Eicosapentaenoic Acid Modifies Cytokine Activity and Inhibits Cell Proliferation in an Oesophageal Cancer Cell Line

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Abstract. *Background/Aim: The present study investigated the effect of eicosapentaenoic acid (EPA) on nuclear factor-kappa B (NF-κB) activation, inflammatory interleukin-6 (IL-6) production, and cell proliferation in a human oesophageal carcinoma cell line (TE-1). Materials and Methods: Lipopolysaccharide (LPS)-induced IL-6 production in TE-1 cells in the presence or absence of EPA was determined using enzyme-linked immunosorbent assay (ELISA). The proliferation of TE-1 cells was determined by the WST-1 assay. TE-1 cells were stained with Hoechst 33342 and propidium iodide to observe apoptosis. Immunohistochemical staining of NF-κB in TE-1 cells was performed. Results: LPS increased IL-6 production in TE-1 cells, and EPA treatment prevented this effect. EPA treatment inhibited NF-κB activation and induced apoptosis of TE-1 cells. Conclusion: EPA inhibits NF-κB activation and IL-6 production in oesophageal cancer cells, their inducing apoptosis. These effects of EPA may be of benefit in improving the outcome of cancer surgery.*

The prognosis of oesophageal carcinoma is poor and surgical stress may contribute to poorer outcomes (1). To improve prognosis and quality of life, new strategies are needed besides conventional therapeutic strategies.

Excessive surgical stress or postoperative complications induce major perioperative release of cytokines, which have been shown to enhance tumour metastasis in experimental models (1). In a previous report we termed this phenomenon surgical oncotaxis (2). Nuclear factor-kappa B (NF-κB) is a transcription factor with a variety of roles in survival, differentiation, and proliferation of cells (3), as well as

inflammatory cytokine release (4). A large body of evidence has demonstrated that NF-κB plays an essential role in tumour survival, chemoresistance, tumour proliferation and tumour metastasis (3, 5). Activation of the NF-κB signalling pathway has been investigated in many types of cancer, including hepatocellular, colonic, pancreatic, and cervical cancers (6). The activation of NF-κB in response to surgical stress may play a key role in surgical oncotaxis. Thus, control of NF-κB activation during the perioperative period may be an important consideration in cancer surgery.

Fish oil, as a third-generation lipid emulsion, is a rich source of bioactive long-chain n-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA), docosapentaenoic acid, and docosahexaenoic acid (DHA). Fish oil has been reported to inhibit NF-κB activation (7, 8). NF-κB has also been shown to modulate the expression of inflammatory cytokines, such as interleukin-6 (IL-6) (4).

We hypothesized that EPA may inhibit the activation of NF-κB in oesophageal cancer cells, resulting in a reduction in inflammatory cytokine production, and inhibition of cancer cell proliferation by induction of apoptosis. The objective of the present study was to investigate the effects of EPA on IL-6 production and induction of apoptosis through NF-κB activation in oesophageal cancer cells.

Materials and Methods

Materials. The TE-1 cell line, an oesophageal squamous cancer cell line, was purchased from RIKEN Bioresource Center Cell Bank (Tsukuba, Ibaragi, Japan). The cells were maintained in RMPI-1 medium supplemented with streptomycin and 10% foetal bovine serum (FBS) in a humid atmosphere with 5% CO_2 at 37°C. 5,8,11,14,17-eicosapentaenoic acid sodium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA). EPA sodium salt was dissolved in phosphate-buffered saline to prepare a stock solution.

IL-6 production. TE-1 cells (2×10^5 cells/ml) were plated in 24-well culture plates and incubated for 24 h. They were then pre-incubated for 3 h with or without 100 μM of EPA before treatment with 0.1-100 μg/ml lipopolysaccharide (LPS) for 24 h. IL-6 was determined using ELISA IL-6: Human IL-6 ELISA Kit (Thermo Fisher

