

Basic nutritional investigation

## $\omega$ -3 Polyunsaturated fatty acids and ionizing radiation: combined cytotoxicity on human colorectal adenocarcinoma cells

Gaëlle Benais-Pont, Ph.D.<sup>a</sup>, Yves M. Dupertuis, Ph.D.<sup>a</sup>, Michel P. Kossovsky, M.D.<sup>b</sup>,  
Philippe Nouet, Ph.D.<sup>c</sup>, Abdelkarim S. Allal, M.D.<sup>c</sup>, Franz Buchegger, M.D.<sup>d</sup>,  
and Claude Pichard, M.D., Ph.D.<sup>a,\*</sup>

<sup>a</sup> *Clinical Nutrition, Geneva University Hospital, Geneva, Switzerland*

<sup>b</sup> *Hospital Care System Research and Analysis Group, Geneva University Hospital, Geneva, Switzerland*

<sup>c</sup> *Radio-oncology, Geneva University Hospital, Geneva, Switzerland*

<sup>d</sup> *Nuclear Medicine, Geneva University Hospital, Geneva, Switzerland*

Manuscript received February 13, 2006; accepted May 10, 2006.

### Abstract

**Objective:** This study evaluated whether  $\omega$ -3 polyunsaturated fatty acids (PUFAs) could enhance the radiosensitivity of three different human colorectal adenocarcinoma cell lines. To understand the underlying mechanisms, the effects of  $\omega$ -3 PUFAs on the cell growth, survival, and apoptosis were evaluated alone or in combination with an antioxidant (vitamin E) and compared with the effects of  $\omega$ -6 PUFAs.

**Methods:** LS174T, CO112, and Caco-2 cell survival was assessed by clonogenic assay after a 3-d pretreatment with  $\omega$ -3/ $\omega$ -6 PUFAs and/or vitamin E before a single X-ray exposure to 4 Gy. Cell growth and viability were measured by double fluorescence-activated cell sorter analyses using propidium iodide and fluorescein isothiocyanate-conjugated annexin V. Student's *t* test or multivariable linear regression analyses were used for comparison.

**Results:** Preincubation with 30 to 100  $\mu$ mol/L of  $\omega$ -3 PUFAs induced a dose-dependent additive decrease in cell survival after irradiation ( $P < 0.05$ ). Evaluation of the underlying mechanisms indicated that  $\omega$ -3 PUFAs mainly decreased the cell number via apoptosis induction. Moreover, formation of lipid peroxidation products and modulation of cyclooxygenase II activity seemed to be involved, because coinubation with 10  $\mu$ mol/L vitamin E abolished the effect of 50  $\mu$ mol/L of  $\omega$ -3 PUFAs ( $P < 0.05$ ), whereas  $\omega$ -6 PUFAs could partly mimic  $\omega$ -3 PUFA effects.

**Conclusion:** These observations suggest that  $\omega$ -3 PUFAs may be potential candidates as nutritional adjuvants to enhance the efficacy of human colorectal cancer radiotherapy. © 2006 Elsevier Inc. All rights reserved.

**Keywords:**  $\omega$ -3 Polyunsaturated fatty acids; Ionizing radiation; Vitamin E; Lipid peroxidation; Human colorectal adenocarcinoma cell lines

### Introduction

Colorectal cancer, with about 1 million cases worldwide diagnosed per year, is one of the most prevalent

This study was supported by a grant of the European Society for Clinical Nutrition and Metabolism (ESPEN-ABBOTT 2004 research grant) and the Nutrition 2000Plus Foundation.

Gaëlle Benais-Pont, Ph.D., and Yves M. Dupertuis, Ph.D., contributed equally to this study.

\* Corresponding author: Tel.: +41-22-372-9345; fax: +41-22-372-9363.

E-mail address: [claudio.pichard@medecine.unige.ch](mailto:claudio.pichard@medecine.unige.ch) (C. Pichard).

malignancies and the second cause of cancers in industrialized countries [1–3]. The main curative strategy remains complete surgical resection of the tumor associated with radiotherapy and/or chemotherapy. Unfortunately, colorectal cancer remains often refractory to these classic therapies and 85% of patients relapse within the first 2 y after surgery and display local recurrence or metastasis [4]. In consequence, the search for new therapeutic modalities with minimal toxicity is of particular interest in colon cancer management.

Recent findings have suggested that  $\omega$ -3 polyunsaturated fatty acids (PUFAs) might be of use as adjuvant for

cancer therapy. In addition to inhibiting the progression of several prevalent cancers [5,6],  $\omega$ -3 PUFAs have been found to enhance the sensitivity of tumor cells to several anticancer drugs, such as mitomycin C, cyclophosphamide, and doxorubicin [7–9]. Regarding colorectal cancer, combination of  $\omega$ -3 PUFA-containing triacylglycerol emulsion with 5-fluorouracil resulted in an additive growth inhibitory effect on the Caco-2 cell line [10].

