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Review Paper

Effect of oil-in-water lipid emulsions prepared with fish oil or soybean oil on the growth of MCF-7 cells and HepG2 cells

Kumiko Ueda, Yukiko Asai, Yoshimizu Yoshimura and Seigo Iwakawa

Abstract

The growth of human breast cancer-derived MCF-7 cells was affected by oil-in-water lipid emulsions prepared with fish oil (FO) rich in n-3 fatty acids (FAs) and egg-yolk phosphatides (EYP) (FO-emulsions), but not by lipid emulsions prepared with soybean oil (SO) and EYP (SO-emulsions). On the other hand, the growth of human hepatocarcinoma HepG2 cells was affected by neither SO-emulsions nor FO-emulsions. The growth inhibition of MCF-7 cells in the presence of FO-emulsions was not affected by trolox, but was inhibited by α-lipoic acid, and was even potentiated by ebselen, which works as an antioxidant as well as a lipoxygenase inhibitor. Since prostaglandin E_3, generated from n-3 FAs by cyclooxygenases, has a suppressive effect on tumour cell growth, and increases when lipoxygenases are inhibited, these findings suggest that lipid emulsions incorporating triglycerides of n-3 FAs might be effective in suppressing the growth of MCF-7 cells, possibly via oxidative stress and through eicosanoid production with anti-proliferating activity against cancer cells.

Introduction

Fish oils rich in n-3 fatty acids (FAs) have attracted attention for their chemopreventive effects on carcinogenesis for decades. Epidemiological studies have shown that consumption of fish is correlated with a lower incidence of cancer (Kaizer et al 1989; Caygill et al 1996). Additionally, decreased consumption of fish and increased intake of vegetable oils rich in n-6 fatty acids among Japanese women during the past decades has been accompanied by increased breast cancer rates (Wynder et al 1991). Animal studies have also shown that the growth of chemically induced cancers and human xenografts could be slowed or completely inhibited by the addition of n-3 FAs in the diet (Reddy & Sugie 1988; Deschner et al 1990; Rose & Connolly 1993).

Despite these epidemiological and in-vivo studies of n-3 FAs, the effects of n-3 and n-6 FAs on cancer cell growth in-vitro are still controversial. Some reports show that not only n-3 FAs but also n-6 FAs exert cytostatic or cytotoxic effects on several types of tumour cells (Chamras et al 2002; Dommels et al 2003). In these studies, non-esterified FAs had been utilized to examine the effects of FAs. Recently, Edwards et al (2004) reported that low-density lipoproteins (LDLs) rich in n-3 FAs, isolated from African Green monkeys fed an n-3 FA-enriched diet, were more effective at inhibiting cell proliferation and inducing apoptosis in MCF-7 cells than were n-3 non-esterified FAs. On the other hand, oil-in-water (O/W) lipid emulsions have long been used in the parenteral delivery of energy and essential FAs for nutritional purposes to patients with diseases such as intestinal failure and sepsis (Wanten & Calder 2007). Recently, fish oil emulsions for parenteral nutrition have gained attention for the rapid supply of n-3 fatty acids to decrease inflammatory responses and cell sensitivity to various stimuli, and to improve endothelial dysfunction (Carpentier & Hacquebard 2006). Therefore, it might be effective and efficient to supply n-3 FAs as fish oil emulsions intravenously for inhibition of tumour growth.

Several hypotheses can be formulated for the anti-proliferating effect of n-3 FAs. One is the cellular uptake of emulsions; the potency of the inhibitory effect of lipid emulsions on cell growth might be also affected by the intracellular level of the target FAs, which would

Department of Pharmaceutics, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

Kumiko Ueda, Yukiko Asai, Yoshimizu Yoshimura, Seigo Iwakawa

Correspondence: K. Ueda, Department of Pharmaceutics, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan. E-mail: ueda@kobepharma-u.ac.jp