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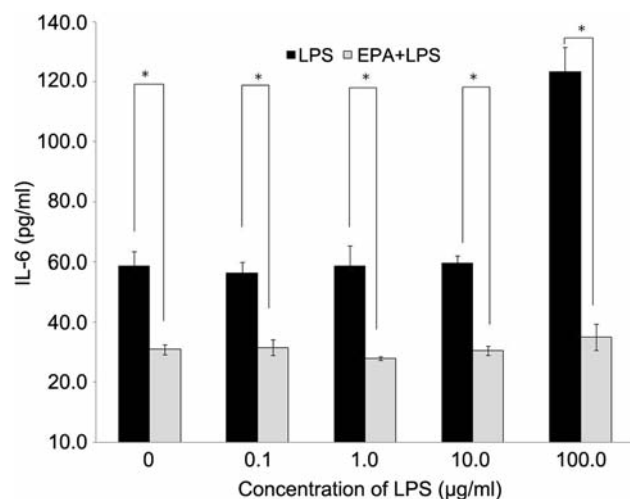
Figure 1. *Interleukin-6 (IL-6) production in TE-1 cells. IL-6 production was markedly increased by the addition of 100 μg/ml lipopolysaccharide (LPS) IL-6 production was suppressed by 100 μM Eicosapentanoic acid (EPA) (*p<0.001). Pre-incubation with 100 μM EPA prevented the LPS-induced increase in IL-6 production. Data are 123.3±8.2pg/ml in 100μg/ml LPS only, and 35.0±4.4pg/ml in pre-incubation with 100mM EPA and 100μg/ml LPS (n=3).*

Scientific K.K. Yokohama, Japan) according to the manufacturer's instructions. The developed color was measured by a microplate reader (Corona Electric, Ibaragi, Japan).

Cell proliferation. TE-1 cells (2×10^4 cells/ml) were plated in 96-well culture plates and incubated for 24 h, then treated for 24 h with control medium or medium containing 1-1000 μM EPA. The proliferation of TE-1 cells was determined by the Premix WST-1 cell proliferation assay system kit (Takara, Tokyo, Japan). WST-1 reagent (10 μl) was added to each well, then cells were incubated at 37°C for 2 h. Absorbance was measured at 450 nm by an automated microplate reader (Thermo Fisher Scientific K.K. Japan).

Morphological changes. TE-1 cells (1×10^4 cells/ml) were plated in 24-well culture plates (IWAKI, Chiba, Japan) and incubated for 24 h, then treated for 24 h with control medium or medium containing 10-1000 μM EPA. After treatment with EPA, cells were examined using an inverted fluorescence microscope, 1X81 (Olympus, Tokyo, Japan).

Hoechst 33342, a blue fluorescent dye (excitation/emission maxima ~350/461 nm when bound to DNA), stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. Propidium iodide (PI), a red fluorescent dye (excitation/emission maxima ~535/617 nm when bound to DNA), only permeates dead cells (8). Hoechst 33342 and PI were purchased from Sigma-Aldrich.

TE-1 cells (1×10^4 cells/ml) were plated in 24-well culture plates (IWAKI) and incubated for 24 h, then were treated for 24 h with control medium or medium containing 1-1000 μM EPA. Hoechst 33342 (final concentration 2 μM) and PI (1 μg/ml) were added to each well and cells then incubated in CO_2 for 15 min in the dark. Cells were examined using an inverted 1X81 microscope.
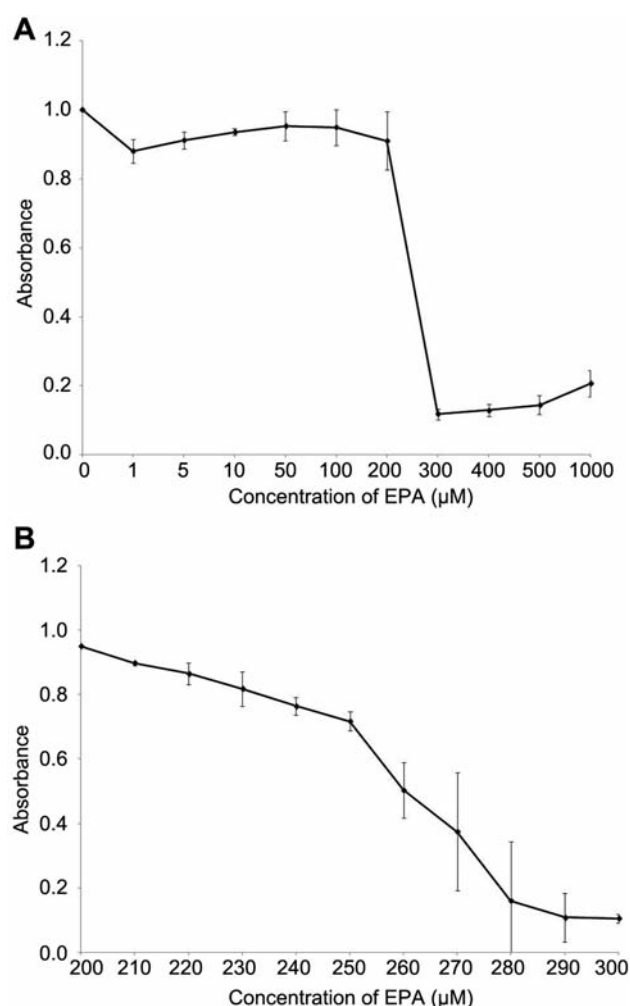