To date the mechanism by which  $\omega$ -3 PUFAs modulate tumor sensitivity to anticancer drugs is not completely elucidated. The biological effects of  $\omega$ -3 PUFAs are frequently attributed to competitive inhibition of eicosanoid production from  $\omega$ -6 PUFA precursors [5]. This may be of particular value in colorectal cancer, where ectopic overexpression of cyclo-oxygenase-2 (COX-2) has been reported to increase prostaglandin E<sub>2</sub> synthesis and to suppress apoptosis through Bcl-2 induction [11]. However, accumulation of  $\omega$ -3 PUFAs in the cell membrane may act at numerous levels, including membrane fluidity, receptor binding, signal transduction, and enzyme activity [5]. It has been suggested that drug transport in tumor cells is correlated with the unsaturation index in membrane phospholipids [12]. In addition, an increase in the unsaturation index may result in greater susceptibility of the tumor to oxidative stress, which may explain why  $\omega$ -3 PUFAs enhance tumor sensitivity to anticancer drugs that generate reactive oxygen species (ROS) [9]. This latter hypothesis is supported by recent findings demonstrating that  $\omega$ -3 PUFAs also enhanced the antitumor effect of ionizing radiation in animal models of head and neck [13] and mammary [14] tumors.

Combination of  $\omega$ -3 PUFAs with radiotherapy may be of particular interest, because the enhanced antitumor effect has been also associated with a reduction of mucosal/epidermal radiotoxicity and a decrease in proinflammatory COX-2 expression in HEP-2 human carcinoma xenografted mice [13]. To our knowledge, there is no study investigating the combined effect of  $\omega$ -3 PUFAs with radiotherapy on colorectal cancer, despite its favorable location being susceptible to dietary intervention.

Therefore, the present study evaluated the interaction between  $\omega$ -3 PUFAs and X-rays on the growth and survival of three human colorectal adenocarcinoma cell lines, which display different radiosensitivities and enterocytic properties. To discriminate between COX-2 pathway modulation and oxidative stress induction, we compared the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with those of arachidonic acid (AA) and challenged them with vitamin E.

Our investigations provide new insight into understanding how  $\omega$ -3 PUFAs mediate their suppressive action on tumor cells and determine whether they could enhance the efficacy of human colorectal cancer radiotherapy.

## Materials and methods

### Cell lines and culture

Three human colorectal adenocarcinoma cell lines, which differ in genetic pattern expression, doubling time, and enterocytic differentiation, were chosen according to the classification of Chantret et al. [15]. The poorly differentiated LS174T and the well-differentiated Caco-2 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Radiosensitive LS174T and Caco-2 cell lines were compared with the radioresistant CO112 cell line (from our laboratory) [16]. They were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, L-glutamine (0.3 mg/L), and penicillin-streptomycin (0.1 mg/mL; all from Life Technologies Inc., Grand Island, NY, USA). The medium was changed twice a week and cells were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub> and maintained in exponential growth phase.

### Cell treatment

The effect of EPA, DHA, and AA was evaluated and challenged with  $\alpha$ -tocopherol palmitate (vitamin E; all from Sigma Chemie AG, Buchs, Switzerland). They were all stored at 10 g/L in ethanol (–20°C). For the experiments, cells were seeded in 24-well plates or 50-mL tissue culture flasks at a density on the order of 10<sup>4</sup> cells/well and 10<sup>5</sup> cells/flask, respectively. After a 3-d incubation in standard culture medium, cells were incubated for 1 to 4 d in culture medium supplemented with 1 to 100  $\mu$ mol/L of PUFAs and/or vitamin E before exposure to a single ionizing radiation dose of 4 Gy, using a linear beam accelerator (X-ray 6 MV). Cell culture medium was daily renewed to maintain adequate nutrient concentrations. In control conditions, ethanol was added in standard culture medium at concentrations similar to those present in the different experimental conditions ( $\leq$ 0.3%, v/v).

### Cell growth

At different treatment days, cells were detached with trypsin/ethylene-diaminetetra-acetic acid (EDTA) 1 $\times$ , resuspended in 400  $\mu$ L of phosphate buffered saline (PBS) and stained with 5  $\mu$ g/mL of propidium iodide (PI; Sigma Aldrich, Buchs, Switzerland) to discriminate between live and dead cells. Fluorescence-activated cell sorter analysis was performed within the following hour by using a flow cytometer (FACStrack, Becton Dickinson, Franklin Lakes, NJ, USA) with a fixed counting time of 30 s, after calibration with a hemocytometer. The population doubling time (PDT) of the different cell lines was calculated in the exponential growth phase as:

$$\text{PDT} = 1/3.32 * [\log (\text{cell number at } t_2) - \log (\text{cell number at } t_1)] / (t_2 - t_1)$$

### Cell survival

Cell survival was evaluated after different treatments with a clonogenic assay. In brief, cells were detached with trypsin/EDTA 1×, resuspended in standard medium, and counted as previously described. The cell suspension was serially diluted and an appropriate number of cells known to produce about 200 colonies from preliminary assays were seeded into 100- × 20-mm tissue culture dishes in 10 mL of fresh PUFA-free culture medium. After a 14-d incubation at 37°C, dishes were washed with PBS and cells were fixed and stained with 0.5% crystal violet in methanol/acetic acid (3:1, v/v). Colonies containing ≥50 cells were counted to compare the different treatments. The survival fraction was calculated relative to untreated controls ( $S/S_0$ ).

