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Antiproliferative effect of docosahexaenoic acid on adult human keratinocytes *in vitro*

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Abstract

Numerous clinical studies demonstrate benefits of dietary supplementation with fish oils in autoimmune diseases and other inflammatory diseases such as psoriasis, multiple sclerosis, systemic lupus erythematodes and so on. Docosahexaenoic acid (DHA) is an omega-3 fatty acid which is abundantly found in fish oil. In the present study we investigated effects of DHA on proliferation of human keratinocytes established from skin of seven adult donors, cultivated in growth medium that allows optimal cell proliferation. We found a dose-dependent inhibition of cell proliferation when keratinocytes were incubated with 6.25, 12.5 and 25 –μM of DHA. Inhibition of proliferative capacity considerably varied in keratinocyte cultures derived from different donors, particularly when incubated with the lowest concentration of the assessed substance. Lactate dehydrogenase-release assay excluded necrosis of cultivated keratinocytes as a cause of decreased proliferation. Our results suggest that DHA may potentially be used as a routine adjuvant therapy, with classical therapy of inflammatory hyperproliferative skin diseases.

Omega-3 fatty acids are a family of unsaturated fatty acids**,** which have a final carbon–carbon double bond in the n–3 position in common, and cannot be synthesized by the human body. The most nutritionally important omega-3 polyunsaturated fatty acids (PUFAs) are: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

PUFAs exhibit several potent immuno-modulatory features (1) and among the omega-3 PUFAs, those derived from fish oil - EPA and docosahexaenoic acid - are particularly biologically potent. Many of the placebo-controlled trials revealed significant benefit of fish oil and PUFAs in chronic inflammatory diseases, including decreased disease activity and decreased needs for use of anti-inflammatory drugs (2). Several of biologic effects of DHA have been demonstrated from feeding studies with fish or fish oil supplements in humans and animals. These include effects on triglycerides, high-density lipoprotein cholesterol, platelet function, endothelial and vascular function, blood pressure, and cardiac excitability, measures of oxidative stress, pro- and anti-inflammatory cytokines, and immune function (3).

Although it is well known that omega-3 fatty acids may affect the inflammatory components of skin diseases, the cellular and molecular basis of their beneficial effects is still not well delineated. Profound changes in the metabolism of eicosanoids, with increased concentrations of free arachidonic acid and its proinflammatory metabolites, have been observed in psoriatic lesions. Free eicosapentaenoic acid may compete with liberated arachidonic acid and result in an anti-inflammatory effect (4).

Effects of PUFAs have been investigated on immortalized HaCaT cell-line and it has been suggested that induction of cyclooxigenase-2 (COX-2) in keratinocytes may be important in the anti-inflammatory and protective mechanism of PUFAs action (5). It has been reported that DHA has antiproliferative effects on some epithelial cells: human

colon epithelial cell-lines and adenocarcinoma (HT-29, HCT-116) origin (7), PC-3 prostate carcinoma cells (8) and human endothelial cells (9).

Until now, the effects of DHA on keratinocytes have been explored in a single study, which demonstrated that DHA has antiproliferative effects on human papillomavirus type 16 (HPV-16) immortalized cervical keratinocytes in the presence of estradiol, a growth stimulator for these cells. The same study indicated that DHA inhibited proliferation of HPV immortalized foreskin cells, but it had no effect on the normal foreskin cell pool (6).

The present study shows for the first time, that DHA inhibits proliferation of adult human keratinocytes *in vitro.* However, substantial individual differences in keratinocyte response to DHA were found particularly when incubated with low concentrations of DHA.

