The Inhibitory Effect of Conjugated and Polyunsaturated Fatty Acids on the Growth of Human Cancer Cell Lines

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Noriko Matsumoto[a], Yasushi Endo[a]*, Kenshiro Fujimoto[a],

Seiji Koike[b] and Wataru Matsumoto[b]

[a] Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba, Sendai 981-8555, Japan
[b] Asahi Denka Kogyo K.K., 7-2-35 Higashiogu, Arakawa, Tokyo 116-8553, Japan

Summary

The cytotoxicity of conjugated and polyunsaturated fatty acids (CPUFAs) was assessed on human breast (MCF-7) and colorectal (HT-29) cancer cells. Cancer cells were incubated with conjugated arachidonic (CAA), eicosapentaenoic (CEPA), docosahexaenoic acids (CDHA), and free fatty acids of tung oil and balsam seed oil as CPUFAs at the concentration of 1 to 100 μM for 24 to 72 h. The proliferation of MCF-7 cell was completely inhibited when supplemented with 100 μM CPUFAs. Especially, CEPA and tung oil fatty acids substantially inhibited the growth of MCF-7 cell at 10 μM after 48 h-incubation. All CPUFAs significantly inhibited the growth of HT-29 cell at 10 μM except for balsam seed oil fatty acids. Among CPUFAs, CEPA and tung oil fatty acids showed the strongest inhibitory effect on the growth of HT-29 cell. The level of TBA-reacting compounds was higher in MCF-7 and HT-29 cells incubated with CPUFAs. When α-tocopherol being an antioxidant was added to the culture medium containing 100 μM CPUFAs at the range of 0.01 to 1.0 μM, the viability of MCF-7 and HT-29 cells was increased with the concentration of α-tocopherol. Especially, MCF-7 cell incubated with CPUFAs was not almost dead by supplementation of α-tocopherol. The strong toxicity of CPUFAs such as CEPA, CDHA and tung oil fatty acids to human cancer cells may be related to lipid peroxidation.

Key words : arachidonic acid, conjugated fatty acid, cytotoxicity, docosahexaenoic acid, eicosapentaenoic acid, human cancer cell, polyunsaturated fatty acid

Introduction

Conjugated linoleic acid (CLA) is a general name for a mixture of isomers of

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linoleic acid with conjugated double bonds at position of 9 and 11 or 10 and 12. CLA is present mainly in dairy products such as milk fat, natural and processed cheeses, and beef (1, 2). It has also been detected in human serum and bile (3). CLA is considered as functional foods and medicinal constituents because it has important physiological properties including, the reduction of fat deposition and body weight gain, hypocholestrolemic and antiatherogenic effects (4-6). Especially, the anticarcinogenic effects of CLA were reported in animal experimental models and cultured cells. Chemoprotective properties of dietary CLA were observed for benzo(a)pyrene-induced mouse forestomach neoplasia (7), and 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mouse epidermal tumors (8). Visonneau *et al*. (9) and Cesano *et al*. (10) also reported that CLA suppressed the growth of human breast adenocarcinoma cells in SCID mice. In cell culture studies, CLA inhibited the proliferation of human malignant melanoma, colorectal and breast cancer cells (11, 12). Although the lipid peroxidation was involved in the anticarcinogenic activity of CLA (13, 14), the potential mechanism for the inhibition of cancer cell growth by CLA has not been elucidated so far. However, the interference of eicosanoid metabolism was suggested (7, 12). It was reported that a part of CLA administered to rats was elongated and desaturated (15). These conjugated and polyunsaturated fatty acids (CPUFAs) may have stronger anticarcinogenic properties because their structures are more simulative to that of arachidonic acid than CLA. However, the anticarcinogenic effects have not been studied for CPUFAs containing conjugated triene and tetraene besides conjugated diene.

In this study, the cytotoxicity of various CPUFAs was assessed using cultured human breast (MCF-7) and colorectal (HT-29) cancer cells.

