

Omega-3 Fatty Acids

Investigations From Cytokine Regulation to Pancreatic Cancer Gene Suppression

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Omega-3 (ω -3) fatty acids have been clinically and experimentally associated with the amelioration of chronic and acute inflammation; however, the mechanisms for these observations have not been well defined. During the past decade, laboratories of nutrition and inflammation have demonstrated that the anti-inflammatory activities of ω -3 fatty acids occur at least in part through the inhibition of macrophage-elaborated tumor necrosis factor production and through inactivation of the nuclear factor- κ B signaling pathway subsequently altering proinflammatory cytokine transcription. These observations led to further experiments that support a role for ω -3 fatty acids in the restoration of apoptosis in various chemoresistant tumor models through a similar inactivation of the nuclear factor- κ B signaling pathway. The potential for nutritional modulation of host inflammation has been an ongoing and expanding area of investigation. An increased emphasis has been placed on the potential for diet and dietary supplements to serve as modulators of host response to disease, injury, and infection.

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Omega 3 (ω -3) fatty acids (FAs) are polyunsaturated fats, commonly referred to as essential FAs because they are not synthesized by mammals and must be obtained through diet.¹ A-Linolenic acid, a precursor of ω -3 FAs, is metabolized in the liver to longer-chain FAs called eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Eicosapentaenoic acid and DHA are considered the most biologically active forms of ω -3 FAs.¹ Consumption of EPA has been associated with the prevention of atherosclerosis, the successful treatment of coronary artery disease, and amelioration of chronic inflammatory diseases.²⁻⁵

ANTI-INFLAMMATORY PROPERTIES OF ω -3 FAs THROUGH TNF

In healthy humans and animal models of experimental infection, EPA has consistently reduced prostaglandin synthesis in

response to inflammatory stimuli; however, a concomitant reduction in proinflammatory cytokine production, especially tumor necrosis factor (TNF) and interleukin 1, has been variably reported.⁶⁻¹⁰ Variability in type and content of experimental EPA preparations may account for some of the inconsistent results observed. For example, EPA salts and EPA-albumin complexes may independently increase baseline levels of macrophage TNF in vitro. These noted discrepancies may be attributable to lipid oxidation, endotoxin contamination, or simply the presence of immunosuppressive components (ie, albumin) in these EPA preparations.¹¹ In the late 1990s, a sterile, pharmaceutical-grade ω -3 FA lipid emulsion (Omegaven; Fresenius Kabi Deutschland GmbH, Hamburg, Germany) containing EPA-DHA mixed in a 1:1 ratio became available in Europe and was used as an anti-inflammatory agent in critically ill patients; however, the mechanism(s) of its anti-inflammatory actions remains unknown.^{12,13} The availability of a similar ω -6 lipid emulsion (Lipoven;

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Fresenius Kabi Deutschland GmbH) for use as a control enabled appropriate experiments to be conducted.

This ω -3 FA emulsion was experimentally used to define ω -3 effects on TNF production in a model of lipopolysaccharide (LPS)-stimulated macrophages (M ϕ s).¹⁴ A murine M ϕ cell line (RAW 264.7 cells) was incubated with media alone (Omegaven) or an isoenergetic ω -6 lipid emulsion for 4 hours before stimulation with LPS (1 mg/mL) or media alone for an additional 3 hours. We demonstrated that 4-hour ω -3 pretreatment significantly reduced TNF production in LPS-stimulated cells, with a 46% reduction in TNF from baseline observed. These data support the contention that anti-inflammatory effects of ω -3 FAs occur at least in part through the inhibition of M ϕ TNF production in response to endotoxin.

ANTIANGIOGENIC PROPERTIES OF ω -3 FAs

The use of ω -3 FAs in the treatment of patients with cancer has been of special interest to many investigators and patients. In vitro studies^{15,16} have shown that malignant murine melanoma, human breast cancer cells, and human fibrosarcoma cancer cells cultured in EPA-rich media are less proliferative and invasive. In various animal models, supplemental EPA decreased the number and size of tumors.^{1,17} Fish oil-fed mice demonstrate reduced tumor growth, metastatic load, blood vascular area, mast cell number, and M ϕ infiltration in solid mammary tumors.¹⁸ In addition, ω -3 FAs have been shown to slow primary tumor growth and decrease metastasis in animal and human models of mammary tumorigenesis.¹⁸⁻²³

Although the mechanisms for the amelioration of symptoms associated with malignant tumors by EPA are not well defined, prior studies^{24,29} have suggested that EPA-induced downregulation of proinflammatory mediators occurs by displacing arachidonic acid in M ϕ membrane, thereby inhibiting the cyclooxygenase 2 pathway and decreasing production of angiogenic growth factors, including vascular endothelial growth factor α .

