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Specific Accumulation and Growth Inhibitory Effects of Hybrid Liposomes to Hepatoma Cells In Vitro

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Abstract—Specific accumulation and growth inhibitory effects of hybrid liposomes composed of 90 mol% dimyristoylphosphatidylcholine and 10 mol% polyoxyethylene(23)dodecyl ether were obtained in human hepatoma cells without affecting normal liver cells at all.

Recently, the great improvement in the technology used in cancer therapy leads to some satisfactory results in the treatment process. These remedies include surgical remedy, radiotherapy, immunotherapy, chemotherapy, laser therapy and hyperthermia treatment. However, none of these yield a totally satisfactory outcome. Thus there is a vital need for a therapy which can detect the presence of cancer at the earliest stage. This is especially the case in the liver, which is a 'silent organ', due to the absence of subjective symptoms.[1,2]

Hybrid liposomes, first developed by Ueoka et al., can be prepared by simply ultrasonicating a mixture of vesicular and micellar molecules in the buffer solution.[3] This preparation of hybrid liposomes should be of great advantage to clinical applications because of the lack of any contamination with organic solvents. The physical properties of these liposomes, such as size, membrane fluidity, phase transition temperature, and hydrophobicity can be controlled by changing the composition of hybrid liposomes.[4] In the course of our study, it has been reported that hybrid liposomes demonstrated remarkable inhibitory effects on lymphoma cells.[5–7] Significantly prolonged survival was also obtained in vivo in mice with carcinomas.[8,9] Interestingly, hybrid liposomes showed no toxicity in normal human cells, and in normal rats in vivo without any side effects. In concern with the anticancer mechanism, it became clear that the apoptosis should be induced using hybrid liposomes on the basis of flow cytometry and DNA agarose gel electrophoresis.[5,10] Furthermore, it is attractive that there were no side effects and therapeutic effects for the patient with malignant lymphoma furuncle after being approved in the bioethics committee.[11]

In this study, the inhibitory effects of hybrid liposomes composed of dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenedodecyl ether [$C_{12}(EO)_{23}$] on the growth of human hepatoma (Hep-G2) and human primary hepatocyte (HC) cells in vitro were examined. The induction of apoptosis for Hep-G2 cells was also observed by flow cytometry. In addition, high accumulation of hybrid liposomes toward Hep-G2 cells was examined by confocal laser microscopy using hybrid liposomes having the fluorescence-label lipid (NBDPC).

The hybrid liposomes were prepared by dissolving both DMPC and $C_{12}(EO)_{23}$ in phosphate-buffered saline with sonication (BRANSONIC Model B2210 apparatus) at 45 °C and 90 W. Hybrid liposomes were sterilized using membrane filter with 0.45 μm pore size. We examined the morphology of hybrid liposomes composed of 90 mol% DMPC and 10 mol% $C_{12}(EO)_{23}$ on the basis of dynamic light scattering measurement. Time course of changes in the hydrodynamic diameter (d_{hy}) of hybrid liposomes estimated at 37 °C are shown in Figure 1. The hybrid liposomes were found to be stable for more than 1 month, having a uniform distribution of d_{hy}. Similarly, the hybrid liposomes having the fluorescence-label lipid were uniform and stable for more than 1 month. In

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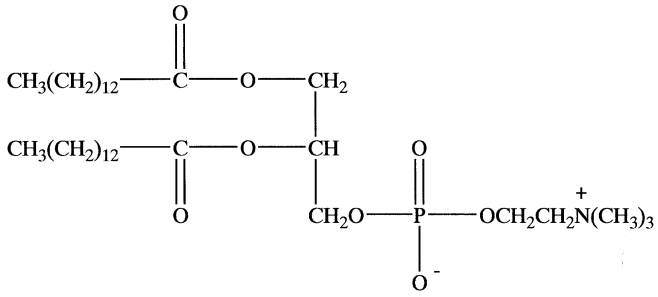
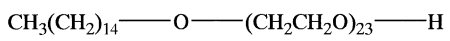
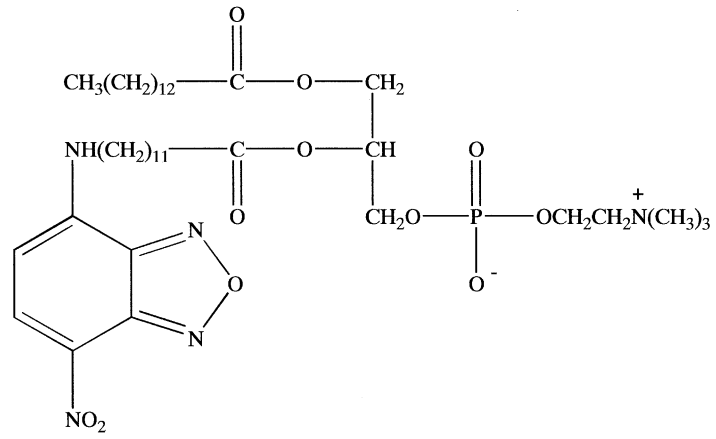


