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Remarkably high inhibitory effects of docosahexaenoic acid incorporated into hybrid liposomes on the growth of tumor cells along with apoptosis

Yoshihiro Tanaka, Koichi Goto [1], Yoko Matsumoto [2], Ryuichi Ueoka *

Division of Applied Life Science, Graduate School of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

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ABSTRACT

Inhibitory effects of hybrid liposomes composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene (20) sorbitan monooleate (Tween 80) including polyunsaturated fatty acids or their ethyl esters (HL-PUFA) on the growth of human tumor cells were examined *in vitro*. Remarkably high inhibitory effects of HL including docosahexaenoic acid (HL-DHA) and α-linolenic acid ethyl ester (HL-ALAE) on the growth of lung carcinoma (RERF-LC-OK and A549) cells, colon tumor (WiDr) cells and stomach tumor (MKN45) cells were obtained. The addition of vitamin E (α-tocopherol) to HL-DHA and -ALAE prevented almost completely the growth inhibition of A549 cells distinct from the other tumor cells used in this study. On the other hand, fluorescence microscopic and flow cytometric analyses indicated that the inhibitory effects of HL-DHA on the growth of RERF-LC-OK, WiDr and MKN45 cells could be attained through the induction of apoptosis.

1. Introduction

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. For example, an epidemiological research suggested that the daily ingestion of seafood rich in PUFA could reduce breast cancer risk in Greenlandic women (Prener et al., 1991). In animal experiments, it was reported that azoxymethane-induced carcinogenesis in rats fed on fish oil rich in n-3 PUFA was considerably reduced as compared with that in rats given corn oil rich in n-6 PUFA (Reddy and Maruyama, 1986). Furthermore, the antitumor effects and the mechanism of PUFA were discussed from the viewpoints of lipid peroxidation and production of eicosanoids on the basis of *in vitro* experiments (Chow et al., 1989; Chiu and Wan, 1999).

On the other hand, we have produced hybrid liposomes (HL) which can be prepared by sonication of vesicular and micellar molecules in buffer solutions (Ueoka et al., 1985, 1988). HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenealkyl ethers have remarkable inhibitory effects on the growth of tumor cells *in vitro* (Matsumoto et al., 1995, 1999; Nagami et al., 2006) and *in vivo* (Kitamura et al., 1996; Kanno et al., 1999; Ueoka et al., 2000). Furthermore, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported (Ueoka et al., 2002). Recently, we elucidated the mechanistic details of apoptosis of tumor cells induced by HL (Matsumoto et al., 2005; Iwamoto et al., 2005) and the correlation between antitumor effects and membrane fluidity of HL (Komizu et al., 2006). On the other hand, the antitumor activity of extracts from the leaves of *Ginkgo biloba* L. (Yamamoto et al., 2002) and the peels of *Citrus natsudaidai* (Kadota et al., 2004) encapsulated in HL composed of DMPC and polyoxyethylene sorbitan monoacylates were significantly enhanced in comparison with those of the free extracts. In particular, it was found that HL including unsaturated fatty acids and their ethyl esters extracted from barley-*koji miso* have markedly inhibitory effects on the growth of human lung carcinoma (RERF-LC-AI) cells *in vitro* (Kadota et al., 2005).

In this study, we report on the inhibitory effects of hybrid liposomes including polyunsaturated fatty acids or their ethyl esters (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, flow cytometry and Western blot analysis.

* Corresponding author. Tel.: +81 96 326 3952; fax: +81 96 326 0522.
E-mail addresses: hep_g2_mem_10fbs_99neaa@hotmail.com (Y. Tanaka), kgoto@life.sojo-u.ac.jp (K. Goto), matumoto@life.sojo-u.ac.jp (Y. Matsumoto), ueoka@life.sojo-u.ac.jp (R. Ueoka).
[1] Tel.: +81 96 326 3974; fax: +81 96 323 1331.
[2] Tel.: +81 96 326 3965; fax: +81 96 323 1331.

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2. Materials and methods

2.1. Materials

Polyunsaturated fatty acids, oleic acid (OA) (Nacalai Tesque, Japan), linoleic acid (LA) (Nacalai Tesque), α-linolenic acid (ALA) (Kanto Chemical, Japan), eicosapentaenoic acid (EPA) (Nacalai Tesque), docosahexaenoic acid (DHA) (Sigma Chemical, St. Louis, MO) and their ethyl esters, oleic acid ethyl ester (OAE) (Nacalai Tesque), linoleic acid ethyl ester (LAE) (Wako Pure Chemical Industries, Japan), α-linolenic acid ethyl ester (ALAE) (Sigma Chemical), eicosapentaenoic acid ethyl ester (EPAE) (Nippon Oil and Fats, Japan), docosahexaenoic acid ethyl ester (DHAE) (Nippon Oil and Fats) were obtained commercially and used without further purification. L-α-Dimyristoylphosphatidylcholine and polyoxyethylene (20) sorbitan monooleate (Tween 80) were purchased from Nippon Oil and Fats. Vitamin E (α-tocopherol) was from Nacalai Tesque. The culture media, RPMI-1640, Dulbecco's modified eagle medium (DMEM), minimum essential medium (MEM) and 1% non-essential amino acids (NEAA) were from Invitrogen (La Jolla, CA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT).

2.2. Preparation of hybrid liposomes including polyunsaturated fatty acids or their ethyl esters (HL-PUFA)

Hybrid liposomes including polyunsaturated fatty acids or their ethyl esters (HL-PUFA) were prepared by the method as described previously (Kadota et al., 2004). Briefly, DMPC, Tween 80 and polyunsaturated fatty acids or their ethyl esters were mixed in 5% glucose solution and sonicated with a sonicator (VS-N300, VELVO-CLEAR, Japan) at 45 °C under a nitrogen atmosphere with 300 W, followed by filtration with 0.20 μm membrane filter. HL-PUFA including vitamin E (HL-PUFA/vitamin E) was prepared by sonication of a mixture containing DMPC, Tween 80, PUFA and α-tocopherol in the same manner as described above.