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be related to the cellular uptake of the emulsions. Previously, we have studied the pharmacokinetics of menatetrenone (vitamin K_2) incorporated into O/W lipid emulsions in rats (Ueda et al 2001, 2002, 2003, 2004). Menatetrenone, a model lipophilic drug, was retained inside the lipid particles even after entering the circulation in rats (Ueda et al 2001). Therefore, menatetrenone would also be retained inside the lipid particles during in-vitro experiments and would be a suitable marker for determining the cellular uptake of the particles. Since menatetrenone is a cofactor for γ-glutamyl carboxylase and is easily converted into menatetrenone epoxide in the liver (Nishimura et al 1990; Suhara et al 2006), we considered it necessary to examine the level of menatetrenone epoxide as well. Another hypothesis is the peroxidation due to n-3 FAs; eicosapentaenoic acid (EPA) and docosahexaenoic acid, two major n-3 FAs, are readily oxidized in the presence of free radicals because they have additional carbon–carbon double bonds compared with other FAs (Yin et al 2007). It has also been shown that antioxidants attenuated the cell growth inhibitory effects of n-3 FAs (Chajès et al 1995). Antioxidants vary in their mechanisms of action (e.g., trolox is a hydrophilic derivative of vitamin E, and lipoic acid, an amphipathic antioxidant, has the ability to scavenge reactive oxygen species and significant effects on the tissue concentrations of reduced forms of other antioxidants) (Bilska & Wlodek 2005). Ebselen is an antioxidant with glutathione peroxidase activity and is an effective scavenger of organic hydroperoxides as well as an inhibitor of enzymes including lipoxygenases (Schewe 1995). Examining the effect of these antioxidants on the inhibition of cell proliferation by lipid emulsions would help clarify the mechanism of the inhibition. Also, n-3 FAs might inhibit the production of n-6 FA-derived eicosanoids that promote cell growth (Calder 2006). Furthermore, n-3 FAs might be converted to eicosanoids with anti-proliferating activity against cancer cells. Prostaglandin E_3 (PGE_3), produced from EPA by cyclooxygenases, inhibited the growth of melanoma (Xia et al 2006). In the presence of ebselen, lipoxygenases are inhibited and more metabolites, such as PGE_3, would be produced via cyclooxygenases.

In this study, we attempted to clarify the effect of the FAs as triglycerides incorporated in the O/W lipid emulsions on the growth of two cancer cell lines, human breast cancer-derived MCF-7 and human hepatocarcinoma HepG2 cells. Fish oil (FO) rich in n-3 FAs and soybean oil (SO) rich in n-6 FAs were utilized as the oil phase of the lipid emulsions. The mean particle size of the lipid emulsions utilized as parenteral nutrition is larger than 200 nm; however, large particles might not penetrate the vascular walls. Therefore, we prepared the lipid emulsions with less oils (5%) and more phosphatides (5%) to make the particle size smaller than 100 nm. We have examined the cellular uptake of the lipid particles by incorporating menatetrenone into the particles and determining the cellular level of menatetrenone and menatetrenone epoxides after incubation with the cells. We have also examined the effect of antioxidants on cell growth to clarify the mechanisms of growth inhibition due to the lipid emulsions, and the mechanisms for the anti-proliferating effects of the emulsions are discussed.

Materials and Methods

Materials

SO and menatetrenone were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). FO (sardine oil (Sanomega 28)) and egg-yolk phosphaticles (EYP (NC-50)) were from Nihon Oil and Fats (Tokyo, Japan). The FA compositions of SO and FO, according to the data sheets provided by the manufacturer, are shown in Table 1. Menatetrenone epoxide was kindly provided by Professor Toshio Okano and Dr Yoshitomo Suhara, Department of Hygienic Sciences, Kobe Pharmaceutical University, Japan. All other chemicals were of reagent grade.

Cell lines

MCF-7 cells were obtained from American Type Culture Collection (VA), and maintained at 37°C under 5% CO_2 in minimum essential medium (MEM; Gibco, Invitrogen Corp., CA) supplemented with 10% FBS (Tissue Culture Biologicals, CA), 1 mM sodium pyruvate, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. They were subcultured every 4–6 days and the culture medium was changed every 2–3 days. HepG2 cells (JCRB1054) were obtained from Health Science Research Resources Bank (Osaka, Japan) and maintained at 37°C under 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., MO) supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were subcultured every 4–6 days and the culture medium was changed every 2–3 days.

Preparation of lipid emulsions

Lipid emulsions (SO-emulsions and FO-emulsions) were prepared using a high-pressure homogenization system as described previously with some modifications (Ueda et al 2001). Briefly, SO or FO (5% w/w) were mixed with EYP (5% w/w) and purified water using a homomixer (model LR-1; Mizuho Industrial Co., Osaka, Japan), and the mixture (100 mL) was emulsified using a microfluidizer system

Table 1 Fatty acid composition of SO and FO utilized for preparation of emulsions

Fatty acids	SO (%)	FO (%)
Myristic acid	—[a]	4.9
Palmitic acid	9–12	7.6
Stearic acid	3–5	6.1
Oleic acid	21–28	3.2
Linoleic acid	50–56	9.0
Linolenic acid	7–10	—
Eicosapentaenoic acid	—	28.0
Docosahexaenoic acid	—	13.4
Others	—	26.7

SO, soybean oil; FO, fish oil. [a]Not determined. The data was provided by the manufacturers.

