

# The Mechanism of the Synergistic Anticancer Effect of CDDP and EPA in the TE1 Cell Line

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**Abstract.** *Background/Aim:* Eicosapentaenoic acid (EPA) is an unsaturated fatty acid with various bioactivities, including antitumor effects. We previously reported a synergistic antitumor effect of cisplatin (CDDP) and EPA. Here, we examined the underlying mechanism. *Materials and Methods:* The human oesophageal cancer cell line TE-1 was treated with the combination of EPA and CDDP. Nuclear translocation of NF- $\kappa$ B, a transcription factor involved in cytokine production, was detected by immunohistochemistry. IL-6 levels were measured by ELISA. Apoptosis and cell cycle distribution were evaluated by flow cytometry. *Results:* Nuclear translocation of NF- $\kappa$ B in TE-1 cells was synergistically decreased by CDDP and EPA. IL-6 production was increased following treatment with CDDP, but treatment with EPA decreased IL-6 levels. Apoptosis was synergistically induced by CDDP and EPA. A G<sub>2</sub>/M cell cycle arrest was observed with the combination of CDDP and 150  $\mu$ M EPA, and S phase arrest with the combination of CDDP and 100  $\mu$ M EPA. *Conclusion:* The combination of CDDP and EPA synergistically suppresses NF- $\kappa$ B nuclear translocation and increases apoptosis by inducing cell cycle arrest at the S or G<sub>2</sub>/M phase.

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid that is abundant in fish oil and exhibits platelet aggregation inhibitory activity (1). EPA is effective in improving lipid metabolism, blood clotting and glucose metabolism

abnormality (1-5). Recent studies showed that EPA has cytostatic or apoptosis induction activity against various types of cancer cells (6-8). Another report using a mouse model revealed that EPA has antitumor activity (9). Furthermore, other studies reported anti-inflammatory action or possibility of weight gain from improved metabolism in cancer patients treated with EPA (10-12). Together these findings suggest that EPA might be useful in cancer treatment.

In our previous study, we reported several effects of EPA on the TE-1 human oesophageal cancer cell line (13). Lipopolysaccharide promotes the nuclear translocation of the NF- $\kappa$ B transcription factor (14). Lipopolysaccharide induces cytokine production in TE-1 cells, and this activity was inhibited by EPA (13). We further found that EPA promoted apoptosis and controlled tumor proliferation through inhibition of NF- $\kappa$ B nuclear translocation and activation. Several studies examined the combined use of EPA and anticancer agents and found that EPA showed a synergistic antitumor effect when combined with docetaxel, paclitaxel or cisplatin (CDDP) (15).

To clarify the mechanism of the synergistic antitumor effect of the combined use of EPA and CDDP, we examined the role of NF- $\kappa$ B and investigated cytokine production, apoptosis and cell cycle arrest in response to the combination treatment.

## Materials and Methods

*Cell lines, cell culture and reagents.* The TE-1 cell line, which is derived from human oesophageal cancer, was purchased from RIKEN (Saitama, Japan). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100  $\mu$ g/ml of streptomycin (Sigma-Aldrich) at 37°C and 5% CO<sub>2</sub>.

EPA (cis-5,8,11,14,17-eicosapentaenoic acid sodium salt) (Sigma-Aldrich) was dissolved in PBS. A cisplatin injection solution (Nichi-Iko Pharmaceutical, Toyama, Japan) was used for experiments with CDDP.

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**Setting of EPA and CDDP concentration.** Our previous studies examining the antitumor effect of EPA on TE-1 cells showed that the IC<sub>50</sub> was 260 µM (13). Based on this result, the EPA concentrations used in experiments in the current study were set to 0, 100, 150 and 200 µM, as these concentrations did not result in any antitumor effect when EPA was applied alone. The blood concentration of CDDP ranged from 3.76 to 1.69 µg/ml when CDDP administered to patients (16), and therefore, CDDP was used at 0, 2 and 5 µg/ml in this study.

