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Modulatory Effects of EPA and DHA on Proliferation and Apoptosis of Pancreatic Cancer Cells*

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Summary: In order to investigate the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the proliferation, apoptosis of pancreatic cancer cell line SW1990 cells and the expression of cyclin E mRNA, the SW1990 cells were treated with different concentrations of EPA or DHA (20, 40, 60 μg/mL) for 0, 12, 24, 36 and 48 h respectively. By using MTT method, the inhibitory effects of EPA or DHA on the cell growth were assayed. Real time PCR was used to detect the expression changes of cyclin E mRNA after the SW1990 cells were treated with 40 μg/mL EPA or DHA for different time. Flow cytometry was used to test the changes of apoptostic rate in the SW1990 cells treated with different concentrations of EPA or DHA for 24 h. The results showed that EPA and DHA could inhibit the growth of SW1990 cells in a time- and concentration-dependent manner (P<0.01). EPA or DHA could also significantly inhibit the expression of cyclin E mRNA in a time-dependent manner (P<0.05). EPA or DHA could induce the apoptosis of SW1990 cells in a concentration-dependent manner (P<0.01). It was concluded that ω-3 fatty acid could inhibit the proliferation of pancreatic cancer cell line SW1990 cells and promote their apoptosis. The down-regulation of the cyclin E expression by ω-3 fatty acid might be one of the mechanisms for its anti-tumor effect on pancreatic cancer.
Key words: ω-3 fatty acid; cyclin E; apoptosis; malignant tumor

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main components of ω-3 fatty acid and belong to the essential fatty acids which are not synthesized by self. Epidemiological investigations revealed that in the population with dietary high ω-3 fatty acid intake the incidence of colon carcinoma, prostate cancer and breast cancer etc was obviously reduced[1]. Tanaka *et al* reported that EPA and DHA could inhibit the proliferation of Ehrich ascite tumor cells by suppressing the DNA synthesis of tumor cells[2], but the study of ω-3 fatty acid on pancreatic cancer and the mechanism was little at present. In this study, the human pancreatic cancer cell line SW1990 cells were treated with different concentrations of EPA and DNA in order to investigate its effects on the proliferation and apoptosis of cells so as to provide the experimental evidence for EPA and DHA clinically treating pancreatic cancer.

1 MATERIALS AND METHODS

1.1 Main Reagents

EPA and DHA (Sigma, USA); MTT (Janssen Chimica, USA); RPMI-1640 medium, fetal calf serum (FCS) and TRIzol (Gibco, USA). Real time PCR kit (Shanghai Jiusheng Medical Product Co., Ltd., China); Annexin V/PI kit (Shenzhen Jingmei Biological Product Co., Ltd., China). Human pancreatic cancer cell line SW1990 (American Type Culture Collection, ATCC, USA). The SW1990 cells were routinely passaged and cultured in low-glucose DMEM medium containing 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a saturated humidified incubator with 5% CO_2. The medium was changed every other day and the cells were passaged once every 3 days.

1.2 Assay of Inhibitory Rate of Cell Proliferation by MTT

The SW1990 cells in logarithmic phase were digested with 0.25% trypsin, adjusted to a density of 5×10^5/mL, and put in a 96-well plate. In each well, 100 μL cell suspension was added and treated with different concentrations of EPA or DHA (20, 40, 60 μg/L, serving as 3 groups). Negative control group was set up by adding nothing. Each group was triplicate. After the cells were cultured for 0, 12, 24, 36 and 48 h, the wells were removed from the incubator, following by addition of MTT (5 mg/mL) 10 μL in each well, continuously cultured for other 4 h. After the cells were centrifuged and the supernatant was discarded, DMSO 100 μL was added into each well and the cells were thoroughly dissolved. In 490nm, A value in each well was determined by ELISA. Growth inhibitory rate of tumor cells=(1−Everage A values in experimental group/A values in control group) ×100%.

1.3 Detection of the cyclin E mRNA Expression by Real-time PCR

SW1990 cells were treated with 40 μg/mL EPA or

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DHA for 0, 12, 24, 36 and 48 h respectively. The cells were harvested and the total RNA was extracted with TRizol according to the instruction of the kit. The RNA was dissolved and its purity and concentration were measured by UV spectrophotometry (A_{260}/A_{280}>1.8). The primers were designed by Premier 5.0 software according to the sequence of cyclin E cDNA in Genebank and synthesized by Shanghai Shenggong Biologic Co., Ltd., China). For cyclin E, up-stream: 5'-AATAGAGAGGA AGTCTGG-3', down-stream, 5'-AGATAGTCAACCTG CATG-3'; For β-actin, up-stream, 5'-CGCTGCGCTG GTCGTCGACT-3', down-stream, 5'-GTCACGCACGA TTTCCCGCT-3'. The reaction conditions were as follows: pre-degeneration at 94°C for 5 min, degeneration at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, 45 cycles; final extension at 72°C for 10 min. cDNA-free negative control group was set up at the same time of amplification. The Ct values (threshold cycle) of the tested samples were measured by real-time PCR. The relative levels of cyclin E mRNA expression were taken as $2^{-\triangle Ct}$, in which, $\triangle Ct=Ct_{Negative\ controls}-Ct_{tested\ samples}$.

