
Docosahexaenoic Acid Induces Death in Murine Leukemia Cells by Activating the Extrinsic Pathway of Apoptosis.

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Abstract

Docosahexaenoic acid (DHA) is a unique fatty acid that is found predominantly in the phospholipids of cell membranes. It has wide-ranging therapeutic effects that are broadly appreciated but poorly understood. Its principal location in the membranes of cells suggests that these myriad effects are manifest there. When cultured in DHA-enriched medium, cells of the murine leukemia cell line T27A took up the fatty acid and incorporated it into cellular phospholipids, particularly those of the plasma membrane. Culture in DHA-enriched media also caused significant dose-dependent cell death accompanied by increased plasma membrane bleb formation. Cysteine-dependent aspartate-directed proteases (caspases)-3 -8 and -9 were also activated, establishing apoptosis as the mechanism of DHA-induced cell death. Inhibition of any one of these caspases rescued the cells from apoptotic death. Caspase inhibition experiments identified T27A cells as belonging to the type II group of apoptotic cells and showed that apoptosis was initiated via the extrinsic pathway. Together these and previous data support the hypothesis that DHA causes cell death in leukemic cells by inducing alterations in the structure of lipid rafts that lead to the ligand-independent activation of death receptors and apoptosis.

Introduction

DOCOSAHEXAENOIC ACID (DHA, 22:6n-3) is a unique fatty acid that is found in the cells of a wide range of organisms from bacteria to humans. It is the longest and most unsaturated of the commonly occurring n-3 (omega-3, ω -3) fatty acids (Salem *et al.* 1986). DHA has diverse therapeutic properties that are acclaimed in both the scientific and lay communities (Stillwell and Wassall 2003; Siddiqui *et al.* 2004; Chapkin *et al.* 2009). A remarkable number of conditions and diseases have been demonstrated to be prevented, mitigated, counteracted or improved by DHA. These include maladies as disparate as cancer, heart disease, cystic fibrosis, diabetes, immune function and even psychiatric disorders (Stillwell and Wassall 2003; Siddiqui *et al.* 2004; Calder 2012; Mischoulon and Freeman 2013). While the relationship between DHA

and improved health is widely appreciated, the basic molecular mechanism underlying this relationship remains unclear. As noted by Stillwell (2008), the assortment of seemingly unrelated biochemical and physiological processes underlying the diseases and conditions that are influenced by DHA suggests that this fatty acid influences a fundamental cellular function or property.

DHA has been shown to have powerful anti-cancer effects in animals and cultured tumor cells (Siddiqui *et al.* 2004). For example, it is effective at reducing the accumulation of leukemic cells *in vitro* and in slowing the rate of progression of leukemia in animals (*e.g.* Jensi *et al.* 1993; Jensi *et al.* 1995; Zerouga *et al.* 1996). DHA has been shown to induce cell death in human and mouse leukemia cells in a dose dependent manner (Kafrawy *et al.* 1998; Yamagami *et al.* 2009) and it has been suggested the anti-leukemia properties of DHA are in general founded on the ability of DHA to induce cell death in tumor cells (Serini *et al.* 2009).

Despite continuing efforts, it is currently unclear precisely how DHA triggers cell death. DHA can be converted into reactive oxygen species that can influence cell survival (Siddiqui *et al.* 2008), and into powerful anti-inflammatory and pro-resolving mediators (resolvins, protectins and maresins) that can influence cell survival and disease etiology (Serhan *et al.* 2014; Colas *et al.* 2014; Dalli *et al.* 2015). DHA can also affect gene expression (Berger *et al.* 2006), the acylation patterns of membrane proteins (Webb *et al.* 2000), and the function of enzymes and ion channels (Matta *et al.* 2007). However a large and growing body of evidence indicates that DHA induces cell death only after it has become incorporated into membrane phospholipids and that the initial triggering event in cell death is a membrane-based phenomenon (Stillwell and Wassall 2003; Stillwell *et al.* 2005; Calder 2012).

There is substantial physiological, biochemical, biophysical, and morphological evidence that DHA-containing phospholipids change the structure of cell membranes (Mitchell *et al.* 2003; Niu and Mitchell 2005; Chapkin *et al.* 2008; Shaikh 2010; Rockett *et al.* 2012; Teague *et al.* 2013; Pinot *et al.* 2014). Indeed, whether provided as a dietary component to an individual organism (Lien 2009) or as a component of the incubation medium of cultured cells (Zerouga *et al.* 1996; Williams *et al.* 1998; Williams *et al.* 1999), DHA is taken up by cells and incorporated into the

phospholipids of membranes. The plasma membrane in particular appears to be a primary location of action for the tumor cell killing properties of DHA (Jenski *et al.* 1993; Pascale *et al.* 1993; Williams *et al.* 1998; Williams *et al.* 1999). Of particular interest in this regard is the influence of DHA-containing phospholipids on the membrane microdomain structures known as lipid rafts. Lipid rafts serve as platforms for the regulation of cell processes and represent a selective cellular compartment that can co-localize and modulate the activities of enzymes, receptors and other proteins (Simons and Ikonen 1997; Lingwood and Simons 2010). There is evidence that DHA-containing phospholipids induce cell death by altering the structure or organization of lipid rafts, and that this influence on membrane structure is the first and most important step in DHA-induced cell death (Stillwell *et al.* 2005; Schley *et al.* 2007; Chapkin *et al.* 2008).