Materials and Methods

Conjugated and polyunsaturated fatty acids (CPUFAs)

CLA was purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A) CLA was a mixture of the following isomers : 9, 11-isomer, 49% ; 10, 12-isomer, 51%. Conjugated arachidonic acid (CAA), eicosapentaenoic acid (CEPA) and docosa-hexaenoic acid (CDHA) were prepared from the corresponding polyunsaturated fatty acids (PUFAs) by reacting them with potassium hydroxide in diethyleneg-lycol at 150°C for 35 min in vacuo. These CPUFAs were purified using a Sep-Pak silica cartridge column with diethyl ether/n-hexane (10 : 90, vol/vol). The composition of fatty acids with conjugated diene, triene, tetraene, and pentaene in CAA, CEPA and CDHA estimated by the spectrophotometric method (16) is shown in Table 1. Free fatty acids obtained from tung oil and balsam seed oil by saponification were used as examples of PUFAs with conjugated triene and tetraene. Fatty acid composition of tung oil and balsam seed oil was estimated

TABLE 1. *Isomeric Composition* (%) *in Conjugated Arachidonic* (*CAA*),
Eicosapentaenoic (*CEPA*), *and Docosahexaenoic Acids* (*CDHA*)

	C2 (%)	C3 (%)	C4 (%)	C5 (%)	Total (%)
CAA	46.7	23.3	2.1	0.0	72.0
CEPA	48.9	27.7	14.1	3.3	93.9
CDHA	52.4	23.0	17.0	7.6	100.0

C2 : conjugated diene fatty acid
C3 : conjugated triene fatty acid
C4 : conjugated tetraene fatty acid
C5 : conjugated pentaene fatty acid

TABLE 2. *Fatty Acid Composition* (%) *of Tung Oil and Balsam Seed Oil*

Fatty acid	Tung oil	Balsam seed oil
16 : 0	2.2	6.4
16 : 1 (n-7)	—	0.3
18 : 0	1.9	5.1
18 : 1 (n-9)	5.9	24.9
18 : 2 (n-6)	7.6	14.9
18 : 3 (n-3)	—	25.1
18 : 3 (conjugated diene)	—	0.3
18 : 3 (conjugated triene)	79.5	1.2
18 : 4 (conjugated tetraene)	—	20.4
Others	2.8	1.3

by a gas chromatographic method (Table 2). Tung oil contained 80% α-eleostearic acid (*cis*-9, *trans*-11, *trans*-13-octadecatrienoic acid) while balsam seed oil contained 20% *cis*-parinaric acid (*cis*-9, *trans*-11, *trans*-13, *trans*-15-octadecatetraenoic acid).

All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except for DL-α-tocopherol (Eisai Co., Tokyo, Japan).

Cell lines and culture condition

Human breast cancer cell MCF-7 was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Human colorectal cancer cell HT-29 was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The MCF-7 and HT-29 cancer cells were grown in the RPMI-1640 containing 10% FBS, 10,000 U/ml penicillin and 10,000 μg/ml streptomycin in Falcon T-25 cm^2 flasks and maintained at 37°C in a humidified atmosphere of 5% CO_2. The pH of the medium was maintained at 7.2–7.4.

Normal human epidermal keratinocyte (NHEK) was purchased from Kurabo Biomedical Business (Osaka, Japan). NHEK cell was grown in the HuMedia-KG2 in Falcon T-25 cm^2 flasks at 37°C in an atmosphere of 5% CO_2 as well as the human cancer cells (12).

Cytotoxic test

Each CPUFA was dissolved in ethanol and was then added to the medium at various concentrations of 1 to 100 μM (final ethanol concentration was 0.1%) with or without α-tocopherol (0.01 to 1 μM). The cells were cultured at 37°C in 5% CO_2 for 24 h to 72 h. Viable cells were determined by measuring the absorbance at 490 nm after incubation with the 2, 3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide (XTT) as a color reagent at 37°C in 5% CO_2 for 4 h (17).

Thiobarbituric acid (TBA) test

TBA test was done by the same method which Schonberg and Krokan have used (14).

All tests were performed in six parallel runs. Data are described as averages $\pm$ standard deviations (SD), and were statistically analyzed by Scheffe's F-test ($p < 0.05$).

