

POLYUNSATURATED FATTY ACIDS INHIBIT MELANOMA CELL GROWTH *IN VITRO*

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Abstract: Human malignant melanoma is a highly aggressive and incurable cancer due to intrinsic resistance to apoptosis and reprogramming proliferation and survival pathways during progression. Numerous studies, including our own, linked arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) supplementation to induction of apoptosis and decreased proliferation of various cancer cells. The cytotoxic effects result from lipid peroxidation and formation of reactive oxygen species (ROS), which modify proteins and nucleic acids. DNA damage by ROS causes mutations and genomic instability, leading to uncontrolled proliferation or cell death. In the present work, four human melanoma cell lines differing in origin, doubling time, metastatic potential, and melanin content (A375, A2058, G361, and C32) were exposed to AA, EPA or DHA added into culture media in the concentrations ranging from 0 (control) to 100 mM. After 24 h incubation cytotoxicity of the analyzed acids was determined with TOX-2 (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) test. The oxidative protein modifications were measured using Aldehyde Site (DNA and Protein) Detection Kit (Cayman). All the acids tested showed marked inhibition of cell proliferation. The observed effects were statistically significant and depended on the concentration. Decrease of proliferation, associated by oxidative protein and DNA damage (measured as aldehyde sites in cells), was observed for EPA and DHA (50 mM and 100 mM) in A375, A2058, and G361 cells. In case of C32 cell line, which is amelanotic melanoma, EPA and DHA inhibited cell proliferation at 100 mM only. The effect of DHA was more pronounced. AA did not show its antiproliferative action in this cell line. The obtained results suggest that antiproliferative effects of the fatty acids in cultured human melanoma cells depend on the type of acid, its concentration and may be diverse when different melanoma cell lines are used.

Keywords: melanoma, A375, A2058, G361, C32 cells, arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA)

Epidemiological studies on the association of polyunsaturated fatty acids (PUFAs) and cancer suggest a protective effect of n-3 PUFAs and a promoting effect of n-6 PUFAs on cancer. However, also the n-6 PUFA arachidonic acid (AA), even though easily metabolized to a series of pro-inflammatory and pro-neoplastic eicosanoids, may exert proapoptotic and anti-neoplastic action when its oxidative metabolism is inhibited and it accumulates intracellularly in an unesterified form. The ability of PUFAs to induce apoptosis in tumor cells has been attributed to the increased susceptibility of these cells to lipid peroxidation. The formation of cytostatic and cytotoxic compounds and reactive oxygen species (ROS) after peroxidation of long chain PUFAs has been proposed as the primary mechanism for their activity against cancers. DNA and proteins damage by ROS and aldehyde lipid peroxi-

dation products cause mutations, and genomic instability leading to uncontrolled proliferation or cell death (1-3). Numerous studies, including our own, linked AA (20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) supplementation to induction of apoptosis and decreased proliferation of cancer cells (2-5).

Oxidative stress may play different roles in the pathogenesis of human skin cancers. In non-melanoma skin cancer, a diminished antioxidant defense caused by chronic UV exposure might contribute to multistep carcinogenesis, whereas melanoma cells exhibit increased oxidative stress which could damage surrounding tissue and thus support the progression of metastasis. Human malignant melanoma is a highly aggressive and incurable cancer due to intrinsic resistance to apoptosis and reprogramming proliferation and survival

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pathways during progression (6, 7). This motivated us to investigate the ability of exogenous AA, EPA, and DHA to modulate viability and oxidative protein and DNA modifications of four human melanoma cell lines (A375, A2058, G361, and C32) differing in origin, doubling time, metastatic potential, and melanin content.

EXPERIMENTAL

Cell culture

A375, A2058, G361, and C32 human melanoma cells were obtained from the American Type Culture Collection (ATCC) and cultured (25000/cm²) in modified Eagle medium (MEM; A375, A2058, C32) or in McCoy's 5A medium (G361) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA The Cell Culture Company), 10 mM HEPES buffer (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell exposure to PUFAs

Arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) were purchased from Sigma. The fatty acids were dissolved in 99% ethanol and stored as stock solutions (100 mM) under nitrogen at -20°C. To achieve experimental conditions, PUFAs were prepared freshly from stock solutions and diluted with the appropriate volumes of the adequate growth medium.