### Apoptosis induction

Cells were doubly stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI using an apoptosis detection kit (BD Biosciences/Pharmingen, Basel, Switzerland) according to the manufacturer's instructions. After different treatments, floating cells were collected with the supernatant, and adherent cells were washed twice with PBS<sup>Ca<sup>-</sup></sup>, Mg<sup>-</sup> and detached with trypsin 10% in 5 mmol/L (v/v) of PBS/EDTA. Floating and detached cells were then pooled, counted, and resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4; 140 mmol/L of NaCl; 2.5 mmol/L of CaCl<sub>2</sub>) at a concentration of  $1 \times 10^6$  cells/mL. Aliquots of 0.1 mL were stained with 5 μL of annexin V-FITC and 5 μL of PI at room temperature for 15 min in the dark. These were then diluted in 400 μL of binding buffer and analyzed within the following 30 min with a flow cytometer (FACStrack, Becton Dickinson). To set up compensation and quadrants, unstained cells, cells stained with annexin V-FITC alone, and cells stained with PI alone were used as controls. Untreated controls were used to define the basal level of apoptotic and dead cells.

### Data analysis

For all flow cytometric experiments, data acquisition and analysis were performed with CellQuest software (Becton Dickinson). All experiments were performed in triplicate, and results were computed as mean ± SD. Statistical significance was determined with Student's *t* test for comparison of means or a multivariable linear regression analysis in which an interaction term was introduced to assess combined effects of PUFAs and radiotherapy. Significance was considered as meaningful at  $P \leq 0.05$ .

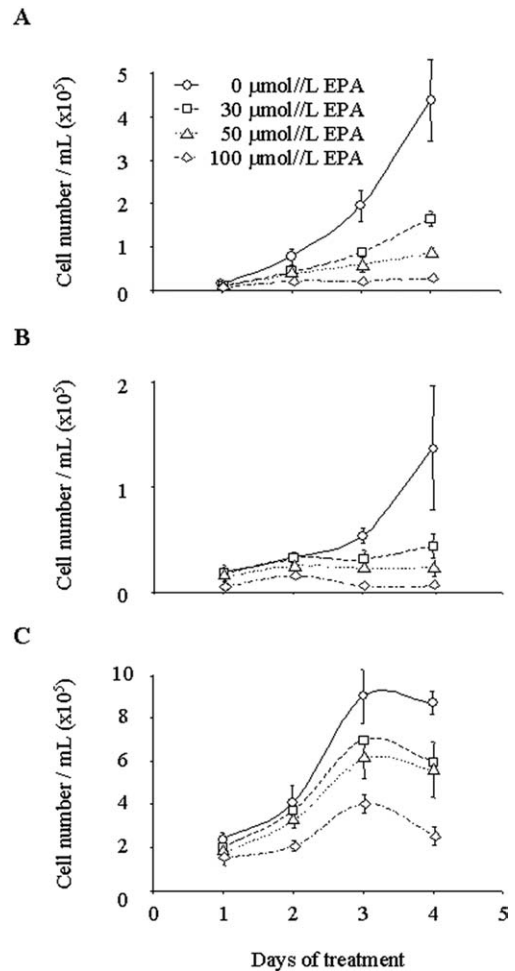


Fig. 1. Evaluation of the growth of LS174T (A), CO112 (B), and Caco-2 (C) cells after a 1- to 4-d incubation with 30, 50, or 100 μmol/L of EPA (renewed daily) using a flow cytometer. Values are means ± SD ( $n = 3$ ). EPA, eicosapentaenoic acid.

## Results

### Cell growth after EPA supplementation

The growth of LS174T (Fig. 1A), CO112 (Fig. 1B), and Caco-2 (Fig. 1C) cells was measured during an incubation of 4 d in culture medium supplemented with EPA. In control condition with EPA-free medium, the most rapidly growing cell line was LS174T (PDT 19 h), whereas CO112 (PDT 23 h) and Caco-2 (PDT 25 h) grew more slowly. A time- and dose-dependent inhibition of cell growth was observed for all cell lines at EPA concentrations ranging from 30 to 100 μmol/L, whereas the effect was less marked at lower EPA concentrations (data not shown). Growth inhibition of all cell lines became significant after 3 d in the presence of 30 μmol/L of EPA (LS174T:  $55 \pm 3\%$ ,  $P < 0.01$ ; CO112:  $42 \pm 15\%$ ,  $P < 0.05$ ; Caco-2:  $22 \pm 2\%$ ,  $P < 0.05$ ) and after only 1 d in the presence of 100 μmol/L of EPA (LS174T:  $47 \pm 9\%$ ,  $P < 0.05$ ; CO112:  $71 \pm 21\%$ ,  $P = 0.06$ ; Caco-2:  $37 \pm 14\%$ ,  $P < 0.05$ ).

Table 1  
Comparative effect of a 2-d incubation with EPA, DHA, or AA on the growth of LS174T, CO112, and Caco-2 cells using a flow cytometer\*

Treatment group	LS174T	CO112	Caco-2
EPA ( $\mu\text{mol/L}$ )			
0	100.0 $\pm$ 4.6	100.0 $\pm$ 30.6	100.0 $\pm$ 28.3
10	92.0 $\pm$ 7.2 <sup>†</sup>	156.8 $\pm$ 33.2 <sup>†</sup>	75.6 $\pm$ 15.4 <sup>†</sup>
100	51.3 $\pm$ 5.3 <sup>§</sup>	47.8 $\pm$ 28.6 <sup>§</sup>	50.9 $\pm$ 24.5 <sup>§</sup>
DHA ( $\mu\text{mol/L}$ )			
0	100.0 $\pm$ 3.4	100.0 $\pm$ 8.8	100.0 $\pm$ 10.4
10	89.5 $\pm$ 8.1 <sup>†</sup>	90.5 $\pm$ 6.1	97.9 $\pm$ 19.6
100	53.7 $\pm$ 9.8 <sup>§</sup>	45.6 $\pm$ 6.1 <sup>§</sup>	57.9 $\pm$ 1.01 <sup>§</sup>
AA ( $\mu\text{mol/L}$ )			
0	100.0 $\pm$ 3.8	100.0 $\pm$ 27.8	100.0 $\pm$ 35.1
10	91.0 $\pm$ 7.0 <sup>†</sup>	90.1 $\pm$ 21.5	101.5 $\pm$ 34.8
100	63.2 $\pm$ 5.6 <sup>§</sup>	32.5 $\pm$ 6.0 <sup>§</sup>	62.6 $\pm$ 20.2 <sup>†</sup>