Material and methods

Cell culture and reagents

Skin samples were obtained from seven healthy volunteers undergoing cosmetic surgery at the plastic surgery unit. The epidermis was separated from the dermis after overnight treatment (4°C) with dispase (5 U/ml), while a single-cell suspension was subsequently obtained upon treatment with trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA) (0.53 mM). Cells were seeded at a density of 5000 cells/cm^2 in 25 cm^2 flasks and further cultivated in keratinocyte growth medium (Invitrogen, Paisley, UK) at 37°C in an incubator with humidified atmosphere containing 5% CO_2. DHA was dissolved in dimethyl sulfoxide (DMSO) and stored in 10 mM aliquots at –20°C and protected from light. All reagents used in the study were from Sigma (St. Louis, MO, USA), unless stated otherwise. The cells were used for experiments after the third or fourth passage. They were seeded in 96-well plates in 200 µl of keratinocyte growth medium, for cell proliferation, and treated as described in the figure legends. The control cell cultures contained the amount of DMSO corresponding to its content in the solution, with the highest concentration of DHA.

Cell proliferation

Cell proliferation was measured by [^{3}H]thymidine incorporation into newly synthesized DNA of cultivated cells. For the assessment of proliferation, keratinocytes were cultivated in 96-well plates (3 × 10^3 cells/well) for 48 h, then washed and cultivated for additional 72 h in fresh medium containing 6.25, 12.5 and 25 µM DHA or 0.25% DMSO (corresponding to DMSO content in cultures treated with 25 µM DHA) as a control. During the last

Table 1. Proliferation of keratinocytes cultivated with various concentrations of DHA

^{3}H Thymidine incorporation (Mean ± Standard deviation of the triplicate)				
DHA (µM)	0**	6,25	12,5	25
I*	6714 ± 1140	1040 ± 256	224 ± 57	243 ± 153
II *	25359 ± 4038	16616 ± 2093	210 ± 16	279 ± 125
III*	3415 ± 653	1378 ± 445	157 ± 44	191 ± 37
IV*	6613 ± 500	2785 ± 320	2667 ± 538	223 ± 31
V*	16809 ± 1898	10784 ± 7522	4559 ± 5917	156 ± 29
VI*	11855 ± 3088	5227 ± 140	3119 ± 1298	209 ± 99
VII*	1534 ± 213	1210 ± 91	196 ± 47	184 ± 36

* different human keratinocyte cultures

**control

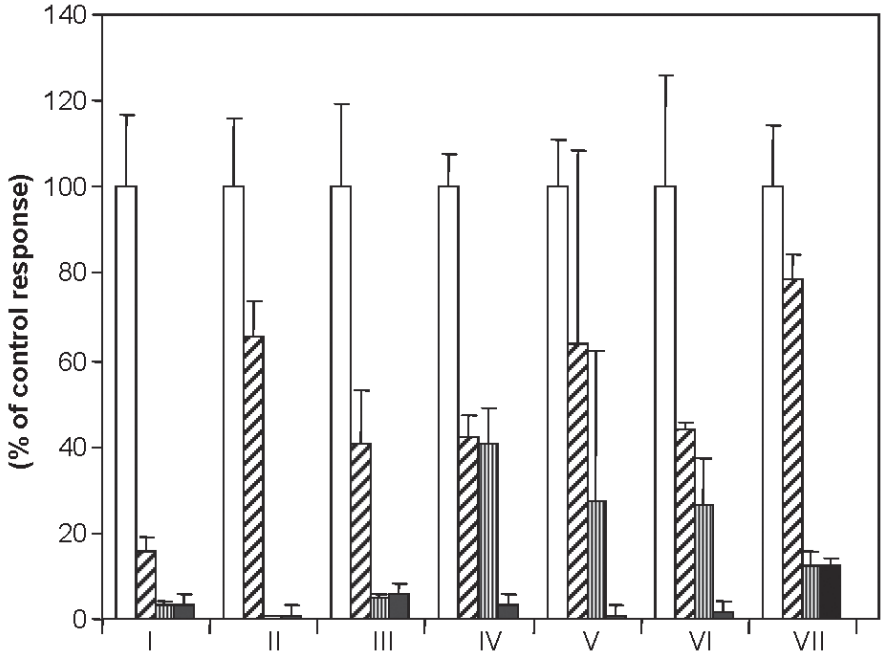

Figure 1. DHA inhibits keratinocyte proliferation *in vitro*

Keratinocytes were incubated without (control, open bars) or with 6.25 μM (cross hatched bars), 12.5 μM (vertical hatched bars), or 25 μM (black bars) of DHA for 72 h. The incorporation of [^{3}H]thymidine (1 μCi/well) was determined during the last 24 h of cultivation. Data are expressed as the percentage of the control response. Error bars represent standard deviations of the triplicate for each culture and treatment.