DMPC



$C_{12}(EO)_{23}$



NBDPC

addition, it was clarified that the physical properties of hybrid liposomes with and without the fluorescence-label lipid were almost the same.

Both Hep-G2 cells (Riken Cell Bank), HC cells (Dainippon Pharmaceutical)[12] were used for the evaluation of inhibitory effects of hybrid liposomes. Inhibitory effects in vitro were examined on the basis of WST-1 assay.[13] The cells (1.0×10^4 viable cells/mL) were inoculated in a 96-well tissue culture plate and incubated in a humidified 5% CO_2 incubator at 37 °C for 24 h. The cells were cultured for 2 days in a humidified 5% CO_2 incubator at 37 °C after adding the hybrid liposomes. The inhibitory effects of hybrid liposomes 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 hybrid liposomes, respectively. The inhibitory effects of hybrid liposomes on the growth of Hep-G2 and HC cells were examined as shown in Figure 2. No inhibitory effects of individual component [DMPC, $C_{12}(EO)_{23}$, NBDPC] on the growth of Hep-G2 cells were obtained. It is

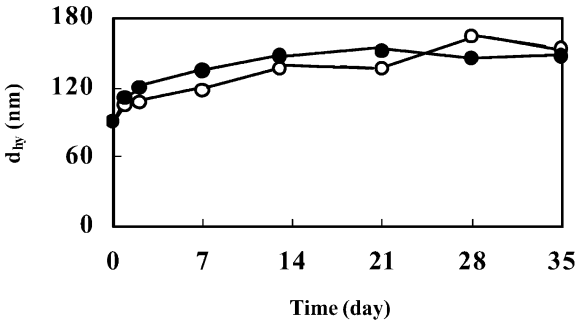


Figure 1. Time courses of d_{hy} change for hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ including NBDPC. ○, DMPC/$C_{12}(EO)_{23}$; ●, DMPC/$C_{12}(EO)_{23}$/NBDPC [DMPC] = 1.0×10^{-3} M, [$C_{12}(EO)_{23}$] = 1.2×10^{-4} M, [NBDPC] = 4.7×10^{-5} M.

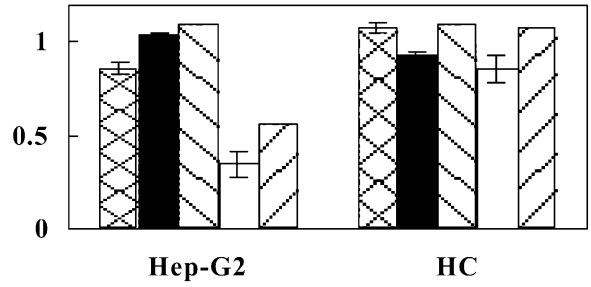


Figure 2. Inhibitory effects of hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ including NBDPC on the growth of HC and Hep-G2 cells in vitro. ⊠, DMPC; ■, $C_{12}(EO)_{23}$; ⧅, NBDPC; □, DMPC/$C_{12}(EO)_{23}$; ⧄, DMPC/$C_{12}(EO)_{23}$/NBDPC [DMPC] = 3.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 3.5×10^{-5} M, [NBDPC] = 1.4×10^{-5} M.