Results

Cytotoxicity of CPUFAs for MCF-7 and HT-29 cells

At first, the effects of nonconjugated PUFAs such as arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) were investigated on the growth of MCF-7 and HT-29 cells. The ratio of viable cells in MCF-7 and HT-29 cells incubated in the culture medium with supplementation of AA, EPA or DHA (100 μM) for 72 h was measured. All natural and non-conjugated PUFAs of AA, EPA and DHA hardly inhibited the growth of MCF-7 and HT-29 cells. No cytotoxicity of AA, EPA and DHA was observed for human cancer cells in this experiment.

MCF-7 and HT-29 cells were incubated with CPUFAs (CAA, CEPA, CDHA, fatty acids of tung oil and balsam seed oil) for 24, 48 and 72 h. The ratio of viable cells in MCF-7 cells after incubation with CPUFAs was compared to the control (Fig. 1). The cytotoxicity of CPUFAs on the MCF-7 cell depended upon the incubation time and the concentration. CPUFAs substantially inhibited the growth of the MCF-7 cell at 100 μM except for the balsam seed oil fatty acids. However, the inhibitory effect of CPUFAs was not observed for the cancer cell cultured for 24 h at 10 μM. MCF-7 cells did not survey after 48 h-incubation with 100 μM CAA, CEPA, CDHA and tung oil fatty acids. Especially, CEPA
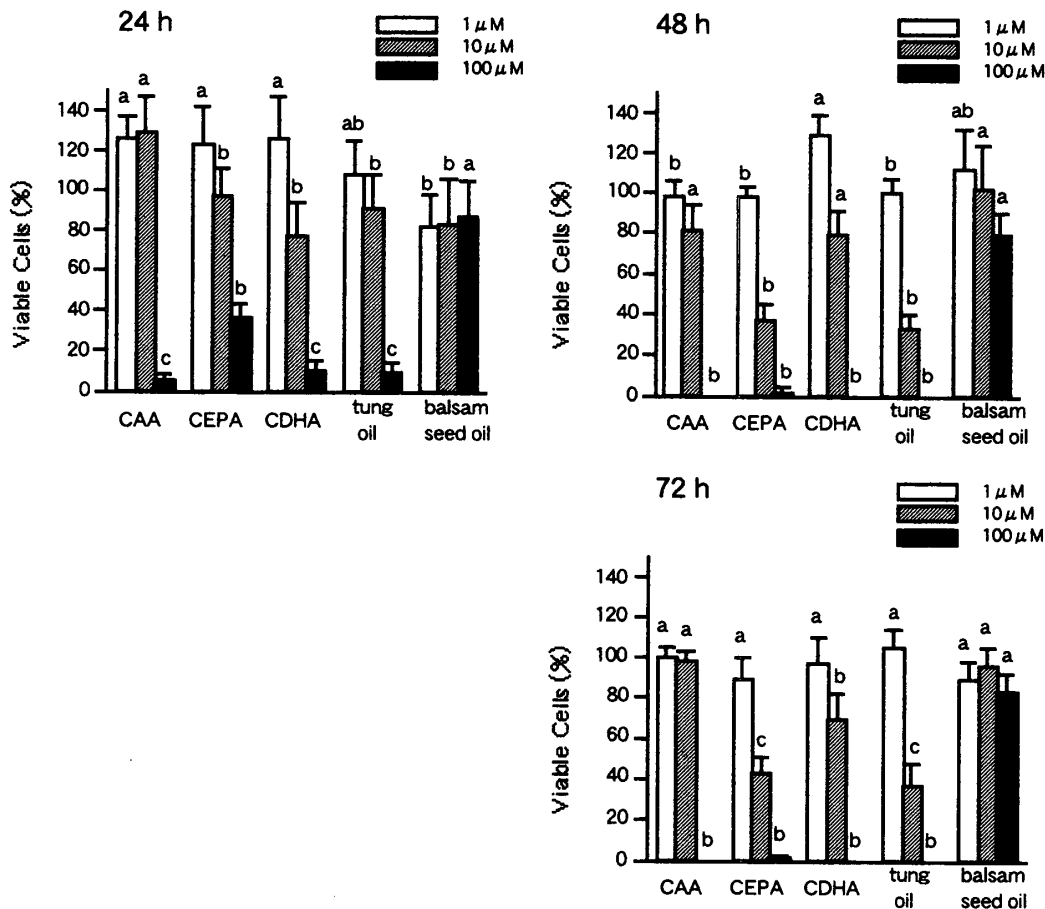
FIG. 1. The inhibitory effect of conjugated and polyunsaturated fatty acids (CPUFAs) on the growth of human breast cancer cell MCF-7. MCF-7 cell was incubated with CPUFAs at the concentration of 1 to 100 μM for 24 h to 72 h. Viability was determined by XTT methods. Values with different superscripts are significantly different ($p < 0.05$) between fatty acids and control at the same concentration. Abbreviations: CAA, conjugated arachidonic acid; CDHA, conjugated docosahexaenoic acid; CEPA, conjugated eicosapentaenoic acid; CPUFAs, conjugated polyunsaturated fatty acids; XTT, 2, 3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide.