Table 2 Particle size and levels of triglycerides, phospholipids and menatetrenone in emulsions

Emulsion	Particle size (nm ± s.d.)	Triglycerides (% w/w)	Phospholipids (% w/w)	Menatetrenone (% w/w)
SO-emulsion	66 ± 30	5.14	5.34	—[a]
FO-emulsion	48 ± 23	5.12	5.26	—
$SO(K_2)$-emulsion	65 ± 28	5.06	4.96	1.00
$FO(K_2)$-emulsion	60 ± 27	5.05	5.02	1.00

[a]Not determined.

(M110-EH; Mizuho Industrial Co., Osaka, Japan – the distributor in Japan) at a pressure of 20000 psi for 30 min at 13°C. For cellular uptake experiments, lipid emulsions incorporating menatetrenone (1% w/w) ($SO(K_2)$-emulsions and $FO(K_2)$-emulsions) were prepared by dissolving menatetrenone into SO or FO. The size and distribution of the lipid emulsions were determined by the dynamic light-scattering method using an FPAR-1000 fibre-optics particle analyser (Otsuka Electronics, Osaka, Japan). Analysis of the lipid emulsions was performed in triplicate, and the findings for the median size were adopted. Typical data are shown in Table 2. The lipid emulsions were stored at 4°C in the dark and were used in the experiments within 2 days of their preparation. Phospholipid and triglyceride levels were determined using a diagnostic kit (Phospholipid C-test Wako and Triglyceride E-test Wako; Wako Pure Chemical Industries, Ltd, Osaka, Japan). The extent of peroxidation of the emulsions was characterized by the level of thiobarbituric acid-reactive substances (TBARS), primarily malondialdehyde (MDA) (Buege & Aust 1987), measured spectrometrically, before the WST-8 assays (Dojindo, Kumamoto, Japan) in the presence of various antioxidants.

Cell proliferation assay

MCF-7 and HepG2 cells were seeded in 96-well plates at a density of 5000 cells per well and, after incubation at 37°C for 24 h, SO-emulsions and FO-emulsions were added to each well following sterilization with filters of 0.22-μm pore size. Antioxidants (trolox, α-lipoic acid and ebselen at a final concentration of 250 μM, 100 μM and 5 μM, respectively) were also added into the well at the same time. The final volume of the experimental media was 200 μL/well. A WST-8 assay was performed after incubation for 72 h as directed.

Size and distribution of lipid emulsions after incubation in the culture media

To examine whether the size of emulsions had changed soon after dilution in the culture media, SO-emulsions and FO-emulsions, after filtration, were incubated with a 20-fold volume of DMEM or MEM at 37°C for 2 or 72 h under sterile conditions, and the particle size and distribution were examined by the dynamic light-scattering method using a FPAR-1000 fibre-optics particle analyser.

Cellular level of menatetrenone and menatetrenone epoxide after incubation with emulsions incorporated with menatetrenone

MCF-7 and HepG2 cells were seeded in 3.5-cm dishes at 2×10^5 cells/dish, and incubated at 37°C under 5% CO_2 for 3 days. The medium was changed to one containing $SO(K_2)$-emulsions or $FO(K_2)$-emulsions at a concentration of 200 μM as menatetrenone, and the cells were incubated for 0 or 2 h at 37°C or 4°C. After incubation, the medium was aspirated, the cells were washed with 1 mL of ice-cold PBS(−) twice and scraped off the dish with cell scrapers, and menatetrenone and menatetrenone epoxide were extracted into 500 μL of ethanol. The cellular menatetrenone level was determined by HPLC as described previously (Ueda et al 2001). The retention time for menatetrenone and menatetrenone epoxide was about 10 min and 5 min, respectively. Since menatetrenone is known to be converted to menatetrenone epoxide in HepG2 cells, the menatetrenone epoxide level was also determined similarly. The menatetrenone and menatetrenone epoxide levels were normalized based on the amount of protein in the cells per dish, determined with a Protein Assay Kit (Bio-Rad Laboratories Inc., CA).