**Immunohistochemistry (IHC) for NF-κB.** TE-1 cells were trypsinized, resuspended at 1×10<sup>5</sup> cells/ml culture medium, and plated at 4×10<sup>4</sup> cells/well (400 µl/well) in LAB-TEK 8-well plates (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured for 24 h in the medium containing 0, 100, 200 µM EPA, or 0, 2 µg/ml CDDP, or both. Then, cells were washed with PBS and fixed in Citesetter (Matsunami Garasu, Osaka, Japan). Cells were then incubated with NF-κB p65 (D14E12) XP®Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA) as a primary antibody overnight at 4°C. On the following day, cells were incubated with a colour coupler using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, and Rabbit/Mouse (Agilent, Santa Clara, CA, USA) secondary antibody (13). Nuclei were stained with Mayer haematoxylin (Muto Kagaku, Tokyo, Japan) for 1 min. Cells were examined and photographed using an IX83 microscope (Olympus, Tokyo, Japan). Five hundred cells were analyzed in one field, and cells with NF-κB-positive nuclear staining were counted. This experiment was repeated three times.

**ELISA.** TE-1 cells were trypsinized, resuspended at 1×10<sup>5</sup> cells/ml in culture medium, and plated at 5×10<sup>4</sup> cells/well (500 µl/well) in 24-well plates. Cells were cultured for 24 h and then cultured in medium containing 0, 100 and 200 µM EPA, or 0, 2 µg/ml CDDP, or both. After 48 h of culture, the supernatant was collected and IL-6 levels were measured using an IL-6 human ELISA Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. The measurement was performed using Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific). This experiment was repeated three times.

**Flow cytometry.** To evaluate apoptosis, TE-1 cells were resuspended at 1×10<sup>6</sup> cells/ml and plated at 2×10<sup>6</sup> cells/well (2 ml/well) in 6-well plates. Cells were cultured for 24 h and then incubated in medium containing 0, 100, 150 µM of EPA, or 0, 2 µg/ml of CDDP, or both. Cells were collected 24 h later and stained using the Zombie Green™ Fixable Viability Kit (BioLegend, San Diego, CA, USA) and Annexin V, Alexa Fluor™ 647 conjugate (Thermo Fisher Scientific). Cells were analysed using the FACS Aria III Cell Sorter, DIVA soft (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and data were examined using FlowJo ver.10 (Becton, Dickinson and Company). The experiment was repeated three times.

For cell cycle analyses, TE-1 cells were resuspended at 1×10<sup>6</sup> cells/ml and plated at 2×10<sup>6</sup> cells/well (2 ml/well) in 6-well plates. They were cultured for 24 h and then incubated in medium containing 0, 100, 150 µM of EPA, or 0, 2 µg/ml of CDDP, or both. Cells were collected 48 h later and stained with PI after RNase processing. Cells were analysed using the FACS Aria III Cell Sorter, DIVA Soft (Becton, Dickinson and Company) and data were examined with FlowJo ver.10. The experiment was repeated three times.

**Statistical analysis.** Effects were considered as synergistic when the effect of the combined treatment was higher than the sum of the effects with EPA alone and CDDP alone.

Two-way factorial analysis was performed using JMP ver.1.2 and *t*-test was conducted for comparison of two groups. *p*-Value<0.05 was considered to indicate a significant difference.

## Results

**Intra-nuclear localization rate of NF-κB.** Representative staining of NF-κB intra-nuclear localization in TE-1 cells treated with EPA, CDDP or their combination is shown in Figure 1A.

The rate of NF-κB nuclear stained cells in TE-1 cells treated with 0, 2 and 5 µg/ml of CDDP was 8.2%, 9.5% and 8.4%, respectively, and there were no significant differences between these rates. In contrast, treatment with 100 and 200 µM EPA resulted in 7.5% and 1.3% NF-κB nuclear stained cells, respectively, with a significant difference in cells treated with 200 µM EPA compared with controls (*p*<0.05). In cells treated with various concentrations of CDDP together with 100 µM EPA, the rate of intranuclear localization of NF-κB was changed in a CDDP concentration-dependent tendency, without a significant difference.

