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# Induction of apoptosis of human tumor cells by hybrid liposomes including docosahexaenoic acid

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**Abstract**—Inhibitory effects of hybrid liposomes (HL) composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(20) sorbitan monooleate (Tween 80) including polyunsaturated fatty acids on the growth of human tumor cells were examined in vitro. Remarkably high inhibitory effects of HL including docosahexaenoic acid (HL-DHA) on the growth of lung carcinoma (RERF-LC-OK), colon tumor (WiDr), and stomach tumor (MKN45) cells were obtained. The induction of apoptosis by HL-DHA was revealed on the basis of fluorescence microscopic and flow cytometric analyses.


Polyunsaturated fatty acids (PUFA) are 'polyene' fatty acids having plural carbon–carbon double bonds in the long hydrocarbon chains and showing auto-oxidation with the generation of free radicals. They are also precursors of eicosanoids such as prostaglandins, leukotrienes, thromboxanes, and lipoxins which lead cells to express specific physiological effects. In addition, the relationships between dietary PUFA and tumorigenesis have been attracting much attention from many researchers in food and medical science.[1,2]

On the other hand, we have produced hybrid liposomes (HL) which can be prepared by sonication of vesicular and micellar molecules in buffer solutions.[3] HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenealkyl ethers have remarkable inhibitory effects on the growth of tumor cells in vitro[4] and in vivo.[5] Furthermore, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported.[6] Recently, we elucidated the mechanistic details of apoptosis of tumor cells induced by HL[7] and the correlation between antitumor effects and membrane fluidity of HL.[8]

In addition, HL composed of DMPC and polyoxyethylene(20) sorbitan monolaurate (Tween 20) including antitumor nitrosoureas (BCNU) have been found to have a highly inhibitory effect on the growth of glioma in vitro and in vivo.[9] The study indicated that the HL had no cytotoxicity and could be effective carriers for improving solubilization and stabilization of hydrophobic BCNU in drug delivery systems. In this study, we report the inhibitory effects of hybrid liposomes composed of DMPC and polyoxyethylene(20) sorbitan monooleate (Tween 80) including polyunsaturated fatty acids (HL-PUFA) on the growth of various human tumor cells in vitro. The mechanism for the inhibitory effects of HL-PUFA on the growth of tumor cells is also discussed on the basis of the results from fluorescence microscopy and flow cytometry.

HL-PUFA were prepared by sonication of a mixture containing DMPC (NOF, Japan) ($1.0 \times 10^{-3}$ M), Tween 80 (Nikko Chemicals, Japan) ($1.1 \times 10^{-4}$ M) and PUFA (oleic acid (OA) (Nacalai Tesque, Japan), linoleic acid (LA) (Nacalai Tesque, Japan), α-linolenic acid (ALA) (Kanto Chemical, Japan), eicosapentaenoic acid (EPA) (Nacalai Tesque, Japan), docosahexaenoic acid (DHA) (Sigma Chemical, MO)) in 5% glucose solution as described previously.[10] The sample solutions were sterilized using membrane filter with 0.20 μm pore size. Dynamic light-scattering measurements with Otsuka Electronics ELS-8000 apparatus (Japan) showed that the size of HL-PUFA was less than 210 nm in diameter and stable for more than 1 month.

With respect to the inhibitory effects of HL-PUFA on the growth of human lung carcinoma RERF-LC-OK

