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Membrane targeted chemotherapy with hybrid liposomes for colon tumor cells leading to apoptosis

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Abstract—A good correlation between 50% inhibitory concentration of hybrid liposomes (HL) composed of 90 mol% dimyristoylphosphatidylcholine and 10 mol% polyoxyethylene(*n*)dodecyl ether on the growth of human colon tumor (WiDr) cells, and membrane fluidity of HL was obtained. HL distinguished between WiDr and normal colon cells and then fused and accumulated into the membranes of WiDr cells leading to apoptosis.

Molecular targeted therapeutics has attracted much attention in recent years as an efficient therapy for cancer on the basis of molecular level studies on human cells. Targeted molecules included protease, growth factor, receptor, oncogene, and so on. Especially, recombinant humanized anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibody produced durable objective responses in patients with HER2 overexpressing breast cancer.[1,2] On the other hand, it is already known that the fluidity of tumor cell membranes as molecular aggregate is generally larger than that of normal cells.[3–5] However, there are few reports on therapy from the viewpoint of membrane fluidity of tumor cells.

We have produced hybrid liposomes (HL-*n*) which can be prepared by sonication of vesicular and micellar molecules in a buffer solution. (Scheme 1)[6,7] The physical properties of HL such as shape, size, membrane fluidity, and the temperature of phase transition can be controlled by changing the constituents and compositional ratios.[7] In the course of our study on the stereoselective hydrolysis of amino acid esters in membrane systems, we emphasized that the stereochemical control is attained by regulating the temperature[8] and ionic strength.[9] In particular, the authors observed almost complete L-enantiomer-selective catalysis, which was attained by controlling the reaction microenvironment in relation to the fluidity of membranes.[10]

In addition, significantly prolonged survival in rats was obtained by using HL as drug carriers in the treatment of brain tumors.[11] HL also inhibited the proliferation of various tumor cells along with apoptosis in vitro and in vivo.[12–16] Recently, lipid-mediated apoptosis for tumor cells has been observed.[17–19] However, as far as we know, there is no report on antitumor effects in relation to membrane fluidity of liposomes. In this study, we report the first successful experiment resulting in a good correlation between antitumor activity on the growth of human colon tumor cells and membrane fluidity of HL composed of dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(*n*)dodecyl ether ($C_{12}(EO)_n$; $n = 4$, 8, 10, 21, 23, and 25).

We examined the effect of HL composed of DMPC and 10 mol% $C_{12}(EO)_n$ on the growth of human colon tumor (WiDr) cells on the basis of WST-1 assay.[20] WiDr was purchased from Health Science Research Resources Bank. WiDr cell line is derived from a human colon adenocarcinoma cell line.[21] HL were prepared by sonication (VELVO VS-N300, 300 W) of a mixture containing 90 mol% DMPC and 10 mol% $C_{12}(EO)_n$ in 5% glucose solution at 45 °C with 300 W, followed by filtration with a 0.20 μm filter. The tumor cells (2.0×10^4 viable cells/ml) were cultured for 48 h in a humidified 5% CO_2 incubator at 37 °C after adding the sample solutions. Then WST-1 solutions were added to the cells and the absorbance at a wavelength of 450 nm was measured by spectrophotometer. The inhibitory concentration was evaluated by $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote the absorbance of water-soluble formazan, which was useful as an indicator of cell viability,

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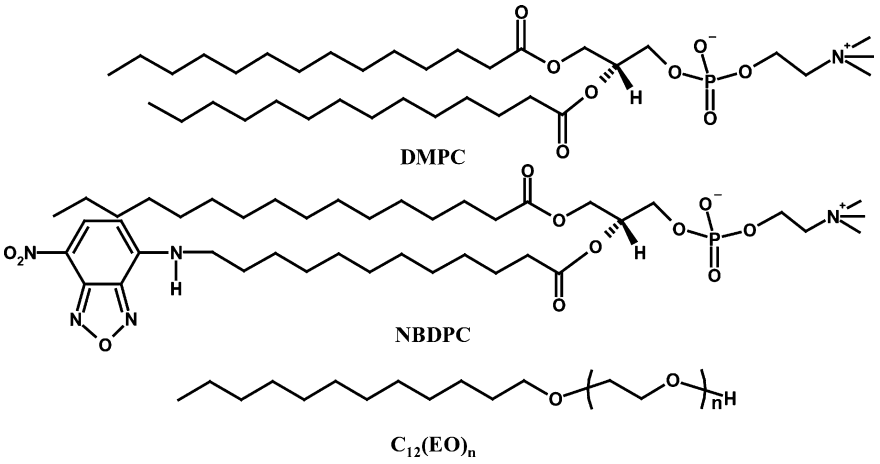


in the presence and absence of sample solutions, respectively. Fifty percent inhibitory concentration (IC_{50}) of HL were 0.29 mM DMPC for HL-8, 0.25 mM DMPC for HL-10, 0.23 mM DMPC for HL-21, 0.26 mM DMPC for HL-23, and 0.30 mM DMPC for HL-25. On the other hand, IC_{50} of DMPC liposomes was greater than 1.2 mM. These results indicate that HL-*n* (*n* = 8, 10, 21, 23, and 25) should be effective for inhibiting the growth of WiDr cells.