Other evidence strongly suggests that DHA causes cell death in tumor cells by the induction of apoptosis (Blanckaert *et al.* 2010; Kang *et al.* 2010). There are two distinct activation pathways for apoptosis. The extrinsic pathway involves plasma membrane-associated death receptors and a cysteine-dependent aspartate-directed protease, caspase-8. The intrinsic pathway involves the release of cytochrome c from mitochondria and the activation of caspase-9. These two initiating events then cause the activation of downstream effector caspases including caspase-3 which in turn cleaves a series of intercellular substrates to continue the apoptotic cascade. Lipid rafts are importantly involved in the extrinsic apoptotic pathway as the death receptors, a subset of the tumor necrosis factor receptor superfamily, are among those receptors regulated by lipid rafts (Gajate *et al.* 2009; Lang *et al.* 2012).

Thus, there is evidence that DHA causes the death of many types of tumor cells, that the cause of cell death in many of these instances is the induction of apoptosis, that DHA alters the structure of lipid rafts, and that lipid rafts regulate the receptors involved in initiating the extrinsic pathway of apoptosis. This study attempts to connect these links by testing the hypothesis that DHA causes cell death in leukemia cells by specifically triggering the extrinsic pathway of apoptosis. We show that DHA is selectively incorporated into the plasma membrane of murine leukemia (T27A) cells. We use both morphological and biochemical

means to demonstrate that DHA induces apoptosis in these cells. By monitoring and manipulating the activities of caspases -8, -9 and -3, we further show that all three caspases are activated by DHA and that the inhibition of any one of them rescues T27A cells from DHA-induced apoptosis. Together these and previous data support the hypothesis that DHA causes cell death by inducing alterations in the structure of lipid rafts that lead to the ligand-independent activation of death receptors and apoptosis.

Methods

Materials

T27A murine leukemia cells were obtained from American Type Culture Collection (Manassas, Va). Fatty acids and fatty acid methyl ester (FAME) reference standards were purchased from Nu-Chek-Prep (Elysian, MN). RPMI-1640 culture medium supplemented with 2 mM glutamine, 25 mM HEPES, 50 $\mu\text{g}/\text{mL}$ streptomycin and 100 units/mL penicillin, was from Cambrex Bio Science (Walkersville, MD). Bovine calf serum was from Hyclone (Logan, UT). Irreversible, cell-permeable inhibitors of caspases -3 (Z-D[O-Me]E[O-Me]VD[O-Me]-FMK), -8 (Z-IE[O-Me]TD[O-Me]-FMK), and -9 (Z-LE[O-Me]HD[O-Me]-FMK) were from Calbiochem (EMD Biosciences, Inc., La Jolla, CA). The colorimetric assay kits for measuring the activities of caspases -3, -8 and -9 were from BioVision (Mountain View, CA.). Staurosporin, SiO₂ (“Celite”), and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were from Sigma or Thermo Fisher Scientific (Waltham, MA).

Cell culture

Except where noted, T27A cells were cultured in RPMI-1640 medium supplemented as described above and with 10% (vol/vol) bovine calf serum in 25 cm² culture flasks maintained at 37°C under an atmosphere of 5% CO₂ in humidified air. As noted previously (Zerouga *et al.* 1996; Williams *et al.* 1998; Williams *et al.* 1999), under these conditions cultures doubled every 12 to 15 hours. Cell viability was monitored by trypan blue exclusion (0.04% in phosphate buffered saline [PBS, 0.154 M NaCl, 0.016 M NaH₂PO₄, pH 7.2]).