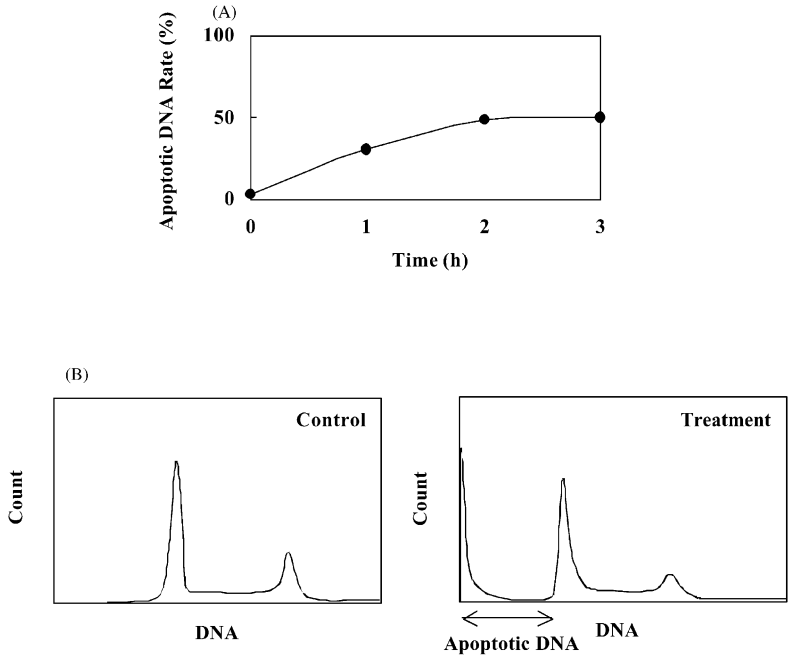


Figure 3. Apoptotic DNA rate (A) and relative DNA contents (B) for Hep-G2 cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$. [DMPC] = 5.0×10^{-3} M, [$C_{12}(EO)_{23}$] = 5.6×10^{-4} M.

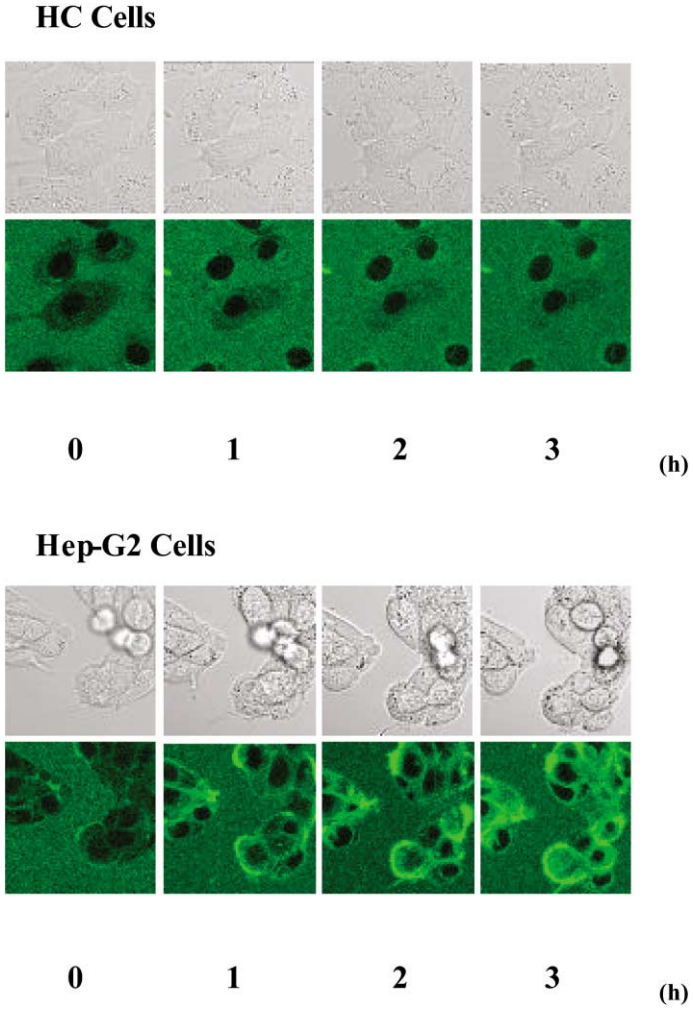


Figure 4. Fluorescence micrographs of HC and Hep-G2 cells treated with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ including NBDPC. [DMPC] = 1.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 1.2×10^{-5} M, [NBDPC] = 4.7×10^{-6} M.