How do HL suppress the growth of tumor cells? We evaluated the fluidity of HL from fluorescence polarization (P) of 1, 6-diphenyl-1,3,5-hexatriene (DPH).[22] The fluorescence depolarization is caused by the molecular motion of the fluorescence probe, which reflects the microviscosity of the surrounding region.[23] The results obtained by fluorescence measurements correspond closely with data from NMR and DSC measurements in terms of the mobility of molecules in microenvironments.[24] The results are shown in Figure 1. It is worthy to note that a good correlation between the *P* values and IC_{50} was obtained. This indicates that HL having larger fluidity could suppress greater the growth of tumor cells. We examined further mechanisms for suppressing the growth of WiDr cells by HL. Apoptotic DNA rates for WiDr cells treated with HL using flow cytometer (Beckman-Coulter, Epics-XL). The results are shown in Figure 2a. HL-23 and 25 were effective for increasing apoptotic DNA rates among all the liposomes employed in this study. So, we next examined caspase cascade in relation to apoptosis induced by HL-23 on the basis of caspase fluorometric protease assay.[25] It is well known that the apoptosis signal is transduced by sequential activation of caspase family cystein protease. The results are shown in Figure 2b. Activation of caspase-8, -9, and -3 was observed for WiDr cells after the treatment with HL-23, indicating that HL23 could induce apoptosis through the activation of caspase-8, -9, and -3. It is well known that the caspase-8 and -9 activities are around 1/10 of caspase-3. However, we have already reported that the activities of caspase-8 and -9 were 1/3–1/2 of caspase-3 for human leukemia,[15] lung tumor,[26] and breast tumor cells[27] after the treatment with hybrid liposomes. So, the results in Figure 2a suggest that the high activities of caspase-8 and -9 for tumor cells could be characterized in the treatment with hybrid liposomes.

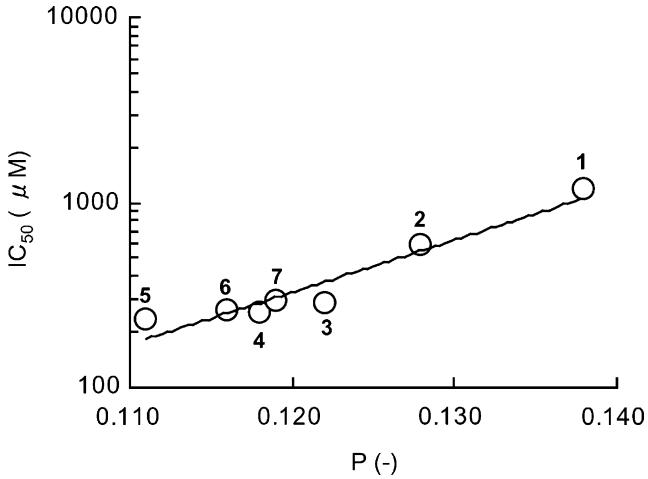
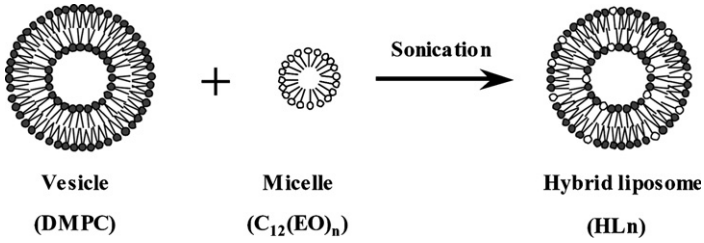


Figure 1. Relationship between P of DPH in hybrid liposomes (HL) and 50% inhibitory concentration of HL on the growth of WiDr cells. 1, DMPC; 2, HL-4; 3, HL-8; 4, HL-10; 5, HL-21; 6, HL-23; 7, HL-25.



Scheme 1. Schematic representation of hybrid liposomes.

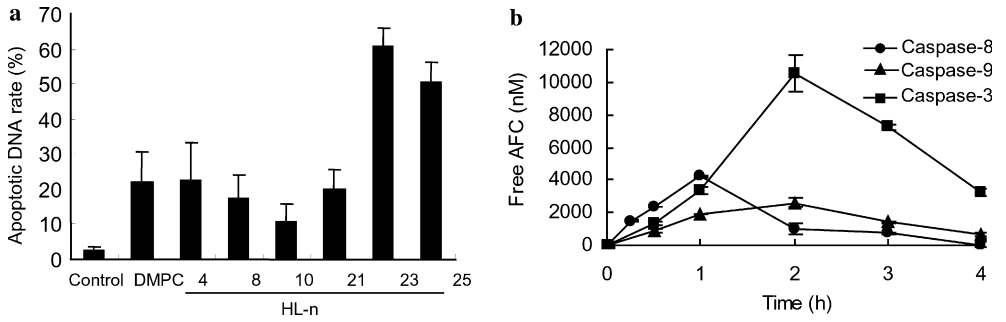


Figure 2. Induction of apoptosis by HL for WiDr cells. (a) Apoptotic DNA rate for WiDr cells treated with HL-*n* for 24 h. (b) Activation of caspases for WiDr cells treated with HL-23.