To assess this hypothesis, we used a rapidly growing tumor model, the methylcholanthrene sarcoma, to examine EPA-associated inhibition of the neoangiogenic factor vascular endothelial growth factor α and its effect on tumor volume.²⁶ Fisher 344 (Charles River Laboratories International Inc, Wilmington, Massachusetts) rats with methylcholanthrene-induced fibrosarcoma were randomly assigned to 3 groups with treatment delivered via oral gavage twice daily: (1) 5 g/kg of EPA plus 10 IU of vitamin E/G fat, (2) 5 g/kg of corn oil plus 10 IU of vitamin E/G fat, and (3) 5 g/kg of saline plus 10 IU of vitamin E/G saline. Rats pretreated with EPA had significant reductions in tumor volume compared with isocaloric corn oil and control saline rats (25%, $P < .01$, and 33%, $P < .01$, respectively).²⁶ Rats receiving EPA demonstrated significantly decreased vascular endothelial growth factor α messenger RNA levels (mean [SD], 0.02 [0.001]) compared with those receiving corn oil (0.13 [0.047]) or saline (0.15 [0.026]; $P < .05$). These results demonstrated that EPA supplementation inhibited tumor growth, potentially through alterations in the expression of the proangiogenic vascular endothelial growth factor α .

The liver plays a central role in modulating nutritional status and cancer-induced cachexia. Using the previously described model, we examined the liver and nutritional parameters indicative of cancer cachexia along with tumor volume in response to EPA.²⁷ The EPA-treated rats showed a notable reduction in tumor volume compared with animals fed corn oil (25% reduction, $P < .01$) and saline (33% reduction, $P < .01$). The EPA rats also demonstrated increased liver weight ($P < .01$) and total liver protein levels ($P < .03$) over saline-treated animals. Interestingly, regression analysis showed that weight and protein differences between treatment groups were not correlated with individual tumor volumes; therefore, tumor growth inhibitory effects and anticachexiogenic effects of EPA are independent phenomena.

MECHANISMS OF ω -3 FA CYTOKINE REGULATIONS

Several hypotheses have been explored in an attempt to identify the specific mechanism(s) for the noted attenuation of proinflammatory cytokines in response to EPA elaboration. Among the several postulated mechanisms are (1) a direct and specific ω -3 lipid signal transduction-mediated action,²⁸ (2) a substrate phenomenon whereby the substitution of ω -3 lipids for the ω -6 arachidonic acid results in the elaboration of 3-series prostaglandins rather than 2-series prostaglandins,²⁹ (3) modulation of signal transduction-associated cascades such as mitogen-activated protein kinase by ω -3 lipids or their cyclooxygenase enzyme-catalyzed products,³⁰ and (4) alteration in the activation of transcriptional factors such as nuclear factor (NF)- κ B or activator protein-1, resulting in decreased cytokine production.^{30,31}

DIRECT SIGNAL TRANSDUCTION EFFECT OF ω -3 FAs ON NF- κ B

Despite previous demonstrations that ω -3 FA emulsions inhibit LPS-mediated TNF expression in murine M ϕ s,¹⁴ the exact mechanisms of action remain unknown. One hypothesis is that ω -3 FAs may modulate NF- κ B transcriptional activator proteins, a principal pathway for M ϕ proinflammatory cytokine elaboration.^{32,33}

The inflammatory cytokine NF- κ B is located in the cytoplasm and usually dimerized to the inhibitory protein (I κ B).³⁴ Under basal conditions, I κ B maintains NF- κ B in the cytoplasm by preventing display of the nuclear localization sequence.^{35,36} When M ϕ s are activated by a large variety of inducers, including endotoxin via the Toll 4 receptor,³⁷ I κ B kinase- α phosphorylates I κ B at 2 serine residues (Ser 32 and 36), allowing dissociation from NF- κ B.³⁸⁻⁴⁰ Phosphorylated I κ B is subsequently targeted for polyubiquitination and degradation through the 26S proteasome pathway. The NF- κ B is then free to localize to the nucleus, initiating transcription of various proinflammatory cytokine genes, most notably TNF.⁴¹ Because the activation of NF- κ B plays a vital role in the elaboration of TNF, we hypothesized that ω -3 FAs may exert inhibitory effects at a specific point along the NF- κ B pathway.