Cells were seeded in standard 96-well plates (8 × 10³ cells/200 µL). Twenty-four hours after seeding, the culture media were removed and replaced by the media containing 0.1% ethanol (control), or PUFAs (25, 50, and 100 µM). Control cells were cultured in the medium containing ethanol in the same concentration (v/v; 0.1%) as the experimental cultures for another 24 h. The ethanol solution in such concentration had no noticeable influence on the proliferation of the experimental cultures.

Cytotoxicity assay

Viability of cells exposed to PUFAs was measured by the tetrazolium salt assay (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) according to the manufacturer's instruction. The method based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner

salt), yielding orange formazan crystals, which are soluble in aqueous solutions. Absorbance of formazan was measured at 450 nm with a plate reader (Triad LT Multimode Detector, Dynex Technologies). Cell viability was expressed as a percentage of absorbance measured in the treated wells relative to that in the untreated control wells.

Oxidative DNA and protein modifications

Oxidative DNA and protein modifications in cells exposed to PUFAs were measured using commercial Aldehyde Site (DNA and Protein) Detection Kit (Cayman) according to the manufacturer's instruction. The aldehyde reactive probe (ARP; O-(biotinylcarbazoymethyl) hydroxylamine) reacts specifically with aldehyde groups that result from protein or DNA modification. By using an excess amount of ARP, aldehyde sites in both protein and DNA can be converted to biotin-tagged aldehyde sites, which can be detected with avidin-FITC complex. Fluorescence intensity with excitation and emission wavelength of 485 nm and 535 nm, respectively, was measured with a plate reader (Triad LT Multimode Detector, Dynex Technologies).

Statistical analysis

The data obtained from 3 independent series of experiments were expressed as mean values ± standard deviations. Statistical significance analysis was based on analysis of variance (ANOVA) followed by Tukey's HSD test. The p-value of less than 0.05 was considered significant. Statistical analysis was performed using Statistica 10 PL software for Windows (StatSoft, Poland).

RESULTS AND DISCUSSION

In this study the comparison of the effects of AA, EPA, and DHA on the viability of melanoma cell lines (A375, A2058, G361, and C32) was made. All the PUFAs tested showed marked inhibition of melanoma cell proliferation. The observed effects were statistically significant and depended on the PUFAs concentration. In case of C32 cell line, which is amelanotic melanoma, EPA and DHA inhibited cell proliferation at 100 µM only. AA did not show its antiproliferative action in this cell line (Tab. 1). It was proved that the n-3 PUFAs can prevent non-melanoma skin cancers (8) and melanoma by enhancing apoptosis and reducing angiogenesis; in turn, lung colonization was inhibited in melanoma-bearing animals (9). In contrary, de Sousa Andrade et al. (10) observed that the toxic effect of fatty acids on melanoma cells did not cor-

relate with the degree of unsaturation and carbon chain length. They found that EPA and DHA were not toxic for melanoma cell lines in any of the conditions tested. In addition, AA was not toxic to SK-Mel 28 human melanoma cells, while in the melanoma cell lines S91 and SK-Mel 23 was the most effective. They suggested that it was unlikely that the toxic effect of AA on the two sensitive melanoma cell lines was due to the oxidative stress.

The toxicity of AA might result from an accumulation of this fatty acid in the melanoma cells, or change in the synthesis of prostaglandins. On the other hand, the lack of toxicity of arachidonic acid on SK-Mel 28 cells could be attributed to high levels of cyclooxygenase (COX) in these cells. The overexpression of this, involved in AA metabolism, enzyme was linked to the apoptosis inhibition in these human tumor cells (10). Xia et al. (11) postu-

