doi:10.1246/cl.2011.90
Published on the web December 16, 2010

Inhibitory Effects of Hybrid Liposomes Composed of Phosphatidylcholine and Docosahexaenoic Acid on the Growth of Colon Cancer Cells along with Apoptosis and Differentiation

Koichi Goto, Keiko Zako, Yuji Komizu, and Ryuichi Ueoka*
Division of Applied Life Science, Graduate School of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082

(Received November 4, 2010; CL-100925; E-mail: ueoka@life.sojo-u.ac.jp)

Novel hybrid liposomes (DMPC/DHA) composed of L-α-dimyristoylphosphatidylcholine and docosahexaenoic acid were produced. DMPC/DHA were smaller and more stable than pure DMPC liposomes. It is attractive that DMPC/DHA could remarkably inhibit the growth of human colon cancer HCT116 cells along with apoptosis and differentiation in vitro.

Docosahexaenoic acid (DHA) is an ω-3 polyunsaturated long-chain fatty acid (ω-3 PUFAs), whose antitumoral actions have been widely demonstrated in epidemiological and experimental studies.[1] For example, diets containing fish oil rich in DHA and eicosapentaenoic acid (EPA) show inhibitory effects against colon tumorigenesis.[2] Furthermore, it has been reported that DHA inhibits the growth of various cancer cells by cytotoxicity of lipid peroxidation[3] and by apoptotic cell death in vitro.[4] In addition, recent studies have indicated that the induction of apoptosis might be caused by oxidative DNA damage[5] and endoplasmic reticulum (ER) stress[6] in cancer cells. This research suggests that DHA could be an effective drug for cancer chemotherapy.

Hybrid liposomes (HL)[7] can be prepared by sonication of a mixture of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(n) alkyl ethers in a buffer solution. HL have remarkably inhibitory effects on the growth of various cancer cells in vitro[8] and in vivo.[9] Furthermore, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported since being approved by the committee of bioethics.[10] On the other hand, significantly prolonged survival for brain tumor model rats was obtained by the administration of antitumor nitrosoureas (BCNU) encapsulated in HL composed of DMPC and polyoxyethylene(20) sorbitan monolaurate (Tween 20).[11] Moreover, it was found that DHA and other PUFAs incorporated in HL composed of DMPC and polyoxyethylene(20) sorbitan monooleate (Tween 80) inhibited the growth of cancer cells through the induction of apoptosis or necrosis in vitro.[12] These studies showed that HL should be effective for improving the solubilization and stabilization of hydrophobic agents in the drug delivery system (DDS).[13]

To extend our study of DHA as an anticancer drug in DDS, we produced hybrid liposomes (DMPC/DHA) composed of DMPC and DHA and investigated the inhibitory effects of DMPC/DHA on the growth of human colon cancer (HCT116) cells in vitro. First, the physical properties of hybrid liposomes (DMPC/DHA) (Figure 1a) were examined on the basis of dynamic light scattering[8c] and fluorescence polarization.[9b] DMPC/DHA were prepared by sonication of a mixture containing DMPC (NOF) and DHA (Sigma Chemical) with a bath type sonicator (VS-N300, VELVO-CLEAR) at 45 °C in 5% glucose solution.[12] The liposomes composed of DMPC alone (DMPC liposomes) were prepared in the same manner as described above. Figure 1b shows the time course of the hydrodynamic diameter (d_{hy}) change for DMPC/DHA using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics). The mean d_{hy} of DMPC/DHA composed of 10–60 mol % DHA were 60–160 nm in diameter with a single and narrow distribution and stable for 21 days, though DMPC liposomes gradually increased with time. On the other hand, the membrane fluidity of DMPC/DHA was evaluated by the fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene (DPH) placed in the hydrophobic membrane domain. The P value of DPH in DMPC/DHA increased with increasing the composition of DHA (Figure 1c). This indicates that membrane fluidity of DMPC/DHA decreased gradually with increasing composition of DHA. Probably, hydrophobic DHA incorporated into the hydrophobic membranes of DMPC liposomes could improve the membrane stability.[12b,13]

