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Induction of Apoptosis by Eicosapentaenoic Acid in Esophageal Squamous Cell Carcinoma

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Abstract. *Background: Eicosapentaenoic acid (EPA) suppresses the proliferation of cell lines derived from colon, pancreatic, breast and other cancers. Few reports have described the effect of EPA on esophageal cancer cell lines. Materials and Methods: We investigated the effect of EPA on the proliferation of the esophageal squamous cell carcinoma cell lines TE11 and KYSE180 with a WST-1 assay. Apoptosis was evaluated with a DNA fragmentation assay. Levels of apoptosis-related proteins (caspase-3, -7, -9 and poly (ADP-ribose) polymerase (PARP)) and cleaved caspase-3, -7, -9 and PARP were evaluated by western blot analysis. Results: After exposure to EPA for 24 h, KYSE180 and TE11 cell proliferation was suppressed in a dose-dependent manner ($p<0.05$). In addition, caspase -3, -7, -9 and PARP were activated. EPA (0.1 μM, 1 μM, 10 μM) induced apoptosis in a dose-dependent manner, as detected by the DNA fragmentation assay. Conclusion: EPA shows potential as a new treatment for esophageal cancer.*

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies in Japan with a 5-year survival rate of 20% to 30% after curative surgery (1, 2). Even in the early disease stages, many patients develop local tumor recurrence or distant metastases within a short period after surgery.

Recently, nutritional aid therapies for esophageal cancer have taken on increased importance since the nourishment state should be optimal when esophageal cancer patients receive medical attention. Above all, patients should be encouraged to take omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (3).

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In addition, omega-3 PUFAs induce the expression of a family of anti-inflammatory mediators called resolvins, making omega-3 PUFAs potentially potent anti-inflammatory agents (4).

EPA plays a role in the inhibition of cell proliferation and, as such, the anti-cancer effect of EPA has been attracting attention in recent years (5-8). Many studies have suggested that EPA induces apoptosis in cancer cells and have proposed mechanisms for this pro-apoptotic function (5, 9, 10). Nonetheless, the biological functions of EPA are not yet fully understood.

Furthermore, to our knowledge, there are few reports of whether EPA can promote apoptosis in ESCC.

In the present study we used ESCC cell lines to clarify whether EPA has anticancer activity in the esophagus that is similar to its effects seen for other cancers. Herein we evaluated the capacity of EPA to affect proliferation of ESCC cell lines and induce apoptosis in these cells.

Materials and Methods

Reagents. 5,8,11,14,17-eicosapentaenoic acid (EPA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in ethanol. Antibodies against poly (ADP-ribose) polymerase (PARP), cleaved PARP, caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, caspase-9, cleaved caspase-9 and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal goat anti-rabbit immunoglobulin/HRP secondary antibodies were purchased from Dako A/S (Glostrup, Denmark).

Cell culture. Two human esophageal carcinoma cell lines were used: the TE series, a kind gift from the Institute of Development, Aging and Cancer, Tohoku University and the KYSE series, obtained from JCRB (Japanese Collection Research Bioresources, Osaka, Japan). The cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Inc., NY, USA) and antibiotic antimycotic Solution (×100) (Sigma). The cell lines were cultured essentially according to the manufacturers' instructions.

Western blot analysis. Equal amounts of total cell lysates solubilized in RIPA buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA) were subjected to SDS-PAGE and then