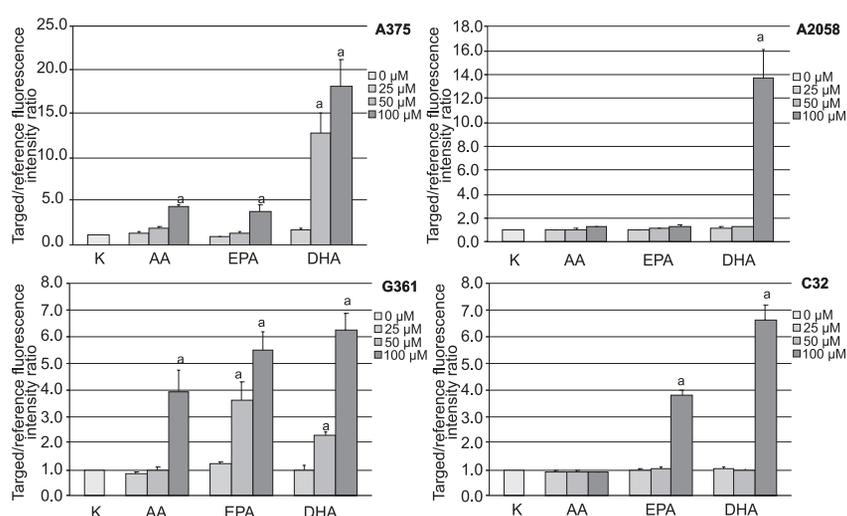


Figure 1. PUFAs-induced alterations of oxidative DNA and protein modifications in A375, A2058, G361, and C32 melanoma cells. Data are expressed as target/reference fluorescence intensity ratio. Data were calculated as the means \pm SD from three independent experiments. * statistically significant difference in comparison with the control; $p < 0.05$

Table 1. Cytotoxicity of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in human melanoma cells A375, A2058, G361, and C32 expressed as a percentage of viability of PUFA-treated cells vs. non-treated cells.

PUFA	PUFA (μ M)	A375	A2058	G361	C32
AA	25	74.39 \pm 1.96 *	110.19 \pm 4.45	115.50 \pm 7.10	109.61 \pm 2.83
	50	51.56 \pm 5.15 *	96.74 \pm 2.44	110.30 \pm 5.25	111.32 \pm 3.28
	100	17.20 \pm 0.17 *	85.73 \pm 1.51	22.91 \pm 2.67 *	109.66 \pm 0.77
EPA	25	111.60 \pm 1.71 *	101.92 \pm 7.10	73.54 \pm 6.84 *	107.61 \pm 4.21
	50	72.76 \pm 1.34 *	89.86 \pm 2.65	27.81 \pm 4.09 *	107.71 \pm 2.86
	100	22.70 \pm 4.54 *	80.65 \pm 5.33 *	17.41 \pm 2.00 *	27.32 \pm 0.51 *
DHA	25	53.94 \pm 3.82 *	88.00 \pm 5.76	109.05 \pm 6.87	104.22 \pm 2.11
	50	6.64 \pm 0.93 *	76.04 \pm 2.19 *	37.68 \pm 1.43 *	105.66 \pm 2.04
	100	4.88 \pm 1.07 *	6.35 \pm 1.08 *	11.17 \pm 0.61 *	13.29 \pm 0.98 *

Data were calculated as the means \pm SD from three independent experiments. * statistically significant difference in comparison with the control; $p < 0.05$.

lated, that modification of the tissue n-6/n-3 fatty acid ratio by increasing n-3 fatty acids and decreasing n-6 fatty acids may be an effective therapeutic approach, because this not only reduces the AA-derived cancer-promoting eicosanoids but also increases the formation of n-3-derived antitumor eicosanoids. Using fat-1 transgenic mice, they demonstrated an antimelanoma effect of n-3 PUFAs and identified PGE3-PTENaa - a potential pathway for their action (11).

Multiple cellular mechanisms have been proposed to explain the anticancer effects of n-3 PUFAs, including alteration in prostaglandin synthesis, influences on transcription factors and gene expression, modifications of signal transduction pathways - cellular proliferation apoptosis and differentiation, changes of antioxidant enzymes expression and activity, generation of free radicals, and increased lipid peroxidation (1, 2, 8, 9). Bachi et al. (12) reported that AA-derived leukotriene B4 (LTB4), the main product of the 5-LOX pathway, was able to induce growth of subtumorigenic inocula of melanoma cells, and a LTB4 receptor antagonist inhibited acute inflammation-associated tumor growth. Addition to the tumor inflammatory microenvironment of EPA, or leukotriene B5 (an EPA-derived leukotriene) significantly inhibited tumor progression (12). Recently, Serini et al. hypothesized that a modulation of COX-2 mRNA stability could also be involved in the pro-apoptotic effect exerted by DHA in the primary WM115 and the metastatic WM266-4 melanoma cell lines (13).