In untreated cells, the percentage of cells with NF-κB intra-nuclear localization was 8.2%. In cells treated with 100 µM EPA alone, the percentage was 7.5% and in cells treated with 5 µg/ml of CDDP alone, the percentage was 8.4%. Thus, the NF-κB intra-nuclear localization rate decreased by 0.7% in cells treated with 100 µM EPA alone and increased by 0.2% in cells treated with 5 µg/ml CDDP alone compared with controls, although the difference was not significant. In comparison, the NF-κB intra-nuclear localization rate was 3% with the combination of 100 µM EPA and 5 µg/ml CDDP. Because the additive effect was a 0.5% decrease, the reduction of NF-κB intra-nuclear localization rate to 3% indicated a synergic effect of the combination treatment. In response to 200 µM EPA, NF-κB intra-nuclear localization was potently inhibited by only EPA alone and thus we were not able to calculate the combined effect with CDDP (Figure 1B).

**IL-6 levels in culture supernatant.** We next examined IL-6 levels in the culture supernatant (Figure 2). In control cells, the IL-6 levels in the culture supernatant were 247.6 pg/ml. The levels of IL-6 increased to 384.2 pg/ml and 326.2 pg/ml with 2 µg/ml and 5 µg/ml of CDDP, respectively, whereas it decreased to 133.4 pg/ml and 136.1 pg/ml with 100 µM and 200 µM EPA, respectively.

When EPA was added together with CDDP, IL-6 levels were reduced. While cells treated with 2 µg/ml CDDP alone showed 384.2 pg/ml IL-6 levels, the concentrations were reduced to 212.6 pg/ml and 197.5 pg/ml with the addition of 100 µM and 200 µM EPA, respectively. While cells treated with 5 µg/ml CDDP alone showed 326.2 pg/ml IL-6 levels, the concentrations were reduced to 266.0 pg/ml and 164.1 pg/ml with 100 µM and 200 µM EPA, respectively. Because