2.3. Dynamic light scattering measurement

The diameter of HL-PUFA was measured by dynamic light scattering measurement using an electrophoretic light scattering spectrophotometer (ELS-8000, Ostuka Electronics, Japan) with a He–Ne laser light source (633 nm, 10 mW) at the scattering angle of 90°. The diameter was calculated by Stokes–Einstein equation (Eq. (1)),

$$d_{hy} = \frac{\kappa T}{3\pi\eta D} \quad (1)$$

where κ is Boltzmann's constant, T is the absolute temperature, η is the viscosity of the solvent and D is the diffusion coefficient.

2.4. Fluorescence depolarization

Membrane fluidity of HL-PUFA was evaluated by the fluorescence depolarization method. Two microliters of 1,6-diphenyl-1,3,5-hexatriene (DPH, 0.10 mM) (Nacalai Tesque) in tetrahydrofuran or 1-[4-trimethylammoniumphenyl]-1,3,5-hexatriene iodide (tma-DPH, 1.0 mM) (Wako Pure Chemical Industries) in methanol was added to 2.0 ml of HL-PUFA solutions at 37 °C. The emission at 430 nm originating from DPH was monitored upon excitation at 357 nm, and the emission at 431 nm from tma-DPH was upon excitation at 361 nm using a fluorescence spectrophotometer (F-2000, Hitachi, Japan). The fluorescence polarization (P) was calculated according to Eq. (2),

$$P = \frac{I_{vv} - C_f I_{vh}}{I_{vv} + C_f I_{vh}} \quad (2)$$

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: e.g., I_{vh} indicates the fluorescence intensity measured with a vertical excitation polarizer and a horizontal analyzer polarizer. C_f is the grating correction factor, given by I_{hv}/I_{hh}.

2.5. Cell culture

Human lung carcinoma (RERF-LC-OK, A549) cell lines, human colon tumor (WiDr) cell line and human stomach tumor (MKN45) cell line were purchased from Riken Cell Bank (Japan). RERF-LC-OK and MKN45, A549, and WiDr cells were maintained in RPMI-1640, DMEM, and MEM (1% NEAA), respectively, supplemented with 10% FBS and antibiotics (100 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin). The cells were cultured in a 5% CO_2 humidified incubator at 37 °C.

2.6. Assessment of inhibitory effects of HL-PUFA on the growth of tumor cells in vitro

WST-1 (2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay was performed as described in literature Ishiyama et al. (1993). The tumor cells in 100 μl of culture medium were seeded at a density of 1.0×10^3 or 2.0×10^3 viable cells in 96-well tissue culture plates (Sumitomo Bakelite, Japan) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, 10 μl of sample solutions were added into each well and the plates were incubated for 48 h. WST-1 solution (Dojindo Laboratories, Japan) was added and incubated for 3 h, and then the absorbance at a wavelength of 450 nm was measured using a spectrophotometer (MAXline Microplate Readers, Molecular Devices, CA). The inhibitory effects of HL-PUFA and HL-PUFA/vitamin E on the growth of tumor 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 sample solutions, respectively. Fifty percent inhibitory concentration (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).

2.7. Fluorescence microscopy

Fluorescence microscopic analysis of cell death was performed using a confocal laser microscope (TCS-SP, Leica, Germany) and Vybrant Apoptosis Assay Kit #4 (Molecular Probes Eugene, OR). The tumor cells in 2.0 ml of culture medium were seeded at a density of 1.0×10^5 or 2.0×10^5 viable cells in 2.0–3.0 ml glass bottom culture dishes (Mat Tek, MA) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, 200 μl of sample solutions were added into the dishes and the dishes were incubated for 42 h. After centrifuging and removing the medium, the cells were washed twice and suspended with cold phosphate buffered-saline (PBS (−)), and then stained with each 2.0 μl of YO-PRO-1 (100 μM, excitation/emission (nm) = 491/509, green color) and propidium iodide (PI) (1.5 mM, excitation/emission (nm) = 493/635, red color) stock solutions on ice for 30 min. After staining, the cells were washed and resuspended with PBS (−). The cells were 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.

2.8. Flow cytometric analysis

Apoptotic DNA rate in tumor cells was measured by flow cytometry. The tumor cells in 15 ml of culture medium were seeded at a density of 2.5×10^6 viable cells in 25 ml Easy Flasks (Nunclon Δ Surface, Denmark) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, 4.5 ml of the sample solutions were added into the flasks and the flasks were incubated for 24 and 48 h. After centrifuging and removing the medium, the cells were washed twice with PBS (−) and fixed in chilled-ethanol. Then, the cells were washed again, treated with RNase solution (0.25 μg ml^{-1}) and stained with PI solution (0.5 mg ml^{-1}) for 30 min. After staining, the cells were washed and resuspended with PBS (−), and then analyzed by a flow cytometer (Epics XL system, Beckman Coulter, Bedford, MA) with a single excitation 488 nm of 15 mW air-cooling Ar laser. The PI signals were detected by FL3 sensor in 605–635 nm.

2.9. Western blot analysis

Expression of Bcl-2 in RERF-LC-OK cells treated with HL-DHA was analyzed by Western blotting method using ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd., England). RERF-LC-OK cells in 15 ml of culture medium were seeded at a density of 3.75×10^6 viable cells in 25 ml Easy Flasks and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, 2.25 ml of the sample solutions were added into the flasks and the flasks were incubated for 24 and 48 h. After centrifuging and removing the medium, the cells were washed with PBS (−) and resuspended in lysis buffer containing 30 mM Tris–HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10% glycerol and 0.5% protease inhibitor cocktail (Sigma Chemical). The lysated cells were incubated on ice for 30 min, homogenized by a polytron homogenizer (POLYTRON PT3100, Kinematica, Switzerland) at 15,000 rpm for 15 s, and centrifuged at 15,000 rpm for 30 min. The concentration of protein in the collected supernatant was determined by using – Proteostain – Protein Quantification Kit-Wide Range (Dojindo Laboratories). Subsequently, protein (49 μg) was separated under reducing conditions on a SDS–polyacrylamide gel (12.5%) and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA). The PVDF membrane was washed with TBS-T (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20) and treated with 5% ECL Blocking Agent in TBS-T for 1 h at room temperature. Then, the PVDF membrane was washed twice with TBS-T, immunoblotted with rabbit anti-human Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-human Cdk4 monoclonal antibody (Calbiochem, La Jolla, CA) for 1 h, washed 3 times with TBS-T, and incubated with the respective peroxidase-conjugated affinity-purified secondary antibodies (ECL Plus Western Blotting Reagent Pack) for 1 h at room temperature. After washing 3 times with TBS-T, the specific signals were detected by AISIN SEIKI LumiVisionPRO (Japan) with ECL Plus Western Blotting Detection Reagents according to the manufacturer's instruction.