In our study, a decrease of proliferation, associated by oxidative protein and DNA damage (measured as aldehyde sites in cells), was observed for AA, EPA, and DHA (50 mM and 100 mM) in A375, A2058, and G361 cells (Fig. 1). Published evidence supports the hypothesis that lipid peroxidation is a crucial part of PUFAs action. The susceptibility of PUFAs to lipid peroxidation renders them capable of rapidly generating lipid peroxides, which may directly cause cytotoxicity or may influence intracellular signaling pathways, resulting in growth inhibition or death of tumor cells (14-16). This hypothesis is supported by experiments showing that n-3 and n-6 PUFAs effects can be attenuated or completely reversed by the antioxidant, vitamin E. Moreover, the cytotoxicity of PUFAs on malignant cells can be potentiated by increasing cellular oxidative stress (2, 3, 14, 17, 18). The present study showed that cytotoxic effect of DHA was the most evident. The antitumoral action of DHA has been widely demonstrated in epidemiological and experimental studies. The proapoptotic effect of

DHA in cancer cells is one of the main biological mechanisms invoked to explain the antitumoral effect of this fatty acid (1, 2, 15). Our own findings agree with the reports that DHA is one of the most anticancer-3 PUFAs due to possessing of a greater number of double bonds than other PUFAs, which increases its susceptibility to peroxidation.

The obtained results suggest that antiproliferative effect of the fatty acids in cultured human melanoma cells depend on the type of acid, its concentration and may vary depending on melanoma cell line used.

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REFERENCES

- Berquin I.M., Edwards I.J., Chen Y.Q.: *Cancer Lett.* 269, 363 (2008).
- Serini S., Piccioni E., Merendino N., Calviello G.: *Apoptosis* 14, 135 (2009).
- Trombetta A., Maggiora M., Martinasso G., Cotogni P., Canuto R.A., Muzio G.: *Chem. Biol. Interact.* 165, 239 (2007).
- Zajdel A., Wilczok A., Latocha M., Dzierżewicz Z.: *Adv. Clin. Exp. Med.* 19, 481 (2010).
- Zajdel A., Wilczok A., Gruchlik A., Padaszyński P., Dzierżewicz Z.: *Adv. Clin. Exp. Med.* 19, 585 (2010).
- Sander C.S., Hamm F., Elsner P., Thiele J.: *Br. J. Dermatol.* 148, 913 (2003).
- Su M.S., Zhang O., Wang X., He P., Zhu Y.J., Zhao J., Rennert O.M., Su Y.A.: *Mol. Cancer Ther.* 8, 1292 (2009).
- Black H.S., Rhodes L.E.: *Cancer Detect. Prev.* 30, 224 (2006).
- Mannini A., Kerstin N., Calorini L., Mugnai G., Ruggieri S.: *Br. J. Nutr.* 101, 688 (2009).
- de Sousa Andrade L.N., de Lima T.M, Curi R., de Lauro Castrucci A.M.: *Toxicol. In Vitro* 19, 553 (2005).
- Xia S., Lu Y., Wang J., He C., Hong S., Serhan C.N., Kang J.X.: *Proc. Natl. Acad. Sci. USA* 103, 12499 (2006).
- Bachi A.L., Kim F.J., Nonogaki S., Carneiro C.R., Lopes J.D., Jasiulionis M.G., Correa M.: *Mol. Cancer Res.* 7, 1417 (2009).
- Serini S., Fasano E., Piccioni E., Monego G., Cittadini A.R., Celleno L., Ranelletti F.O., Calviello G.: *Carcinogenesis* 33, 164 (2011).

14. Siddiqui R.A., Harvey K., Stillwell W.: *Chem. Phys. Lipids* 153, 47 (2008).
15. Anderson B.M., Ma D.W.: *Lipids Health Dis.* 8, 33 (2009).
16. Rogers K.R., Kikawa K.D., Mouradian M., Hernandez K., McKinnon K.M., Ahwah S.M., Pardini R.S.: *Carcinogenesis* 31, 1523 (2010).
17. Gadjeva V., Dimov A., Georgieva N.: *J. Clin. Pharm. Ther.* 33, 179 (2008).
18. Leonardi F., Attorri L., Benedetto R.D., Biase A.D., Sanchez M., Tregno F.P., Nardini M., Salvati S.: *Free Radic. Res.* 41, 748 (2007).