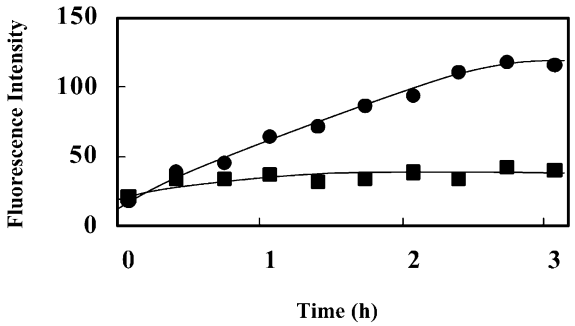


Figure 5. Fluorescence intensity of NBDPC incorporated into hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ in HC and Hep-G2 cells using confocal laser microscope. ●, Hep-G2 cells; ■, HC cells [DMPC] = 1.0×10^{-4} M, [$C_{12}(EO)_{23}$] = 1.2×10^{-5} M, [NBDPC] = 4.7×10^{-6} M.

noteworthy that high inhibitory effects of the hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ and 86 mol% DMPC/10 mol% $C_{12}(EO)_{23}$/4 mol% NBDPC for Hep-G2 cells without toxicity for HC cells were obtained.

The apoptotic DNA rate by hybrid liposomes in Hep-G2 cells was verified on the basis of flow cytometry with the fragmented DNA-staining method. The cells (5.0×10^5 viable cells/mL) were inoculated in a tissue culture dish and incubated in a humidified 5% CO_2 incubator at 37 °C for 24 h. The hybrid liposomes were added to the cells and incubated for 3 h. Afterwards, the cells were washed in the PBS(−). The cells were incubated in RNase for 30 min after tissue fixation in cold 70% ethanol, and stained by PI solution for 30 min. DNA content measurement were performed by flow cytometry (Coulter EPICS-XL) with 488 nm laser line of Ar laser for excitation of PI (fluorescent wavelength 635±4 nm). Time course of changes in the apoptotic DNA rate of Hep-G2 cells was examined as shown in Figure 3(A).[14] The time dependent apoptotic DNA rate was increased using 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ hybrid liposomes. Figure 3(B) shows relative DNA contents for Hep-G2 cells after the treatment with hybrid liposomes of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ for 2 h.

The accumulation of hybrid liposomes toward tumor cell membrane and normal cell membrane were examined with the confocal laser microscopy using hybrid liposomes having the fluorescence-label lipid. The cell membrane accumulation of hybrid liposomes in Hep-G2 and HC cells were observed with the confocal laser microscopy (Leica TSC SP) using hybrid liposomes having the fluorescence-label lipid (NBDPC, fluorescent wavelength 534 nm) as shown in Figure 4. In addition, fluorescence intensity of NBDPC incorporated into hybrid liposomes was measured. The results are shown in Figure 5. Interestingly, fluorescence intensity of NBDPC after adding to tumor Hep-G2 cells gradually increased as the time proceeded, though that after adding to normal HC cells was low and almost constant. As a result, the fluorescence intensity for tumor Hep-G2 cells was 3-fold higher than that for normal HC cells 3 h after adding hybrid liposomes including NBDPC. The specific high accumulation of 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ hybrid liposomes might be caused by the larger fluidity of Hep-G2 cell membranes as compared with HC cell membranes.

In conclusion, the induction of apoptosis for Hep-G2 cells by 90 mol% DMPC/10 mol% $C_{12}(EO)_{23}$ hybrid liposomes was clarified on the basis of flow cytometry. It is worthy to note that highly selective accumulation and growth inhibitory effects of these hybrid liposomes was obtained in Hep-G2 cells without affecting normal HC cells at all for the first time.

Acknowledgements

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14. Apoptotic DNA rate was calculated according to eq 1.

$$\text{Apoptotic DNA rate} = (\text{apoptotic DNA contents}/\text{DNA contents}) \times 100 \quad (1)$$