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Phospholipid Class as a Determinant in Docosahexaenoic Acid's Effect on Tumor Cell Viability

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Abstract

Here we explore how incorporation of the omega-3 fatty acid docosahexaenoic acid (DHA) into murine leukemia cells (T27A) may alter membrane structure and function. When cells were cultured in DHA-supplemented medium, DHA incorporated rapidly and preferentially into phosphatidylethanolamine (PE), with lesser and slower incorporation into phosphatidylcholine (PC). DHA at low concentrations preferred PE over neutral lipids, but in DHA excess accumulation in neutral lipids outstripped that of phospholipids. High DHA levels reduced cell growth in the apparent absence of lipid peroxidation. To study the importance of DHA's phospholipid class, cells were fused with lipid vesicles of either 18:O;22:6 PE or 18:O;22.6 PC. DHA-containing PC vesicles produced a dose dependent decrease in cell viability, whereas PE-containing vesicles had little effect although they appeared more fusogenic. These results provoke the interesting speculation that T27A cells can safely accumulate DHA in PE, but are vulnerable if excessive DHA is incorporated into PC

Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:10 omega-9, oleic acid; 18:2 omega-6, linoleic acid; 20:4 omega-6, arachidonic acid; 22:6 omega-3, docosahexaenoic acid; BCS, bovine calf serum; BSA, bovine serum albumin; DHA, docosahexaenoic acid; NBD-PE, nitrobenzoxadiazole-phosphatidylethanolamine; PA, phospnatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SUV, small unilamellar vesicles; TBA-RS, thiobarbituric acid-reactive substances.

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Introduction

Omega-3 polyunsaturated fatty acids are reported to benefit human health in such diverse areas as vision, brain development, atherosclerosis, arthritis, and cancer. Aside from their influence in leukotriene and prostaglandin production, omega-3 fatty acids likely contribute to membrane structure and function. Docosahexaenoic acid (DHA), 1 the longest and most unsaturated omega-3 fatty acid commonly found in biological systems, is routinely found in abundance in only a few tissues (retinal rod outer segment, brain gray matter, and sperm) but its concentration can be increased in both normal and neoplastic cells through supplementation in diet or culture medium [1,2]. Once incorporated, DHA is tenaciously retained, typically at the expense of omega-6 unsaturated fatty acids [1]. Its high proportion in certain tissues and persistence in most tissues suggest that DHA has a vital role in membrane function.

DHA is found in phosphatidylethanolamine (PE) of brain gray matter, synaptosome plasma membrane, and rod outer segment at levels of 24 to - 40% of total fatty acids, and in phosphatidylserine (PS) of the same tissues at the same relative percent [1]. In contrast, DHA is found in phosphatidylcholine (PC) at much lower levels, from <5% in brain to 18.5% in rod outer segment; however, in sperm, DHA has been reported in PC at levels of 77% [3], although this may be an overestimate [1]. Because membrane DHA levels may vary by tissue type and fatty acid availability, monitoring DHA incorporation into the various phospholipid classes is an important component of the investigation of DHA's effects on cell function.

We reported previously that the tumor line T27A shows both structural [4-6] and functional alterations [7,8] when enriched in DHA, alterations that may be associated with the putative anticancer properties of DHA [9]. Fusion of DHA-containing PC vesicles to T27A plasma membranes produced rapid cell death whereas equimolar incorporation of oleic acid containing PC vesicles had no such detrimental effects on T27A [8]. These observations imply that DHA may be safely incorporated at high levels in some cells but in other cells is detrimental in small amounts. To test whether DHA's phospholipid class rather than its absolute amount determines cytotoxicity, here we investigate the phospholipid location of newly incorporated DHA, the kinetics of its incorporation into various phospholipid classes, and the differential effect of DHA on T27A tumor cell viability when present in one or the other of the two major phospholipid classes (PC, PE). With these data we may better interpret the mechanism of DHA's anti-cancer properties.

Materials and Methods

Tumor line. The murine leukemia T27A (American Type Culture Collection. Rockville, MD) was cultured routinely in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 units of penicillin/ml, 100 [micro g of streptomycin/ml and 10% bovine calf serum (BCS; HyClone Labs., Logan,

UT), unless otherwise stated, in a humidified 5% CO2 atmosphere. Cell viability was determined by trypan blue exclusion and tritiated thymidine incorporation into DNA [10].