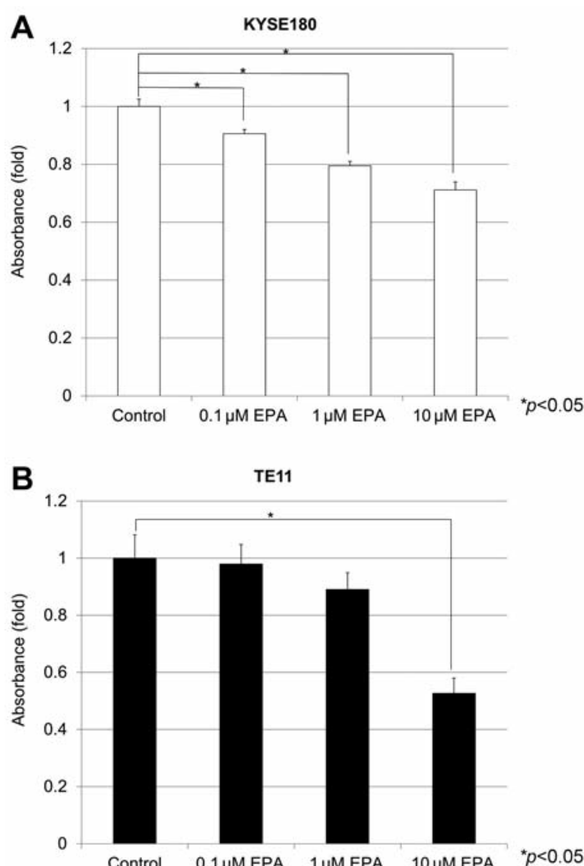

Figure 1. *A, B. The effect of EPA on (A) KYSE180 and (B) TE11 cells. WST-1 assay shows the effects of EPA on KYSE180 and TE11 cells. KYSE180 and TE11 cells were stimulated with EPA for 24 h at the indicated concentration (0.1 μM, 1 μM, 10 μM). Results are expressed as the absorbance ratio relative to untreated control cells.*

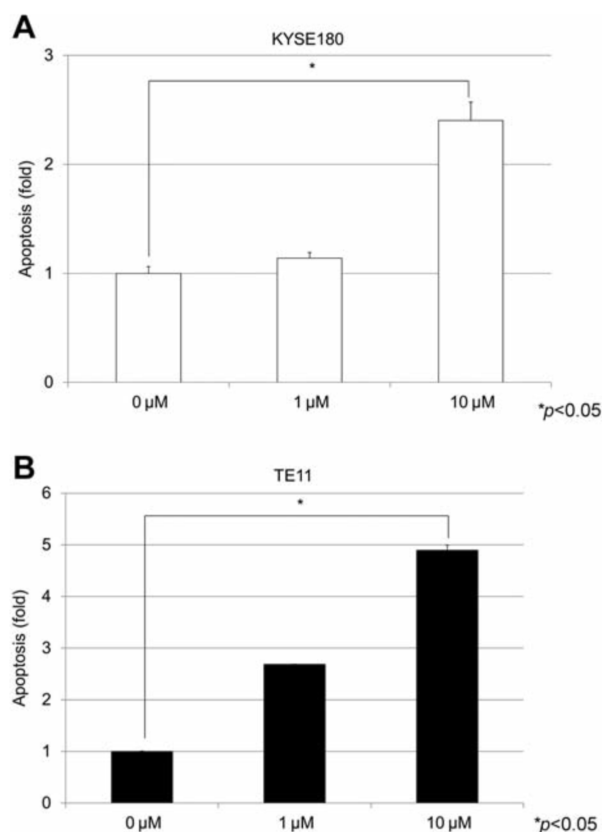

Figure 2. *A, B. EPA-induced apoptosis of (A) KYSE180 and (B) TE11 cells. TE11 and KYSE180 cells were treated with EPA for 24 h. After 24 h, the fold-change in apoptotic cells was measured using the Cell Death Detection ELISAplus kit. Results are representative of three independent experiments and presented as mean±SD (*p<0.05).*

transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 30 min in PBS containing 5% milk. The filters were first incubated overnight at 4˚C with primary antibodies for caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, caspase-9, cleaved caspase-9, PARP, cleaved PARP or GAPDH, which were all diluted 1:1,000 in TBS-T (0.1% Tween 20) containing 1% milk. The membranes were then incubated with polyclonal G anti-rabbit immunoglobulin/HRP secondary antibodies for 60 min in 1% milk in TBS-T (0.1% Tween 20) at room temperature. The membranes carrying the protein and antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Thermo) and SuperSignal West Femto Chemiluminescent Substrate (Thermo).

Cell proliferation. Cell viability was determined using a WST-1 assay (Takara Bio Inc., Otsu, Japan). Cells were seeded in 96-well plates at 1.0×10^3 cells/well and allocated to a control group or stimulus groups (EPA concentration: 0.1 μM, 1 μM, 10 μM). The medium was changed to serum-free medium 24 h after cell seeding. Various EPA concentrations (0.1 μM, 1 μM, 10 μM) were added to the wells 24 h later.