The RAW 264.7 cells (murine MØ cell line) were pre-treated with isocaloric emulsions of ω -3 FAs (Omega-ven), ω -6 FAs (Lipoven), or Dulbecco modified Eagle medium and subsequently exposed to LPS.³¹ Phosphorylation of I κ B- α was assayed using Western blotting. Binding of NF- κ B was assessed using the electromobility shift assay, and activity was measured using a luciferase reporter vector. Reverse transcriptase-polymerase chain reaction and enzyme-linked immunosorbent assay quantified TNF messenger RNA and protein levels, respectively. These experiments demonstrated that murine MØs treated with ω -3 FAs significantly decreased I κ B phosphorylation at serine 32 and consequently reduced the ability of NF- κ B to bind to the TNF-specific consensus sequence. As a result, the NF- κ B signal transduction cascade is inhibited, and this decreased NF- κ B activity is translated into a concomitant decrease in TNF messenger RNA and protein expression by 47% and 46%, respectively. Moreover, the ω -6 FA-treated MØs exhibit effects similar to MØs exposed to media alone in all experiments, validating observations that the anti-inflammatory effects on MØs are exclusive to ω -3 FAs and not a result of a general lipid effect.

ω -3 FAs AND CANCER

Recent evidence has shown that the modern Western diet contains a low amount of ω -3 FAs.¹⁷ In early human diets, the ratio of ω -6 FAs to ω -3 FAs was approximately equal. With the evolution of the modern Western diets, the ratio of ω -6 FAs to ω -3 FAs largely favors ω -6 FAs. This distressing trend has contributed to an increase in the risk of cardiovascular disease and certain cancers, such as those of the breast and colon. Inversely changing this ratio to favor ω -3 FAs has been shown to have suppressive growth effects on cancerous cells within in vitro and in vivo animal models.¹

Investigators have previously reported that the coincubation of various cancer cell lines with ω -3 FAs leads to a reduction in cell number in a time-dependent and dose-dependent manner.⁴²⁻⁴⁴ This effect has been noted to occur in pancreatic cancer cell culture, suggesting a potential antiproliferative role for ω -3 FAs; however, the mechanism of action remains unknown.^{45,46}

ROLE OF ω -3 FAs IN PANCREATIC CANCER

We hypothesized that the noted reduction in cell numbers observed after pancreatic cancer cell coincubation with ω -3 FAs resulted from decreased cell proliferation, alteration in cell cycle progression, and the induction of apoptosis. In a set of experiments, MIA PaCa-2 cells (human pancreatic ductal adenocarcinoma cell line) were treated with ω -3 FA emulsion, ω -6 FA emulsion, or media for all experiments.⁴⁷ Cellular proliferation was evaluated with a water-soluble tetrazolium salt reagent. Cells were stained with propidium iodide and analyzed by flow cytometry for cell cycle arrest, which was further analyzed by cell cycle regulator expression. Membrane and media lipid concentrations were analyzed by high-performance liquid chromatography. Apoptosis was evaluated by Annexin V fluorescein isothiocyanate

flow cytometry and reconfirmed by poly (adenosine diphosphate ribose) polymerase (PARP) cleavage and Bcl-2 expression.

We noted that propidium iodide flow cytometry of MIA PaCa-2 dosed with ω -3 FAs showed a decrease in cells in the G1 phase (11%-17%) and an increase in cells in the G2 phase (7%-13%) compared with controls. In addition, cell cycle regulator expression was decreased at 24 hours compared with controls. Annexin V staining of ω -3 FA-treated cells demonstrated time-dependent increased apoptosis, and PARP cleavage was present only in the ω -3 FA treatment group. Also decreased in the ω -3 FA-treated cells compared with controls was phospho-Bcl-2. This report supports the presence of specific antiproliferative and proapoptotic mechanisms in this pancreatic cancer cell line, which parallel the ω -3 FA-associated experimental observations that have been made in other nonpancreatic cancer in vitro models.

