Enhancement of human cancer cell radiosensitivity by conjugated eicosapentaenoic acid - a mammalian DNA polymerase inhibitor

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Abstract. We previously found that conjugated eicosapentaenoic acid (cEPA) selectively inhibited the activities of mammalian DNA polymerases (pols), and suppressed human cancer cell growth. The aim of the present study was to evaluate the efficacy of concurrent radiation with cEPA in a human colon carcinoma cell line, HCT 116. Furthermore, we examined the most effective timing of irradiation. The post-irradiation addition of cEPA significantly enhanced HCT116 cell radiosensitivity by decreasing the expression of pols β, δ and ε, increasing damaged DNA, such as DNA double-strand breaks, inhibiting clonogenic survival, and inducing apoptosis. However, cells treated by pre-irradiation addition of cEPA did not influence radiosensitive survival and radiation-induced apoptosis. cEPA inhibited the activities of pols needed for DNA repair, thereby DNA damage must be augmented by cEPA and irradiation. These results suggested that the combination of inhibitors of DNA repair-related pols/radiation could be an effective anticancer therapy.

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Abbreviations: cEPA, conjugated eicosapentaenoic acid; pol, DNA polymerase (EC 2.7.7.7); DSB, DNA double-strand break; PUFAs, polyunsaturated fatty acid; ATR, ataxia-telangiectasia mutated- and Rad3-related protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction

Key words: conjugated eicosapentaenoic acid, DNA polymerase, enzyme inhibitor, radiosensitivity, DNA damage, anticancer therapy

Introduction

DNA polymerases (pol, i.e., DNA-dependent DNA polymerases, EC 2.7.7.7) catalyze the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of primed double-stranded DNA molecules, and are involved in producing vital cellular processes, such as DNA replication, repair and recombination (1). The human genome encodes at least 15 pols to conduct cellular DNA synthesis (2,3). Eukaryotic cells contain three replicative pols (α, β and ε), mitochondrial pol γ, and at least 13 repair-related pols; β, δ, ε, ζ, η, θ, τ, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT) and REV1 (2-4). Pols have recently emerged as important cellular targets for chemical intervention in the development of anticancer agents.

We have been screening for pol inhibitors from natural products (5,6), and found that mammalian pols α and β are inhibited by linear-chain fatty acids with the following characteristics: 1) C18- or more carbon chains, 2) a free carboxylic group, and 3) double bonds of cis-configuration, n-3 polyunsaturated fatty acid (PUFA) having the strongest inhibitory effect of any fatty acid tested (7.8). Eicosapentaenoic acid (EPA; 5Z8Z11Z14Z17Z-20:5) and docosahexaenoic acid (DHA; 4Z7Z10Z13Z16Z19Z-22:6), both n-3 PUFAs, exert significant inhibitory effects on colon carcinoma cell growth at the primary site and metastases (9,10). PUFA are present at high concentrations in some fish oils, and have been evaluated in various clinical trials in which they have proved to be safe and well tolerated.

Conjugated fatty acids are positional and geometrical isomers with several conjugated double bonds. Fatty acids with conjugated double bonds exist in nature; seaweeds such as red and green algae contain highly n-3 unsaturated conjugated fatty acids, i.e., conjugated eicosapentaenoic acid (cEPA; 5Z7E9E14Z17Z-20:5), bosseopentaenoic acid (5Z8Z10E12E14Z-20:5) and stellaheptaenoic acid (4Z7Z9 E11E13Z16Z19Z-22:7) (11,12). As n-3 PUFAs have been shown to have anticarcinogenic activity, conjugated fatty acids converted from n-3 PUFAs may show higher tumor-
inhibiting activity than n-3 PUFAs themselves (13,14). We previously realized the importance of the two classes of n-3 PUFAs: EPA and DHA, normal and conjugated, and the inhibitory effect of cEPA on pol was stronger than that of EPA, DHA and cDHA (15). Furthermore, cEPA regulates the cell cycle by DNA damage-response proteins, including the ataxia-telangiectasia mutated- and Rad3-related protein kinase (ATR)-Chk1/2 pathway without influencing the proliferation of normal cells (16,17).