Figure 2. *TE-1 cell proliferation A: Cell proliferation after treatment with 1-1000 μM EPA. The WST-1 assay demonstrated that EPA significantly inhibited the growth of TE-1 cells in a dose-dependent manner (F=318.446; p=0.001). B: After treatment of TE-1 cells with 200-300 μM EPA, the EPA was determined to be 260 μM.*

Immunohistochemical (IHC) staining of NF-κB in TE-1 cells. TE-1 cells (2×10^4 cells/ml) were plated in Nunc Lab-Tec Chambered Coverglass (Thermo Scientific) for 24 h, then treated for 3 h with control medium or medium containing 100, 200 and 300 μM EPA. After fixation of the samples with 4% paraformaldehyde, a rabbit monoclonal antibody against p65 (D14E12) (Cell Signalling Technology, Boston, MA, USA) was used as the primary antibody and cells were incubated overnight at 4°C. Then incubation with the secondary antibody, ChemMate Envision Kit, (Dako, Tokyo, Japan) was performed for 60 min, followed by application of diaminobenzidine chromagen for 5 min. Subsequently, the slides were counterstained with Meyer's haematoxylin for 1 min, dehydrated in a graded series of alcohol solutions, treated with xylene and coverslipped. This analysis was repeated nine times, and cells with stained nuclei were counted as having activation of NF-κB.
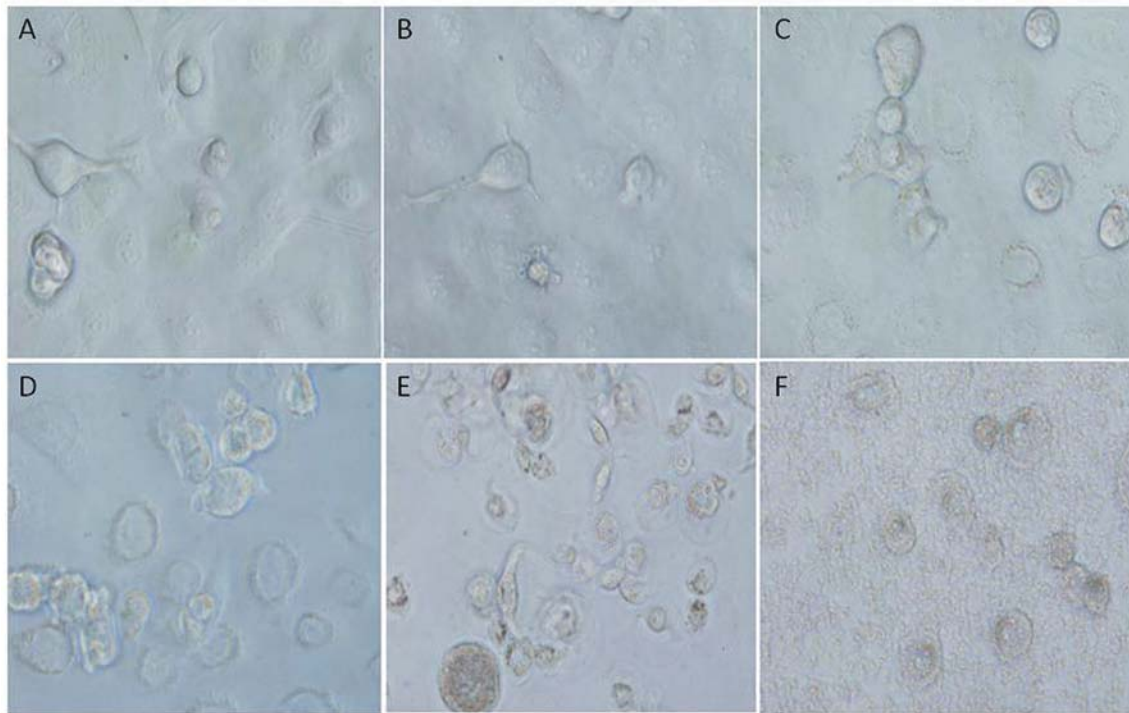
Figure 3. *Morphological changes in TE-1 cells induced by Eicosapentanoic acid (EPA). EPA concentration: (A) 0 μM, (B) 10 μM, (C) 50 μM, (D) 100 μM, (E) 500 μM, (F) 1000 μM.*

