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DHA-mediated enhancement of TRAIL-induced apoptosis in colon cancer cells is associated with engagement of mitochondria and specific alterations in sphingolipid metabolism

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ABSTRACT

Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid present in fish oil, may exert cytotoxic and/or cytostatic effects on colon cancer cells when applied individually or in combination with some anticancer drugs. Here we demonstrate a selective ability of subtoxic doses of DHA to enhance antiproliferative and apoptotic effects of clinically useful cytokine TRAIL (tumor necrosis factor-related apoptosis inducing ligand) in cancer but not normal human colon cells. DHA-mediated stimulation of TRAIL-induced apoptosis was associated with extensive engagement of mitochondrial pathway (Bax/Bak activation, drop of mitochondrial membrane potential, cytochrome c release), activation of endoplasmic reticulum stress response (CHOP upregulation, changes in PERK level), decrease of cellular inhibitor of apoptosis protein (XIAP, cIAP1) levels and significant changes in sphingolipid metabolism (intracellular levels of ceramides, hexosyl ceramides, sphingomyelines, sphingosines; HPLC/MS/MS). Interestingly, we found significant differences in representation of various classes of ceramides (especially C16:0, C24:1) between the cancer and normal colon cells treated with DHA and TRAIL, and suggested their potential role in the regulation of the cell response to the drug combination. These study outcomes highlight the potential of DHA for a new combination therapy with TRAIL for selective elimination of colon cancer cells via simultaneous targeting of multiple steps in apoptotic pathways.

1. Introduction

Increased incidence of colorectal cancer is often associated with imbalance between intake and output of energy, and excessive fat consumption. Not only the quantity, but especially the quality of dietary fat may significantly affect the risk of cancer development [1]. It has been demonstrated that algal and fish oils containing n-3 polyunsaturated fatty acids (PUFAs) may have protective and suppressive effects on colon cancer [2,3]. Docosahexaenoic acid (DHA, 22:6), a highly unsaturated representative of n-3 PUFAs, has unique structure and abilities, which allow it to affect diverse physiological processes. Potential mechanisms comprise mainly alterations of cellular lipid composition and metabolism, which may consequently influence membrane properties and functions [4], production of eicosanoids [5], and modulation of different cell signalling pathways regulating cell growth and death [6]. DHA was shown to impact on the total level and/or activity of numerous proteins involved in apoptosis regulation such as the Bcl-2 family members [7], caspases [8] or their endogenous inhibitors such as cIAPs or cFLIP [9–11]. We and others also showed that DHA may affect production of reactive oxygen species (ROS), lipid peroxides or expression/activity of antioxidant enzymes, which may significantly interfere with apoptosis regulation [12,13]. In addition, DHA triggered the endoplasmic reticulum (ER) stress and unfolded protein response, thus contributing to further co-ordination of apoptosis or cell cycle progression [14,15]. DHA effects were also reported to be associated with decreased signalling through the PI3K/Akt cell survival pathway or altered activity of various MAPKs (mitogen-activated protein kinases) [16].

Abbreviations: Bcl-2, B-cell lymphoma 2 protein; C, ceramide; cFLIP, cellular FLICE-like inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; CK18, cytokeratin 18; DHA, docosahexaenoic acid; ER, endoplasmatic reticulum; ERK, extracellular signal-regulated kinase; CHOP, C/EBP homology protein (also known as GADD153); MAPK, mitogen-activated protein kinases; MMP, mitochondrial membrane potential; PARP, poly(ADP)ribose polymerase; PERK, protein kinase RNA-like ER kinase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SM, sphingomyeline; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis protein

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Although DHA can trigger dose- and time-dependent apoptotic signalling in cancer cells by itself, it also enhances colon cancer cell responsiveness to various chemotherapeutic drugs, including 5-fluorouracil, arsenic trioxide, paclitaxel, celecoxib, or sulindac sulphide [17,18], while protecting the gut from chemotherapy-induced toxicity [19]. In addition, DHA may also act as a potent agent sensitising colon cancer cells to the action of endogenous apoptosis regulators present in the gut. Our previous results documented enhanced antiproliferative and apoptotic effects in human colon epithelial cells after combined treatment with subtoxic doses of DHA and short-chain fatty acid butyrate or TNF-family cytokines such as TNF-alpha or anti-Fas antibody [20]. We also reported on an efficient co-operation of DHA with cytokine TRAIL (tumor necrosis factor-related apoptosis inducing ligand) in apoptosis induction of HT-29 human colon cancer cells [21], but relevant molecular mechanisms still remain to be elucidated.

TRAIL exerts antitumor activity in a wide range of cancer cell types, while being minimally toxic to most normal tissues [22,23]. TRAIL-induced apoptosis is mediated through interaction with its two death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), followed by the death-inducing signalling complex (DISC) formation. As a result, caspase-8 is activated and can further cleave effector caspases (e.g. caspase-3) and/or Bid protein, which in turn leads to amplification of the apoptotic signal via mitochondria. Changes of mitochondrial membrane permeability, sophisticated interplay among Bcl-2 family proteins, and other related processes then lead to cytochrome c release, apoptosome formation, and caspase-9 activation. In the final stages, the effector caspase-mediated cleavage of numerous cellular substrates (cytokeratin-18, PARP, etc.) culminates in apoptosis execution [24]. Unfortunately, there is increasing evidence that numerous cancer cells including the colon cells are resistant to TRAIL-mediated toxicity. This resistance to TRAIL can be manifested at different points in its signalling pathways, e.g. due to dysfunctions of DR4 and DR5, overexpression of cellular FLIP (cFLIP) [25], Bcl-2, Bcl-X_L, Mcl-1 [26,27], cIAPs, [28] loss of Bax [29], or reduced mitochondrial release of proapoptotic mediators [30]. In addition, modulation of the activity of different MAPKs [27] or changes in the production of specific lipid mediators such as ceramides [31] may significantly contribute to potent regulation of cancer cell responses to TRAIL. Therefore, understanding the molecular basis of cancer cell resistance and introduction of new strategies for its overcoming are crucial prerequisites for the future successful clinical application of TRAIL. Because of DHA's outstanding ability to modulate the expression or activity of molecules with relevance to apoptotic signalling pathways triggered by TRAIL, DHA seems to be a promising candidate for cancer cell sensitisation to the killing effects of this cytokine.