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

\* Values are means  $\pm$  SD ( $n = 3/\text{group}$ ), percentage of control.

<sup>†</sup>  $P < 0.05$ ;

<sup>‡</sup>  $P < 0.01$ ;

<sup>§</sup>  $P < 0.001$ , different from untreated controls (Student's  $t$  test).

### Comparison $\omega$ -3/ $\omega$ -6 PUFAs

Because antagonism between  $\omega$ -3 and  $\omega$ -6 PUFAs has been often reported, we compared the inhibitory effect of EPA and DHA with that of the  $\omega$ -6 PUFA, AA, on the growth of LS174T, CO112, and Caco-2 cells (Table 1). No significant difference could be observed among these three PUFAs when using a multivariable linear regression analysis. A 2-d incubation with 10 to 100  $\mu\text{mol/L}$  of EPA, DHA, or AA resulted into a dose-dependent inhibition of growth, which became significant at 100  $\mu\text{mol/L}$  for all cell lines ( $P < 0.05$ ). Thus,  $\omega$ -3 and  $\omega$ -6 PUFAs inhibited cell growth with the same magnitude in all cell lines and in a dose-dependent manner.

### Combination of PUFAs and ionizing radiation

Different radiosensitivities were observed from one cell line to another after exposure to a single dose of 4 Gy. Overall, LS174T and Caco-2 cells were the most sensitive, with mean decreases in cell survival of  $83 \pm 10\%$  and  $67 \pm 18\%$ , respectively, whereas CO112 cells were more radioresistant, with a mean decrease in cell survival of  $45 \pm 12\%$ . When X-rays were combined with a 3-d pretreatment with 30 to 100  $\mu\text{mol/L}$  of EPA, a dose-dependent additive decrease in cell survival was observed in all cell lines ( $P < 0.05$ ; Fig. 2). Multivariable linear regression analysis even indicated a slight supra-additive effect between X-rays and EPA pretreatment in LS174T cells at all EPA concentrations tested (Fig. 2A), in Caco-2 cells at 50 and 100  $\mu\text{mol/L}$  of EPA (Fig. 2C), and to a lesser extent in CO112 cells at 100  $\mu\text{mol/L}$  of EPA (Fig. 2B). As for EPA, a 3-d pretreatment with AA before X-rays induced a dose-dependent additive de-

crease in cell survival. However, in contrast to EPA, multivariable linear regression analysis indicated a partly additive effect on cell survival after pretreatment with 50 to 100  $\mu\text{mol/L}$  of AA ( $P < 0.05$ ) in LS174T and CO112 cell lines (Table 2).

### Interaction between PUFAs and vitamin E

As previously shown for EPA and AA, a 3-d incubation with 50  $\mu\text{mol/L}$  of DHA significantly decreased cell survival from 100% to  $50 \pm 6.9\%$  ( $P < 0.01$ ) in CO112 (Fig. 3). When combined with X-rays, DHA pretreatment