Supplementation of culture media with fatty acids

DHA and oleic acid (OA, 18:1n-9) were added to RPMI culture medium using the methods of Spector and Hoak (1969) exactly as described by Williams *et al.* (1998). The fatty acid was dissolved in hexane and transferred to an Erlenmeyer flask containing SiO₂. Ten g of SiO₂ were used per mmol of fatty acid. The hexane was removed completely by a gentle stream of N₂ before the dry mixture was transferred to a solution of fatty acid free bovine serum albumin (1% fatty acid free BSA in RPMI supplemented as above, but excluding serum). After stirring for 30 min in the dark, the RPMI/fatty acid mixture was centrifuged for 30 min at 600 grav to remove the SiO₂ and the medium was sterilized by filtration (0.22 μm). Bovine calf serum was added to 10% (vol/vol) of the total just before use. Calf serum contributes a small amount of fatty acids to the final culture medium, but less than 1% of that is DHA (Williams *et al.* 1998). Unless noted otherwise, cells were incubated in fatty acid-enriched medium for 3 days (68-76 hours). Under these conditions T27A cells take up considerable DHA, and at DHA concentrations below 0.61 mM they remain >90% viable (Williams *et al.* 1998; Williams *et al.* 1999; and see below).

Assay of caspase activity and caspase inhibition

The activities of caspases -3, -8, and -9 were measured spectrophotometrically in 90-well plates. For each assay, T27A cells were cultured in RPMI medium containing no additions, 1.3 μM staurosporin, or 0.61 mM DHA. After 16 h of culture, cells from each flask were harvested by low-speed centrifugation. Cell viability (always greater than 90% in control cells) was assessed by trypan blue exclusion and cell density was determined by duplicate counts on a hemacytometer. For each treatment, 3×10^6 cells were treated with 50 μL of lysis buffer according to the manufacturer's instructions. After centrifugation, 30 μL of cell lysate were mixed with 20 μL of caspase assay medium in a well of the plate, mixed, and allowed to incubate at 37°C for 1 hour before the absorbance was read at 405 nm. Background values were subtracted from all absorbances and all treatment values were expressed as percentage of the control. For caspase inhibition experiments, cells were exposed to 10 μM inhibitor in DMSO (0.1% final concentration) for 30 min before exposure to control or fatty acid-enriched medium.

The linearity of the caspase assays was confirmed using p-nitroaniline as a standard. Regression analyses of the resulting standard curves yielded lines with $r_2 > 0.990$. Staurosporin was used as a positive control and only those assays that showed staurosporin-induced caspase activity were analyzed further.

Isolation of plasma membranes

After 48 h of culture in either normal (control) medium or medium enriched with 0.3 mM DHA, cell cultures were disrupted by sonication and the resulting homogenate was fractionated by the centrifugation protocol of Kaduce *et al.* (1977) using the buffers of Molnar *et al.* (1969) as described by Williams *et al.* (1998; 1999). Briefly, T27A cells were collected by centrifugation (500 grav for 15 min), resuspended in 0.25 M sucrose buffer (0.25 M sucrose, 40 mM NaCl, 100 mM KCl, 5 mM MgSO₄ · 7 H₂O, 20 mM Trizma base, pH 7.2 with HCl), and disrupted (on ice) by sonication for 2 x 35 sec using a tip-type sonicator (Fisher Scientific Model 500, 35 seconds, pulse on 1 sec, pulse off 1.5 sec). The cell homogenate was centrifuged at 27 kgrav for 10 min to remove undisrupted cells and cellular debris and the supernatant over the resulting pellet was spun for 1 hour at 105 kgrav to produce a mixed membrane pellet. The mixed membrane pellet was layered onto a pad of 1.1 M sucrose (remaining composition as above) and spun at 107 kgrav for 16 hours. The white interfacial material was collected and washed twice in excess PBS. The resulting membrane represents a better than 8-fold purification of plasma membrane over the mixed membrane fraction (Kaduce *et al.* 1977) and has been used in previous studies to determine the effects of DHA on membrane structure and composition in T27A cells (Williams *et al.* 1998; Williams *et al.* 1999).

Lipid extraction and gas chromatography of membrane fatty acids

Total lipids were extracted from whole cell preparations and from isolated plasma membranes using CHCl₃/CH₃OH (Bligh and Dyer 1959) and concentrated under a stream of dry N₂ gas. Phospholipids separated from neutral lipids (*e.g.*, triacylglycerols) by silicic acid chromatography (Wren 1960; Williams and Somero 1996) were transesterified into FAMES using methanolic sodium methoxide (Eder *et al.* 1992). FAMES were resolved using a 0.25 mm x 30 m HP-23 cis/trans

FAME column in a Hewlett-Packard 6890 gas chromatograph. The instrument was programmed to produce a temperature ramp from 180°C to 240° at 2°C/min starting 2 minutes after sample injection. Peaks corresponding to individual FAMES were identified by comparison of retention times to those of authentic standards. Peak areas were calculated using Hewlett-Packard's ChemStation software.