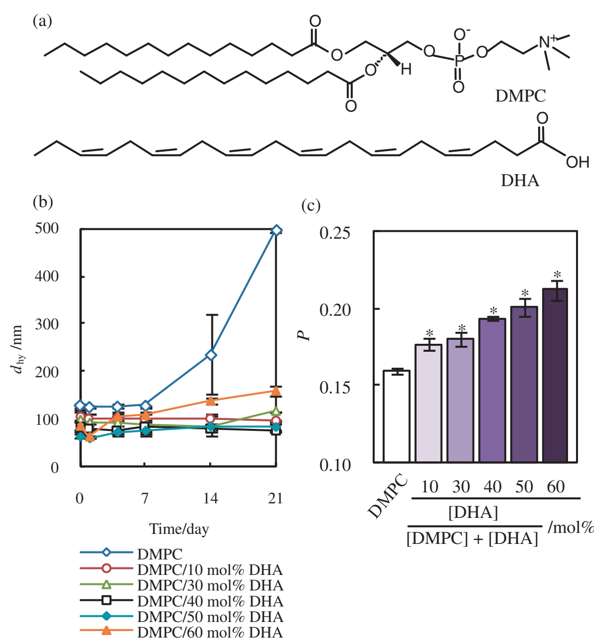

Figure 1. Hybrid liposomes (DMPC/DHA) composed of DMPC and DHA. (a) Molecular structures of DMPC and DHA. (b) Time course of hydrodynamic diameter (d_{hy}) change for DMPC/DHA at 25 °C. (c) Fluorescence polarization (P) of DPH in DMPC/DHA at 37 °C. [DMPC] = 1000 μM; [DHA] = 111 (10 mol %), 429 (30 mol %), 667 (40 mol %), 1000 (50 mol %), and 1500 μM (60 mol %); [DPH] = 0.10 μM. *Significant difference ($p < 0.05$) compared with DMPC liposomes.

© 2011 The Chemical Society of Japan

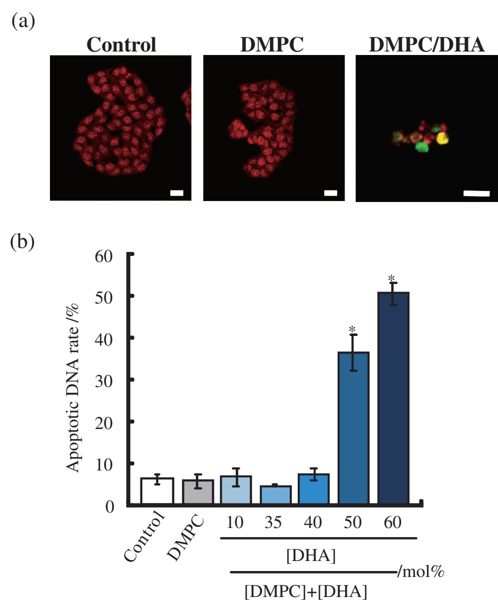

Figure 2. Induction of apoptosis for HCT116 cells treated with DMPC/DHA. (a) Fluorescence micrograph images of HCT116 cells stained by TUNEL after the treatment with DMPC/DHA for 48 h. [DMPC] = 100 μM, [DHA] = 100 μM. Scale bar: 10 μm. (b) Apoptotic DNA rate for HCT116 cells after the treatment with DMPC/DHA for 48 h. [DMPC] = 100 μM; [DHA] = 11.1 (10 mol %), 53.8 (35 mol %), 66.7 (40 mol %), 100 (50 mol %), and 150 μM (60 mol %). Data represent the mean ± SE ($n = 3$). *Significant difference ($p < 0.05$) compared with the control.

With respect to the inhibitory effects of DMPC/DHA on the growth of human colon cancer (HCT116) cells, we examined the fifty percent inhibitory concentration (IC_{50}) of DMPC/DHA on the basis of WST-8 assay[9b] in vitro. The IC_{50} value of DHA in DMPC/DHA ([DMPC] = 100 μM) was 53 ± 2 μM, whereas that of DMPC liposomes was 405 ± 3 μM. It was obvious that DMPC/DHA markedly inhibited the growth of HCT116 cells, as similarly observed in the case of DHA included in HL for other cancer cells in vitro.[12] In our previous studies, it was also shown that the inhibitory effects of HL including DHA on the growth of lung carcinoma RERF-LC-OK cells, stomach tumor MKN45 cells, and colon tumor WiDr cells could be attained through the induction of apoptosis in vitro.[12] Therefore, we examined the induction of apoptosis in HCT116 cells exposed to DMPC/DHA composed of 50 mol % DHA (100 μM) by TUNEL using a confocal laser microscope.[9b] The fluorescence micrograph images of HCT116 cells are shown in Figure 2a. The nuclei of all cells were stained with TOPRO-3, which emitted red fluorescence. As regards the TUNEL staining, HCT116 cells were dyed in green (or yellow) by the addition of DMPC/DHA, though no green fluorescence was observed in the case of DMPC liposomes. The observations indicated that exposure of HCT116 cells to DMPC/DHA induced the DNA fragmentation of apoptosis. In addition, the DNA fragmentation in apoptotic HCT116 cells was surveyed by flow cytometry[8c] and the results are shown in Figure 2b. High apoptotic DNA rates of HCT116 cells were obtained for DMPC/DHA composed of 50 and 60 mol % DHA (100 and 150 μM) as compared with those for the control and DMPC liposomes. This demonstrated that the markedly inhibitory effects of DMPC/DHA on the growth of HCT116 cells are caused by the induction of apoptosis in vitro. However, the DNA fragmentation in HCT116 cells was not detected in the presence of DMPC/DHA composed of 10–40 mol % DHA (11.1–66.7 μM), which suggested another mechanism for the inhibitory effects of DMPC/DHA on the growth of HCT116 cells.