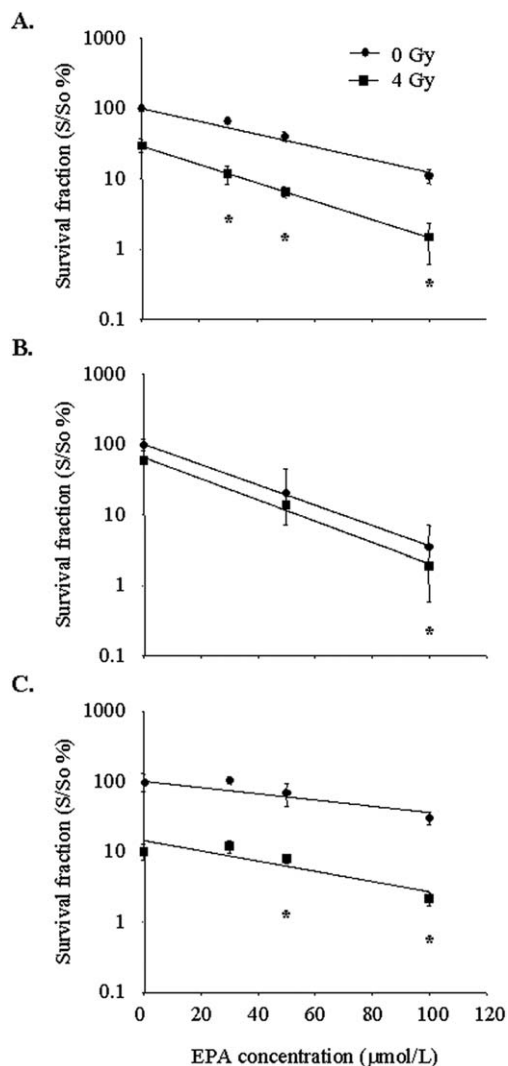


Fig. 2. Evaluation of the survival of LS174T (A), CO112 (B), and Caco-2 (C) cells after a 3-d pretreatment with 30, 50, or 100  $\mu\text{mol/L}$  of EPA (renewed daily) followed or not by X-ray exposure to 4 Gy using a 14-d clonogenic assay. Values are expressed as survival fractions (%) relative to untreated controls. Values at each concentration represent means  $\pm$  SD ( $n = 3$  in A,  $n = 9$  in B,  $n = 6$  in C). Asterisk indicates a supra-additive effect after X-rays and EPA pretreatment (multivariable linear regression analysis,  $P < 0.05$ ). EPA, eicosapentaenoic acid; S/S<sub>0</sub>, survival fraction in relation to untreated controls.

Table 2

Evaluation of the survival fraction of LS174T, CO112, and Caco-2 cells after a 3-d incubation with AA followed by X-ray exposure to 4 Gy using a 14-d clonogenic assay\*

Treatment group	LS174T	CO112	Caco-2
AA ( $\mu\text{mol/L}$ ), 0 Gy			
0	100.0 $\pm$ 17.7	100.0 $\pm$ 16	100.0 $\pm$ 33.9
0.1	95.6 $\pm$ 22.8	97.7 $\pm$ 10.8	92.7 $\pm$ 35.8
50	68.7 $\pm$ 14.6 <sup>‡</sup>	43.4 $\pm$ 31.0 <sup>‡</sup>	91.4 $\pm$ 31.7
100	52.4 $\pm$ 13.0 <sup>‡</sup>	3.5 $\pm$ 2.4 <sup>‡</sup>	42.1 $\pm$ 18.5 <sup>‡</sup>
AA ( $\mu\text{mol/L}$ ), 4 Gy			
0	17.4 $\pm$ 7.9	49.3 $\pm$ 7.8	38.2 $\pm$ 13.7
0.1	16.1 $\pm$ 4.2	53.1 $\pm$ 10.9	34.2 $\pm$ 11.3
50	13.7 $\pm$ 4.1 <sup>§</sup>	23.8 $\pm$ 15.5 <sup>§§</sup>	23.3 $\pm$ 5.3 <sup>†</sup>
100	12.2 $\pm$ 4.2 <sup>†§</sup>	2.4 $\pm$ 1.5 <sup>§§</sup>	14.3 $\pm$ 6.6 <sup>‡</sup>

AA, arachidonic acid

\* Values are means  $\pm$  SD ( $n = 9/\text{group}$ ), except for LS174T cells ( $n = 3/\text{group}$ ); percentage of control.

<sup>†</sup>  $P < 0.05$ ;

<sup>‡</sup>  $P < 0.01$ , different from untreated controls (Student's  $t$  test).

<sup>§</sup> Partly additive effect between X-rays and AA pretreatment (multivariable linear regression analysis,  $P < 0.05$ ).

induced a further decrease in cell survival from  $50 \pm 6.9\%$  to  $40 \pm 2.8\%$  ( $P < 0.05$ ). Coincubation with  $10 \mu\text{mol/L}$  of vitamin E reversed the cytotoxic effect of DHA regardless of treatment combination, but did not counteract the effect of X-rays on cell survival. Similar results were found with the other cell lines tested (data not shown).

#### Apoptosis induction by PUFAs

Compared with CO112 cells incubated in PUFA-free culture medium (Fig. 4A), a 3-d incubation with  $50 \mu\text{mol/L}$  of DHA did not induce a significant change in the number of dead cells (Fig. 4B). However, an increase in intermediate peak from  $7.7 \pm 1.3\%$  to  $26.3 \pm 0.6\%$  ( $P < 0.01$ ) between living and dead cells suggested that DHA incubation induces cell apoptosis (Fig. 4B). Although incubation with  $10 \mu\text{mol/L}$  of vitamin E alone did not affect cell viability (Fig. 4C), coincubation with vitamin E completely abolished the DHA-induced intermediate peak ( $P < 0.01$ ; Fig. 4D). Induction of apoptosis by  $\omega$ -3 PUFA pretreatment was confirmed by annexin V/FITC detection, which showed a decrease in living cells by induction of early and late apoptosis in the presence of  $50 \mu\text{mol/L}$  of DHA (Fig. 5). Such an apoptotic process was found in LS174T cells ( $P < 0.05$ ) and a same tendency was observed in Caco-2 cells (Table 3). In addition, detailed results indicated that a necrotic process might also be involved in CO112 and Caco-2 cell lines (Table 3). The addition of vitamin E counteracted apoptosis and necrosis induction by DHA in LS174T and CO112 cells and, to a lesser extent, in Caco-2 cells (Table 3).

## Discussion

Previous studies have suggested that PUFAs inhibit the development of several prevalent cancers [5,17–19] and might be used as adjuvants for cancer therapy [8–10,13,14]. Our results demonstrated that  $\omega$ -3 PUFAs are able to inhibit the proliferation of the three human colonic cell lines studied. These observations confirm previous findings showing a PUFA inhibitory effect on colorectal tumor cell growth in vitro [20,21] and in vivo in murine and human xenografted tumor models [22] and in human colorectal tumors in athymic mice [23]. Because we observed that  $\omega$ -3 PUFAs inhibited the growth of cell lines displaying different doubling times and enterocytic differentiation levels, it is probable that the underlying mechanism is a general feature independent of the cell genetic expression pattern.