3. Results and discussion

3.1. Physical properties of hybrid liposomes including polyunsaturated fatty acids or their ethyl esters (HL-PUFA)

It has been reported that hybrid liposomes (HL) composed of DMPC and polyoxyethylene sorbitan monoacylates could be effective carriers for improving solubilization and stabilization of

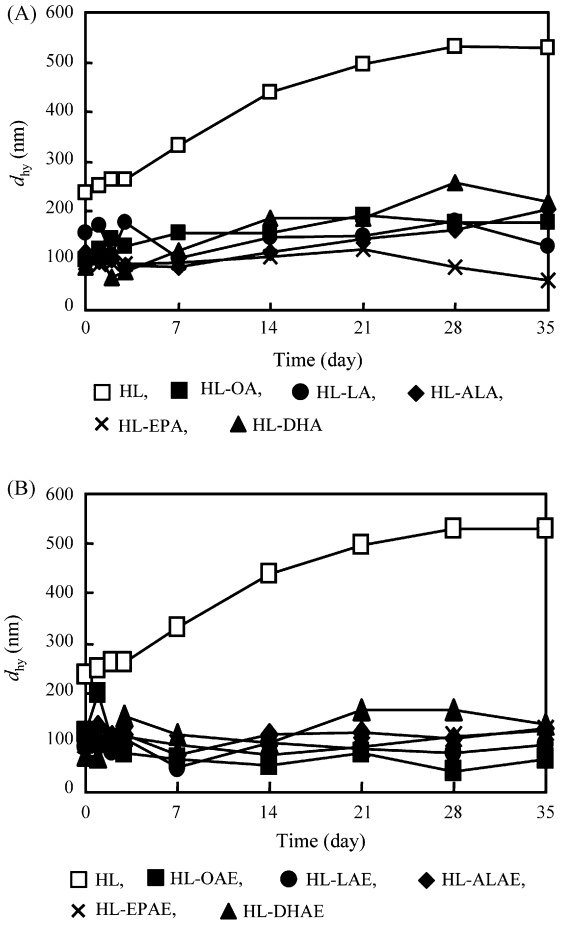

Fig. 1. Time courses of d_{hy} change for HL-PUFA. HL-PUFA was prepared by sonication of a mixture containing DMPC, Tween 80 and PUFA using a bath type sonicator in 5% glucose solution. The diameter of HL-PUFA was measured by dynamic light scattering measurement using an electrophoretic light scattering spectrophotometer at 37 °C. [DMPC] = 1000 μM; [Tween 80] = 111 μM; [PUFA] = 100 μg ml^{-1}.

hydrophobic compounds in aqueous solutions (Kitamura et al., 1996; Yamamoto et al., 2002; Kadota et al., 2004, 2005). In this study, we investigated the physical properties of hybrid liposomes including polyunsaturated fatty acids (oleic acid, linoleic acid, α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid) or their ethyl esters (oleic acid ethyl ester, linoleic acid ethyl ester, α-linolenic acid ethyl ester, eicosapentaenoic acid ethyl ester, docosahexaenoic acid ethyl ester) (HL-PUFA) on the basis of dynamic light scattering method and fluorescence depolarization measurement. Fig. 1 shows the time courses of the mean hydrodynamic diameter (d_{hy}) change for HL-PUFA composed of DMPC (1000 μM), Tween 80 (111 μM) and PUFA (100 μg ml^{-1}). HL alone was about 240 nm in diameter immediately after the sonication, but increased gradually with time and became over 500 nm after 30 days. On the other hand, HL including polyunsaturated fatty acids (Fig. 1(A)) and their ethyl esters (Fig. 1(B)) were smaller than HL and kept the sizes of 50–210 nm over 1 month. It was proved that the clear aqueous solutions of HL including polyunsaturated fatty acids or their ethyl esters could be obtained and remained stable for more than 1 month. As for the morphology of HL, we have reported that d_{hy} of HL depends on the structure of included agents (Yamamoto et al., 2002). Plausibly, hydrophobic PUFA was readily incorporated into the hydrophobic membrane domain of HL. As a result, the membrane stability of HL was improved and the size was kept almost constant. On the other hand, the membrane