Statistics

Statistical analyses were carried out using version 2.15.3 of R (R Development Core Team, 2008; <http://www.r-project.org/>). Probabilities ≤ 0.05 were considered significant (and labeled *). Percent data were arcsine transformed ($\sin^{-1}\sqrt{\text{proportion}}$) before statistical analyses as recommended (Sokal and Rohlf 1981). The normality of distribution of each data set was assessed using the Shapiro-Wilk test. The homogeneity of variances among data sets was tested using Fligner-Killeen test as it has been shown to be least sensitive to departures from normality (Conover *et al.* 1981). The slopes of regression lines were compared to each other and to slope = 0 using the linear model function of R. Group means were compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD mean separation test, or where appropriate, the Kruskal-Wallis test followed by Wilcoxon rank sum tests.

Results

When cells of the murine leukemia line T27A were cultured in media supplemented with DHA, they took up the fatty acid and incorporated it into cellular phospholipids (Table 1). In phospholipids isolated from whole cells, DHA levels were 25 times that found in control cells. The increased proportion of DHA was associated with a large reduction in the proportions of stearic acid (18:0) and the n-6 isomer of 18:3. Proportions of palmitic acid (16:0) and oleic acid (18:1) increased. By contrast, DHA incorporation into phospholipids of the plasma membrane represented an 18-fold increase over that of control cells and resulted in a final proportion of DHA almost twice that observed in phospholipids isolated from whole cells. In the plasma membrane, DHA largely displaced oleic acid (18:1), as well as arachidonic acid (20:4) and other long chain polyunsaturated fatty acids. The DHA-induced alteration of membrane lipid composition of both whole cells and plasma membrane

is reflected in the near inversion of the n-6/n-3 ratios (Table 1) after treatment with DHA.

The dramatic accumulation of DHA in phospholipids of the plasma membrane of T27A cells can also be clearly seen when comparing the ratios of palmitate to stearate (16:0/18:0) and of DHA to stearate (22:6/18:0) in phospholipids extracted from plasma membrane and whole cells cultured in control versus DHA-enriched media (Figure 1). The results shown in Table 1 and Figure 1 closely mirror previously reported observations on the effects of DHA on the lipid composition of plasma membranes isolated from these cells (Zerouga *et al.* 1996; Williams *et al.* 1998; Williams *et al.* 1999) and indicate that the experiments presented here both compliment and expand those earlier works.

Table 1. The distribution of phospholipid fatty acids, as percent of total fatty acids, extracted from whole cells and from isolated plasma membranes after 48 h of culture in control or DHA-enriched (0.3 mM) medium. Minor fatty acids, *i.e.* those comprising less than 1% of the total, are excluded from the analysis. The data represent the means of two independent experiments.

Fatty Acid	Whole Cells		Plasma Membranes	
	Control	+ DHA	Control	+ DHA
14:0	1.1	1.4	1.2	1.1
14:1	1.1	0.4	0.2	0.1
16:0	12.2	17.5	15.0	16.9
16:1	0.8	0.8	1.3	0.8
18:0	73.5	59.1	49.4	50.6
18:1	3.6	6.4	20.3	7.7
18:2 n-6	1.2	1.9	4.9	2.9
18:3 n-6	4.6	1.2	0.0	0.0
20:3 n-6	0.0	0.0	1.6	0.4
20:4 n-3	0.4	0.0	3.6	1.0
22:5 n-3	0.0	0.0	1.3	0.0
22:6 n-3	0.4	10.3	1.1	18.5
total n-6	5.8	3.1	6.5	3.3
total n-3	0.8	10.3	6.0	19.5
n-6/n-3	7.25	0.30	1.08	0.17

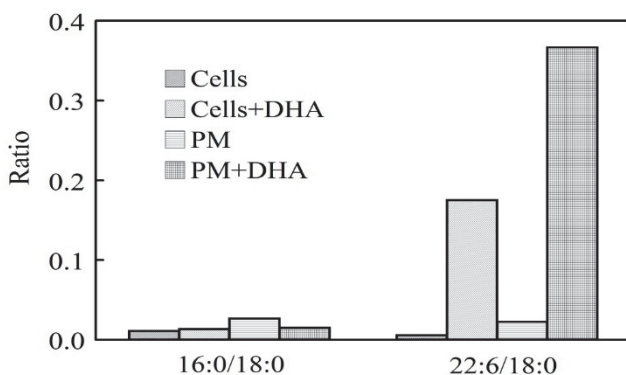


Figure 1. The ratios of mean values of palmitate to stearate (16:0/18:0) and of DHA to stearate (22:6/18:0) in phospholipids extracted from whole cells or plasma membrane (PM) after the cells had been cultured for 48 h in control medium or in medium containing 0.3 mM DHA.