Our study demonstrates the selective ability of DHA to increase the sensitivity of various types of colon cancer epithelial cells to the apoptotic effects of TRAIL, while leaving the non-cancer human colon cells unaffected. A particular focus was paid to the molecular mechanisms of the interaction of the signalling pathways triggered by DHA and TRAIL, especially at the level of caspases, mitochondria, and endoplasmic reticulum. Moreover, a detailed analysis of changes in the individual sphingolipids following DHA and TRAIL treatment was performed, compared in cancer and normal colon cells, and correlated with the overall apoptotic response.

2. Material and methods

2.1. Cell culture and treatment

The SW480 cell line was established from a primary colon adenocarcinoma, while the SW620 cell line was derived from a lymph node metastasis from the same patient one year later. Both of them were purchased from ATCC (LGC Standards, Lomianki, Poland) and maintained in McCoy's 5A modified medium (Sigma-Aldrich, Prague, Czech Republic). DLD1 human colon adenocarcinoma cells (ATCC) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA). The media were supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10% fetal bovine serum (FBS, PAA, Pasching, Austria). The NCM460 cell line derived from the normal human adult colon epithelium was obtained by a Material transfer agreement with INCELL Corporation (San Antonio, Texas, USA). The cells were routinely propagated under standard conditions in M3:10 ™ medium (INCELL), supplemented with 10 % of FBS [32]. Within the experiments, the cells were treated with the following agents: docosahexaenoic acid (DHA, #90310, Cayman Chemicals, Michigan, USA, dissolved in 96% ethanol), TRAIL (human recombinant Killer TRAIL, kindly provided by Dr. L. Anděra, Institute of Molecular Genetics, AS CR, Prague, Czech Republic), pan-caspase inhibitor z-VAD-fmk (#550377, BD Pharmingen, New Jersey, USA), MEK1/2 inhibitor U0126 (#9903, Cell Signaling Technology, Danvers, MA, USA), ceramide synthases inhibitor fumonisin B1 (#62580, Cayman Chemicals, Michigan, USA), or the appropriate vehicle (DMSO, ethanol, methanol) for the time points and at concentrations as indicated in the relevant Figure legends.

2.2. Real-time cell impedance analysis

The xCELLigence System monitors cellular events in real time without the incorporation of labels. The system measures electrical impedance across microelectrodes integrated on the bottom of tissue culture E-plates. The impedance measurement provides quantitative information (cell index) about the biological status of the cells, including cell number, viability, adhesion, and morphology (Roche Applied Science, Prague, Czech Republic). The cells were seeded on E-plates, cultivated for 24 h, and then treated with DHA, TRAIL or their combination for the times and at the concentrations as indicated in Figures. The cells were monitored continuously every 15 min over a time period of 80 h and analysed using the xCELLigence RTCA SP system including RTCA software v1.2 (Roche Applied Science) as described previously [33].

2.3. Cell number determination

Adherent and floating cell numbers were quantified using a Coulter Counter (model ZM, Coulter Electronics, Luton, UK) as described previously [21], and their changes following the specific treatments were presented as fold of control.

2.4. Immunoblotting

The cells were harvested, washed in PBS, whole cell lysates were prepared, separated by electrophoresis, and Western blot analysis was performed as described previously [20]. Immunodetection was carried out with the following primary antibodies: anti-caspase-8 (#9746), anti-poly(ADP-ribose) polymerase (PARP) (#7150), anti-caspase-3 (#7272), anti-cIAP-2 (#7944) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cleaved caspase-9 (#9505), anti-p38 (#9212), anti-phospho-p38 (#9211), anti-phospho-ERK (#9101), anti-XIAP (#2042), anti-Bid (#2002), anti-cIAP-1 (#4952), anti-Bcl-xL (#2762), anti-ERK (#9102), anti-caspase-2 (#2224), anti-PERK (#5683), anti-CHOP (#2895), anti-calnexin (#2679), anti-PDI (#3501), anti-BiP (#3177) (all from Cell Signaling Technology), anti-caspase-10 (#M059-3, MBL, Woburn, MA, USA), anti-Bax (#556467), anti-Bak (#556396) (all from BD Pharmingen, San Jose, CA, USA), and anti-Mcl-1 (#M8434, Sigma-Aldrich, Czech Republic). The protein complexes were recognised by horseradish peroxidase-labelled secondary antibodies: anti-mouse IgG (#NA931), anti-rabbit IgG (#NA934) (Amersham Biosciences, Bucks., UK), anti-goat IgG (#A4174, Sigma-Aldrich), and detected by an enhanced chemiluminescence kit (ECL, Amersham Biosciences or Immobilon Western HRP Substrate, Millipore Corp., Darmstadt, Germany). An equal loading was verified using β-actin (#A5441, Sigma-Aldrich) antibody.