Preparation of fatty acid-supplemented medium. DHA (Nu-Chek Prep., Elysian, MN) was introduced into RPMI 1640 medium supplemented with 1% (w/v) fatty acid-free bovine serum albumin (BSA, Sigma Chemical Co.) as described by Spector and Hoak [11] with modifications we have reported [12]. The medium was supplemented with BCS in which the major fatty acids were 16:0 (14% of total fatty acids), 18:0 (14%),18:l omega-9 (16%), and 18:2 omega-6 (47%).

Phospholipid isolation and fatty acid analysis. T27A cells cultured in DHA-supplemented or control medium were harvested by centrifugation and washed free of unincorporated fatty acid with physiological saline containing 5% fatty acid-free BSA. Lipids were extracted according to the technique of Foich et al [13] and individual phospholipid classes were sequentially eluted from aminopropyl minicolumn bonded silica according to the method of Pietsch and Lorenz [14] and recovery quantified as described [15]. The fatty acid composition of phospholipid classes was determined after hydrolysis and methylation according to Morrison and Smith [16], and the fatty acid methyl esters were analyzed with a Shimadzu GC-17A gas chromatograph equipped with a 0.25 mm x 30 m Stabilwax capillary column (Restek, Bellefonte, PA). Acyl glycerol in the neutral lipid fraction was determined enzymatically by measuring released glycerol [17].

Peroxidation estimation. Thiobarbituric acid reactive substances (TBARS) were measured with a fluorescence method [9] based on techniques described by Esterbauer and Cheeseman [18].

Preparation of small unilamellar vesicles and fusion with cells. Cells were modified by fusion with small unilamellar vesicles (SUV) made from 18:0;18:1 PC; 18:0;22:6 PC; and 18:0;22:6 PE (Avanti Polar Lipids, Alabaster, AL) as described earlier [8]. To monitor fusion, the lipids were spiked with nitrobenzoxadiazole-phosphatidylethanolamine (NBDPE; Molecular Probes, Eugene, OR) at a 200:1 molar ratio.

Flow cytometry. Live cells identified by forward scatter and side scatter were gated and cell-associated NBD-PE resistant to exhaustive washing was monitored by fluorescence at 525 nm with an argon laser-equipped Coulter Elite ESP cell sorter. A linear gate from the origin encompassing 95% of the cells was set in the negative control and electronically replicated onto histograms from SUV-treated samples. The net increase in NBD-PE+ cells was calculated from the difference between gated cells in negative control and treated samples.

Results

Kinetics of DHA incorporation into phospholipid classes. To test the hypothesis that the phospholipid class in which DHA resides influences its effect on cell function, we first monitored DHA's incorporation from culture medium into the various phospholipid classes. As shown in Figure 1 (top panel), DHA incorporation was highest in PE, ranging from 3.3% of total fatty acids (in the control) to 21% at 12 hours and 29% at 48 hours. DHA in PC rose from undetectable levels to 5.9% at 12 hours and an apparent plateau value of 9.6% at 24 hours. DHA was not detectable in PI and PS+PA until after 12 hours, and by 48 hours reached levels of 7.0% and 4.7%, respectively. The major phospholipid in T27A was PC (48%), followed by PE (23%), PS+PA (13%), SM (9%), and PI (7%) (Figure 1, bottom panel). After 48 hours exposure, T27A cells exhibited new DHA incorporation into PE about 2.7 times greater than into PC; when the 2.1-fold excess of membrane PC to PE is factored in, it can be estimated that a new incoming DHA molecule preferred PE over PC by about 5.7 times.

Dose-response of DHA incorporation. Based on the preferential incorporation of DHA into PE, we predicted that DHA's appearance in other phospholipid classes would occur principally under conditions of DHA excess, a prediction borne out by the results shown in Figure 2 . At low DHA concentrations, DHA preferred PE, whereas at high DHA concentrations DHA accumulated in the other phospholipid classes (again with a small preference for PC). In the major phospholipids PC and PE, the principal changes in other fatty acids were decreases of 18:2 omega-6 and 18:1 omega-9 in PC and PE, 20:4 omega-6 in PE, and 18:0 in PC, and an increase of 16:0 in PC (Table 1). Not unexpectedly, DHA also accumulated in neutral lipids (Figure 2 , middle panel). At the lowest DHA concentration (0.038 mM), PE contained approximately 13% more DHA on a per cell basis than did the neutral lipid fraction. At the higher DHA doses, where DHA incorporation into PE was no longer linearly related to dose, accumulation of DHA into neutral lipids outstripped incorporation into PE. DHA in fact became a substantial proportion of the cells' lipid reserves, occupying 15% of the neutral lipid acyl chains in cells grown in 0.30 mM DHA (Figure 2 , bottom panel).