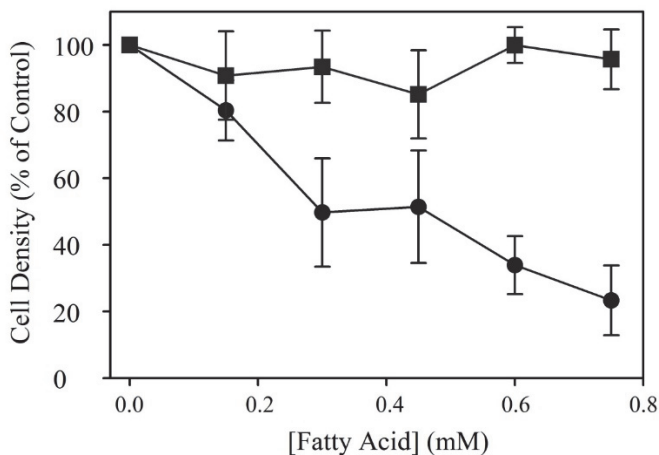


Figure 2. The density of T27A cells three days after exposure to the indicated concentrations of fatty acid and expressed as a percentage of control. Squares, OA; circles, DHA. Linear modeling revealed that the slope of the regression of the OA response is not significantly different from zero. The slope of the regression of the DHA response is highly significantly different from both slope = 0 and the OA response ($p < 0.001$ in both cases). Each point represents the mean \pm 1 standard error of the mean from $n = 7-14$ (DHA) or $n = 3-6$ (OA) independent determinations of different cultures.

Culture in DHA-enriched medium caused a significant reduction in the rate of leukemic cell proliferation. Figure 2 shows a DHA-dose dependent reduction in cell density compared to control cultures and to cultures similarly exposed to OA. The proportion of viable cells in the DHA-enriched cultures also fell significantly, while the viability of cells in cultures exposed to OA remained indistinguishable from that of the controls (Figure 3). Together these data show that DHA caused significant cell death over a three day exposure to concentrations of DHA in the culture medium from 0.3 to 0.9 mM.

Phase contrast microscopy revealed that unlike control cells or cells cultured in OA-enriched medium, cells cultured in DHA-enriched medium were irregularly shaped and exhibited conspicuously higher internal complexity including extensive cytoplasmic vacuolization. In addition, the external surfaces of control cells and of cells cultured in OA-enriched medium were even and regular, whereas the surfaces of cells cultured in DHA-enriched medium were uneven and displayed numerous exvaginations of the plasma membrane (commonly referred to as “blebs”; *e.g.* Charras 2008). Figure 4 shows that the percentage of T27A cells exhibiting blebs increased steadily with DHA dose until at the highest doses tested these structures appeared on nearly 75% of all cells present in the culture.

Culture of T27A cells for 16 h in a medium containing 0.61 mM DHA resulted in a significant elevation of the activities of caspases-3, -8, and -9 (Figure 5). When cell cultures were individually treated with 10 μ M of an inhibitor specific for each of these caspases for 30 min prior to culture in DHA-enriched medium they did not undergo cell death and cell densities were similar to those of control cultures (Figure 6).

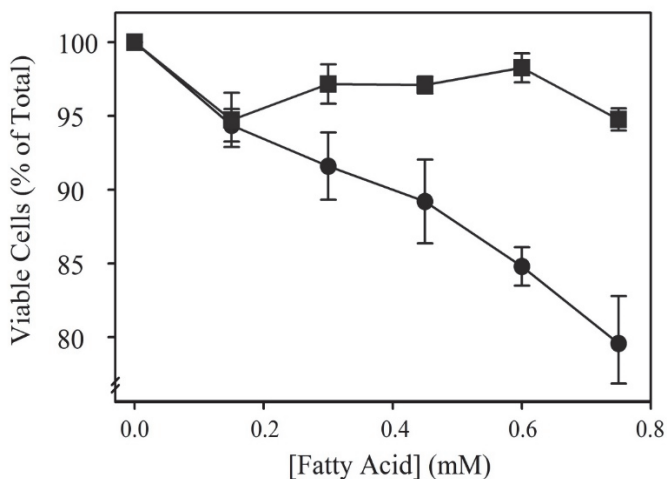


Figure 3. The viability of T27A cells as assessed by trypan blue exclusion three days after exposure to the indicated concentrations of fatty acid. The slope of the regression of the OA response is not significantly different from zero and that of the regression of the DHA response is highly significantly different from both slope = 0 and the OA response ($p < 0.001$ in both cases). Squares, OA; circles, DHA. Each point represents the mean \pm 1 standard error of the mean from $n = 3$ independent determinations of different cultures.