The inhibitory effects of  $\omega$ -3 PUFAs have been frequently attributed to COX-2 downregulation and decreased production of prostaglandin  $E_2$  through  $\omega$ -6/ $\omega$ -3 PUFA competition [24]. In contrast to this hypothesis, our findings demonstrated that AA was able to inhibit cell growth with the same magnitude as EPA and DHA. This observation, in line with a previous work describing a PUFA-induced cell growth inhibitory effect on a colon tumor cell line that does not express COX [25], indicates that eicosanoid synthesis modulation cannot be the only mechanism of action. A COX-independent pathway involving activation of peroxisome proliferator-activated receptor- $\gamma$  was reported to play a predominant role in PUFA antitumor action by inducing cell cycle arrest and apoptosis or by reprogramming cell differentiation [26–29]. In the present study, coadministration of an antioxidant (vitamin E) counteracted the effect of DHA on cell apoptosis. Therefore, it seems likely that a

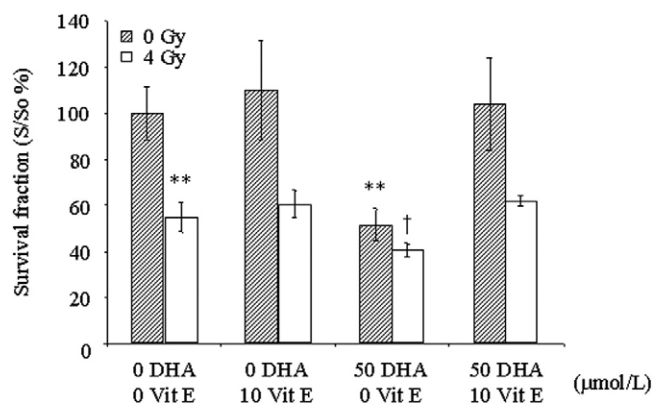


Fig. 3. Evaluation of the interference of vitamin E on DHA and/or X-ray cytotoxicity. Survival of CO112 cells was evaluated after a 3-d pretreatment with  $50 \mu\text{mol/L}$  of DHA and/or  $10 \mu\text{mol/L}$  of vitamin E followed or not by exposure to 4 Gy using a 14-d clonogenic assay. Values are expressed as survival fractions (%) relative to untreated controls. Values are means  $\pm$  SD ( $n = 3$ ). Asterisks indicate difference from untreated controls (Student's  $t$  test, \* $P < 0.05$ , \*\* $P < 0.01$ ). <sup>†</sup> $P < 0.05$ , supra-additive effect after X-rays and DHA pretreatment (multivariable linear regression analysis). DHA, docosahexaenoic acid; S/S<sub>0</sub>, survival fraction in relation to untreated controls; Vit E, vitamin E.

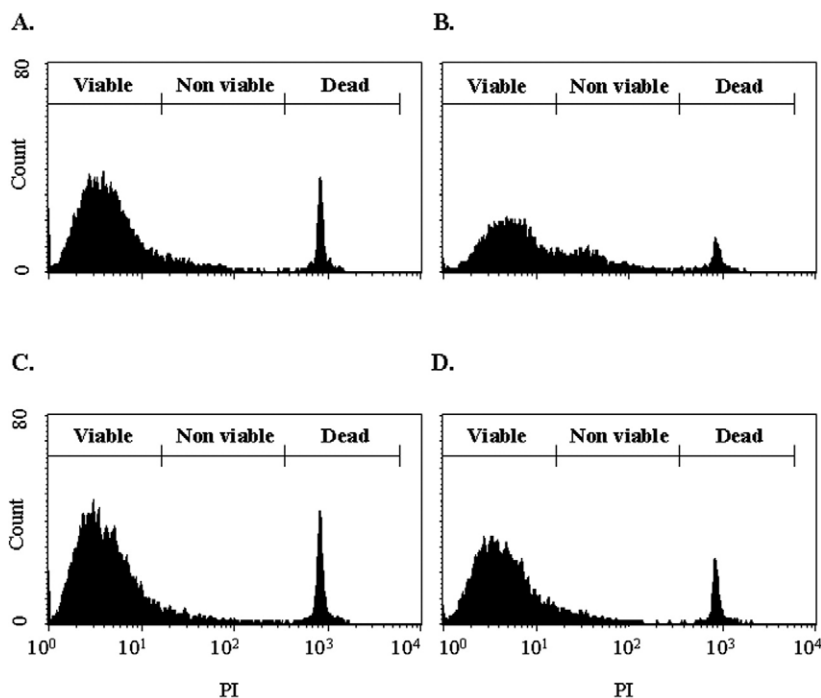


Fig. 4. Evaluation by fluorescence-activated cell sorter analysis of the interference of vitamin E on the viability of CO112 cells after treatment with docosahexaenoic acid using the dead cell discriminatory dye PI. Living, non-viable, and dead cells were sorted after a 3-d incubation in standard culture medium (A), supplemented with 50  $\mu\text{mol/L}$  of docosahexaenoic acid (B), 10  $\mu\text{mol/L}$  of vitamin E (C), or 50  $\mu\text{mol/L}$  of docosahexaenoic acid + 10  $\mu\text{mol/L}$  of vitamin E (D). PI, propidium iodide.