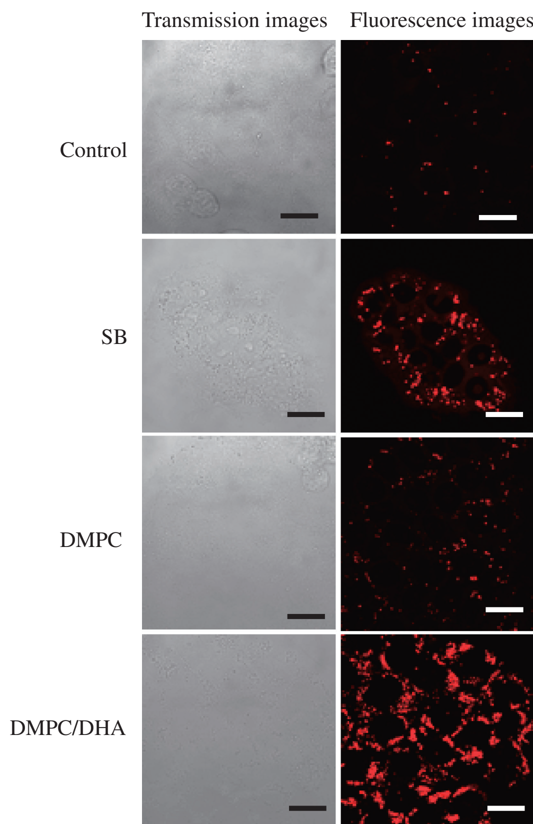

Figure 3. Fluorescence micrographs of HCT116 cells stained with Nile Red after the treatment with SB, DMPC liposomes, or DMPC/DHA for 48 h. [SB] = 1.0 mM, [DMPC] = 100 μM, [DHA] = 53.8 μM, [Nile Red] = 0.31 μM. Scale bar: 20 μm.

Recently, some studies reported that DHA could induce cell differentiation toward certain cancer cell lines. For example, DHA inhibited the growth of human breast MCF-7 cancer cells[14] and colon CaCo-2, SW480, SW620 cancer cells[15,16] accompanied with the differentiation in vitro. The differentiation of cancer cells could be evaluated by measuring the accumulation of cytoplasmic lipid droplets, activation of alkaline phosphatase, upregulation of E-cadherin, and so on.[17–19] Here, we observed the induction of differentiation in HCT116 cells treated with DMPC/DHA composed of 35 mol % DHA (53.8 μM) by lipid droplet staining with Nile Red[20] and alkaline phosphatase (AP) activity assay with *p*-nitrophenyl phosphate substrate.[22] Sodium butyrate (SB) was used as a differentiation-inducing agent in positive control experiments.[23,24] The fluorescence micrographs of HCT116 cells stained with Nile Red are shown in Figure 3. The accumulation of lipid droplets stained with Nile Red (red fluorescence) was observed in the cytoplasm of HCT116 cells treated with SB and DMPC/DHA, though the lipid droplets