DHA inhibition of tumor cell growth. Figure 3 (top panel) demonstrates that T27A growth is severely hindered in medium with high DHA concentrations. In unsupplemented medium and medium prepared with 0.15 mM DHA there was a 30-fold increase in cell number over a 3 day period, translating to the expected generation time of about 15 hours. In contrast, medium prepared with 0.38, 0.55, and 0.76 mM DHA showed lower overall population growth (19.5, 13.5, and 5-fold increases in cell number, respectively) and longer apparent generation times (18, 20.5, and 29 hours, respectively). There was not an accumulation of dead cells (i.e., viability was >90%), suggesting that the high DHA levels retarded cell growth rather than causing death after considerable proliferation. These results were strengthened by the observation that tritiated thymidine incorporation into DNA, an estimate of cell proliferation, was

inhibited when T27A cells were cultured in medium containing high DHA concentrations (Figure 3, bottom panel).

Cytotoxicity of 18:0;22:6 PC but not PE. Having demonstrated that T27A preferentially incorporates DHA into PE rather than PC, we proposed that high concentrations of DHA in PC and sudden enrichment of DHA in PC without compensatory changes in membrane lipids are incompatible with viability of T27A tumor cells. To test this prediction, T27A cells were fused with SUV of 18:0;22:6 PC or 18:0;22:6 PE, and cell viability was measured by trypan blue exclusion. Figure 4 (top panel) demonstrates reduced viability of T27A cells treated with 18:0;22:6 PC whereas 18:0;22:6 PE-treated cells were essentially unaffected. The bottom panel of Figure 4 demonstrates that not only did both 18:0;22:6 PE and 18:0;22:6 PC SUV fuse with cells (i.e., become tightly cell associated), but also the fusion of 18:0;22:6 PE with cells was more efficient than 18:0;22:6 PC (based on the almost 3-fold steeper slope of the PE dose-response curve).

Measurement of peroxidation products. To determine whether peroxidation may be contributing to growth inhibition, we measured TBA-RS in control and DHA (0.15 mM) supplemented media before and after a 3-day culture and found no evidence for excessive accumulation of peroxidation products. The values for TBA-RS ranged from 0.12 to 0.44 nmoles MDA per ml of culture medium, and the values did not correlate with either the presence of DHA or incubation at 37 degrees C. We estimate that less than 0.001% of the added DHA oxidized to TBA-RS. Additionally, cytotoxicity mediated by 18:0;22:6 PC could not be ascribed to lipid peroxidation, as there were more detectable peroxidation products in SUV composed of 18:0;18:1 PC plus 18:0;22:6 PE (3:1) compared to SUV composed of 18:0;18:1 PC plus 18:0;22:6 PC (3: 1), i.e., 2.72 and 1.57 nmoles MDA/mg DHAcontaining lipid, respectively. In both cases peroxidation was low, representing only 0.1-0.2 mol% of the polyunsaturated phospholipid. Thus, the data are consistent with the hypothesis that DHA accumulation in PC leads to structural membrane changes that are detrimental to cell viability.

Discussion

We show in this paper that DHA from culture medium was preferentially and rapidly incorporated at high levels into PE by the murine myeloid leukemia T27A. More slowly and under conditions of DHA excess, DHA accumulated in neutral lipids, PC, and to a small extent in other phospholipid classes. Because excess DHA reduced T27A cell growth, we speculated that the appearance of DHA-containing PC in T27A's plasma membrane may be detrimental to the cell, and this prediction was borne out by the reduced viability of cells fused with 18:0;22:6 PC but not 18:0;22:6 PE. Our results suggest that natural or artificial introduction of significant levels of DHA-containing PC molecules into cancer cell plasma membranes may serve as an anti-cancer therapy.

Preferential DHA accumulation in PE has been reported for some other cell types, but there is great diversity in the processing of fatty acids by cultured cells, making

evaluation of fatty acids' intracellular fates critical to understanding their biological roles [19,20]. The diversity of phospholipid classes and acvl chains strongly implies unique or specialized roles for the different lipid species. DHA's proposed functions include: i) reduction in eicosanoid (prostaglandin) production from 20:4, which has not yet been investigated in the T27A model system; ii) direct or indirect modulation of second messengers; iii) increased nonenzymatic peroxidation; and iv) alteration of membrane structure and function. Clearly there are instances in which peroxidation correlates very well with suppressive effects of omega-3 fatty acids on tumor growth [21,22], but we have observed in this study as have other investigators [23,24] that DHA may have a growth inhibitory effect distinct from lipoperoxidation. We have focused on DHA's role as a structural membrane element, and suggest that the experiments reported here may be understood in terms of DHA-induced lipid domains.