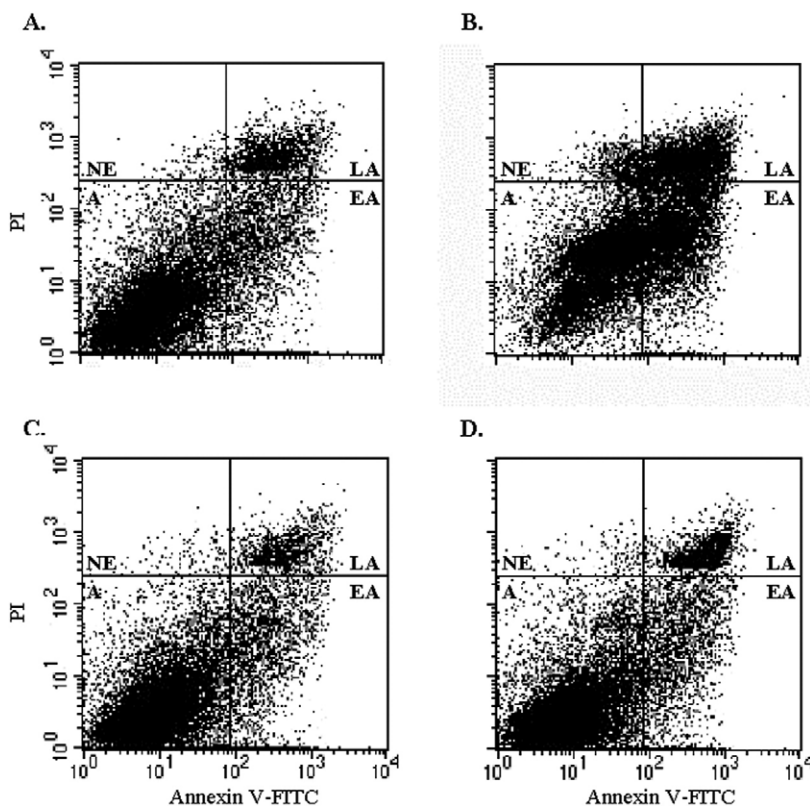


Fig. 5. Evaluation of the interference of vitamin E on apoptosis induction in CO112 cells after docosahexaenoic acid pretreatment using fluorescence-activated cell sorter analysis with FITC-conjugated annexin V and PI. Cells in NE, EA, and LA were sorted after a 3-d incubation in standard culture medium (A), supplemented with 50  $\mu\text{mol/L}$  of docosahexaenoic acid (B), 10  $\mu\text{mol/L}$  of vitamin E (C) or 50  $\mu\text{mol/L}$  of docosahexaenoic acid + 10  $\mu\text{mol/L}$  of vitamin E (D). A, alive; EA, early apoptosis; FITC, fluorescein isothiocyanate; LA, late apoptosis; NE, necrosis; PI, propidium iodide.

Table 3

Evaluation of the viability of LS174T, CO112, and Caco-2 cells after a 2-d incubation with 50  $\mu\text{mol/L}$  of DHA and/or 10  $\mu\text{mol/L}$  of vitamin E using fluorescence-activated cell sorter analysis with annexin V/fluorescein isothiocyanate labeling\*

Treatment group	Alive	Early apoptotic	Late apoptotic	Necrotic
LS174T				
Controls	75.0 $\pm$ 2.8	10.8 $\pm$ 1.0	12.7 $\pm$ 1.5	1.5 $\pm$ 0.1
Vitamin E	70.1 $\pm$ 4.2	10.5 $\pm$ 2.0	17.7 $\pm$ 2.3	1.8 $\pm$ 0.3
DHA	53.3 $\pm$ 6.2 <sup>‡</sup>	25.7 $\pm$ 2.8 <sup>‡</sup>	19.0 $\pm$ 3.6 <sup>†</sup>	1.9 $\pm$ 0.2
DHA + vitamin E	68.2 $\pm$ 1.3 <sup>‡</sup>	13.5 $\pm$ 1.4 <sup>‡</sup>	15.8 $\pm$ 1.2 <sup>†</sup>	2.4 $\pm$ 1.4
CO112				
Controls	80.4 $\pm$ 2.5	11.4 $\pm$ 0.6	7.4 $\pm$ 1.7	0.7 $\pm$ 0.3
Vitamin E	80.7 $\pm$ 1.8	10.7 $\pm$ 0.3	8.1 $\pm$ 2.0	0.6 $\pm$ 0.1
DHA	55.5 $\pm$ 4.8 <sup>‡</sup>	26.9 $\pm$ 1.2 <sup>‡</sup>	14.9 $\pm$ 3.0 <sup>‡</sup>	2.7 $\pm$ 0.7 <sup>‡</sup>
DHA + vitamin E	77.7 $\pm$ 2.1 <sup>‡</sup>	13.5 $\pm$ 1.5 <sup>‡</sup>	8.3 $\pm$ 1.0 <sup>†</sup>	0.5 $\pm$ 0.1 <sup>†</sup>
Caco-2				
Controls	92.4 $\pm$ 0.9	3.5 $\pm$ 0.8	3.2 $\pm$ 0.4	0.9 $\pm$ 0.1
Vitamin E	90.5 $\pm$ 2.5	3.9 $\pm$ 0.8	4.5 $\pm$ 2.0	1.1 $\pm$ 0.4
DHA	89.1 $\pm$ 2.3	3.8 $\pm$ 0.8	5.5 $\pm$ 1.5	1.6 $\pm$ 0.1 <sup>†</sup>
DHA + vitamin E	91.1 $\pm$ 1.5	2.9 $\pm$ 0.5	4.9 $\pm$ 0.8	0.9 $\pm$ 0.2

DHA, docosahexaenoic acid

\* Values are means  $\pm$  SD ( $n = 3/\text{group}$ ), percentage of total cell number.