and tung oil fatty acids were more toxic to MCF-7 cell than CAA and CDHA. Both CEPA and tung oil fatty acids inhibited the growth of MCF-7 by about 60% after 48 and 72 h-cultivation at the concentration of 10 μM.

The comparative cytotoxicity of CPUFAs to HT-29 cell is shown in Fig. 2. All CPUFAs strongly inhibited the growth of the HT-29 cells except for the balsam seed oil fatty acids at the concentration of 10 μM for 48 h and 72 h-cultivation. Balsam seed oil fatty acids are toxic to HT-29 cells at 100 μM although it did not inhibit the growth of MCF-7 cells at the same concentration. CEPA and tung oil fatty acids showed the strongest cytotoxicity among CPUFAs
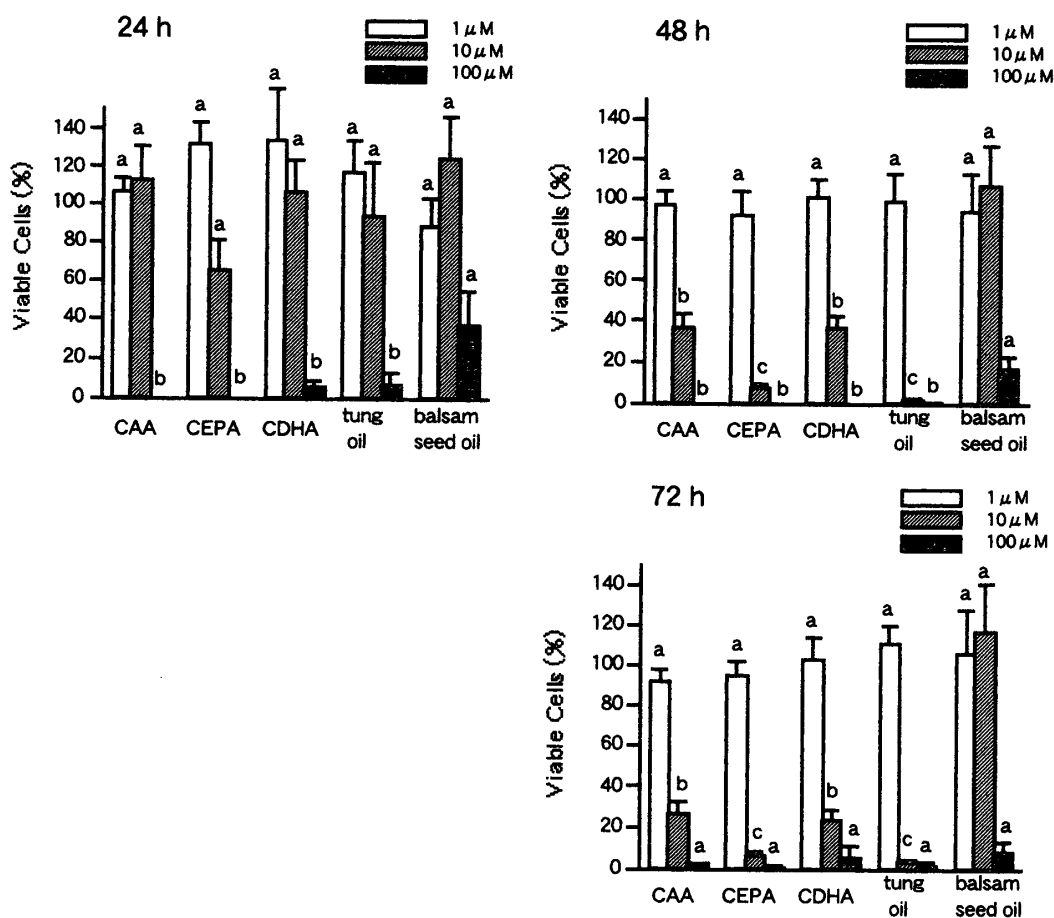
Fig. 2 The inhibitory effect of conjugated and polyunsaturated fatty acids
(CPUFAs) on the growth of human colorectal cancer cell HT-29. HT-29 cell
was incubated with CPUFAs at the concentration of 1 to 100 μM for 24 h to
72 h. Viability was determined by XTT methods. Values with different
superscripts are significantly different ($p < 0.05$) between fatty acids and
control at the same concentration. Abbreviations are shown in Fig. 1.