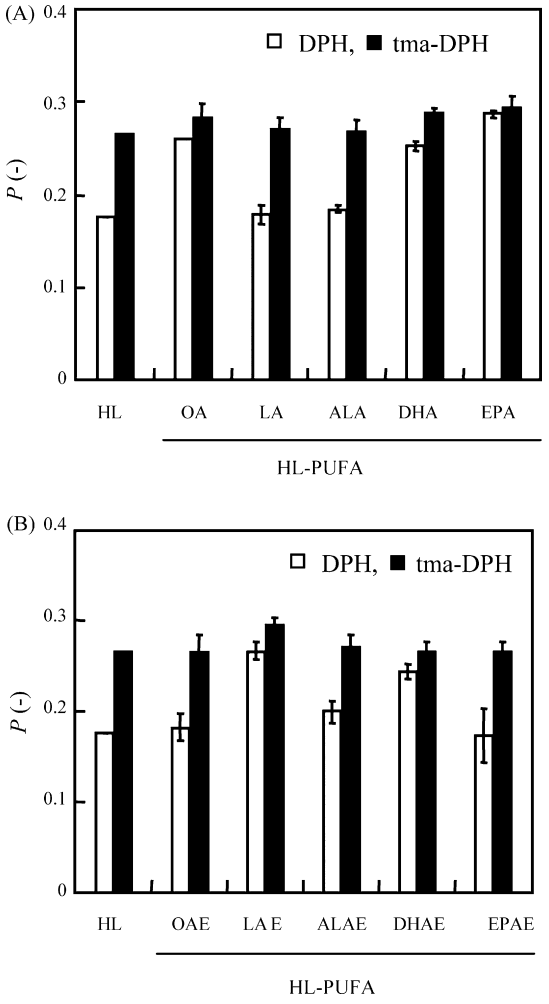

Fig. 2. Fluorescence polarization (*P*) of DPH and tma-DPH in HL-PUFA. The tetrahydrofuran solution of DPH or the methanol solution of tma-DPH was added to HL-PUFA solutions and stirred at 37 °C. The emission at 430 nm originating from DPH was monitored upon excitation at 357 nm, and the emission at 431 nm from tma-DPH was upon excitation at 361 nm using a fluorescence spectrophotometer. [DMPC] = 1000 μM; [Tween 80] = 111 μM; [PUFA] = 100 μg ml^{-1}; [DPH] = 0.10 μM; [tma-DPH] = 1.0 μM. Data presented are means; bars, S.D.s.

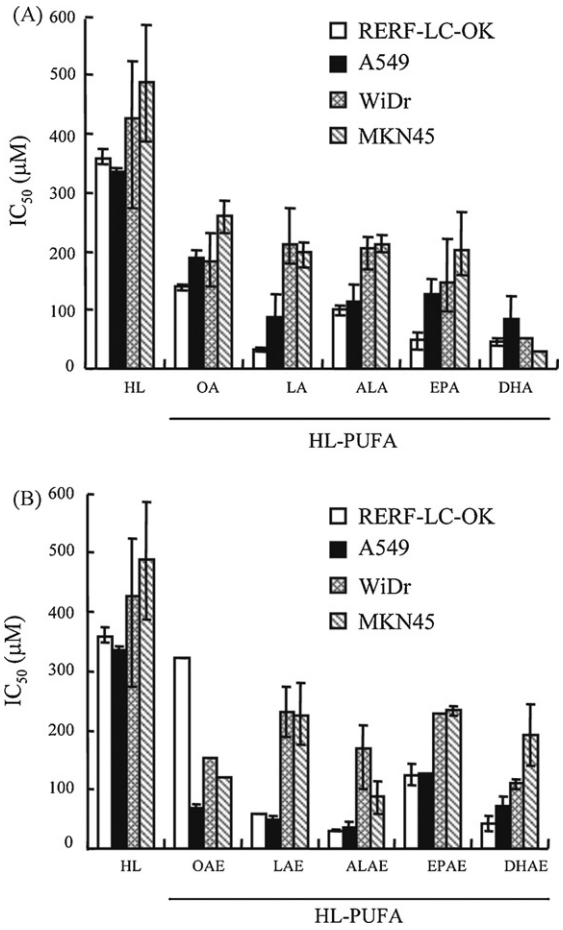

Fig. 3. 50% inhibitory concentration (IC_{50}) of HL-PUFA on the growth of tumor cells for 48 h *in vitro*. The tumor cells were seeded at a density of 1.0×10^4 cells ml^{-1} (RERF-LC-OK, A549) or 2.0×10^4 cells ml^{-1} (WiDr, MKN45) in 96-well tissue culture plates and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into each well and the plates were incubated. After 48 h, WST-1 solution was added and the absorbance at a wavelength of 450 nm was measured using a spectrophotometer. The inhibitory effects of HL-PUFA on the growth of tumor 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 sample solutions, respectively. IC_{50} of HL-PUFA was determined by the concentration-dependence of $A_{Mean}/A_{Control}$. [DMPC] = 100 μM; [Tween 80] = 11 μM. Data presented are means; bars, S.D.s.

fluidity of HL-PUFA was evaluated by the fluorescence polarizations (*P*) of DPH placed in the inner hydrophobic domain and tma-DPH placed in the pseudo-hydrophobic domain near the membrane surface (Prendergast et al., 1981). The results are shown in Fig. 2. The *P* values of DPH were increased in HL-OA, -DHA, -EPA (Fig. 2(A)) and HL-LAE, -DHAE (Fig. 2(B)) as compared with that in HL, though all of the *P* values of tma-DPH in HL-PUFA were almost the same in the case of HL. These results indicate that the fluidity of the hydrophobic domain in HL-OA, -DHA, -EPA, -LAE and -DHAE were smaller than that of HL.

3.2. Inhibitory effects of HL-PUFA on the growth of tumor cells in vitro

With respect to the inhibitory effects of HL-PUFA on the growth of human tumor cells (lung carcinoma RERF-LC-OK and A549 cells, colon tumor WiDr cells and stomach tumor MKN45 cells) *in vitro*, we examined the 50% inhibitory concentrations (IC_{50}) of HL-PUFA on the basis of WST-1 assay. As shown in Fig. 3, the IC_{50} values of HL-PUFA were even smaller than those of HL alone, though no significant inhibitory effect of HL-OAE on the growth of RERF-LC-OK cells was observed. Most of the HL-PUFA indicated growth inhibition of tumor cells, but no distinguished relationship between the inhibitory effect on the growth of tumor cells and the membrane fluidity of HL-PUFA were observed apart form hybrid liposomes composed of DMPC and PEG surfactants (Komizu et al., 2006). Previously, some studies reported that n-9 PUFA such as OA did not show the growth inhibition toward tumor cells *in vitro* (Chow et al., 1989; Finstad et al., 1994). 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 (Rose and Connolly, 1990). In this study, we obtained significant inhibitory effects of HL-n-9 PUFA (OA and OAE) as well as HL-n-6 PUFA (LA and LAE) 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 (Fig. 3(A)). The IC_{50} values were 46.4 μM for RERF-LC-OK cells, 83.6 μM for A549 cells, 53.3 μM for WiDr cells and 30.3 μM for MKN45 cells. Furthermore, fairly high inhibitory effects of HL-ALAE on the growth of RERF-LC-

OK (IC_{50} = 30.4 μM) and A549 cells (IC_{50} = 35.6 μM) were attained (Fig. 3(B)). These results suggest that HLs including n-9, n-6, and especially n-3 polyunsaturated fatty acids or their ethyl esters could be effective generally in the growth inhibition of human tumor cells *in vitro*. Recently, we reported that HL distinguished between tumor and normal cells, and then fused and accumulated into the membranes of tumor cells only (Nakano et al., 2002; Komizu et al., 2006). 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*.