24h of incubation, keratinocytes were pulsed with 1 μCi of [^{3}H]thymidine per well, harvested and counted in a liquid scintillation counter as described previously (10). Results are expressed as a percentage of proliferation measured in control cultures.

Lactate dehydrogenase (LDH) release assay

LDH release assay was employed to assess the cell leakage of cytoplasm through damaged membrane that occurs during necrosis. To assess the LDH release, keratinocytes were cultivated under the same conditions as for the proliferation assay.

Measurement of lactate dehydrogenase activity was performed exactly as previously described (11, 12).

Statistical analysis

All experiments with keratinocyte cultures were performed in triplicates. The results are presented as mean ±SD of triplicate cultures, or as the percentage of inhibition of control response, as indicated in figure legends. Differences in proliferation of control vs. treated keratinocytes in the group of 7 donors, were analyzed by paired t-test.

Results

DHA inhibits keratinocyte proliferation

Effects of DHA on keratinocyte proliferation were first assessed. Although there was a considerable difference in inhibition of proliferation upon incubation with DHA among individual cultures (Table 1) (Figure 1), it was established that even in lowest tested concentrations, DHA significantly decreased the proliferative capacity of cultivated keratinocytes from 100 ± 15.8 in mock treated cultures, to 50.1 ± 11.7 (mean % of control culture proliferation ± standard deviation) in cultures treated with 6.25 μM DHA ($p=0.04$). DHA in concentration of 12.5 and 25 μM, decreased the proliferation of cultivated keratinocytes up to 16.5% ± 8.5% and 4.1% ± 1.1% of ^{3}H incorporation in control cultures, respectively ($p<0.01$). There was a significant difference between cultures treated with 6.25 μM DHA, and those treated with 12.5 μM DHA ($p=0.01$), whereas there was no significant difference between cultures treated with 12.5 and 25 μM DHA ($p=0.09$).