tested at the concentration of 10 μM and they completely killed HT-29 cells after
incubation for 48 h.

Lipid peroxidation and cytotoxicity of MCF-7 and HT-29 cells

The cytotoxic action of some conjugated fatty acids and PUFAs was reported
to be in part due to lipid peroxidation (16-18). Since the cytotoxicity of
CPUFAs might also be related to lipid peroxidation, the formation of TBA-
reacting compounds, secondary lipid oxidation products, was investigated for
MCF-7 and HT-29 cells. TBA values of MCF-7 and HT-29 cells incubated with
100 μM CEPA and tung oil fatty acids for 24 h were 0.9~1.1 nmol/culture, and
they were 1.7~2.1 times as high as control (0.5 nmol/culture). TBA value of
MCF-7 and HT-29 cells after the incubation was higher with increased CPUFAs
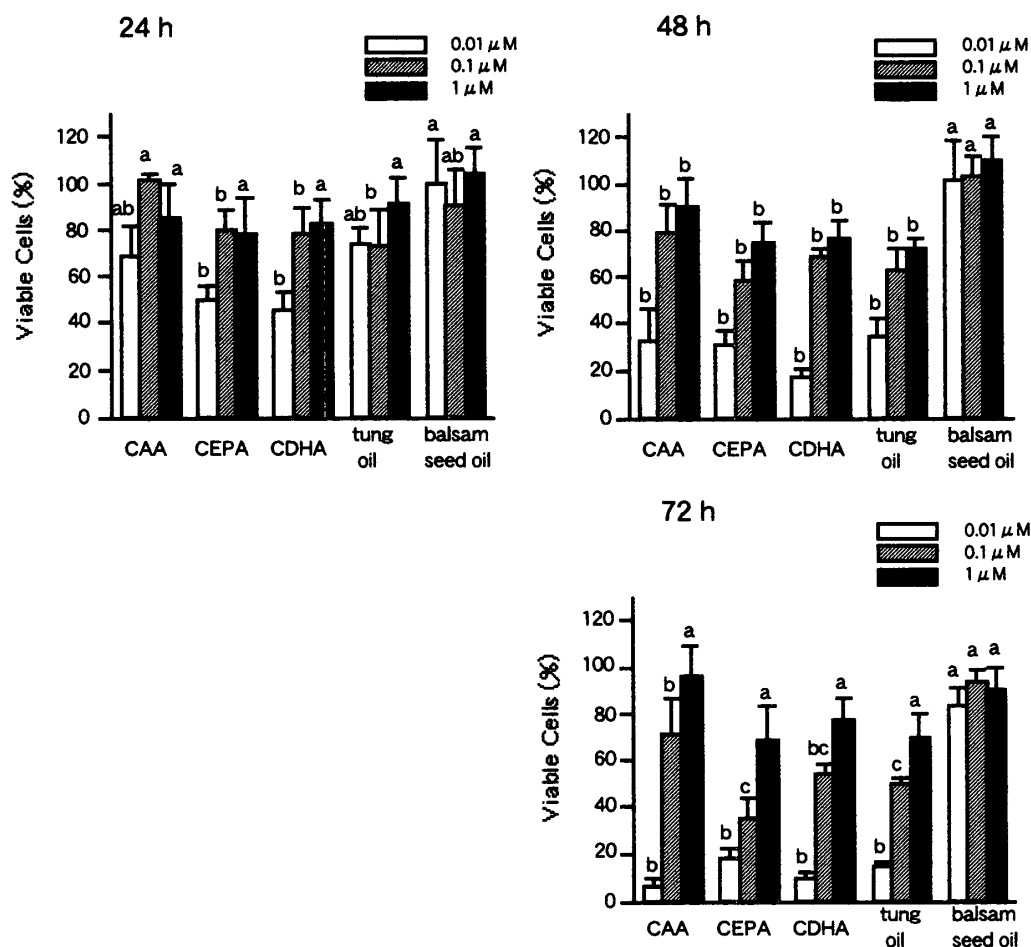
Fig. 3 The effect of α-tocopherol on the growth of human breast cancer cell MCF-7 in the presence of CPUFAs. MCF-7 cell was incubated with α-tocopherol at the concentrations of 0.01 to 1.0 μM together with 100 μM CPUFAs for 24 h to 72 h. Viability was determined by XTT methods. Values with different superscripts are significantly different ($p < 0.05$) between fatty acids and control at the same concentration. Abbreviations are shown in Fig. 1.

