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ORIGINAL CONTRIBUTION

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Effect of an omega-3 fatty acid containing lipid emulsion alone and in combination with 5-fluorouracil (5-FU) on growth of the colon cancer cell line Caco-2

Summary *Background* In this study we examined the effects of a fish oil-based lipid emulsion (FO) rich in omega-3 fatty acids, which is used in humans as a component of parenteral nutrition, on the growth of the colon cancer cell line Caco-2. *Aim of the study* The aim of the present study was to investigate whether the FO influences growth and chemosensitivity of the colon cancer cell line Caco-2. FO was tested alone and in combination with the anticancer drug 5-fluorouracil (5-FU). *Methods* Cell numbers were determined with crystal violet staining, cell cycle distribution was assessed using a flow cytometer and apoptosis was visualized by staining nuclei with diamino-phenylindole hydrochloride. *Results* FO inhibited growth of Caco-2 cells in a time and dose dependent manner. FO treatment evoked apoptosis as confirmed by cell morphology. Cell cycle analysis identified an accumulation of cells in the G_2/M phase after incubation with FO. The combined treatment of the cells with FO and 5-FU resulted in a significant enhancement of the growth inhibition seen after exposure to either substance alone. Treatment of the cells with 5-FU specifically blocked the cell cycle in the S phase. The combined treatment of 5-FU with FO showed a further increase in the accumulation of cells in the S phase. *Conclusions* In conclusion, FO has a potent antiproliferative effect on Caco-2 cells, at least in part, due to a decrease in the progression of the cell cycle and the induction of apoptosis. The combination of FO with 5-FU results in an additive growth inhibitory effect.

Key words fish oil – cell proliferation – cell cycle – apoptosis

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in Western societies [1]. Dietary factors, including fat consumption, are thought to modify the risk for CRC [2]. Recent studies have shown that the individual families of fatty acids have different effects on colon carcinogenesis, and it is currently believed that the pattern of lipids consumed is more important than the total intake of fat [3]. A number of epidemiological, clinical and experimental studies indicate that omega-3 fatty acids derived from fish oil, in particular eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), may exert a protective effect in colon carcinogenesis [4–6] and, moreover, may be beneficial for treatment of CRC [7]. The mechanism of action of omega-3 fatty acids is not yet fully understood and is likely to be very complex. Polyunsaturated fatty acids (PUFAs) are important structural constituents of all cellular membranes. Changes in plasma membrane fatty acid composition by increasing the proportion of omega-3 fatty acids may affect membrane fluidity, receptor binding, signal transduction processes, activity of membrane-associated enzymes and eicosanoid production [reviewed in 8, 9].

Administration of omega-3 fatty acids has been proven to be a relatively non-toxic form of cancer therapy [10] and several clinical trials focusing on the effect of fish oil on tumor cachexia have been performed [11,

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12]. Dietary modifications that have been used so far in cancer therapy are extreme and cannot be achieved with ordinary foods.

This implicates the need for special fish oil preparations, e. g., fish oil capsules and liquid formulas enriched with omega-3 fatty acids. For specialized needs intravenously applied solutions, which contain stable polyunsaturated triacylglycerol emulsions, are available.

There are some advantages of the intravenous application over the oral administration: first, intestinal loss due to lipid remodelling is bypassed [13] and, second, it provides the possibility to administer relatively high doses of omega-3 fatty acids.

Recent findings suggest that omega-3 fatty acids modulate the sensitivity of tumor cells to several anticancer drugs. *In vitro* studies showed an increase in the cytotoxic activity of the anticancer drug doxorubicin on L1210 murine leukemia cells [14], on small-cell lung carcinoma cell lines [15] and on the human breast cancer cell line MDA-MB-231 [16] when given in combination with PUFAs, especially polyunsaturated fatty acids of the omega-3 series. A synergistic action of eicosapentaenoic acid and the angiogenesis inhibitor TNP-470 was found in various breast cancer cell lines [17]. In experimental mammary tumors an increased tumor response to mitomycin C [18, 19] and cyclophosphamide [20] was found in athymic mice that were fed a diet enriched in fish oil.