3.3. Effects of vitamin E on the growth inhibition of tumor cells by HL-PUFA

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 (Bégin et al., 1985; Das et al., 1987). Thus, the inhibitory effects of PUFA were prevented in the presence of anti-oxidants such as vitamin E (Chow et al., 1989). Therefore, we examined the inhibitory effects of HL-PUFA (LA, DHA and ALAE) 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 Fig. 4. 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 were decreased in the absence of vitamin E as shown in Fig. 4(A). On the other hand, the $A_{Mean}/A_{Control}$ value 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 (Fig. 4(A)). This result indicates that vitamin E prevents the growth inhibition of tumor cells by HL-LA via the lipid peroxidation. Interestingly, the $A_{Mean}/A_{Control}$ value of HL-DHA including vitamin E (HL-DHA/vitamin E) for RERF-LC-OK, WiDr and MKN45 cells were slightly increased, while the $A_{Mean}/A_{Control}$ value for A549 cells was increased to the value for HL/vitamin E (Fig. 4(B)). Similar tendencies were observed in the case of HL-ALAE including vitamin E (HL-ALAE/vitamin E) (Fig. 4(C)), that is, the $A_{Mean}/A_{Control}$ value of HL-ALAE/vitamin E for RERF-LC-OK cells was slightly increased and that for A549 cells was increased to the value for HL/vitamin E. The $A_{Mean}/A_{Control}$ values for WiDr and MKN45 cells were increased moderately, but not to the value for HL/vitamin E. These results suggest that the inhibition of HL-LA, -DHA and -ALAE on the growth of A549 cells could be attributable to the cytotoxicity via the lipid peroxidation of PUFA. On the other hand, the marked inhibitory effects of HL-DHA and -ALAE on the growth of the other tumor cells could not be due to only the lipid peroxidation in this study.

3.4. Induction of cell death by HL-PUFA toward tumor cells

We next examined the further mechanism of the inhibitory effects of HL-n-3 PUFA on the growth of tumor cells, using double staining assay fluorescence microscopic analysis. Figs. 5–7 show the fluorescence micrographs of RERF-LC-OK (A), A549 (B), WiDr (C) and MKN45 (D) cells dyed with YO-PRO-1 and PI after the treatment with HL-PUFA (LA, DHA and ALAE). The double staining assay of cell death with YO-PRO-1 (Idziorek et al., 1995) and PI detects apoptotic and necrotic cells as green and red (or orange) fluorescent cells, respectively. As shown in Fig. 5, most of the tumor cells were dyed in red after adding HL-LA, thought 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, RERF-LC-OK, WiDr and MKN45 cells treated with HL-DHA (Fig. 6) and HL-ALAE (Fig. 7) exhibited the green fluorescence, indicating

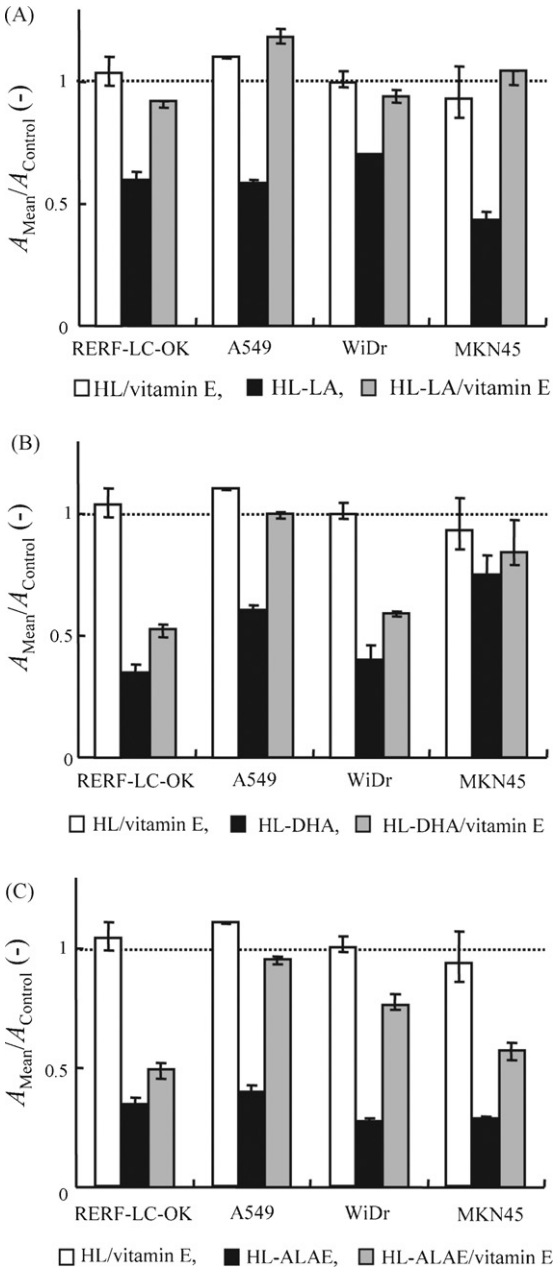

Fig. 4. Inhibitory effects of HL-PUFA ((A) HL-LA; (B) HL-DHA; (C) HL-ALAE) including vitamin E on the growth of tumor cells for 48 h *in vitro*. The tumor cells were seeded at a density of 1.0×10^4 cells ml^{-1} (RERF-LC-OK, A549) or 2.0×10^4 cells ml^{-1} (WiDr, MKN45) in 96-well tissue culture plates and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into each well and the plates were incubated. After 48 h, WST-1 solution was added and the absorbance at a wavelength of 450 nm was measured using a spectrophotometer. The inhibitory effects of HL-PUFA/vitamin E on the growth of tumor 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 sample solutions, respectively. [DMPC] = 100 μM; [Tween 80] = 11 μM; [PUFA] = IC_{50} ([LA] = 93 μg ml^{-1} (RERF-LC-OK), 245 μg ml^{-1} (A549), 591 μg ml^{-1} (WiDr), 554 μg ml^{-1} (MKN45); [DHA] = 152 μg ml^{-1} (RERF-LC-OK), 275 μg ml^{-1} (A549), 105 μg ml^{-1} (WiDr), 100 μg ml^{-1} (MKN45); [ALAE] = 93 μg ml^{-1} (RERF-LC-OK), 109 μg ml^{-1} (A549), 517 μg ml^{-1} (WiDr), 266 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, S.D.s.