After 24 h of exposure to EPA, the WST-1 reagent was added to each well and incubated at 37˚C for one additional hour and cell viability was determined by measuring the absorbance of the culture medium at 450 nm.

DNA fragmentation assay. Cells were seeded in 6-well plates at 1.0×10^5 cells/well and incubated for 24 h. The medium was changed to serum-free medium 24 h after seeding and before incubation for another 6 hours.

The cells were then exposed to different EPA concentrations (0.1 μM 1 μM 10 μM) in serum-free medium for 24 h. DNA fragmentation was analyzed using the Cell Death Detection ELISA^{plus} kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The extent of apoptosis was presented as a ratio between treated and untreated cells (mU of treated cells/mU of non-treated cells).

Statistical analysis. Biostatistical analyses were performed using the R software. One-way analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment was used to analyze the significance of difference between the groups. A value less than 0.05 ($p<0.05$) was considered to be statistically significant.

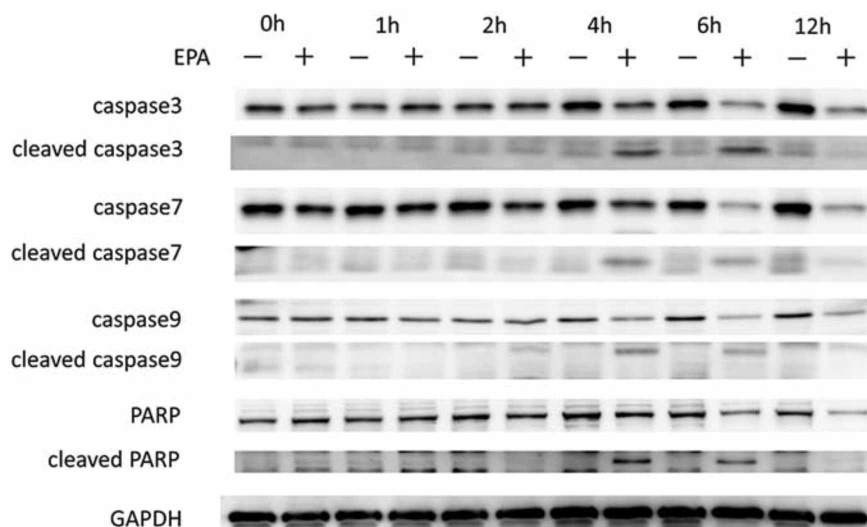

Figure 3. *Apoptosis-related proteins are activated by EPA. Western blotting of caspase-3, -7, -9 and PARP in KYSE180 cells treated with 10 μM EPA for 1, 2, 4, 6 or 12 h. Proteins were extracted at each time point with proteins from cells treated only with ethanol used as a control. Apoptosis-related proteins caspase-3, -7, -9 and PARP were activated following stimulation with EPA. After treatment with EPA for 4 and 6 h, cleaved caspase-3, caspase-7, caspase-9 and PARP were detected.*

Results

Growth suppression in ESCC cell lines following exposure to EPA. We used human ESCC cell lines to evaluate the anti-cancer effect of EPA. Two cell lines (TE11 and KYSE180) were exposed to different concentrations of EPA (0.1 μM, 1 μM, 10 μM). After 24 h of exposure, we performed a WST-1 assay. Cell growth was dose-dependently suppressed by EPA ($p<0.05$) with proliferation of TE11 cells being inhibited to a larger extent than KYSE180 cells at 10 μM EPA (Figures 1A and B).

EPA induced apoptosis of ESCC cells. Next, to confirm whether EPA induces apoptosis in esophageal cancer cell lines, we performed a DNA fragmentation analysis using the Cell Death Detection ELISA^{plus} kit. Cells were treated with 10 μM EPA for 24 h, which was selected based on the results of the cell proliferation experiment. Apoptosis was induced to a greater degree in TE11 and KYSE180 cells after EPA stimulation compared with the untreated control cells ($p<0.05$) (Figures 2A and B).