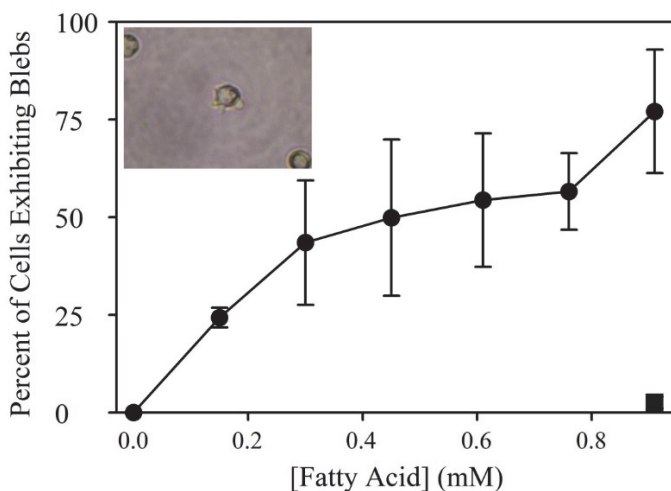


Figure 4. The percentage of T27A cells exhibiting plasma membrane exvaginations ("blebs", inset) after 16 h as a function of the concentration of fatty acid in the culture medium. Square, OA; circles, DHA. Each point represents the mean \pm 1 standard error of the mean from $n = 3$ independent determinations of different cultures.

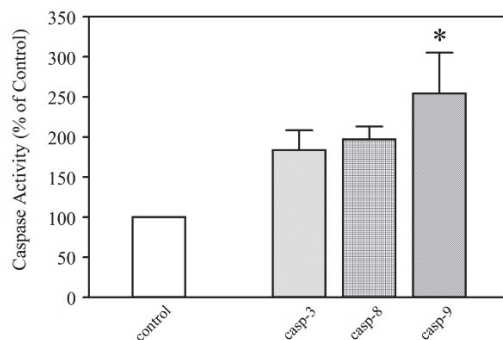


Figure 5. The effect of DHA on cellular caspases. The activities of caspases (casp-) 3, 8, and 9 in T27A cells after 16 h of culture in normal medium (control) and in medium containing 0.61 mM DHA. The activity of caspase-9 is significantly (*, $p < 0.05$) different from the control value. The activities of caspases -3 and -8 are not significantly different from caspase-9 and are marginally significantly ($0.05 < p < 0.1$) different from the controls. The data are presented as percent of activity found in control cells and represent the means \pm 1 standard error of the mean from $n = 3$ (caspase-3) or $n = 4$ independent assays using separate cell cultures.

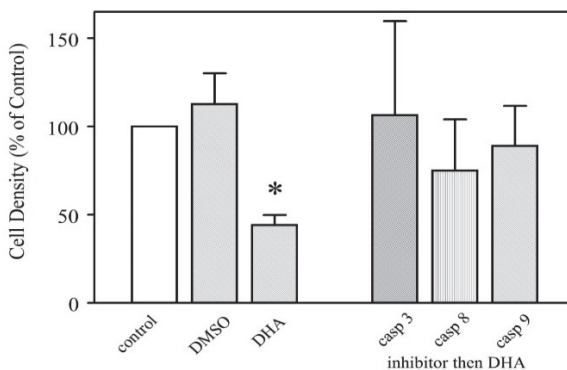


Figure 6. Density of T27A cell cultures expressed as a percentage of that in control flasks after 48 hours in the presence of medium containing no additions, 0.1% (vol/vol) DMSO (carrier control), and medium enriched with 0.61 mM DHA. These are compared to the densities of cell cultures exposed for 30 min to 10 μ M of an inhibitor specific to each one of the indicated caspases before the 48 h exposure to medium containing 0.61 mM DHA. Each bar represent the mean \pm 1 standard error of the mean from $n = 12$ (control, DMSO, and DHA) or $n = 4$ (inhibitors) independent cultures and assays. The bar labeled with the asterisks is significantly ($p \leq 0.05$) different from the control value.

Discussion

T27A is a line of murine B lymphoblast cells with well-described susceptibility to DHA-induced cell death (Zerouga *et al.* 1996; Kafrawy *et al.* 1998). Under normal growth conditions they possess very little DHA (Table 1). During culture in media supplemented with DHA, T27A cells incorporated considerable amounts of the fatty acid into phospholipids of their plasma membrane (Table 1, Figure 1). Delivering exogenous metabolites and drugs to cells as albumin conjugates is thought to simulate physiological delivery conditions and buffers the availability of the delivered substance. Other advantages of using albumin as a biological carrier molecule are described elsewhere (Kratz 2008; Elsadek and Kratz 2012).