Radiotherapy is one of the most commonly used therapeutic modalities in cancer treatment. Radiation ionizes the molecules of tumor cells, and damages their DNA (18). These effects are not limited to tumor cells but also affect normal cells within the tumor stroma (19,20). The cytotoxicity of radiation is mostly mediated through the generation of DNA double-strand breaks (DSBs) as demonstrated by the radiosensitivity of cells and organisms defective in the machinery of DSB repair (21-23). Ataxia-telangiectasia mutated (ATM) protein kinase is a component of these pathways and integrates the cellular response to damage by phosphorylating some key proteins involved in cell cycle regulation and DSB repair (24,25). Thus, cEPA seems to be an ideal model for the study not only of the molecular mechanisms that inhibit pol activity for the development of new anticancer drugs, but also of cellular proliferation processes such as DNA replication and repair of damaged DNA, such as DSBs, by X-ray irradiation.

In the present study, we investigated whether the radiosensitizing effect of cEPA on human colon carcinoma HCT116 cells and further analyzed the mechanisms of radiosensitization by cEPA.

Materials and methods

Materials. EPA was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). Nucleotides and chemically synthesized DNA template primers, such as poly(dA) and oligo(dT)12-18, and radioisotope reagents, such as \([^{3}H]\)dTTP (2'-deoxythymidine 5'-triphosphate) (43 Ci/mmol), were purchased from GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). All other reagents were of analytical grade and purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Preparation of cEPA by alkaline treatment. cEPA was prepared by alkaline treatment following the AOAC method with slight modifications (26). Potassium hydroxide at a concentration of 6.6 or 21% (w/w) in ethylene glycol was prepared and the KOH solution was bubbled for 5 min with nitrogen gas. 10 mg of EPA was added to 1 ml of 6.6% or 21% KOH solution in a test tube (10 ml volume). The mixture was bubbled with nitrogen gas and then screw-capped and allowed to stand for 5 or 10 min at 180°C. The reaction mixture was cooled, and 1 ml methanol was added. The mixture was acidified to below pH 2 with 2 ml of 6 N HCl. After dilution with 2 ml distilled water, the conjugated fatty acid was extracted with 5 ml hexane. The hexane extract was then washed with 3 ml of 30% methanol and with 3 ml distilled water before being evaporated under a nitrogen gas stream. The conjugated fatty acids were stored at -20°C after being purged with nitrogen gas. UV/VIS spectrophotometric analysis was performed with a Shimadzu UV-2400PC. Spectrophotometric readings confirmed the conjugation of fatty acids of pentane (345 nm) (27). cEPA was dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec.

Cell culture. Human colon carcinoma cell line, HCT116, was a kind gift from Dr Bert Vogelstein (Johns Hopkins University, Baltimore). The cells were maintained in McCoy's 5A medium supplemented with 10% FBS, sodium bicarbonate (2 mg/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂/95% air.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. HCT116 cells were trypsinized and resuspended in McCoy's 5A medium with 10% FBS, and 5x10³ cells were seeded in 96-well tissue culture plates for 24 h. The cells were then stimulated with different concentrations of cEPA containing DMSO of final concentration of 1% for 24 h. After treatment, MTT solution was added (final concentration 0.5 mg/ml MTT in PBS) for 3 h (28). The medium was discarded and the cells were lysed in acidified 2-propanol. Absorbance was measured at 570 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

DNA polymerase assay of the cell extract. HCT116 cells were plated at 3x10⁴ into a 100-mm culture dish with or without 30 μM of cEPA. After 24 h incubation, the cells were washed with PBS, collected by centrifugation, and the pellets were sonicated for 3 min in lysis buffer (10 mM NaCl, 10 mM Tris- HCl, 1 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 7.5% glycerol, 1% Triton X). The pol activity of the cell extract was measured as described previously (7,8). Poly(dA)/oligo(dT)₁₂,₁₈ and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol dNTP (2'-deoxyribonucleotide 5'-triphosphate), i.e. dTTP into the synthetic DNA template-primers [i.e. poly(dA)/oligo(dT)₁₂,₁₈] in 60 min at 37°C under normal reaction conditions for the enzyme (7,8).

X-ray irradiation. Cultures of HCT116 cells were irradiated using an X-ray irradiator (MBR-1505R2; Hitachi Medico, Tokyo, Japan) at a dose rate of 7.8 Gy/min. Dosimetry was carried out using an ionization chamber connected to an electrometer system.