© 2011 The Chemical Society of Japan

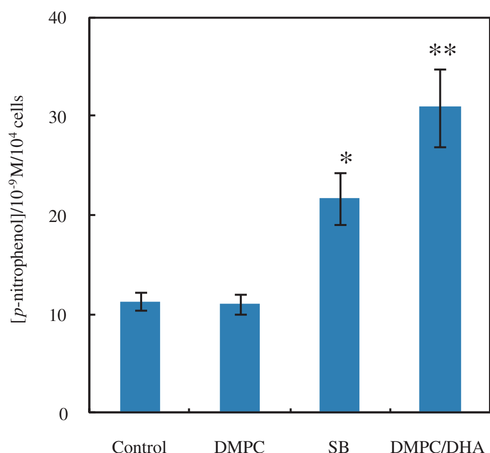

Figure 4. Alkaline phosphatase (AP) activity of HCT116 after treatment with SB, DMPC liposomes, or DMPC/DHA for 48 h. [SB] = 1.0 mM, [DMPC] = 100 µM, [DHA] = 53.8 µM. AP activity of HCT116 cells is expressed as the concentration of *p*-nitrophenol produced by the hydrolysis of *p*-nitrophenyl phosphate for 30 min. Data represent the mean ± SE ($n = 3$). *, **Significantly difference ($p < 0.05$, $p < 0.01$) compared with the control.

were less in those treated with DMPC liposomes. Figure 4 shows the AP activity of HCT116 cells, which is expressed as the concentration of *p*-nitrophenol produced by the hydrolysis of *p*-nitrophenyl phosphate for 30 min. The AP activity was significantly increased in the HCT116 cells treated with SB and DMPC/DHA compared to that for the control and DMPC liposomes. These observations indicate that DMPC/DHA could induce the differentiation toward HCT116 cells and inhibit the growth of HCT116 cells through the induction of not only apoptosis but also differentiation.

In conclusion, this study demonstrated for the first time that markedly inhibitory effects of hybrid liposomes composed of DMPC and DHA on the growth of human colon cancer HCT116 cells were attained through the induction of apoptosis and differentiation.

This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 20107007, 20360377, and 21560813).