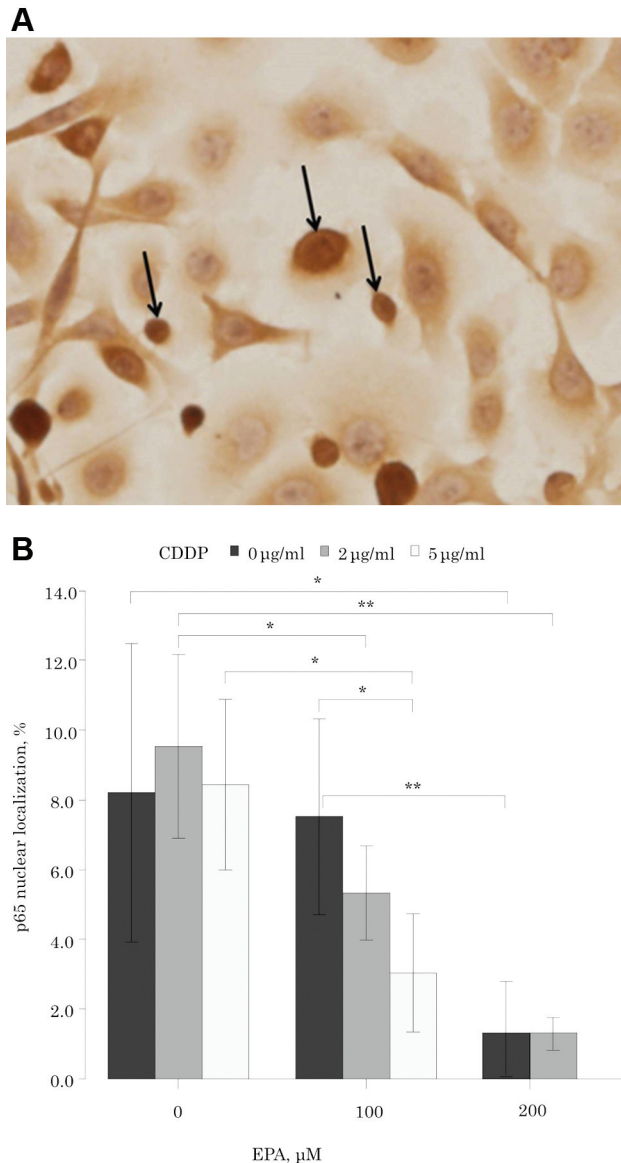


Figure 1. NF-κB p65 nuclear localization and activation in TE-1 cells. (A) NF-κB nuclear localization in TE-1 cells. Black arrows indicate cells with nuclear NF-κB p65 staining. (B) NF-κB nuclear localization rates in TE-1 cells treated with EPA combined with CDDP for 24 h. (n=3). \* $p<0.05$ , \*\* $p<0.01$ .

IL-6 production was increased with CDDP alone, we were not able to evaluate an additional or synergic effect with the combination treatment.

**Apoptosis.** We next examined the levels of apoptosis in TE-1 cells exposed to the single or combined treatments for 24 h (Figure 3). The apoptosis rates were as follows: 0 μM EPA/0 μg/ml CDDP: 6.7% (Figure 3A); 100 μM EPA/0 μg/ml CDDP: 10.1% (Figure 3B), 150 μM EPA/0 μg/ml

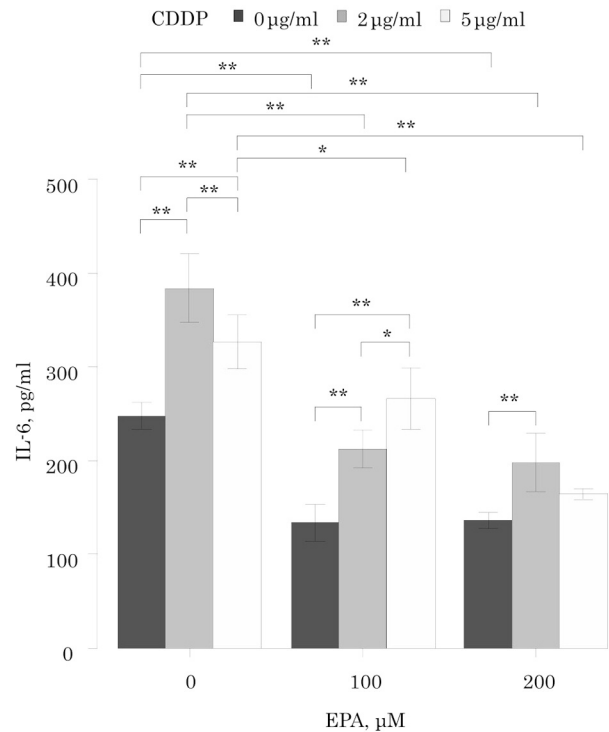


Figure 2. IL-6 levels in culture supernatant in TE-1 cells treated with EPA combined with CDDP for 48 h. \* $p<0.05$ , \*\* $p<0.01$  (n=3).

CDDP: 11.1% (Figure 3C); 0 μM EPA/2 μg/ml CDDP: 12.3% (Figure 3D); 100 μM EPA/2 μg/ml CDDP: 19.9% (Figure 3E), and 150 μM EPA/2 μg/ml CDDP: 22.8% (Figure 3F).

These results showed that apoptosis increased by 3.4% and 4.4% in cells treated with 100 μM and 150 μM EPA, respectively, compared with controls, without a significant difference. In contrast, apoptosis increased by 5.6% in response to 2 μg/ml CDDP, which was statistically significant. Notably, the combination of EPA together with 2 μg/ml CDDP increased apoptosis to 19.9% and 22.8% in the presence of 100 μM and 150 μM EPA, respectively. These results indicate a synergistic effect of EPA combined with CDDP on apoptosis (Figure 3G).