1.4 Detection of Apoptosis by Flow Cytometry

SW1990 cells were treated with EPA or DHA (0, 20, 40, 60 μg/mL) for 24 h, harvested, washed with PBS buffer and resuspended. By using AnnexinV/PI-conjugated flow cytometry, the apoptosis was detected according to the instruction of the kit. The cells were adjusted to a density of 2×10^5/mL, and 195 μL cell suspension was added into fluorescein isothiocyanate (FITC)-labeled AnnexinV, incubated for 10 min at room temperature, washed, re-suspended with 190 μL/mL PBS buffer, added with 5 μL PI, and assayed by flow cytometer (Becton Dickinson, USA).

1.5 Statistical Analysis

The experimental data expressed as $\bar{x}\pm s$ and tested by analysis of variance. The intergroup comparison was done by LSD method. All statistical analyses were performed by SPSS11.5 statistical software. P<0.05 was considered to be statistically significant.

2 RESULTS

2.1 Effects of EPA and DHA on the Proliferation of SW1990 Cells

MTT results revealed that after the SW1990 cells were treated with different concentrations of EPA or DHA (20, 40 and 60 μg/mL) for 0, 12, 24, 36 and 48 h respectively, the proliferation of SW1990 cells could be inhibited by EPA or DHA in a time- and concentration-dependent manner. There was significant difference in the inhibitory rate between experimental group and negative control group (P<0.01, table 1).

Table 1 Anti-proliferative effects of EPA and DHA on SW1990 cells by MTT (n=3, %)

Groups	Inhibitory rate (%)					
	12 h	24 h	36 h	48 h	F values	P values
Control	0	0	0	0		
EPA (μg/mL)						
20	3.12±0.23	5.37±0.38	6.75±0.49	8.52±0.61		
40	10.34±0.75	22.71±1.29	31.12±1.66	40.56±2.83	4.56	0.016
60	14.93±0.72	29.05±1.60	38.43±2.1	50.11±2.35		
DHA (μg/mL)						
20	5.38±0.62	9.53±0.55	11.99±0.62	14.25±1.01		
40	12.82±0.70	24.36±1.17	32.63±1.72	45.21±2.37	5.28	0.012
60	16.83±0.86	35.19±1.82	44.93±2.31	56.74±2.66		

P<0.05, intergroup comparison in the experimental groups

2.2 Effects of EPA and DHA on the Expression of cyclin E mRNA in SW1990 Cells

After the SW1990 cells were treated with EPA or DHA (40 μg/mL) for 0, 12, 24, 36 and 48 h respectively, the expression of cyclin E mRNA was down-regulated in a time-dependent manner. There was significant difference between experimental groups and negative control group (P<0.05, fig. 1).

2.3 Effects of EPA and DHA on the Apoptosis of SW1990 Cells

After the cells were treated with 20 μg/mL EPA or DHA for 24 h respectively, there was no significant difference in the apoptotic rate of SW1990 cells between the experimental group and negative group. But when the EPA or DHA concentration was increased to 40 and 60 μg/mL, EPA or DHA could induce the apoptosis of SW1990 cells in a concentration-dependent manner, and there was significant difference in comparison to that in negative control group (P<0.01, fig. 2).

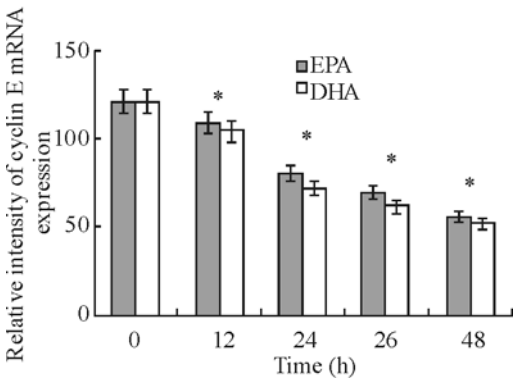

Fig. 1 Modulatory effects of EPA (40 μg/mL) and DHA (40 μg/mL) on the expression of Cyclin E mRNA in SW1990 cells