References and Notes

1 R. S. Chapkin, J. Seo, D. N. McMurray, J. R. Lupton, *Chem. Phys. Lipids* **2008**, *153*, 14.
2 a) B. S. Reddy, H. Maruyama, *Cancer Res.* **1986**, *46*, 3367. b) C. P. J. Caygill, A. Charlett, M. J. Hill, *Br. J. Cancer* **1996**, *74*, 159. c) W.-C. L. Chang, R. S. Chapkin, J. R. Lupton, *J. Nutr.* **1998**, *128*, 491. d) E. Fernandez, L. Chatenoud, C. L. Vecchia, E. Negri, S. Franceschi, *Am. J. Clin. Nutr.* **1999**, *70*, 85.
3 W.-Q. Ding, J. L. Vaught, H. Yamauchi, S. E. Lind, *Mol. Cancer Ther.* **2004**, *3*, 1109.
4 S. Serini, E. Piccioni, N. Merendino, G. Calviello, *Apoptosis* **2009**, *14*, 135.
5 J. Pan, J. Keffer, A. Emami, X. Ma, R. Lan, R. Goldman, F.-L. Chung, *Chem. Res. Toxicol.* **2009**, *22*, 798.
6 C. H. Jakobsen, G. L. Størvold, H. Bremseth, T. Follestad, K. Sand, M. Mack, K. S. Olsen, A. G. Lundemo, J. G. Iversen, H. E. Krokan, S. A. Schønberg, *J. Lipid Res.* **2008**, *49*, 2089.
7 R. Ueoka, Y. Matsumoto, R. A. Moss, S. Swarup, A. Sugii, K. Harada, J. Kikuchi, Y. Murakami, *J. Am. Chem. Soc.* **1988**, *110*, 1588.
8 a) Y. Matsumoto, C. Imamura, T. Ito, C. Taniguchi, R. Ueoka, *Biol. Pharm. Bull.* **1995**, *18*, 1456. b) Y. Matsumoto, T. Kato, Y. Kemura, M. Tsuchiya, M. Yamamoto, R. Ueoka, *Chem. Lett.* **1999**, 53. c) Y. Matsumoto, Y. Iwamoto, T. Matsushita, R. Ueoka, *Int. J. Cancer* **2005**, *115*, 377. d) Y. Komizu, S. Nakata, K. Goto, Y. Matsumoto, R. Ueoka, *Chem. Lett.* **2010**, *39*, 1291.
9 a) A. Kanno, K. Tsuzaki, M. Miyagi, Y. Matsumoto, R. Ueoka, *Biol. Pharm. Bull.* **1999**, *22*, 1013. b) H. Nagami, Y. Matsumoto, R. Ueoka, *Int. J. Pharm.* **2006**, *315*, 167. c) S. Shimoda, H. Ichihara, Y. Matsumoto, R. Ueoka, *Chem. Lett.* **2009**, *38*, 134.
10 H. Ichihara, H. Nagami, T. Kiyokawa, Y. Matsumoto, R. Ueoka, *Anticancer Res.* **2008**, *28*, 1187.
11 I. Kitamura, M. Kochi, Y. Matsumoto, R. Ueoka, J. Kuratsu, Y. Ushio, *Cancer Res.* **1996**, *56*, 3986.
12 a) K. Goto, Y. Tanaka, Y. Matsumoto, R. Ueoka, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1880. b) Y. Tanaka, K. Goto, Y. Matsumoto, R. Ueoka, *Int. J. Pharm.* **2008**, *359*, 264.
13 a) S. Yamamoto, K. Nakano, C. Ishikawa, M. Yamamoto, Y. Matsumoto, M. Iwahara, S. Furusaki, R. Ueoka, *Biochem. Eng. J.* **2002**, *12*, 125. b) Y. Kadota, C. Taniguchi, S. Masuhara, S. Yamamoto, S. Furusaki, M. Iwahara, K. Goto, Y. Matsumoto, R. Ueoka, *Biol. Pharm. Bull.* **2004**, *27*, 1465. c) Y. Kadota, T. Ibuki, K. Goto, Y. Matsumoto, R. Ueoka, *Chem. Lett.* **2005**, *34*, 370.
14 H. Chamras, A. Ardashian, D. Heber, J. A. Glaspy, *J. Nutr. Biochem.* **2002**, *13*, 711.
15 B. A. Narayanan, N. K. Narayanan, B. Simi, B. S. Reddy, *Cancer Res.* **2003**, *63*, 972.
16 S. A. Schønberg, A. G. Lundemo, T. Fladvad, K. Holmgren, H. Bremseth, A. Nilsen, O. Gederaas, K. E. Tvedt, K. W. Egeberg, H. E. Krokan, *FEBS J.* **2006**, *273*, 2749.
17 S. Akare, S. Jean-Louis, W. Chen, D. J. Wood, A. A. Powell, J. D. Martinez, *Int. J. Cancer* **2006**, *119*, 2958.
18 O. Carter, G. S. Bailey, R. H. Dashwood, *J. Nutr.* **2004**, *134*, 3441S.
19 J. R. Gum, W. K. Kam, J. C. Byrd, J. W. Hicks, M. H. Sleisenger, Y. S. Kim, *J. Biol. Chem.* **1987**, *262*, 1092.
20 Lipid droplets in HCT116 cells were observed by staining with Nile Red as described in ref. 21. After pre-culturing HCT116 cells (2.0×10^4 cells/mL) in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h, the HCT116 cells were incubated in the presence of SB (1.0 mM), DMPC liposomes (100 µM), or DMPC/DHA ([DMPC] = 100 µM, [DHA] = 53.8 µM) for 48 h. Subsequently, the HTC116 cells were fixed with 10% neutral formalin, treated with 0.4% Triton X-100, stained with Nile Red (Sigma Chemical) (0.31 µM), and observed using a confocal laser microscope (Epics XL system II, Beckman Coulter) (Ex/Em = 552 nm/636 nm).
21 Q. Zhou, Z. K. Melkoumian, A. Lucktong, M. Moniwa, J. R. Davie, J. S. Strobl, *J. Biol. Chem.* **2000**, *275*, 35256.
22 After pre-culturing HCT116 cells (2.0×10^4 cells/mL) in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h, the HCT116 cells were incubated in the presence of SB (1.0 mM), DMPC liposomes (100 µM), or DMPC/DHA ([DMPC] = 100 µM, [DHA] = 53.8 µM) for 48 h. Then, the alkaline phosphatase (AP) activity of HCT116 cells was measured with StemTAG™ Alkaline Phosphatase Activity Assay Kit (Cell Biolabs) according to the manufacturer's instruction.
23 X. Leschelle, S. Delpal, M. Goubern, H. M. Blottière, F. Blachier, *Eur. J. Biochem.* **2000**, *267*, 6435.
24 A. Orchel, Z. Dzierżewicz, B. Parfiniewicz, L. Węglarz, T. Wilczok, *Dig. Dis. Sci.* **2005**, *50*, 490.

 © 2011 The Chemical Society of Japan