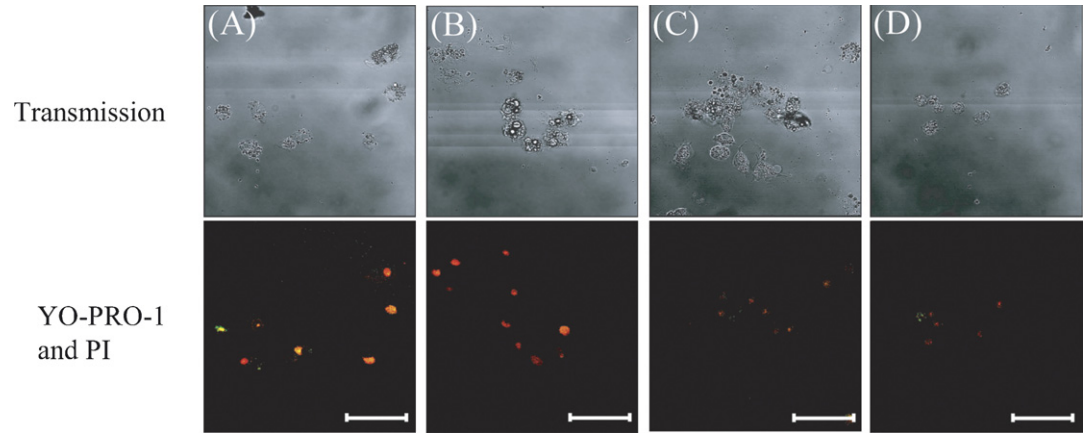

Fig. 5. Fluorescence micrographs of tumor cells ((A) RERF-LC-OK; (B) A549; (C) WiDr; (D) MKN45) stained with YO-PRO-1 and PI after the treatment with HL-LA for 42 h. Scale bar: 100 μm. The tumor cells were seeded at a density of 5.0×10^4 cells ml^{-1} (RERF-LC-OK, A549) or 1.0×10^5 cells ml^{-1} (WiDr, MKN45) in glass bottom culture dishes and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into the dishes and the dishes were incubated. After 42 h, the cells were stained with YO-PRO-1 (green color) and PI (red color) and observed using a confocal laser microscope with an Ar laser of excitation 488 nm for YO-PRO-1 and 543 nm for PI, respectively. [DMPC] = 100 μM; [Tween 80] = 11 μM; [LA] = 50 μg ml^{-1}.

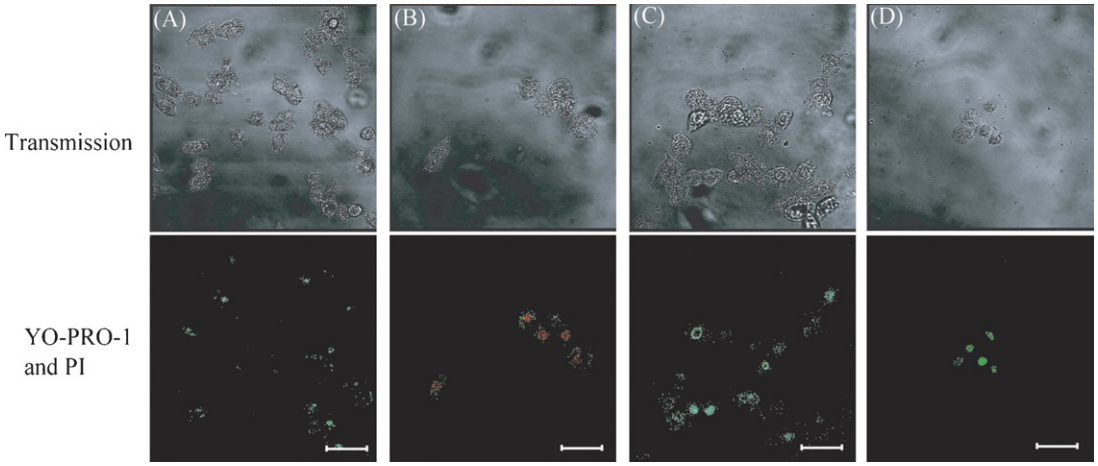

Fig. 6. Fluorescence micrographs of tumor cells ((A) RERF-LC-OK; (B) A549; (C) WiDr; (D) MKN45) stained with YO-PRO-1 and PI after the treatment with HL-DHA for 42 h. Scale bar: 100 μm. The tumor cells were seeded at a density of 5.0×10^4 cells ml^{-1} (RERF-LC-OK, A549) or 1.0×10^5 cells ml^{-1} (WiDr, MKN45) in glass bottom culture dishes and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into the dishes and the dishes were incubated. After 42 h, the cells were stained with YO-PRO-1 (green color) and PI (red color) and observed using a confocal laser microscope with an Ar laser of excitation 488 nm for YO-PRO-1 and 543 nm for PI, respectively. [DMPC] = 100 μM; [Tween 80] = 11 μM; [DHA] = 50 μg ml^{-1}.