*P<0.05 as compared with that at 0 h

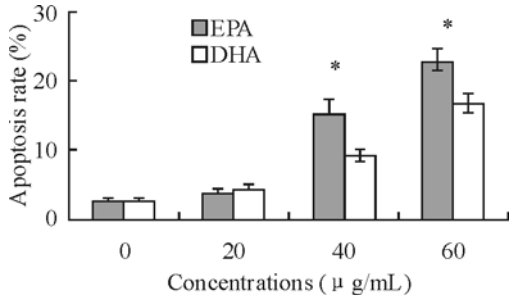

Fig. 2 Inducting effects of EPA or DHA on apoptosis in SW1990 cells by flow cytometry
$^{*}P<0.01$ as compared with control group

3 CONCLUSION

It is recognized at present that EAP and DHA, as the main components of ω-3 fatty acid, can prevent the genesis of cardiovascular diseases, and at the same the clinical practice also verifies that the application of EPA and DHA can decline the occurrence of infective complication after surgical operation and promote the wound healing[2]. Recent studies demonstrated that EPA and DHA possessed the inhibitory effects on the growth of various tumor cells, but the anti-tumor mechanisms have not been clear yet. It was inferred that the anti-tumor mechanism of EPA or DHA might be related to the direct inhibition of tumor cell proliferation, inhibition of tumor cell metastasis and the enhancement of body immune function[1].

In this study, human pancreatic cancer cell line SW1990 with high metastastic potential was chosen to observe the effects of EPA and DHA on the proliferation of tumor cells. The results revealed that both of them could inhibit the growth of SW1990 cells in a time- and concentration-dependent manner. Ding *et al* reported EPA and DHA could increase the lipid peroxidation levels of membrane in tumor cells to produce more lipid peroxides and active groups, which leads to membrane perforation, protein cross linking and nucleotide degeneration, etc., resulting in the necrosis of tumor cells. However, normal cells possess the anti-oxidative reaction system absent in tumor cells and are not injured easily[4].

Cell cycle abnormality is one of the characteristics of tumor cells, which can make the cell proliferation uncontrol, subsequently inducing tumorigenesis. G1 phase is an important check point of cell cycle and is a sole phase by which cells can receive proliferative or inhibitory signals from external environment into the cells. Cyclin E is an important cell cycle modulatory protein and can bind with corresponding Cyclin-dependent kinase (CDK) to form Cyclin-CDK compound[5], the key of transformation from G1 phase to S phase, which can phosphorylate Rb protein, induce the release of transcription factor, and activate a series of downstream genes to make the cells break through the cell cycle check point to directly enter into S phase[6]. The abnormal overexpression of cyclin E can cause the modulatory disturbance of G1 phase, accelerate the progress of cell cycle, and finally result in the tumorigenesis of a variety of malignancies. The studies by Rosen *et al* revealed that there was a relationship between the overexpression of cyclin E in the patients with ovary carcinoma and the worse prognosis to varying degrees[7]. In addition, Skalicky *et al* observed the abnormal overexpression of cyclin E in pancreatic cancer cells[8]. This study also found that in the absence of EPA or DHA, the expression of cyclin mRNA in SW1990 cells was up-regulated, but after treatment with EPA or DHA, real time PCR revealed that the expression of cyclin E was inhibited by both of them in a time-dependent manner.

Apoptosis induction of tumor cells plays an important role in the chemotherapy of malignant tumors. It has been verified that in the culture *in vitro*, EPA and DHA could induce the apoptosis of breast cancer and leukemia cells etc[9]. In this experiment, SW1990 cell line was treated with different concentrations of EPA or DHA respectively for 24 h, Annexin V/PI-conjugated cytometry revealed that both of them could induce apoptosis in a concentration-dependent manner. The mitochondria pathway controlled by Bcl-2 gene family is an important one modulating cell apoptosis, and the ratio of the expression product of apoptosis-enhanced genes and apoptosis-suppressed genes is one of the important causes determining the cell death ways. Danbara *et al* believed that the induction of apoptosis by EPA and DHA was achieved by up-regulating the expression of Bak and Bcl-xS in Bcl-2 family, and simultaneously down-regulating the expression of Bcl-xL and Bcl-2.

To sum up, this study demonstrated that EPA and DHA had obvious anti-proliferative and apoptosis inducing effects on pancreatic cancer cell line SW1990 and inhibited the expression of cyclin E in a time- and concentration-dependent manner. It was suggested that the inhibition of the cyclin E expression might be one of the mechanisms by which EPA and DHA suppressed the proliferation and promoted the apoptosis of pancreatic cancer cells, which provided a novel clue for the treatment of pancreatic cancer.

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