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cells, colon tumor WiDr cells, and stomach tumor MKN45 cells (RIKEN Cell Bank, Japan) in vitro, we examined the 50% inhibitory concentrations ($IC_{50}$) of HL-PUFA on the basis of WST-1 assay.[11] The tumor cells were cultured for 48 h in a humidified 5% $CO_2$ incubator at 37 °C after adding the sample solutions. WST-1 solution (Dojindo Laboratories, Japan) was added and the absorbance at a wavelength of 450 nm was measured using a spectrophotometer (MAXline Microplate Readers, Molecular Devices, CA). The inhibitory effects were 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, in the presence and absence of sample solutions, respectively. The $IC_{50}$ of HL-PUFA was determined from the PUFA concentration-dependence of $A_{Mean}/A_{Control}$ under the condition of a constant HL concentration ([DMPC] = 100 μM, [Tween 80] = 11 μM). As shown in Figure 1, the $IC_{50}$ values of HL-PUFA were even smaller than those of HL alone. Previously, some studies reported that n-9 PUFA such as OA did not show the growth inhibition toward tumor cells in vitro.[12,13] Also, other studies reported that n-9 and n-6 PUFA such as LA showed both the effects on growth stimulation and on growth inhibition in response to the concentration.[14] In this study, we obtained significant inhibitory effects of HL-n-9 PUFA (OA) as well as HL-n-6 PUFA (LA) on the growth of tumor cells in vitro. This may be caused by the differences in experimental conditions: tumor cell lines, cultivation time, and/or state and concentration of PUFA in the sample solutions. As regards HL-n-3 PUFA, marked inhibitory effects of HL-DHA on the growth of all tumor cells were obtained. The $IC_{50}$ values were 46.4 μM for RERF-LC-OK cells, 53.3 μM for WiDr cells, and 30.3 μM for MKN45 cells. These results suggest that HLs including n-9, n-6, and especially n-3 polyunsaturated fatty acids could be effective generally in the growth inhibition of human tumor cells in vitro. Recently, we reported that hybrid liposomes distinguished between tumor and normal cells, and then fused and accumulated into the membranes of tumor cells only.[8,15] By including PUFA into the HL, it is possible that PUFA could be effectively delivered to tumor cells and should inhibit the growth of tumor cells not only in vitro but also in vivo.

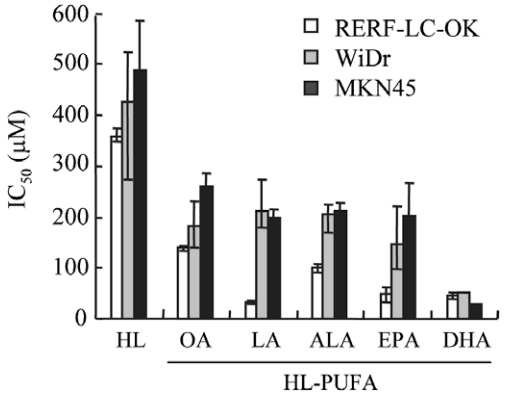


**Figure 1.** 50% inhibitory concentration ($IC_{50}$) of HL-PUFA on the growth of tumor cells for 48 h in vitro. Initial cell number: $1.0 \times 10^4$ cells $ml^{-1}$ (RERF-LC-OK), $2.0 \times 10^4$ cells $ml^{-1}$ (WiDr, MKN45), [DMPC] = 100 μM, [Tween 80] = 11 μM. Data presented are means; bars, SDs.

It is known that PUFA could be effective for inhibiting the growth of human tumor cells depending on the lipid peroxidation which produces several radicals, peroxides, and aldehydes.[16,17] Thus, the inhibitory effects of PUFA were prevented in the presence of anti-oxidants such as vitamin E.[12] Therefore, we examined the inhibitory effects of HL-PUFA (LA and DHA) including vitamin E (α-tocopherol) on the growth of tumor cells in vitro. The concentrations of PUFA and α-tocopherol in sample solutions were the same as the $IC_{50}$ of PUFA in μM unit. The results are shown in Figure 2. The $A_{Mean}/A_{Control}$ values of HL including vitamin E (HL/vitamin E) for all the tumor cells used in this study were nearly 1, which showed that vitamin E itself did not affect the proliferation of tumor cells. The $A_{Mean}/A_{Control}$ values of HL-LA were decreased in the absence of vitamin E as shown in Figure 2(A). On the other hand, the $A_{Mean}/A_{Control}$ values of HL-LA including vitamin E (HL-LA/vitamin E) were increased to the values for HL/vitamin E around 1 for all of the tumor cells. These results indicate that vitamin E prevents the growth inhibition of tumor cells by HL-LA via the lipid peroxidation. Interestingly, the $A_{Mean}/A_{Control}$ values