Statistical analysis

Statistical analyses were performed with the Student's *t*-test and Kruskal–Wallis test followed by Dunn's test for two and multiple comparisons, respectively.

Results

Preparation of lipid emulsions

The particle size and phospholipid, triglyceride and menatetrenone levels of the lipid emulsions are shown in Table 2. Typical data for 3–6 preparations for each formulation were adopted. The mean particle size of the emulsions was 48–66 nm, and the measured levels of phospholipids, triglycerides and menatetrenone were within ±10% of the amounts indicated for all of the emulsions prepared.

Effect of SO- and FO-emulsions on the proliferation of MCF-7 and HepG2 cells

Figure 1 shows the effects of SO- and FO-emulsions on the growth of MCF-7 and HepG2 cells. The growth of MCF-7

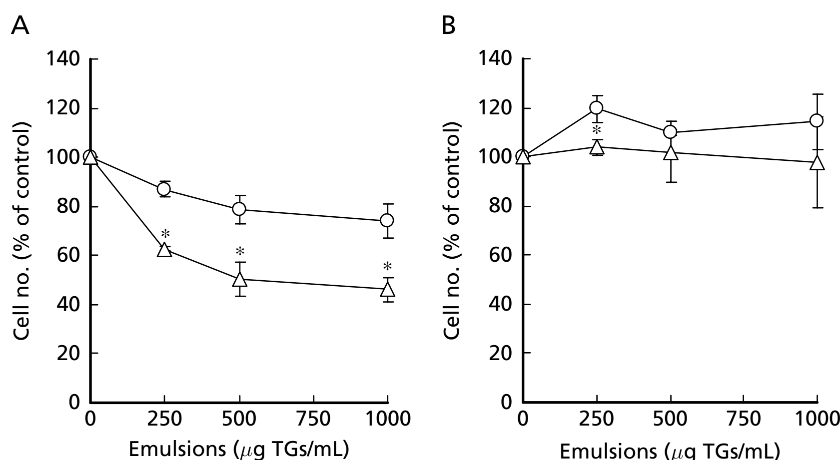

Figure 1 Effect of SO- and FO-emulsions on the growth of MCF-7 and HepG2 cells. WST-8 assays were performed in the presence of SO-emulsions (circles) and FO-emulsions (triangles) with MCF-7 (A) and HepG2 (B) cells. Results are expressed as a percentage of the control without emulsions. Each point represents the mean ± s.d. of 3 independent experiments. $^{*}P < 0.05$ versus SO-emulsions (Student's *t*-test).

cells was reduced to 50% in the presence of FO-emulsions, while it decreased only to 80% in the presence of SO-emulsions, at a concentration of 1000 μg triglycerides/mL (Figure 1A). On the other hand, the proliferation of HepG2 cells was affected by neither SO- nor FO-emulsions (Figure 1B).

Effect of culture media on the particle size of emulsions

To examine whether or not the lipid emulsions were aggregated during the incubation in the culture media, the mean particle size of emulsions after 0, 2 and 72 h of incubation with MEM or DMEM was determined with the particle analyser. Typical values of the particle size of SO-emulsions after 0, 2 and 72 h of incubation with MEM were 67 ± 33, 62 ± 24 and 66 ± 19 nm, respectively, and those after 0, 2 and 72 h of incubation with DMEM were 67 ± 32, 63 ± 20 and 66 ± 27 nm, respectively. Typical values of the particle size of FO-emulsions after 0, 2 and 72 h of incubation with MEM were 51 ± 23, 60 ± 25 and 101 ± 44 nm, respectively, and those after 0, 2 and 72 h of incubation with DMEM were 51 ± 22, 58 ± 14 and 98 ± 46, respectively. The particle size of FO-emulsions was twice as large after incubation with the culture media for 72 h, but no phase separation or coagulation of the emulsions was observed.