(data not shown).

Effect of antioxidants on the growth of MCF-7 and HT-29 cells incubated with CPUFAs were investigated using α-tocopherol (Figs. 3 and 4). α-Tocopherol was added to the culture medium containing 100 μM CPUFAs at the range of 0.01 to 1.0 μM. α-Tocopherol did not affect the growth of MCF-7 cells at 1.0 μM when incubated without CPUFAs. However, the viability of MCF-7 cells incubated with CPUFAs increased with the concentration of α-tocopherol, as shown in Fig. 3. These results suggested that the cytotoxicity of CPUFAs to MCF-7 cells was mainly due to lipid peroxidation during cultivation.

Figure 4 shows the viability of HT-29 cells incubated for 24 h to 72 h after supplementation of α-tocopherol together with CPUFAs. The viability of
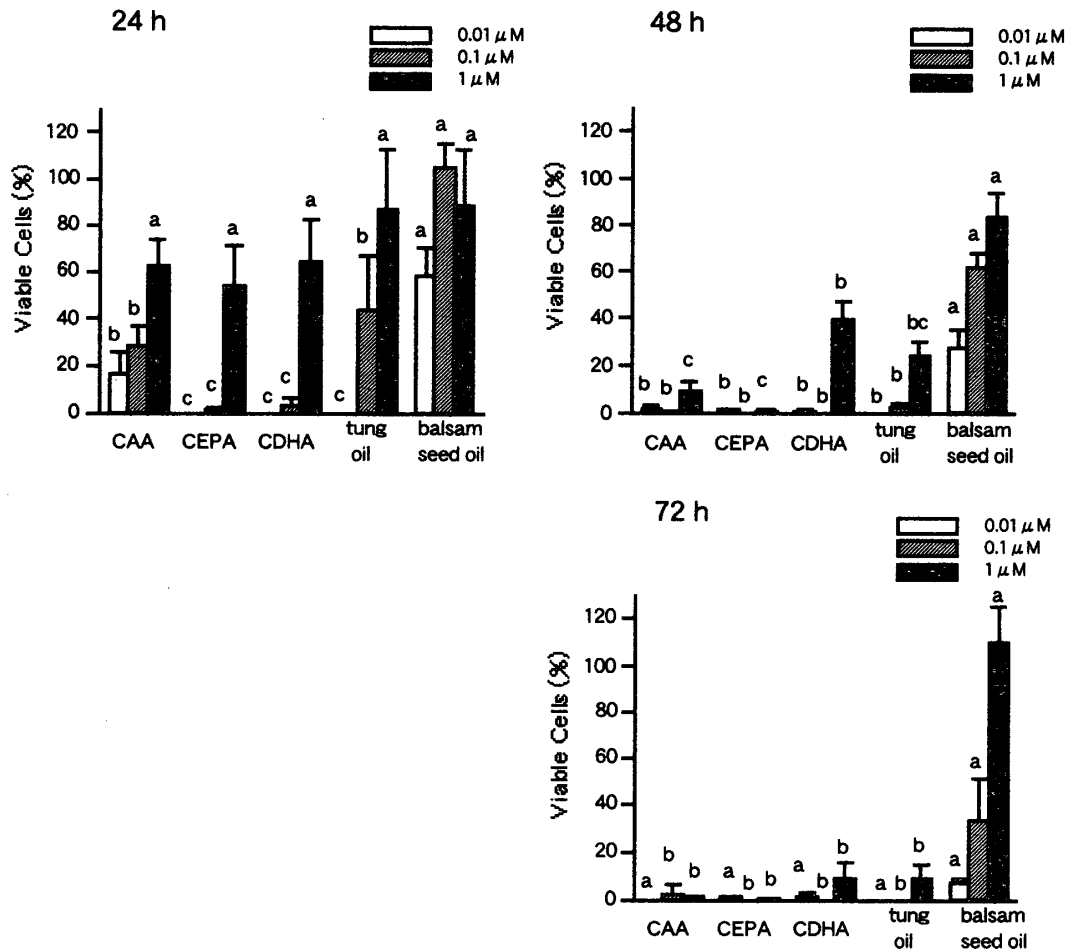
FIG. 4   The effect of α-tocopherol on the growth of human colorectal cancer cell
HT-29 in the presence of CPUFAs. HT-29 cell was incubated with α-
tocopherol at the concentrations of 0.01 to 1.0 μM together with 100 μM
CPUFAs for 24 h to 72 h. Viability was determined by XTT methods.
Values with different superscripts are significantly different ($p < 0.05$) between
fatty acids and control at the same concentration. Abbreviations are shown
in FIG. 1.

HT-29 cells incubated with CPUFAs was very low, regardless of addition of the
α-tocopherol. Most HT-29 cells were dead after 72 h-incubation. The mecha-
nism of cytotoxicity of CPUFAs to HT-29 cells may be different from that of
MCF-7 cells.

Cytotoxicity of CPUFAs for normal human cells

Normal human cells (NHEK) were incubated with CEPA and tung oil fatty
acid (100 μM) for 24 h to understand the effects of CPUFAs on the growth of