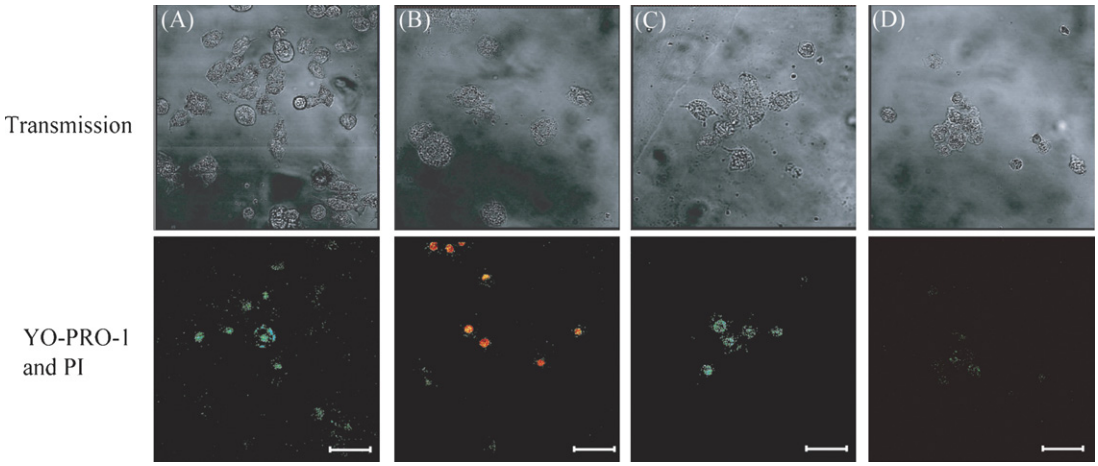

Fig. 7. Fluorescence micrographs of tumor cells ((A) RERF-LC-OK; (B) A549; (C) WiDr; (D) MKN45) stained with YO-PRO-1 and PI after the treatment with HL-ALAE for 42 h. Scale bar: 100 μm. The tumor cells were seeded at a density of 5.0×10^4 cells ml^{-1} (RERF-LC-OK, A549) or 1.0×10^5 cells ml^{-1} (WiDr, MKN45) in glass bottom culture dishes and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into the dishes and the dishes were incubated. After 42 h, the cells were stained with YO-PRO-1 (green color) and PI (red color) and observed using a confocal laser microscope with an Ar laser of excitation 488 nm for YO-PRO-1 and 543 nm for PI, respectively. [DMPC] = 100 μM; [Tween 80] = 11 μM; [ALAE] = 50 μg ml^{-1}.

the presence of nuclear condensation and fragmentation in apoptotic cells. On the other hand, red color was observed in A549 cells treated with HL-DHA and HL-ALAE, indicating that HL-DHA and -ALAE induced necrosis for A549 cells. These microscopic observations revealed that HL-DHA and HL-ALAE should principally induce apoptosis toward RERF-LC-OK, WiDr and MKN45 cells and necrosis toward A549 cells.

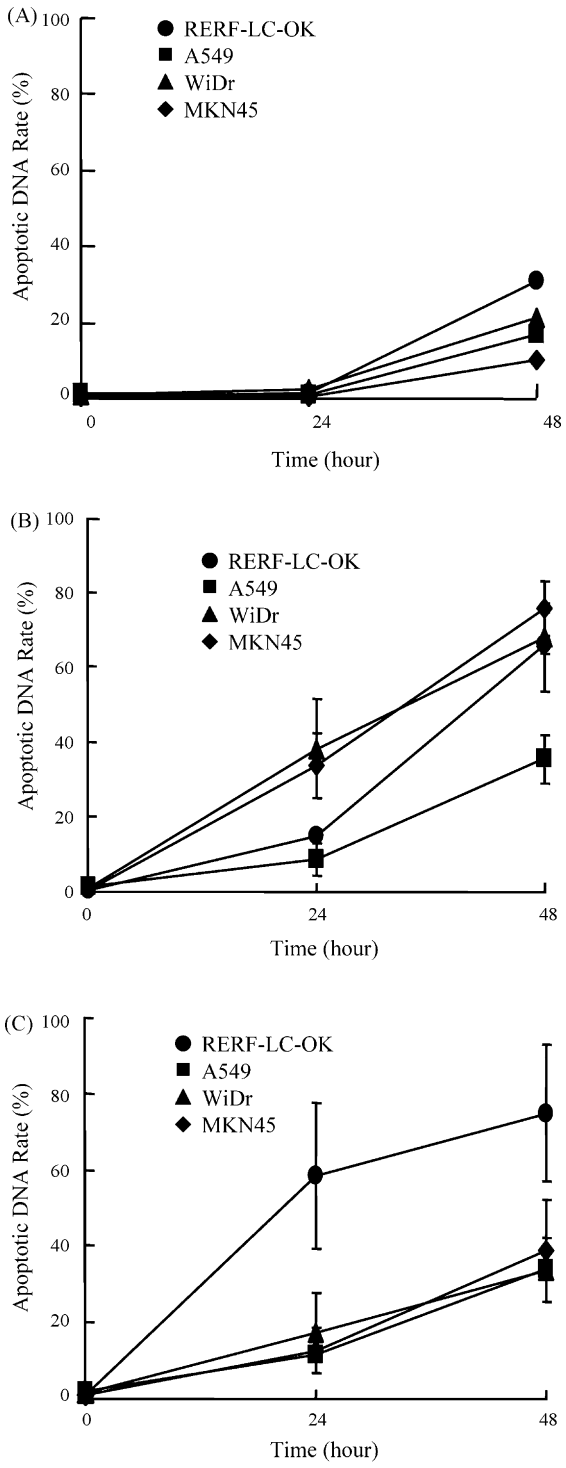

Fig. 8. Apoptotic DNA rate for tumor cells treated with HL-LA (A), HL-DHA (B) and HL-ALAE (C) for 24 and 48 h. The tumor cells were seeded at a density of 1.7×10^5 cells ml^{-1} in culture flasks and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into the flasks and the flasks were incubated. After 24 and 48 h, the cells were stained with PI and analyzed by a flow cytometer with a single excitation 488 nm of an Ar laser. [DMPC] = 100 μM; [Tween 80] = 11 μM; [PUFA] = 50 μg ml^{-1}. Data presented are means; bars, S.D.s.