Cellular levels of menatetrenone and menatetrenone epoxide after incubation with emulsions incorporated with menatetrenone in MCF-7 and HepG2 cells

The levels of menatetrenone and menatetrenone epoxide after incubation with SO(K_2)-emulsions and FO(K_2)-emulsions were examined in MCF-7 and HepG2 cells (Figure 2). In MCF-7 cells, the levels after incubation with SO(K_2)-emulsions and FO(K_2)-emulsions at 37°C were 1.9–2.5 and 0.37–0.40 nmol (mg protein)$^{-1}$ for menatetrenone and menatetrenone epoxide at 120 min, respectively. On the other hand, in HepG2 cells, the levels after incubation with SO(K_2)-emulsions and FO(K_2)-emulsions at 37°C were 0.87–1.0 and 1.4–1.6 nmol (mg protein)$^{-1}$ for menatetrenone and menatetrenone epoxide at 120 min, respectively. Menatetrenone and menatetrenone epoxide levels were low at 4°C even after 120 min of incubation with SO(K_2)-emulsions and FO(K_2)-emulsions in either MCF-7 or HepG2 cells. There were no differences among the cellular levels of menatetrenone and menatetrenone epoxide within the emulsions used in either cell line.

Effect of antioxidants on the growth inhibition by the emulsions in MCF-7 cells

Since oxidative stress has been proposed as one of the mechanisms for cancer cell death due to n-3 FAs, the TBARS level, an index of lipid peroxidation, was determined for emulsions (Figure 3). The TBARS level of FO-emulsions was about 10-fold higher than that of SO-emulsions. The effects of three antioxidants, trolox, α-lipoic acid and ebselen, on the growth inhibition of MCF-7 cells by emulsions were examined using WST-8 assay (Figure 4). Although the growth inhibition in the presence of FO-emulsions was not affected by 250 μM trolox, it was attenuated to the control level by 100 μM α-lipoic acid. On the other hand, the growth inhibition in the presence of FO-emulsions was enhanced to about 20% of the control by 5 μM ebselen. The growth of MCF-7 cells in the presence of SO-emulsions was not affected by either of the antioxidants.

Discussion

In this study, we found that the growth of human breast cancer-derived MCF-7 cells was inhibited by FO-emulsions but not by SO-emulsions, although the growth of human hepatocarcinoma HepG2 cells was unaffected in the presence of either SO- or FO-emulsions. Furthermore, the growth inhibition of MCF-7 cells in the presence of FO-emulsions was