CAN ω -3 FA CONTROL PANCREATIC CANCER RESISTANCE TO GEMCITABINE THROUGH NF- κ B?

Altered cellular apoptosis is known to be an important mechanism in pancreatic cancer chemoresistance and is a current active area of research. Recently, the potential of the STAT family of proteins and NF- κ B to serve as important targets for anticancer and proapoptotic therapies has been identified.⁴⁸⁻⁵⁰

Based on its role in the initiation, progression, and dysfunction of malignant cells, as well as the elaboration of inflammatory cascades associated with malignant transformation, NF- κ B is considered a significant component in the regulation of cellular activity.⁵¹ Furthermore, NF- κ B has been demonstrated to be a key mediator in pancreatic cancer cell chemoresistance to gemcitabine hydrochloride (Gemzar; Eli Lilly and Co, Indianapolis, Indiana).⁵²

Clinical data suggest a potential role for ω -3 FAs in the treatment of patients with pancreatic adenocarcinoma to improve functional status, weight gain, and treatment-related consequences.⁵³⁻⁵⁵ We and others^{43,44,56} also previously documented that ω -3 FAs when incubated with various cancer cell types in vitro have been shown to have antiproliferative and proapoptotic effects. At present, the criterion standard pancreatic cancer chemotherapeutic agent, gemcitabine, has been shown in experimental models to induce NF- κ B activation in pancreatic cell lines, which is likely a paradoxically pro-proliferative and antiapoptotic event. On the basis of the known biologic activities for ω -3 FAs, the potential to overcome gemcitabine-associated NF- κ B activation was investigated further.^{14,57}

Four pancreatic cancer cell lines (MIA PaCa-2, BxPC-3, PANC-1, and L3.6), each with distinct basal NF- κ B and differing gemcitabine sensitivity profiles, were administered: 100 μ M of (1) ω -3 FAs, (2) ω -6 FAs, (3) gemcitabine, (4) ω -3 FA and gemcitabine, or (5) ω -6 FAs and gemcitabine for 24 and 48 hours. Proliferation was assessed using water-soluble tetrazolium salt assays. To define the mechanism(s) of altered proliferation, electromobility shift assay for NF- κ B activity and Western blots

of phosphoSTAT3, phospho-I κ B, and PARP cleavage were performed in the MIA PaCa-2 cell line. All cell lines demonstrated a time-dependent and dose-dependent inhibition of proliferation in response to ω -3 FAs. For MIA PaCa-2 cells, ω -3 FA and ω -3 FA plus gemcitabine treatment resulted in reduction of I κ B phosphorylation and NF- κ B activation when compared with the ω -6 FA control. The ω -3 FA and combination treatment also demonstrated significantly decreased STAT3 phosphorylation, whereas gemcitabine alone had no such effect. The ω -3 FA and ω -3 FA plus gemcitabine groups demonstrated increased PARP cleavage, mirroring NF- κ B activity and STAT3 phosphorylation.

GENETIC EXPRESSION PROFILING of ω -3 FA TREATMENT ON PANCREATIC CANCER

Recent studies have begun to characterize the complex molecular changes associated with pancreatic carcinogenesis. The aggressive, chemoresistant behavior is thought to be owing to a complex alteration in multiple signal transduction pathways, including Ras-MAPK, PI3K-AKT, and hedgehog.

Using a 118-gene microarray designed to assess 18 signal transduction pathways, our laboratory used ω -3 FAs, gemcitabine, and a combination treatment to study the effects on the pancreatic cancer cell line, Mia PaCa-2. The cells were treated for 12 hours with 100 μ M of ω -3 FA emulsion (94 μ M EPA and 87 μ M DHA), 100 μ M of gemcitabine, or a combination of 100 μ M of ω -3 FA emulsion and 100 μ M of gemcitabine.

Of 118 genes assayed, ω -3 FA treatment had 10 significantly expressed genes, 7 unique to ω -3 FA treatment alone. Gemcitabine treatment was associated with 15 significantly expressed genes, 6 unique to gemcitabine. Combination treatment was associated with 14 significantly expressed genes and, interestingly, 8 genes uniquely expressed in combination alone. Also of interest, there were 3 significantly expressed genes shared by ω -3 FA and gemcitabine treatment not present in combination treatment.