Real-time PCR analysis. Real-time polymerase chain reaction (PCR) analyses were conducted using standard assays according to Heintel et al (29). HCT116 cells were pre-cultured for 24 h, treated with or without 30 μM cEPA for 24 h, radiated at 8 Gy X-ray and subsequently incubated for 10 min. Total RNA was isolated from treated HCT116 cells using the RNeasy mini kit (Qiagen, Hilden, Germany). The extracted total RNA was reverse-transcribed into single-stranded cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Warrington, UK). Aliquots of cDNA were used as templates for real-time PCR reactions containing
primers of either the target gene or the control. All oligonucleotide primers for the endogenous control (18s rRNA) and the target five pol genes (i.e., human pols α, β, δ, ε, and λ) were synthesized by Applied Biosystems, and were as follows: accession number (common name), 4308329 (18s rRNA); Hs00415835 (polymerase α); Hs00160263 (polymerase β); Hs00172491 (polymerase δ); Hs00173030 (polymerase ε); Hs00203191 (polymerase λ). Each amplification was performed using first-strand cDNA with TaqMan First Universal PCR Master Mix (Applied Biosystems). The PCR reactions, TaqMan analyses, and subsequent calculations were performed with the StepOne™ Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. All reactions were performed in a 20 μl reaction volume in triplicate. The mRNA expression level was determined using the 2−ΔΔCT method.

Comet assay. To assess the generation of DSB in cultured cancer cells, the standard protocol for the single-cell electrophoresis (comet) preparation and analysis was adopted (30). Briefly, HCT116 cells were seeded, cultured in 6-well plates for 24 h, and then incubated with or without 30 μM cEPA for 30 min. The plates were immediately radiated with 8 Gy X-ray. After the irradiated cells with or without cEPA were harvested for 0, 15 and 30 min, the cells were lysed in neutral (pH 7.0) buffer, electrophoresed, and stained following the manufacturer’s instructions. All reactions were performed in a 20 μl reaction volume in triplicate. The mRNA expression level was determined using the 2−ΔΔCT method.

Radiation clonogenic assay. Survival following radiation exposure was defined as the ability of cells to maintain clonogenic capacity and form colonies. After HCT116 cells were treated with vehicle (DMSO) alone, cEPA alone, irradiation alone, or cEPA plus irradiation for 48 h, the cells were trypsinized, counted, and appropriate dilutions were made. The appropriate number of cells was plated in fresh medium without cEPA for colony formation into 100 mm dishes. After incubation intervals of 14 days, the colonies (containing ≥50 cells) were stained with methylene blue, and the numbers of colonies were counted. The surviving fraction (SF) was calculated as a ratio of the number of colonies to the number of cells plated (plating efficiency) divided by the same ratio calculated for the non-irradiated group. Experiments were performed in duplicate.

Assessment of apoptosis by annexin V staining. Exactly 48 h after treatment with cEPA (30 μM) alone, irradiation (8 Gy) alone, or cEPA/irradiation combination, HCT 116 cells were harvested, and then apoptotic cells were detected by annexin V staining using a commercially available kit (PharMingen Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. The stained cells, which are apoptotic, were analyzed using a FACSCanto II flow cytometer in combination with FACSDiVa software (BD Biosciences).

Results

The effect of cEPA on cellular growth and DNA polymerase activity. First, to determine the effects of cEPA on cultured human cancer cells, we tested their influence on cell growth in human colon carcinoma HCT116 cells. cEPA efficiently inhibited cell growth in a dose-dependent manner, and the LD50 value was 30.1 μM for 24 h incubation (Fig. 1). The total pol activity of the HCT116 cell extract, which was treated with cEPA for 24 h, was lower than that of non-treated cells (Fig. 1B). The enzyme activity was dose-dependently
decreased with an increase in the concentrations of cEPA, and the inhibitory effect of cEPA on cell growth showed the same tendency as that on pol activity in the cells (Fig. 1). Since we previously reported that cEPA selectively inhibited the activities of purified mammalian pols in vitro with IC50 values of 11.0-31.8 μM (31), the inhibited enzyme by cEPA in the HCT116 cell extract may consist of both DNA replicative pols (i.e., pols α, β, δ, and ε) and DNA repair-related pols (i.e., pols β, δ, ε and λ). cEPA was more cytotoxic than normal EPA to HCT116 cells, and this tendency of cell growth inhibition was the same as the inhibitory effect of mammalian pols (data not shown).

Effect on expression of pols in HCT116 cells treated with cEPA. Next, we investigated in more detail the effect of cEPA as a selective mammalian pol inhibitor on HCT116 proliferating cells when combined with X-ray radiation. We examined whether the inhibitory activity of pols by 30 μM cEPA/8 Gy radiation combination was associated with the expression of mRNA of pols using real-time PCR. As shown in Fig. 2, 30 μM cEPA (i.e., the LD50 value of HCT116 cell growth in Fig. 1A) suppressed the expression of all pols, and especially the mRNA amounts of pols α and ε, which are replicative pols, were less than half against non-treated cEPA (i.e., control). cEPA was more cytotoxic than normal EPA to HCT116 cells, and this tendency of cell growth inhibition was the same as the inhibitory effect of mammalian pols (data not shown).

