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RESEARCH ARTICLE

Omega-6 fatty acids have a negative impact on the proliferation and survival of three different neural cell lines

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Abstract

Polyunsaturated fatty acids (n-3 PUFA) have been implicated in various types of anticancer effects. In the present study, we have used three neural cancer cell lines, namely, C6 glia, Neura 2a and SHSY5Y and incubated with 100nM Stearic acid (SA), Docosahexanoic acid (DHA) and Eicosapentanoic acid (EPA). It was observed that these cells lines were not able to proliferate when incubated with EPA/DHA. The cell lines were not able to survive in presence of EPA/DHA. Inversely, incubation with 100nM SA did not had any effect on the survival and cell proliferation of the above mentioned cancer cell lines.

Introduction

Omega-3 polyunsaturated fatty acids (PUFA) exert selective cytotoxicity against various types of cancer cells (Begin et al, 1986). There is an inverse association between consumption of n-3 PUFAs and incidence of breast, colon and prostrate cancers (Nkondjock et al, 2003). Polyunsaturated fatty acids generally induce apoptosis and increase the efficiency of drugs (Diggle et al., 2002). Multiple cellular mechanisms have been proposed to explain the anticancer effects of n-3 PUFAs (Hardman, 2004), but lipid peroxidation is believed to be a crucial part of their action (Gonzalez et al., 1991). Factors responsible in the PUFA mediated effects are modification of tumor cell membranes affecting cell signaling pathways (Lauritzen et al., 2001), lipid peroxidation and oxidative stress (Schonberg et al., 1997), eicosanoid production (Needleman, 1979), fatty acid metabolism (Hague, 1984) and the regulation of gene expression (Narayanan, 2001).

EPA (eicosapentanoic acid, 20:5n-3) showed a moderate enhancement of drug efficacy and DHA (docosahexaenoic acid, 22:6n-3) was found to be more efficient in enhancing doxorubicin efficacy in breast cancer cell lines (Germain et al., 1998). It has also been described that changes in the activity of antioxidant enzymes like Glutamine Cysteine Synthetase (γGCS) (Lemaitre et al., 1997), Superoxide Dismutase (SOD) (Zelko et al., 2002), Catalase (CAT) (Kirkman, 2007) and Glutathione Peroxidase (GPx) (Arthur, 2007). DHA has an antitumor effect in glioma cell line U251 and mouse hepatoma (Feng et al., 2010). It has also been found that DHA can strongly induce apoptosis in human MCF-7 breast cancer cells both in vivo and in vitro (Kang et al., 2010). It is also reported that omega-3 PUFAs are protective against cancer progression, which omega-6 PUFAs do not (Larsson et al., 2004). Researchers at the New York Medical College and the Americam Health Foundation have found that docosahexaenoic acid is highly effective in inhibiting the growth of human melanoma cells (Albino et al., 2000). Researchers also speculate that fish oils exert their beneficial effect by decreasing the body's production of prostaglandin E2 which plays an important role in the initiation and progression of cancer (Gogos et al., 1998).

It is therefore, important to understand whether the effect of DHA and/or EPA has a similar effect on neural cell lines. In this study, we sought to investigate the effects of DHA and EPA in three different neural cell lines, namely, C6 glioma, Neura 2a and SHSY5Y. Details of the cell lines are given below:

C6 glioma cells- This rat glial tumour cell line represent a mixed culture of astrocytes and oligodendrocytes with 50-60 passage cells expressing both cell properties (Panchal et al., 2008). The cell line was maintained in DMEM + 10% serum in a Forma-CO_2 Incubator (5% CO_2/95%air) at 37 ^{0}C.

Neura 2a- This is a mouse neural crest derived cell line that has been extensively used to study neuronal differentiation, axonal growth and signaling pathways (Trembley et al., 2009). The cell line was maintained in

DMEM + 10% serum and 1% Non essential amino acids (NEAA) in a Forma-CO_2 Incubator (5% CO_2/95%air) at 37 ^{0}C.

SHSY5Y- This is a thrice-cloned neuroblastoma, originally from SK-N-SH and reported in 1978. A neuroblast like subclone of SK-N-SH, named SH-SY, was subcloned as SH-SY5, which was subcloned again as SH-SH5Y (Beidler et al., 1978). The cell line was maintained in DMEM + 10% serum in a Forma-CO_2 Incubator (5% CO_2/95% air) at 37 ^{0}C.