The aim of the present study was to investigate whether a complex omega-3 fatty acid containing polyunsaturated triacylglycerol emulsion, derived from fish oil and used in human parenteral nutrition, may influence growth and chemosensitivity of the colon cancer cell line Caco-2. We tested the effects of FO alone and in combination with a commonly used anticancer drug, 5-fluorouracil (5-FU).

Materials and methods

Cell culture

The colon cancer cell line Caco-2 (European Collection of Cell Cultures, Porton Down, UK) was routinely grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 5% non-essential amino acids, 5% sodium pyruvate, 100 U/ml penicillin and 100 mg/l streptomycin (all obtained from Gibco, Eggenstein, Germany), at 37 °C in the presence of humidified 5% carbon dioxide in air. Cells were passaged at 80% confluency using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA (Gibco, Eggenstein, Germany). The fish oil-based lipid emulsion (FO) (Omegaven 10%®; Fresenius-Kabi, Bad Homburg, Germany) consists of fish oil 100 g/l and glycerol 25 g/l. The amount of EPA and DHA was 21 g/l and 17 g/l, respectively. FO dosage was calculated corresponding to an amount of EPA varying from 10 to 100 µM, shown to inhibit growth in a variety of cancer cell lines [21, 22], which resulted in the final concentrations of FO ranging from 0.14 to 1.44 ml/l. The corresponding DHA concentrations were 7.5 to 75 µM. To exclude a non-specific effect of lipid emulsion on the cells, control experiments were carried out with a lipid emulsion on the basis of soybean oil (SO) consisting of soybean oil 100 g/l and glycerol 25 g/l (Lipovenoes 10% PLR®; Fresenius-Kabi, Bad Homburg, Germany). This lipid emulsion contained no EPA or DHA and the predominant fatty acids were linoleic and oleic acids. The fatty acid composition of both lipid emulsions is shown in Table 1. In addition controls were run with medium only (no additives). Cytotoxicity of the substances was excluded by lactate dehydrogenase (LDH) release assay (Boehringer Mannheim, Mannheim, Germany).

For combination experiments 5-fluorouracil (5-FU) (Medac, Hamburg, Germany) and FO were added in increasing concentrations: 1) 0.25 µM of 5-FU with 10, 25 und 50 µM EPA (0.14, 0.36 and 0.72 µl ml^{-1} FO), 2) 0.5 µM of 5-FU with 10, 25 und 50 µM EPA (0.14, 0.36 und 0.72 µl ml^{-1} FO), 3) 1.0 µM of 5-FU with 10, 25 und 50 µM EPA (0.14, 0.36 und 0.72 µl ml^{-1} FO).

Growth experiments

Cells were cultured in 96-well tissue culture plates at a density of 5×10^3 cells per well in 100 µl of medium for the indicated time. At the end of the incubation period the adherent cells were fixed to the plate with 5% formaldehyde in phosphate-buffered saline (PBS) and the cell numbers were determined by staining with a 0.5% aqueous solution of crystal violet followed by the elution of the dye with 33% aqueous acetic acid [23]. The absorbance at 620 nm was determined with a rainbow SLT Tecan microplate reader (SLT Tecan, Crailsheim, Germany). Cell proliferation was assessed by using a colorimetric immunoassay based on the measurement of 5-bromo-2'deoxyuridine (BrdU) incorporation during DNA synthesis (Boehringer, Mannheim, Germany).

Table 1 Fatty acid composition of the lipid emulsions (*SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *FA* fatty acids)

Fatty acids (%)	FO	SO
SFA	17	15
MUFA	23	23
PUFA	49	62
n-3-FA	88	9
n-6-FA	12	91
Others	11	–

Cell cycle analysis

Flow cytometric analysis was performed with a FACScalibur (Becton Dickinson, Heidelberg, Germany). Cells were seeded on 6-well plates at a density of 2×10^5 cells per well in 2 ml medium. After incubation with the lipid emulsion for 72 h, cells were trypsinized, washed with PBS and prepared with DNA cycle test plus® (Becton Dickinson, Heidelberg, Germany). Propidium iodide (PI) stained nuclei were obtained according to the manufacturer's instructions. For each experimental condition a minimum of 1×10^4 cells was analyzed. Data were acquired using CellQuest Software (Becton Dickinson, Heidelberg, Germany). Calculation of cell cycle distributions was performed by using ModFit 2.0 software (Verity Software House, Topsham, ME).