Figure 2. Regulation of pol expression in HCT116 cells by combining cEPA and X-ray radiation. Human colon carcinoma cells (HCT116 cells) were pre-cultured for 24 h, treated with or without 30 μM cEPA for 24 h, radiated at 8 Gy X-ray, and subsequently incubated for 10 min. mRNA was extracted and subjected to real-time PCR as described in Materials and methods. The rate of mRNA expression in both non-treated cEPA and non-irradiated cells (i.e., control) is 1.0. IR is X-ray irradiation. Values are the means ± SEM of three independent experiments.

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Inhibitory effect of cEPA on the repair activity of damaged DNA by X-ray radiation. A critical determinant of radiation-induced lethality is the induction and repair of DNA damage, specifically DSBs (32). To determine the effect of cEPA on DNA damage in irradiated HCT116 cells, we evaluated tailing DNA consisting of DSBs by single-cell electrophoresis (comet) assay in neutral buffer. As shown in Fig. 3, in the cells treated with 30 μM cEPA alone, DNA tailing reflecting the formation of DSBs was not observed, suggesting that cEPA does not make DSBs. When cells were exposed to X-rays at 8 Gy alone, DNA tailing was immediately observed in most cells (93.5±7.9%). DNA tailing disappeared gradually with proceeding incubation period, and the cells bearing DNA tailing decreased to 6.4±0.6% at 30 min after irradiation. However, the population of cells bearing DNA tailing, which were treated with a combination of X-rays (8 Gy) and cEPA (30 μM), remained relatively constant 0, 15 and 30 min after irradiation, and the rate of DNA-damaged cells was 94.6±8.2%. These results suggest that HCT116 cells have the ability to quickly repair damaged DNA by inhibiting the activities of repair-related pols, such as pols β, δ, ε and λ.
Effect of cEPA on survival of HCT116 cell radiosensitivity.

To determine whether cEPA enhances cellular sensitivity to X-ray radiation, the HCT116 cell line was cultured receiving the combined cEPA/radiation treatment, and then clonogenic survival analysis was performed. HCT116 cells were treated with 1% DMSO (vehicle control) or 30 μM cEPA for 48 h with 8 Gy of radiation 0, 24 or 48 h after treatment of cEPA [(a), (b) or (c) in Fig. 4A, respectively], and subsequently plated in 100-mm dishes at different densities based on the stringency of treatments. This protocol was used in an attempt to eliminate any effects of trypsinization on post-irradiation signaling/recovery processes. The surviving fractions obtained after 30 μM cEPA treatment only and 8 Gy X-ray radiation exposure only were 0.89±0.08 and 0.12±0.01 for HCT116 cells, respectively. As shown in Fig. 4B, cEPA exposure for 48 h after irradiation [i.e., ‘post-irradiation’ = (a) of Fig. 4A] resulted in an increase in radiation-induced cell killing for HCT116 cells, and the surviving fraction was 0.017±0.002; therefore, the survival rate of radiosensitive enhancement was >7. Furthermore, treatment combining cEPA with radiation therapy was enhanced with the cEPA dose, and 40 μM cEPA showed an approximately 10-fold reduction in clonogenic survival compared with the control (0 μM cEPA) (Fig. 4C). In this treatment protocol, when cEPA was treated for 48 h with radiation 24 or 48 h after cEPA treatment ['mid-irradiation' = (b) or 'pre-irradiation' = (c) in Fig. 4A, respectively], the radiosensitizing effect had no influence compared with no treatment with cEPA. These data indicate that radiosensitization induced by cEPA is mediated through a post-irradiation process or event.

Effect of cEPA on enhancement of radiation-induced apoptosis.