normal human cells. The viability of NHEK cell was about 60% of the control
after incubation with CEPA and tung oil fatty acids. The inhibitory effects of
CEPA and tung oil fatty acids on the growth of normal cells were lower than those

of cancer cells. These results showed that human cancer cells could be sensitive to the cytotoxicity of CPUFAs.

Discussion

Antitumor effects of PUFAs such as EPA and DHA have been reported on cultured cells (13, 19, 20). Begin *et al.* (19) found the selective cytotoxicity of γ-linolenic acid (GLA), dihomo-γ-linolenic acid, AA, and EPA to human breast and lung cancer cells. Das (13) reported the cytotoxicity of AA, GLA and EPA to human breast cancer cell ZR-75-1. Ramesh and Das (20) also observed that *cis*-PUFAs inhibited the growth of methylcnolanthrene-induced sarcoma cells. Especially, DHA had the strongest inhibitory effect. However, the cytotoxicity of PUFAs was not observed for human breast (MCF-7) and colorectal (HT-29) cancer cells in this experiment. Therefore, the inhibitory effects of CPUFAs on the growth of both MCF-7 and HT-29 cells were investigated. All CPUFAs except for balsam seed oil fatty acids strongly inhibited the growth of MCF-7 and HT-29 cells at 100 μM although the inhibitory effect of CLA was not observed in this level. Shultz *et al.* (11) reported the potential inhibitory effect of CLA on the growth of MCF-7 and HT-29 cells. Our results were different from theirs. They incubated MCF-7 and HT-29 cells in the culture medium supplemented with CLA for more than 8 days whereas we incubated them for less than 3 days. The antitumor effect of CLA may need a long-time incubation.