Furthermore, the time courses of DNA fragmentation in RERF-LC-OK, A549, WiDr and MKN45 cells treated with HL-PUFA (LA, DHA and ALAE) were examined by flow cytometry. As shown in Fig. 8, the apoptotic DNA rates of tumor cells treated with HL-PUFA were increased gradually for 48 h. However, all of the tumor cells treated with HL-LA showed rates less than 32% at 48 h. In contrast, the rates in RERF-LC-OK cells treated with HL-DHA and -ALAE reached high values of more than 65% at 48 h. Such high rates were also obtained for the rates of WiDr and MKN45 cells treated with HL-DHA. These findings on DNA fragmentation of the tumor cells in the presence of HL-DHA were in good agreement with the marked growth inhibition and the induction of apoptosis for those tumor cells observed in WST-1 assay and fluorescent microscopic analysis, respectively. On the other hand, low rates of the DNA fragmentation were observed in A549 cells treated with HL-DHA and -ALAE (less than 40% at 48 h), as well as in all of the tumor cells treated with HL-LA. We consider that these observations should be compatible with the considerable growth inhibitions of the tumor cells induced by necrosis via the lipid peroxidation of PUFA. It was shown that HL-n-3 PUFA could primarily induce necrosis in A549 cells and apoptosis in other tumor cells. Similar findings were obtained in human leukemic cell lines HL-60 and K-562, in which n-3 PUFA EPA induced apoptosis only in HL-60 cells but not in K-562 cells, though the inductions of necrosis by EPA were observed in both tumor cells (Chiu and Wan, 1999). The study showed that the apoptosis-inducing property of EPA was cell type specific and the differential response to EPA might be due to the metabolic events which exist in different cell types. In this study, the mechanistic details are not clear, but the difference in metabolic characteristics between A549 cells and other tumor cells could cause differential inductions of cell death by HL-n-3 PUFA.

As mentioned above, it was clarified that HL-n-3 PUFA (DHA and ALAE) induced cell death toward tumor cells through two different mechanisms. One should be necrosis depending on the lipid peroxidation of PUFA and the other could be apoptosis not depending on the lipid peroxidation. With respect to apoptosis induced by n-3 PUFA, a possible mechanism related to the production of eicosanoids in arachidonate metabolic cascade has been postulated (Sheng et al., 1998; Chiu and Wan, 1999). That is, n-3 PUFA such as EPA inhibits the production of prostaglandin E_2 (PGE_2) catalyzed by prostaglandin endoperoxide synthase 2, referred to as cyclooxygenase 2 (COX-2), and PGE_2 is associated with the expression of Bcl-2 gene which codes the apoptosis suppression proteins. It is possible that n-3 PUFA delivered to the tumor cells by HL would

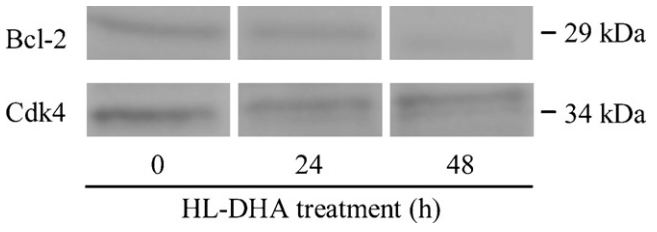

Fig. 9. Expression of Bcl-2 in RERF-LC-OK cells treated with HL-DHA for 24 and 48 h. RERF-LC-OK cells were seeded at a density of 2.5×10^5 cells ml^{-1} in culture flasks and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The sample solutions were added into the flasks and the flasks were incubated. After 24 and 48 h, the supernatant of cell lysates was subjected to SDS-PAGE followed by Western blotting using antibodies for anti-Bcl-2 (29 kDa) and anti-Cdk4 (34 kDa). Cdk4 protein level was measured as control for protein loading on the gel. Bcl-2 and Cdk4 antibodies were used at a dilution of 1:200 and 1:40, respectively. [DMPC] = 130 μM; [Tween 80] = 14 μM; [DHA] = 130 μg ml^{-1}.

act as COX-2 competitive inhibitors and lead to the downregulation of Bcl-2 expression. As a result, HL-n-3 PUFA could effectively induce apoptosis toward RERF-LC-OK cells. In order to clarify the relationship between the induction of apoptosis and expression of Bcl-2 in tumor cells, we performed Western blot analysis for RERF-LC-OK cells treated with HL-DHA. As shown in Fig. 9, the protein level of Bcl-2 in RERF-LC-OK cells was gradually decreased after the treatment with HL-DHA. This observation supported the assumption that HL-n-3 PUFA could induce apoptosis toward tumor cells by downregulation of Bcl-2 expression. Furthermore, other mechanisms of the growth inhibition of tumor cells through the metabolism of n-3 PUFA in lipoxygenase pathway were considered (Rose and Connolly, 1990). Probably, apoptosis induced by HL-n-3 PUFA might be attributed to the regulation of Bcl-2 or other proteins related to apoptosis by metabolites of n-3 PUFA in tumor cells.

4. Conclusion

In conclusion, we clearly demonstrated the inhibitory effects of hybrid liposomes including polyunsaturated fatty acids or their ethyl esters (HL-PUFA) on the growth of human tumor cells (lung carcinoma RERF-LC-OK and A549 cells, colon tumor WiDr cells and stomach tumor MKN45 cells) *in vitro*. The noteworthy aspects are as follows. (a) Clear aqueous solutions of HL-PUFA could be obtained and kept stable for more than 1 month. (b) Remarkably high inhibitory effects of HL including docosahexaenoic acid (HL-DHA) or α-linolenic acid ethyl ester (HL-ALAE) on the growth of human tumor cells were obtained. (c) The inhibitory effects of HL-DHA and HL-ALAE on the growth of A549 cells were closely related to the lipid peroxidation of PUFA. (d) HL-DHA and HL-ALAE could principally induce apoptosis toward RERF-LC-OK, WiDr and MKN45 cells. It is worthy to note 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 (ester) for three-component hybrid liposomes should be important in order to induce apoptosis in full consideration of future clinical applications.

Acknowledgments

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