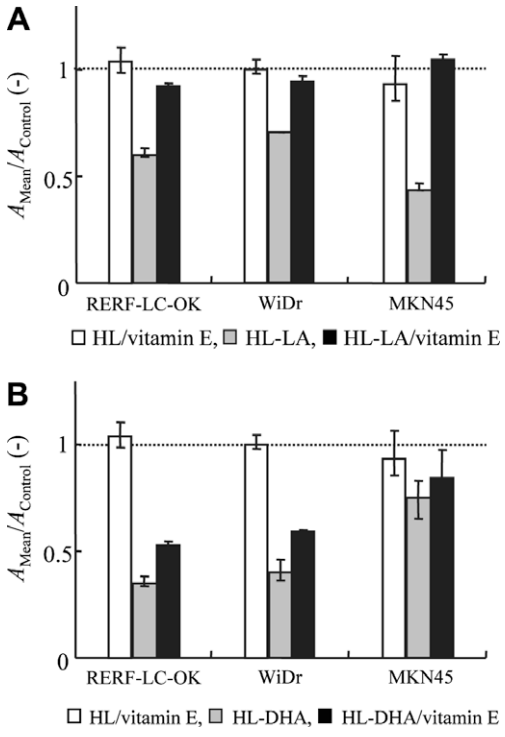


**Figure 2.** Inhibitory effects of HL-PUFA ((A) HL-LA, (B) HL-DHA) including vitamin E on the growth of tumor cells for 48 h in vitro. Initial cell number: $1.0 \times 10^4$ cells $ml^{-1}$ (RERF-LC-OK), $2.0 \times 10^4$ cells $ml^{-1}$ (WiDr, MKN45), [DMPC] = 100 μM, [Tween 80] = 11 μM, [PUFA] = $IC_{50}$ ([LA] = 93 μg $ml^{-1}$ (RERF-LC-OK), 591 μg $ml^{-1}$ (WiDr), 554 μg $ml^{-1}$ (MKN45), [DHA] = 152 μg $ml^{-1}$ (RERF-LC-OK), 105 μg $ml^{-1}$ (WiDr), 100 μg $ml^{-1}$ (MKN45)). Each concentration of α-tocopherol in HL-PUFA was the same as the $IC_{50}$ of PUFA as μM unit. Data presented are means; bars, SDs.

of HL-DHA including vitamin E (HL-DHA/vitamin E) for RERF-LC-OK, WiDr, and MKN45 cells were slightly higher than those of HL-DHA (Fig. 2(B)). These results suggest that the marked inhibitory effects of HL-DHA on the growth of tumor cells could not be due to only the lipid peroxidation of DHA.

We next examined the further mechanism of the inhibitory effects of HL-DHA on the growth of tumor cells, using double staining assay fluorescence microscopic analysis of cell death. The fluorescence microscopic analysis was performed with a confocal laser microscope (TCS-SP, Leica, Germany) and Vybrant Apoptosis Assay Kit #4 (Molecular Probes Eugene, OR). After the cultivation of tumor cells with HL-PUFA in a humidified 5% $CO_2$ incubator at 37 °C for 42 h, the tumor cells were centrifuged to remove the medium, washed, and resuspended with phosphate buffered-saline (PBS (−)), and then stained with YO-PRO-1 (100 μM, excitation/emission (nm) = 491/509) and propidium iodide (PI) (1.5 mM, excitation/emission (nm) = 493/635) stock solutions. The cells were again washed and resuspended with PBS (−) and observed using a fluorescence microscope with a 75 mW Ar laser of excitation 488 nm for YO-PRO-1 and 543 nm for PI, respectively. Figures 3 and 4 show the fluorescence micrographs of RERF-LC-OK (A), WiDr (B) and MKN45 (C) cells dyed with YO-PRO-1 and PI after the treatment with HL-LA and -DHA, respectively. The double staining assay of cell death with YO-PRO-1[18] and PI detects apoptotic and necrotic cells as green and red (or orange) fluorescent cells, respectively. As shown in Figure 3, most of the tumor cells were dyed in red after adding HL-LA, though a few cells dyed in green or yellow were observed. These observations indicate the induction of necrosis by HL-LA toward most tumor cells. On the other hand, all the tumor cells treated with HL-DHA exhibited the green fluorescence, indicating the presence of nuclear condensation and fragmentation in apoptotic cells (Fig. 4). Furthermore, we performed the preliminary analysis of DNA fragmentation in the tumor cells treated with HL-PUFA using a flow cytometer (EPICS XL, Beckman Coulter, MA). The observations showed that the apoptosis DNA rates of RERF-LC-OK, WiDr, and MKN45 cells treated with HL-DHA reached high values more than 65% at 48 h, while those treated with HL-LA were less than 32%. These results were in agreement with the inductions of apoptosis for those tumor cells by HL-DHA observed in the fluorescent microscopic analysis. With respect to apoptosis induced by n-3 PUFA, it was reported that n-3 PUFA such as EPA inhibited the production of prostaglandin $E_2$ ($PGE_2$) catalyzed by prostaglandin endoperoxide synthase 2, referred to as cyclooxygenase 2 (COX-2), and $PGE_2$ was associated with the expression of Bcl-2 gene which codes the apoptosis suppression proteins.[19,20] Probably, apoptosis induced by HL-DHA might be attributed to the regulation of Bcl-2 or other proteins