In phospholipids extracted from whole cells, DHA increased from less than one-half of 1% of the total in control cells to over 10% of total phospholipid fatty acids in cells cultured with supplemental DHA. In purified plasma membrane preparations the percentage rose from close to 1% to over 18%. These results agree well with previous data from these cells (Williams *et al.* 1998) and with reports showing that DHA has a powerful effect on both the composition and structure of their plasma membranes (Zerouga *et al.* 1996; Zerouga *et al.* 1997; Williams *et al.* 1998; Williams *et al.* 1999). These observations suggest that the metabolism of fatty acids in these leukemic cells favors the non-random incorporation of DHA into cell membranes with a preferential incorporation of DHA into phospholipids of the plasma membrane. Preferential incorporation of DHA into the plasma membranes of T27A cells has been observed previously (Jenski *et al.* 1993; Pascale *et al.* 1993; Williams *et al.* 1998; Williams *et al.* 1999).

Culture of T27A cells in DHA-enriched media caused a dose-dependent decrease in cell density and cell viability, an increase in the percent of cells exhibiting blebs, and the activation of cellular caspases. OA did not induce these effects (Figures 2 and 3). We chose OA as the control fatty acid for this study because it is the most abundant fatty acid in many cell types, it is not toxic to T27A cells (Kafrawy *et al.* 1998) and because in other cells types and in model membranes it neither induces apoptosis nor influences membrane raft function or structure (Kishida *et al.* 2006; Shaikh *et al.* 2009; Shaikh *et al.* 2009a). Figures 2 and 3 show

that when cells were cultured in media containing DHA at concentrations above approximately 0.3 mM, their rate of proliferation was slowed and significant numbers of cells died. The observation of induction of cell death near 0.3 mM is consistent with what we and others have found in this cell line (Zerouga *et al.* 1996; Williams *et al.* 1998; Williams *et al.* 1999), and may have implications for human health. In a study of healthy men and women the serum concentration of DHA-phospholipids was found to be near 0.15 mM. That concentration rose to over 0.35 mM after six weeks of dietary supplementation with DHA capsules (Conquer and Holub 1998). In a separate study of 234 healthy men, the mean serum concentration of DHA-phospholipids was 0.18 mM and was elevated to over 0.31 mM by similar DHA capsule supplementation (Grimsgaard *et al.* 1997). These studies show that the DHA levels used to reduce the growth and proliferation of mouse leukemia cells *in vitro* can be achieved in humans by dietary manipulation.

At all concentrations of DHA examined, cells exhibited distinct exvaginations or blebs on their plasma membranes (Figure 4). Though the significance of these structures is not well understood, they are widely recognized as a hallmark of apoptosis (Charras 2008). The definitive indicator of apoptosis is the presence of active caspases (Galluzzi *et al.* 2011) and in these cells culture in medium containing 0.61 mM DHA resulted in the activation of caspases -3, -8, and -9 (Figure 5). These observations establish that DHA induces apoptosis in T27A cells.

In general, apoptosis can be triggered by two separate, but linked, pathways: the intrinsic and extrinsic pathways (Portt *et al.* 2011; Galluzzi *et al.* 2011). The intrinsic pathway originates with mitochondria and involves the release from the intermembrane space of pro-apoptotic molecules, particularly cytochrome c. The released cytochrome c initiates a series of events that result in the conversion of inactive procaspase-9 into active caspase-9. Caspase-9 then activates caspase-3 which is responsible for setting off the series of down-stream events characteristic of apoptosis. The extrinsic pathway involves death receptors located in the plasma membrane of the cell. Binding of an appropriate ligand to a death receptor initiates an apoptotic cascade that begins with the conversion of inactive procaspase-8 into active caspase-8. Depending on the type of cell, caspase-8 then activates caspase-3 directly or indirectly

by converting the protein Bid into tBid which activates caspase-9 (Portt *et al.* 2011; Galluzzi *et al.* 2011).

Caspases -3, -8, and -9 are all active in T27A cells after exposure to DHA (Figure 5) and the inhibition of any one of them prevents the cells from undergoing apoptosis (Figure 6). Since caspase-3 is an effector caspase acting downstream of the initiator caspases -8 and -9, it appears that a linear cascade of activation events occurs whereby one initiator caspase activates the other (*i.e.* either caspase-8 activates caspase-9 or vice versa) and the latter then activates caspase-3. In some cell types both caspases -8 and -9 are able to activate caspases-3 directly (Slee *et al.* 1999; Peter and Krammer 2003), but apparently in T27A cells under the conditions used here one of these caspases is unable to do so. It is possible that one of the initiator caspases (-8 or -9) activates caspase-3, then caspase-3 activates the remaining initiator caspase (Özören and El-Deiry 2003), but this is also not the case here because the activation of caspase-3 initiates the irreversible stages of apoptosis (the execution pathway) and thus the inhibition of the initiator caspase that was activated by caspases-3 would not result in the rescue from cell death shown in Figure 5. The data presented here suggest that one of the initiator caspases activates the other yet is itself unable to activate caspase-3. These results are consistent with T27A cells belonging to the type II group of apoptotic cells (Scaffidi *et al.* 1998; Özören and El-Deiry 2002). In cells able to undergo type I apoptosis, death receptor/ligand binding results in the direct activation of effector caspases like caspase-3. Most cells undergo type II apoptosis, in which death receptor/ligand binding is indirectly linked to the activation of effector caspases through the mitochondrion-dependent pathways via Bid and tBid (Scaffidi *et al.* 1998; Özören and El-Deiry 2002; Blarova *et al.* 2011). These observations are consistent with a pathway in T27A cells in which DHA induces apoptosis by first triggering caspase-8 which in turn activates caspase-9 to initiate the effector caspases.