Cell morphological characterization

Apoptosis was proven by nuclear staining with DAPI (diamino-phenylindole hydrochloride). Both adherent and floating cells were stained with 10 µl DAPI and viewed directly with a fluorescence ultraviolet light microscope. This allowed unequivocal identification of apoptotic cells using established criteria [24, 25].

Calculations and statistics

For each variable at least 3 independent experiments were performed. Data are given as the mean ± SD. Statistical analysis was carried out using Mann-Whitney U test (Microsoft Excel, Microsoft, Roselle, IL). A *P*-value < 0.05 was considered significant.

Results

Effect of FO on proliferation and number of Caco-2 cells

The effect of the lipid emulsions in various concentrations was tested after 24, 48 and 72 h. Incubation of the cells with FO inhibited growth in a time and dose dependent manner.

Growth inhibition curves for a 72 h treatment with FO showed that there was a low growth promoting effect of FO at a concentration of 0.14 ml/l corresponding to 10 µM EPA, whereas increasing concentrations exerted a highly significant growth inhibition (Fig. 1A). At a concentration of 100 µM EPA (1.44 ml/l FO) it induced a reduction of the cell number greater than 80 % (85 ± 7 % of control, $P < 0.001$). The IC_{50} was 45 µM EPA and 34 µM DHA, respectively (0.65 ml/l FO). The control lipid emulsion with a similar lipid load had only a slight inhibitory effect on growth of the Caco-2 cells.

After 24 h a highly significant growth inhibition of –21 ± 6 % of control ($P < 0.001$) was seen in cells exposed to 50 µM EPA (0.72 ml/l FO). This growth inhibition progressively increased up to –42 ± 4 % of control ($P < 0.001$) at 48 h and up to –53 ± 5 % of control ($P < 0.001$) at 72 h of incubation with FO (Fig. 1B).

Comparable results were determined for BrdU uptake used as an index of DNA synthesis (data not shown).

Morphology of cell death

As a first approach to determine the mechanism by which the growth inhibition may be transmitted, we