Statistical analysis. Experiments were repeated three times using different isolations of TE-1 cells. All data are expressed as the mean standard deviation. The estimated model was considered significant when the two-tailed *p*-value obtained by analysis of variance was less than 0.05. All analyses were performed on a personal computer using the statistical package JMP version 9 for Windows (SAS Institute, Cary, NC, USA). When analysis of variance indicated significant differences, the treatment means were compared in pairs using Fisher's least significant difference procedure. A *p*-value 0.05 was considered significant.

Results

LPS-induced IL-6 production. TE-1 cells produced IL-6, which was significantly reduced by pre-incubation with 100 μM EPA (*p*=0.001) (Figure 1). In the group without EPA, IL-6 production was markedly increased by 100 μg/ml LPS. In the group pre-incubated with EPA, there was no increase in IL-6 level even after addition of 100 μg/ml LPS. This difference was significant (*p* 0.001).

Cell proliferation. The inhibition rate was calculated based on 0 μM EPA. The WST-1 assay demonstrated that EPA significantly inhibited the growth of TE-1 cells in a dose-dependent manner (F=318.446, *p*=0.001) (Figure 2A). TE-1 cell growth was inhibited by 88.3% with 300 μM EPA. The IC_{50} of EPA for inhibition of TE-1 cell growth was 260 μM (Figure 2B).

Morphological changes after treatment with EPA. Apoptosis was induced in TE-1 cells after treatment with EPA for 24 h (Figure 3). TE-1 cells underwent blebbing when treated with 500 μM EPA (Figure 3E), and fragmentation with 1000 μM EPA (Figure 3F).

In the immunohistochemical analysis using Hoechst 33342 and PI, apoptosis of TE-1 cells increased after treatment with 50-500 μM EPA. The DNA of the TE-1 cells was fragmented and/or the nuclei underwent condensation (Figure 4).

Inhibition of NF-κB activation. IHC staining of TE-1 cells is shown in Figure 5. Stained nuclei were counted to determine the activation of NF-κB (Figure 5). Activation occurred in 18.8±4.94% of the untreated group and in 10.51±2.79% of the group treated with 300 μM EPA group (Figure 6). EPA significantly inhibited NF-κB activation in TE-1 cells (F=8.4977, *p*=0.00162).

Discussion

Esophagectomy for oesophageal cancer is a very stressful procedure, resulting in major perioperative cytokine release. The phenomenon of surgical oncotaxis, which leads to poorer clinical outcomes, is thought to result from excessive corticosteroid secretion, coagulopathy in the peripheral
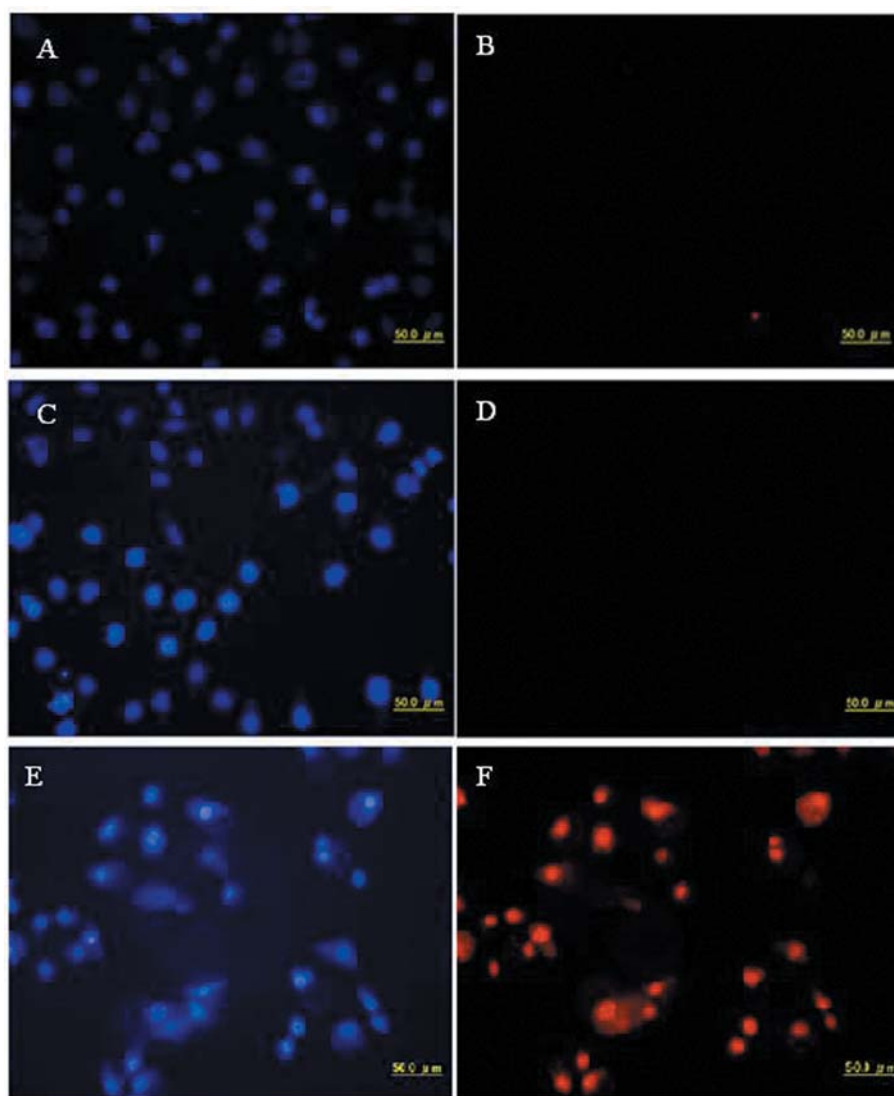
Figure 4. *Immunohistochemical detection of Eicosapentanoic acid (EPA)-induced apoptosis in TE-1 cells. Hoechst 33342, a blue fluorescent dye, stained the condensed chromatin, and propidium iodide, a red fluorescent dye, stained only apoptotic cells. EPA concentration: (A, B) 0 μM, (C, D) 50 μM, (E, F) 500 μM.*