In terms of potential synergy between these 2 agents, the 8 significantly expressed genes unique to combination treatment and 3 significantly expressed genes lost in combination treatment represent 11 genetic-level changes that only occur with concomitant treatment of ω -3 FA and gemcitabine. The 8 significantly expressed genes unique to combination treatment are *Birc5*, *CDKN2B*, *CYP19A1*, *FN1*, *IL-2*, *IRF-1*, *NF- κ BIA*, and *TP53*. These genes are represented in the following pathways (in no particular order): Wnt, TGF β , cyclic adenosine diphosphate responsive element binding, PI3K/AKT, NF- κ B, nuclear factor of activated T cells, calcium/PKC, Jak-STAT, stress, and P53. The 3 genes absent in combination are *TRAILR2* (*TNFRSF10*), *EGR1*, and *NAB2*, representing the P53, MAPK, PLC, and cyclic adenosine diphosphate responsive element binding pathways.

In all, these 11 gene-level alterations encompass 12 signal transduction pathways. This indicates that the potential synergy between ω -3 FAs and gemcitabine not only alters the expected pathways, NF- κ B, MAPK, and PI3-AKT, but also has numerous cellular effects not previ-

ously described. The previously observed antiproliferative, proapoptotic effects seen with ω -3 FA treatment, alone and in combination with gemcitabine, can be explained by these wide-ranging and complex changes at the molecular level. Certainly, the host-beneficial effects seen thus far with ω -3 FA treatment may only be a precursor to the power of this natural adjuvant.

The completed experiments support several important and novel findings with possible clinical implications. First, the proliferation data demonstrate that when pancreatic cancer cells of varying genetic expressions are treated with ω -3 FAs, baseline proliferation is significantly reduced. Even PANC-1, the cell line that proliferated in the presence of gemcitabine alone, demonstrated inhibited proliferation with ω -3 FA treatment. Data regarding I κ B and NF- κ B support an ω -3 FA attenuation of NF- κ B-associated transcriptional sequelae that has been previously described.⁵⁸ Although gemcitabine treatment did not significantly change the phosphorylation of I κ B, there was a significant change in the combination treatment results as demonstrated by the NF- κ B electromobility shift assay data. Also, the phosphorylation of STAT3 was observed to be significantly decreased subsequent to ω -3 FA treatment, denoting an ω -3 FA-associated attenuation of the cytokine-induced antiapoptotic STAT3 signaling cascade. Similarly, gemcitabine treatment produced related effects on the phosphorylation of STAT3. Last, the presence of the PARP cleavage product supports progression through apoptosis rather than toxicity. In this model, ω -3 FA treatment shows a strong positive correlation with PARP cleavage. Although there does not seem to be a synergistic effect supported by the PARP cleavage product data, there is a mild proapoptotic effect noted with gemcitabine-only treatment.

A proposed mechanism of ω -3 FA activity on pancreatic cancer chemoresistance is that apoptotic regulatory processes are restored to a system that responds to apoptotic signals through the downregulation of activated NF- κ B and STAT3. This concept of "restored apoptosis" has been described in the literature in other pancreatic cancer models, and this approach has the potential to overcome tumor chemoresistance, as was observed in our ω -3 FA model.^{59,60}

Taken as a whole, these data suggest that pancreatic cancer cells treated with ω -3 FAs alone or in combination with gemcitabine produce an increased inhibition of proliferation, and the mechanism of this inhibition is through progression to apoptosis, not toxicity. An important future clinical implication for these findings is that there is no known negative effect of ω -3 FAs as an adjunct with gemcitabine; specifically, gemcitabine-induced cytotoxicity is not lessened when combined with ω -3 FA in vitro.

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of the data and the accuracy of the data analysis. *Study concept and design:* Boutros, Somasundar, Helton, and Espot. *Acquisition of data:* Boutros, Razzak, Helton, and Espot. *Analysis and interpretation of data:* Boutros and Espot. *Drafting of the manuscript:* Boutros, Somasundar, Razzak, and Espot. *Critical revision of the manuscript for important intellectual content:* Helton and Espot. *Statistical analysis:* Boutros. *Obtained funding:* Espot. *Administrative, technical, and material support:* Boutros, Razzak, Helton, and Espot. *Study supervision:* Somasundar and Helton.

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