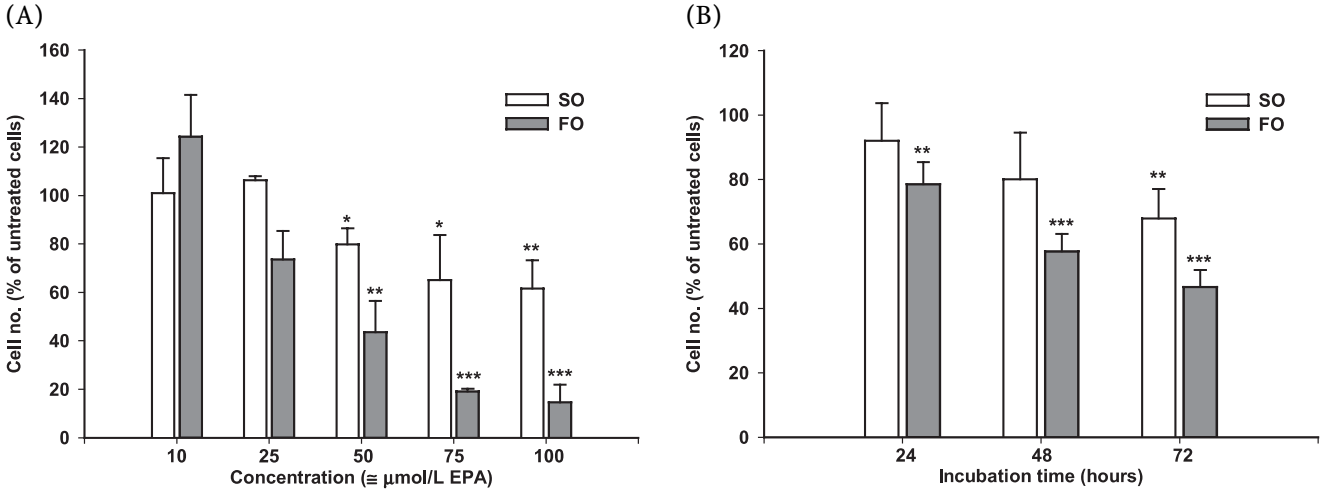

Fig. 1 Effect of FO on cell proliferation of Caco-2 cells (**A**) dose dependent after 72 h-exposure to increasing concentrations of the lipid emulsions (0.14–1.44 ml/l corresponding to 10–100 µM EPA in FO) and (**B**) time dependent after incubation of the cells with the lipid emulsions (0.72 ml/l FO corresponding to 50 µM EPA in FO) for 24, 48 and 72 h. Results are expressed as percentage of the growth of cells exposed to medium alone. Values are means ± SD, n = 8. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control

studied the test compounds with respect to the induction of apoptosis in the Caco-2 cells. Following the nuclear staining of Caco-2 cells exposed to FO with DAPI, the typical apoptotic morphology of the cells was confirmed with fluorescence microscopy (Fig. 2). The death by apoptosis of Caco-2 cells, exposed to 50 µM EPA (0.72 ml/l FO) for 72 h, was reflected by a marked reduction in size of both the nucleus and the whole volume of the cell, and by the appearance of a highly condensed chromatin and irregular nuclear DNA.

Effect of FO on cell cycle progression

The effect of the test compounds on the cell cycle progression was analyzed by flow cytometry. The cell cycle analysis of Caco-2 cells grown in medium alone revealed a percentage of cells of 42 ± 6 % in the G_0/G_1 phase, 47 ± 5 % in the S phase and 11 ± 1 % in the G_2/M phase (Fig. 3). Treatment with FO decreased cell cycle progression in the G_2/M phase. Incubation of the cells with increasing concentrations of EPA (0–50 µM corresponding to 0–0.72 ml/l FO) resulted in an increase in the percentage of DNA staining up to 2.2-fold in the G_2/M phase (25 ± 6 % versus 11 ± 1 %) while the proportion of cells in the G_0/G_1 and the S phase decreased (35 ± 4 % versus 42 ± 6 % and 40 ± 8 % versus 47 ± 5 %). No changes in the cell cycle distribution were observed when the cells were incubated with the control lipid emulsion (data not shown).

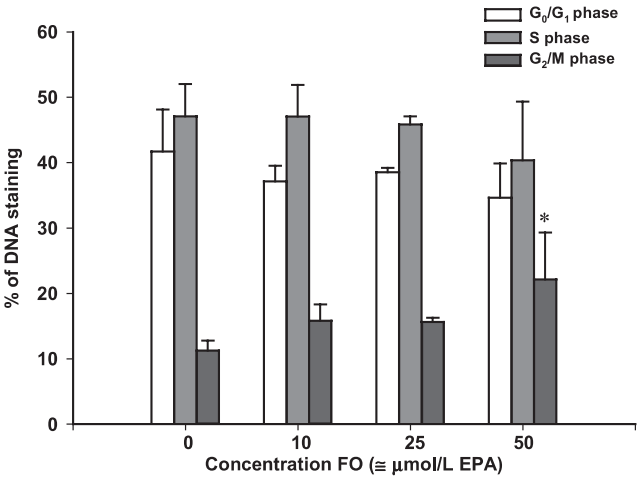

Fig. 3 Effect of FO on cell cycle distribution of Caco-2 cells. Cells were exposed to increasing concentrations of EPA (10–50 µM EPA corresponding to 0.14–0.72 ml/l FO) for 72 h. The percentage of DNA staining increased up to 2.2-fold in the G_2/M phase while the proportion of cells in the G_0/G_1 and the S phase decreased. Values are means ± SD, n = 3. * P < 0.05 vs. control