**Cell cycle analysis.** We next examined the cell cycle distribution of TE-1 cells exposed to the single or combined treatments for 48 h (Figure 4). In TE-1 cells incubated with 2 μg/ml CDDP alone for 48 h, the number of G<sub>0</sub>/G<sub>1</sub> phase cells decreased 40.7%, while the number of S and G<sub>2</sub>/M phase cells increased 34.4% and 8.1%, respectively ( $p<0.01$ ). No changes were observed in the cell cycle distribution of TE-1 cells exposed to EPA alone. In cells treated with 2 μg/ml CDDP combined with 100 μM EPA

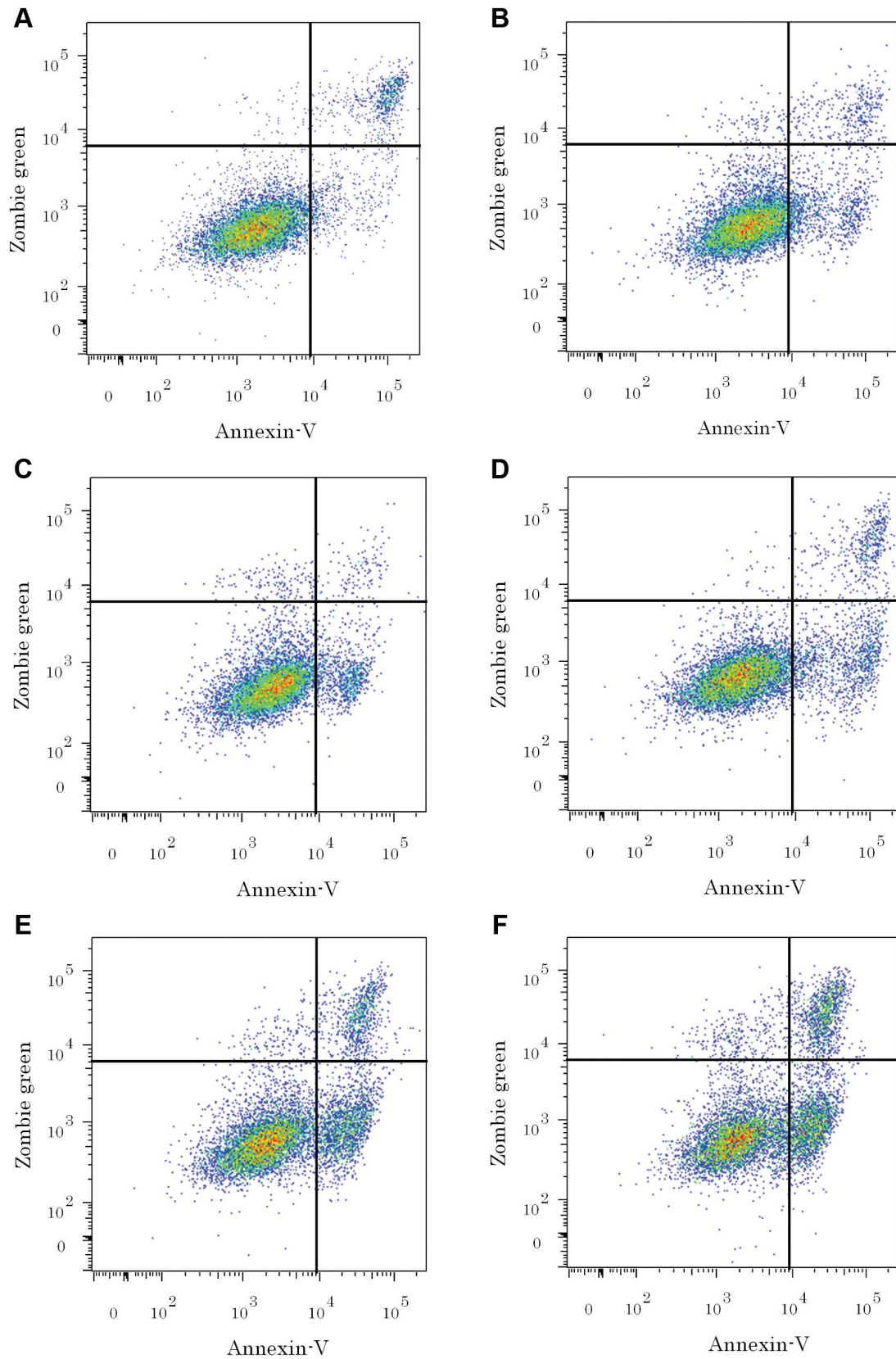


Figure 3. Continued



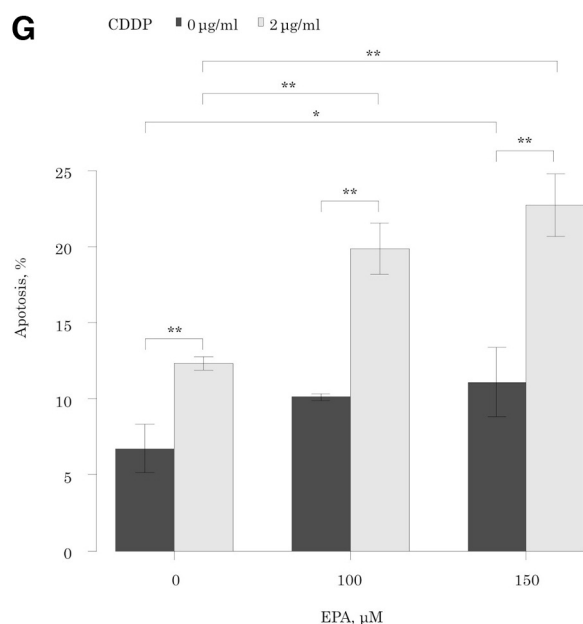


Figure 3. Apoptosis of TE-1 cells treated with EPA combined with CDDP for 24 h. (A) EPA 0  $\mu$ M/CDDP 0  $\mu$ g/ml, (B) EPA 100  $\mu$ M/CDDP 0  $\mu$ g/ml, (C) EPA 150  $\mu$ M/CDDP 0  $\mu$ g/ml, (D) EPA 0  $\mu$ M/CDDP 2  $\mu$ g/ml, (E) EPA 100  $\mu$ M/CDDP 2  $\mu$ g/ml, (F) EPA 150  $\mu$ M/CDDP 2  $\mu$ g/ml. (G) The percentages of apoptotic cells in the indicated treatment groups. \* $p$ <0.05, \*\* $p$ <0.01 ( $n$ =3).

showed an 8.2% increase in the number of S phase cells ( $p$ <0.01) and 5.0% decrease of G<sub>2</sub>/M phase cells ( $p$ <0.01). In cells co-treated with 150  $\mu$ M EPA, the S phase population decreased 5.1% ( $p$ <0.05) and the G<sub>2</sub>/M phase population increased 10% ( $p$ <0.01).

