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Original article

Interaction of ω-3 polyunsaturated fatty acids with radiation therapy in two different colorectal cancer cell lines☆

Fang Cai [a], Olivier Sorg [b], Virginie Granci [a], Elena Lecumberri [a], Raymond Miralbell [c], Yves M. Dupertuis [a], Claude Pichard [a,*]

[a] Clinical Nutrition, Geneva University Hospital, Geneva 1211, Switzerland
[b] Swiss Centre for Applied Human Toxicology, University of Geneva, Switzerland
[c] Radio-Oncology, Geneva University Hospital, Switzerland

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SUMMARY

Background & aims: This study aims at evaluating if docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) increases the efficacy of radiation therapy (RT) on two human colorectal cancer cell lines with different radio-sensitivity.
Methods: LS174T and HT-29 cells were treated with 20 or 50 μmol/L EPA or DHA followed by single X-ray RT of 0, 2 or 4 Gy, to evaluate cell survival, apoptosis, peroxide and malondialdehyde productions. Inflammation- and apoptosis-related proteins were analyzed by Western Blot. ANOVAs were used for statistical analysis.
Results: LS174T was more sensitive to RT than HT-29. DHA and to a lesser extent EPA increased cell death, apoptosis and peroxide production after RT in LS174T and to a lesser extent in HT-29 ($p < 0.05$). This was associated with increased expression of heat shock protein 70, decreased expression of NF-kB p65, COX-2 and Bcl-2 proteins.
Conclusions: The effect of RT combination with DHA and to a lesser extent EPA was synergistic in the radio-sensitive LS174T cells, but additive in the radio-resistant HT-29 cells. This enhanced cytotoxicity was provoked at least partly by lipid peroxidation, which consequently modulated inflammatory response and induced apoptosis.
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1. Introduction

Colorectal cancer (CRC) is currently one of the most prevalent causes of cancer death.[1] CRC treatment consists mainly in complete surgical resection with or without adjuvant radiation therapy (RT) and/or chemotherapy. Even though, CRC frequently remains refractory to these conventional treatments. More than 50% of patients develop local relapse or metastasis within two years after surgery.[2] New modalities to improve CRC treatment outcome are therefore of great interest.

ω-3 Polyunsaturated fatty acids (PUFAs) might be used as adjuvant for cancer therapy. They could inhibit tumor progression in breast, skin and prostate cancers.[3,4] The adjuvant use of ω-3 PUFAs increased chemo-[5] and radio-sensitivity in different cancers.[6,7] In our study, we investigated if ω-3 PUFAs could enhance radio-sensitivity in CRC cell lines.

Among the ω-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are the most studied. Both have been described to increase the therapeutic efficacy of RT in breast cancer cell lines,[8] rodent model[9] and also in patients.[10] In CRC, EPA or DHA, on its own, inhibits HT-29 cell growth, but DHA is more efficient than EPA due to its rapid cellular absorption and integration into cell membrane.[11] However, the combined effect of EPA/DHA and RT is rarely reported. We previously observed that EPA and DHA were able to increase radio-toxicity in different CRC cell lines, *i.e.* LS174T, Caco-2 and CO112. This synergic toxicity was negatively correlated with the differentiation degree of the cell lines.[6]

Oxidative stress might contribute to the cytotoxicity of PUFAs, because of an increase in oxidative stress markers, such as glutathione, disulfide glutathione and malondialdehyde (MDA) observed

Abbreviations: CRC, colorectal adenocarcinoma; DHA, docosahexaenoic acid; DNPH, 2,4-dinitrophenylhydrazine; EDTA, Ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PBS, phosphate-buffered saline; RT, radiation therapy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ω-3 PUFAs, ω-3 polyunsaturated fatty acids.

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* Corresponding author. Tel.: +41 22 372 93 49; fax: +41 22 372 93 63.
E-mail address: Claude.Pichard@unige.ch (C. Pichard).

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during DHA or EPA combination with chemotherapy in breast carcinoma cells.[5] When combined with RT, the mechanism of cytotoxicity of ω-3 PUFAs has not yet been evaluated in depth, especially in CRC. This study therefore aimed at evaluating the effect of RT combination with EPA or DHA on LS174T and HT29 CRC cell survival. These two cell lines have different proliferation rates and genetic backgrounds, especially in p53 and Bax protein expressions. The p53 and Bax positive HT29 cell line is more radioresistant than the p53 and Bax negative LS174T cell line. Lipid peroxidation and its downstream pathways such as inflammation and apoptosis, which cause cell survival inhibition, were also investigated.

2. Materials and methods

2.1. Reagents

EPA, DHA were obtained from Sigma–Aldrich (Buchs, Switzerland), diluted in ethanol and stored at 10 g/L at −20 °C under argon. All other chemicals were also purchased from Sigma–Aldrich (Buchs, Switzerland) unless otherwise stated.

2.2. Cell line

The human CRC cell line LS174T (ATCC no. CL-188™) and HT-29 (ATCC no. HTB-38™, USA) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Switzerland). The medium was changed twice a week to keep the cells in exponential growth phase at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used no more than 10 passages in order to maintain their biological characteristics. According to ATCC product description, HT29 cells express p53 and Bax, while LS174T cells are Bax mutated and p53 mRNA positive but without p53 protein expression.