X-ray radiation is known to be a strong inducer of apoptosis (33). To examine whether susceptibility to apoptosis can be a determinant of cEPA treatment, the effect of cEPA on radiation-induced apoptosis in HCT116 cells was investigated using flow cytometry. In this experiment, the three combinations of 30 μM cEPA and 8 Gy X-ray irradiation were the same schedules as in Fig. 4A (a-c). After cells were treated with cEPA for 48 h with irradiation, flow cytometric analysis was performed instead of the clonogenic assay in Fig. 4A. As shown in Fig. 5, a lower concentration of cEPA (i.e., 30 μM of the LD50 value of HCT116 cell growth in Fig. 2A) alone did not induce apoptosis, although apoptotic cells were induced by 8 Gy X-ray radiation. Radiation-induced apoptosis depended on the incubation time after irradiation, and cells incubated for 48 h after irradiation [i.e., (a) of Fig. 4A] alone did not induce apoptosis, although apoptotic cells were induced by 8 Gy X-ray radiation. Radiation-induced apoptosis depended on the incubation time after irradiation, and cells incubated for 48 h after irradiation [i.e., (a) of Fig. 4A] showed the highest apoptosis induction compared with other cells incubated for 24 and 0 h after irradiation; i.e., (b) and (c) of Fig. 4A. In HCT116 cells, DNA damage occurred immediately with irradiation (Fig. 3), although apoptosis was induced 24 h or later after irradiation. The apoptotic level in cultures receiving combined radiation/cEPA treatment was greater than in the radiation-only group; therefore, the cEPA-mediated increase in radiosensitivity could be attributed to enhanced susceptibility to apoptosis.
Discussion

We reported previously that conjugated PUFA, such as cEPA prepared by alkaline treatment of PUFA, was a two-fold stronger pol inhibitor than normal PUFA (31). cEPA did not influence pol activities from plants and prokaryotes and other DNA metabolic enzyme activities, and no interaction of cEPA with DNA was detected in an independent DNA-binding assay (i.e., Tm of double-stranded DNA measurements) (23). These results suggested that selective inhibitory action by cEPA might be due to specific binding to pol enzymes. Furthermore, the mechanisms by which cEPA suppresses human cancer cell growth were investigated, and it was revealed that the inhibition of pol activity by cEPA influenced not only cell proliferation but also the cell cycle (31). Cell cycle arrest in the G1 phase by cEPA was considered to be induced by the p53/p21 pathway from the ATR-Chk1/2-signaling pathway in HCT116 cells (16). Since cEPA did not influence the proliferation of normal cells (data not shown), cEPA should also be considered the lead compound of a group of potentially useful agents for cancer chemotherapy.

In this study, cEPA (conjugated C20:5 fatty acid) was prepared from EPA by alkaline treatment, as described in Materials and methods, and the chemically synthesized fraction was used. This fraction contained 98% cEPA, which had conjugated double bonds, but did not contain hydroperoxy- and/or hydroxy-fatty acids (data not shown); however, each cEPA isomer, which has a cis- or trans-double bond compound, was not separated and purified. Jain et al reported that trans-arachidonic acid (C20:4 fatty acid) isomers (especially 5,6- trans-arachidonic acid) showed distinct activity by targeting cell progression through the cell cycle (arrest in the G1 phase) and inducing apoptosis (34).
These results suggested that the trans-cEPA isomer in the fraction might have bio-activities, such as pol inhibition, prevention of cancer cell growth, G1 phase arrest of the cancer cell cycle, and apoptosis induction.

Radiotherapy remains a primary cancer treatment modality for solid tumors, and the ability to enhance its efficacy is thus likely to impact a significant number of cancer patients. DNA repair-related pols, especially pol β, have been shown to be altered in around 30% of tumors, suggesting a role in tumor formation (35). These changes include overexpression, truncation and mutations modulating the activity of these enzymes and are postulated to influence DNA repair and therefore possibly tumorigenesis (36-38). This is the initial investigation into the combination of radiation and cEPA, which is a selective mammalian pol inhibitor. The results of this study indicate that cEPA enhanced the radiosensitivity of human colon carcinoma HCT116 cells, which inhibited the activities of pols in the cells (Fig. 1), reduced the expression of pols, especially DNA repair-related pols such as pols β, δ and ε (Fig. 2), and caused consequent DNA repair inactivation of damaged DNA, including DSBs by X-ray irradiation (Fig. 3). Furthermore, the cell treatment of post-irradiation addition of cEPA showed significantly greater anticancer effects, such as inductions of cancer cell killing and apoptosis, than other combined cEPA/radiation schedules (Figs. 4 and 5). In conclusion, combining selective inhibitors of DNA repair-related pols, such as cEPA, and radiation might have clinical potential as a cancer treatment strategy.

Figure 5. Detection of apoptosis in HCT116 cells by combining cEPA and X-ray radiation. Treatment schedules (a) to (c) combining 30 μM cEPA and 8 Gy X-ray radiation of human colon carcinoma HCT116 cells are shown in Fig. 4A. Treated cells were cultured for 48 h, harvested, stained with annexin V, and then analyzed using flow cytometry.

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