## Discussion

Carcinoma of the oesophagus is more prevalent in Japan than in western countries, and most oesophageal cancers are squamous cell carcinoma (17). Oesophageal cancer often develops in patients over 60 years old, and these patients represent approximately 70% of oesophageal cancer patients (18). In Japan, the percentage of the population over 65 years of age was 27.7% in 2017, and it is expected to increase to 35.3% in 2040 (19). Therefore, as the older population continues to increase, the percentage of patients with carcinoma of the oesophagus is also expected to rise. Older patients are often diagnosed with various diseases, which can be accompanied by a risk for surgical management. In cases in which surgical treatment is not an option, chemo-radiotherapy might be selected. However, chemo-radiotherapy is associated with multiple side effects. Therefore, as a method to reinforce the effect without increasing the harmful effects of chemo-radiotherapy, the co-

administration of polyunsaturated fatty acids (PUFAs) such as EPA is considered. Thus, we explored the potential effects of EPA on CDDP efficacy.

EPA is an n-3 PUFA that shows various bioactivities with minimum side effects. We previously reported that EPA regulated NF- $\kappa$ B nuclear localization, and therefore activation, and induced apoptosis in TE-1 cells (13). While the mechanism has not been fully elucidated, previous studies in gastric or colon cancer cell lines have suggested the involvement of activation of caspase-3, -7 and -9 or poly (ADP-ribose) polymerase (20) along with nuclear receptors including constitutive androstane receptor or pregnane X receptor (21).