Combination of FO with the anticancer drug 5-FU

In additional experiments we examined the effect of combining FO with the conventional chemotherapeutic agent 5-FU on the growth of Caco-2 cells. To determine the cytotoxic activity of 5-FU on Caco-2 cells, dose-effect experiments were performed (data not shown). For the combined experiments sub-optimal doses of 5-FU (0.25–1 µM) and FO (10–50 µM EPA corresponding to 0.14–0.72 ml/l FO) were used. When the cells were exposed to the combination of FO and 5-FU, more cells were killed compared to the exposure of the cells to either agent alone (Fig. 4). FO enhanced the growth-inhibitory activity of 5-FU in a dose dependent manner. The combined treatment of 0.25 µM 5-FU with 10 µM EPA (0.14 ml/l FO) resulted in a growth inhibition of –8 ± 4 % ($P < 0.05$; combined treatment vs. both substances alone). The combination with 25 and 50 µM EPA (0.36 and 0.72 ml/l FO) exhibited a further growth inhibition of –61 ± 2 % ($P < 0.001$; combined treatment vs.

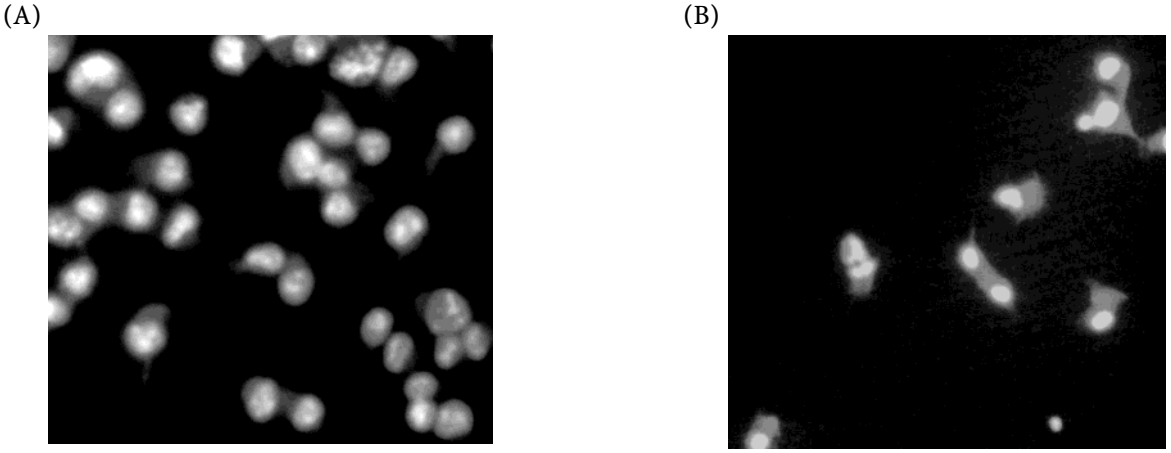

Fig. 2 Caco-2 cells, stained with DAPI and viewed by fluorescence microscopy. **A** Untreated cells; **B** cells exposed to 50 µM EPA (0.72 ml/l FO). In (**B**) cells treated with FO showed classic characteristics of apoptosis, which are highly condensed chromatin and irregular nuclear DNA

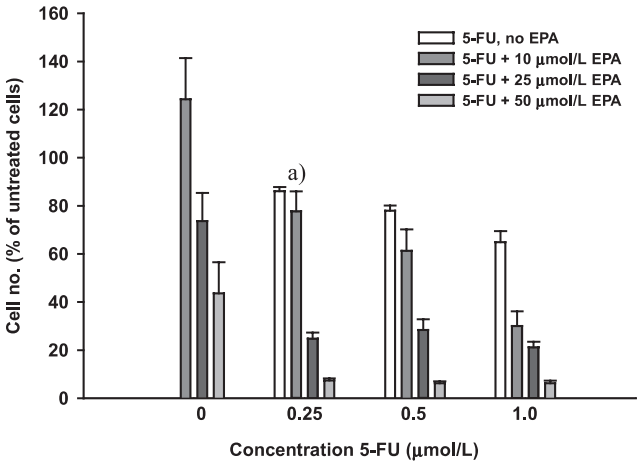

Fig. 4 Effect of 5-FU in combination with FO on cell proliferation of Caco-2 cells. Cells were exposed to increasing concentrations of EPA (10–50 µM corresponding to 0.14–0.72 ml/l FO) and 5-FU (0.25–1 µM). Cell counts were determined after 72 h of incubation. Results are expressed as percentage of the growth of cells exposed to medium alone. Values are means ± SD, n = 8. All values were found highly significant ($P < 0.001$; combined treatment vs. both substances alone) except for a) $P < 0.05$