Lipid rafts are dynamic and ephemeral laterally segregated assemblies of the plasma membrane that are rich in sphingolipids, cholesterol, and acylated and glycosylphosphatidylinositol (GPI)-anchored proteins (Simons and Ikonen 1997; Lingwood and Simons 2010). Lipid rafts serve as important platforms for the regulation of cell processes by confining and concentrating receptors and enzymes from the

surrounding membrane. They represent another selective cellular compartment that can co-localize and modulate the activities of these proteins (Simons and Ikonen 1997; Lingwood and Simons 2010). Death receptors, a subset of the tumor necrosis factor receptor superfamily, are among those receptors that have been shown to be regulated by lipid rafts. They include tumor necrosis factor receptor-1 (TNF-R1, p55), death receptor (DR) 3 (WSL-1/APO-3), DR4 (tumor necrosis factor-related apoptosis-inducing ligand receptor-1 [TRAIL-R1]), DR5 (TRAIL-R2/APO-2), DR6 and CD95 (Fas/APO-1) (Ashkenazi and Dixit 1998; Lavrik 2011). These receptors initiate extrinsic apoptosis after ligand binding or ligand-independent clustering of receptors (Fumarola *et al.* 2001; Scheel-Toellner *et al.* 2004). Only when located within lipid rafts do death receptors facilitate the activation of caspase-8 and down-stream events leading to apoptosis. Death receptors do not activate caspase-8 when located in non-raft regions of the membrane (Xu *et al.* 2009; Gajate *et al.* 2009; Blanarova *et al.* 2011).

Lipid raft dysfunction has previously been implicated in the DHA-induced cell death of T27A cells (Williams *et al.* 1998; Williams *et al.* 1999). Other work has shown that DHA alters the structure (Wassall and Stillwell 2008), size (Chapkin *et al.* 2008; Rockett *et al.* 2012) and protein composition (Rogers *et al.* 2010) of lipid rafts. Recently, Shaikh's group has shown that DHA has profound effects on mammalian immune function and that these effects arise from the influence of DHA on the lipid rafts of B cells (Rockett *et al.* 2012; Gurzell *et al.* 2013). Other evidence convincingly shows that DHA alters the raft-localization of epidermal growth factor receptor (Schley *et al.* 2007; Rogers *et al.* 2010), caveolin-1 (Li *et al.* 2007), toll-like receptors (Wong *et al.* 2009), the major histocompatibility complex (MHC) class I proteins (Ruth *et al.* 2009; Shaikh *et al.* 2009), the signaling molecules SFK, Lck, Fyn, and c-Yes (Stulnig *et al.* 1998; Stulnig *et al.* 2001; Chen *et al.* 2007), the interleukin-2 receptor (Li *et al.* 2005), phospholipase D1 (Diaz *et al.* 2002), endothelial nitric oxide synthase (Li *et al.* 2007; Matesanz *et al.* 2010), and protein kinase C (Fan *et al.* 2004). Combined with the data presented here, these observations suggest that DHA has an influence on death receptor-mediated apoptosis via an action on lipid rafts. This conclusion is reinforced by studies showing that a number of structurally diverse anti-tumor agents selectively induce apoptosis in cancer cells by

triggering apoptosis thorough Fas-clustering in lipid rafts (Xu *et al.* 2009; Mollinedo *et al.* 2010; Blanarova *et al.* 2011).

Conclusions

When T27A leukemic cells are cultured in media enriched with DHA, the cells take up the fatty acid and incorporate it into their membranes, particularly the plasma membrane. Culture in DHA-enriched medium also causes cell death by inducing apoptosis. This induction of apoptosis is caused by the initiation of the extrinsic apoptotic pathway and with a linear activation of caspases in the sequence caspase-8, caspase-9, then caspase-3. Coupled with previous observations by us and others, the data suggest that the first step in DHA-induced cell death in T27A leukemia cells involves the activation of plasma membrane-associated death receptors by an influence of DHA on lipid rafts.

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