vasculature, immune suppression and excessive production of reactive oxygen species (2). The release of cytokines in response to operative stress, especially after major surgery such as thoraco-laparotomy, was found to be caused by activation of NF-κB in lung, liver, and spleen cells in rats (10).

Activation of NF-κB has been linked to various cellular processes in cancer, including inflammation, cell transformation and proliferation, angiogenesis, invasion, metastasis, chemoresistance and radioresistance (5). In cancer, NF-κB plays a pivotal role in facilitating oncogenesis, as well as metastasis (5, 11). The activation of NF-κB leads to an increase in the expression of pro-inflammatory cytokines required for cell proliferation (4). In

surgical oncotaxis, hyper-inflammation is characterized by the production of inflammatory cytokines, arachidonic acid-derived eicosanoids, and other inflammatory mediators, while immunosuppression is characterized by impairment of antigen presentation and helper T-cell type I responses. Inhibition of surgical oncotaxis may be a valid aim to improve outcomes after surgery.

The n-3 PUFAs are important dietary components that are involved in a number of diverse physiological processes. The preventive effect of n-3 PUFAs on cardiovascular disease and metabolic syndromes such as obesity and diabetes has been extensively investigated. There is evidence that EPA is beneficial in conditions with a prominent inflammatory
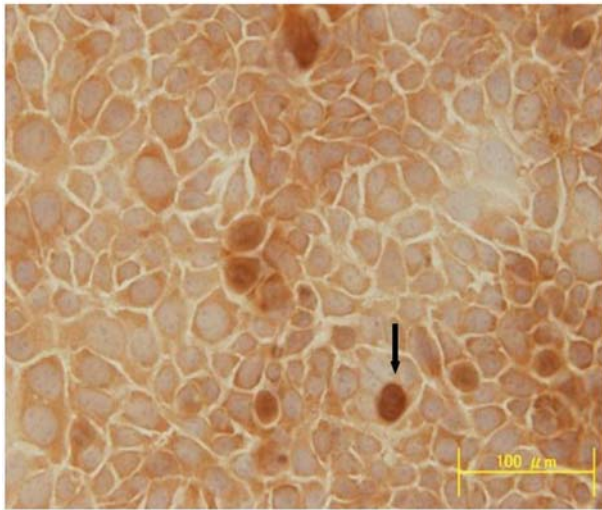
Figure 5. *Positive staining for nuclear factor-kappa B in the nuclei of TE-1 cells (black arrow).*
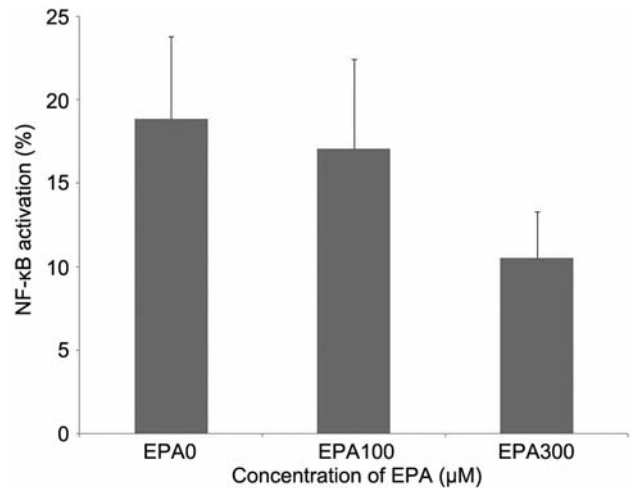