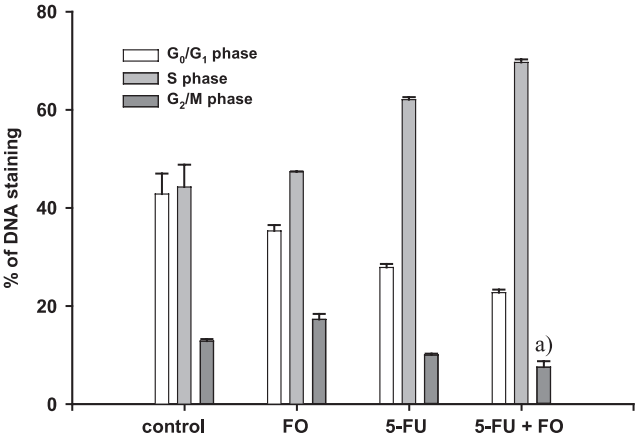

Fig. 5 Cell cycle analysis of Mia-Paca-2 cells after combined treatment with FO and 5-FU for 72 h. Representative data for exposure to 25 µM EPA (0.36 ml/l FO) and 5-FU (1 µM). Incubation of the cells with 5-FU resulted in an increase of the cells in the S phase fraction of up to 62 ± 1 %. The combined treatment of 5-FU and FO showed a further increase in the accumulation of cells in the S phase (70 ± 1 %). Values are means ± SD, n = 3. All values were found highly significant ($P < 0.001$; combined treatment vs. both substances alone) except for a) $P < 0.05$

both substances alone) and −79 ± 1 % ($P < 0.001$; combined treatment vs. both substances alone), respectively.

Effect of a combined treatment with FO and 5-FU on the cell cycle progression of Caco-2 cells

5-FU is an antimetabolite known to specifically block cells in the S phase. In our experiments incubation of the cells with 1 µM 5-FU resulted in an increase of the cells in the S phase fraction of up to 62 ± 1 % compared to 44 ± 3 % in untreated cells. G_0/G_1- and G_2/M phase were 28 ± 1 % (versus 43 ± 3 %) and 10 ± 1 % (versus 12 ± 1 %), respectively (Fig. 5). The combined treatment of 5-FU (1 µM) and FO (25 µM EPA corresponding to 0.36 ml/l FO) showed a further increase in the accumulation of cells in the S phase (70 ± 1 %). The number of the cells in the G_0/G_1- and G_2/M phase decreased to 23 ± 1 % and 7 ± 2 %, respectively.

Discussion

It has been reported that omega-3 polyunsaturated fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are present in fish oil, may inhibit the initiation and promotion, as well as the progression of experimental neoplasia [26–29]. The fish oil preparation used in the present study inhibited proliferation of Caco-2 cells in a time and concentration dependent manner. This growth inhibition is consistent with the previously reported growth inhibitory effect of EPA and DHA on a variety of malignant cells *in vitro* [21, 30–33].

This study differs from previous investigations in the form of the fatty acid preparation. The substances we used were complex lipid emulsions. The colonic cancer cell line Caco-2 is reported to possess an endogenous lipase present in the cytosolic cell fraction as well as in the apical brush border membrane that permits an uptake of structured triglycerides (TG) [34]. The lipid emulsions used in these experiments contain the specific fatty acid pattern of the native source (fish oil versus soybean oil). The predominant PUFAs in the fish oil preparation are the omega-3 fatty acids EPA and DHA, whereas in soybean oil, linoleic acid, which belongs to the omega-6 family, predominates.