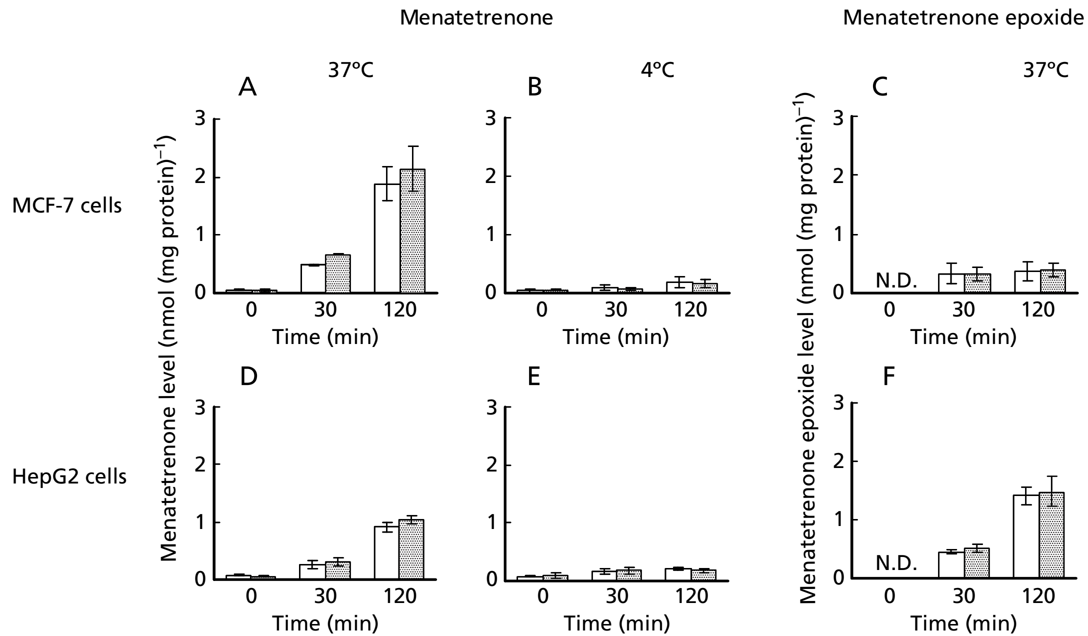

Figure 2 Levels of menatetrenone and menatetrenone epoxide after incubation with emulsions incorporated with menatetrenone in MCF-7 and HepG2 cells. Cellular levels of menatetrenone (A, B, D, E) and menatetrenone epoxide (C, F) were determined after incubation with 500 μg triglyceride/mL of SO-emulsions (open columns) and FO-emulsions (hatched columns) at 37°C (A, C, D, F) and 4°C (B, E) for 0, 30 and 120 min in MCF-7 (A, B, C) and HepG2 (D, E, F) cells. Menatetrenone epoxide was not detected in either MCF-7 or HepG2 cells after the incubation with emulsions at 4°C. Each point represents the mean ± s.d. of 3 independent experiments. The statistical analysis was done with Student's *t*-test, but no significant differences were observed between the emulsions. N.D., not detected.

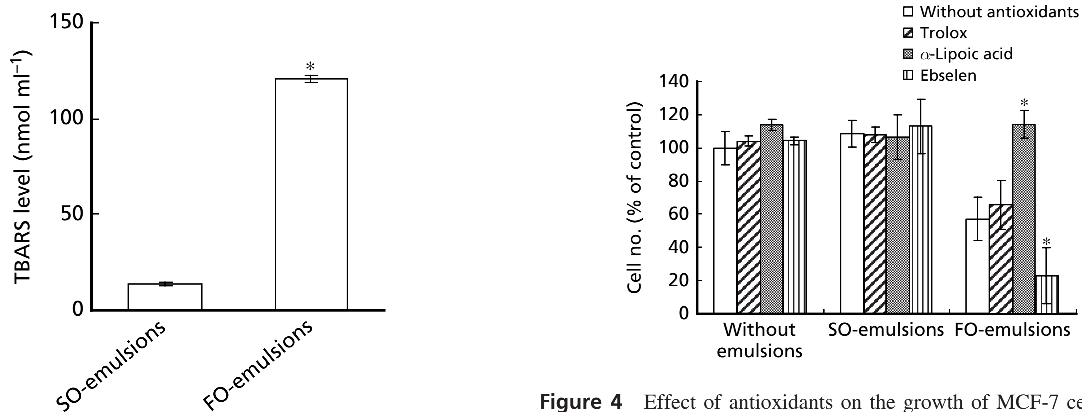

Figure 3 TBARS levels of SO- and FO-emulsions determined spectrometrically. Each point represents the mean ± s.d. for 3 different preparations. *$P < 0.05$ versus SO-emulsions (Student's *t*-test).

Figure 4 Effect of antioxidants on the growth of MCF-7 cells in the presence of SO- and FO-emulsions. The WST-8 assay was performed with 500 μg triglyceride/mL of SO- and FO-emulsions in the absence or presence of 250 μM trolox, 100 μM α-lipoic acid or 5 μM ebselen. Results are expressed as a percentage of the value without emulsions. Each point represents the mean ± s.d. of 3 independent experiments. *$P < 0.05$ versus value without antioxidants (Dunn's test).

not affected by trolox, but was inhibited by α-lipoic acid and, interestingly, enhanced by ebselen. Since PGE_3, generated from n-3 FAs by cyclooxygenases, has a suppressive effect on tumour cell growth, these findings suggest that lipid emulsions prepared with triglycerides of n-3 polyunsaturated fatty acids would be effective in suppressing the growth of MCF-7 cells, at least partly via oxidative stress and through eicosanoid production with anti-proliferating activity against cancer cells.

Lipid emulsions, after intravenous administration in-vivo, are eliminated from the circulation largely via direct particle uptake (Carpentier & Hacquebard 2006). When emulsion particles enter the blood stream, they quickly acquire apolipoproteins by transfer from HDL lipoproteins. Emulsion particles that acquire apolipoprotein CII are recognized by lipoprotein lipase (LPL) and triglycerides inside the lipid