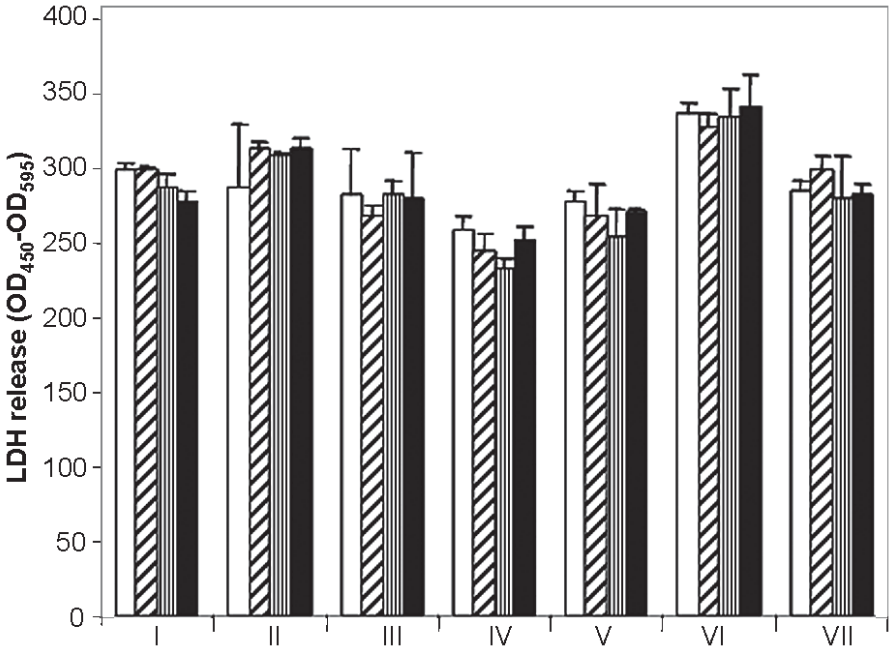

Figure 2. DHA does not induce necrosis in keratinocyte cultures

Keratinocytes were incubated without (control, open bars) or with 6.25 μM (cross hatched bars), 12.5 μM (vertical hatched bars), or 25 μM (black bars) of DHA for 72 h. The LDH release in the supernatant of cultures is expressed as OD_{450}-OD_{595}. Results are presented as mean ± SD for the triplicate of each culture and treatment.

DHA does not induce necrosis in cultivated keratinocytes

In order to test whether decreased proliferation of keratinocytes upon incubation with DHA is a result of necrotic death of cultivated keratinocytes, LDH release assay was performed. It was established that LDH activity in supernatants of cultures incubated with DHA was not significantly higher ($p>0.05$) than in control cultures (Figure 2), hence excluding that DHA induced necrosis in tested cultures.

Discussion

Omega-3 PUFAs are reported to reduce inflammation in various disorders including cardiovascular disease, ulcerative colitis, rheumatoid arthritis, and psoriasis (4, 13, 14). Potential beneficial mechanisms include modulation of pro-inflammatory cytokines production (15, 16) and n-6 eicosanoids synthesis (17).

Until now, the effects of DHA on keratinocyte proliferation have been tested in a single study which included normal foreskin keratinocytes, HPV 16 immortalized cervical keratinocytes, HPV 16 immortalized foreskin keratinocytes, normal laryngeal keratinocytes and keratinocytes derived from laryngeal papillomas (6). Study results revealed antiproliferative effects on HPV 16 immortalized cervical keratinocytes in the presence of estradiol, a growth stimulator for these cells. The same study indicated that DHA inhibited HPV immortalized foreskin cells, but did not affect normal cells and authors concluded that DHA has a profound growth inhibitory effect on HPV containing cells, but has no such effect on normal cells. Although pooled foreskin keratinocytes and transformed keratinocyte cultures are valuable systems for studying the biology of keratinocytes, they also have shortcomings: the former neglect individual variability by using pooled samples, while the latter disregard multiple molecular alterations in immortalized cell lines that can lead to different responses compared to primary cell cultures. Our previous study demonstrated that keratinocyte responsiveness to antiproliferative action of Vitamin A and Vitamin D derivates may be individual, if keratinocyte cultures are derived from different

donors (18).

Furthermore, lack of DHA effect on neonatal keratinocytes, demonstrated by Chen and Auburn (6), may be at least partially explained by the fact that neonatal keratinocytes in comparison with those obtained from adult donors, have higher proliferative capacity that is maintained until the age of 10, while later it gradually decreases (19).

In the present study, we demonstrated a dose-dependent inhibitory effect of DHA on proliferation of adult human skin keratinocytes *in vitro,* with a DHA concentration one order of magnitude lower than reported by Chen and Auburn (6). The final DHA concentration used in our study was in the range of 6.25 - 25μM, based on findings that these concentrations may be achieved in plasma after daily intake of dietary or therapeutic doses of DHA (200 mg to 1,6g/ day) (20, 21). These concentrations may seem high, since individuals consuming typical Western diets have very low blood PUFA levels, but it has been shown that temporary levels of DHA, as high as 100 μM, can be achieved in plasma with dietary supplements (22).

Our results also indicate a considerable variability in responsiveness of keratinocytes from different adult donors to DHA. While a clear antiproliferative effect was uniformly obtained with 25 μM of DHA, a substantial variability in keratinocyte response was observed upon treatment with lower drug concentrations (6.25 and 12.5 μM).