The order of inhibitory potency of CPUFAs on the growth of the MCF-7 cell was CEPA, tung oil fatty acids > CDHA > CAA. The inhibitory effect was not observed for balsam seed oil fatty acids in this experiment although Cornelius *et al.* (18) found the cytotoxicity of *cis*-parinaric acid (PA) being one component of balsam seed oil fatty acids for cultured malignant cells. Fatty acids, other than PA, present in balsam seed oil fatty acids might reduce the cytotoxicity of PA.

On the other hand, the order of inhibitory potency of CPUFAs on HT-29 cells was CEPA, tung oil fatty acids > CDHA, CAA > balsam seed oil fatty acids. CEPA and tung oil fatty acids showed strong inhibitory effects on the proliferation of both MCF-7 and HT-29 cells. These observations showed that the carbon number and the double bond in CPUFAs were not always proportional to their inhibitory potency. The composition of conjugated compounds in CEPA was compared with those in CAA and CDHA. As shown in Table 1, the content of conjugated triene fatty acids in CEPA (28%) was more than those of CAA (23%) and CDHA (23%) although the main components of CEPA, CAA and CDHA were conjugated diene fatty acids. Moreover, a major component of tung oil fatty acids was octadecatrienoic acid with conjugated triene (80%), as shown in Table 2. Therefore, the cytotoxicity of CPUFAs to human cancer cells may be mainly due to conjugated triene fatty acids contained in CPUFAs.

In order to know the mechanism of the antitumor effect of CPUFAs, MCF-7 and HT-29 cancer cells were incubated in the culture medium supplemented with α-tocopherol together with CPUFAs. Cornelius et al. (18) reported that the cytotoxicity of PA to human monocytic leukemia was reduced by BHT acting as an artificial antioxidant. Schonberg and Krokan (14) found that malondialdehydes being lipid peroxidation products were formed in human lung adenocarcinoma cell after incubation with CLA. They also reported that the antitumor effect of CLA and the formation of malondialdehyde were inhibited by the addition of vitamin E (α-tocopherol). Moreover, the prooxidant activity and unstability of CLA was reported in a neat system (21, 22). Igarashi and Miyazawa (23) have reported that CEPA and CDHA could induce apoptosis due to lipid peroxidation on cultured human tumor cells including hepatoma (HepG2), lung (A-549) and colorectal (DLD-1) adenocarcinoma. We also observed that prooxidant effect and cytotoxicity of CPUFAs on MCF-7 cells could be reduced by the addition of α-tocopherol. Especially, the strongly inhibitory effect of tung oil fatty acids on the growth of MCF-7 was reduced by additional α-tocopherol. Peroxidation products or prooxidant actions of CPUFAs including tung oil fatty acids could be related in their cytotoxicity to the MCF-7 cell.

On the other hand, the inhibitory effect of CPUFAs on the growth of the HT-29 cell could not be reduced by α-tocopherol. The cytotoxicity of CPUFAs to HT-29 cells may not be always due to lipid peroxidation. It was reported that CLA could modulate the eicosanoid biosynthesis (5, 12). The stronger antitumor effect of CPUFAs including tung oil fatty acids may be related in part in eicosanoid metabolism in cancer cells, but the mechanism is unknown at the present.

In conclusion, CPUFAs such as CEPA, CDHA and tung oil fatty acids are expected as functional foods and medicines with potentially anticarcinogenic properties because they are selectively cytotoxic to human cancer cells.

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