Material and Methods

Treatment of cell lines with fatty acids

The cell lines were maintained in DMEM with 10% sera. After 2 days, medium was replaced with serum-free medium containing DMEM and F12 (1:1 ratio). At day 4, free FAs, namely, SA, EPA and DHA, were added, and the cells were cultured for a further 48 h. Untreated controls received vehicle only. Ascorbic acid (40 mM) was used as an antioxidant agent. Cell cultures were treated with FA solubilized in 0.1% ethanol and 1% serum (Joardar et al., 2006).

[^{3}H]-thymidine incorporation

Cell proliferation was evaluated by measuring the incorporation of ^{3}H-thymidine during the S phase of the cell cycle (Gago et al., 2003). Cells were incubated with ^{3}H-thymidine (1 μCi/ml) at 37°C for the last 3 h of treatment with drugs in a 5% CO_2 incubator in a 24-well tissue culture plates. Supernatant was removed and cells washed twice with PBS. Cells were fixed twice with 500 μl 100% methanol for 5 min each and washed with water. They were then incubated with 5% cold trichloric acid for 10 min and rinsed thrice with water. Cells were solubilized with 250 μl of 0.1 N NaOH. 50 μl aliquots were used to measure protein by Lowry *et al*. (1951) and radioactivity of 100 μl lysate was counted in a liquid scintillation analyzer (Wallac, model 1409-411, Perkin Elmer, USA). ^{3}H-Thymidine incorporation (TI) was expressed in counts per minute (cpm)/ mg protein.

Assessment of cell viability

Cell viability was determined by the reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) into a purple formazan product by mitochondrial dehydrogenases of metabolically active cells (Joardar et al., 2006). Cells were grown on 24-well plates, and FA treatment (100nM) was carried out after 4 days of culture for different time intervals. After treatment, the medium was removed. 400 μl of fresh medium was added to each well followed by 40 μl of MTT (5 mg/ml). After 4 h, the MTT solution was carefully removed, the purple crystals were solubilized in 1.4 ml of DMSO, and absorbance was measured at a test wavelength of 550 nm with a reference wavelength of 620 nm. The absorbances obtained from treated cells were expressed as percentages of the absorbances obtained from untreated cells.

Statistical analysis

Data were summarised as mean ± SEM and analysed by one way analysis of variance (ANOVA) after Bonferroni's Multiple Comparison test. A significance level of 0.01 was used for all comparisons.

Results

It was observed previously that 100 nM concentration of fatty acids are optimal for the growth and proliferation of primary astrocyte cell culture (Joardar et al., 2006). Furthermore, the cell lines, when incubated with different concentrations of SA, DHA and EPA separately, it was observed that they could tolerate and grow considerably in presence of said fatty acids at lower concentrations (data not shown). From the above observation, it was concluded that 100 nM concentration of the fatty acids are suitable for our further experiments.

Determination of proliferation of C6G, N2A and SH-SY5Y cell lines were carried out using tritiated thymidine assay (Table 1). The cells were grown in presence of 10% serum, without serum, DHA, EPA and SA for 48h. Then thymidine assay was carried out. It was found that cell proliferation of cell lines were maximum in case of cells grown in presence of 10% sera. When the cells were grown in absence of sera (in F12 media), the proliferation of cells was reduced drastically and significantly. When the cell lines were grown in presence of 100 nM DHA or EPA, there was a significant decrease in cell proliferation as compared to cells grown in presence and absence of sera. The cell lines, when grown in presence of SA, did not showed any significant change in cell proliferation as compared to cells grown in absence of sera. However, all the cell lines showed significant decrease in proliferation in presence of fatty acids, when compared with cells grown in presence of 10% sera.

Similarly, the survival of C6G, N2A and SH-SY5Y cell lines were carried out using MTT assay (Table 2). The cells were grown in presence of 10% serum, without serum, DHA, EPA and SA for 48 hours. Then MTT survival assay was carried out. It was observed that cell survival was significantly decreased when grown in absence

of 10% sera (in presence F12 media). In presence of DHA, all the cell lines showed significantly reduced survival. In presence of EPA, C6G and SH-SY5Y showed significant decrease in survival but N2A did not showed any significant change in survival as compared to absence of sera. Cells when grown in presence of SA, did not showed any significant change in survival of the cell lines. However, all the cell lines showed significant decrease in survival in presence of fatty acids, when compared with cells grown in presence of 10% sera.

Table 1. Thymidine assay of different neural cell lines.