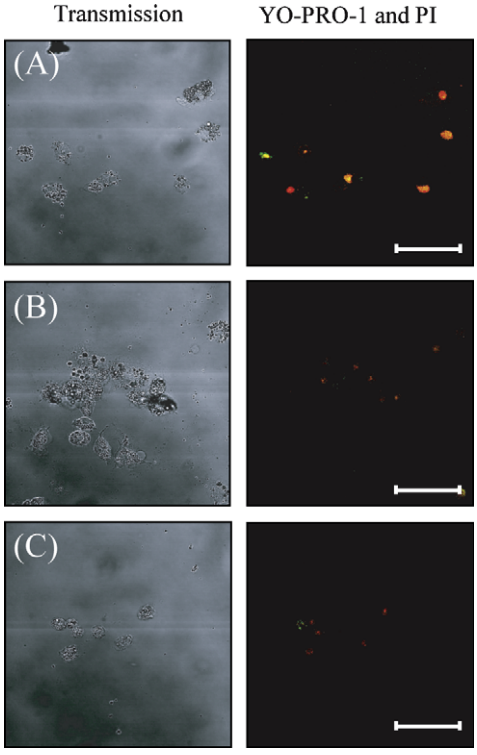


**Figure 3.** Fluorescence micrographs of tumor cells ((A) RERF-LC-OK, (B) WiDr, (C) MKN45) stained with YO-PRO-1 and PI after the treatment with HL-LA for 42 h. Scale bar: 100 μm. Initial cell number: $5.0 \times 10^4$ cells $ml^{-1}$ (RERF-LC-OK), $1.0 \times 10^5$ cells $ml^{-1}$ (WiDr, MKN45), [DMPC] = 100 μM, [Tween 80] = 11 μM, [LA] = 50 μg $ml^{-1}$.

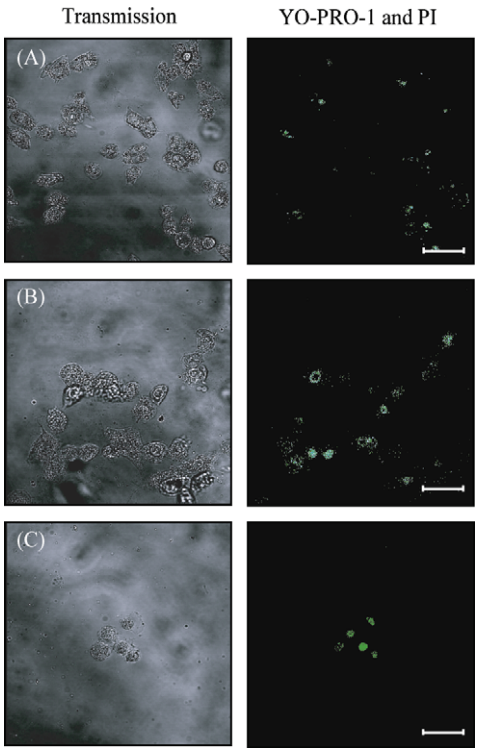


**Figure 4.** Fluorescence micrographs of tumor cells ((A) RERF-LC-OK, (B) WiDr, (C) MKN45) stained with YO-PRO-1 and PI after the treatment with HL-DHA for 42 h. Scale bar: 100 μm. Initial cell number: $5.0 \times 10^4$ cells $ml^{-1}$ (RERF-LC-OK), $1.0 \times 10^5$ cells $ml^{-1}$ (WiDr, MKN45), [DMPC] = 100 μM, [Tween 80] = 11 μM, [DHA] = 50 μg $ml^{-1}$.

related to apoptosis by metabolites of DHA delivered to the tumor cells by HL.