Western blot analysis. In order to examine which apoptosis-related proteins are activated by EPA, we performed western blot analysis using various protein antibodies. Caspase-9, caspase-7, caspase-3 and PARP were activated from 4 to 12 h after EPA stimulation. Caspase-3 expression levels were lower in cells that were not treated with EPA compared to those in cells exposed to EPA for 6 h (Figure 3).

Discussion

EPA is an omega-3 PUFA that has been shown to have anti-cancer activity and anti-inflammatory effects (11-13). Recently, EPA was reported to have an anticancer effect on pancreatic, breast and colorectal cancer, as well as hepatocellular carcinoma and cancers of the prostate and lung (5, 14-18). Most studies indicating a suppressant effect of EPA on cancer cell proliferation noted that this compound could induce apoptosis.

In the present study, we showed that EPA suppressed proliferation of human ESCC cell lines and induced apoptosis. To the best of our knowledge, our data are the first to report EPA-dependent changes in the expression of apoptotic proteins in ESCC cell lines.

Other articles concerning the suppression of cell growth of ESCC cell lines indicated a role for NF-κB inhibition in these effects (19).

Our cell proliferation assay showed that cell growth was inhibited by EPA in a dose-dependent manner across a concentration range of 0.1 to 10 μM. In another study, proliferation of the human pancreatic cancer cell lines BxPC3 and HPAF-II was suppressed by an EPA concentration of 10 μM (20). We confirmed a similar action of EPA on ESCC cells at the same concentration, as well as at two other concentrations and obtained results that were consistent with the findings of other studies that also showed a dose-dependent effect of EPA in pancreatic cancer cells and cholangiocarcinoma cells (14, 21). Also in

agreement with our results, another report using brain microvascular endothelial cells showed suppressed proliferation in the presence of 10 μM EPA (22). For cholangiocarcinoma cells, apoptosis was seen with 20 μM EPA, while other cancer cells required much higher concentrations of EPA (260 μM) before an effect was observed (21). Taken together these results indicate that EPA acts across a wide range of concentrations, although further investigation will be needed to determine the optimal effective doses of this compound.

Caspases are cysteine proteases that participate in signal transmission pathways that lead to cell apoptosis (23, 24). Caspases exist as pro-caspases in the cell that, upon apoptosis induction, are activated (25) and in turn activate other downstream caspases (26, 27). The activation of these effector caspases promotes the subsequently seen apoptosis (14, 28).

Herein, expression of apoptotic proteins, including cleaved caspase-3, -7, -9 and PARP was detected following exposure of ESCCs to EPA, indicating that induction of apoptosis may be an important mechanism by which this compound acts.

In our study, we demonstrated the activation of caspase-3, -7, -9 and PARP upon stimulation of cells with EPA, again indicating that EPA may be able to induce apoptosis of ESCCs, a function that may be useful as an effective method for treating esophageal cancer.

In patients with esophageal cancer, prognostic markers including cyclin-D1 (29-31), *E-cadherin* (29, 31), *MDM2* (29, 31), *fascin* (32) and *HGF* (33) have been reported. We previously showed that the expression of *survivin* (34), pituitary tumor transforming gene 1 (PTTG1) (35), DNA fragmentation factor 45 (*DFF45*) (36), excision repair cross complementing 3 (*ERCC3*) (37), *CD44v6* (38) and *FBXW7* (39) may also be prognostic markers for ESCC. However, whether the prognosis of esophageal cancer patients can be improved simply by relying on these molecules as biomarkers or targeting the effects of these proteins is unclear, particularly given the difficulties in drug development and drug delivery. In addition to current treatment methods including surgery, chemotherapy and radiation, new strategies are needed that can improve the prognosis of esophageal cancer patients. Herein we explored the potential of nutrition therapy in treating esophageal cancer and evaluated the potential of EPA supplementation as a key component of therapy for this cancer type.

Although the precise molecular mechanism by which EPA acts in cancers requires clarification, our data indicate that EPA is a good candidate for inclusion as a therapeutic nutrition reagent for patients with this intractable disease.

EPA has the potential to become a new treatment for esophageal cancer that could complement surgical, chemotherapeutic and radiation therapies.

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Conflicts of Interest

The Authors did not receive a financial grant nor are they in cooperation with industry and they have no conflicts of interest to declare.

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