A previous review reported that PUFAs enhanced the effect of 15 kinds of anticancer agents in various cancers (22). The tumor types included breast cancer, prostate cancer, colon cancer, lung cancer, cervical cancer, ovarian cancer, neuroblastoma, leukaemia and lymphoma. One study showed a high synergic effect of PUFAs on the survival of MDA-MB-231 breast cancer cells, which are highly metastatic, when DHA was used together with paclitaxel or docetaxel (23). We also examined the combined effect of EPA together with four antitumor agents (paclitaxel, docetaxel, 5-FU, CDDP) on TE-1 cells and found that paclitaxel, docetaxel and CDDP showed a synergistic antitumor effect with EPA in a dose-dependent manner (15). However, the mechanism underlying this effect was not determined.

To examine the mechanism of the synergistic effect of EPA and CDDP, we focused on NF- $\kappa$ B, as it is a key protein in cell proliferation. Activation of NF- $\kappa$ B affects production of cytokines such as IL-6, apoptosis and the cell cycle. NF- $\kappa$ B is present in the cytoplasm in an inactivated state and undergoes nuclear localization and activation in response to cytotoxic signals or TNF $\alpha$  stimulation. We reported that EPA induced NF- $\kappa$ B activation in TE-1 cells (13). Another study showed that CDDP regulated NF- $\kappa$ B activation (23). Therefore, we examined whether NF- $\kappa$ B activation was synergistically enhanced in response to the combination of CDDP and EPA. To evaluate the potential synergistic effect of CDDP and EPA, the EPA concentration was set at a level that does not induce antitumor effects (13). Notably, our results showed that NF- $\kappa$ B activation was suppressed synergistically with the combination of EPA with CDDP.

We further examined the anti-inflammatory effect of the combination treatment in TE1 cells. Because inflammatory cytokines, such as IL-6, promote tumor growth or metastasis, the control of cytokine production is important in tumor management (24). EPA alone suppressed IL-6 production, while CDDP alone increased IL-6 production. EPA together with CDDP further suppressed IL-6 in a concentration-dependent manner. Therefore, no synergistic effect was determined. A previous study reported that head and neck squamous cell carcinoma patients with high level of IL-6