Figure 6. *Percentage of nuclear factor-kappa B (NF-κB) activation. Eicosapentanoic acid (EPA) significantly inhibited NF-κB activation in TE-1 cells (F=804977, p=0.00162).*

component, such as autoimmune disease and critical illness, and in cancer, fish oil consumption has been shown to reduce experimental and spontaneous metastases (12, 13). The n-3 PUFAs have been associated with maintenance of lean body mass, increased physical activity, and improved appetite and weight gain (14). In addition, one study showed that nutritional intervention with fish oil appeared to prevent a reduction in weight and muscle mass during chemotherapy in patients with non-small cell lung cancer (15). Furthermore, n-3 PUFAs reduce the release of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), IL-1 and IL-6, by inhibiting the activation of transcription factors such as NF-κB (16). In patients with oesophageal cancer, EPA-supplemented early enteral nutrition was associated with preservation of lean body mass after oesophagectomy compared with a loss of body mass with standard enteral nutrition (17).

Ingestion of n-3 fatty acid-enriched energy and protein at the recommended dose (1.65-2.2 g EPA, 24-32 g protein) by patients with cancer cachexia increased their lean body mass and improved their quality of life (18). Administration of EPA has been shown to inhibit cancer growth and increase weight gain and quality of life in patients with pancreatic cancer (19). These effects may be related to control of cytokine production. Furthermore, EPA reduced the release of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6, by inhibiting the activation of NF-κB (20, 21, 22). Thus, the use of n-3 PUFAs perioperatively could contribute to control of the stress-induced cytokine storm through inhibition of NF-κB activation.

Our unpublished clinical experience showed that after daily intake of two packs of EPA-rich enteral nutrition for two weeks, the EPA concentration in serum increased to over 300 μM. In addition, the physical activity of some patients was increased and tumour marker levels were decreased.

In our study, cell proliferation was markedly suppressed by 300 μM or higher concentrations of EPA, with an IC_{50} of 260 μM. This study showed that 300 μM EPA inhibited TE-1 cell proliferation and induced TE-1 cell apoptosis. Our results also showed that this was associated with inhibition of NF-κB activation. Other studies have shown that EPA and DHA appear to be cytotoxic to neoplastic cells by inducing oxidative stress and preventing NF-κB-dependent escape from apoptosis, amongst other mechanisms (13, 17, 21, 22). Our results indicate that EPA inhibits NF-κB activation in TE-1 cells, resulting in induction of apoptosis. This means that EPA has an anticancer effect on TE-1 cells.

The production of IL-6 in TE-1 cells was also suppressed by pre-incubation with EPA. Patients with cancer are frequently exposed to high levels of inflammatory cytokines, resulting in loss of body mass. More cytokines are produced after the stress of an operation, which might encourage cancer cell proliferation and promote metastasis, resulting in cancer recurrence. As mentioned above, we found that 2 g/day of EPA for two weeks increased the blood concentration of EPA to over 300 μM (unpublished data). Such a concentration of EPA was sufficient to inhibit NF-κB activation, IL-6 production and cancer cell proliferation in the present study. This suggests that intake of an EPA-rich supplement before surgery could help to suppress surgical oncotaxis.

In summary, EPA treatment inhibited cell proliferation and induced apoptosis of TE-1 cells. EPA suppressed IL-6 production in TE-1 cells, and also prevented LPS-induced IL-6 production. The effect of EPA was associated with a reduction of NF-κB activation. Thus, EPA supplementation may contribute to better outcomes after cancer surgery.

Acknowledgements

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