particles are hydrolysed to FAs. Concomitantly, through cholesteryl ester transfer protein, emulsion particles gain cholesteryl esters from, and transfer triglycerides to, endogenous cholesterol-rich lipoproteins. These processes form remnant particles, which are largely taken up by the liver and several other tissues. HepG2 cells take up very-low-density lipoproteins (VLDL) through LDL receptors, LDL receptor-related protein (LRP) or hepatic scavenger receptor class B type I (SR-BI) (Mahley & Ji 1999; Rhainds et al 2003). MCF-7 cells have also been reported to express LDL and VLDL receptors on their surface (Webb et al 1999; Chamras et al 2002). Lipid emulsions are also taken up as a whole without being metabolized by LPL (Hultin et al 1995). On the other hand, fish oil emulsions are eliminated by a route different from that of soybean oil emulsions. The hydrolytic rate of fish oil emulsions is slower than that of soybean oil emulsions (Oliveira 1997). Elimination of fish oil emulsions is independent of apolipoprotein E, LDL receptors and lactoferrin-sensitive pathways, but may depend on CD36 and proteoglycan-mediated pathways (Qi et al 2002; Densupsoontorn et al 2008). In this study, the cellular uptake of the emulsions, examined by the cellular level of menatetrenone, was similar between SO- and FO-emulsions, and was temperature dependent for both MCF-7 and HepG2 cells. Because we did not add apolipoproteins or LPL in the experimental media, the uptake process observed might not mimic in-vivo situations. However, our findings suggest that the difference in cell proliferation observed in MCF-7 cells is not due to the amount of the emulsion particles taken up by the cells. We could not compare the amount of the emulsions taken up by the cells between the two cell lines, because menatetrenone was partly converted to menatetrenone epoxide in HepG2 cells. The emulsion particles that were taken up by HepG2 cells might have been underestimated compared with the in-vivo situation in the liver, since it has been reported that, firstly, LDL receptors are upregulated in cultured hepatocytes by the co-culture of sinusoidal endothelial cells (Nahmias et al 2006), secondly, addition of apoE enhances the uptake of oil particles in HepG2 cells (Morita et al 2003) and, thirdly, LPL on the cell surface is required for the intracellular uptake of lipoproteins (Augustus et al 2003). Moreover, it should be noted that, as we have utilized oils that are mixtures of various compounds, the possibility of a suppressive effect on cell growth due to compounds other than the triglycerides of n-3 FAs could not be completely excluded.

Several mechanisms, such as lipid peroxidation and suppression of n-6 FA-derived eicosanoids, have been proposed for the growth inhibition of cancer cells by n-3 FAs (Chajès et al 1995; Hardman 2004). In this study, the TBARS level of FO-emulsions was higher than that of SO-emulsions, suggesting that the lipid peroxidation might have contributed to the inhibition of MCF-7 cell growth. However, the effects of antioxidants on the growth of MCF-7 cells in the presence of FO-emulsions were not simple: trolox did not affect, α-lipoic acid attenuated and ebselen potentiated the growth inhibitory effect of FO-emulsions (Figure 4). Since trolox acts on the cytosol, while α-lipoic acid acts on both lipids and the cytosol (Bilska & Wlodek 2005), one of the mechanisms for cell growth inhibition by FO-emulsions would be lipid peroxidation, probably at the lipid membrane of MCF-7 cells. Furthermore, ebselen functions as an antioxidant as well as a lipoxygenase inhibitor (Schewe 1995), and n-3 FAs inhibit the production of n-6 FA-derived eicosanoids via inhibition of cyclooxygenases and lipoxygenases (Hardman 2004). Also, it was reported recently that n-3 FAs reduced the extent to which melanomas form and grow via PGE_3 produced from EPA by cyclooxygenases in transgenic mice that convert n-6 FAs to n-3 FAs in the body (Xia et al 2006). Therefore, it is probable that the production of PGE_3 from n-3 FAs by cyclooxygenases was enhanced when lipoxygenases were inhibited by ebselen, resulting in strong inhibition of cell growth or the promotion of cell death, or both. Also, it might be that n-3 FAs inhibited the production of n-6 FA-derived eicosanoids that promote cell proliferation (Calder 2006). Further study is required to clarify the precise mechanisms for cell growth inhibition by FO-emulsions and cell death due to co-incubation of FO-emulsions and ebselen.

Conclusion

In conclusion, we found that FO-emulsions suppressed the growth of MCF-7 cells but not HepG2 cells. The suppression was not due to an increase in the uptake of the lipid emulsions but partly due to the lipid peroxidation of the emulsions. Furthermore, the growth inhibition of MCF-7 cells by FO-emulsions was potentiated by ebselen, which suggested that the lipoxygenases inhibition resulted in increased production of PGE_3, a prostaglandin derivative with the capacity to inhibit cancer cell growth, by cyclooxygenase. These findings suggest that the n-3 FAs in lipid emulsions affect cancer cell growth, possibly via oxidative stress and eicosanoid production with anti-proliferating activity against cancer cells.

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