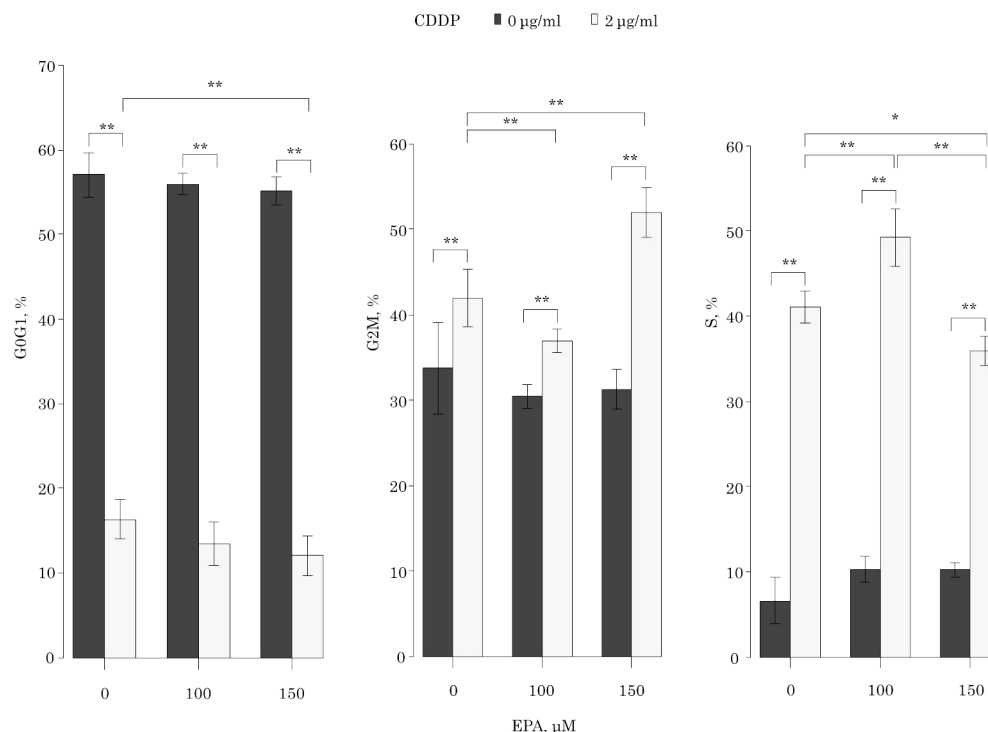


Figure 4. Cell cycle distributions of TE-1 cells treated with EPA combined with CDDP for 48 h. \* $p<0.05$ , \*\* $p<0.01$  ( $n=3$ ).

showed CDDP-resistance and poor prognosis (25). Therefore, EPA intake during therapy with CDDP might be useful from the viewpoint of prevention of CDDP resistance.

NF- $\kappa$ B activation is related to apoptosis. We previously showed that EPA promoted apoptosis in TE-1 cells (13). Because the combined use of CDDP and EPA synergistically suppressed NF- $\kappa$ B, we further evaluated the effects of the combination treatment on apoptosis. Our results showed a 19.9% increase in cells treated with EPA 150  $\mu$ M/CDDP 2  $\mu$ g/ml and 22.8% increase in cells treated with EPA 100  $\mu$ M/CDDP 2  $\mu$ g/ml. These results indicate that the increase in apoptosis in response to co-treatment of EPA and CDDP was synergistic.

The cell cycle is closely related with cell proliferation and apoptosis. Previous studies showed that inhibition of NF- $\kappa$ B activation suppresses the cell cycle (26-28). CDDP binds to DNA and stops DNA duplication. Therefore, we evaluated cell cycle changes in TE1 cells treated with the combination of CDDP and EPA. No alterations in the cell cycle were observed in response to EPA only. However, in cells treated with the combination of 2  $\mu$ g/ml CDDP and EPA, cells in S phase increased significantly (8.2%) with EPA 100  $\mu$ M, while cells in G<sub>2</sub>/M increased significantly (10%) with EPA 150  $\mu$ M, compared with CDDP alone. These results indicate an increase of apoptosis with the combined treatment; however, the

difference in cell cycle phase distributions between the two EPA concentrations is not yet clear. In the human pancreatic cancer cell line MIA PaCa-2, EPA promoted apoptosis with an increase in the number of cells in the sub-G<sub>1</sub> and G<sub>2</sub>/M phase (29). In the human breast cancer cell lines MCF-7 and MDA-MB-231, genistein increased apoptosis with a G<sub>2</sub>/M phase arrest, an increase in Bax and Caspase 3 and decrease in Bcl-XL (30). In addition, a G<sub>2</sub>/M arrest and increase in apoptosis were reported with various drugs in human cancer cell lines such as colon cancer (31), prostate cancer (32, 33) and other cancer cells (34), but the detailed mechanism was not identified. However, apoptosis-inducing drugs exert their effects by regulating proteins such as Bax, Bcl-xL, Bcl-2, Caspase3, Caspase8, cdc2 or cdk2 and causing a G<sub>2</sub>/M arrest (31-33). In addition, DHA, an n-3 unsaturated fatty acid similar to EPA, regulates cell cycle-related proteins and induces a G<sub>2</sub>/M phase arrest as well as a G<sub>1</sub> arrest (34). Therefore, future studies are needed to more precisely clarify the mechanism by which the combination of CDDP and EPA exhibits a synergistic effect on apoptosis.

## Conclusion

The combination of EPA and CDDP showed a synergic effect on NF- $\kappa$ B activation and cytostatic action in the TE-1 cell line and induced IL-6 production and cell cycle arrest.

## Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

## Authors' Contributions

Ayako Ogo: Investigation, Visualization, Writing. Sachi Miyake: Resources. Hisako Kubota: Supervision. Masaharu Higashida: Supervision. Hideo Matsumoto: Supervision. Fusako Teramoto: Supervision. Toshihiro Hirai: Conceptualization, Supervision, Writing, review & editing. Tomio Ueno: Supervision, review & editing.

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