It is now universally accepted that biological membranes are not homogeneous mixtures of lipid and protein but instead are composed of rapidly changing arrangements of widely differing membrane patches called domains [25,26]. We have previously proposed that the role of DHA may be in affecting lipid domain structure of plasma membranes through interaction with other membrane lipids particularly cholesterol [5,27]. The distribution of DHA and cholesterol in membranes is thought to be heterogeneous [28-30], implying the existence of lipid domains. Cholesterol is known to associate more strongly with PC than PE [31] to the extent that cholesterol has been reported to be excluded from PE-rich membranes [32]. Cholesterol-phospholipid affinities may also be affected by the hydrophobic center of the bilayer as cholesterol associates very strongly with oleic acid and very poorly with DHA [5,33]. When cells are allowed to slowly accumulate free DHA into membranes, the fatty acid appears primarily in PE, a phospholipid that demonstrates little affinity for cholesterol. However, if membranes are forced to accept DHA linked to PC, the normally favorable interaction between cholesterol and the PC head group would be offset by the now unfavorable interaction with the DHA tail. New lipid microdomains would be produced, attracting a different protein population and activity. Therefore, we predict that if given the opportunity, cells would prefer to safely accumulate DHA into PE where it would minimize the effect on lipid domain structure.

For our model system (T27A), we propose that the lipid class into which DHA is channeled will influence whether DHA has a detrimental (cytostatic or cytotoxic) effect on cells, and as a corollary to this prediction we further suggest that the availability (i.e., concentration) of DHA will determine the lipid class into which it is incorporated.

Specifically, our results suggest that significant DHA incorporation into PC corresponds to reduced cell growth and direct cytotoxicity. To the best of our knowledge, the current study is the first to show a clear effect of phospholipid class on DHA-associated cancer cell killing. This finding suggests a valuable application for DHA-containing PC in cancer therapies.

Acknowledgments

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Table I. *Fatty acid analysis of phospholipids of T27A cells cultured in DHA-supplemented medium*[a].

Fatty acid	PE		PC		PI		PS/PA	
	-DHA	+DHA	-DHA	+DHA	-DHA	+DHA	-DHA	+DHA
16:0	17.7 ± 0.3	19.0 ± 1.2	22.0 ± 0.6	32.0 ± 1.5[c]	9.7 ± 0.4	14.0 ± 0.6[b]	21.3 ± 0.9	19.3 ± 1.5[c]
18:0	14.0 ± 0.6	15.7 ± 0.9	20.0 ± 0.9	11.9 ± 0.5[c]	27.0 ± 1.2	23.3 ± 2.5[c]	18.0 ± 0.6	20.3 ± 1.9
18:1ω9	21.0 ± 0.6	13.7 ± 1.2[c]	18.3 ± 0.7	14.3 ± 0.3[c]	15.5 ± 0.6	13.5 ± 1.2	19.0 ± 1.2	15.0 ± 0.6[b]
18:2ω6	17.0 ± 0.6	8.0 ± 0.6[b]	24.0 ± 1.2	14.5 ± 0.3[c]	16.3 ± 1.5	11.0 ± 0.6[b]	23.0 ± 1.5	20.3 ± 0.3
20:4ω6	13.7 ± 0.9	9.6 ± 0.3[c]	5.2 ± 0.1	5.9 ± 0.4	16.0 ± 0.9	17.0 ± 1.3	3.3 ± 0.1	5.0 ± 0.6
22:6ω3	2.0 ± 0.1	20.3 ± 0.3[b]	0	5.5 ± 0.6[b]	0	3.3 ± 0.0[b]	0	4.2 ± 0.1[b]