The classic pathway influenced by omega-3 fatty acids is the generation of eicosanoids. It has been suggested that EPA, as a competitive inhibitor of arachidonic acid in the cyclooxygenase (COX) pathway, may reduce the production of eicosanoids such as prostaglandin E_2 (PGE_2), which seems to play an important role in the colon carcinogenesis [35–37]. Rigas et al. (1993) found increased concentrations of PGE_2 in human surgically excised colon cancers compared with the corresponding normal colonic mucosa [38]. In rats a reduction in the PGE_2 content of colonic tumors which was associated with a suppression of rat colon carcinogenesis could be seen after treatment with EPA [39].

The elevated levels of PG in colon cancers are associated with an upregulation of COX-2 [40]. Several experimental studies have shown the effectiveness of selective COX-2 inhibitors as chemopreventive agents [41, 42]. In rats this enzyme induction could be enhanced further

by feeding a diet rich in omega-6 fatty acids while an omega-3 fatty acid rich-diet suppresses the COX-2 expression [43]. Thus, the suppressive effect of omega-3 fatty acids on colon carcinogenesis appears to be intimately related to the down-regulation of COX-2.

Treatment of Caco-2 cells with the fish oil-based lipid emulsion resulted in apoptosis, as confirmed morphologically by the presence of characteristic apoptosis-related features like cell shrinkage, formation of apoptotic bodies and irregular nuclear DNA. A close relationship exists between apoptotic cell death and cell cycle block. The onset of apoptosis appears to follow the arrest of the cells in the G_2/M phase [44]. Flow cytometric analysis revealed that exposure of the cells to the fish oil-based lipid emulsion resulted in an increase of the proportion of cells in the G_2/M phase, while a marked decrease of cells occurred in the G_0/G_1 and S phase.

Various experimental studies have reported the ability of exogenous fatty acids to modulate the cytotoxic activity of several anticancer drugs in cell culture as well as in tumor-bearing animals [16, 20]. The single most effective chemotherapeutic agent for advanced CRC is 5-fluorouracil (5-FU). It achieves clinical benefit and symptom improvement in 20–30 % of patients [45]. Despite the established single-agent activity there is a need for combination therapy schedules in order to improve the efficiency of the therapy. This should ideally include agents with negligible side effects, e. g., natural substances like omega-3 fatty acids. As shown in this study, a combination of FO and 5-FU induced a significantly greater cytotoxicity than either agent alone. This finding complements previous studies showing that the effect of omega-3 fatty acid therapy is additive to those of other classical chemotherapeutic agents, e. g., doxorubicin, cis-platinum and mitomycin C [16, 20].

The action of 5-FU is mediated via the pyrimidine salvage pathway, mainly by inhibition of the thymidilate synthase resulting in an arrest of cells in the S phase of the cell cycle. Our results from the flow cytometric analysis of cells treated with 5-FU confirmed the specificity of the agent to arrest cells in the S phase of the cell cycle. After the combined treatment of 5-FU with FO the specific S phase accumulation was significantly greater, which proves FO to be a candidate for potentiating the effect of 5-FU.

To date no precise mechanism of the action of omega-3 fatty acids in the modulation of tumor chemosensitivity is known. It has been proposed that the increase in anticancer drug efficacy could result from alterations in the biophysical properties and functions of the cell membrane after supplementation of fats rich in omega-3 fatty acids. Membrane fluidity and drug transport are influenced by the ratio of saturated to unsaturated fatty acids [46]. Since omega-3 fatty acids are highly unsaturated, their intake could increase the level of unsaturation in tumor membrane phospholipids, which then correlates with an increased cellular accumulation of cytotoxic drugs [46, 47]. In addition, modification of the unsaturation index results in a higher susceptibility of the tumor to oxidative stress [19].

In summary, the fish oil-based lipid emulsion had a significant growth inhibitory effect on the colon cancer cell line Caco-2. This effect was at least in part due to the induction of apoptosis and a decrease in DNA synthesis. In addition, the fish oil-based lipid emulsion potentiated the cytotoxic activity of 5-fluorouracil. As omega-3 fatty acids are being tested as relatively non-toxic, anticancer therapeutic agents, this knowledge of the mode of cell death and the potential for combining two different modes of therapy, may help to design improved treatments for patients with some refractory forms of cancer, such as colorectal cancer.

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