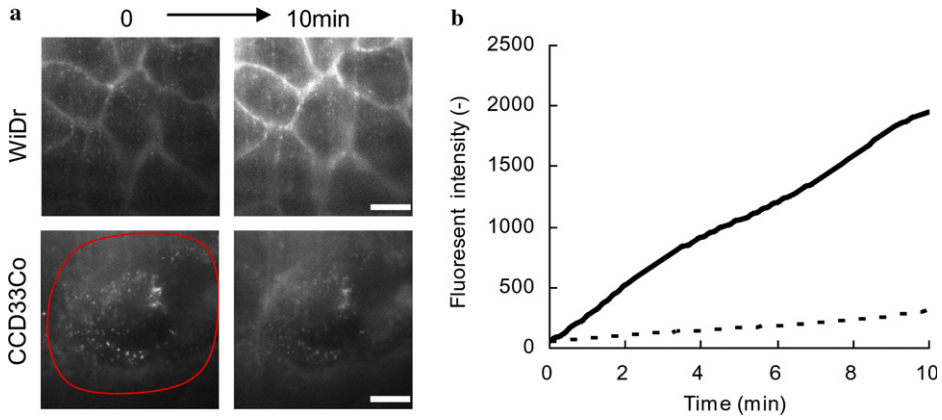


Figure 3. Specific accumulation of hybrid liposomes including NBDPC in WiDr cells. (a) TIRF micrographs of WiDr and CCD33Co cells after the treatment with HL. The red circle indicates a CCD33Co cell. (b) Fluorescent intensity of NBDPC incorporated into HL in WiDr (solid line) and CCD33Co (dot line) cells.

We have already elucidated the pathways of apoptosis induced by HL for human promyelocytic leukemia (HL-60) cells. That is, HL fused and accumulated in HL-60 cell membranes, and the apoptosis signal passed through caspases and then reached the nucleus.[15] Here, to get evidence about the fusion and accumulation of HL into WiDr cell membrane, total internal reflection fluorescence (TIRF) microscopy[28] was examined using 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl]-*sn*-glycero-3-phosphocholine (NBDPC) as a fluorescence probe with a IX71 Olympus fluorescence microscope.[29] TIRF micrographs of WiDr and normal colon (CCD33Co) cells after the treatment with HL-23 including NBDPC are shown in Figure 3a. CCD33Co was purchased from American Type Culture Collection and originated from human. In addition, fluorescence intensity of NBDPC incorporated into HL was measured as shown in Figure 3b. Interestingly, the fluorescence intensity of NBDPC incorporated into HL-23 in WiDr cell membrane drastically increased after 10 min (Fig. 3b), though that in CCD33Co cell was almost constant. The specific high accumulation of HL-23 in WiDr cell membrane was for the first time observed using TIRF microscopy.

In conclusion, a good correlation between membrane fluidity of HL and IC_{50} for the growth of WiDr cells was observed for the first time. In addition, we clarified that HL induced apoptosis for WiDr cells through the activation of caspases. It is also worthy to note that TIRF micrographs showed that HL distinguished between tumor (WiDr) and normal (CCD33Co) colon cells, and then fused and accumulated into WiDr cells after the treatment with HL. Thus, this study demonstrated that growth inhibition and apoptosis for tumor cells by HL provides the possibility of therapy from a viewpoint of biophysical characteristics of tumor cell membranes and that we should consider both IC_{50} and apoptotic DNA rates for clinical application.

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22. The emission at 430 nm originating from DPH was monitored upon excitation at 360 nm using a fluorescence polarization spectrophotometer (Hitachi F-2000). P of DPH into HL was calculated by

$$P = (I_{vv} - C_f I_{vh})/(I_{vv} + C_f I_{vh})$$

where I is the fluorescence intensity and the subscripts v and h refer to the orientations, vertical and horizontal, respectively, for the excitation and analyzer polarizers in this sequence: for example, I_{vh} indicates the fluorescence intensity measured with a vertical excitation polarizer and a horizontal analyzer polarizer.[22] C_f is the grating correction factor, given by I_{hv}/I_{hh}.
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29. We used HL23 including NBDPC (DMPC: $C_{12}(EO)_{23}$:NBDPC = 8.6:1.0:0.4) for TIRF microscopy. TIRF images were captured every 3 seconds with a cooled CCD camera (EM-CCD, Hamamatsu Photonics) and the fluorescent intensity was measured with a software (Aquacosmos, Hamamatsu Photonics).