<sup>†</sup>  $P < 0.05$ ;

<sup>‡</sup>  $P < 0.01$ , different from untreated controls (multivariable linear regression analysis).

mechanism involving oxidative stress also plays a major role in this phenomenon. It has been reported that PUFAs may induce cell death through generation of ROS and lipid peroxidation products [30]. This latter mechanism is supported by the ability of PUFAs to enhance the efficacy of cytotoxic drugs generating ROS synthesis [9,18] and by the finding of the direct involvement of ROS in the mechanism of PUFA-induced apoptosis in HL-60 cells [31]. In addition, lipid peroxide generation was reported to induce cell necrosis through oxidative nucleic acid base modification and DNA strand breaks [32,33]. In agreement with these findings, our results demonstrated that DHA increased early and late apoptoses and necrosis. These latter observations, associated with the ability of vitamin E to counteract PUFA cell growth inhibitory effects, indicate that lipid peroxidation is involved in PUFA antitumor properties.

It has been demonstrated that  $\omega$ -3 PUFAs increase the antitumor effect of ionizing radiation in animal models of human head and neck cancer [13] and induced mammary tumors [14]. Our results confirmed that EPA, DHA, and AA pretreatments increased colorectal cancer cell death after exposure to ionizing radiations. In addition, EPA was able to potentiate slightly the cell response to ionizing radiation. Such a supra-additive effect was reported for the ROS-generating drug procarbazine, which was described to act synergistically with ionizing radiation by reducing intracellular glutathione and cleaving DNA in vitro [34]. Recently, Higushi [33] postulated that hydroperoxides generated by PUFA peroxidation cause and amplify ROS-mediated DNA fragmentation through glutathione depletion. Because ionizing radiation produces ROS, this may explain our observation of increased cell toxicity in PUFA pretreated cells. Moreover, it may explain why cell survival inhibition was

observed for radiosensitive and radioresistant cell lines. The finding that AA pretreatment caused only a partly additive effect compared with EPA or DHA may also reflect involvement of COX-2 pathway in this process. The intrinsic property of ionizing radiation to upregulate COX-2 expression [13] may reinforce  $\omega$ -6 PUFA-induced COX-2 upregulation, which participates in inhibition of programmed cell death via prostaglandin E<sub>2</sub> eicosanoid production and anti-apoptotic Bcl2 expression [35]. On the other hand, EPA-induced COX-2 downregulation was reported to be improved by the combination with irradiation [13]. Moreover, it is conceivable that EPA increases lipid peroxidation susceptibility due to its structural conformation displaying more double bonds than AA. Thus, these two last features may also explain the slight difference between EPA and AA efficiency observed in our results.

In our study, vitamin E supplementation abolished the additive cytotoxicity of DHA after ionizing radiation without affecting the intrinsic inhibitory effect of a 4-Gy exposure on cell survival. These findings suggest that vitamin E was able to prevent only DHA peroxidation events and that ROS-mediated ionizing radiation exceeded the cellular antioxidant scavengers and enzymes, leading to cellular death processes. Based on the literature and our results, we hypothesize that PUFA hydroperoxides, generated from ionizing radiations may have caused an increase in nucleic acid base oxidative modification such as 8-hydroxy-2'-deoxyguanosine [36] and DNA strand breaks, leading to cell apoptosis and necrosis.

These investigations suggest that EPA, DHA, and AA enhance radiotherapy efficacy mainly via a pathway that involves ROS generation and lipid peroxidation. Hence, a general mechanism by which PUFAs may improve ionizing

radiation efficacy is described here, even though a COX-dependent pathway is also probably involved to a lesser extent. It might be worthwhile to note that we used an *in vitro* model, in which some *in vivo* parameters were not taken into account. This is the case, for instance, of tumor angiogenesis and inflammatory response, in which COX-2 expression modulation was found to be involved directly [37]. In a human tumor xenografted model, Wen et al. [13] observed that  $\omega$ -3 PUFA-enhanced radiotherapy was associated with angiogenesis inhibition and decreased mucosal response. Although our study confirms that  $\omega$ -3 PUFAs enhances the antitumor effect of ionizing radiation and proposes a mechanism of action, the additional properties of  $\omega$ -3 PUFAs on angiogenesis and inflammation, probably through the COX-2 pathway, might further enhance their potential as an adjuvant for radiotherapy.

## Conclusion

Our observations strongly suggest that  $\omega$ -3 PUFAs are potential candidates for optimizing human colorectal cancer therapy. However, further *in vivo* experiments are needed to determine the optimal timing of  $\omega$ -3 PUFA supplementation before radiotherapy and to evaluate side effects on healthy tissues and potential induction of angiogenesis and inflammation.

## Acknowledgments

The authors gratefully acknowledge the technical assistance of Véronique Karsegard and the availability of the Nuclear Medicine Laboratory team.

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