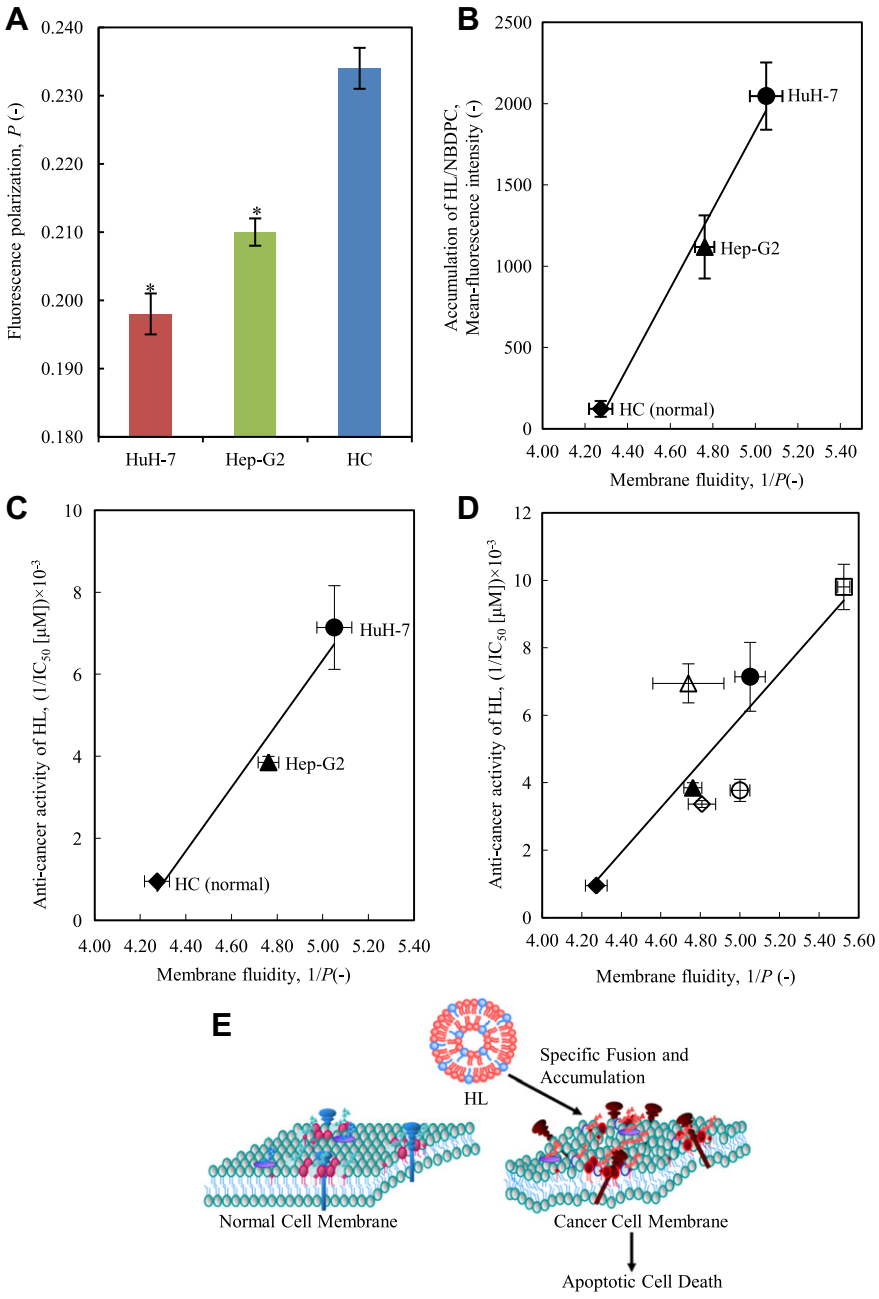

Fig. 3. Anti-cancer activity of HLs correlates well with the fluidity of HCC cell-membranes. (A) Membrane fluidity of intact cells, HCC cells and normal hepatocytes (HC) at 37 °C. As the *P* value of DPH in the cell membranes becomes smaller, the cell membranes have larger fluidity. *Significant difference from the HC cells, $p < 0.05$. Data are means ± S.E. ($n = 3$). (B) Correlation between accumulation of HLs/NBDPC (mean-fluorescence intensity) into the cell membranes in Fig. 2B and membrane fluidity (1/*P*) of these cells in Fig. 3A ($r = 0.99$, $p < 0.01$). (C) Correlation between anti-cancer activity ($1/IC_{50}$) of HLs in Fig. 1B and membrane fluidity (1/*P*) of these cells in Fig. 3A ($r = 0.98$, $p < 0.01$). (D) Correlation between anti-cancer activity ($1/IC_{50}$) of HLs for seven distinct cells and their membrane fluidity (1/*P*) in Supplementary Table S1 ($r = 0.85$, $p < 0.01$). HuH-7 (filled circles), HepG-2 (filled squares), HC (filled rhombuses), U251 (open circles), KP-4 (open triangles), MOLT-4 (open squares) and Marcus (open rhombuses) cells. (E) A schematic representation of HLs target cancer cell-membranes that have their grater fluidity as compared to normal cell-membranes, and trigger apoptotic cell death.

to determine the degree of fluidity of cellular membrane lipids in normal cells and cancer ones [6,10]. Recently, we have found that the degree of membrane fluidity of human adult T-cell leukemia cells was greater than that of normal lymphocyte cells on the basis of fluorescence depolarization method [32]. On the other hand, it is well known that a membrane fusion of lipid vesicles with mammalian cells could influence lipid fluidity in these membranes [37]. Therefore, we focused on the membrane fluidity of HCC cells vs. normal hepatocytes, and attempted to investigate the relationship among three parameters of the fluidity of cell membranes: the degree of accumulation of HLs into cell membranes and the selective inhibitory effects of HLs on the cell growth for HCC. For the determination of membrane fluidity in intact cells, normal hepatocytes and HCC cells were first labeled DPH and the degree of fluorescence polarization (*P*) of DPH embedded in these membranes was evaluated on the basis of fluorescence depolarization method. The fluorescence depolarization is caused by the molecular motion of the fluorescence probe, which reflects the microviscosity of the surrounding region. As shown in Fig. 3A, the mean *P* values of DPH-labeled both HCC cells were significantly decreased as compared to