[a] Cells were cultured in unsupplemented medium or medium supplemented with 100 mg DHA/ml for 72 hours at which time they were harvested and processed for phospholipid isolation and fatty acid analysis. For gas chromatography, the programmed oven temperature ramp was: 180 °C for 2 min, 3° C/min from 180 °C to 240, 240 °C for 5 min, 1° C/min from 240 °C to 245 °C, and 245 °C for 5 min. Peaks were identified by comparison of retention times of methyl ester standards (Nu-Chek Prep, Elysian, MN). Only the major fatty acids likely to play a significant structural role are shown. The data are presented as mean ± SE (n=3).
[b] $p<0.001$, [c] $p<0.01$ compared to the control (Student's t test)

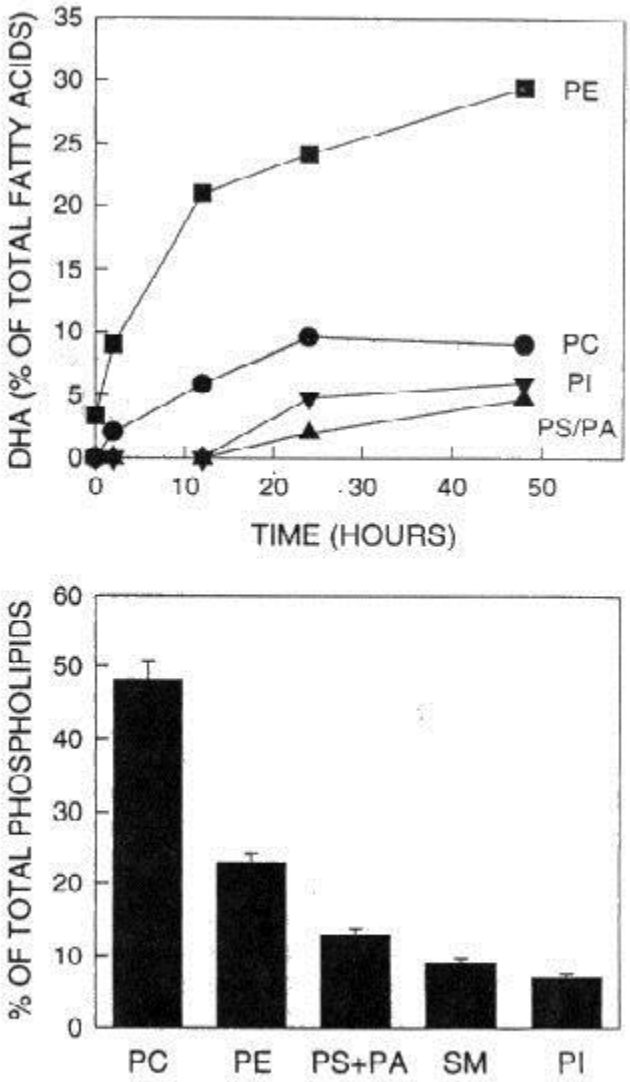

Figure 1. *Top Panel: DHA incorporation into individual phospholipid classes of T27A cells as a function of incubation time (culture medium was supplemented with 0.015 mM DHA). Bottom Panel: Distribution of phospholipid classes in T27A cells grown in control medium. The data are presented as mean ± SE (n=3).*