In conclusion, we clearly demonstrated the inhibitory effects of hybrid liposomes including polyunsaturated fatty acids (HL-PUFA) on the growth of human tumor cells (lung carcinoma RERF-LC-OK cells, colon tumor WiDr cells and stomach tumor MKN45 cells) in vitro. The noteworthy aspects are as follows. (a) HL including linoleic acid (HL-LA) showed the growth inhibition of all the tumor cells induced by necrosis via the lipid peroxidation. (b) HL including docosahexaenoic acid (HL-DHA) indicated the markedly high inhibition effects not depending on the lipid peroxidation on the growth of the tumor cells. (c) It was clarified that the HL-DHA could induce apoptosis toward the tumor cells on the basis of the microscopic and flow cytometric analyses. It is attractive that significantly inhibitory effects of HL-DHA on the growth of human tumor cells could be attained through the induction of apoptosis. The selection of polyunsaturated fatty acid for three-component hybrid liposomes should be important in order to induce apoptosis in full consideration of future clinical applications.

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## References and notes

1. Prener, A.; Nielsen, N. H.; Storm, H. H.; Hansen, J. P. H.; Jensen, O. M. *APMIS Suppl.* **1991**, *99*, 7.
2. Reddy, B. S.; Maruyama, H. *Cancer Res.* **1986**, *46*, 3367.
3. Ueoka, R.; Matsumoto, Y.; Moss, R. A.; Swarup, S.; Sugii, A.; Harada, K.; Kikuchi, J.; Murakami, Y. *J. Am. Chem. Soc.* **1988**, *110*, 1588.
4. Nagami, H.; Nakano, K.; Ichihara, H.; Matsumoto, Y.; Ueoka, R. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 782.
5. Kanno, A.; Terada, Y.; Tsuzaki, K.; Matsumoto, Y.; Ueoka, R. *Drug Delivery Syst.* **1999**, *14*, 37.
6. Ueoka, R.; Matsumoto Y.; Ichihara H.; Kiyokawa T. In Am. Chem. Soc. Symposium Series 830. Chemotherapy with hybrid liposomes composed of dimyristoylphosphatidylcholine and polyoxyethylenealkyl ether without drugs; Marten, M. R., Park, T. H., Nagamune, T., Eds.; Am. Chem. Soc. Books: Washington, 2002; pp 177 189.
7. Iwamoto, Y.; Matsumoto, Y.; Ueoka, R. *Int. J. Pharm.* **2005**, *292*, 231.
8. Komizu, Y.; Matsumoto, Y.; Ueoka, R. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6131.
9. Kitamura, I.; Kochi, M.; Matsumoto, Y.; Ueoka, R.; Kuratsu, J.; Ushio, Y. *Cancer Res.* **1996**, *56*, 3986.
10. Kadota, Y.; Ibuki, T.; Goto, K.; Matsumoto, Y.; Ueoka, R. *Chem. Lett.* **2005**, *34*, 370.
11. Ishiyama, M.; Shiga, M.; Sasamoto, K.; Mizoguchi, M.; Pin-gang, H. *Chem. Pharm. Bull.* **1993**, *41*, 1118.
12. Chow, S. C.; Sisfontes, L.; Björkhem, I.; Jondal, M. *Lipids* **1989**, *24*, 700.
13. Finstad, H. S.; Kolset, S. O.; Holme, J. A.; Wiger, R.; Farrants, A.-K. Ö.; Blomhoff, R.; Drevon, C. A. *Blood* **1994**, *84*, 3799.
14. Rose, D. P.; Connolly, J. M. *Cancer Res.* **1990**, *50*, 7139.
15. Nakano, K.; Iwamoto, Y.; Takata, W.; Matsumoto, Y.; Ueoka, R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3251.
16. Bégin, M. E.; Das, U. N.; Ells, G.; Horrobin, D. F. *Prostagl. Leukotr. Med.* **1985**, *19*, 177.
17. Das, U. N.; Bégin, M. E.; Ells, G.; Huang, Y. S.; Horrobin, D. F. *Biochem. Biophys. Res. Commun.* **1987**, *145*, 15.
18. Idziorek, T.; Estaquier, J.; De Bels, F.; Ameisen, J.-C. *J. Immounol. Meth.* **1995**, *185*, 249.
19. Chiu, L. C. M.; Wan, J. M. F. *Cancer Lett.* **1999**, *145*, 17.
20. Sheng, H.; Shao, J.; Morrow, J. D.; Beauchamp, R. D.; DuBois, R. N. *Cancer Res.* **1998**, *58*, 362.