normal HC cells, which suggest that both HCC cell-membranes are more fluid than those of normal hepatocytes. This observation agrees with the results of our and other studies that reported increased fluidity of plasma membranes in adult T-cell leukemia cells [32], human lung cancer cells [13,14] and rat hepatoma cells [11,12], and the correlation between higher fluidity and malignancy of cancer cells from patients with HCC [16]. Next, the degree of membrane fluidity ($1/P$) was plotted against the accumulation (mean-fluorescence intensity) of HLs into the HCC cells as shown in Fig. 3B. It was found that the degree of accumulation of HLs/NBDPC into HCC cells was positively correlated well with the fluidity of cell membranes ($r = 0.99$, $p < 0.01$). This result suggests that HLs could selectively fuse and accumulate into the plasma membranes of HCC cells due to their greater fluidity compared to those of normal hepatocytes. Finally, the degree of membrane fluidity ($1/P$) was plotted against the anti-cancer activity ($1/IC_{50}$) of HLs as shown in Fig. 3C. Interestingly, a significant positive correlation between the anti-cancer activity of HLs and membrane fluidity in HCC cells was obtained ($r = 0.98$, $p < 0.01$). Additionally, for six cancer cells and normal hepatocytes, similar results were obtained (Fig. 3D, Supplementary Table S1 and Fig. S1, $r = 0.85$, $p < 0.01$). These results strongly suggest that the selective growth-inhibition of HLs for HCC cells could be caused by the specific accumulation of HLs into the membranes of HCC cells that had a larger fluidity as compared to those of normal hepatocytes. Thus, the findings in this study demonstrate that HLs might be a new type of nanomedicinal anti-cancer agent that targets cancer cell-membranes to trigger apoptotic cell death for HCC and other cancer cells.

In conclusion, we clarified that nano-sized hybrid liposomes (HLs) composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ could selectively inhibit the cell growth and induce apoptosis via caspase-3 for hepatocellular carcinoma (HCC) cells through the specific accumulation of HLs into the plasma membranes of HCC cells that have their greater fluidity as compared to those of normal hepatocytes (Fig. 3E). The results in this study indicate that HLs could provide the possibility of novel nanotherapy for intractable cancers targeting the cell membranes from the viewpoint of biophysical characteristics of cancer vs. normal cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.134.

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