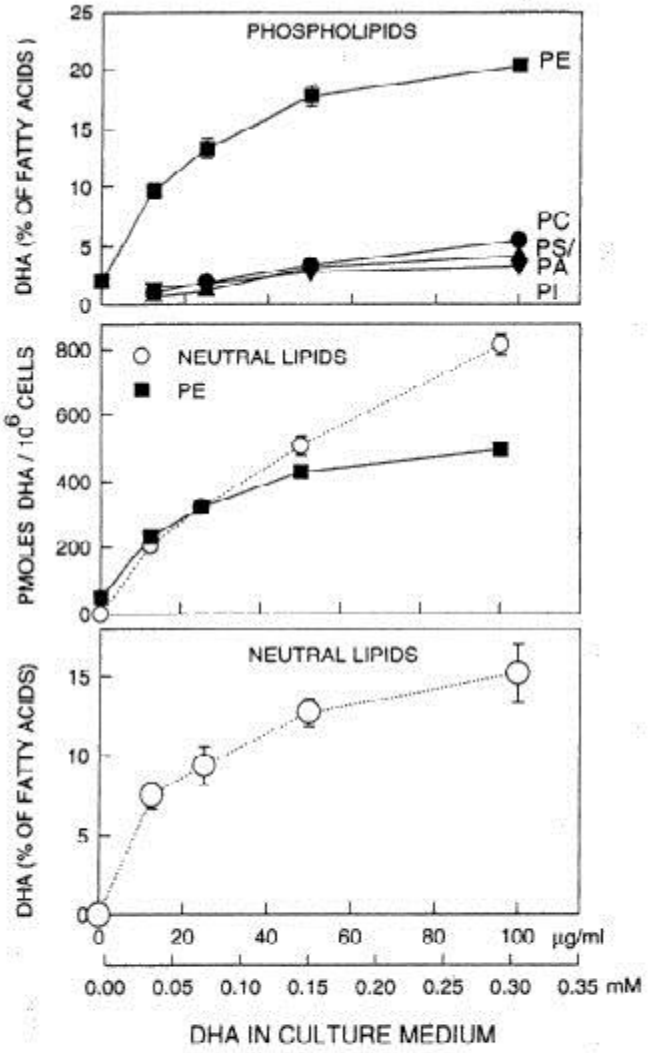

Figure 2. *T27A cells were grown in medium supplemented with various DHA concentrations for 72 hours, lipids were extracted and fatty acids were analyzed. Top Panel: Dose dependence of DHA incorporation into phospholipids. Data are presented as mean ± standard error (n=3). Middle Panel: DHA content of PE and neutral lipids. Bottom Panel: DHA content as percent of total fatty acids in neutral lipids. Mean ± SE (n=3).*

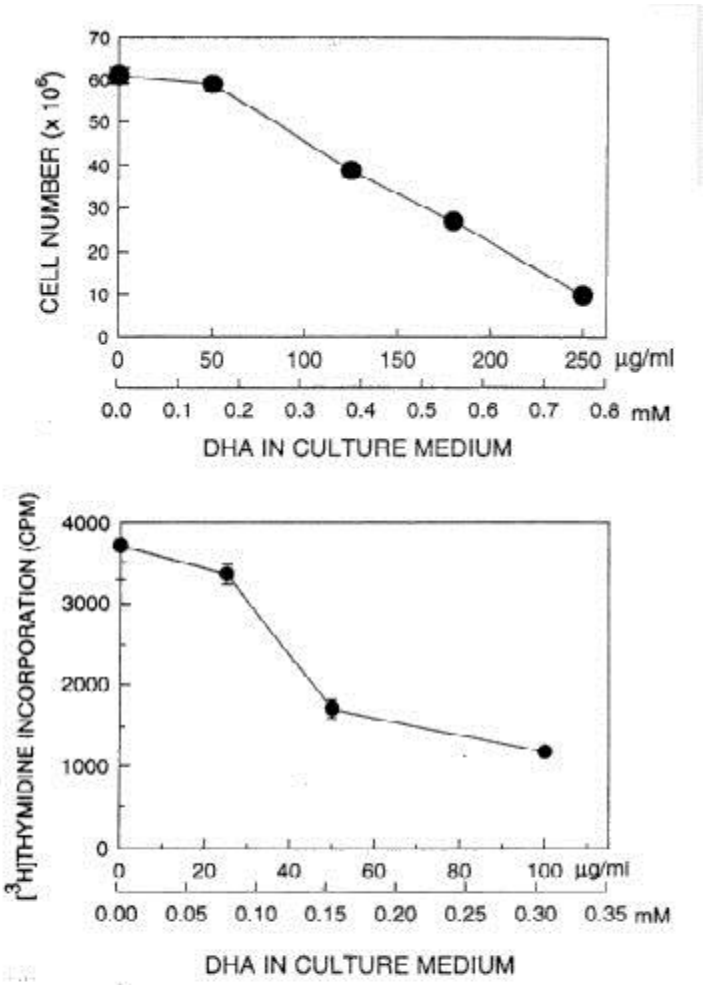

Figure 3. *High DHA concentrations inhibit T27A growth. Top Panel: Culture flasks were seeded with 5x104 T27A cells/ml of medium supplemented with 10% BCS and DHA (concentration shown on the abscissa). After 3 days, the cultures were harvested and the cell number (shown on the ordinate) and viability (>90% in each culture) were determined. Bottom Panel: T27A cells were synchronized by serum depletion (20 hrs in 0.5% BCS) and then cultured in microtiter plates at 5×10^3 cells/well in medium supplemented with 1% BCS (serum content reduced to accentuate the effect) and DHA. [^{3}H]Thymidine (0.5 mCi/well) was added at t=0 and the cells were harvested onto glass fiber filters 24 hrs later. Radioactivity incorporated into DNA and present on the filters was measured by scintillation counting. Mean ± SD (n=3; some error bars are smaller than the symbols).*

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