2.3. Cell treatment with EPA or DHA in combination with radiotherapy

Cells were treated with EPA, DHA at 20 or 50 μmol/L for 4 h before a single RT of 2 or 4 Gy, using a linear beam accelerator (X-rays, 6 MV). Non-treated cells were incubated with the same concentration of ethanol found in a treatment corresponding to 20 or 50 μmol/L EPA or DHA.

2.4. Cell survival

LS174T and HT29 cells were seeded in 6-well plates at a density of 250 cells/well. After the previously described treatment, cells were kept for 14 days at 37 °C, and then washed with phosphate-buffered saline (PBS) solution, fixed with methanol/acetic acid (3:1, v/v) for 20 min at 4 °C, and stained with 0.5% crystal violet in methanol/acetic acid (3:1, v/v). Colonies containing $\geq$ 50 cells were counted. The survival fraction was calculated as relative to untreated controls (S/So). The sensitizer enhancement ratio (SER) was defined as the ratio of required X-ray dose to reduce 80% of the survival fraction without ω-3 PUFAs, to the dose required to obtain the same survival fraction with PUFAs. The required dose was calculated based on the linear-quadratic regression model.[12]

2.5. Lipid peroxidation

Cells were seeded at a density of 600 000/well in 6-well plates 24 h before treatment of 50 μmol/L EPA or DHA followed by a 4-Gy RT. Four hours after irradiation, cells were collected and 50 μl cell lysate (in 50 mmol/L Tris–HCl pH 7.3, 150 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1.0% Triton X-100) or standard (hydrogen peroxide 30%, Merck, Germany) was mixed with 50 μl xylenol orange reactive (50 mmol/L sulphuric acid, 500 μmol/L ammonium iron (II) sulfate, 200 μmol/L xylenol orange, 200 mmol/L D-sorbitol) and then incubated at room temperature (protected from light) for 1 h. The absorbance of the mixture was read using a colorimeter (Model 680, Bio-Rad Laboratories AG) at 595 nm.

MDA level was measured following a derivatization with 2,4-dinitrophenylhydrazine (DNPH) and HPLC analysis[13] with minor modifications. Fifty μl of reconstructed samples were injected into HPLC system (Agilent 1100 series) equipped with an autosampler, a quaternary pump, a UV diod-array detector, and a C18 column (Nucleodur® C18 Pyramid 3 μmol/L, Macherey–Nagel). The flow-rate of the mobile phase was 1 ml/min. The DNPH derivatization products of MDA (1-(2,4-dinitrophenyl) pyrazole) and methyl-MDA (4-methyl-1-(2,4-dinitrophenyl) pyrazole, internal standard, 100 μmol/L, synthesized by heating a mixture of 0.25 g 3-dimethylamino-2-methyl-2-propenal, 0.1 g sodium hydroxide and 350 μl HPLC grade water at 70 °C for 25 min) were analyzed at 306 nm and 322 nm, respectively. The calibration of the derivatization products was done using that of MDA and methyl-MDA standard solutions with molar extinction coefficients of 12'800 (M^{-1} cm^{-1}) at 249 nm for MDA in acidic medium and 22'800 (M^{-1} cm^{-1}) at 279 nm for methyl-MDA in alkaline medium.

2.6. Apoptosis quantification

Cells were seeded at a density of 50 000/well in 24-well plates for 24 h before treatment of 50 μmol/L EPA or DHA for 4 h followed by a 4-Gy RT. Apoptosis was quantified 2, 4 or 24 h after RT using a two-parameter fluorescence-activated cell sorting (FACS) analysis with annexin V/propidium iodide detection kit according to manufacturer's instructions (BD Biosciences, Switzerland). Briefly, cells were detached with trypsin, washed with 1×PBS and then resuspended in binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) at a concentration of 1×10^6 cells/ml. Samples were stained with 5 μL annexin V conjugated with fluorescein isothiocyanate (FITC) and 5 μL propidum iodide at room temperature for 15 min in the dark. They were then diluted in 400 μL of binding buffer and analyzed within 1 h using a flow cytometer (Accuri C6 personal flow cytometer, BD Biosciences; excitation 488 nm; emission 530 nm).

2.7. Western Blot

Two hours after a 4-Gy RT, cells were washed with PBS and collected with 100 μl of RIPA lysate buffer (NaCl 150 mmol/L, Tris–HCl pH8 50 mmol/L, NP40 1%, Triton X100 1%, SDS 0.1%)), and supplemented with proteinase inhibitors. The supernatant was then collected and denaturated at 95 °C for 5 min. 25 μg of protein from each sample were loaded on 9%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred, and immunoblotted onto PVDF membrane. Anti-NF-kB p65, anti-COX-2, anti-HSP-70, anti-Bcl-2, anti-Bax, anti-p53 antibodies (Santa-Cruz, USA) were used as primary antibodies. Anti-GAPDH antibody (Millipore, USA) was used as internal standard for protein.

2.8. Data analysis

For apoptosis analysis, data were acquired with CFlow® Plus software (BD biosciences, USA). All experiments were reproduced in triplicate and results were presented as means ± SD. Normality of data was tested using Shapiro–Wilk. Three-way ANOVA was used for clonogenic assay, apoptosis and xylenol orange, while two-way

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