	C6G	N2A	SH-SY5Y
control	51.00^{ay}±0.365	18.50^{az}±0.223	54.17^{ax}±0.307
F12	20.50^{by}±0.428	11.83^{bz}±0.307	43.83^{bx}±0.307
DHA	9.33^{dx}±0.494	2.17^{cz}±0.477	6.83^{cy}±0.307
EPA	7.83^{ex}±0.307	2.33^{cz}±0.557	7.00^{cy}±0.365
SA	19.83^{by}±0.307	12.33^{bz}±0.333	43.00^{bx}±0.516

Cells were incubated with ^{3}H-thymidine (1 μCi/ml) at 37°C for the last 3 h of treatment with drugs in a 5% CO_2 incubator in a 24-well tissue culture plates. Cells were solubilized with 250 μl of 0.1 N NaOH. Radioactivity of 100 μl lysate was counted in a liquid scintillation analyzer ^{3}H-Thymidine incorporation (TI) was expressed in counts per minute (cpm)/ mg protein. Means showing different superscripts differ significantly from each other with a significance level of 0.01.

Table 2. MTT survival assay of different neural cell lines.

	C6G	N2A	SH-SY5Y
control	81.83^{ax}±0.477	31.83^{ay}±0.477	16.17^{az}±0.307
F12	43.83^{cx}±0.307	27.83^{by}±0.401	14.17^{bz}±0.401
DHA	9.50^{ey}±0.562	27.50^{bx}±0.223	8.67^{cz}±0.210
EPA	14.83^{dy}±0.307	28.33^{bx}±0.210	6.33^{dz}±0.333
SA	45.50^{bx}±0.619	27.67^{by}±0.333	14.00^{bz}±0.447

Cell viability was determined by the reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) into a purple formazan product by mitochondrial dehydrogenases of metabolically active cells. After 48 h of fatty acid incubation, 400 μl of fresh medium was added to each well followed by 40 μl of MTT (5 mg/ml). After 4 h, the purple crystals were solubilized in 1.4 ml of DMSO, and absorbance was measured at a test wavelength of 550 nm with a reference wavelength of 620 nm. The absorbances obtained from treated cells were expressed as percentages of the absorbances obtained from untreated cells. Means showing different superscripts differ significantly from each other with a significance level of 0.01.

Discussion

The cell lines were grown in presence of F12 medium:DMEM (1:1) without any sera as sera contains many fatty acids. The involvement of a single fatty acid could not be ascertained without removing it. From our study, it was established that the survival and proliferation of C6 glioma, Neura 2a and SHSY5Y cells were stalled when incubated with omega-6 fatty acids, namely, EPA and DHA. This result is similar to results already published by different authors. In many a published data, cytotoxicity induced by DHA on cancer cell lines were discussed. Five human cancer cell lines were found to be differentially sensitive to DHA, they are MCF-7, MDA-MB-231, SiHa, Raji, DHL-4 (Ding et al., 2004). Also DHA induces apoptosis and growth inhibition in human Paca-44 pancreatic

cancer cell line (Merendino et al., 2003). Researchers have also found that regular supplementation with EPA results in decrease in COX-2 enzyme, which is associated with colon cancer (Mehta et al., 2008).

Researchers have also found that DHA hyperperoxide is an inducer of apoptosis in human neuroblastoma SH-SY5Y cells, which may be mediated by mitochondrial dysfunction pathway (Liu et al., 2008). Role of DHA was also established in methyl mercury induced neurotoxicity in C6 glial and B35-neuronal cell lines (Kaur et al., 2007). Also, EPA, which is an omega 3 fatty acid, induces apoptosis in human pancreatic cancer cells (Fukui et al., 2013).

In our case, there may be various causes which may inhibit the growth of neural cancer cell lines. Among them apoptosis appears to be one of the common and most probable causes. In our study, it has been found that the exposure of various neural cell lines to DHA and EPA, results in death of the cells, with only exception being Neura 2A cell line whose survival is not significantly affected by EPA and DHA. This discrepancy may be due to the fact that the growth of this cell line is very slow and steady. So, although its proliferation is adversely affected by the fatty acids, its survival is not. An exciting point to note is that DHA, in primary astrocyte cell culture has been shown to have exactly an opposite effect i.e. it helps in the growth and differentiation of primary culture of astrocytes (Joardar et al., 2006) after 48 hours of incubation of the same with 100 nM DHA. There must be some underlying cause of the change in outcome. Further work is required to ascertain that exactly how the ω-6 fatty acids are contributing to the death of neural cancer cell lines.

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