In order to evaluate whether antiproliferative effect is a result of keratinocyte necrosis, LDH release assay was performed. LDH activity in supernatants was employed to assess leakage of cell cytoplasm through damaged membrane that occurs during the necrosis process. Our results indicate that antiproliferative effect of DHA is not due to keratinocyte necrosis induction, and that another process was involved in lower proliferative response of keratinocytes. Chen and Auburn suggested that DHA-induced lipid peroxidation may lead to arrest of proliferation in several types of keratinocytes. This finding may also apply to skin keratinocyte cultures. Further investigations are necessary to determine the exact mechanism of inhibition of skin keratinocyte proliferation.

Therefore, studies on keratinocytes obtained from different adult donors may be more suitable to examine the antiproliferative potential of DHA, as well as to predict their potential therapeutic effects in individual patients.

Conclusion

In conclusion, this study has demonstrated for the first time that DHA exhibits antiproliferative effects on adult human skin keratinocytes *in vitro*. Furthermore, our results imply that keratinocyte responsiveness to antiproliferative action of DHA is individual to certain extent, thus warranting further studies combining clinical research with dietary supplementation of DHA, and *in vitro* research to investigate whether *in vitro* keratinocyte response can possibly be a predictor of the therapeutic efficacy of DHA in hyperproliferative inflammatory skin diseases.

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Abbreviations

ALA - Alpha-Linolenic Acid
COX-2 - Cyclooxygenase-2
DHA - Docosahexaenoic Acid
DMSO - Dimethyl Sulfoxide
EDTA - Ethylenediaminetetraacetic Acid
EPA - Eicosapentaenoic Acid
HPV - Human Papillomavirus
LDH - Lactate Dehydrogenase
PUFA - Polyunsaturated Fatty Acids

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Antiproliferativni efekat dokosaheksanoične kiseline na adultne humane keratinocite *in vitro*

Sažetak

Uvod: Brojne kliničke studije ukazale su na povoljan efekat ishrane obogaćene ribljim uljem na tok autoimunih i zapaljenskih bolesti kao što su multipla skleroza, sistemski eritemski lupus, psorijaza itd. Dokosaheksanoična kiselina (DHA) je omega-3 masna kiselina koja je u visokoj koncentraciji prisutna u ribljem ulju.
Cilj: Zadatak ovog istraživanja bio je ispitivanje uticaja dokosaheksanoične kiseline na proliferaciju humanih keratinocita *in vitro.*
Materijal i metode: Proliferacija je određivana na osnovu ugradnje ^{3}H timidina u novosintetisanu DNK proliferisanih ćelija u kulturama keratinocita poreklom od 7 zdravih volontera. Za ispitivanje nekroze korišćen je test oslobađanja laktat-dehidrogenaze (LDH).
Rezultati: Dobijeni rezultati ukazuju da DHA značajno smanjuje prolifertivni kapacitet kultivisanih keratinocita i to od 100±15,8 u netretiranim kulturama, do 50,1±11,7 (% kontrolnog odgovora ± standardna devijacija) u kulturama tretiranim sa 6,25 μM DHA (p=0,04); dok u koncentraciji 12,5 i 25 μM smanjuje proliferaciju kultivisanih keratinocita na 16,5±8,5% i 4,1±1,1%, (p<0,01). Ustanovljena je statistički značajna razlika između kultura tretiranih sa 6,25 μM DHA i onih tretiranih sa 12,5 i 25 μM DHA (p=0,01), dok nije ustanovljeno postojanje statistički značajne razlike između kultura tretiranih sa 12,5 i 25 μM DHA (p=0,09).
Aktivnost LDH u supernatantima kultura nije bila

statistički značajno veća (p>0,05) od aktivnosti u kontrolnim, netretiranim kulturama.
Zaključak: Dobijeni rezultati ukazuju da DHA ispoljava antiproliferativni efekat na humane adultne keratinocite *in vitro* i da sniženje proliferacije nije posledica indukcije procesa nekroze u ovim ćelijama.

